A Clarification of the Aetiology and Pathogenesis of Renal Tubular Epithelial Cell Vacuolization in Diabetic Ketoacidosis

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DECLARATION

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ABSTRACT

Diabetes mellitus is an increasingly prevalent disease amongst Western populations. A serious and potentially fatal sequela of diabetes is diabetic ketoacidosis which often relies on post-mortem examination for diagnosis in the context of unwitnessed deaths. One of the major end-organs affected by diabetes is the kidneys, thus, post-mortem examination of the kidneys serves as a major diagnostic tool.

Renal tubular vacuolization represents a standard response to a variety of metabolic insults, and analysis of vacuole morphology can assist in determining the nature of the underlying metabolic derangement. The Armanni-Ebstein phenomenon refers to a pattern of renal tubular epithelial cell vacuolization noted in diabetic coma with a demonstrated relationship to the extent of hyperglycaemia. It involves a pattern of cytoplasmic clearing by a single large vacuole with luminal displacement of nuclei by intra-vacuolar glycogen. In more recent literature, the Armanni-Ebstein phenomenon has also been used to describe basal/subnuclear vacuoles containing lipids and triglycerides, thus causing confusion. However, there are limited studies surrounding basal vacuolization in renal tubular epithelial cells beyond case reports, thus, its aetiology remains largely unknown.

This thesis aims to clarify the terminology surrounding patterns of renal tubular vacuolization in diabetic ketoacidosis, investigate the aetiology of basal vacuolizations in human and animal models, and further define useful markers at autopsy for significant metabolic derangements. These studies will facilitate greater consistency in the reporting of findings by pathologists,

and improve the linking of specific morphological findings to underlying pathophysiological processes and diagnoses.

AIMS

Chapter 1: Clarification of terminology

1. To clarify the terminology surrounding patterns of renal tubular epithelial cell vacuolization in diabetic ketoacidosis.

Chapter 2: Histopathological studies to confirm or refute associations between histomorphology and pathological conditions

2. To investigate the occurrence of basal vacuolizations in non-diabetic causes of ketoacidosis.
3. To investigate the relationship between basal vacuolizations in renal tubular epithelial cells and hyperlipidaemia.
4. To investigate the relationship between basal vacuolizations in renal tubular epithelial cells and hypothermia.
5. To investigate if macroscopic renal cortical pallor can be a useful sign for underlying metabolic derangements at autopsy.
6. To investigate if characteristic formalin pigment deposition can be used as a surrogate marker for basal vacuolizations.

Chapter 3: Determining incidence and further defining diagnostic utility

7. To determine the incidence of Armanni-Ebstein lesions and investigate their relationship with hyperglycaemia.
8. To determine the incidence of basal vacuolizations and investigate their relationship with ketoacidosis.

Chapter 4: Establishing and utilizing animal models to facilitate further investigation

9. To investigate the relationship between basal vacuolizations in renal tubular epithelial cells and terminal hypothermia using an animal model.
10. To investigate the role of osmotic effect in the aetiology of Armanni-Ebstein lesions.
11. To develop an isolated perfused kidney model capable of reproducing renal tubular epithelial vacuolizations secondary to metabolic derangements.
    a. To utilize the isolated perfused kidney model to reproduce Armanni-Ebstein lesions.
    b. To utilize the isolated perfused kidney model to reproduce basal vacuolizations.

HYPOTHESES

Chapter 1: Clarification of terminology

1. That Armanni-Ebstein lesions refer to cytoplasmic clearing with luminal displacement of nuclei and is a distinct histological change from basal vacuolizations.

Chapter 2: Histopathological studies to confirm or refute associations between histomorphology and pathologic conditions

2. That basal vacuolizations in renal tubular epithelial cells may occur in non-diabetic causes of ketoacidosis.
3. That basal vacuolizations in renal tubular epithelial cells are associated with hyperlipidaemia.
4. That basal vacuolizations in renal tubular epithelial cells are not associated with hypothermia *per se*.
5. That macroscopic renal cortical pallor at autopsy may be an early indication of basal vacuolization of renal tubular epithelial cells.
6. That formalin pigment deposition in a characteristic basal distribution may be used as a surrogate marker for basal vacuolization.

Chapter 3: Determining incidence and further defining diagnostic utility

7. That Armanni-Ebstein lesions are uncommon in hyperglycaemia and their incidence correlates with the degree of glucose elevation.
8. That basal vacuolizations are common in ketoacidosis and their incidence correlates with the degree of ketone elevation.

Chapter 4: Establishing and utilizing animal models to facilitate further investigation

9. That basal vacuolizations in renal tubular epithelial cells are not associated with hypothermia.
10. That Armanni-Ebstein lesions result from the metabolic sequelae of hyperglycaemia and not the osmotic effect of glucose.
11. That the isolated perfused kidney model is a viable ex-vivo model for the investigation of renal tubular epithelial vacuolizations, i.e.
    a. That Armanni-Ebstein lesions are reproducible in an isolated perfused kidney model.
    b. That basal vacuolizations are reproducible in an isolated perfused kidney model.

CHAPTER 1: CLARIFICATION OF TERMINOLOGY

ARMANNI-EBSTEIN LESIONS: A NEED FOR CLARIFICATION

CONTEXTUAL STATEMENT

AIM: To clarify the terminology surrounding patterns of renal tubular epithelial cell vacuolization in diabetic ketoacidosis

HYPOTHESIS: That Armanni-Ebstein lesions refer to cytoplasmic clearing with luminal displacement of nuclei and is a distinct histological change from basal vacuolizations.

COMMENTARY: The Armanni-Ebstein phenomenon describes a pattern of cytoplasmic clearing with glycogen deposition in renal tubular epithelial cells, noted in deaths due to diabetic coma. Its pathogenesis has been firmly established to hyperglycaemia through animal studies. In recent literature, Armanni-Ebstein lesions have also been used to describe basal vacuolizations which stain positively for lipids with a possible pathogenesis involving hyperlipidaemia. Thus, this study was conducted with the aim of clarifying the terminology of the ‘Armanni-Ebstein phenomenon’ through a systematic and chronological review of current literature.

This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSION: Armanni-Ebstein lesions and basal vacuolizations represent two distinct histologic changes in renal tubular epithelial cells and are associated with different metabolic derangements which overlap in diabetic ketoacidosis.

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Author Contributions

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Armanni-Ebstein Lesions: A Need For Clarification

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ABSTRACT: Armanni-Ebstein lesions were first described by Luciano Armanni, a pathologist at the University of Naples, during autopsy studies undertaken in 1872, as a unique vacuolar nephropathy associated with poorly controlled diabetes that involves selective renal epithelial cell glycogen accumulation. However, within the last two decades a broader range of vacuolar changes, including lipid deposition, have also been termed Armanni-Ebstein lesions, creating some confusion on possible etiology. We would suggest that the term Armanni-Ebstein phenomenon would be best reserved for the original clear cell change associated with glycogen deposition, and that this should be clearly distinguished from subnuclear lipid vacuolization ("basal vacuolization"). Although there is obvious interrelation between these two types of vacuoles, they appear morphologically and biochemically distinct from each other. More precise classification may assist in clarifying the causal processes and possible diagnostic significance of different types of renal epithelial vacuolization at autopsy.

Keywords: forensic science, Armanni-Ebstein, basal vacuolization, renal epithelial cell change, diabetes mellitus, ketoacidosis

Origins of the Armanni Ebstein lesion

In the late 1800s, Armanni observed that the morphology of epithelial cells in the proximal tubules of the deep cortex and outer medulla of the kidneys had altered; the cells had lost their normal polarized cuboidal shapes and were swollen, rounded and transparent, with small dark nuclei that were often displaced to the periphery, rather than in their normal central position. This observation was presented to medical students in 1872 and first appeared in Italian in Cantani's textbook *Patologia e Terapia del Ricambio Materiale* in 1875, with the large polygonal cells described on page 257 and illustrated by a sketch in Figure 6 (reproduced here as Figure 1) (1). Armanni's initial study was later published in German in 1877 (2). Giordano's translation of Armanni's description of large polygonal epithelial cells filled with hyaline material appeared in the English literature in 1987 (3). Ten years after Armanni's initial observations, Ebstein reported similar lesions in the German literature in 1882, describing edematous cells with remnants of cytoplasm (4).

Contemporary literature

One of the first analyses of Armanni-Ebstein lesions in the English literature in more recent times was in a study by Ritchie and Waugh in 1957 (5). These authors described the lesions as occurring rarely in poorly controlled diabetic states after the advent of insulin therapy, and appearing as markedly swollen epithelial cells with a normal appearing central nucleus but with "virtually total conversion of the cytoplasm into a single large vacuole". Affected cells bulged into, and sometimes occluded the tubular lumen. This was consistent with the original descriptions by Armanni and Ebstein, as illustrated in their paper in Figures 2 and 4 (5). The lesions principally affected the terminal straight portion of the proximal convoluted tubule, with a consistent localization at the corticomedullary junction. The

affected area involved mainly the outer medulla with some extension into the inner cortex, but did not affect tubules in the middle or outer cortices. Vacuoles in affected cells stained positively for glycogen with no stainable lipids (5). Of interest, the authors also mentioned that fatty vacuolization found in some of their cases was usually basal and present only in the upper proximal tubule regions, not in the areas typical of Armanni-Ebstein (5).

In 1978, Armanni-Ebstein lesions were described in Meadows' textbook, *Renal Histopathology* as a rare tubular disorder principally affecting long-term diabetics. The lesions appeared as swollen cells that often occluded tubular lumina with vacuoles containing abundant glycogen (6) (Figures 2 & 3). Rasch described Armanni-Ebstein lesions in streptozotocin-induced diabetic rats in 1984. In agreement with the findings of Ritchie and colleagues, the lesions were confined to the cortex and outer medulla with abnormal cells appearing "empty" or full of PAS-positive material but with an intact nucleus. Electron microscopy showed the cytoplasm of these cells to be filled with glycogen (7). In 1986, Bendon and Hug described typical Armanni-Ebstein lesions in five patients with Fanconi Syndrome and commented that the common feature of Fanconi syndrome and diabetes mellitus was glycosuria. The lesions were localized in tubular epithelial cells at the corticomedullary junction which were abnormal in being filled with glycogen but which retained distinct cell membranes, clear cytoplasm and normal nuclei (8).

Animal models

Ishizaki et al noted Armanni-Ebstein lesions in the ascending limbs of the loop of Henle in all twenty of their spontaneously diabetic rats, with affected cells characterized by the presence of vacuoles filled with glycogen (9). In the same year, 1987, Orloff et al monitored the presence of Armanni-Ebstein lesions, characterized by vacuolization and

distension of renal tubular cells due to glycogen accumulation, in alloxan-treated diabetic rats receiving pancreatic transplants. In their study, Armanni-Ebstein lesions appeared 1-3 months after the induction of diabetes with alloxan, and progressively increased over the next 24 months (10).

In 1990, Reyes et al found that cholesterol administration in streptozotocin-induced diabetic rats prevented the development of Armanni-Ebstein lesions despite persistent hyperglycemia. As with previous studies, the observed lesions were described as swelling of tubular epithelial cells with clear cytoplasm, which were shown by electron microscopy to contain diffusely distributed granules characteristic of glycogen (11). A study by Dobashi et al in 1991 on streptozotocin-induced diabetic rats further refined the timeline for the development of Armanni-Ebstein lesions by showing that the lesions were not present at two weeks after treatment, but had appeared by eight weeks (12).

Due to the consistent demonstration of glycogen within vacuoles, Armanni-Ebstein lesions have also been referred to as ‘glycogen nephrosis’ (13-16), in keeping with the original work in the field linking the condition to hyperglycemia associated with diabetes. In a study of alloxan-diabetic rats, Curtis et al concluded that the appearance of these lesions was solely dependent upon the terminal blood glucose level, with Armanni-Ebstein changes invariably present with levels above 350mg/100mls and consistently absent below 300mg/100mls (13).

Broadening of the concept

The situation changed somewhat in the mid 1990s with work by Kock and Vestergaard expanding the range of lesion phenotypes. They found eight out of 47 cases of insulin-dependent diabetics had tubular epithelial changes that were interpreted as Armanni-

Ebstein lesions with two morphological classes of lesions described. One type of lesion consisted of tubular cells that had nuclei "completely surrounded by the vacuolation" that was glycogen-positive based on Best's carmine staining. Affected cells were located in the outer zone of the medulla, consistent with previous descriptions of Armanni-Ebstein lesions. The second type of lesion was composed of cells that had basally-located vacuoles with luminally-displaced nuclei, not characteristic of the classic Amanni-Ebstein phenomenon. PAS staining for glycogen was negative (14) (Figure 4). A drawing of these basally-located, non-glycogenic lesions is shown in Figure 5 and contrasts with the illustration of the original Armanni-Ebstein findings shown in Figure 1. Another contrasting feature of the second category was that vacuolization was observed in all regions of the cortex as well as in the outer zone of the medulla, although the middle and outer cortices had been previously described as being Armanni-Ebstein "immune" (5). With mixed data on the presence of glycogen and two morphologically distinct patterns of vacuolization, the authors concluded that "a combination of glycogen nephrosis and hydropic vacuolization might well have caused the widespread changes of the tubular epithelium, which seemed to be more extensive than described in the characteristic AE lesions in the literature" (14). These observations effectively expanded the morphological spectrum of Armanni-Ebstein lesions to include PAS-negative tubular epithelial cells with basal vacuolization that were located in the middle and outer cortex.

Several years later Thomsen and Hansen described a 47-year-old insulin-dependent diabetic woman with Armanni-Ebstein phenomenon where the epithelial vacuoles (which were located mainly in the proximal tubules) were PAS-negative but stained strongly for neutral lipid (15) (Figure 6). This led to a new hypothesis that the Armanni-Ebstein phenomenon was due to the accumulation of triglycerides. It was noted that some tubules

astride the corticomedullary junction did stain positively for glycogen (15), suggesting in retrospect that more than one class of pathology was present. Neilsen and colleagues further described the Armanni-Ebstein phenomenon occurring in vacuolated tubular epithelial cells within the renal cortex as being "seen as a line of empty spaces in contact with the peritubular basement membrane". This again diverged from the original descriptions of cellular ballooning with a clear cell change (16). These authors also reported a tripling of renal cortical lipid due to a 60-100 fold increase in triglycerides which suggested that these lesions were caused by accumulated lipids. This supported the proposal of Kock and Vestergaard (14) that the Armanni-Ebstein phenomenon encompassed two types of lesions: one in the cortex associated with triglyceride accumulation and the other in the outer medulla due to glycogen deposition (16) (Figure 7). In a further study by Thomsen and colleagues, the role of the original "glycogen nephrosis" was downplayed further with the statement that "in all probability the Armanni-Ebstein phenomenon is caused by the accumulation of lipids in the proximal tubules". The authors suggested that a more appropriate name would be "fatty kidney in diabetics" (17).

Possible association with hypothermia

In 2004, Preuβ et al noted similar basal lipid vacuoles in 87% of cases of fatal hypothermia, suggesting that these lesions were "a very reliable histologic diagnostic criterium in cases of hypothermia", comparable to Wischnewsky spots (18). Wischnewsky spots are superficial gastric lesions characterized histologically by necrosis of the mucosa with hematin formation (19), reportedly present in 40-90% of fatal hypothermia cases (20). This newly attributed significance of basal lipid vacuolization has subsequently been incorporated into forensic texts (19).

Recent findings

In our investigations (21-24), we have found evidence of glycogen nephrosis consistent with the classic Armanni-Ebstein phenomenon in the tubules of the outer medulla, but have concentrated on cases of basal vacuolization of the renal tubular epithelium, which we also termed Armanni-Ebstein phenomena. In a retrospective review of deaths with terminal hyperglycemia, we observed basal vacuolization in deaths due to diabetic ketoacidosis and determined that there was no correlation between the extent of these lesions and the degree of terminal hyperglycemia (21). Similar lesions were observed in 33% of our hypothermic cases; however, given the significant number of diabetics in this group it was suggested that these changes might have arisen due to underlying metabolic disturbances related to diabetes rather than to hypothermia per se (22). Renal cortical pallor was found to be a useful macroscopic marker for the presence of basal vacuolization at autopsy. Histologically, numerous subnuclear epithelial cell vacuoles were identified which contained lipid on electron microscopy and did not stain with PAS, consistent with the results of other work (20). Finally, we analysed subnuclear vacuolization in deaths due to alcoholic ketoacidosis and found this to be a feature of non-diabetic ketoacidosis in the setting of hypo- or normoglycemia (23). Similar findings, again described as Armanni-Ebstein lesions, were recently reported in a child who died of ketoacidosis and starvation in the absence of hyperglycemia, further strengthening the association between basal (subnuclear) vacuolization and ketoacidosis (24).

Clarification

It appears, therefore, that there has been broadening of the definition of Armanni-Ebstein lesions in the more recent literature. Whereas classical studies described specific pathological swelling of cells, loss of shape, clearing of cytoplasm, and accumulation of cytoplasmic glycogen that was directly linked to hyperglycemia and subsequent glucosuria, the more recent literature includes other conditions of basal vacuolization with preservation of cellular integrity, luminal displacement of nuclei, accumulation of lipids and triglycerides within vacuoles, and an association with ketoacidosis.

How is this disparity best dealt with? We considered that as the terminology used to describe renal tubular epithelial vacuolization has altered over time with changing of original definitions, a review of early morphologic descriptions was timely. Having obtained translations of the original nineteenth century texts by Armanni and Ebstein and extensively reviewed the literature, we suggest that the term Armanni-Ebstein phenomenon would be best reserved for the original clear cell change described in their original work (Figures 1 and 3). This is not to suggest that subnuclear lipid vacuolization of renal tubular epithelial cells (Figures 2 and 4) is not an important finding at autopsy, as it indicates that there has been a significant metabolic derangement. These vacuoles, however, appear morphologically and biochemically distinct from the lesions described by Armanni and Ebstein and might be best referred to as “basal vacuolization”.

Conclusion

Neilsen et al (2003) and Kock and Vestergaard (1994) were correct in noting that tubular epithelial lesions in diabetic ketoacidosis are of two kinds. For purposes of clarity, it would seem logical to refine the nomenclature to distinguish these conditions and to restore the classical definition of Armanni-Ebstein to avoid any confusion that might have

inadvertently arisen (16,17,20,24). The benefit of clarifying terminology and separating these two lesions (i.e Armanni-Ebstein and basal vacuolization) will be in creating greater consistency in the reporting of findings which will allow better linking of specific morphological findings at autopsy to unique, although possibly related, underlying pathophysiological processes and diagnoses. The elucidation of different types of renal vacuolar change with more precise classification should help to further clarify the composition, causal processes and possible diagnostic significance of particular types of renal epithelial vacuolization at autopsy. This may then assist in determining possible lethal mechanisms and metabolic derangements in forensic cases.

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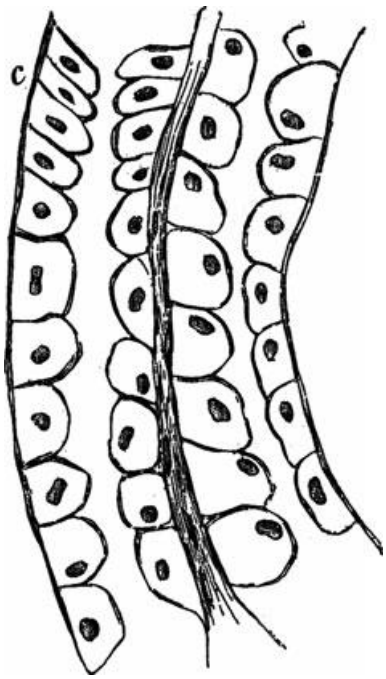

FIG. 1-*A sketch showing typical Armanni Ebstein vacuolization of renal tubular epithelial cells, depicted by Luciano Armanni in Cantani's 1875 textbook Patologia e Terapia del Ricambio Materiale, showing swollen polygonal cells with normal nuclei.*

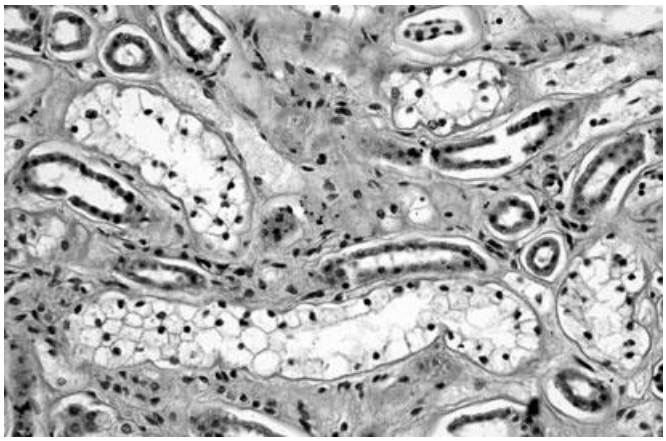

FIG. 2- *Classical Armanni-Ebstein changes in the proximal convoluted tubule, at the corticomedullary junction showing markedly swollen epithelial cells with almost total conversion of the cytoplasm of individual epithelial cells into a single large vacuole with normal appearing nuclei. The affected cells bulge into the tubular lumen.*

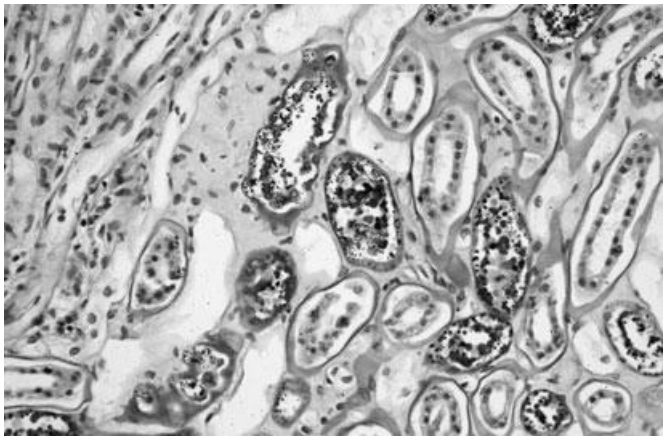

FIG. 3-*Special staining for glycogen in cases of classical Armanni-Ebstein is positive (PAS x 200).*

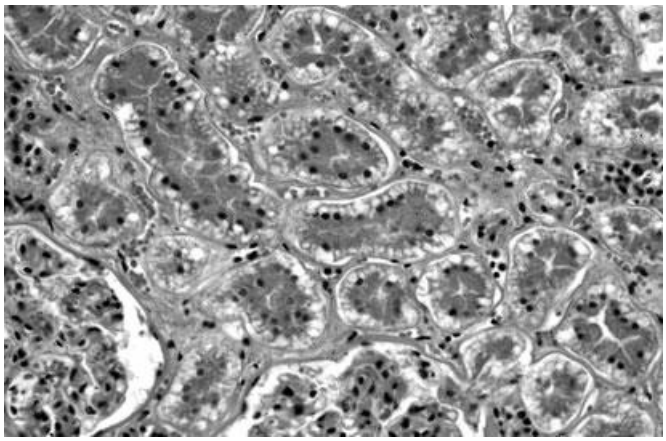

FIG. 4- *A lesion that has more recently also been called Armanni-Ebstein with orderly basal vacuolization of tubular epithelial cells and luminal displacement of nuclei. Special staining for glycogen in these cases is negative (Hematoxylin & eosin, H&E x 200).*

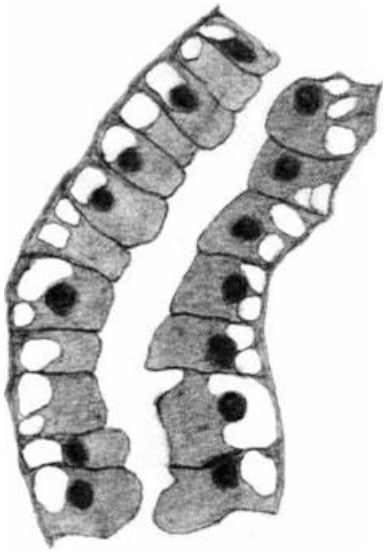

FIG. 5-*A sketch depicting vacuolization of the basal portions of renal tubular epithelial cells with luminal displacement of nuclei, a change that has been referred to in recent times as typical of Armanni-Ebstein.*

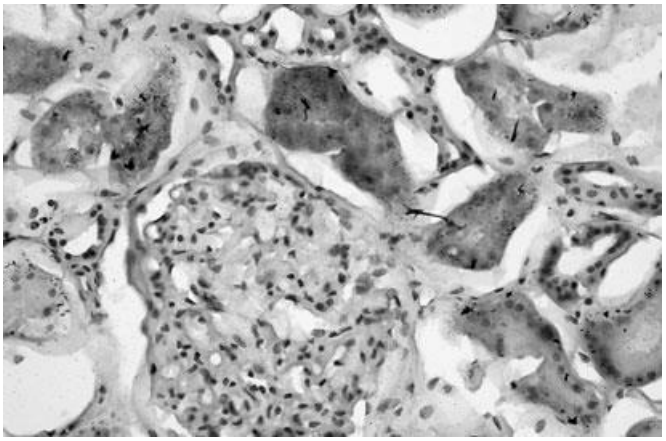

FIG. 6- *Positive staining of basal vacuoles in tubular epithelial cells for neutral lipid (oil-red-O x 200).*

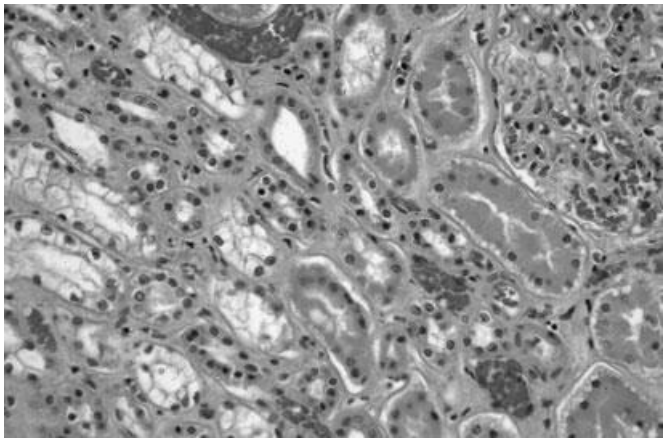

FIG. 7-*The corticomedullary junction showing tubules with distinctive basal vacuolization next to a glomerulus, with Armanni Ebstein vacuolization in tubules deeper within the medulla (Hematoxylin & eosin, H&E x 200).*

CHAPTER 2: HISTOPATHOLOGICAL STUDIES TO CONFIRM OR REFUTE ASSOCIATIONS BETWEEN HISTOMORPHOLOGY AND PATHOLOGICAL CONDITIONS

BASAL RENAL TUBULAR EPITHELIAL CELL VACUOLIZATION AND ALCHOLIC KETOACIDOSIS

CONTEXTUAL STATEMENT

AIM: To investigate the occurrence of basal vacuolizations in non-diabetic causes of ketoacidosis.

HYPOTHESIS: That basal vacuolizations in renal tubular epithelial cells may occur in non-diabetic causes of ketoacidosis.

COMMENTARY: Basal lipid vacuolization of renal tubular epithelial cells has been reported in diabetic ketoacidosis, however, it appears to be a distinct histologic phenomenon compared to Armanni-Ebstein lesions which have traditionally been regarded as being pathognomonic for diabetes. Thus, the authors wondered whether basal lipid vacuolization could be observed in non-diabetic causes of ketoacidosis, such as alcoholic ketoacidosis, in which there is no elevation in glucose levels.

Literature review establishing the field of knowledge is discussed within the paper.

This manuscript preceded the manuscript titled “Armanni-Ebstein lesions: a need for clarification” in chapter 1, therefore, the terminology of “Armanni-Ebstein” lesions/phenomenon was still used to refer to these basal vacuolizations. The manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSION: Basal vacuolizations in renal tubular epithelial cells are caused by alcoholic ketoacidosis.

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Author Contributions

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Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Conceptualization of work, performed literature review, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	3/7/14

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscript evaluation and editing, acted as corresponding author		
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PUBLISHED MANUSCRIPT

Basal Renal Tubular Epithelial Cell Vacuolization and Alcoholic Ketoacidosis

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ABSTRACT: Subnuclear renal tubular epithelial cell vacuolization is a marker for diabetic ketoacidosis. Whether it is because of hyperglycemia or of ketoacidosis is unclear. To examine the effect of ketoacidosis on renal cells in isolation, five cases of lethal alcoholic ketoacidosis without hyperglycemia were examined (vitreous humor β-hydroxybutyrate: 6.42-8.75 mM, mean 7.66 mM; glucose: 0.1-4.2 mM, mean 1.46 mM). Microscopic examination of the kidneys revealed basal vacuoles in three cases (60%). Seven control cases with acute alcohol toxicity without ketoacidosis (blood alcohol: 0.18-0.43%, mean 0.31%; and β-hydroxybutyrate: 0.12-0.42 mM, mean 0.21 mM) did not have these changes. In this study, basal epithelial vacuolization was found only in cases with significant ketoacidosis. Although the numbers are small, the finding of basal renal tubular epithelial vacuolization in normoglycemic cases with elevated β-hydroxybutyrate levels provide further evidence that disordered lipid metabolism may be involved in the pathogenesis this phenomenon.

Keywords: Forensic science, alcoholic ketoacidosis, intoxication, Armanni Ebstein phenomenon, diabetes mellitus, alcoholism

Introduction

The term “Armanni-Ebstein phenomenon”, first described in 1877 by Luciano Armanni, has been used to refer to subnuclear vacuolization of renal tubular epithelial cells, which when severe enough may present macroscopically as renal cortical pallor. First recognized in poorly controlled diabetic states, it was initially thought to be a direct effect of hyperglycemia causing cytoplasmic glycogen accumulation (1-5). However there is now evidence that the intracellular vacuoles contain lipid (6-8). A recent study also failed to demonstrate a significant correlation between the degree of hyperglycemia and these morphological changes, suggesting a more complicated etiology possibly involving the effect of ketoacidosis or lipiduria on tubular cells (9). Although this subnuclear vacuolization has been reported in cases of lethal hypothermia, it appears that in at least some of these cases it may be related to underlying diabetic ketoacidosis, that is, basal epithelial vacuolization in these cases may be merely a marker of diabetic metabolic derangement rather than to hypothermia *per se* (10,11). To examine the effect of ketoacidosis on renal cells in isolation, without concomitant hyperglycemia, a series of deaths because of alcoholic ketoacidosis were reviewed. A control group consisted of cases with significantly elevated blood alcohol levels, but no evidence of ketoacidosis.

Materials and methods

Case files over a 7-year-period from 2004 to 2010 at Forensic Science SA, Adelaide, South Australia, were retrospectively reviewed for all cases of nontraumatic deaths where alcohol was listed as a direct cause of death. Cases where deaths were attributed to alcoholic ketoacidosis or where acute alcohol intoxication had played a role were then selected. All cases had full coronial and police investigations with complete forensic autopsies. Alcoholic

ketoacidosis was diagnosed when the vitreous humor β-hydroxybutyrate was ≥ 5 mM with normoglycemia or hypoglycemia (vitreous glucose ≤ 11 mM), in conjunction with a history of alcohol abuse and/or autopsy findings suggestive of chronic alcoholism. Alcohol toxicity was taken as a blood alcohol level of >0.15%. Cases where vitreous humor biochemistry and full toxicology had not been performed were excluded. Case files were summarized and all available microscopic slides of the kidneys were then blindly reviewed for basal epithelial vacuolization; there were no histologic sampling differences between the alcoholic ketoacidosis and the acute alcohol toxicity group, with one section per kidney being reviewed. Cases where the kidneys were too autolysed for accurate assessment were also excluded.

Results

A total of 26 cases were identified, consisting of 12 deaths because of alcoholic ketoacidosis, and 14 because of acute alcohol toxicity. Of the 12 cases of alcoholic ketoacidosis, six were excluded due to incomplete vitreous humor biochemical evaluations, and one was excluded due to insufficiently raised β-hydroxybutyrate (<5 mM). Of the 14 deaths where alcohol toxicity had contributed to the lethal episode, five were excluded because of lack of vitreous biochemistry, and two were excluded because of insufficiently raised blood alcohol concentrations (<0.15%).

All five cases where death was because of alcoholic ketoacidosis had a history of chronic alcohol abuse, two with hepatomegaly, four with steatosis and periportal fibrosis, and one with splenomegaly. The age range was from 51 to 72 years (mean 61 years) and all were men. Vitreous humor β-hydroxybutyrate levels ranged from 6.42 to 8.75 mM (mean 7.66 mM) with no elevation in glucose levels (range 0.10-4.20 mM; mean 1.46 mM). In one case, the blood alcohol concentration was 0.20%, and alcohol was not detected in the others. The

post-mortem interval in which samples were drawn ranged from 4 to 8 days, with a mean of 5.6 days. No cases showed evidence of significant putrefaction. Microscopic examination of the kidneys revealed basal epithelial vacuolization in three cases (Fig. 1). There was no history of diabetes mellitus in any of these cases.

A total of seven cases with acute alcohol toxicity without ketoacidosis were studied; six had a history of chronic alcohol abuse with hepatomegaly at autopsy in four cases, marked cirrhosis in one case, early cirrhotic changes in four cases, mild steatosis in one case, and splenomegaly in four cases. The ages ranged from 37 to 68 years (mean 51.1 years). The male to female ratio was 3:4. In one case there was a medical history of insulin-dependent diabetes mellitus. Blood alcohol concentrations ranged from 0.18 to 0.43% (mean 0.31%). β-hydroxybutyrate levels ranged from 0.12 to 0.42 mM (mean 0.21 mM). Vitreous glucose was tested in three cases and ranged from 0.1 to 1.3 mM (mean 0.5 mM). The post-mortem interval in which samples were withdrawn ranged from 2 to 6 days, with a mean of 3.9 days. No cases showed evidence of significant putrefaction. None of the cases exhibited basal epithelial vacuolization in the kidneys (Fig. 2).

Discussion

Ethanol is a commonly used substance, the excessive consumption of which can have a variety of effects on the body with forensic repercussions. Ethanol readily crosses the blood-brain-barrier into the cerebral extracellular fluid, and has a direct depressant effect on neurons. Chronic excessive alcohol intake is associated with many significant conditions including malnutrition, cirrhosis with liver failure, esophageal varices, chronic pancreatitis, and cardiomyopathy (12,13). Alcohol is also frequently an indirect cause of death with

excessive alcohol consumption associated with aspiration of gastric contents and a variety of unnatural deaths that include drowning, falls, burns, and other forms of trauma (13).

Alcoholic ketoacidosis has been reported in 7-10% of alcoholic patients suffering sudden death (14). β-hydroxybutyrate levels >2.5 mM are considered lethal (15), and a typical episode involves binge drinking with subsequent reduced caloric intake (16). Ethanol levels are frequently low or absent (<10 mg/dL) at autopsy, as the victims have often stopped drinking after the onset of symptoms (17,18). Alcoholic ketoacidosis accounts for up to 25% of cases of ketoacidosis (19), occurring once for every four cases of diabetic ketoacidosis (17). It results from low protein and carbohydrate stores, and/or alcoholic liver disease with subsequent hepatic glycogen depletion, a situation that causes a fall in blood glucose concentrations with suppression of insulin secretion (14,19). Volume depletion caused by vomiting, decreased fluid intake, and/or diaphoresis, can lead to hypotension and a sympathetic response, further decreasing insulin production and increasing catecholamines, cortisol, growth hormone, and glucagon levels (14,18). Starvation and hypothermia may also lead to an elevation in these hormone levels, which in conjunction with the direct affect of ethanol, promotes lipolysis, and increases the supply of fatty acids to the liver (14). When this exceeds the rate of oxidation, a surplus of acetyl-CoA results and is converted to acetoacetate (20). In addition, ketogenesis is favoured by the inhibition of gluconeogenesis and decreased pyruvate levels. Subsequent accumulation results, as clearance of ketoacids is also impaired by volume depletion and low plasma insulin (17).

In the present series, typical basal epithelial vacuolization in renal tubular epithelial cells was found in three of the five cases (60%) of alcoholic ketoacidosis that fulfilled the criteria for the study. In addition, these so-called Armanni-Ebstein changes were present only in cases where there was significant ketoacidosis and were not related to blood ethanol levels.

While vitreous glucose levels decline after death, in our experience markedly elevated levels can still be detected for many days after death (10), and thus the glucose levels in the three cases with basal vacuolizations were not considered to have been significantly raised prior to death. In addition, there was no history of diabetes mellitus in any of these victims. Although the numbers are small, the detection of renal tubular epithelial vacuolization in cases where β-hydroxybutyrate levels were elevated in the absence of hyperglycemia may provide further evidence for the role of disordered lipid metabolism in the pathogenesis of this phenomenon.

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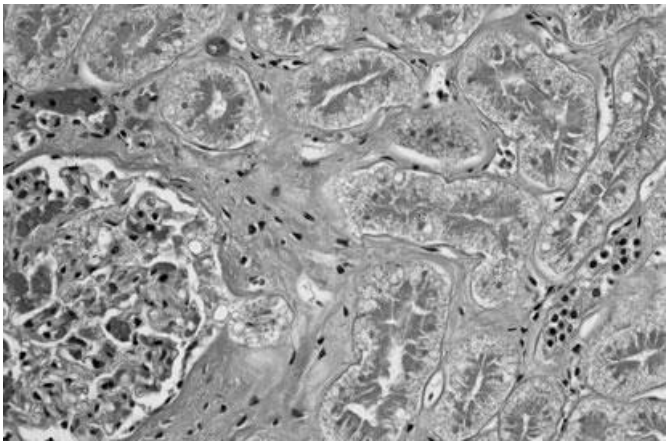

FIG. 1-*Typical vacuolization in the basal portions of renal tubular epithelial cells in a case of normoglycemic alcoholic ketoacidosis. Mild autolytic change is present (hematoxylin and eosin x280).*

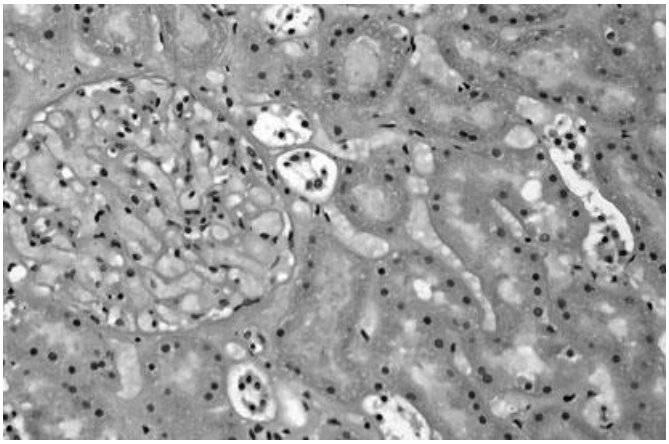

FIG. 2-*Normal renal tubular epithelial cells in a case of acute alcoholic toxicity (Blood alcohol = 0.43%) with no evidence of vacuolization (hematoxylin and eosin x 280).*

SEPTIC KETOACIDOSIS – A POTENTIALLY LETHAL ENTITY WITH RENAL TUBULAR EPITHELIAL VACUOLIZATION

CONTEXTUAL STATEMENT

AIM: To investigate the occurrence of basal vacuolizations in non-diabetic causes of ketoacidosis.

HYPOTHESIS: That basal vacuolizations in renal tubular epithelial cells may occur in septic ketoacidosis.

COMMENTARY: Basal lipid vacuolization of renal tubular epithelial cells has been reported in diabetic, alcoholic, and starvation ketoacidosis, suggesting a possible relationship to elevated ketones. Therefore, the same pattern of vacuolization should also be observed in ketoacidosis secondary to sepsis or severe infections.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSION: Basal vacuolizations in renal tubular epithelial cells are caused by septic ketoacidosis.

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Conceptualization of work, performed literature review, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
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Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
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Septic ketoacidosis – a potentially lethal entity with renal tubular epithelial vacuolization

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ABSTRACT: Fatal ketoacidosis due to diabetes mellitus, alcoholism and starvation may produce characteristic basal vacuolization of renal tubular epithelial cells (RTEC). Septic ketoacidosis has recently been recognized clinically as a distinct condition in which septicemia can lead to elevation of ketones and various anions unrelated to diabetes mellitus, alcoholism, or caloric deprivation. We report four lethal cases with significantly elevated vitreous ketones secondary to sepsis and/or severe localized infection in individuals with no history of diabetes mellitus, alcoholism, or starvation. Three of four cases exhibited typical basal vacuolization of RTEC. We suggest that septic ketoacidosis is an appropriate cause of death in the forensic setting where sepsis or severe localized infection is found with significant ketoacidosis (β-hydroxybutyrate>5mmol/l) - in the absence of diabetes mellitus, alcoholism, starvation, or other states associated with accelerated ketogenesis. The finding of basal vacuolization of RTEC in such cases provides morphological support for the underlying metabolic derangement.

Keywords: Forensic science, ketoacidosis, sepsis, basal vacuolization, subnuclear vacuolization, diabetes mellitus, alcoholism, starvation

Introduction

Ketoacidosis can lead to death via many mechanisms including severe extracellular volume contraction, electrolyte disturbances such as hypokalemia, cerebral edema, and sudden cardiac arrest (1,2). The cause of ketoacidosis is most commonly diabetes mellitus, but it may also be related to alcohol abuse, starvation, non-insulin mediated hypoglycemia (including inherent errors of fatty acid metabolism), and drugs (e.g. those that inhibit insulin release or acetyl-CoA carboxylase activity)(1). Septic ketoacidosis has recently been recognized in clinical settings as a separate pathological condition which can lead to marked ketoacidosis independent of diabetes mellitus, alcoholism, starvation, and drugs (3); it is, therefore, logical that sepsis or severe localized infections causing ketoacidosis could be a cause of death in forensic cases.

The post-mortem diagnosis of ketoacidosis relies heavily on detecting elevations in vitreous β-hydroxybutyrate and acetoacetate, as often there is no gross pathology present at autopsy. Basal vacuolizations in renal tubular epithelial cells and characteristic formalin pigment deposition in the region of the basal vacuoles are, however, useful histological observations supporting significant ketoacidosis (4). These vacuoles have been reported in ketoacidosis due to diabetes mellitus, alcoholism, and starvation. We report characteristic basal vacuolizations found at autopsy in three of four cases of lethal septic ketoacidosis.

Materials and methods

Case files over a 5-year-period from 2010 to 2014 at Forensic Science SA, Adelaide, South Australia were retrospectively reviewed for all cases of sepsis and/or where death had been attributed to severe infection. All cases had full coronial and police investigations with

complete forensic autopsies. Cases with a history of diabetes mellitus, alcoholism, recent starvation, or autopsy findings supporting any of these conditions were excluded. Septic ketoacidosis was diagnosed when vitreous humor β-hydroxybutyrate ≥ 5mmol/l with normoglycemia or hypoglycemia (vitreous glucose ≤ 11mmol/l), in conjunction with positive bacterial cultures and autopsy findings of localized infection. Cases with insufficiently raised β-hydroxybutyrate levels, elevated glucose levels (>11mmol/l), or where vitreous humor biochemistry had not been performed were excluded. Case files were summarized and microscopic slides of the kidneys were then blindly reviewed for basal vacuolization of renal tubular epithelial cells. Cases where autolysis precluded accurate histological assessment were also excluded.

Case reports

Case 1: A 41–year-old Caucasian male with a history of Human Immunodeficiency Virus (HIV) and hepatitis C positivity was found dead lying face down outdoors. There were reports of him suffering from poor concentration and a change in behavior leading up to death. He had no history of diabetes mellitus, alcoholism, or recent starvation. At autopsy, he appeared adequately nourished with a BMI of 22; bilateral pneumonia was found in the lungs with *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Penicillium*, and *Mycobacterium heckeshornense* identified on culture. The heart was unremarkable with no significant coronary artery disease, and the liver was also normal with no steatosis or cirrhosis. Subnuclear vacuolization of renal tubular epithelial cells was present in the kidneys, with no evidence of diabetic nephropathy. Vitreous biochemistry showed significant ketoacidosis with a β-hydroxybutyrate concentration of 13.32 mmol/l and lactate of 7.63 mmol/l. There was no evidence of hyperglycemia (vitreous glucose <0.6 mmol/l). Toxicology

detected acetone in the blood but was otherwise unremarkable. *Staphylococcus aureus* was also cultured from the spleen, thus, death was attributed to *Staphylococcus aureus* sepsis arising from pneumonia, with HIV as a likely significant underlying factor predisposing to infection.

Case 2: A 78-year-old Caucasian male (BMI = 21.5) with a past medical history of epilepsy, dementia, and mobility problems was found dead lying supine on a concrete floor at his home address. His medications include lamotrigine, aspirin, and ostelin. There was no history of diabetes mellitus, alcoholism, or starvation. At autopsy, extensive acute bilateral confluent bronchopneumonia was present on a background of chronic obstructive pulmonary disease. Post-mortem bronchial swab and lung tissue showed a mixed growth of *Klebsiella oxytoca*, *Citrobacter sp.*, *Hemophilus influenzae*, and *Streptococcus pneumoniae*. Examination of the heart revealed severe triple vessel coronary artery disease with left ventricular hypertrophy and myocardial scarring; no acute changes were identified. Sections of the kidneys showed mild to moderate generalized nephrosclerosis and subnuclear vacuolization of renal tubular epithelial cells. Scattered mucosal erosions were noted in the lower stomach consistent with hypothermic changes. Neuropathological changes of Alzheimer's disease were present in the brain, with no evidence of acute trauma or ischemic injury. Multiple minor areas of bruising, abrasions, and underlying right rib fractures (8^{th}, 9^{th}, and 10^{th}) were present, likely relating to a fall around the time of death. Vitreous biochemistry showed significant ketoacidosis with a β-hydroxybutyrate level of 8.13 mmol/l and no hyperglycemia (glucose < 0.6 mmol/l). Blood toxicology was unremarkable. Death was attributed to multi-organ failure, ketoacidosis, hypothermia and dehydration due to ischemic heart disease and bilateral lower lobe pneumonia.

Case 3: A 78-year-old Caucasian male (BMI = 22) was found dead on the floor of his residence. He had no history of diabetes mellitus, alcoholism or starvation. Acute bronchopneumonia was found at autopsy and post-mortem lung swabs grew mixed organisms including *Hemophilus influenzae*. The heart was unremarkable with no significant coronary artery disease. Wischnewsky-type mucosal hemorrhages were present in the stomach and proximal small intestine. The liver showed moderate steatosis. There was no evidence of diabetic nephropathy or subnuclear vacuolization in the kidneys. There was a fractured left humerus with extensive bruising around the left shoulder most likely due to a fall associated with the terminal episode. There was no other evidence of trauma. Vitreous biochemistry showed a raised β-hydroxybutyrate level of 8.07 mmol/l with normoglycemia (1.4 mmol/l). Acetone was found in the blood on toxicology. Death was attributed to acute bronchopneumonia complicated by hypothermia with ketosis and fracture of the left humerus.

Case 4: A 72-year-old underweight Caucasian female (BMI = 14) was found dead on the floor of her lounge room. It appeared that she had collapsed striking her chin. She had a history of breast carcinoma 20 years previously, arthritis, and possible overuse of analgesics. She did not have a history of diabetes mellitus, alcoholism, or starvation. At autopsy, florid suppurative meningitis was found with chronic inflammatory cells suggesting that the process had been present for some time. *Streptococcus agalactiae* was cultured from the brain. Bacterial endocarditis involved the mitral valve, and abrasions were present on her chin and limbs consistent with terminal collapse. Examination of the heart showed mild (up to 30%) stenosis of the epicardial coronary arteries but no evidence of recent or old ischemic changes. The liver was unremarkable with no steatosis or cirrhosis. Histopathological examination of the kidneys showed bacterial overgrowth and focal collections of chronic inflammatory cells, as well as subnuclear vacuolization of renal tubular epithelial cells (Figure 1); there was no

evidence of diabetic nephropathy. Vitreous biochemistry showed ketoacidosis (β-hydroxybutyrate 6.26 mmol/l) but no hyperglycemia (glucose <0.6 mmol/l). Blood toxicology was unremarkable. Death was attributed to *Streptococcus agalactiae* meningitis, and endocarditis.

Discussion

Ketoacidosis is a form of metabolic acidosis which is known to be a complication in diabetics, alcoholics, and those with prolonged starvation. Recently, septic ketoacidosis has been suggested as a separate pathological condition in the clinical setting by Nakamura and colleagues who reported a case of significant ketoacidosis (β-hydroxybutyrate 9.86 mmol/l, acetoacetate 2.55 mmol/l) caused by *Klebsiella pneumonia* sepsis due to acute obstructive cholangitis in a non-diabetic and non-alcoholic patient with no history of starvation (3). Previous studies have also shown that the septic state is associated with a decreased arterial ketone body ratio (acetoacetate/hydroxybutyrate)(5), as is seen in diabetic and alcoholic ketoacidosis (6,7). Although post-mortem blood cultures were not undertaken in our cases, the pathological findings, in conjunction with the available microbiological results in case 1 and 4, would be in keeping with disseminated sepsis. In cases 2 and 3, it was clear that a severe burden of infection was present as both were complicated by hypothermia and ketoacidosis, and case 2 was complicated by multi-organ failure, however, as only the post-mortem lung tissue or lung swab was submitted for microbiology, disseminated sepsis was not confirmed.

The mechanism by which sepsis causes ketoacidosis is likely multifactorial. Sepsis is associated with a relative increase in the level of glucagon and impairs the actions of insulin on endogenous glucose production and utilization, thus causing a functional insulin

deficiency. (3,8). Sepsis can also lead to ketogenesis through acetic acid overload from bacterial metabolism in the gastrointestinal tract, and inhibition of acetyl CoA carboxylase by adrenalin which is elevated during sepsis. The latter can lead to ketogenesis even without relative or absolute insulin deficiency (1,9). Furthermore, sepsis, particularly in its early stages, is associated with a hypermetabolic state, thus there is a relative increase in carbohydrate demands compared to normal individuals (10,11). This can contribute to and exaggerate ketogenesis by inducing a relative state of starvation with increased lipolysis, increased circulating free fatty acids presented to the liver, and subsequent increase in hepatic ketone production (12). Approximately half of the ketoacids produced are removed by oxidation in the brain and one quarter by oxidation in the kidneys (1). Other mechanisms of ketone removal include urinary excretion, conversion to acetone, and oxidation in the gastrointestinal tract (1). Sepsis is a known cause and precipitant of multi-organ failure, as in Case 2, and can therefore also significantly impair the removal of circulating ketones by affected organs.

On retrospective review of autopsy reports, the cause of ketoacidosis in all four cases had been presumed to be dehydration and inadequate food intake in the pre-terminal period of the infective illnesses. However, although fasting may result in mild to moderate ketosis, it is not known to cause a marked elevation of ketoacids to the levels seen in diabetics and alcoholics. This may be because although low, plasma insulin levels are still consistently detectable in the fasted state (12), whereas diabetic ketoacidosis is associated with absolute insulin deficiency. Diets with over 100g of carbohydrates a day are sufficient to prevent ketosis (β-hydroxybutyrate < 0.1 mmol/l), and an intake of approximately 20-40g of glucose per day is associated with a β-hydroxybutyrate level of around 1 mmol/l (13). The average normal-weight adult only reaches a β-hydroxybutyrate level of 2.5-4.5 mmol/l after 5 to 7

days of starvation (12), and levels between 4-7 mmol/l after 2 weeks of fasting (13). There have, however, been two case reports of starvation ketoacidosis after approximately 3 days, with β-hydroxybutyrate levels of 4.5 and 5.21 mmol/l, respectively (1,14). In comparison, obese individuals take longer (10 to 14 days) to reach a β-hydroxybutyrate level of 2.5-4.5 mmol/l (12). Similarly, only mild ketosis has been reported in animal studies with small rodents achieving a β-hydroxybutyrate level of 2-3 mmol/l with starvation, and most rodents having levels < 1 mmol/l (13,15). Starvation ketoacidosis with β-hydroxybutyrate levels of >5mmol/l may be seen in children, possibly related to the increased rate of carbohydrate utilization by extrahepatic tissues and lower glycogen stores compared to adults (16).

As there is also accelerated hepatic ketogenesis in pregnancy and lactation, (pregnancy is a diabetogenic state associated with relative insulin resistance, increased lipolysis, and elevated free fatty acids), a shorter period of starvation may precipitate ketoacidosis (17). For example, a report of a 22-year-old woman at 32 weeks gestation revealed a blood ketone level of 4.0 mmol/l after only 24 hours of starvation (18); the highest reported β-hydroxybutyrate level of 7.1 mmol/l associated with starvation ketoacidosis was in a 32-year-old lactating woman 10 days after commencing a low carbohydrate, high fat diet, with an estimated carbohydrate intake of less than 20g per day (19). The only case report of starvation ketoacidosis in a forensic setting was of a 3-year-old girl who had been starved by her parents for over two days with a vitreous β-hydroxybutyrate level of 3.97 mmol/l (20). Thus, starvation rarely causes ketoacidosis of >5mmol/l in adults, particularly in the absence of pregnancy and/or lactation. Furthermore, as even small amounts of glucose, such as 7.5g, can decrease ketone production (13), it may be incorrect to attribute significant ketoacidosis to starvation in the absence of known absolute or prolonged fasting, or conditions that predispose to accelerated ketogenesis.

In our cases, the β-hydroxybutyrate levels ranged from 6.26 to 13.32 mmol/l (mean = 8.95 mmol/l). None of these cases had a history of diabetes mellitus, alcohol abuse, prolonged fasting, or were pregnant/lactating. Vitreous glucose was not elevated in any case. Three cases had normal BMIs of 21.5 to 22 and only Case 4 having a low BMI of 14. Although the decedent in Case 4 was underweight, a comment was made in the autopsy report that "she appeared thin but in good general condition and well kept". While starvation as a cause of ketoacidosis is an important differential diagnosis to consider, the observed degree of ketoacidosis in these cases is beyond that which can be accounted for by fasting alone. In contrast, sepsis in the absence of fasting, has been reported to show severe ketoacidosis with β-hydroxybutyrate of 9.86 mmol/l (3). Cases 2 and 3 also had evidence of hypothermia in the form of Wischnewsky ulcers (21,22). This could have been contributory to ketogenesis, as ketoacidosis can be aggravated by hypothermia (23), and hypothermia is a known complication of sepsis and severe infections associated with increased mortality (24-26). Therefore, the combination of an absence of documented fasting and the presence of sepsis or a severe burden of infection at autopsy makes septic ketoacidosis a more likely diagnosis in the reported cases.

Postmortem diagnosis of ketoacidosis can be a challenge as there are often little or no significant findings at autopsy. The diagnosis has traditionally relied upon detection of raised β-hydroxybutyrate levels on vitreous biochemistry, although vitreous humor may not always be available for analysis. Histological examination of the kidneys may also help in diagnosing ketoacidosis when characteristic basal vacuolizations in renal tubular epithelial cells are identified. These vacuoles contain lipid, and appear as a single row beneath the nucleus in contact with the basement membrane, with possible luminal displacement of nuclei. These vacuoles have recently been identified as a separate phenomenon from the

Armanni-Ebstein lesion (27), to which they have previously been referred (28). Their presence has been reported in diabetic, alcoholic, and starvation ketoacidosis (20,29,30).

In the current study, we observed characteristic basal vacuolization (Figure 1) in 3 out of 4 cases, which is in keeping with the association between these vacuoles and ketoacidotic states. Importantly, however to the best of our knowledge this is the first report of renal epithelial tubular basal vacuolization occurring in the setting of lethal septic ketoacidosis. It may, therefore, be a useful marker in the autopsy assessment of such cases.

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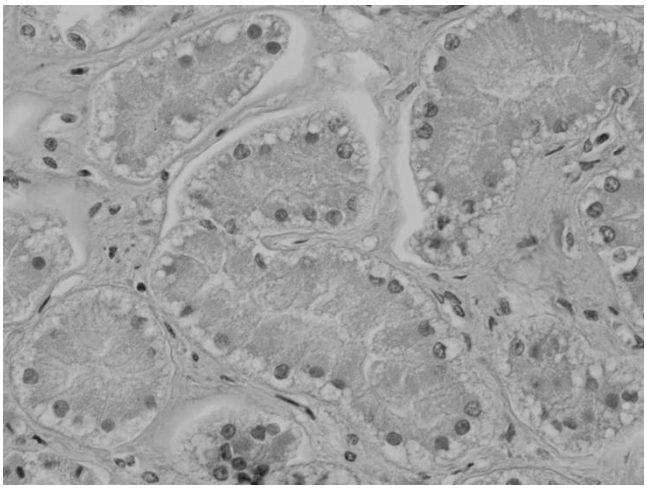

FIG. 1- Characteristic basal vacuolizations of renal tubular epithelial cells in a 72 year old female with septic ketoacidosis (Hematoxylin and eosin x400).

RENAL TUBULAR EPITHELIAL VACUOLES – A MARKER FOR BOTH HYPERLIPIDEMIA AND KETOACIDOSIS AT AUTOPSY

CONTEXTUAL STATEMENT

AIM: To investigate the relationship between basal vacuolizations in renal tubular epithelial cells and hyperlipidaemia.

HYPOTHESIS: That basal vacuolizations in renal tubular epithelial cells are associated with hyperlipidaemia.

COMMENTARY: Basal vacuolization of renal tubular epithelial cells has been reported in diabetic, alcoholic, septic and starvation ketoacidosis, suggesting a possible relationship to elevated ketones. However, the constituents of these vacuoles have been consistently demonstrated to contain lipids by methods including the Oil-Red-O stain and electron microscopy. Therefore, the authors wondered if hyperlipidaemia in the absence of ketoacidosis could produce basal lipid vacuolizations.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSION: Basal lipid vacuolizations in renal tubular epithelial cells are caused by hyperlipidaemic conditions such as nephrotic syndrome, however, the vacuoles appear to be of a different morphology to those observed in ketoacidotic states.

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the Paper	Conceptualization of work, performed literature review, wrote ethics application, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	3/7/14

Name of Co-Author	Assoc. Prof. Lynette Moore		
Contribution to the Paper	Supervised development of work, helped in data collection and interpretation, helped in manuscript evaluation and editing		
Signature		**Date**	2-7-14

Name of Co-Author	Prof. Andrea J. Yool		
Contribution to the Paper	Helped to evaluate and edit the manuscript		
Signature		**Date**	3/7/2014

Name of Co-Author	Alvis Jaunzems		
Contribution to the Paper	Helped in examination of study cases under electron microscopy and taking of original photographs		
Signature		**Date**	3/7/2014

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing		
Signature		**Date**	3/7/14

PUBLISHED MANUSCRIPT

Renal Tubular Epithelial Vacuoles – A Marker for Both Hyperlipidemia and Ketoacidosis at Autopsy

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ABSTRACT: Review of 15 cases of nephrotic syndrome found that eight had significant hyperlipidemia with serum cholesterol levels ranging between 10.59 and 18.60 mmol/L (mean 12.88) and serum triglyceride levels between 2.30 and 9.92 mmol/L (mean 4.58); all of these cases displayed basal lipid vacuolization. Seven of the 15 study cases had normal–mild hyperlipidemia with serum cholesterol levels ranging between 4.71 and 7.54 mmol/L (mean 6.02) and serum triglyceride levels between 0.65 and 4.1 mmol/L (mean 1.57). Six of the seven cases had basal lipid vacuoles (86%). Of these, five cases were hyperlipidemic and one case had borderline hyperlipidemia with a serum cholesterol level of 4.71 mmol/L. Although hyperlipidemia was associated with renal tubular epithelial vacuolization, the vacuoles appeared morphologically different to those found in ketoacidosis. This study has shown that while hyperlipidemia in isolation may result in basal lipid vacuolization within renal tubular epithelial cells, the phenotype differs from that observed in ketoacidosis.

Keywords: forensic science, hyperlipidemia, renal tubular epithelial vacuoles, ketoacidosis, Armanni Ebstein phenomenon, diabetes mellitus, alcoholism

Introduction

A variety of metabolic derangements cause pathological changes in renal tubular epithelial cells. Very recently, Armanni–Ebstein phenomenon and basal vacuolization have been distinguished from each other due to different morphologies and probable distinct etiologies (1). While the pathogenesis of Armanni–Ebstein lesions has been clearly attributed to hyperglycemia (2,3), the exact cause of basal vacuolization remains unclear; basal lipid vacuolization has been reported in ketoacidosis due to diabetes, alcoholism, and starvation (4–7). These vacuoles stain positively with Oil-red O, contain lipids on electron microscopy (8,9), and demonstrate formalin pigment artifact deposition (10). In addition, their pathogenesis appears to be independent of glucose levels (4–7), suggesting that their etiology instead involves elevation of serum lipid levels (8,9). This study reviewed renal biopsies in cases of nephrotic syndrome to test the hypothesis that hyperlipidemia, in the absence of hyperglycemia and ketoacidosis, could result in the basal vacuolization similar to that observed in ketoacidotic states.

Materials and methods

A search of renal biopsy reports was retrospectively conducted over an 8-year period from 2005 to 2012 at the Department of Histopathology at the Women's and Children's Hospital, Adelaide, South Australia, where the clinical summary contained the keyword 'nephrotic'. Patients who did not have lipid studies, 24-h urine collection, serum biochemistry, or an acid-base profile within 1 month from the date of renal biopsy were excluded from the study.

Hospital case notes and blood/urine test results were then reviewed. Inclusion criteria involved a clinical presentation consistent with nephrotic syndrome (i.e., peripheral edema,

weight gain, pleural effusion, ascites, etc.), proteinuria (total urinary protein >0.16 g/L, protein/creatinine ratio >20, and albumin/creatinine ratio >3), hypoalbuminemia (<35 g/L), and hyperlipidemia as defined by the American Academy of Pediatrics (borderline ≥4.40 mmol/L, high ≥5.17 mmol/L) (11). Cases that did not satisfy these criteria were excluded from the study. Ketoacidosis was excluded based on an absence of suggestive clinical signs and symptoms (e.g., history of diabetes, polyuria/polydipsia, alcohol intake, altered mental state, coma, Kussmaul breathing), the absence of urinary glucose and ketones, and normal acid– base profile.

Study cases were then divided into two groups: those with significant hyperlipidemia (serum cholesterol ≥6.5 mmol/L and hypertriglyceridemia ≥2.3 mmol/L) and those with normal–mild elevation in lipid profiles (serum cholesterol <6.5 mmol/L or triglycerides <2.3 mmol/L).

Renal biopsy slides stained with hematoxylin and eosin were then reviewed for vacuolization of tubular epithelial cells and graded “mild-moderate”, where vacuoles were limited to a basal involvement, and “marked” if one or more tubules had epithelial cells completely effaced by vacuoles. Lipid was confirmed within the vacuoles by Oil-Red O staining when frozen sections of renal biopsies were available, or by electron microscopy in the other cases. The severity of lipid vacuolization was then plotted against the level of hyperlipidemia and hypertriglyceridema to determine if a relationship existed.

Approval was obtained from the Women’s and Children’s Hospital Human Research Ethics Committee prior to commencement of this study.

Results

A total of 15 cases of nephrotic syndrome fulfilling the above criteria were found. The age range was 2–15 years (mean 8.3 years) with a male to female ratio of 8:7. The interval between the date of renal biopsy and lipid studies ranged from 0 to 29 days (mean 9.9 days), 24-h urine collection ranged from 0 to 12 days (mean 2.7 days), and serum biochemistry including acid–base profile ranged from 0 to 12 days (mean 3.9 days). Eight of the 15 study cases had significant hyperlipidemia. The age range was 2–14 years (mean 7.1) with a male to female ratio of 1:1. Serum cholesterol levels ranged from 10.59 to 18.6 mmol/L (mean 12.88) and serum triglyceride levels from 2.30 to 9.92 mmol/L (mean 4.58). The patients were all clinically edematous, severely proteinuric with a total urinary protein of 2.60–31.18 g/L (mean 9.16), protein/creatinine ratio of 295–2006 (mean 1191.6), albumin/creatinine ratio of 15.1–1863.7 (mean 986.7), and hypoalbuminemic with a serum albumin range of 13–30 g/L (mean 19.8). Three patients had no treatment prior to biopsy; three patients were treated with prednisolone, one with tacrolimus, and one with tacrolimus and prednisolone. Basal epithelial cell vacuolization was present in all cases including three graded as mild–moderate and five graded as severe. Basal vacuolization ranged from clusters of basally located vacuoles (Fig. 1A) to cases where vacuoles occupied virtually the entire cytoplasm of the affected cells (Fig. 1B). Oil- red O staining was undertaken in five cases, all of which showed positive staining for lipids (Fig. 2). Three cases were reviewed by electron microscopy, and intravacuolar lipid was identified in all of these (Fig. 3).

Seven of the 15 study cases had normal–mild hyperlipidemia. The age range was 5–15 years (mean 9.7) with a male to female ratio of 4:3. Serum cholesterol ranged from 4.71 to 7.54 mmol/L (mean 6.02) and serum triglycerides from 0.65 to 4.1 mmol/L (mean 1.57). One case had an acute presentation with peripheral edema, and two biopsies were

done for incomplete remission, two for relapse after initial remission, and the other two to monitor response to treatment and drug toxicity. Total urinary protein in these cases ranged from 0.06 to 27.93 g/L (mean 7.02), protein/creatinine ratio ranged from 50 to 1961 (mean 715.4), albumin/creatinine ratio ranged from 25.8 to 1693.3 (mean 514.2), and serum albumin ranged from 15 to 36 g/L (mean 24.6). One patient was not on treatment, two were on prednisolone, two were on tacrolimus, one was on prednisolone and tacrolimus, and one was on a combination of prednisolone, tacrolimus, and mycophenolate. Basal epithelial cell vacuolization was absent in one case and present in six cases; two were graded as mild– moderate and four graded as severe. Of these six cases, five cases were hyperlipidemic and one case had borderline hyperlipidemia with a serum cholesterol level of 4.71 mmol/L 10 days after the date of renal biopsy. Oil-red O staining was performed on one case which stained positively for lipids. Electron microscopy demonstrated intravacuolar lipid in all three cases where material was available.

Plotting the degree of hypercholesterolemia and hypertriglyceridemia against the severity of lipid vacuolization revealed no significant relationship.

Discussion

Renal tubular epithelial cells display varying patterns of vacuolization in response to different metabolic stimuli. Although basal vacuolization has been referred to as “Armanni-Ebstein” lesions due to the association with diabetic ketoacidosis, the two entities should be separated as they most likely have different constituents and etiology (1). The pathogenesis of Armanni-Ebstein lesions due to hyperglycemia has been clearly established in animal models (12); however, the pathogenesis of basal vacuolization remains unclear. Typically, basal vacuolization occurs with ketoacidosis in diabetes, alcoholism, and starvation, independent of

blood glucose levels. The vacuoles stain positively with Oil-Red O, and intravacuolar lipids have been demonstrated on electron microscopy (13). Because of this observation, Thomsen et al. (9) have suggested that hyperlipidemia, lipiduria, and subsequent reabsorption in the renal tubules are the causes of these vacuoles. The association between ketoacidosis and hyperlipidemia, which has also been demonstrated in previous studies (14-17), lends support to this hypothesis. In the current study, patients with nephrotic syndrome were chosen for evaluation as hyperlipidemia occurs without a concurrent rise in serum ketones or glucose thus, enabling determination as to whether hyperlipidemia, independent of hyperglycemia and ketoacidosis, could result in basal lipid vacuolization. A limitation of this study was the time interval between lipid studies and the date of renal biopsy, and so a dose-response relationship could not be confidently established.

Hyperlipidemia is a feature of nephrotic syndrome with elevated concentrations of serum cholesterol, triglycerides, and phospholipids confined to lipoproteins that contain apoprotein B (18). One study of 207 adults with nephrotic syndrome due to nondiabetic renal disease showed a mean total cholesterol concentration of 203 mg/dL (7.8 mmol/L) (19), and another study with 100 patients revealed total serum cholesterol concentrations >200 mg/dL (5.2 mmol/L) in 87%, 300 mg/dL (7.8 mmol/L) in 53%, and 400 mg/dL (10.3 mmol/L) in 25% (20). The pathogenesis of hyperlipidemia is directly related to low plasma oncotic pressure secondary to proteinuria and hypoalbuminemia. There is an inverse relationship between serum albumin concentration, plasma oncotic pressure, and serum lipid concentrations (18,21). Furthermore, it has also been demonstrated that an increase in oncotic pressure secondary to albumin or dextran reverses these changes in vitro and decreases lipid levels (22). Decreased oncotic pressure leads to a compensatory increase in hepatic lipoprotein synthesis (23,24) through stimulation of hepatic apoprotein B gene

transcription (22). Clearance of lipoproteins from the circulation may also be reduced in nephrotic syndrome as hypoalbuminemia causes decreased removal of free fatty acids and lysolecthin, which in turn leads to reduced activity of lipoprotein lipase and lectothin acyltransferase, respectively (24).

Although intracellular lipid vacuoles were observed in the patients with nephrotic syndrome, they appeared morphologically different to the typical lipid vacuoles found in ketoacidosis. Aggregated lipid in hyperlipidemia appeared as collections of circular vacuoles within the cytoplasm with well-defined rims that began basally and then spread to involve the entire cell (Fig. 1), whereas those in ketoacidosis tended to be purely basally located (Fig. 4) as a single large vacuole under the nucleus against the basement membrane. The numerous small subnuclear vacuoles in hyperlipidemia were also not necessarily in contact with the basement membrane. On light microscopy, vacuoles in ketoacidosis appeared more opaque than the translucent vacuoles in pure hyperlipidemia.

As basal lipid vacuolization was found in fourteen of the 15 study cases, it can be concluded that hyperlipidemia in the absence of hyperglycemia and ketoacidosis can be related to this type of change in renal tubular epithelial cells. However, as the vacuoles appeared morphologically different to those found in ketoacidosis, future studies will be needed to determine whether hyperlipidemia, independent of increased permeability in nephrotic syndrome, can result in lipid vacuoles that are similar structurally to those associated with ketoacidosis, or whether the synergistic effect of elevated serum ketones and lower pH is required to cause this particular phenotype. Despite these disparities, the differential diagnosis of renal tubular epithelial cell vacuolization detected in postmortem sections should include the possibility of hyperlipidemic states, rather than just ketoacidosis.

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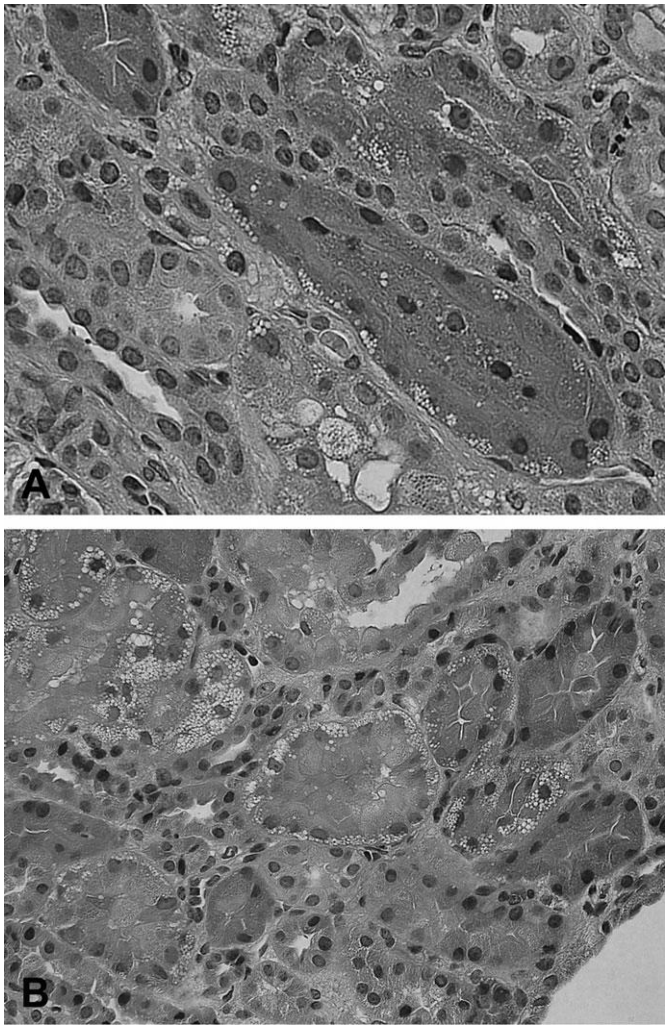

Fig 1-*Vacuolization of renal tubular epithelial cells in a case of nephrotic syndrome with hypercholesterolemia and hypertriglyceridemia demonstrating multiple basally oriented vacuoles (A). Vacuolization in a case of nephrotic syndrome with severe hypercholesterolemia and hypertriglyceridemia, demonstrating the progression of vacuoles from a basal location to involvement of the entire cell (B). (Hematoxylin and Eosin [H&E] x100).*

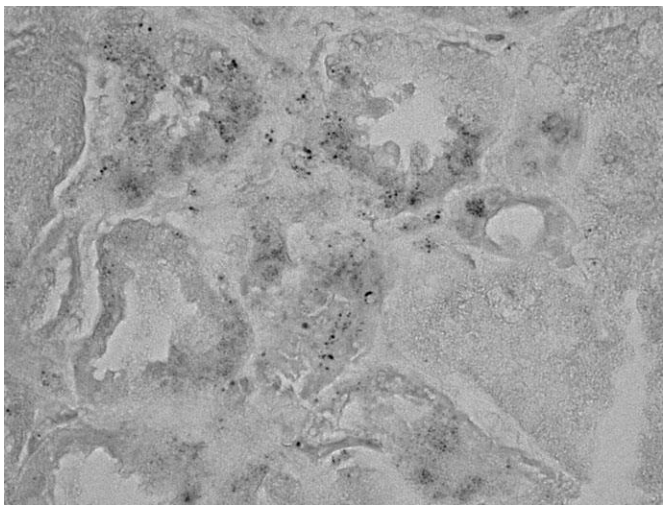

Fig 2-*Positive lipid staining with Oil-Red O in hyperlipidemic basal vacuoles of nephrotic syndrome (black dots) (Oil-Red O x 100).*

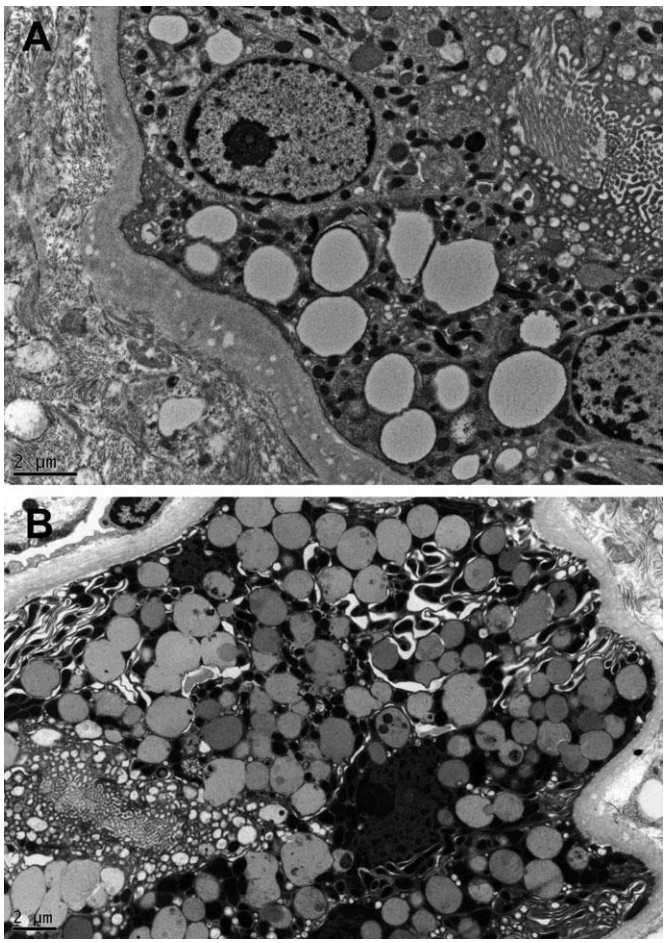

Fig 3-*Electron microscopy of basal vacuoles in nephrotic syndrome demonstrating intravacuolar lipid close to, but separate from the basement membrane (A). Filling of the entire cell with vacuoles (B) (Magnifications x 8000, x 6000).*

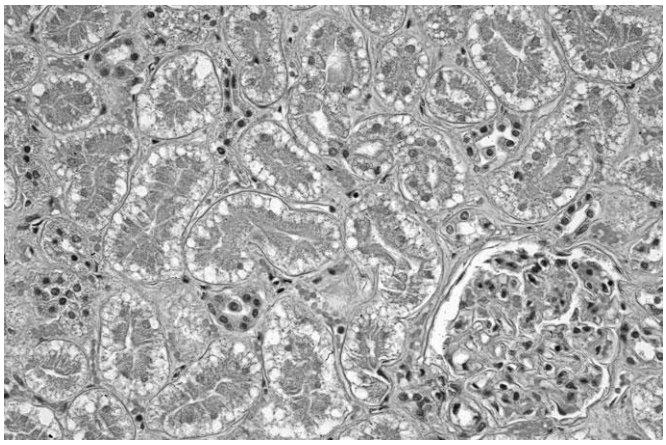

Fig 4-*Typical subnuclear vacuoles in a case of diabetic ketoacidosis for comparison.*

ARMANNI-EBSTEIN PHENOMENON AND HYPOTHERMIA

CONTEXTUAL STATEMENT

AIM: To investigate the relationship between basal vacuolizations in renal tubular epithelial cells and hypothermia.

HYPOTHESIS: That basal vacuolizations in renal tubular epithelial cells are not associated with hypothermia *per se*.

COMMENTARY: Basal vacuolization of renal tubular epithelial cells has been suggested by Preuβ et al as "*a very reliable histologic diagnostic criterium in cases of hypothermia, comparable to the significance of Wischnewsky ulcers*" (1). Therefore, the authors conducted a retrospective analysis of hypothermic deaths in an attempt to elucidate any possible underlying metabolic conditions which may be contributed to the development of basal lipid vacuoles in this context.

Literature review establishing the field of knowledge is discussed within the paper.

This manuscript preceded the manuscript titled "Armanni-Ebstein lesions: a need for clarification" in chapter 1, therefore, the terminology of "Armanni-Ebstein" lesions/phenomenon was still used to refer to these basal vacuolizations. The manuscript was written in American English to satisfy the publication requirements of Forensic Science International.

CONCLUSION: Basal vacuolizations in renal tubular epithelial cells can be seen in deaths due to hypothermia, but the aetiology in some cases may be a manifestation of underlying metabolic derangements predisposing to the development of hypothermia, rather than to the hypothermia on its own.

1. Preuβ J, Dettmeyer R, Lignitz E, Madea B. Fatty degeneration in renal tubule epithelium in accidental hypothermia victims. Forensic Sci Int 2004;141:131-5.

STATEMENT OF AUTHORSHIP

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Conceptualization of work, performed literature review, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	3/7/14

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
Signature		**Date**	3/7/14

PUBLISHED MANUSCRIPT

Armanni-Ebstein Phenomenon and Hypothermia

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ABSTRACT Retrospective review was undertaken of 46 cases of lethal hypothermia for the presence of subnuclear vacuolization of renal tubular epithelial cells. Fifteen of the 46 cases (33%) had renal tubular vacuolization typical of the Armanni-Ebstein phenomenon. The age range was 30-87 years (average 59 years) with a male to female ratio of 6:9. Nine of the 15 cases with Armanni-Ebstein changes (60%) had a history of diabetes mellitus, and in seven of these, vitreous humour biochemical analyses were performed, all of which revealed diabetic ketoacidosis (vitreous glucose levels = 32.9-85.3 mmol/L; β-hydroxybutyrate = 7.4-20 mmol/L). This study has confirmed the association between hypothermia and renal tubular epithelial vacuolization, but in addition raises the prospect that this may be contributed to in some cases by underlying diabetic ketoacidosis. Hypothermic deaths should, therefore, raise the possibility of diabetes mellitus and initiate postmortem biochemical measurement of vitreous humor glucose and β-hydroxybutyrate levels.

Keywords: Armanni-Ebstein phenomenon, renal tubular vacuolization, hypothermia, diabetes mellitus, ketoacidosis

Introduction

Armanni-Ebstein is the term used to describe subnuclear vacuolization of renal tubular epithelial cells that is most often seen in poorly controlled diabetic states. Debate has occurred as to whether the accumulated material consists of glycogen or lipid, with recent data supporting the latter [1-3]. In addition to diabetes mellitus, renal tubular vacuolization has also been reported in cases of lethal hypothermia. The following study was undertaken to further investigate this association.

Materials and methods

Case files from Forensic Science SA, Adelaide, Australia were retrospectively reviewed over a six-year period from April 2004 to March 2010 for cases where deaths were due to, or contributed to, by hypothermia. All cases had undergone full police and coronial investigations with complete autopsy examinations being performed. Hypothermia had been diagnosed when there was a recorded antemortem core temperature less than 30°C, or if erosive gastritis with so-called Wischnewsky spots was found at autopsy in an individual who had been alone for some time in a cool environment.

Autopsy reports were reviewed and the age, gender and pathological findings were summarized. Specific details of diabetic status, blood alcohol concentration and the results of vitreous biochemistry if performed, were recorded. Histological sections of the kidneys were then examined for the presence of Armanni-Ebstein lesions. Cases where putrefaction and autolysis precluded accurate histological assessment were excluded from the series.

Results

A total of 62 cases with terminal hypothermia were identified, 16 of which were excluded due to poor renal preservation. Of the remaining 46 cases, Wischnewsky spots were present in 44 (96%) and antemortem core temperatures were taken in 7 cases (ranging from 22.5 to 29.7°C). Blood alcohol levels were measured in 31 cases ranging from 0 to 0.19%, with a mean of 0.02%. Fifteen of the 46 cases (33%) had renal tubular vacuolization typical of the Armanni-Ebstein phenomenon (Fig. 1). PAS/PAS-D staining did not reveal glycogen, although oil-red O staining and electron microscopy (Fig. 1 inset and Fig. 2) demonstrated lipid droplets. The age range was 30-87 years (average 59 years) with a male to female ratio of 6:9. Blood alcohol levels were measured in five of these cases and were negative. Nine of the 15 cases with Armanni-Ebstein changes (60%) had a documented medical history of diabetes mellitus; in the remaining six cases the clinical history was unknown and vitreous humour biochemical analyses had not been performed. Of the nine cases with a known history of diabetes mellitus, five had nodular glomerulosclerosis and seven had vitreous humour biochemical analyses performed, all of which revealed diabetic ketoacidosis (vitreous glucose levels = 32.9-85.3 mmol/L; β-hydroxybutyrate = 7.4-20 mmol/L); this was not tested for in the remaining two cases (Fig. 3). The postmortem interval when vitreous humor was withdrawn ranged from 2 to 6 days, mean 5.2 days.

A total of 31 of the 46 cases (67%) did not have Armanni-Ebstein lesions. The ages ranged from 38 to 89 years (average 69.23 years) with a male to female ratio of 13:18. A history of diabetes mellitus had been documented in four cases (13%) and was unknown in the remaining 27 cases. Diabetic ketoacidosis had not been tested for at autopsy.

Only one of the victims who had antemortem core temperatures taken (indicating survival for some time) had Armanni-Ebstein changes, and this was a woman who had died of diabetic ketoacidosis with significantly elevated glucose (47.6 mmol/L) and β-

hydroxybutyrate (8.87 mmol/L) levels, and no detectable blood alcohol (Brief details of this case have been previously reported [4]). Deaths in the remaining six cases were due to: ischemic heart disease and dementia, ischemic heart disease, subdural hemorrhage, epilepsy and cardiomegaly, and bronchopneumonia (N = 2).

Discussion

Hypothermia results from the body core temperature falling to less than 35°C and occurs when counter-regulatory mechanisms such as vasoconstriction and heat production are exceeded by heat loss to the environment [5,6]. Hypothermia is a significant event and has been associated with greater than 70% mortality when the core temperature drops to 30°C, and 90% at 26°C. Lethal mechanisms include ventricular fibrillation or asystole, contributed to by myocardial ischemia, hypoxia, electrolyte abnormalities, and elevated catecholamine levels [5].

The most common cause of severe hypothermia is accidental exposure to low ambient temperatures associated with a number of exacerbating factors such as damp conditions, air movement, inadequate or wet clothing, low muscle mass, alcohol ingestion and alcoholism, certain medications and drugs, trauma, open injuries, immobility, neurological, endocrine and cardiovascular disorders, and psychiatric illness [7]. Children and the elderly are at highest risk [5].

Erosive gastritis or Wischnewsky spots are present in 40-90% of fatal hypothermia cases, but this may be artificially high if this finding has been used as a diagnostic parameter, as in the current series. The lesions consist of superficial gastric erosions characterized histologically by necrosis of the mucosa with acid hematin formation. Acute pancreatitis with hemorrhage and surrounding fat necrosis may also rarely be present [5,6].

Fatty changes on routine histology have been associated with fatal hypothermia, with vacuolization of hepatocytes and cardiac myocytes occasionally being documented [6]. In terms of renal tubules, Preuβ *et al* demonstrated basal vacuolization of tubular epithelial cells in 87% of cases of terminal hypothermia leading to the conclusion that these changes represented "a very reliable histologic diagnostic criterium in cases of hypothermia, comparable to the significance of Wischnewsky ulcers" [8]. The vacuoles stain positively for lipids and are morphologically similar to Armanni-Ebstein lesions that are strongly associated with poorly controlled diabetic states and deaths due to diabetic ketoacidosis [9,10]. While the vacuoles in Armanni-Ebstein lesions complicating diabetes were initially thought to contain to glycogen [9-13], recent studies have demonstrated positive staining for lipids, suggesting that the vacuoles consist of accumulated triglycerides [1-3]. Occasionally the vacuolization may be so profound that it can be observed macroscopically as cortical pallor [4]. In our series, a case of hypothermia and diabetic ketoacidosis demonstrated lipid on both histological staining and on electron microscopy (Figs 1 and 2).

. Although it has been asserted that "fatty degeneration of renal tubules cannot be regarded as a sign of diabetes" [8], it is unclear from this study what the levels of glucose and β-hydroxybutyrate were. Certainly while many individuals with a history of diabetes mellitus do not manifest Armanni-Ebstein phenomenon, due to lack of terminal ketoacidosis, others do [1-3,9-13,14]. Thus, as the renal tubular vacuoles in hypothermia and in diabetic ketoacidosis appear similar in morphology and composition, the current study was undertaken to ascertain whether there might be a relationship between cases of hypothermia with Armanni-Ebstein phenomenon, and diabetes mellitus, as has been suggested in a case report that was not included in the present series [15].

The diagnosis of Armanni-Ebstein phenomenon at autopsy may be difficult if there has been significant putrefaction and/or autolysis, as this may cause nonspecific cytoplasmic vacuolization and artefactual separation of proximal tubular epithelial cells from the basement membrane, features that resemble Armanni-Ebstein lesions. This may be predisposed to in hypothermic deaths when there has been social isolation and delay in finding the victim, as this may enhance putrefactive changes [16].

The present study has shown that more than half of the cases of terminal hypothermia where Armanni-Ebstein change was observed (60%) had an established history of diabetes mellitus. This may well be a significant underestimation, as a history of diabetes mellitus may not have been volunteered in the remaining cases, and so vitreous biochemical testing was not routinely undertaken. Significantly, of the nine known diabetic victims with Armanni-Ebstein lesions, all who were tested (N = 7) had biochemical evidence of diabetic ketoacidosis. This suggests that renal tubular epithelial vacuolization in cases of hypothermia may have a significant association with diabetic ketoacidosis.

In turning to the literature, there is certainly a recognized association between diabetes mellitus and hypothermia, with diabetic individuals being more susceptible to dropping their core temperature [7] resulting in hospital admissions for hypothermia being more common among diabetic patients compared to the general population [17,18]. This may be influenced by the greater frequency of pathological conditions that are associated with an increased risk of hypothermia among the diabetic population, such as other endocrinopathies [18]. Autonomic neuropathy, which is a common complication of diabetes mellitus, also places individuals at increased risk of developing hypothermia due to impaired physiologic thermoregulatory mechanisms, including peripheral vasoconstriction [17,19,20].

Metabolic complications of diabetes mellitus can cause secondary hypothermia. For example, hypoglycaemia may result in increased heat loss from sweating, inhibition of shivering, and peripheral vasodilation [17-19]. Hypothermia may also be a cause and a complication of diabetic ketoacidosis [21] with Gale and Tattersall reporting diabetic ketoacidosis as the basis for 11.8% of all hospital admissions for severe hypothermia; a rate higher than that for hypothyroidism (8%) [22]. Acidosis can interfere with thermoregulation and cause peripheral vasodilation with a reduced ability to maintain core body temperature when exposed to cold ambient temperatures [23,24]. Insulin deficiency in diabetic ketoacidosis also decreases the uptake of glucose into muscles and adipose tissue, leading to decreased substrate availability for heat production and impaired chemical thermogenesis [7, 21,23].

A reverse relationship between hypothermia and diabetes mellitus also exists as primary hypothermia can worsen a decompensated diabetic state. When the core temperature decreases below 32°C, insulin activity and release are both markedly reduced, resistance to exogenous insulin develops, and peripheral utilization of glucose declines. Additionally, there is also increased secretion of catecholamines and cortisol during hypothermic states, which exacerbate diabetic ketoacidosis [7,22,24]. Consequently, concurrent hypothermia and diabetic ketoacidosis may initiate a vicious metabolic cycle associated with a high mortality rate exceeding 30% [25].

Although in the current study, Armanni-Ebstein lesions were found in only approximately a third of cases with terminal hypothermia, considerably less than other studies [8], the association between renal tubular epithelial vacuolization and hypothermia was confirmed. While the numbers in the study are relatively small and the review was retrospective, a history of diabetes mellitus was recorded in autopsy files in over a half of

those with Armanni-Ebstein changes, and all of those from this group who were tested exhibited biochemical evidence of diabetic ketoacidosis. Thus, we concur with other authors that Armanni-Ebstein lesions are a feature of hypothermic deaths [5,6,8], but would also suggest that in some cases this may occur due to an association with diabetic ketoacidosis, rather than as a result of a significant reduction in body temperature *per se*. Thus when hypothermia is suspected at autopsy, specific enquiry should be made regarding a possible history of diabetes mellitus and vitreous humour should be tested for elevated glucose, β-hydroxybutyrate and lactate to determine whether there is biochemical evidence of underlying diabetic ketoacidosis.

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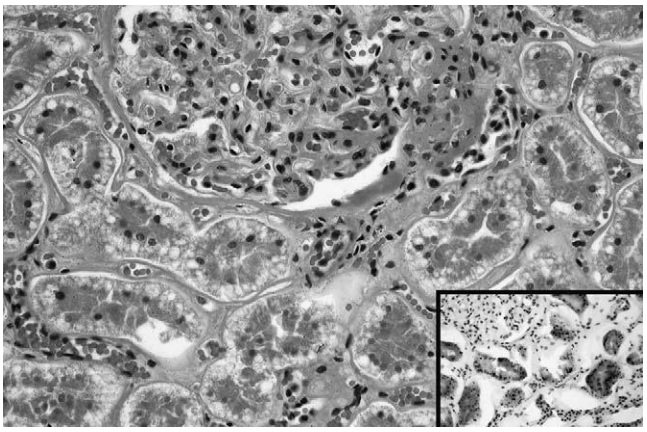

FIG. 1-*Characteristic subnuclear vacuolization of renal tubular epithelial cells in a case of hypothermia and ketoacidosis (Hematoxylin & eosin x 100). Inset shows patchy oil-red O staining of lipid droplets within tubular epithelial cells (x 60)*

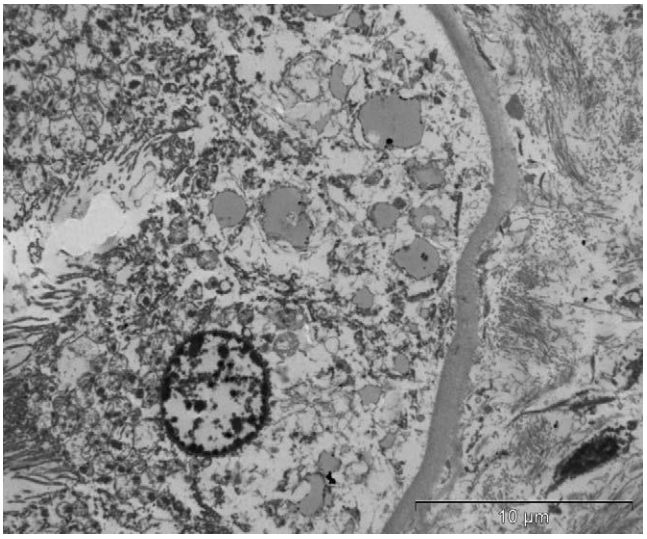

FIG. 2-*Electron microscopy of the case shown in Figure 1 demonstrating intraepithelial lipid accumulation.*

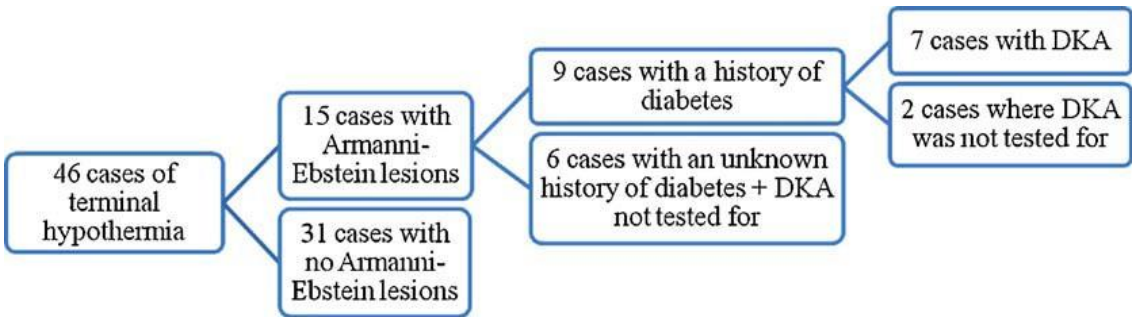

FIG. 3-*An analysis of 46 cases with hypothermia showing the number of cases with Armanni-Ebstein phenomenon and the relationship to diabetes mellitus and ketoacidosis (DKA) on vitreous humor testing.*

RENAL CORTICAL PALLOR – A USEFUL MACROSCOPIC MARKER FOR METABOLIC DERANGEMENTS AT AUTOPSY

CONTEXTUAL STATEMENT

AIM: To investigate if macroscopic renal cortical pallor can be a useful sign for underlying metabolic derangements at autopsy.

HYPOTHESIS: That macroscopic renal cortical pallor at autopsy may be an early indication of basal vacuolization of renal tubular epithelial cells.

COMMENTARY: The authors have previously reported a case of diabetic ketoacidosis where marked pallor of the renal cortices was associated with underlying prominent basal vacuolization of the proximal convoluted tubules (1). Following on from this, a retrospective review of cases demonstrating macroscopic renal cortical pallor was conducted to further define the usefulness of this sign at autopsy.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSION: Macroscopic renal cortical pallor is a useful marker for significant metabolic derangements and the underlying spectrum of histologic changes includes basal vacuolization, Armanni-Ebstein lesions, and osmotic nephrosis. Therefore, it’s presence at

autopsy should prompt measurement of vitreous humour glucose and β-hydroxybutyrate levels.

1. Zhou C, Gilbert JD, Byard RW. Early diagnosis of Armanni-Ebstein phenomenon at autopsy. Forensic Sci Med Pathol 2010;6:133-4.

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Performed literature review, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	3/7/14

Name of Co-Author	Prof. Andrea Yool		
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Signature		**Date**	3/7/2014

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Conceptualization of work, supervised development of work, helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
Signature		**Date**	3/7/14

PUBLISHED MANUSCRIPT

Renal Cortical Pallor – a Useful Macroscopic Marker for Metabolic Derangements at Autopsy

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ABSTRACT Renal cortical pallor was studied as a potential marker at autopsy of diabetic ketoacidosis in 23 cases, hyperglycemic non-ketotic coma in eight cases, and alcoholic ketoacidosis in five cases (vitreous humor glucose $\geq$11.1 mM; β-hydroxybutyrate $\geq$5 mM). Renal cortical pallor was noted on macroscopic examination in 10 of 23 cases of lethal diabetic ketoacidosis (43.5%), three of eight cases of fatal hyperglycemic non-ketotic coma (37.5%) and in two of five cases of alcoholic ketoacidosis (40%). Histological examination revealed basal vacuolization of renal tubular epithelial cells in 12 cases, Armanni-Ebstein lesions in 10, and osmotic nephrosis in three. Although renal cortical pallor did not appear to be a particularly sensitive marker for hyperglycemia or ketoacidosis, and did not correlate with the severity of these parameters, it may still represent a useful macroscopic marker for underlying metabolic conditions at autopsy and should therefore prompt measurement of vitreous humor glucose and β-hydroxybutyrate levels.

Keywords: Forensic science, renal cortical pallor, ketoacidosis, diabetes mellitus, alcoholism, hyperglycemia, epithelial vacuolization, Armanni-Ebstein

Introduction

In previous studies we have investigated the histologic manifestations of ketoacidosis and hyperglycemia in the tubular epithelium of the kidney, where it appears that at least two distinct changes may occur involving basal vacuolization and Armanni-Ebstein change (1,2). Although there has been some confusion in the literature regarding terminology, swelling of cells with loss of normal shape, clearing of cytoplasm, and accumulation of cytoplasmic glycogen represent the classical changes first described by Armanni and Ebstein due to glycogen nephrosis associated with hyperglycemia and glucosuria (3). In addition, basal vacuolization due to lipid accumulation with luminal displacement of nuclei may be present, and linked to ketoacidosis (3). Osmotic nephrosis is another change that may occur in tubular epithelial cells exposed to high solute concentrations with diffuse intracytoplasmic vacuolization. One of the associated changes that we observed macroscopically in some of these cases was marked renal cortical pallor (4,5). The following study was undertaken to determine how useful this feature is at the time of autopsy dissection as a marker of underlying metabolic disturbance, and whether it is of value in the assessment of lethal diabetic ketoacidosis, hyperglycemic non-ketotic coma, and alcoholic ketoacidosis.

Materials and Methods

Case files over a 6-year period from 2004 to 2009 at Forensic Science SA, Adelaide, South Australia, were retrospectively reviewed for all cases in which the cause of death was listed as diabetic ketoacidosis, hyperglycemic non-ketotic coma, or alcoholic ketoacidosis. All cases had full coronial and police investigations with complete forensic autopsies. Significant diabetic ketoacidosis was recorded when the vitreous humor glucose was $\geq$11.1 mM, and β-hydroxybutyrate was $\geq$5 mM. Hyperglycemic coma was recorded when there was

vitreous hyperglycemia (≥11.1 mM) with no elevation of β-hydroxybutyrate levels. Significant alcoholic ketoacidosis was diagnosed when the vitreous humor β-hydroxybutyrate was ≥5 mM with normo- or hypoglycemia (vitreous glucose ≤11 mM) in conjunction with a history of alcohol abuse and/or autopsy findings suggestive of chronic alcoholism. Cases that did not meet these criteria, those with an incomplete vitreous humor biochemical screen, and those in which kidneys were autolysed to a level that prevented accurate histological assessment were excluded from the series.

The autopsy reports of the study cases were then reviewed with special attention to the documentation of renal cortical pallor. When pallor was present, histological slides of the kidneys were examined. Statistical analysis was conducted using unpaired Student's *t*-test, and statistical significance was defined as $p<0.05$.

Results

A total of 36 study cases were identified with 23 deaths due to diabetic ketoacidosis, eight due to hyperglycemic non-ketotic coma, and five due to alcoholic ketoacidosis.

The 23 cases of lethal diabetic ketoacidosis consisted of 13 men and 10 women (age range 21-80 years; mean 47.6 years). Ten had renal cortical pallor documented at autopsy (43.5%; age range 21-80 years; mean 43.4 years; M:F = 1:1; Fig. 1). In this group, vitreous humor glucose ranged from 32.9 to 71.8 mM (mean 47.33 mM) and β-hydroxybutyrate ranged from 8.85 to 20.40 mM (mean 13.15 mM). The remaining 13 cases did not have pallor of the kidneys documented. These consisted of eight men and five women, whose age ranged from 25 to 80 years (mean 50.8 years). Their vitreous glucose levels ranged from 22.2 to 91.9 mM (mean 54.73 mM) and β-hydroxybutyrate levels ranged from 6.3 to 20.0 mM (mean 12.06 mM). There was no statistically significant difference in the glucose ($p = 0.35$)

and β-hydroxybutyrate levels ($p = 0.53$) between the cases with renal cortical pallor and those without. Seven cases with renal cortical pallor showed superficial gastric erosions, or Wischnewsky spots, indicating that hypothermia played a role in the terminal episodes.

The eight cases of fatal hyperglycemic non-ketotic coma were aged between 14 and 60 years (mean 43.1 years; M:F = 5:3). Three of these cases had pallor of the renal cortices (37.5%; age range 37-60 years; mean 50.3 years; all were men). The vitreous glucose levels ranged from 19.7 to 35.8 mM (mean 28.3 mM), with normal levels β-hydroxybutyrate (range 0.2-2.55 mM; mean 1.25). The remaining five cases that did not have noticeably pale renal cortices were aged from 14 to 52 years (mean 38.8 years; M:F = 2:3) with vitreous glucose levels ranging from 17.0 to 49.7 mM (mean 28.88 mM), and β-hydroxybutyrate levels ranging from 0.02 to 1.65 mM (mean 0.7 mM). There was no statistically significant difference in the glucose ($p = 0.95$) and β-hydroxybutyrate levels ($p = 0.41$) between the cases with and without renal cortical pallor.

All five cases where death was due to alcoholic ketoacidosis had a history of chronic alcohol abuse, two with hepatomegaly, three with steatosis and periportal fibrosis, and one each with macrovesicular steatosis and splenomegaly. The age ranged from 51 to 72 years (mean 61 years), and all were men. Two cases had renal cortical pallor (40%), both were men aged 63 and 51 years, respectively. Vitreous humor glucose levels were 2.6 and 4.2 mM, and β-hydroxybutyrate levels were 7.40 and 6.42 mM, respectively. The remaining three cases without pallor of the kidneys were aged from 51 to 72 years (mean 63.7 years). There was no elevation in glucose levels (range 0.1-0.3 mM; mean 0.17), with elevated β-hydroxybutyrate levels ranging from 7.55 to 8.75 mM (mean 8.17). Given the low numbers of these cases, statistical evaluations were not performed. One case with renal cortical pallor had superficial gastric erosions (Wischnewsky spots) in keeping with hypothermia.

Histological examination of slides from the cases with diabetic ketoacidosis and cortical pallor revealed basal vacuolization of tubular epithelial cells in all 10 cases (Fig. 2), with Armanni-Ebstein changes in seven (Fig. 3). In the three cases of hyperglycemic non-ketotic coma and cortical pallor, there were two cases with Armanni-Ebstein change and no cases with basal vacuolization. Both cases of alcoholic ketoacidosis had basal vacuolization with one case having Armanni-Ebstein changes. In this case, there was demonstrable pancreatic fibrosis with a 4-day post mortem interval. Three cases had diffuse vacuolization and swelling typical of osmotic nephrosis.

Discussion

Pallor of the kidneys involving both the cortex and medulla may be observed in situations of acute blood loss. Differential pallor of the cortex compared to the medulla is, however, suggestive of a more specific and localized effect, which may be a marker for local cell necrosis. For example, pallor of the renal cortices is a feature of renal cortical necrosis, in which coagulative necrosis results in the development of patchy or diffuse sharply demarcated zones of pallor with hyperemic rims (6,7). It may also be seen in acute tubular necrosis (8,9) and has been reported in cases of severe asphyxia (10) and septicemia (11,12). Cortical pallor may also be a feature of acute renal failure, possibly due to the redistribution of blood flow away from cortical glomeruli (13). Renal cortical pallor may also be observed in cases where death involves diabetic ketoacidosis and hypothermia, due to underlying metabolic derangements that may merely alter the composition and structure of renal tubular epithelial cells, rather than inducing necrosis (4,5).

Three different metabolic disorders commonly seen in the forensic context were investigated in this study, including diabetic ketoacidosis, hyperglycemic nonketotic coma,

and alcoholic ketoacidosis. Diabetic ketoacidosis arises when counterregulatory hormone excess in conjunction with insulin deficiency leads to increased gluconeogenesis, increased glycogenolysis, and decreased glyconeogenesis. Ketogenesis also occurs as oxaloacetate is directed to gluconeogenesis, resulting in a relative lack of oxaloacetate for use in the Krebs cycle, and a corresponding rise in acetyl-CoA, which would otherwise have been consumed. Consequently, acetyl-CoA is used for the production of ketones (14,15), and elevated levels of both glucose and β-hydroxybutyrate may be detected in the vitreous humor at autopsy.

In contrast, ketoacidosis does not occur in most type 2 diabetics with metabolic decompensation; instead, they progress to a hyperglycemic hyperosmolar nonketotic state. This is most likely due to the presence of residual pancreatic function with secretion of small amounts of insulin that depress the release of counter-regulatory hormones and the mobilization of free fatty acids, thus inhibiting ketogenesis (16). The vitreous biochemistry profile in these cases is, therefore, one of elevated glucose levels in the absence of elevated β-hydroxybutyrate.

Alcoholic ketoacidosis tends to occur in malnourished, chronic alcoholics who have low protein and carbohydrate stores, and/or alcoholic liver disease with subsequent hepatic glycogen depletion (17,18). This, in addition to volume depletion caused by vomiting, decreased fluid intake, and/or diaphoresis, leads to a fall in blood glucose levels, the suppression of insulin, and a sympathetic response causing increases in catecholamines, cortisol, growth hormone, and glucagon levels (17,19). These hormones, in conjunction with the direct effect of ethanol, promote the mobilization of free fatty acids to the liver (17). When the supply exceeds the rate of oxidation, a surplus of acetyl-CoA results, which is converted into acetoacetate (20). Additionally, alcohol dehydrogenase preferentially uses NAD^+ to convert ethanol to acetaldehyde, leading to less NAD^+ being available to convert

lactate to pyruvate, with subsequent decreased production of oxaloacetate required for gluconeogenesis; thus gluconeogenesis is inhibited. Alcoholic ketoacidosis can be identified on post mortem vitreous biochemistry by an elevated β-hydroxybutyrate levels in the setting of hypo- or normoglycemia.

Macroscopic pallor of the renal cortices was found in all three of these metabolic disorders, specifically in 43.5% of those with diabetic ketoacidosis, in 37.5% of those with hyperglycemic nonketotic coma, and in 40% of those with alcoholic ketoacidosis (Table 1). In the cases of diabetic ketoacidosis and those dying from hyperglycemic nonketotic coma, no statistically significant difference could be demonstrated between the cases that had renal cortical pallor, and those that did not, in the glucose ($p = 0.35$ and 0.95, respectively) and β-hydroxybutyrate levels ($p = 0.53$ and 0.41, respectively). Thus, the presence of macroscopic pallor did not appear to correlate with the severity of the underlying metabolic disturbance.

Histologic investigation was also carried out in those cases with pale renal cortices to identify corresponding morphological abnormalities. Subnuclear vacuolization of tubular epithelial cells, as described by Thomsen et al. (20,21), was found in all of the cases of diabetic and alcoholic ketoacidosis ($N = 12$), in keeping with the effects of ketoacidosis. Characteristic Armanni-Ebstein changes were seen in the tubules of 70% (7/10) of those who had died of diabetic ketoacidosis, and in 67% (2/3) of those who had died from hyperglycemic nonketotic coma whose kidneys displayed renal cortical pallor. This would be in keeping with the effects of hyperglycemia. It is apparent, therefore, that renal cortical pallor may be caused by different subcellular morphological abnormalities.

An unusual finding was that of Armanni-Ebstein change in renal tubular epithelial cells in a case of alcoholic ketocacidosis without an elevated glucose level. However, in this case, it is possible that the glucose level was artifactually reduced following death as there

was a post mortem interval of 4 days. The presence of pancreatic fibrosis could be further evidence to suggest that there may have been an underlying disturbance in pancreatic function.

A potential problem with this study is its retrospective nature, in that not all cases of renal cortical pallor may have been documented. However, alternatively this also means that at the time of assessment of renal color there was no skewing by potential observer bias.

An interesting observation made during the study was that concurrent hypothermia was present in seven out of the 10 individuals who died from diabetic ketoacidosis with renal cortical pallor. It was also present in the single case of alcoholic ketoacidosis with pallor. Although it has been proposed that one of the substrates of cortical pallor, renal epithelial subnuclear vacuolization, may be caused by hypothermia in isolation (22), we consider that it is more likely to involve a more complex interaction between both diabetic ketoacidosis and reduced body core temperature (23). The concurrence of the two in the present study may suggest that hypothermia may add to the underlying metabolic disturbance, and therefore could contribute to the development of macroscopic pallor.

In conclusion, this study has shown that renal cortical pallor in cases of suspected ketoacidosis or hyperglycemia represents a marker for significant metabolic derangement in only 37.5-43.5% of cases. Once observed at autopsy, however, full biochemical screening may provide further useful information. The histologic basis for pallor was underlying glycogen nephrosis (Armanni-Ebstein phenomenon), subnuclear lipid vacuolization, and/or osmotic nephrosis. The presence of renal cortical pallor did not appear to be a reflection of the severity of the underlying metabolic derangement and may have been contributed to by hypothermia. The sensitivity and specificity of renal cortical pallor in the general autopsy population as a marker for metabolic disturbance remains to be determined.

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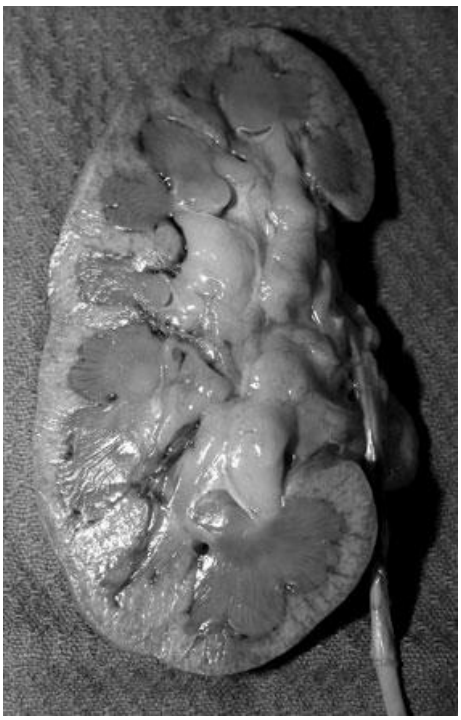

FIG 1-*Typical pallor of the renal cortex relative to the medulla that may be observed in cases of diabetic ketoacidosis, hyperglycemic nonketotic coma and alcoholic ketoacidosis.*

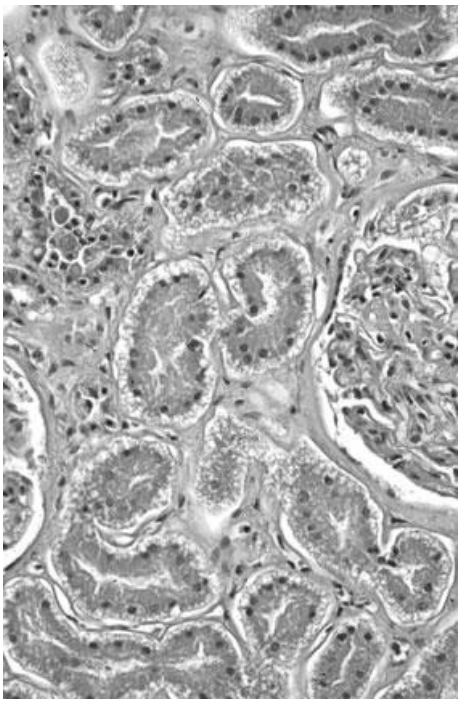

FIG 2- *Diffuse basal subnuclear vacuolization of renal tubular epithelial cells in a case of ketoacidosis with macroscopic renal cortical pallor (hematoxylin and eosin x 150).*

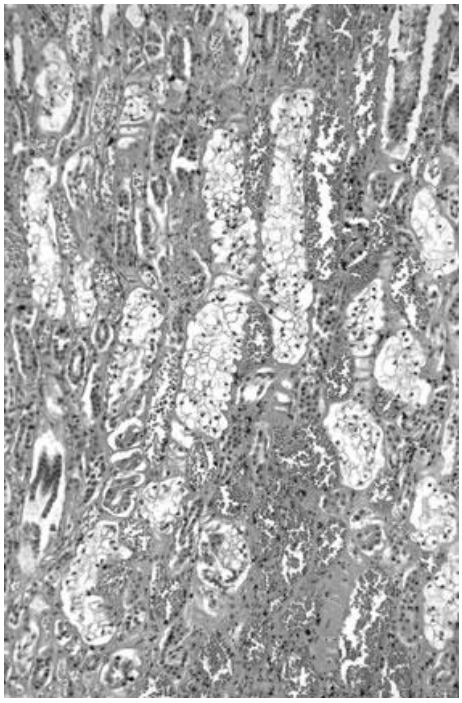

FIG 3- Armanni-Ebstein lesions of renal tubules with ballooning of epithelial cells in a case of diabetic ketoacidosis with macroscopic renal cortical pallor (hematoxylin and eosin x 100).

TABLE 1—*Numbers and percentages of cases of diabetic ketoacidosis, hyperglycemic nonketotic coma, and alcoholic ketoacidosis with macroscopically noted renal cortical pallor at autopsy.*

	Renal Cortical Pallor		
	+	–	*N*
Diabetic ketoacidosis	10 (43.5%)	13	23
Hyperglycemic nonketotic coma	3 (37.5%)	5	8
Alcoholic ketoacidosis	2 (40%)	3	5

BASAL EPITHELIAL FORMAIN PIGMENT DEPOSITION IN THE KIDNEYS – A USEFUL MARKER FOR KETOACIDOSIS AT AUTOPSY

CONTEXTUAL STATEMENT

AIM: To investigate if characteristic formalin pigment deposition can be used as a surrogate marker for basal vacuolizations.

HYPOTHESIS: That formalin pigment deposition in a characteristic basal distribution may be used as a surrogate marker for basal vacuolization.

COMMENTARY: Formalin pigment deposition is an artefact seen on histologic sections which is not known to have any diagnostic utility. However, occasionally they have been noted to deposit in a characteristic basal distribution in the area corresponding to basal vacuolizations. Therefore, a retrospective review of ketoacidotic deaths was conducted to further define the usefulness of this sign.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic and Legal Medicine.

CONCLUSION: Formalin pigment deposition in a characteristic basal distribution is a useful surrogate marker for basal vacuolization, especially in cases where significant putrefaction or autolysis precludes accurate morphologic assessment of vacuoles.

STATEMENT OF AUTHORSHIP

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Performed literature review, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	3/7/14

Name of Co-Author	Dr. John D. Gilbert		
Contribution to the Paper	Conceptualization of work, helped in manuscript evaluation and editing		
Signature		**Date**	30.6.2014

Name of Co-Author	Andrea Yool		
Contribution to the Paper	Helped to evaluate and edit the manuscript		
Signature		**Date**	3/7/2014

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
Signature		**Date**	3/7/14

PUBLISHED MANUSCRIPT

Basal Epithelial Formalin Pigment Deposition in the Kidneys– A Useful Marker for Ketoacidosis at Autopsy

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ABSTRACT Basal vacuolization of renal epithelial cells occurs in diabetic and alcoholic ketoacidosis, hypothermia and starvation. The vacuoles contain triglycerides. Following a case where formalin pigment deposition within these vacuoles led to the identification of ketoacidosis, a retrospective review of a further 31 cases with ketoacidosis, was undertaken. There were 24 diabetics and 7 alcoholics (age range 21-80 yrs; mean 50.9yrs; M:F ratio = 2:1. The post-mortem interval was 1-12 days (mean – 4.5 days). Characteristic basally-located pigment surrounding vacuoles was found in 16 cases (51.6%) (14 diabetic ketoacidosis; 2 alcoholic ketoacidosis). Fifteen cases had no formalin pigment deposition. No relationship could be found between the intensity of staining and the postmortem interval, degree of putrefaction, or level of vitreous humor β-hydroxybutyrate. No staining was demonstrated in control cases matched for postmortem interval. Although formalin pigment deposition occurred in only 51.6% of cases with proven ketoacidosis at autopsy, it appeared to be a highly specific phenomenon. As these deposits were identifiable after recognizable cellular morphology had been lost due to autolysis and putrefaction, this artefact of fixation may be of particular use in suggesting the possibility of ketoacidosis in decomposed bodies with compromised histology.

Keywords: formalin pigment, basal vacuolization, diabetes, alcoholism, ketoacidosis, decomposition

Introduction

The aetiology of basal vacuolization of epithelial cells of the proximal convoluted tubules of the kidney in the cortex and outer medulla remains unclear[1]. Cases have been reported in association with diabetic and alcoholic ketoacidosis, hypothermia and starvation[2-12], and may be identified macroscopically at autopsy by renal cortical pallor[13,14]. Vacuoles have been shown to contain the neutral lipid, triglyceride[3,4]. As this could be the only indication of ketoacidosis at autopsy, this may be a very significant diagnostic finding. Unfortunately, cellular morphology is progressively lost after death; a process which may be exacerbated by the high glucose levels in diabetics[15], resulting in almost complete loss of microscopic detail in badly decomposed bodies. One of the authors (JDG) has observed, however, that formalin pigment preferentially deposits in the areas of basal vacuolization. The results of the following case and study are reported to demonstrate the usefulness of this finding as a marker of basal vacuolization from ketoacidosis, particularly when tissue preservation is suboptimal.

Case report

A case of unexpected death is reported in an adult male who was found dead in bed at his home address. At autopsy there were moderate putrefactive changes. Toxicology did not reveal any lethal drugs or poisons. There were no injuries present. Although extensive putrefactive changes hindered accurate assessment of the histology (postmortem interval = 6 days), the pancreas showed patchy lobular atrophy and fibrosis and the liver focal severe steatosis, in keeping with alcohol related damage. In addition, deposits of birefringent formalin pigment were noted outlining subnuclear basal spaces in proximal convoluted tubular epithelial cells of the kidneys (Fig. 1). As this had been previously observed by one

of the authors (JDG) in cases of ketoacidosis, biochemical analysis of vitreous humor was undertaken. This showed a markedly elevated glucose level of (51.3 mmol/L) and a raised level of β-hydroxybutyrate (8.33 mmol/L). Death was therefore attributed to diabetic ketoacidosis. Subsequent information indicated that the deceased had been complaining of polyuria and polydipsia in the week preceding death and had a family history of diabetes mellitus.

Materials and methods

In view of these results, case files were retrospectively reviewed at Forensic Science SA, Adelaide, South Australia, over a six-year period from 2004 to 2009 for other cases of diabetic and alcoholic ketoacidosis. All cases had full coronial and police investigations with complete forensic autopsies. Ketoacidosis was diagnosed when the vitreous humor β-hydroxybutyrate was $\geq$ 5 mmol/L. Postmortem interval was noted. A control group matched for post-mortem interval was randomly selected from case files from the same time period for comparison. All cases had renal tissue routinely sampled at autopsy and fixed in 10% buffered formalin solution for 24-48 h prior to processing for histology.

A single renal slide stained with haematoxylin and eosin from each case was evaluated blind for basal vacuolization and the presence of characteristic formalin pigment deposition: i.e. along the base of epithelial cells and outlining basal vacuoles within the proximal convoluted tubules of the kidney in the cortex and outer medulla. Cases were graded as 0 if there was no evidence of this type of basal tubular epithelial cell formalin pigment deposition, 1+ if there was mild focal deposition, 2+ if there was moderate-severe focal, or diffuse mild deposition, and 3+ if there was moderate to marked diffuse deposition. Cases were also graded from 0 to 3+ for autolytic/putrefactive changes with 0 being no autolysis or

putrefaction, 1+ mild, 2+ moderate and 3+ marked, the latter with complete loss of normal cellular detail.

The amount of formalin pigment deposition was compared between the cases with ketoacidosis and the controls, and was also plotted against postmortem interval, degree of autolysis/putrefaction and β-hydroxybutyrate levels.

Results

A total of 31 cases were found with ketoacidosis, consisting of 24 individuals with diabetes mellitus and seven with alcoholism. The age range was 21-80 years (mean 50.9 years) with a male to female ratio of 20:11. The post-mortem interval ranged from 1 to 12 days (mean – 4.5 days). A frequent feature of basal epithelial cell formalin pigment deposition was geographic variation, with no staining in some areas of the cortex contrasting with marked staining in other areas in the same case (Fig. 2). Formalin pigment was found in 16 cases (51.6%) (in 14 cases of diabetic ketoacidosis [58.3%] and in 2 cases of alcoholic ketoacidosis [28.6%] [Fig. 3]). The number of cases without formalin pigment deposition was 15. There were 13 cases with no signs of decomposition, 7 cases with 1+ decomposition, 11 cases with 2+ decomposition. Staining was intense ($\geq$2+) in cases with short postmortem intervals and no autolysis/putrefaction (Fig. 4), and also in cases with long postmortem intervals and marked autolysis/putrefaction, as in the reported case. No staining was demonstrated in any of the 31 control cases.

Plotting the degree of formalin pigment deposition against postmortem interval revealed no significant relationship. Similarly, no relationship could be demonstrated between the degree of autolytic/putrefactive changes and degree of formalin pigment deposition, or β-hydroxybutyrate levels and formalin pigment deposition.

Discussion

A variety of different types of vacuoles occur in renal tubules. Clear cell change of the proximal tubular epithelium may be caused by hyperglycemia and is known as glycogen nephrosis or Armanni Ebstein change[16-18]. Ketoacidosis from a variety of causes may result in basal vacuolization of tubular cells, with displacement of nuclei towards the lumina[2,3]. The detection of these changes on postmortem histology should raise the suspicion of an underlying metabolic disturbance and initiate biochemical analysis of vitreous humor to test for this possibility[14]. However, renal tubular epithelium quickly degrades after death due to the combined effects of autolysis and putrefaction. Thus, basal vacuolization may be obscured by cytoplasmic degeneration and lifting of cells away from the basement membrane. In the absence of a history or other indications of underlying metabolic derangements, such as alcohol abuse or diabetes mellitus, these diagnoses may go undiscovered.

Formaldehyde is used as a tissue preservative in most histology laboratories, and is commonly utilized in buffered preparations at 10% concentration. This fixative has been known to produce an artefact known as ‘formalin pigment’ or ‘acid formaldehyde haematin’, which appears as dark brown to black, finely granular, birefringent microcrystals[19-21]. These pigments are iron-free derivatives of haemoglobin, and are formed when blood-rich tissues are fixed with aqueous solutions of formaldehyde at pH less than 6.0[20,22]. Historically, these pigments have been deemed an artefact with no pathologic significance, and many techniques have been described for their prevention and removal[19,23-25].

In 1971, Holmes reported an affinity of formalin pigment for fat, noting that it was often localized to cells which contained fat before processing of tissues in solvents. These included fatty vacuoles within cells of the liver, kidneys, and lungs[21]. The current study has demonstrated that formalin pigment also preferentially deposits in tissue sections of the

kidney during fixation in areas of accumulated triglyceride. This striking aggregation of formalin pigment to the areas of triglycerides may occur because of the breakdown of triglycerides into component fatty acids, providing a localized area of reduced pH that provokes pigment deposition.

The observation that formalin pigment preferentially deposits in the vacuolated epithelial cells of the renal tubules in cases of ketoacidosis (either diabetic or alcoholic) may be significant as this may enhance the postmortem detection of metabolic disturbances. As pigment deposition may be very focal in nature, it may be useful to examine multiple areas of the cortex and outer medulla. The reason for the lack of correlation with postmortem interval and degree of decomposition is unclear, but may be related to factors that were not controlled for in this study such as minor differences in concentration of the formalin solution, amount of buffer, or time of fixation. While formalin deposition does not appear to be a particularly sensitive marker of ketoacidotic basal vacuolization (occurring in only 51.6% of cases), it does appear to be highly specific (100%). Given that these deposits are present even after autolysis and putrefaction have destroyed recognizable cellular morphology, this finding may therefore be of particular use in decomposed bodies, as was clearly demonstrated in the reported case.

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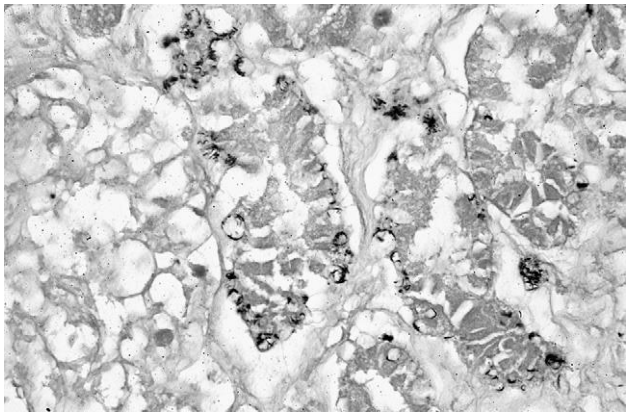

FIG. 1-*Prominent formalin pigment deposition in the reported case with a prolonged postmortem interval (6 days) and marked loss of cellular detail demonstrating the usefulness of the finding in cases where cellular morphology is lacking (Haematoxylin and Eosin [H&E] x 100).*

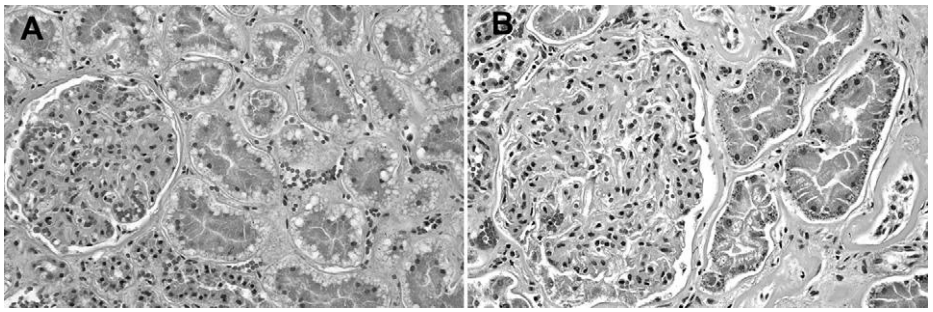

FIG. 2-*Characteristic basal vacuolization of the epithelial cells of the proximal convoluted tubules of the kidney in the cortex in a case of diabetic ketoacidosis with no formalin pigment deposition (A), contrasting with adjacent areas where there was prominent deposition of pigment (B) (H&E x 100).*

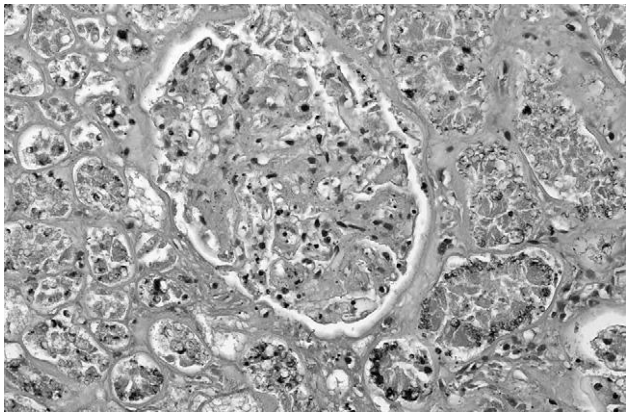

FIG. 3- *Characteristic basal vacuolization of the epithelial cells of the proximal convoluted tubules of the kidney in the cortex in a case of alcoholic ketoacidosis with prominent formalin pigment deposition (H&E x 100).*

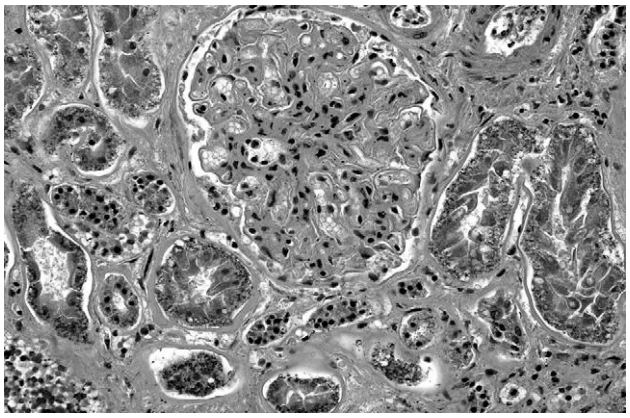

FIG. 4- *Prominent formalin pigment deposition in a case with a short postmortem interval (1 day) and no autolysis or putrefaction (H&E x 100).*

CHAPTER 3: DETERMINING INCIDENCE AND FURTHER DEFINING DIAGNOSTIC UTILITY

ARMANNI-EBSTEIN LESIONS IN TERMINAL HYPERGLYCEMIA

CONTEXTUAL STATEMENT

AIM: To determine the incidence of Armanni-Ebstein lesions and investigate their relationship with hyperglycaemia.

HYPOTHESIS: That Armanni-Ebstein lesions are uncommon in hyperglycaemia and their incidence correlates with the degree of glucose elevation.

COMMENTARY: Armanni-Ebstein lesions refer to a phenomenon of cytoplasmic clearing due to glycogen accumulation within renal tubular epithelial cells. Prior animal studies have demonstrated a direct relationship between glucose levels and the appearance of Armanni-Ebstein lesions. Therefore, a retrospective review of all cases with terminal hyperglycaemia (≥11.1 mmol/l) was conducted to determine if the results of previous animal studies translated to humans.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSIONS:

1. Armanni-Ebstein lesions are present in 38% of cases with terminal hyperglycaemia (≥11.1 mmol/l).
2. There is no significant difference in vitreous glucose levels between cases with and without Armanni-Ebstein lesions.

3. Cases with Armanni-Ebstein lesions have a significantly higher level of β-hydroxybutyrate compared to cases without, suggesting that ketoacidosis may facilitate the development of Armanni-Ebstein lesions.

STATEMENT OF AUTHORSHIP

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By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Conceptualization of work, performed literature review, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	20/10/16

Name of Co-Author	Prof. Andrea J. Yool		
Contribution to the Paper	Helped to evaluate and edit the manuscript		
Signature		**Date**	20 Oct 2016

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
Signature		**Date**	24/10/16

PUBLISHED MANUSCRIPT

Armmani-Ebstein lesions in terminal hyperglycemia

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ABSTRACT: Armanni-Ebstein lesions (AEL) occur in deaths related to uncontrolled diabetes mellitus. To investigate the relationship between AEL and terminal hyperglycemia, we retrospectively reviewed 71 cases with vitreous glucose levels ≥11.1mmol/l; 27 (38%) cases had AEL (vitreous glucose 14.0-77.3mmol/l) and 44 cases (62%) did not (vitreous glucose 11.1-91.9mmol/l). There was no significant difference (p=0.271) in vitreous glucose levels between the cases with AEL (mean 39.2, SD 16.7mmol/l) and those without (mean 34.2, SD 19.8mmol/l). Similarly, there was no difference in the degree of dehydration, renal failure, or osmolality. However, there was a significantly higher level of β-hydroxybutyrate among the cases with AEL compared to those without (p=0.007), suggesting that ketoacidosis may facilitate the development of AEL. Given the possible synergistic role of β-hydroxybutyrate, the correlation between AEL and terminal hyperglycemia in animal studies may not be applicable to humans. AEL may also possibly occur with sub-lethal elevations in glucose.

Keywords: Forensic science, Armanni Ebstein phenomenon, glycogen nephrosis, diabetes mellitus, hyperglycemia, renal tubular vacuolization

Introduction

The Armanni-Ebstein phenomenon refers to a pattern of renal tubular epithelial vacuolization which has been regarded as pathognomonic for hyperglycemia in diabetes mellitus (1). It is characterized by markedly swollen epithelial cells with clear cytoplasm, representing virtual total conversion of the cytoplasm into a single large vacuole filled with PAS-positive material (1). Microdissection studies have localized this lesion to the corticomedullary junction, mainly in the outer medulla, with the middle and outer cortex being spared (1). Electron microscopy has confirmed β-glycogen as the constituent of these vacuoles (2-4), hence the alternative name of glycogen nephrosis (5). Curtis et al. demonstrated that the presence of Armanni-Ebstein lesions in the kidneys in alloxan-induced diabetic rats depended solely upon the degree of terminal hyperglycemia, with lesions invariably present at levels above 350 mg/dl (19.4 mmol/l), and consistently absent at levels below 300mg/dl (16.7 mmol/l) (5). No relationship was demonstrated between the presence of Armanni-Ebstein lesions and the initial blood glucose level after induction of diabetes, or with the maximum glucose level attained by the rats (5).

Although there have been numerous animal studies in diabetic rats (3,5-8), there are limited reports of Armanni-Ebstein lesions in a forensic context, and none that have investigated its correlation with terminal glucose levels. The phenomenon was originally observed in autopsy studies performed by Armanni in 1872 and appeared in Cantani's Textbook of Internal Medicine in 1875 only as an observation without correlations to glucose levels at death (9,10). Ritchie and Waugh conducted microdissection studies on the kidneys of five selected autopsy cases of diabetic decedents with Armanni-Ebstein lesions in order to localize these lesions, but the terminal blood glucose level was only reported in 3 out of the 5 cases, ranging from 424 to 945 mg/dl (23.6 to 52.5 mmol/l). Smith and Glickman investigated

diabetic vacuolization of the iris pigment in 57 postmortem cases, and incidentally found 19 cases with terminal blood glucose levels > 200mg/100ml (11.1 mmol/l) that displayed Armanni-Ebstein lesions in the kidneys, however, the degree of hyperglycemia in these cases was not reported (11). Armanni-Ebstein lesions have also been reported in 5 autopsy cases of severe Fanconi syndrome with glucosuria and death in infancy, however, again the blood glucose levels were not presented (2). Additionally, many of the more recent forensic studies and case reports of Armanni-Ebstein lesions refer to basal lipid vacuolization of renal tubular epithelial cells (12-18), which we now recognize as distinct from the Armanni-Ebstein phenomenon (19). Therefore, the current study was undertaken to investigate the incidence of traditional Armanni-Ebstein lesions in terminal hyperglycemia, its correlation with vitreous glucose levels, and to identify potential factors which may have contributed to its pathogenesis.

Materials and methods

All case files which included vitreous humor analysis over an 11-year-period from 2004 to 2014 at Forensic Science SA, Adelaide, South Australia were retrospectively reviewed for cases with terminal hyperglycemia, defined as vitreous glucose ≥11.1 mmol/l (199.8 mg/dl). All available microscopic slides of the kidneys in study cases were then blindly reviewed with respect to Armanni-Ebstein lesions, and cases where autolysis and putrefaction precluded accurate histological assessment were excluded from the study. All cases had full coronial and police investigations with complete forensic autopsies.

Cases were then classified into two groups based on the presence or absence of Armanni-Ebstein lesions. Vitreous glucose, β-hydroxybutyrate, urea, creatinine and sodium levels, and osmolality were then compared between the two groups to determine if a

statistically significant difference existed. This was done via F-tests for the equality of variances followed by the appropriate two-tailed t-test (with or without equal variance).

Results

A total of 71 cases with terminal hyperglycemia fulfilling the study criteria were identified. Their ages ranged from 14 to 80 (mean 47.5) years with a male to female ratio of 43:28. Vitreous glucose levels ranged from 11.1 to 91.9 mmol/l (199.8-1654.2 mg/dl) with a mean of 36.1 mmol/l (649.8 mg/dl), and β-hydroxybutyrate ranged from 0.1 to 29.35 mmol/l (1.04-305.7 mg/dl) with a mean of 9.09 mmol/l (94.7 mg/dl).

Twenty-seven out of 71 cases (38%) had Armanni-Ebstein lesions of classic morphology (Figure 1) and PAS-positive material was demonstrated within vacuoles in representative cases (Figure 2). Their ages ranged from 17 to 63 (mean 39.9) years with a male to female ratio of 14:13. Postmortem interval ranged from 1 to 13 (mean 4.3) days. Vitreous glucose levels ranged from 14.0 to 77.3 mmol/l (252.0-1391.4 mg/dl) with a mean of 39.2 mmol/l (705.6 mg/dl) and β-hydroxybutyrate ranged from 0.1 to 23.18 mmol/l (1.04-241.5 mg/dl) with a mean of 12.33 mmol/l (128.4 mg/dl). Twenty-one out of the 27 cases (77.8%) had elevated ketone levels of > 5 mmol/l (52.1 mg/dl). Nineteen cases had vitreous sodium levels between 118 and 159 (mean 134.1) mmol/l (mEq/l), sixteen cases had urea levels ranging from 5.4 to 44.4 mmol/l (32.5-267.5 mg/dl) with a mean of 17.1 mmol/l (103.0 mg/dl), thirteen cases had creatinine levels ranging from 32 to 332 µmol/l (0.36-3.76 mg/dl) with a mean of 143.9 µmol/l (1.63 mg/dl), and ten cases had osmolality ranging from 319 to 495 (mean 391.1) mosmol/l. Causes of death were diabetic ketoacidosis (n=21), ischemic heart disease (n=3), cardiomegaly (n=1), pulmonary thromboembolism (n=1), and hanging (n=1). Seventeen cases were known to be insulin-dependent diabetics (two known to be type

1), 2 cases had a history of diabetes but it was unclear which type or if they required insulin therapy, 2 cases were type 2 diabetics on oral hypoglycemic agents, and 6 cases had no known history of diabetes.

Forty-four out of 71 cases (62%) did not have Armanni-Ebstein lesions. Their ages ranged from 14 to 80 (mean 52.3) years with a male to female ratio of 29:15. Postmortem interval ranged from 1 to 11 (mean 3.8) days. Vitreous glucose levels ranged from 11.1 to 91.9 mmol/l (199.8-1654.2 mg/dl) with a mean of 34.2 mmol/l (615.6 mg/dl), and β-hydroxybutyrate levels ranged from 0.1 to 29.35 mmol/l (1.04-305.7 mg/dl) with a mean of 7.09 mmol/l (73.85 mg/dl). Twenty-one out of the 44 cases (47.7%) had elevated ketone levels of > 5 mmol/l (52.1 mg/dl). Twenty-nine cases had vitreous sodium levels between 112 and 188 (mean 137.7) mmol/l (mEq/l), twenty-seven cases had urea levels ranging from 5.1 to 45.3 mmol/l (30.7-272.9 mg/dl) with a mean of 17.6 mmol/l (106.0 mg/dl), twenty-five cases had creatinine levels ranging from 27 to 360 µmol/l (0.31-4.07 mg/dl) with a mean of 138.6 µmol/l (1.57 mg/dl), and twelve cases had osmolality ranging from 318 to 425 (mean 372) mosmol/l. Causes of death included diabetic ketoacidosis (n=21), ischemic heart disease (n=9), drug toxicity (n=3), drowning (n=2), multiple injuries (n=2), congestive cardiac failure (n=1), myocarditis (n=1), pericarditis and hemopericardium complicating stab wounds (n=1), dehydration and hyperglycemia (n=1), hypothermia (n=1), acute subdural hemorrhage (n=1), and upper gastrointestinal hemorrhage (n=1). Twenty-four decedents had been insulin-dependent diabetics (six known to be type 1), 8 cases were type 2 diabetics, 5 cases had a history of diabetes recorded but it was unclear which type, and 7 cases had no previous history of diabetes.

F-tests indicated that the variances in the post-mortem interval ($F=1.55$, $p=0.100$), vitreous glucose ($F=0.715$, $p=0.183$), β-hydroxybutyrate ($F=0.900$, $p=0.395$), sodium

(F=0.547, p=0.092), urea (F=1.080, p=0.418), creatinine (F=1.049, p=0.440), and osmolality (F=2.49, p=0.078) of the cases with and without Armanni-Ebstein lesions were not different. There was also no statistically significant difference between the post-mortem interval (p=0.433), vitreous glucose (p=0.271), sodium (p=0.355), urea (p=0.900), creatinine (p=0.853), and osmolality (p=0.359) of the cases with and without Armanni-Ebstein lesions. However, there were significantly higher levels of ketoacidosis in the cases with Armanni-Ebstein lesions compared to those without (p=0.007). The quantitative comparison of the vitreous glucose and β-hydroxybutyrate levels are summarized in Figure 3 and Figure 4, respectively.

Discussion

In the recent literature, the term Armanni-Ebstein phenomenon has been expanded to include basal vacuolization as well as the traditional cytoplasmic clear-cell change, however, a recent review by our group has suggested that basal vacuolization is a separate and distinct entity from the originally described Armanni-Ebstein lesion, based on morphology, constituents, and etiology (19). For example, in contrast to Armanni-Ebstein lesions, basal vacuoles contain lipids instead of glycogen, and are associated with a variety of ketoacidotic states (19). It appears that basal vacuolizations were included under the umbrella of the "Armanni-Ebstein lesion" after a study by Ritchie and Waugh in 1957 which noted fine basal fat droplets in the cytoplasm of proximal tubular epithelial cells not involved by the Armanni-Ebstein lesion (1,19). Thus, many of the recent reports on the Armanni-Ebstein phenomenon in a forensic context have referred to this type of basal lipid vacuolization, rather than to the originally described clear-cell change (12-18,20). Similarly, the only prior study investigating the relationship between Armanni-Ebstein lesions and hyperglycemia referred to basal

vacuolizations rather than to cytoplasmic glycogen accumulation, and concluded that no relationship existed between terminal hyperglycemia and basal vacuolization (12).

The etiology of the traditional Armanni-Ebstein phenomenon is thought to involve hyperglycemia and glucosuria (1), however, results of the current study suggest that underlying mechanisms may be more complicated. To our knowledge, there has only been one study which investigated the relationship of Armanni-Ebstein lesions to terminal blood glucose levels which used alloxan to induce diabetes in 207 rats, of which 62 survived and met the study criteria (5). The authors observed that lesions were invariably present with glucose levels above 350 mg. per cent (19.4 mmol/l) and that no lesions were present with levels below 300 mg. per cent (16.7 mmol/l). However, the upper threshold level and a direct relationship with the level of hyperglycemia were not observed in our study, as only 31 out of the 44 cases (70.5%) without Armanni-Ebstein lesions had a glucose level above 19.4 mmol/l (349.2 mg/dl). Additionally, there was no significant difference in the degree of terminal hyperglycemia between the cases with and without Armanni-Ebstein lesions ($p=0.271$), and the case with the highest observed glucose level of 91.9 mmol/l (1654.2 mg/dl) did not have typical vacuoles. It is unclear why the direct relationship observed in rats was not observed in human subjects. A possible explanation may be that animal studies tend to be conducted on healthy rats of a selected breed, similar age, and with similar environmental parameters, with diabetes being induced in a uniform manner with a set dose of the same drug (i.e. alloxan or streptozotocin). In contrast, human cases in a forensic environment have more varied demographics, and often have other significant illnesses, impairments, and/or metabolic derangements. For example, 6 out of the 27 cases (22.2%) with Armanni-Ebstein lesions had hyperglycemia incidental to a cause of death other than decompensated diabetes. Therefore,

the variable appearance of Armanni-Ebstein lesions at similar glucose values may reflect other complex organic factors.

Ketoacidosis may, therefore, be contributory to the development of Armanni-Ebstein lesions. In this study, 21 out of the 27 cases (77.8%) exhibiting Armanni-Ebstein lesions had concurrent ketoacidosis demonstrated by elevations in vitreous β-hydroxybutyrate. Although Armanni-Ebstein lesions also occurred in cases with hyperglycemia without elevations in ketones (n=6), an overall significantly higher level of β-hydroxybutyrate was seen in the cases exhibiting Armanni-Ebstein lesions compared to those that did not ($p=0.007$).

The exact mechanism for the development of Armanni-Ebstein lesions is unclear, but it is thought to represent excess reabsorption of glucose in the distal tubules secondary to hyperglycemia (3). However, injury to the pars recta has also been proposed as a mechanism for glycogen accumulation (2), as well as excessive glycogenesis or defective glycogenolysis as a result of cellular dysfunction (1). Ketoacidosis can contribute to this as it may cause renal tubular dysfunction and injury, evidenced by significantly increased urinary excretion of proximal tubular marker alpha 1-microglobulin and distal tubular marker Tamm-Horsfall protein compared to metabolically stable controls (21). The toxic effects of ketone bodies on renal tubular cells were also shown by Asami and colleagues with significantly elevated urinary beta-D-N-acetyl glucosamine and beta 2-microglobulin in patients with non-diabetic ketoacidosis (22). Thus, it is possible that the resultant tubular dysfunction in ketoacidosis may facilitate the intracellular accumulation of glycogen.

Higher levels of glucose in the current post-mortem study were observed compared to previous animal studies. A number of diabetic rat models have demonstrated Armanni-Ebstein lesions in the renal tubules, some were spontaneously diabetic, and others

were treated with alloxan or streptozotocin (3,5-8,23-29). Serum glucose levels reported in these rats ranged from 13.9 to approximately 40.0 mmol/l (250.2-720.0 mg/dl) (3,5-8,23-29) with only one study reporting a glucose level of 66.7 mmol/l (1200.6 mg/dl) in one rat (5). In contrast, the highest vitreous glucose level associated with Armanni-Ebstein lesions in the current study was 77.3 mmol/l (1391.4 mg/dl), and 10 out of the 27 cases (37%) with Armanni-Ebstein lesions had a vitreous glucose level of >40 mmol/l (720 mg/dl). This may be because of more severe metabolic derangements in forensic cases.

A major limitation in attempting to correlate the presence of Armanni-Ebstein lesions with terminal glucose levels is the reduction in glucose after death due to continued consumption by cells. Therefore, the true terminal glucose level will be higher than the vitreous level obtained at the time of autopsy by an amount that depends on the post-mortem interval. For example, in a series of 3076 cases by Zilg et al, the post-mortem vitreous glucose level was observed to drop by approximately 3 mmol/l (54.0 mg/dl) in the very early post-mortem period with levels remaining relatively stable for up to 4 days (30). This was attributed to initial survival of hyalocytes and inner retinal cells with gradual equilibration thereafter (30). Therefore, the observations made in this study are likely to be valid as the majority (65%) of our cases had a post-mortem interval of 4 days or less, and there was no significant difference in post-mortem interval between cases with and without Armanni-Ebstein lesions (p=0.433). Furthermore, exclusion of the 25 cases (35%) with a postmortem interval of greater than 4 days leaves 18 cases with Armanni-Ebstein lesions [vitreous glucose mean 39.5 mmol/l (711.0 mg/dl), standard deviation 16.6 mmol/l (298.8 mg/dl)] and 28 cases without [vitreous glucose mean 31.6 mmol/l (568.8 mg/dl), standard deviation 15.6 mmol/l (280.8 mg/dl)]. Repeating the quantitative analysis similarly shows no statistically significant difference in vitreous glucose levels (p=0.108) between these groups.

In summary, in the present series typical Armanni-Ebstein lesions were found in 38% of all cases with terminal hyperglycemia, and no significant differences in vitreous glucose levels could be demonstrated between cases with or without these lesions (p=0.271). Additionally, the threshold glucose level for the invariable appearance of Armanni-Ebstein lesions established in animal studies did not correspond to the current human data, as 70.5% of cases without Armanni-Ebstein lesions had glucose levels exceeding 19.4mmol/l (349.2 mg/dl). However, a significantly greater degree of ketoacidosis was present among cases with Armanni-Ebstein lesions (p=0.007), suggesting that ketoacidosis may facilitate its development. Additional available biochemical parameters, including vitreous sodium, urea, and creatinine levels, and osmolality, were also compared between cases with and without Armanni-Ebstein lesions and no significant differences were present. Although Armanni-Ebstein lesions can be used as a marker for terminal hyperglycemia at autopsy, its presence does not appear to correlate with the level of glucose as it can be seen with mild sub-lethal elevations. Conversely, its absence did not exclude significant hyperglycemia capable of inducing coma.

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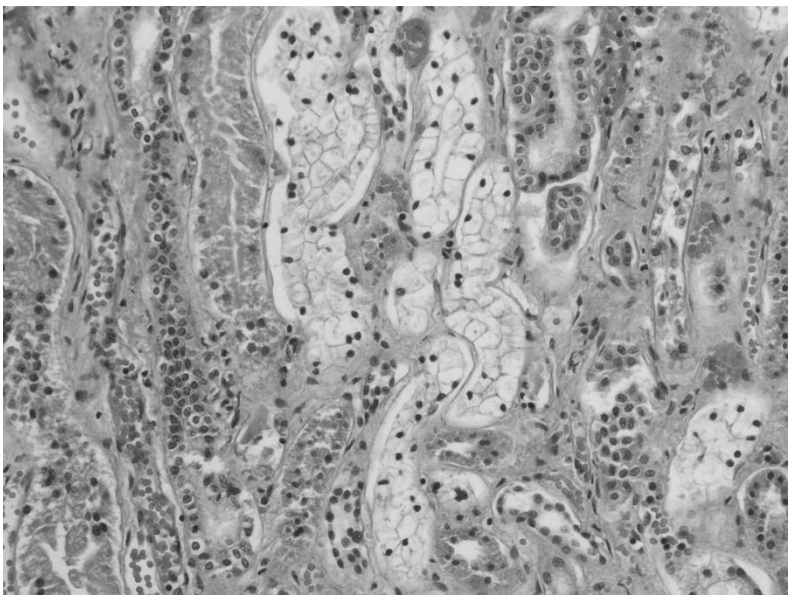

FIG. 1- *Typical Armanni-Ebstein vacuolization in a case of diabetic ketoacidosis (Hematoxylin and eosin x400).*

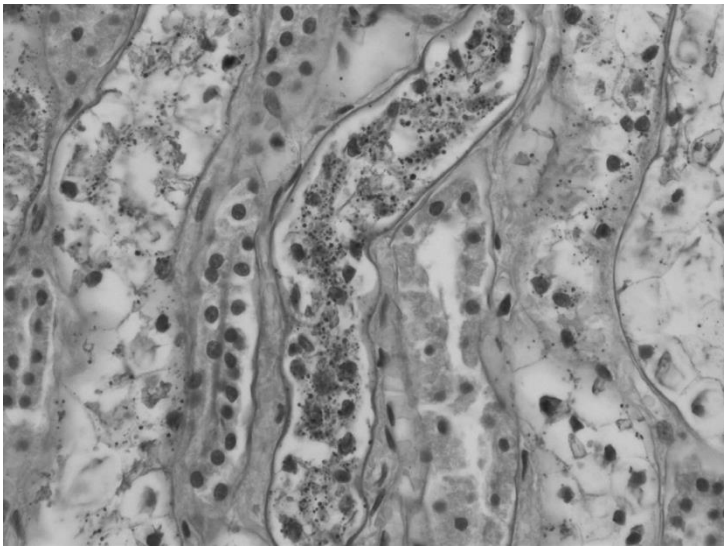

FIG. 2- *PAS-positive material within the vacuolated tubules (Periodic Acid-Schiff x400).*

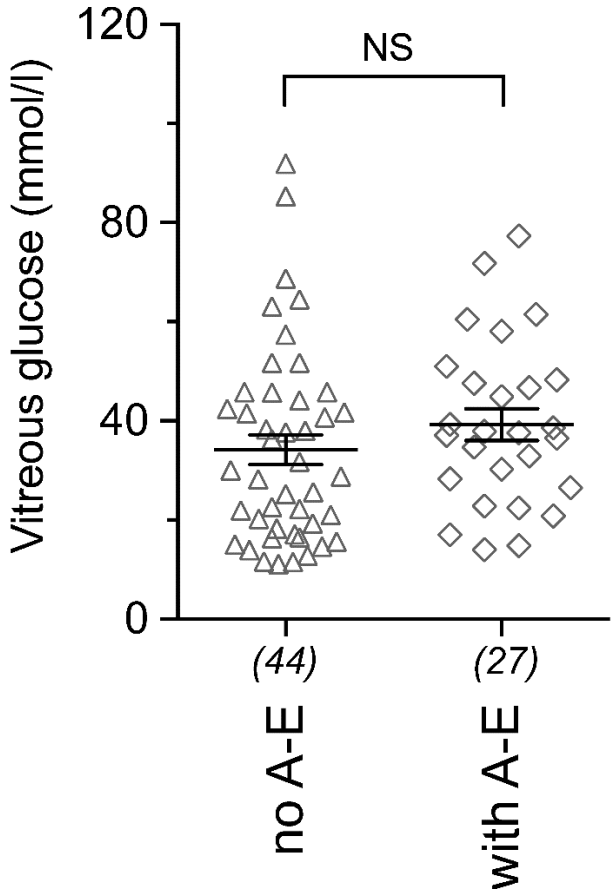

FIG. 3- *Scatterplot histogram summarizing the vitreous glucose levels in cases with and without Armanni-Ebstein lesions; NS is not significant.*

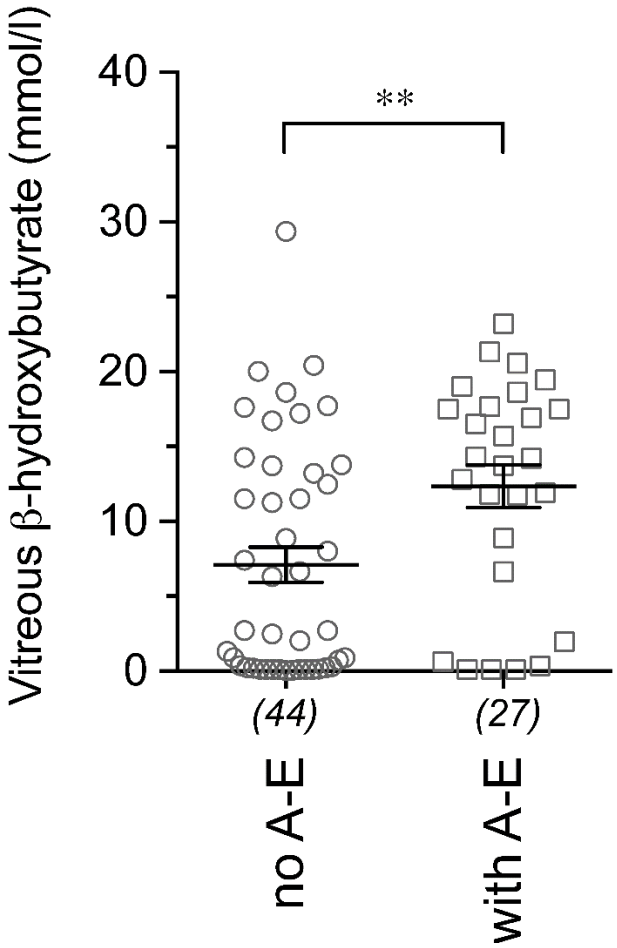

FIG. 4- *Scatterplot histogram summarizing the vitreous β-hydroxybutyrate in cases with and without Armanni-Ebstein lesions. Statistically significant differences are indicated as (**) for p=0.007*

BASAL VACUOLIZATION IN RENAL TUBULAR EPITHELIAL CELLS AT AUTOPSY AND THEIR RELATION TO KETOACIDOSIS

CONTEXTUAL STATEMENT

AIM: To determine the incidence of basal vacuolizations and investigate their relationship with ketoacidosis.

HYPOTHESIS: That basal vacuolizations are common in ketoacidosis and their incidence correlates with the degree of ketone elevation.

COMMENTARY: Basal vacuolization in renal tubular epithelial cells has been reported in diabetic, alcoholic, septic, and starvation ketoacidosis. Although it can be seen along with Armanni-Ebstein lesions in diabetic ketoacidosis, their presence in other normo-glycaemic ketoacidotic conditions strongly suggest a relationship with elevated ketones, and no relationship to serum glucose levels. To prove this, a retrospective review of all deaths featuring elevated β-hydroxybutyrate of >1mmol/l was conducted to investigate the incidence and relationship of basal vacuolizations to ketoacidosis and hyperglycaemia.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSIONS:

1. Basal vacuolizations in renal tubular epithelial cells are present in 43% of cases with terminal ketoacidosis (>1mmol/l).
2. Basal vacuolizations are associated with ketoacidosis, and their incidence increases with the degree of ketone elevation.
3. Basal vacuolizations are not associated with hyperglycaemia.

STATEMENT OF AUTHORSHIP

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Conceptualization of work, performed literature review, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	20/10/16

Name of Co-Author	Prof. Andrea J. Yool		
Contribution to the Paper	Helped to evaluate and edit the manuscript		
Signature		**Date**	20 OCT 2016

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Supervised development of work, helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
Signature		**Date**	24/10/16

PUBLISHED MANUSCRIPT

Basal Vacuolization in Renal Tubular Epithelial Cells at Autopsy and Their Relation to Ketoacidosis

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ABSTRACT: Basal vacuolization of renal tubular epithelial cells is a useful post-mortem marker for ketoacidosis. To investigate its incidence and relationship to the severity of ketoacidosis, 158 autopsy cases with elevated β-hydroxybutyrate (>1mmol/l) over a 7-year-period were retrospectively reviewed. Sixty-eight cases (43%) exhibited basal vacuolizations (vitreous β-hydroxybutyrate: 1.16-29.35mmol/l, mean 10.28mmol/l) and 90 cases (57%) did not (vitreous β-hydroxybutyrate: 1.03-13.7mmol/l, mean 2.84mmol/l). Quantitative analysis revealed on average a four-fold elevation in β-hydroxybutyrate in cases with basal vacuolizations compared to those without. 10.3% of cases with β-hydroxybutyrate concentrations between 1.01-2.00mmol/l had basal vacuolizations, and this incidence increased to 33.3% with concentrations between 4.01-6.00mmol/l. A marked increase in incidence to >70% was observed with concentrations >6.00mmol/l, and basal vacuoles were invariably present (100%) with concentrations >14.01mmol/l. This study demonstrates that basal vacuolizations are a sensitive marker for significant ketoacidosis, and reaffirms its use as an indicator for likely cases of fatal ketoacidosis at autopsy.

Keywords: Forensic science, basal vacuolization, diabetic ketoacidosis, starvation ketoacidosis, alcoholic ketoacidosis, septic ketoacidosis, diabetes mellitus

Introduction

Basal vacuolizations of renal tubular epithelial cells appear as a row of clear spaces beneath the nucleus in close proximity to the basement membrane. Intravacuolar lipids have been demonstrated on Oil-Red O staining and electron microscopy (1-3). Recently, this pattern of vacuolization has been referred to as the 'Armanni-Ebstein phenomenon' (1,2,4-9), possibly after a report on autopsy cases of diabetics with Armanni-Ebstein lesions by Ritchie and Waugh who mentioned fine basal fat droplets in the cytoplasm of proximal tubules not affected by the Armanni-Ebstein lesion in 2 out of their 5 cases (10). However, a recent review of the literature showed that the originally reported Armanni-Ebstein lesion instead represented a clear-cell change with intra-cytoplasmic glycogen accumulation in hyperglycemic states, therefore suggesting that basal vacuolizations should be recognized as a distinct entity from the Armanni-Ebstein lesion on the basis of different morphology, constituents, and etiology (11).

Basal vacuolizations have been reported in deaths due to diabetic, alcoholic, starvation, and septic ketoacidosis (4,6,12,13). They have also been put forward as a marker for hypothermia (3,14), although, a review of hypothermic deaths suggested that a significant proportion of cases had underlying metabolic derangements which may have been the cause of the basal vacuolizations rather than simply decreased core temperature in isolation (15). This concept was supported by an animal model inducing fatal hypothermia in rats where none of the cases demonstrated basal vacuolizations (16). However, to date there have been no studies investigating the overall incidence of basal vacuolizations in ketoacidosis or its relationship to the degree of ketosis.

Materials and methods

All case files which included vitreous humor analysis over a 7-year-period from 2008 to 2014 at Forensic Science SA, Adelaide, South Australia were retrospectively reviewed for cases featuring ketosis, defined as β-hydroxybutyrate ≥ 1 mmol/L (10.3 mg/dl); the cause of ketoacidosis was determined in each case. Cases were classified as diabetic ketoacidosis if concurrent hyperglycemia (vitreous glucose ≥11.1 mmol/l or 200mg/dl) was present. Alcoholic ketoacidosis was diagnosed if there was a previous history combined with signs of chronic alcoholism at autopsy and normoglycemia. Starvation was considered the cause of ketosis when a documented history of decreased oral intake was present in the absence of elevated glucose concentrations or alcoholism. Septic ketoacidosis was diagnosed when proven sepsis or severe infection was present at autopsy in the absence of raised glucose concentrations or a history of alcoholism, diabetes, or starvation. The cause of ketosis in the remainder of cases was unclear and may have been due to a combination of the above factors.

All available microscopic slides of the kidneys were blindly screened for basal vacuolizations of renal tubular epithelial cells and/or formalin pigment deposition around basal vacuolar spaces (17). Cases exhibiting significant autolysis precluding accurate histological assessment were excluded from the series. All cases had full coronial and police investigations with complete forensic autopsies. Study cases were divided into two groups based on the presence or absence of characteristic basal vacuolizations. The vitreous β-hydroxybutyrate concentrationss of the two groups, as well as other biochemical parameters such as glucose, urea, creatinine, and sodium levels, and osmolality, were compared to determine if a statistically significant difference existed. This was done by F-tests for the equality of variances followed by the appropriate t-test (with or without equal variance). The incidence of basal vacuolizations at different β-hydroxybutyrate concentrations was also

plotted to determine if increasing ketosis correlated with an increased incidence of basal vacuolizations.

Results

A total of 158 cases fulfilling the study criteria were identified, including 44 cases of diabetic ketoacidosis, 17 cases of alcoholic ketoacidosis, 5 cases of starvation ketoacidosis, 5 cases of septic ketoacidosis, and 87 cases where the cause of ketosis was unclear and a single causative mechanism could not be determined.

Sixty-eight out of the 158 study cases (43%) displayed characteristic basal vacuolization of the renal tubular epithelial cells (Figure 1). Their ages ranged from 15 to 92 (mean 54.2) years and they comprised of 40 males and 28 females. The post-mortem interval ranged from 1 to 13 (mean 4.7) days. The cause of ketosis was diabetic in 36 cases, alcoholic in 11 cases, septic in 3 cases, starvation in 1 case, and a single cause of ketosis could not be identified in 17 cases. Vitreous β-hydroxybutyrate concentrations ranged from 1.16 to 29.35 (mean 10.28) mmol/l and vitreous glucose concentrations ranged from 0.1 to 91.9 (mean 23.3) mmol/l. Thirty-two of the 68 cases (47.1%) also displayed characteristic basal formalin pigment deposition in the vicinity of the vacuoles (17).

Ninety out of the 158 study cases (57%) did not have characteristic basal vacuolization of renal tubular epithelial cells. Their ages ranged from 5 to 94 (mean 64.1) years and they comprised of 58 males and 32 females. Postmortem intervals ranged from 1 to 12 (mean 4.3) days. The cause of the ketosis was diabetic in 8 cases, alcoholic in 6 cases, starvation in 4 cases, and septic in 2 cases. A single cause for the elevation in ketones could not be determined in the remainder of the 70 cases. Vitreous β-hydroxybutyrate ranged from 1.03 to 13.7 (mean 2.84) mmol/l, and vitreous glucose concentrations ranged from 0.1 to 72.7

(mean 4.5) mmol/l. None of these cases displayed characteristic basal formalin pigment deposition.

Quantitative Analysis

The F-test indicated that the variances in the vitreous β-hydroxybutyrate concentrations ($F=7.731$, $p<0.001$) and postmortem interval ($F=1.540$, $p=0.034$) in cases exhibiting basal vacuolizations were not equivalent to cases without basal vacuolizations. Subsequent t-tests assuming unequal variances demonstrated a statistically significantly higher vitreous β-hydroxybutyrate concentration ($p<0.001$) in cases with basal vacuolizations compared to those without, with no significant difference in the postmortem interval ($p=0.22$). The quantitative comparisons of vitreous β-hydroxybutyrate concentrations between cases with and without basal vacuolizations are summarized in Figure 2.

The incidence of characteristic basal vacuolizations observed at different β-hydroxybutyrate concentrations is summarized in Table 1 and Figure 3. The incidence of basal vacuolizations markedly increased when β-hydroxybutyrate concentration exceeded 6.0 mmol/l from 33.3% to above 70%. Basal vacuolizations were seen in >80% of cases with β-hydroxybutyrate concentration >10.0 mmol/l and were invariably present (100%) with concentrations > 14.0 mmol/l. The incidence of basal vacuolizations among different causes of ketoacidosis was 81.8% in diabetic, 64.7% in alcoholic, 60% in septic, and 20% in starvation ketoacidosis. Basal vacuolizations were present in 19.5% of the cases in which the exact cause of ketoacidosis was unclear or multi-factorial.

Data sets were each fitted with a Gaussian distribution function using GraphPad Prism software; plotting this data on a frequency histogram (Figure 4) demonstrates that cases without basal vacuolization were tightly clustered at low concentrations of β-hydroxybutyrate

whilst cases with basal vacuolizations were widely distributed over a broad range. Additionally, cases exhibiting basal vacuoles had on average a four-fold elevation in β-hydroxybutyrate concentration (mean 5.91 +/- 4.64 mmol/l) compared to cases lacking basal vacuoles (mean 1.43 +/- 0.10 mmol/l).

Discussion

Lethal ketoacidosis encountered in the forensic context is most commonly due to decompensated diabetes mellitus, but elevations in ketones may also occur in alcohol abusers, and in cases of starvation, sepsis, non-insulin mediated hypoglycemia, drug abuse, or in cases involving a combination of these factors (18,19). The mechanism in which ketoacidosis may lead to death includes extracellular volume contraction, electrolyte disturbances (including hypokalemia), sudden cardiac arrest, and cerebral edema (18,20). In the current study, basal vacuolizations were seen in all categories of ketoacidosis including diabetic, alcoholic, septic, starvation-induced, and in cases where the cause of ketoacidosis was unclear or multi-factorial.

Most prior studies have reported on vitreous acetone rather than β-hydroxybutyrate concentrations. However, β-hydroxybutyrate is the major ketone body produced in ketosis accounting for approximately 78% of total ketones, with acetoacetate (20%) and acetone (2%) produced in much smaller amounts (21). The ratio of β-hydroxybutyrate to acetoacetate is normally 1:1, however, this ratio can increase to as high as 10:1 in diabetic and alcoholic ketoacidosis (22,23). Additionally, β-hydroxybutyrate concentrations have also been shown to correlate more closely than serum ketones with the degree of anion gap elevation in ketoacidotic states (21), therefore making it the ideal biochemical marker to reflect the severity of ketoacidosis. However, there has only been one

previous retrospective review of basal vacuolizations in alcoholic ketoacidosis which reported β-hydroxybutyrate concentrations ranging from 6.42 to 8.75 mmol/l (12), and one case report on starvation ketoacidosis which had a vitreous β-hydroxybutyrate of 3.966 mmol/l (6). In the current study, basal vacuolizations were seen at a β-hydroxybutyrate level of 1.16 mmol/l with the highest level being 29.35 mmol/l, representing a wider range than previously reported. Three prior studies reported on vitreous acetone levels which ranged from 0.5 to 12.8mmol/l in decedents with basal vacuolization who died of starvation and diabetic ketoacidosis (4,7,9). At Forensic Science SA, acetone can be detected in the blood on toxicological screening but it does not appear as a quantitative measurement. An acetoacetate screen is also occasionally conducted on vitreous humor biochemistry which appears in a semi-quantitative manner as ‘small’, ‘moderate’, or ‘large’. Therefore, these measurements were not reported in the current study.

Basal vacuolizations are a useful marker for lethal ketoacidosis, with the incidence increasing with the extent of metabolic derangement. A large retrospective review of 1795 forensic cases recommended that β-hydroxybutyrate levels < 0.4 mmol/l should be interpreted as normal, 0.41-1.2 mmol/l to be slightly elevated and rarely (<1%) of concern, 1.21-2.0 mmol/l to be moderately elevated and less rarely (2.5%) of concern, 2.01-6.0 mmol/l to be significantly elevated and frequently (12-48%) of concern, and concentrations above 6.0 mmol/l to indicate life-threatening conditions (24). In this study, we divided the cases according to incremental increases in vitreous β-hydroxybutyrate levels and found that the lowest β-hydroxybutyrate concentration in which basal vacuolizations could be identified was 1.16mmol/l. Above this concentration, and regardless of the cause of ketoacidosis, the incidence of basal vacuolizations increased with greater elevations in β-hydroxybutyrate (summarized in Table 1 and Figure 3). Interestingly, 6.0 mmol/l also seemed to be a threshold

concentration in this study, above which the incidence of basal vacuolizations more than doubled from 33.3% to over 70%. The incidence of basal vacuolizations was >80% with β-hydroxybutyrate concentrations of 10.0-14.0 mmol/l, and 100% when levels exceeded 14.0 mmol/l. Therefore, basal vacuolizations were certainly found to be sensitive markers for fatal ketoacidosis.

In the current study, the incidence of basal vacuolizations in diabetic ketoacidosis was slightly lower than previously reported figures, whereas the incidence in alcoholic ketoacidosis is slightly higher. Basal vacuolizations have been reported in 30.8% of deaths in individuals with a history of diabetes (4) and 84-100% of deaths due to diabetic ketoacidosis (1,9), whereas it was noted in 81.8% in the present series. Two previous studies on alcoholic ketoacidosis reported basal vacuolizations in 47.8-60.0% of cases (7,12), whereas it was seen in 64.7% in the present study. There have been no previous reports on the incidence of basal vacuolizations in septic or starvation ketoacidosis, which in this study, was found to be 60% and 20% respectively. The higher incidences found in diabetic, alcoholic, and septic ketoacidosis is likely a reflection of the typically higher degrees of ketone elevation seen in these conditions compared to starvation (19,25).

Vitreous humor has been proven as a reliable alternative to blood for the determination of β-hydroxybutyrate levels (26-28). Additionally, studies suggest that decompositional changes are not associated with β-hydroxybutyrate production, therefore the concentration detected at autopsy can be considered a reflection of concentrations at the time of death (29,30). This includes a study by Iten and Meier demonstrating no statistically significant increase in serum β-hydroxybutyrate concentration with increasing post-mortem interval (30), and another study by Palmiere et al whom observed lower concentrations of serum β-hydroxybutyrate in 50 cases exhibiting advanced decomposition compared to 400

cases that did not (31). These observations are supported in the present study as no statistically significant difference in post-mortem interval could be demonstrated between cases with and without basal vacuolizations (p=0.22). The exact role that micro-organisms play in β-hydroxybutyrate metabolism after death is unclear, however, it seems that vitreous humor is a relatively protected environment from post-mortem microbiological contamination (32), and has an inherent antibacterial capacity in vitro which does not support bacterial growth (33). Therefore, any potential alterations micro-organisms may have on β-hydroxybutyrate concentrations would be reduced in vitreous humor compared to blood.

The current study is limited by its retrospective nature. Although selection bias was eliminated by conducting a search for all cases containing keywords "vitreous humor" biochemistry and "vitreous biochemistry" rather than specific causes of ketosis, it is possible that a small number of cases may have been missed if these keywords were not used in a report. Cases with β-hydroxybutyrate concentrations <1.0 mmol/l were not reviewed in the present study. Review of these cases in the future may, however, be useful to establish whether basal vacuolization can occur with normal ketone concentrations, although there have been no reports of this in the literature to date. Review of larger numbers of cases would also assist in more accurately quantifying the incidence of basal vacuolization in septic and starvation ketoacidosis as the current study had relatively low case numbers due to the infrequent nature of these conditions leading to death.

In conclusion, this study has demonstrated that basal vacuolization in renal tubular epithelial cells may be found in autopsy material from all categories of ketoacidosis including diabetic, alcoholic, septic, and starvation. It is also present in both minor and severe ketosis, with β-hydroxybutyrate concentrations ranging from 1.16 to 29.35 mmol/l. Additionally, the incidence is related to the degree of ketoacidosis (Table 1, Figure 3), with a significant

increase in incidence to >70% at β-hydroxybutyrate concentrations of >6.0 mmol/l, and vacuoles invariably (100%) present at concentrations >14.0 mmol/l. Therefore, basal vacuolization in renal tubular epithelial cells in autopsy tissues is a very useful marker for ketosis, even in putrefied and/or autolytic kidneys. Once it has been identified it should initiate investigation into the quite diverse potential underlying causes, with full vitreous humor biochemistry (including quantitative β-hydroxybutyrate levels).

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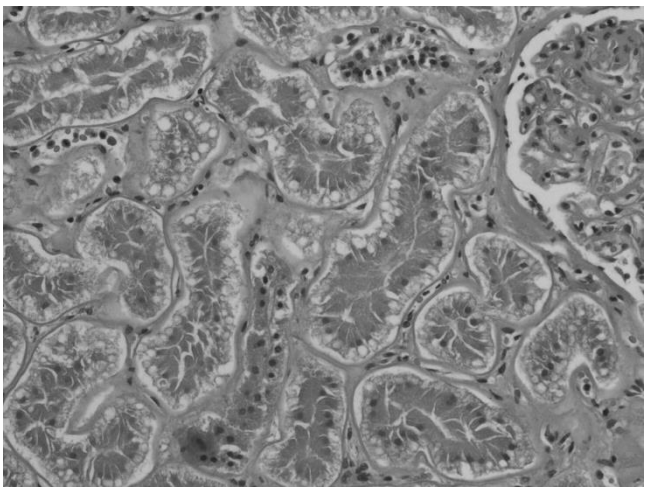

FIG. 1- *Characteristic basal vacuolizations in renal tubular epithelial cells in a case of diabetic ketoacidosis (Hematoxylin and eosin x400).*

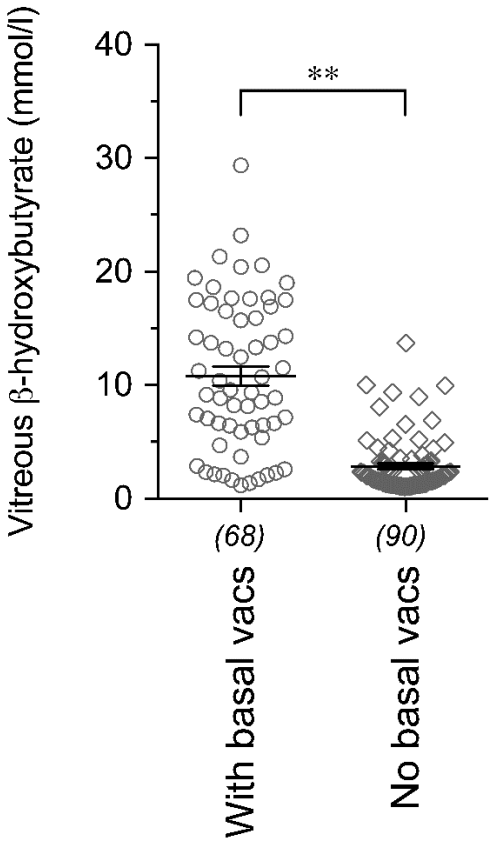

FIG. 2- *Scatterplot histogram summarizing the vitreous β-hydroxybutyrate in cases with and without basal vacuolizations. [Horizontal bars indicate mean +/- standard error of the mean (SEM). Statistically significant differences are indicated as (**) for p<0.001].*

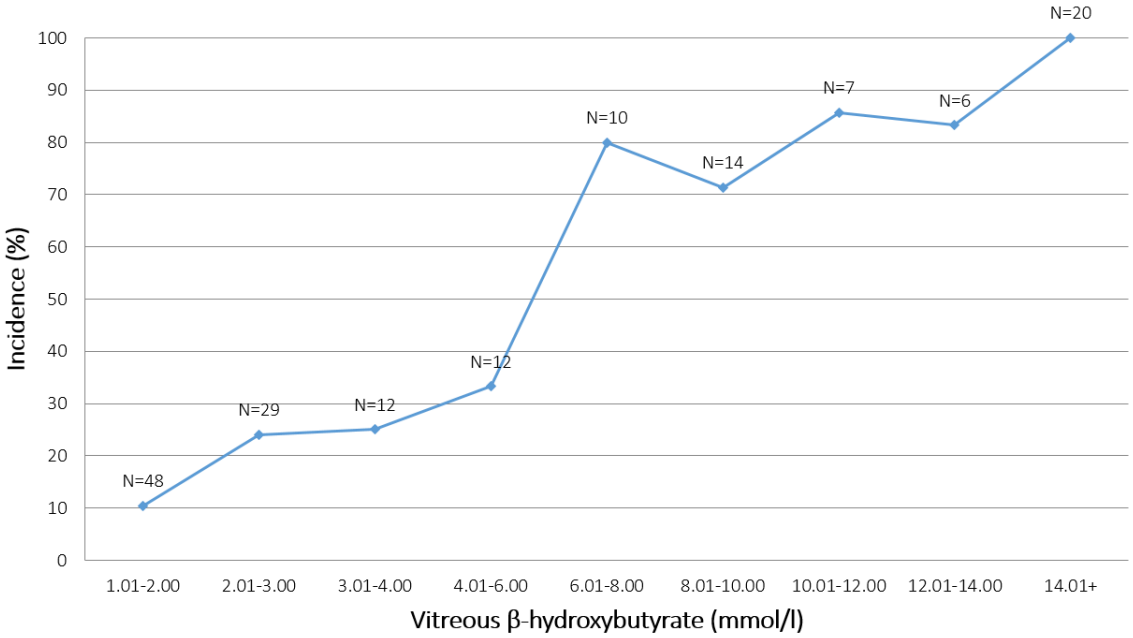

FIG. 3- *Plotting the incidence of basal vacuolizations in renal tubular epithelial cells against incremental increases in vitreous β-hydroxybutyrate levels in 158 autopsy cases with ketosis (β-hydroxybutyrate > 1.0 mmol/l).*

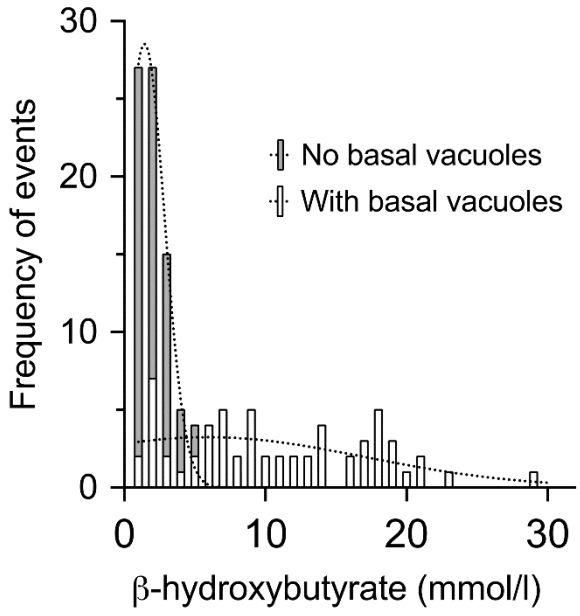

FIG. 4- *Frequency histogram summarizing vitreous β-hydroxybutyrate in cases with and without basal vacuolizations [Dotted line indicates the Gaussian distribution].*

[175]

β-hydroxybutyrate level (mmol/l)	Incidence of basal vacuolizations (%)
1.01-2.00	10.4
2.01-3.00	24.1
3.01-4.00	25.0
4.01-6.00	33.3
6.01-8.00	80.0
8.01-10.00	71.4
10.01-12.00	85.7
12.01-14.00	83.3
14.01+	100

TABLE. 1- *The incidence of basal vacuolizations in renal tubular epithelial cells at different vitreous β-hydroxybutyrate levels in 158 autopsy cases with ketosis (β-hydroxybutyrate > 1.0 mmol/l).*

CHAPTER 4: ESTABLISHING AND UTILIZING ANIMAL MODELS TO FACILITATE FURTHER INVESTIGATION

LETHAL HYPOTHERMIA IN AN ANIMAL MODEL, NOT ASSOCIATED WITH BASAL RENAL EPITHELIAL VACUOLIZATION

CONTEXTUAL STATEMENT

AIM: To investigate the relationship between basal vacuolizations in renal tubular epithelial cells and terminal hypothermia using an animal model.

HYPOTHESIS: That basal vacuolizations in renal tubular epithelial cells are not associated with hypothermia.

COMMENTARY: Basal vacuolization of renal tubular epithelial cells has been suggested by Preuβ et al as "*a very reliable histologic diagnostic criterium in cases of hypothermia, comparable to the significance of Wischnewsky ulcers*" (1). However, our retrospective review of hypothermic deaths (see Chapter 2) revealed that a significant percentage of these cases had a documented history of diabetes and all cases in which vitreous biochemistry was done showed diabetic ketoacidosis. Therefore, it remains unclear if low core body temperature alone, in the absence of underlying metabolic derangements, can cause basal vacuolizations. Therefore, an animal model was employed to investigate the effects of hypothermia in isolation.

Literature review establishing the field of knowledge is discussed within the paper.

CONCLUSION: Basal vacuolizations in renal tubular epithelial cells are not associated with terminal hypothermia in an animal model.

1. Preuβ J, Dettmeyer R, Lignitz E, Madea B. Fatty degeneration in renal tubule epithelium in accidental hypothermia victims. Forensic Sci Int 2004;141:131-5.

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the Paper	Conceptualization of work, performed literature review, conducted data analysis on all samples, interpreted data, and wrote manuscript		
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PUBLISHED MANUSCRIPT

Lethal Hypothermia in an Animal Model, Not Associated with Basal Renal Epithelial Vacuolization

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ABSTRACT A rodent model was used to evaluate the association between hypothermia and basal vacuolization in renal tubular epithelial cells. 28 Sprague Dawley rats were anaesthetized in non-stressful conditions and placed two at a time into a cooling chamber. Body core temperatures dropped to a minimum of 7-10°C, causing death under anaesthesia at times varying from 120 to 240 minutes. The animals were then subjected to necropsy; the kidneys were removed and placed in 10% buffered formalin. Examination of haematoxylin and eosin-stained renal sections failed to reveal basal vacuolization of renal tubular epithelial cells in any of the 28 animals. In this model, no evidence of subnuclear lipid vacuolization of renal tubular cells could be demonstrated despite significant and eventually lethal hypothermia. These results lend support to the hypothesis that the basal vacuolization in hypothermia may be a manifestation of a more complex pathophysiological pathway rather than being due simply to low body core temperatures.

Key words: hypothermia, basal vacuolization, Armanni-Ebstein, death, ketoacidosis, diabetes

Introduction

Deaths from hypothermia are encountered in forensic mortuaries in both temperate and cold climates[1]. Unfortunately the findings at autopsy, although characteristic, are not diagnostic, and their underlying pathophysiology is poorly understood[2]. Typical features include Wischnewsky spots of the gastric mucosa in 40-90% of cases, pinkish discoloration of the skin over larger joints, and acute pancreatitis with haemorrhage and surrounding fat necrosis[3,4]. Microscopic findings have included vacuolization of hepatocytes, cardiac myocytes and renal tubular epithelial cells[4,5]. It has been suggested that basal lipid vacuolization in renal epithelial cells could serve as a potential diagnostic tool for identifying hypothermic deaths, as it has an "equal value of diagnostic sensitivity compared to that of Wischnewski-ulcers"[5].

However, basal vacuolization of renal tubular epithelial cells may be also caused by underlying metabolic derangements, rather than by the direct effects of hypothermia[6-9]. A recent study by the authors demonstrating basal vacuolization in 15 out of 46 cases of hypothermia (33%), revealed that 9 of the 15 cases (60%) had a documented history of diabetes mellitus (which is known to be associated with basal vacuolization)[6]. Vitreous humour biochemical analyses were performed in seven of these cases, all of which demonstrated diabetic ketoacidosis[6]. Based on these findings we suggested that diabetic ketoacidosis may be a more significant factor in the generation of basal vacuolization than hypothermia in isolation[6]. We also noted that hypothermia might, in fact, be both a cause and a complication of diabetic ketoacidosis, which accounts for 11.8% of hospital admissions of all patients with markedly low core temperatures[6,10]. The same may apply to alcoholic ketoacidosis, which is also known to be associated with the formation of renal basal vacuolizations[13,14]; e.g. a study of 51 fatal hypothermic deaths in Sweden revealed that in 47%

of cases the subjects were long-term alcoholics, and that 65% had detectable levels of ethanol in the blood and/or urine[11].

We chose to use a rat model to specifically test whether basal vacuolization of renal tubular epithelial cells could be caused by lethal hypothermia in isolation.

Materials and methods

A model using male Sprague Dawley rats was developed to assess the effects of hypothermia in anaesthetized, minimally-stressed animals[12]. Twenty–eight rats were given an acclimatization period of one week with free access to food, water, and social interaction to reduce psychological and physiological stress. They were then sequentially anaesthetised with isoflurane at a flow rate of 3-5% and an oxygen flow rate of 1-2ml/min prior to placement into a cooling chamber. The isoflurane flow rate was decreased to 1-2% during the procedure to avoid significant respiratory depression whilst maintaining deep sleep. Constant monitoring of body core temperatures was achieved using a rectal temperature probe until death occurred. Core temperatures dropped over time to a minimum of 7-10°C which resulted in death of the animals under anaesthesia at times ranging from 120 to 240 minutes after placement in the cooling chamber. The animals were then subjected to necropsy and the kidneys removed and immediately placed in 10% buffered formalin. Routine haematoxylin and eosin stained slides were then prepared and examined. This study was fully approved by the Animal Ethics Committee at The University of Adelaide.

Results

Microscopic examination of coronal sections through each kidney from all 28 animals failed to reveal any evidence of basal vacuolization in the renal tubular epithelial cells.

Although occasional sections displayed areas of non-specific, irregular cytoplasmic vacuolization, sections of the kidneys were generally unremarkable. (The latter vacuolization was thought to represent an artefact due to delayed fixation of deeper tissues.)

Discussion

Although lipid-containing vacuoles have been found in the basal portions of renal tubular epithelial cells in cases of hypothermia in humans[5,6] the pathophysiology remains uncertain. It appears likely that such deposits may occur when there has been a significant metabolic disruption resulting in lipid mobilization from tissue stores, as occurs with ketoacidosis arising from a number of different conditions including diabetes mellitus, alcoholism and starvation[13-16]. Over the last decade, the term "Armanni-Ebstein phenomenon" has been applied broadly to include descriptions of subnuclear lipid vacuolizations of renal tubular epithelial cells in the outer medulla; however, a recent review has clarified the precise use of the Armanni-Ebstein term, indicating that this classification should refer specifically to clear cell changes of swollen, rounded and transparent cells having peripherally displaced nuclei associated with hyperglycemia[17].

In the present study there was no evidence of Armanni-Ebstein changes, nor of subnuclear lipid vacuolization of tubular cells, despite the imposition of significant and eventually lethal hypothermia. The rats used in this study had been subject to minimal psychological and physiological stress, and had been in good physical condition, therefore creating a suitable model to study the effect of hypothermia in isolation[12].

A possible limitation of this study is that the rat model might not develop the subnuclear vacuolization phenotype. However, studies have shown that rats exposed to hypothermic conditions under stress do demonstrate other features associated with

hypothermia such as Wischnewsky spots of the gastric mucosa[18-22]. Sprague Dawley rats have also been successfully used in previous studies to manifest other renal histological changes such as Armanni-Ebstein lesions[23-26]. These studies suggest the rat is a valid animal model for exploring renal sequelae of pathophysiological conditions. A second possible limitation is that the time of cold exposure may have been too short to allow for the development of subnuclear vacuolization; however some animals did survive for hours with extremely low core temperatures. Of note, the rats in this study also did not develop Wischnewsky spots, which could be directly related to the role that stress may have in the pathological manifestations of hypothermia[12].

This study suggests that basal renal tubular cell vacuolization may not be caused solely by lethal hypothermia. In cases where renal pathology is observed following significant hypothermia, additional studies testing for underlying metabolic disturbances, perhaps involving lipid mobilization due to ketoacidosis, will be required to determine the potential role of disturbances of glucose metabolism associated with lowered core temperatures, in creating this phenotype.

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HYPEROSMOLARITY INDUCES ARMANNI-EBSTEIN-LIKE RENAL TUBULAR EPITHELIAL SWELLING AND CYTOPLASMIC VACUOLIZATION

CONTEXTUAL STATEMENT

AIM: To investigate the role of osmotic effect in the aetiology of Armanni-Ebstein lesions.

HYPOTHESIS: That Armanni-Ebstein lesions result from the metabolic sequelae of hyperglycaemia and not the osmotic effect of glucose.

COMMENTARY: Armanni-Ebstein lesions have been reported in hyperglycaemic conditions including diabetic ketoacidosis and hyperglycaemic hyperosmolar nonketotic coma. The vacuoles in Armanni-Ebstein lesions contain accumulated glycogen, which suggests that their pathogenesis is related to glucose metabolism. However, glucose molecules are also osmotically active, and hyperglycaemia also causes serum hyperosmolarity which alone, is known to cause vacuolization in renal tubular epithelial cells (termed “osmotic nephrosis”). Therefore, this study uses intravenous mannitol to simulate the osmotic effect of glucose and determine if Armanni-Ebstein lesions are on the spectrum of changes induced by hyperosmolarity, or whether the phenomenon is specific to glucose metabolism.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript is written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSION: Armanni-Ebstein lesions were not reproducible by simulating the osmotic effects of glucose, suggesting that these lesions likely result from the metabolic sequelae of hyperglycaemia.

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Hyperosmolarity Induces Armanni-Ebstein-like Renal Tubular Epithelial Swelling and Cytoplasmic Vacuolization

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ABSTRACT: Armanni-Ebstein lesions have been considered pathognomonic for diabetes mellitus, and appear as markedly swollen renal tubular epithelial cells with cytoplasmic clearing and glycogen accumulation. However, the extent to which hyperosmolarity contributes to the Armanni-Ebstein phenotype is unclear. Ten sheep were injected intravenously with 20% mannitol at 11mOsm/kg and subsequent histological evaluation of the kidneys showed variable degrees of osmotic nephrosis and cytoplasmic clearing of renal tubular epithelial cells similar to that seen with Armanni-Ebstein lesions. However, although morphological changes similar to Armanni-Ebstein lesions could be produced, no intracytoplasmic glycogen was demonstrated with periodic-Acid-Schiff (PAS) stain. This suggests that while hyperosmolarity may contribute to the development of an Armanni-Ebstein phenotype, glycogen accumulation may result from the more complex metabolic effects of glucose on renal tubular epithelial cells. Thus, when Armanni-Ebstein-like vacuolizations are seen at autopsy, a confirmatory PAS stain is recommended because of the potential effect of hyperosmolar states.

Keywords: Forensic science, Armanni Ebstein phenomenon, diabetes mellitus, mannitol, osmotic nephrosis

Introduction

The Armanni-Ebstein phenomenon refers to markedly swollen renal tubular epithelial cells with small dark nuclei that are often peripherally displaced, and almost complete conversion of the cytoplasm into a single large vacuole filled with glycogen (1,2). These lesions occur in poorly controlled diabetic states such as diabetic ketoacidosis. Although their pathogenesis has been consistently attributed to hyperglycemia and subsequent glucosuria in both autopsy and animal studies (2-5), it is unclear whether the vacuoles occur secondary to the osmotic effect exerted by glucose, or whether the pathogenesis is specific to the chemical properties of glucose molecules. To investigate this, serum hyperosmolarity was induced in a sheep model using mannitol, a structurally similar osmotic agent to glucose, with subsequent histological analysis of the kidneys to look for the presence of Armanni-Ebstein lesions.

Materials and methods

This project used scavenged sheep kidneys from a study approved by the local animal ethics committees of SA Pathology and The University of Adelaide, South Australia (6). Animal studies were performed according to the guidelines established by the National Health and Medical Research Council (NHMRC).

As part of a neurotrauma study (6), a subgroup of ten anesthetized male merino sheep were intravenously administered 20% mannitol (11 mOsm/kg) over a 10-15min period and euthanized by barbiturate overdose 4 hours later for tissue analysis. Animals were initially anesthetized by intravenous injection of thiopentone before intubation and ventilation (4L/min) with oxygen enriched (30-35%) air containing 2.5% isoflurane. Ventilation parameters were adjusted as necessary on the basis of arterial blood gases sampled at regular intervals. Isoflurane was then lowered to 1.0-1.5% subsequent to insertion of a femoral

catheter, and an intravenous infusion of ketamine (4mg/kg/h) was initiated for ongoing anesthesia. Mean arterial blood pressure was continuously monitored via a femoral arterial catheter using a MacLab data acquisition system, while core body temperature was maintained using a thermostatically controlled heating pad.

After 4 hours, animals were euthanized by barbiturate overdose. Sheep were then intravenously perfused with 10% buffered formalin for 15 minutes before the kidneys were surgically removed and immediately immersed in 10% formalin for fixation. Representative sections of the kidneys were taken, and routinely processed, embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E). Periodic Acid-Schiff (PAS) stain for glycogen was done in representative cases.

Results

Histological examination of the kidneys showed variable degrees of osmotic nephrosis with a uniform degree of non-specific vacuolization of renal tubular epithelial cells (Figure 1). There was also an associated variable degree of cytoplasmic clearing of tubular epithelial cells in all cases ranging from incomplete clearing with undisplaced central and normal appearing nuclei (Figure 2), to complete cytoplasmic clearing with small dark peripherally displaced nuclei (Figure 3) suggestive of Armanni-Ebstein lesions. PAS staining in two cases with Armanni-Ebstein-like lesions did not show any PAS-positive material within the affected tubules (Figure 4).

Discussion

The Armanni-Ebstein phenomenon refers to vacuolated renal tubular epithelial cells, and was first described by Luciano Armanni in 1877 (7). Microscopically, these cells show a

loss of their normal cuboidal shapes, are markedly swollen with small dark nuclei that were often peripherally displaced, and have almost complete conversion of the cytoplasm into a single large vacuole (1,2). Affected cells often bulge into and occlude the tubular lumen (2). Intravacuolar glycogen accumulation can be demonstrated on periodic Acid-Schiff (PAS) staining and on electron microscopy (3). Microdissection studies have shown that Armanni-Ebstein lesions were consistently localized astride the corticomedullary junction, and principally affect the terminal straight portion of the proximal convoluted tubule (2). These lesions have been reported in poorly controlled diabetic states such as diabetic ketoacidosis (2,8), with a demonstrated correlation with hyperglycemia in animal studies (3-5,9). Thus, their presence has been deemed pathognomonic for diabetes (2). It is proposed that vacuolizations reflect marked glucosuria and the excessive reabsorption of glucose (8), however, the extent to which hyperosmolarity contributes to the development of these lesions is unclear.

The current study used scavenged sheep kidneys from a neurotrauma study investigating the effects of substance P antagonists as agents to reduce intracranial pressure (ICP) following traumatic brain injury (6). Four study groups were involved including uninjured controls, and injured animals that were administered intravenous normal saline, 20% mannitol (11 mOsm/kg), or 2.5mg/kg n-acetyl-tryptophan (substance P antagonists). ICP monitoring revealed significantly increased ICP ($p<0.05$) to approximately 20 mmHg by 30 minutes after traumatic brain injury compared to the uninjured controls (ICP 7 ± 2 mmHg). The authors found a continued increase in ICP in saline treated animals, and a reduction in ICP following administration of mannitol or substance P antagonist, concluding that substance P antagonists may offer a novel therapeutic approach to the treatment of acute brain

injury (6). The kidneys from the group administered with 20% intravenous mannitol were analyzed in this study.

This study is subject to limitations as a result of the scavenged nature of the tissues. Kidneys from the uninjured control group or the group administered with saline were not obtained at the time of the experiments, therefore there is an absence of a control group to compare the histological changes induced by mannitol. In hindsight, this would have been greatly beneficial to the study. Serum osmolarity was not measured in the sheep post administration of mannitol as it was not deemed relevant to the initial study from which the kidneys were scavenged (6). In the absence of this, a surrogate marker for increased serum osmolarity can be drawn from the significantly reduced ICP at 3 ($p<0.05$) and 4 hours ($p<0.01$) in mannitol treated animals compared to saline treated animals (6), although ideally, the availability of serum osmolarity as a direct correlate would be better. Animals were also given other medications as part of the anesthetics including intravenous thiopentone and ketamine, and inhalation of isoflurane. The histological effects of these agents on the renal tubular epithelium, if any, is not well documented in the literature so it is not known whether they could have impacted on the observed pathology. The current study is also limited by low numbers and further studies are warranted.

Mannitol was used as a substitute for glucose to induce hyperosmolarity and investigate whether the phenotypic features of Armanni-Ebstein lesions could be induced by an osmotic effect. Mannitol ($C_6H_8(OH)_6$) is a six-carbon polyol (sugar alcohol) of the sugar mannose with a molecular mass of 182, and a chemical structure very similar to that of glucose and sorbitol (10,11). After intravenous injection, however, mannitol is metabolically inert, distributes almost entirely in the extracellular space, is filtered at the glomerulus and then excreted unchanged in the urine (10). Studies have shown that the elimination half-life

after a single dose of 0.5 to 0.71g/kg of mannitol varies widely in humans from 39 to 103 minutes with a mean of 71 minutes (12). Due to its osmotic properties, intravenous mannitol has been used clinically to reduce intracranial and intraocular pressures, as a diuretic agent, for renal protection in transplant surgery, and for the prevention of nephrotoxic renal failure caused by radiocontrast, myoglobin, and cisplatin (10,11,13).

The glucose molecule ($C_6H_{12}O_6$) is structurally similar to mannitol, and it is a key fuel for mammalian cells, as well as being an important metabolic substrate (14). In contrast to mannitol, approximately 90% of filtered glucose is reabsorbed in the proximal convoluted tubules via a low affinity, high capacity sodium glucose transporter, SGLT2, located on the apical membrane. Residual glucose is then absorbed by the high affinity and low capacity SGLT1 located in the proximal straight tubules (14,15). Two basolateral membrane glucose transporters, the low-affinity GLUT2 and high affinity GLUT1, then facilitate transcellular glucose transport (14,15). There is also evidence to suggest that there is increased expression of renal glucose transporters in diabetics (15), with hyperglycemia in diabetes leading to enhanced proximal tubular glucose reabsorption (16). Glucosuria only develops once the filtered glucose exceeds the renal threshold for reabsorption. Additionally, glucose molecules are metabolically active and induce a range of effects on proximal tubular cells, including increase synthesis and secretion of vasoactive hormones (e.g. angiotensin II), cytokines (e.g. transforming growth factor-β), and extracellular matrix proteins (16). Hyperglycemia has also been shown to increase the formation and accumulation of intracellular sorbitol via metabolism of glucose by aldose reductase (16).

In the present study, intravenous injection of 20% mannitol in sheep induced vacuolization of renal tubular epithelial cells in all ten cases with variable degrees of osmotic nephrosis and some tubules exhibiting cytoplasmic clearing and Armanni-Ebstein-like

changes. Compared to the classical Armanni-Ebstein lesion, the membranes of the individual swollen and vacuolated epithelial cells were, however, less distinct, with variable nuclear changes ranging from normal appearing undisplaced nuclei to more characteristic peripherally displaced small dark nuclei. Mannitol-induced cytoplasmic clearing was also more commonly observed as a field effect with many adjacent tubules also affected; this is in contrast to traditional Armanni-Ebstein lesions in which the affected tubule is surrounded by normal unaffected tubules. PAS staining of affected tubules did not demonstrate any intravacuolar glycogen presumably because filtered mannitol is excreted and remains metabolically inert.

This study has demonstrated that the histological appearance of Armanni-Ebstein lesions can be reproduced by mannitol infusion, although no intravacuolar glycogen was present. This suggests that hyperosmolarity may contribute to the formation of Armanni-Ebstein lesions, but that the accumulation of glycogen is more specific to the metabolic effects of the glucose molecule on renal tubular epithelial cells. Thus, when Armanni-Ebstein-like tubules are seen on routine sections of the kidneys, a subsequent PAS stain should be conducted to demonstrate glycogen because of the possibility of these changes being induced by non-diabetic hyperosmolar conditions. Further controlled studies are warranted as the current study was subject to limitations due to the scavenged nature of the kidneys.

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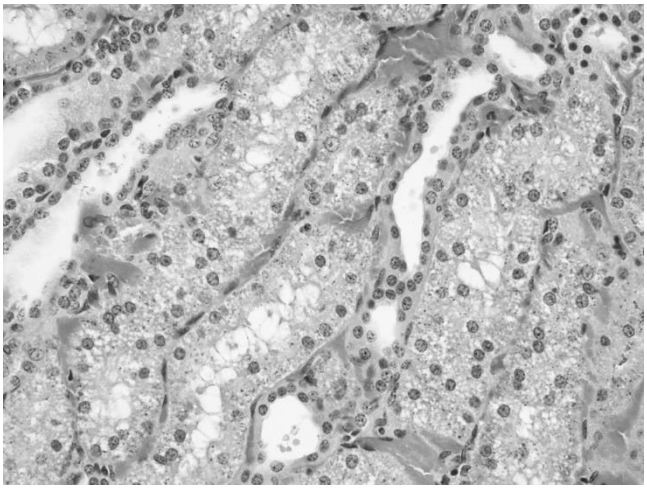

FIG. 1-*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). There is non-specific uniform vacuolization of renal tubular epithelial cells consistent with osmotic nephrosis (Hematoxylin & eosin, H&E x 40).*

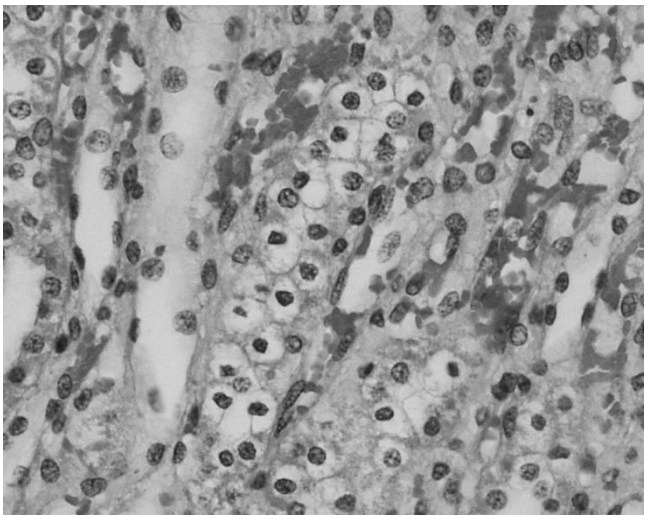

FIG. 2-*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). There is incomplete cytoplasmic clearing with undisplaced normal appearing nuclei (Hematoxylin & eosin, H&E x 40).*

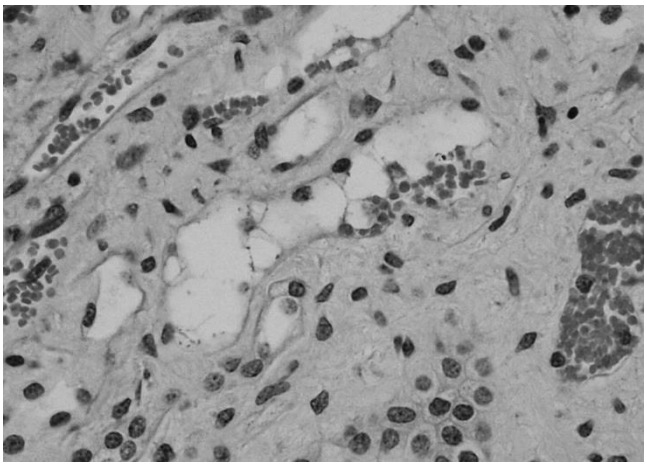

FIG. 3-*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). There is complete cytoplasmic clearing with peripherally displaced small dark nuclei resembling the Armanni-Ebstein lesion (Hematoxylin & eosin, H&E x 40).*

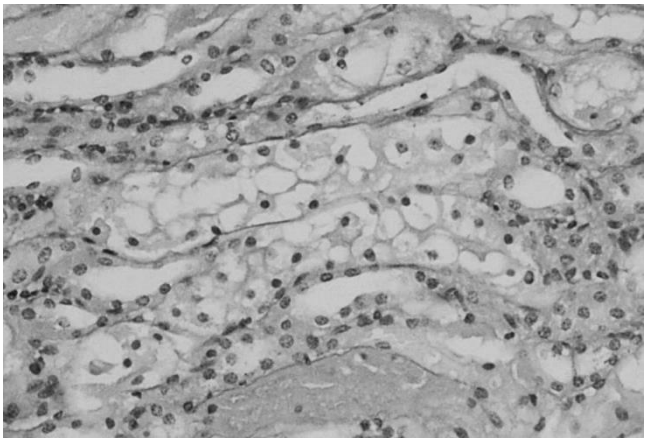

FIG. 4-*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). No glycogen can be demonstrated within the vacuolated tubular cells (Period Acid-Schiff, PAS x 40).*

AN ISOLATED PERFUSED RAT KIDNEY MODEL FOR THE EVALUATION OF THE EFFECT OF GLUCOSE ON RENAL TUBULAR EPITHELIAL MORPHOLOGY

CONTEXTUAL STATEMENT

AIMS:

1. To develop an isolated perfused kidney model capable of reproducing renal tubular epithelial vacuolizations secondary to metabolic derangements.
2. To utilize the isolated perfused kidney model to reproduce Armanni-Ebstein lesions.

HYPOTHESES:

1. That the isolated perfused kidney model is a viable ex-vivo model for the investigation of renal tubular epithelial vacuolizations
2. That Armanni-Ebstein lesions are reproducible in an isolated perfused kidney model.

COMMENTARY: The isolated perfused kidney model has been used for pharmacologic investigations into drug metabolism and excretion, however, to our knowledge it has never been used for histologic studies. This model allows for a single rat kidney to be perfused with physiologic concentrations of electrolytes, with or without the addition of controlled concentrations of other substrates. Therefore, this model was tested to determine if it could be a viable means for the evaluation of renal tubular epithelial histology, and whether Armanni-Ebstein lesions could be reproduced by perfusing kidneys with high concentrations of glucose.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSIONS:

1. The isolated perfused kidney model is a viable ex-vivo model for the investigation of renal tubular epithelial vacuolization.
2. Armanni-Ebstein lesions were not reproduced in the isolated perfused kidney model, suggesting that this change requires more time to develop or involves factors in addition to hyperglycaemia.

STATEMENT OF AUTHORSHIP

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Conceptualization of work, wrote animal ethics application, performed literature review, established isolated perfused kidney model, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	20/10/16

Name of Co-Author	Prof. Andrea J. Yool		
Contribution to the Paper	Supervised development of work, helped to obtain ethics approval, helped in establishing isolated perfused kidney model and data collection, helped to evaluate and edit the manuscript		
Signature		**Date**	20 Oct 2016

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
Signature		**Date**	24/10/16

PUBLISHED MANUSCRIPT

An Isolated Perfused Rat Kidney Model for the Evaluation of the Effect of Glucose on Renal Tubular Epithelial Morphology

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ABSTRACT: An isolated perfused kidney model was used to evaluate the effect of hyperglycemia on renal tubular epithelial cell morphology. Ten Sprague Dawley rat kidneys were perfused with Krebs Henseleit buffer containing 70mmol/l of glucose (5 for 1 hour, 5 for 2 hours). Two control groups consisted of 10 kidneys perfused with Krebs Henseleit Buffer without hyperglycemia (5 for 1 hour, 5 for 2 hours), and 10 non-perfused contralateral kidneys placed in the same environment for the same duration. The hyperglycemia group had significantly increased renal tubular vacuolization ($p<0.001$) compared to both control groups at 1 and 2 hours. The isolated perfused kidney model recapitulates the renal tubular vacuolization phenotype found in hyperglycemia and may be a potential tool for the investigation of causal factors in renal histology. The full pattern of the Armanni-Ebstein phenomenon was not, however, reproduced, suggesting that this change requires more time or involves more complex factors.

Keywords: Forensic science, Armanni Ebstein phenomenon, nephrotic syndrome, isolated perfused kidney, diabetes mellitus, hyperglycemia

Introduction

Identifying histologic changes in renal tubular cells is useful in a variety of clinical situations, such as in the diagnosis of calcineurin inhibitor nephrotoxicity (1) and acute tubular necrosis. In addition, it has been shown to be useful in a forensic context in the diagnosis of diabetic, alcoholic, and starvation ketoacidosis (2-4). Studies have reported the morphology, constituents, and associated metabolic conditions of various types of renal tubular epithelial changes, such as in Armanni-Ebstein lesions and basal lipid vacuolization; however, the pathogenesis of such lesions remains unclear.

The Armanni-Ebstein lesion appears as swollen epithelial cells with almost complete conversion of the cytoplasm into a single large vacuole filled with glycogen (2,5). Although these lesions appear in hyperglycemic states (2,6,7), it has remained uncertain whether elevated glucose levels alone can directly cause this change. Renal biopsies are not routinely undertaken in metabolic states such as diabetic ketoacidosis and hyperglycemic hyperosmolar nonketotic coma, since the outcomes would not affect patient management, thus making controlled studies difficult. Previous investigations have focused solely on in vivo or post-mortem studies, thus the histological consequence of hyperglycemia in isolation on renal tubular epithelial cells is unknown.

The current study utilized an in vitro isolated perfused rat kidney model to directly control the duration and magnitude of hyperglycemia, in the absence of other confounding metabolic derangements (such as hyperlipidemia and ketoacidosis), to determine if a viable model could be established for linking physiological conditions to renal histological outcomes, and to evaluate whether Armanni-Ebstein lesions could be reproduced by high glucose exposure alone.

Materials and methods

The use of scavenged Sprague Dawley rats for this study was approved by the local University Animal Ethics Committees at both SA Pathology and The University of Adelaide, South Australia. Animal studies were performed with approved protocols, in accordance with the guidelines established by the National Health and Medical Research Council (NHMRC).

Dextran (MW = 48,000-90,000), bovine serum albumin (MW = 66,000), sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), monopotassium phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), L-cysteine ($C_3H_7NO_2S$), glycine ($C_2H_5NO_2$), L-glutamic acid ($C_5H_9NO_4$), D-glucose ($C_6H_{12}O_6$), and heparin sodium salt (50mg/ml) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The isolated perfused kidney system and perfusion medium parameters were based on previously described methods (8). The perfusate for the control treatment group (n=10) consisted of Krebs-Henseleit buffer (pH 7.4) containing sodium chloride (118 mmol/l), potassium chloride (4.7 mmol/l), calcium chloride (1.25 mmol/l), magnesium sulfate (1.24 mmol/l), monopotassium phosphate (1.2 mmol/l), sodium carbonate (25 mmol/l), D-glucose (5 mmol/l), L-cysteine (0.5 mmol/l), glycine (2.3 mmol/l), L-glutamic acid (0.5 mmol/l), bovine serum albumin (6.5 g/l), and dextran (36 g/l). The perfusate for the hyperglycemia treatment group (n=10) contained the above, with the addition of 70 mmol/l of D-glucose. 1mg (200 units) of heparin sodium salt in 1ml was added to the perfusates at the beginning of each experiment. The perfusion media were filtered through 1.2 and 0.45 μm filters (Millipore, Bedford, MA, USA) prior to use and equilibrated with a mixture of moistened 95% oxygen and 5% carbon dioxide for 1 hour before and during the kidney perfusion process. The non-perfused group (n=10) consisted of the contralateral kidneys of the control

group placed in the same environment for the same duration of time without being connected to the recirculating perfusion system.

Male and female Sprague Dawley rats >200g were euthanized individually using carbon dioxide (CO_2) inhalation. The abdominal cavities were opened and the intestines and liver were ligated and removed. Retroperitoneal soft tissue was dissected to expose the vena cavae, abdominal aorta, and to free the kidneys (Figure 1). The renal artery was then cannulated through its ostia in the abdominal aorta with a 20 gauge intravenous cannula pre-infused with perfusate including heparin (Figure 2). Both the cannulated kidney and the contralateral kidney were removed and immediately placed on a tray on ice for transportation to the isolated perfusion system.

The kidneys were placed into a thermostatically controlled cabinet at 34-37.5°C. The cannulated kidney was connected to the recirculating system containing 180ml of perfusate, recycled using a peristaltic pump at approximately 32-38 ml/min with continuous adjustments as necessary to maintain renal artery perfusion pressure at 110 ± 20 mmHg, which was monitored using a manometer. The post-mortem interval between death and the initiation of perfusion was recorded for each case. Perfusion of the kidney was monitored by the macroscopic color change of the parenchyma from dark maroon to yellow/tan and the change in the perfusate color from dark red to clear as the residual red blood cells were flushed out. The temperature, flow rate, renal arterial pressure, and macroscopic color changes were routinely recorded at 5 minute intervals. After the designated perfusion time of 1-2 hours, both the perfused and non-perfused kidneys were bisected and immediately placed in 10% buffered formalin for overnight fixation. Kidneys were then routinely processed, embedded in paraffin wax, sectioned and stained with Hematoxylin and Eosin (H&E) and Period Acid-Schiff (PAS) for histological assessment. In the perfused kidney, tissue areas that showed no

remaining red blood cells within the vessels were considered to have been adequately perfused and were assessed histologically for vacuolization.

Ten adjacent fields of each kidney were photographed at 400x magnification, and assessed for the degree of vacuolization using previously described methods (9) to determine the percentage area of unstained tissue. Differences between the high glucose concentration group and the two control groups were assessed by one-way ANOVA, followed by post hoc Tukey HSD test. To determine whether there were significant differences between the percentage area of vacuolization at 1 and 2 hours within each group, initial tests for the equality of variances were conducted using an F-test followed by the appropriate t-test (with or without equal variance).

Results

Control Treatment Group

Ten Sprague Dawley rats, with a male to female ratio of 4:7, and whole animal weights ranging from 206 to 420g (mean=284.8g) were used in the perfused control group, in which kidneys were perfused with Krebs Henseleit Buffer without added glucose. Kidneys from five rats (3 male, 2 female; weight range from 226 to 350g (mean 293.5g) were perfused for 1 hour. Their post-mortem interval ranged from 20 to 30 minutes (mean = 27 minutes). During the perfusion, the temperature was maintained between 34°C to 37°C (mean = 36.1°C), with a flow rate of 34 to 36 ml/min (mean = 35.2 ml/min) required to maintain a renal arterial pressure of 100 to 122 mmHg (mean 110.4 mmHg). The time required for the efferent perfusate to become clear and for the majority of the visible surface of the kidney to change to a yellow/tan color ranged from 10 to 15 minutes (mean 14 minutes). Non-specific

tubular epithelial vacuolizations were observed histologically, and the unstained area per high power field ranged from 3.14 to 10.59% (mean 5.73%).

Kidneys from five female rats weighing from 206 to 320g (mean = 255g) and with a post-mortem interval ranging from 20 to 40mins (mean 26.6 minutes) were perfused for 2 hours. Their temperatures were maintained between 34 to 37.5°C (mean = 36.8°C), with a flow rate of 33.4 to 35.8 ml/min (mean = 35.1 ml/min) required to maintain a perfusion pressure of 100 to 128 mmHg (mean = 111.1 mmHg). The time taken for efferent perfusate to clear ranged from 10 to 15 minutes (mean = 11 minutes), and the majority of the kidneys changed color in 2 to 10 minutes (mean = 8.4 minutes). Non-specific tubular vacuolizations (Figure 3) and occasional tubules with cytoplasmic clearing and normal nuclei were observed, with an unstained area ranging from 12.33 to 33.75% (mean 22.15%).

Hyperglycemia Treatment Group

Kidneys from ten female Sprague Dawley rats weighing between 202 to 260g (mean = 234.7g) were perfused with Krebs Henseleit Buffer with 70 mmol/l of D-glucose. Five kidneys were perfused for 1 hour. In this group, whole animal weights ranged from 202 to 247g (mean 226.4g), and the post-mortem intervals were 25 to 50 minutes (mean = 33 minutes). During perfusion, temperature was maintained between 34°C to 37°C (mean = 36.4°C), with a flow rate between 34 to 36.2 ml/min (mean = 35.1 ml/min) to maintain a renal arterial pressure of 102 to 126 mmHg (mean 112.7 mmHg). The time for the efferent perfusate to clear ranged from 5 to 15 minutes (mean 10.2 minutes) and the time for the majority of the kidney to change to yellow/tan ranged from 5 to 25 minutes (mean = 14.2 minutes). Histologically, isometric fine vacuolizations of tubular epithelial cells consistent with osmotic nephrosis and occasional cytoplasmic clearing with normal appearing nuclei

were seen, however PAS stain did not demonstrate any glycogen deposition. The percentage of unstained area per high power field ranged from 15.83 to 43.90% (mean 31.23%).

Kidneys from five female rats weighing from 210 to 246g (mean = 243g) with a post-mortem interval ranging from 20 to 25mins (mean 21.6 minutes) were perfused for 2 hours. Their temperatures were maintained between 34 to 37°C (mean = 36.6°C), with a flow rate of 34 to 36.2 ml/min (mean = 35.5 ml/min) to maintain a perfusion pressure of 102 to 122 mmHg (mean = 112.8 mmHg). The time taken for efferent perfusate to clear ranged from 3 to 30 minutes (mean = 14 minutes), and the majority of the kidney changed color in 3 to 35 minutes (mean = 15 minutes). Isometric vacuolizations of osmotic nephrosis were observed histologically (Figure 3), along with numerous tubules demonstrating cytoplasmic clearing with normal appearing nuclei surrounded by non-vacuolated tubules (Figure 4). PAS staining in all cases did not demonstrate any accumulated glycogen (Figure 5). The unstained areas ranged from 15.24 to 44.67% (mean 34.64%) per high power field.

Non-perfused Group

Ten contralateral kidneys from animals used in the control and hyperglycemia treatment groups were placed adjacent to their corresponding perfused kidney, in the same perfusion cabinet, with the same temperature and environment, for the same amount of time. Analyses of non-perfused kidneys in the 1 and 2 hour experiments showed a total cleared area ranging between 1.069 to 18.09% (mean 4.79%), and 0.92 to 23.30% (mean 5.57%) per high power field, respectively. In both groups, occasional small non-specific cytoplasmic vacuoles were observed; additionally, tubules with cytoplasmic clearing and pyknotic nuclei were observed in the 2 hour group.

Quantitative Analyses

Analysis via one-way ANOVA revealed a statistically significant difference in renal tubular epithelial vacuolization in the group infused with 70 mmol/l of glucose compared with both control groups at 1 ($F(2,147)=697.47$, $p=<0.001$) and 2 hours ($F(2,147)=438.78$, $p=<0.001$). Quantitative comparisons of epithelial cell vacuolization between the different study groups are summarized in Figure 6.

The magnitude of the response correlated with glucose treatment. Post hoc comparisons using the Tukey HSD test revealed a significantly greater unstained area ($p<0.001$) in the high glucose group (M=31.23, SD=5.91) compared to the perfused control group (M=5.73, SD=1.53). The high glucose group also showed a significantly greater clear area than the non-perfused group (M=4.79, SD=3.34) after 1 hour. Similarly, after 2 hours, there was a significantly greater amount of cellular clearing in the high glucose group (M=34.64, SD=5.83), compared with the perfused control (M=22.15, SD 4.75), and non-perfused control (M=5.57, SD=4.02) groups. There were no statistically significant differences between the perfused control treatment group and the non-perfused control groups at one hour.

The F-tests indicated that the variances in the hyperglycemic groups ($F=0.104$, $p<0.001$) were not equivalent to those in the control groups ($F=0.104$, $p<0.001$). However, the variances for the 1 and 2 hour non-perfused kidneys ($F=0.690$, $p=0.099$) were not different.

The magnitude of the response in the hyperglycemic groups correlated with the time of exposure. The F-test indicated no significant difference in variances between the kidneys perfused for 1 hour and those perfused for 2 hours with hyperglycemic perfusate ($F=1.026$, $p=0.465$). Subsequent two-sample t-test assuming equal variances showed that the mean unstained area per high power field in the hyperglycemia treatment group at 1 hour (M=

31.23, SD=5.91) was significantly less than at 2 hours (M=34.64, SD=5.83), t(98)=-2.910, p=0.002.

Similarly, kidneys perfused with non-hyperglycemic Krebs Henseleit Buffer for 1 hour (M=5.73, SD=1.53) also showed significantly less clearing of tubules compared to the 2 hour group (M=22.15, SD=4.75), t(59)=-23.275, p<0.001. There was no significant difference in vacuolization amongst the 1 hour (M=4.79, SD=3.34) and 2 hour (M=5.57, SD=4.02) non-perfused kidneys, t(98)=-1.053, p=0.147.

Discussion

The in vitro isolated perfused rat kidney model has been extensively utilized for investigating renal drug handling (8,10-12), and the effects of drugs and various substances on renal vasculature (13,14), glomerular function (15,16), renal cellular metabolism (17), and gluconeogenesis (18). However, the model has not been tested for the investigation of etiologic factors causing changes in renal histology. Rodents are appropriate candidates for establishing a new model for renal histological studies as histological abnormalities noted in human kidneys, including the Armanni-Ebstein phenomenon, have been demonstrated previously with in vivo studies in rats (7,19,20).

The current study perfused kidneys with Krebs Henseleit Buffer with and without 70mmol/l of glucose. This concentration was chosen as it represents a ten-fold elevation in the threshold fasting glucose level of 7.0 mmol/l which is diagnostic for diabetes in humans (21). The degree of vacuolization after hyperglycemic treatment was significantly greater than that observed in two different control groups: (i) a perfused group without excess glucose which controlled for any histological changes induced by the perfusion system; and (ii) a non-perfused group which controlled for non-specific vacuolizations induced by post-mortem

autolysis. Results showed a statistically significant increase in renal tubular epithelial vacuolization in the group infused with 70 mmol/l of glucose compared to both control groups at 1 and 2 hours. Additionally, there was a significant increase in vacuolization among the kidneys perfused with high glucose for 2 hours compared to those perfused for 1 hour. This suggests that the isolated perfused kidney system is capable of inducing renal tubular epithelial vacuolizations that increase with perfusion time. The absence of accumulated glycogen (as determined by PAS staining) in the hyperglycemic treatment group may suggest that the pathogenesis of the Armanni-Ebstein lesion requires factors more complicated than simple exposure to hyperglycemia alone, or that cytoplasmic glycogen accumulation requires more than 2 hours of exposure to high glucose concentrations to develop.

Limitations of the system are the need to prevent thrombosis, and the limited duration of the treatment period that can be used to simulate a chronic disease condition (here not exceeding 2 hours). Prevention of thrombosis was addressed by the addition of 1mg (200 units) of heparin to the perfusate in each experiment. This dose was based on previous studies injecting 100 units of heparin into the penile vein of rats weighing between 346-459g (8) or 200 units injected into the renal vein (18). With sporadic thrombi, individual regions and tubules of the kidneys might have been perfused for less than the specified time. The reason why thrombi developed in some cases and not in others is unclear; perhaps more immediate delivery of heparin (e.g. antemortem) or a higher dose would facilitate better perfusion. While morphological changes that emulate human postmortem histology do develop in the ex vivo kidney model over time; the temporal limitation of this model is that the interpretation of vacuolization becomes increasingly more difficult with the onset of autolytic changes associated with increasing post-mortem interval.

To optimize the model, this study used dextran as an oncotic agent to substitute for a large portion of bovine serum albumin (BSA) in a ratio that has been shown previously to confer higher functional viability of the perfused kidney as compared with BSA alone; this was demonstrated by the preservation of the tubular reabsorption of water, glucose, and sodium (8). Dextran is preferred over BSA due to the significantly lower cost, and in pharmacokinetic investigations of drugs that are extensively bound to BSA (22). However, the histological consequences of this substitution are unclear.

The current study has demonstrated that the isolated perfused kidney model is a viable model for the study of renal tubular epithelial cell histology. A significantly greater degree of vacuolization was observed after treatment with high glucose compared to both control groups, which also correlated with the duration of exposure. The morphological features of Armanni-Ebstein lesions (i.e. swelling and cytoplasmic clearing of renal tubular epithelial cells) were observed in the hyperglycemia treatment group, however, intravacuolar glycogen which is a defining feature of classical Armanni-Ebstein lesions, was not demonstrated. Therefore, further studies need to be conducted, perhaps with the addition of ketones and/or regulatory/counter-regulatory hormones (e.g. insulin and glucagon), and over longer periods of time to reproduce glycogen accumulation.

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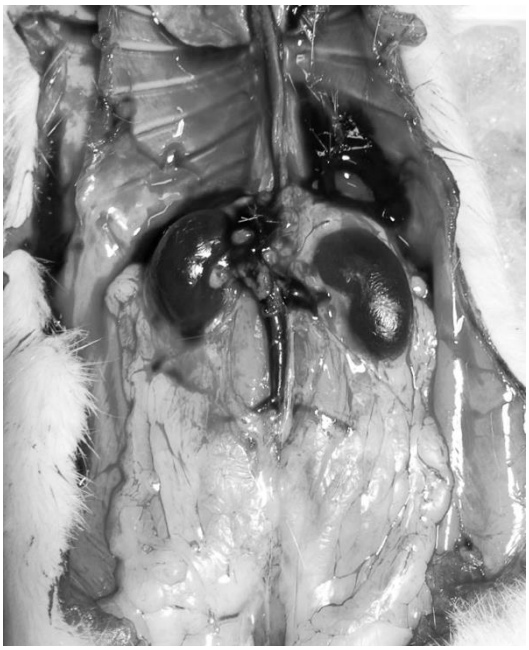

FIG. 1-*Dissection of Sprague-Dawley rat to expose the aorta, vena cava, and bilateral kidneys.*

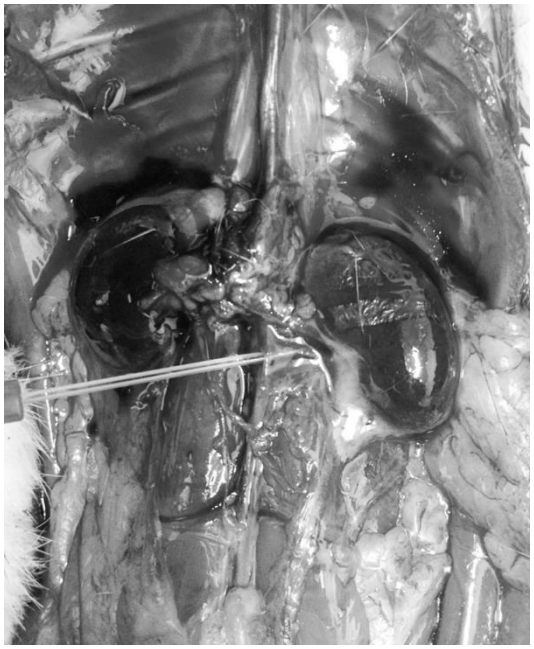

FIG. 2- *Cannulation of the left renal artery through its ostia in the abdominal aorta.*

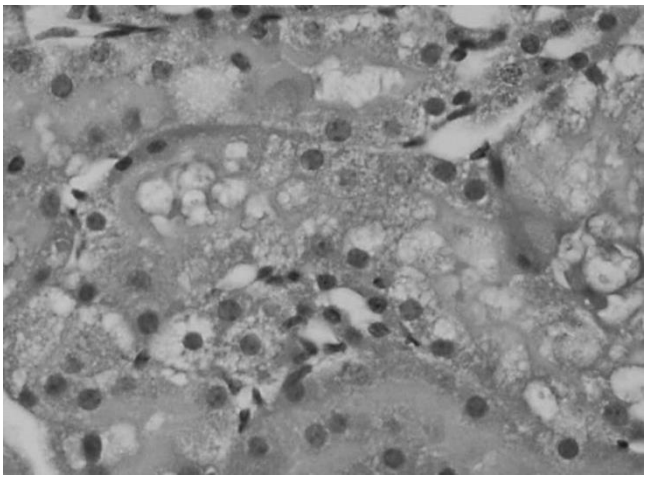

FIG. 3- *Osmotic nephrosis in a rat kidney perfused with Krebs Henseleit Buffer and 70mmol/l of glucose for 2 hours (Hematoxylin and eosin x400).*

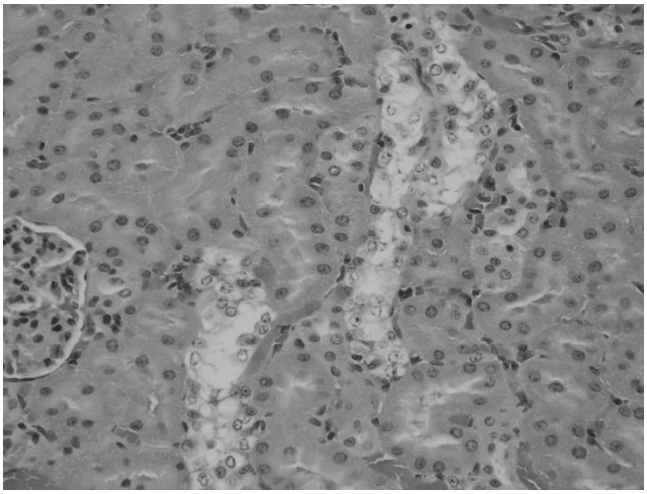

FIG. 4- *Swelling and cytoplasmic clearing of renal tubules in a rat kidney perfused with Krebs Henseleit Buffer and 70mmol/l of glucose for 2 hours (Hematoxylin and eosin x400).*

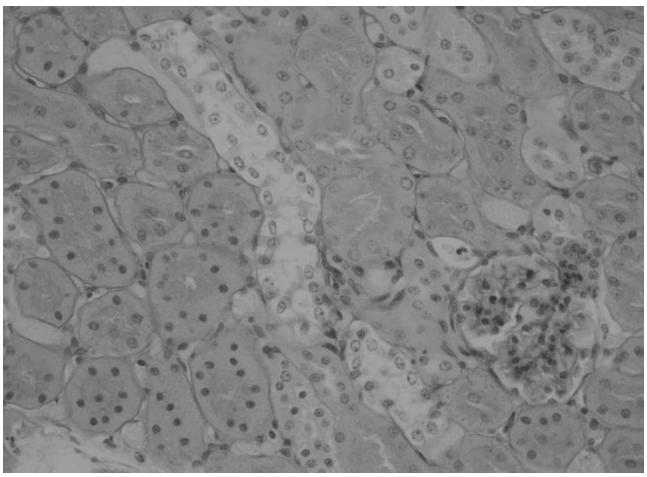

FIG. 5- *No glycogen within vacuolated tubular epithelial cells in a rat kidney perfused with Krebs Henseleit Buffer and 70mmol/l of glucose for 2 hours (Periodic Acid-Schiff x400).*

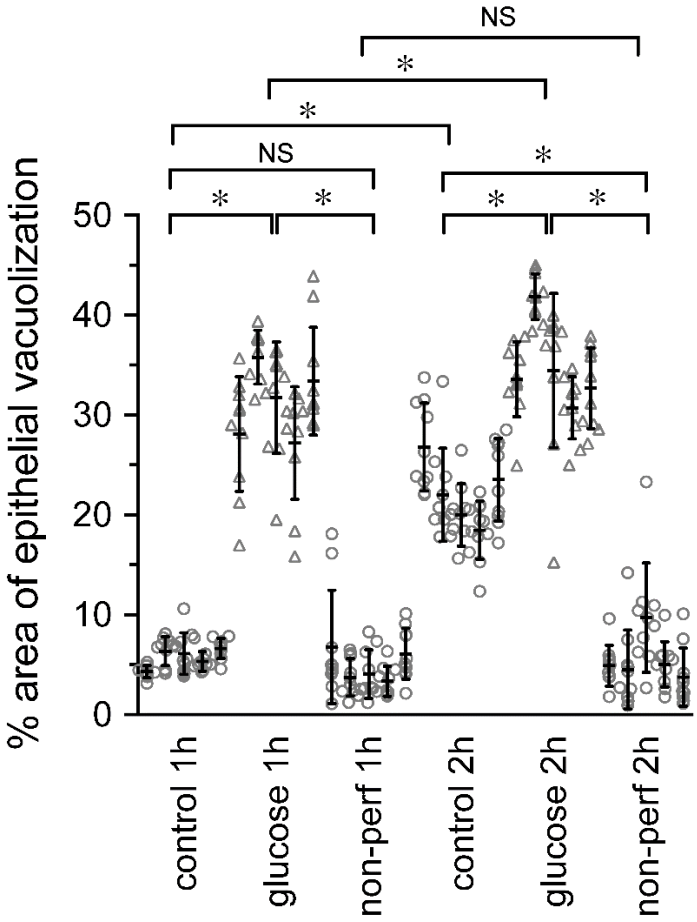

FIG. 6- *Scatterplot histogram summarizing the degree of renal tubular vacuolization between study groups. Statistically significant differences are indicated as (*) for p<0.001; NS is not significant.*

THE ETIOLOGY OF BASAL VACUOLIZATIONS IN RENAL TUBULAR EPITHELIAL CELLS EVALUATED IN AN ISOLATED PERFUSED KIDNEY MODEL

CONTEXTUAL STATEMENT

AIM: To utilize the isolated perfused kidney model to reproduce basal vacuolizations.

HYPOTHESIS: That basal vacuolizations are reproducible in an isolated perfused kidney model.

COMMENTARY: Hyperlipidaermic conditions, such as nephrotic syndrome, have been associated with basal lipid vacuolizations but of a different morphology to those observed in ketoacidosis (see Chapter 2). Therefore, the isolated perfused kidney model was utilized to determine if basal vacuolizations could be reproduced by adding increased concentrations of triglycerides to the perfusate, and whether the morphology of these vacuoles changed with the addition of ketones.

Literature review establishing the field of knowledge is discussed within the paper. This manuscript was written in American English to satisfy the publication requirements of the Journal of Forensic Sciences.

CONCLUSION: Isolated hypertriglyceridemia can cause basal lipid vacuolizations in an isolated perfused kidney model, but of a different morphology to those observed in

ketoacidosis. The addition of ketones did not alter the morphology of the vacuoles, suggesting that other factors are involved in the pathogenesis of basal vacuoles in ketoacidosis.

STATEMENT OF AUTHORSHIP

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Author Contributions

By signing the State of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principle Author (candidate)	Dr. Chong Zhou		
Contribution to the Paper	Conceptualization of work, wrote animal ethics application, performed literature review, established isolated perfused kidney model, conducted data collection and analysis on all samples, interpreted data, and wrote manuscript		
Signature		**Date**	20/10/16

Name of Co-Author	Prof. Andrea J. Yool		
Contribution to the Paper	Supervised development of work, helped to obtain ethics approval, helped in establishing isolated perfused kidney model and data collection, helped to evaluate and edit the manuscript		
Signature		**Date**	20 Oct 2016

Name of Co-Author	Prof. Roger W. Byard		
Contribution to the Paper	Helped in data interpretation, manuscript evaluation and editing, acted as corresponding author		
Signature		**Date**	24/10/16

PUBLISHED MANUSCRIPT

The Etiology of Basal Vacuolizations in Renal Tubular Epithelial Cells Evaluated in an Isolated Perfused Kidney Model

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ABSTRACT: To determine whether basal lipid vacuolization characteristic of ketoacidosis could be induced with short-term hypertriglyceridemia, adult Sprague-Dawley rat kidneys were perfused in an isolated perfused kidney model with, and without, 11.3mmol/l (10g/l) of triglycerides in Krebs-Henseleit Buffer, for 1 and 2 hours (n=5/group). Additional treatments included perfusion with triglycerides with 20mmol/l of β-hydroxybutyrate and 2mmol/l of acetoacetate (n=5); and perfusion with triglycerides with 70mmol/L of glucose (n=1). Basal vacuolization was produced in all groups, but differed in morphology to that reported in postmortem studies. There was no further increase in vacuolization after 2 hours of perfusion compared to 1 hour (p=0.24), and the addition of ketones did not alter the morphology or extent of vacuolization. This study using an ex-vivo model has confirmed that isolated hypertriglyceridemia is sufficient to cause basal lipid vacuolization in renal tubular epithelial cells, but with different morphology to vacuoles observed in lethal ketoacidosis at autopsy.

Keywords: Forensic science, basal vacuolization, subnuclear vacuolization, isolated perfused kidney, nephrotic syndrome, ketoacidosis, hyperlipidemia

Introduction

Renal tubular epithelial cells respond to metabolic insults with a variety of distinctive patterns of vacuolization, as is seen in osmotic nephrosis secondary to hyperosmolarity (1), the Armanni-Ebstein phenomenon secondary to hyperglycemia (2), hypokalemia with coarse irregular vacuolization (3), and calcineurin-inhibitor toxicity with secondary isometric vacuolization (4). Basal vacuolization, characterized by a row of subnuclear vacuoles in close proximity to the basement membrane, has been reported in postmortem samples from subjects with diabetic (5,6), alcoholic (7), septic (8) and starvation (9) ketoacidosis. Basal vacuolization of a different morphology, appearing as numerous circular vacuoles which begins basally and then spreads to involve the entire cell, has also been observed in cases of pediatric nephrotic syndrome (10). A consistent feature in all these conditions has been the demonstration of intravacuolar lipids with Oil Red O staining and by electron microscopy (5,9,10), suggesting that the etiology of the pathological phenotypes is linked to hyperlipidemia (5). However, all studies to date have involved post-mortem tissues, which have made the determination of a direct association between basal vacuolization and hyperlipidemia in isolation challenging. The use of a perfused kidney model has allowed separation of possible causal factors, with minimization of other confounding metabolic abnormalities that are often present in these conditions in vivo (11).

Materials and methods

This use of scavenged Sprague Dawley rats for this study was approved by the Animal Ethics Committees of SA Pathology and The University of Adelaide, South Australia. Animal studies were performed according to the guidelines established by the National Health and Medical Research Council (NHMRC).

The isolated perfused kidney system and perfusion medium composition were based on previously described methods (12). The perfusate consisted of Krebs-Henseleit buffer (pH 7.4) containing 118mmol/l of sodium chloride (NaCl), 4.70mmol/l of potassium chloride (KCl), 1.25mmol/l of calcium chloride ($CaCl_2$), 1.24mmol/l of magnesium sulphate ($MgSO_4$), 1.20mmol/l of monopotassium phosphate (KH_2PO_4), 25mmol/l of sodium bicarbonate ($NaHCO_3$), 5mmol/l of D-glucose ($C_6H_{12}O_6$), 0.5mmol/l of L-cysteine ($C_3H_7NO_2S$), 2.3mmol/l of glycine ($C_2H_5NO_2$), 0.5mmol/l of L-glutamic acid ($C_5H_9NO_4$), 6.5g/l of bovine serum albumin (MW = 66,000), and 36g/l of dextran (MW = 48,000-90,000) (Sigma-Aldrich, St. Louis, MO, USA). Intralipid 20% (Fresenius Kabi AB, Sweden), which contains triglyceride 20%, phospholipid 1.2%, and glycerol 2.2%, was added to the perfusate medium to achieve a triglyceride concentration of 11.3mmol/l (10g/l) (n=10). An additional treatment group (n=5), used to simulate concurrent ketoacidosis, was exposed to perfusate containing 20mmol/l of β-hydroxybutyric acid ($C_4H_8O_3$) and 2mmol/l methyl acetoacetate ($C_5H_8O_3$). A pilot treatment assay (n=1) used to simulate concurrent hyperglycemia contained 70mmol/l of D-glucose instead of the standard 5mmol/l in the perfusate medium. 1mg (200 units) of heparin sodium salt in 1ml was added to the perfusate solutions at the beginning of each experiment. The perfusion media were successively filtered through 1.2 and 0.45 µm filters (Millipore, Bedford, MA, USA) prior to use and equilibrated with a gaseous mixture of moistened 95% oxygen and 5% carbon dioxide for 1 hour before and throughout the kidney perfusion.

Adult Sprague Dawley rats (>200g each) were euthanized using carbon dioxide (CO_2) inhalation. The kidneys were subsequently cannulated through the renal artery via the previously described dissection techniques (11) and placed into a thermostatically controlled cabinet at 34-37.5°C. The cannulated kidney was connected to the recirculating system

containing 180ml of perfusate pumped using a peristaltic pump at approximately 32-38 ml/min with continuous adjustments as necessary to maintain renal artery perfusion pressure at 110 ± 20 mmHg. The post-mortem interval between time of death and the initiation of perfusion was recorded for each case. Renal perfusion was monitored via macroscopic color change from dark maroon to yellow/tan and a change in the efferent perfusate from dark red to clear as red blood cells within the kidney were flushed. The temperature, flow rate, renal arterial pressure, and macroscopic color changes were routinely recorded at 5 minute intervals. After perfusion for the designated time of 1-2 hours, a small portion of the kidney was excised and immediately placed in gluteraldehyde for fixation. The remainder of the kidney was bisected and placed in 10% buffered formalin for overnight fixation. Kidneys were then routinely processed, embedded in paraffin wax, sectioned and stained with Hematoxylin and Eosin (H&E) for histological assessment of basal vacuolization. Only perfused areas of the kidney (those with a microscopic absence of red blood cells) were assessed for vacuolization area and morphology.

The average area of vacuolization per high power field was assessed by photographing ten consecutive fields of each kidney at 40x magnification and calculating the percentage of negative staining in ImageJ using previously described methods (13). Analysis of statistically significant differences between the means of all the treatment groups began with a preliminary test for the equality of variances (F-test), and followed by the appropriate two-tailed t-test which either assumes or does not assume equal variances.

Results

Controls

The control group was taken from a previous study using the same perfusion system with the same concentration of constituents making up the Krebs-Henseleit buffer (11). This consisted of ten Sprague Dawley rats, with body weights ranging from 206 to 420g (mean 284.8g) which were perfused for one and two hours with Krebs-Henseleit buffer in the absence of added triglycerides or ketones. Non-specific tubular epithelial vacuolizations consistent with osmotic nephrosis were observed in all cases, with the unstained area per high power field ranging from 3.14 to 10.59% (mean 5.73%) in the kidneys perfused for 1 hour, and 12.33 to 33.75% (mean 22.15%) in the kidneys perfused for 2 hours. Separation of tubules from the basement membrane was focally observed in some cases, but no discrete subnuclear vacuolizations were present (11).

Hypertriglyceridemia in isolation

Ten Sprague Dawley rats, with body weights ranging from 210 to 342g (mean 270.2g) were used in the groups perfused with hypertriglyceridemia alone, at one and two hours. Five rats weighing between 210 to 246g (mean 225.2g) were perfused for 1 hour. Their post-mortem interval ranged from 25 to 35 minutes (mean 30 minutes). Perfusion temperature was maintained between 34°C to 37°C (mean 36.0°C), and a flow rate of 33.2 to 37.2 ml/min (mean 34.9 ml/min) was required to maintain a renal arterial pressure of 100 to 130 mmHg (mean 111.6 mmHg). The time for the efferent perfusate drops to become clear and for the majority of the kidney to change to a yellow/tan color ranged from 1 to 15 minutes (mean 11.2 minutes), and 1 to 20 minutes (mean 9.6 minutes), respectively. Subnuclear vacuolizations were observed histologically in all cases, and the mean unstained area ranged from 14.8 to 19.7% (mean 17.7%).

Five rats weighing from 292 to 342g (mean 315.2g) and with a post-mortem interval ranging from 15 to 20mins (mean 18.4 minutes) were perfused for 2 hours. Environmental temperature was maintained between 35 to 37.5°C (mean 36.8°C), and a flow rate of 33.6 to 36 ml/min (mean 35.3 ml/min) was required to maintain a perfusion pressure of 100 to 120 mmHg (mean 110.6 mmHg). The time taken for efferent perfusate to clear and the change in kidney color to occur ranged from 10 to 20 minutes with a mean of 17 and 15 minutes respectively. Subnuclear vacuolizations of renal tubular epithelial cells were observed in all cases, with a mean unstained area ranging from 15.9 to 22.3% (mean 19.7%).

Hypertriglyceridemia with concurrent ketoacidosis

Kidneys from five Sprague Dawley rats weighing between 280 to 380g (mean 323g) were perfused with added triglycerides and ketones for 2 hours. Their post-mortem interval ranged from 15 to 30 minutes (mean 19.6 minutes), and the perfusion temperature was maintained between 34°C to 37°C (mean 36.7°C). A flow rate of 32 to 36 ml/min (mean 34.9 ml/min) was required to maintain a renal arterial pressure of 100 to 120 mmHg (mean 110.4 mmHg). The time for the efferent perfusate drops to clear and the time for the majority of the kidneys to change to yellow/tan ranged from 10 to 20 minutes with a mean of 17 and 15 minutes, respectively. Histologically, subnuclear vacuolizations of similar morphology to the ones observed in the hypertriglyceridemia in isolation group were seen in all cases. The mean percentage of unstained area ranged from 19.4 to 24.8% (mean 22.0%).

Hypertriglyceridemia with concurrent hyperglycemia

One kidney from a Sprague Dawley rat with a weight of 316g and a post-mortem interval of 11 minutes was perfused for 2 hours with added triglycerides and glucose. The

perfusion temperature was maintained between 35-37°C (mean 36.8°C) and a flow rate of 34.8 to 35.6 ml/min (mean 35.3 ml/min) was required to maintain renal arterial pressure between 104 to 117 mmHg (mean 111.2 mmHg). Efferent perfusate drops cleared in 20 minutes, and the vast majority of the kidney changed color to yellow/tan in 15 minutes. Subnuclear vacuolization of the same morphology as the other two treatment groups was noted.

Histological observations

Subnuclear vacuolizations of similar morphology were observed in all study groups (Figures 1 and 2). The dominant pattern of vacuolization appeared as a single vacuole in contact with and beneath normal appearing nuclei but not in contact with the basement membrane (Figure 1). As these vacuoles enlarged, they displaced nuclei luminally, and also engulfed the nucleus and involved the entire cell, giving the appearance of cellular cytoplasmic clearing (Figure 3). Occasional focal subnuclear vacuoles in contact with the basement membranes were also observed (Figure 4), and in a few cases, these vacuoles were seen to simultaneously affect the same tubules as the other subnuclear vacuoles (Figure 5). The addition of ketones into the perfusate did not change the overall morphology of the basal vacuolization. Electron microscopy was undertaken on one kidney from the hypertriglyceridemia in isolation group perfused for 1 hour, and one kidney from the hypertriglyceridemia with concurrent ketoacidosis group. Both kidneys showed intracytoplasmic lipids within vacuoles which were in contact with the nucleus but not with the basement membrane (Figure 6).

Quantitative Analyses

F-tests for the equality of variances showed no statistically significant difference in variances between the 1 and 2 hour hyperlipidemia in isolation group ($F=0.39$, $p=0.19$), nor between the 2 hour hyperlipidemia and hyperlipidemia with concurrent ketoacidosis groups ($F=1.49$, $p=0.35$). Quantitative comparisons of areas of epithelial vacuolization in the different treatment conditions are summarized in Figure 7. T-tests assuming equal variances showed no statistically significant differences in the mean unstained areas of the kidneys perfused with added triglycerides for 1 hour ($M=17.65$, $SD=1.92$) and 2 hours ($M=19.69$, $SD = 3.06$), $t(8)=-1.26$, $p=0.24$. Similarly, the degree of vacuolization in the kidneys perfused for 2 hours with elevated triglycerides alone ($M=19.69$, $SD=3.06$) as compared to those perfused with elevated triglycerides and ketones ($M=21.95$, $SD=2.51$) were not statistically different [$t(8)=-1.28$, $p=0.24$].

Discussion

An ex-vivo isolated perfused rat kidney model was used in this study to investigate the etiology of basal vacuolizations as it allows for control of a single metabolic derangement (e.g. hypertriglyceridemia in isolation), and the addition of other known derangements (e.g. ketosis). This is difficult to investigate with post-mortem material as individuals with ketoacidosis can often have other known or unknown co-occurring metabolic abnormalities. This model has previously been validated for the investigation of drug effects on glomerular function (14,15), renal vasculature (16,17), cellular metabolism (18), and gluconeogenesis (19). A recent study has also shown that it is a viable model for the investigation of renal tubular epithelial histology, showing the induction of osmotic nephrosis subsequent to hyperglycemic perfusate with a statistically significant increase in vacuolization in the hyperglycemia treatment as compared with control perfused kidneys (11).

In the current study, hyperlipidemia was induced by the addition of triglycerides, following on studies by Nielsen and colleagues that demonstrated triglycerides as the major constituent of the basal vacuolizations in diabetic ketoacidosis (20). Hypertriglyceridemia is also a feature of nephrotic syndrome; however, the hyperlipidemia seen in nephrotic syndrome usually consists of both elevated cholesterol as well as triglycerides (21). The dual effect is thought to result from proteinuria and subsequent hypoalbuminemia, leading to decreased plasma oncotic pressure, and stimulation of hepatic apoprotein B gene transcription that in turn mediates a compensatory increase in hepatic lipoprotein synthesis (22). The absence of hypercholesterolemia within the perfusate may, therefore, explain the difference in morphology of the subnuclear vacuoles that were observed compared to basal vacuoles in nephrotic syndrome.

Hypertriglyceridemia commonly occurs in diabetic ketoacidosis. For example, a study of 46 patients hospitalized with diabetic ketosis and ketoacidosis showed that 64% had moderate hypertriglyceridemia with levels above the 95th percentile; 28% had severe hypertriglyceridemia (>5.65mmol/l) including 6 patients (13%) with triglyceride levels >11.29mmol/l (23). Treatment of the ketoacidosis was also usually associated with a rapid decrease in plasma triglyceride levels. Hypercholesterolemia was less frequent, with 36% of patients having values above the 95th percentile (23). In a study of plasma endogenous triglycerides in 74 patients with different types of diabetes, the highest rates of triglyceride synthesis were recorded in those with ketoacidotic diabetes (24). Hypertriglyceridemia in diabetic ketoacidosis occurs because insulin deficiency activates lipolysis in adipose tissue, thus releasing free fatty acids (FFA) which stimulate hepatic production of very low density lipoprotein (VLDL) (24,25). Insulin deficiency also increases the esterification of FFAs, and impairs removal of triglycerides from the circulation by inhibiting lipoprotein lipase activity

in adipose tissue (24,26). It has been suggested that co-existing genetic abnormalities such as mutations on the lipoprotein lipase gene, may play a role in ketoacidotic hypertriglyceridemia (26); however, a study of 15 patients with ketosis and severe hypertriglyceridemia of >11.3mmol/l showed that at least 10 and perhaps 12 of these patients did not have an underlying genetic abnormality (27). There are no reports in the current literature on lipid levels in alcoholic, starvation, or septic ketoacidosis, but Fulop and Eder reported that only 11 of their 15 patients with ketosis and severe hypertriglyceridemia (>11.3mmol/l) had definite or probable insulin-dependent diabetes mellitus (27), implying that other causes of ketoacidosis can also be associated with significant derangements in serum triglycerides.

The current study used a hyperlipidemic perfusate with the addition of 11.3mmol/l (10g/L) of triglycerides. This concentration was chosen as it represents double the threshold concentration for the highest tier of hypertriglyceridemia ("Very high"; 500mg/dL) as set by the American Heart Association guidelines (28). β-hydroxybutyrate and acetoacetate are the main ketones in ketoacidosis, and they have a ratio of 1:1 in normal circulating blood. In diabetic ketoacidosis, this ketone body ratio is initially 3:1 or greater, but can be as high as 10:1 as a result of the highly reduced state of hepatic mitochondria (29). Similarly, a high ratio (7:1 to 10:1) of β-hydroxybutyrate to acetoacetate is seen in alcoholic ketoacidosis (30). The concentration of 20mmol/l of β-hydroxybutyrate was chosen as this reflected the highest observed range seen in autopsy studies (31), and 2mmol/l of acetoacetate was chosen to reflect a 10:1 ratio of ketone derangement seen in severe ketoacidosis.

The predominant pattern of basal vacuolization observed in this study differed from that seen in autopsy cases of ketoacidosis, as vacuoles did not abut the basement membrane unless they had expanded to involve the entire cell. Additionally, basal vacuolization was only seen as a focal effect, with vacuolated tubules surrounded by normal

unaffected tubules. In contrast, subnuclear vacuoles in post-mortem cases of ketoacidosis were often observed as a diffuse effect which could occur with β-hydroxybutyrate levels less than 20mmol/l (7). A possible shortcoming of this study is that the concentration of triglycerides was insufficient to cause diffuse basal vacuolization, however, more marked basal vacuolization with clusters of tubules affected has been noted in nephrotic syndrome cases with lower levels of hypertriglyceridemia (<9.92mmol/l)(10). This may suggest that a perfusion time of greater than 2 hours is required for more widespread changes to develop. It may also suggest that additional factors or other concurrent metabolic derangements, such as hypercholesterolemia, might be required to cause the morphological changes and extent of basal vacuolization seen in ketoacidosis. The basal vacuoles which were in contact with the basement membrane (Figure 5) also appeared morphologically different to basal vacuoles in ketoacidosis in that they were elongated perpendicular to the basement membrane, and were frequently confluent, as opposed to discrete and circular. No demonstrable lipids were present within these vacuoles on electron microscopy, therefore, it is likely that these vacuoles may represent a non-specific early autolytic change with separation from the basement membrane.

The study is also limited by the low numbers of animals in each treatment group. To maximize utility and preserve animal usage, experiments in the hypertriglyceridemia treatment group was limited to ten kidneys, and the hypertriglyceridemia with ketosis treatment group was limited to five kidneys as they all demonstrated the same pattern of subnuclear vacuolization (Figure 1), and it was thought further experiments in the same group was unlikely to yield different results. However, larger studies would add more statistical significance to these findings. The single kidney perfused with added glucose was intended to be a pilot study, which did not demonstrate a difference in the pattern of vacuolization, however, further trials in this group are clearly needed.

The current study has demonstrated that hypertriglyceridemia in isolation is able to cause subnuclear lipid vacuolization in renal tubular epithelial cells, however, the morphology of these vacuoles differs from those observed in post mortem tissues that have been exposed to ketoacidosis in vivo (2,7-9). This may suggest that a longer duration of exposure (> 2 hours) or metabolic derangements other than hypertriglyceridemia, ketosis, and hyperglycemia, are involved in the pathogenesis of ketoacidotic basal vacuoles. Further studies will be required to determine the precise contribution of each metabolic derangement, in isolation and also synergistically, to the renal phenotype characteristically observed in autopsy tissues from cases of lethal ketoacidosis.

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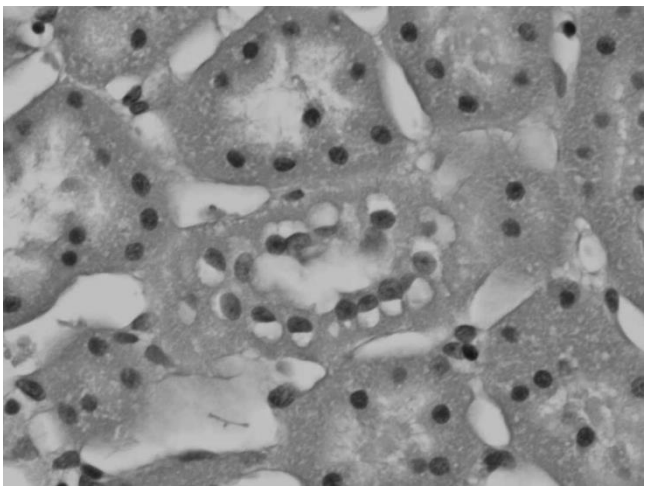

FIG. 1- *Subnuclear vacuolization in a kidney perfused with 11.3mmol/l triglycerides for 2 hours (Hematoxylin and eosin x400).*

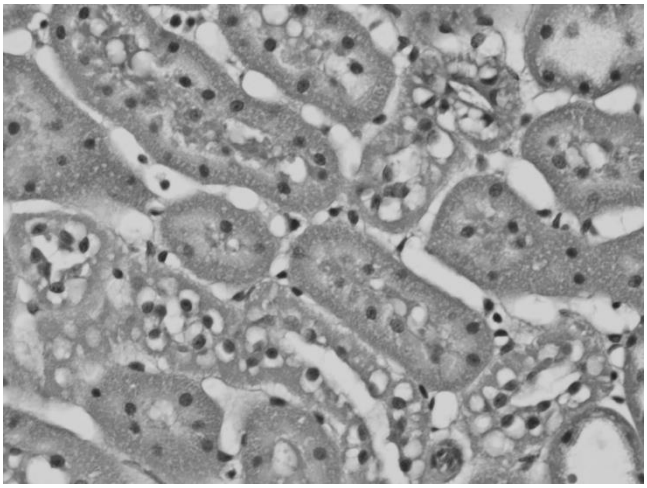

FIG. 2- *Subnuclear vacuolization in a kidney perfused with 11.3mmol/l triglycerides, 20mmol/l β-hydroxybutyrate and 2mmol/l acetoacetate for 2 hours (Hematoxylin and eosin x400).*

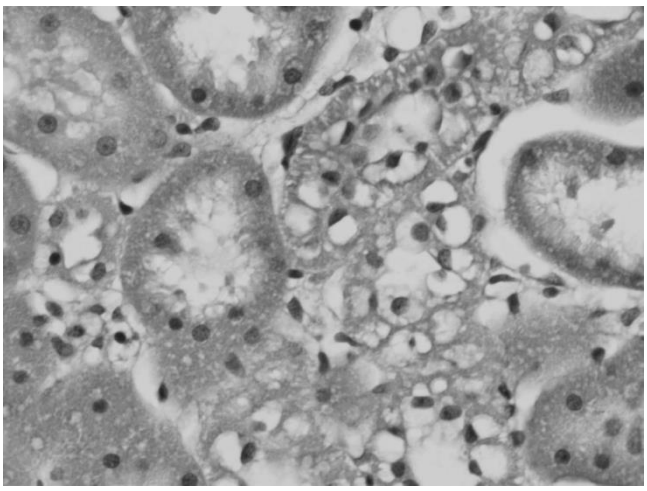

FIG. 3- *Subnuclear vacuolization with focal expansion of vacuoles to involve entire cells in a kidney perfused with 11.3mmol/l triglycerides, 20mmol/l β-hydroxybutyrate and 2mmol/l acetoacetate for 2 hours (Hematoxylin and eosin x400).*

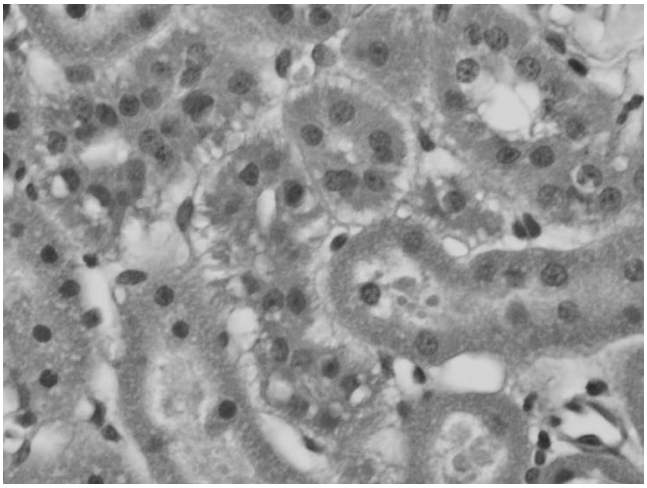

FIG. 4- *Basal vacuolization with vacuoles in contact with the basement membrane in a kidney perfused with 11.3mmol/ triglycerides for 1 hour (Hematoxylin and eosin x400).*

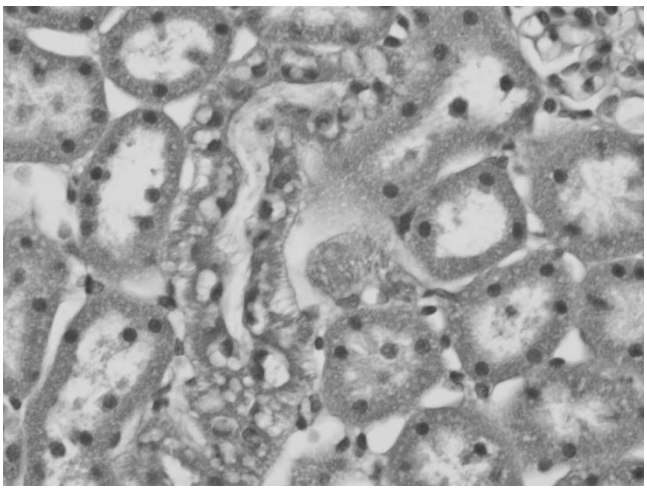

FIG. 5- *Two concurrent patterns of subnuclear vacuolization in a kidney perfused with 11.3mmol/l triglycerides for 1 hour (Hematoxylin and eosin x400).*

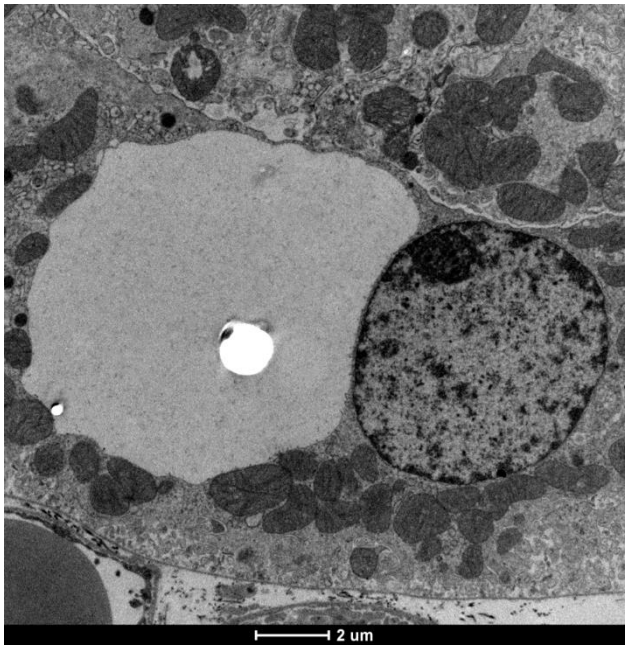

FIG. 6- *Intravacuolar lipid on electron microscopy within the cytoplasm of a renal tubular epithelial cell perfused with 11.3mmol/l triglycerides for 1 hour.*

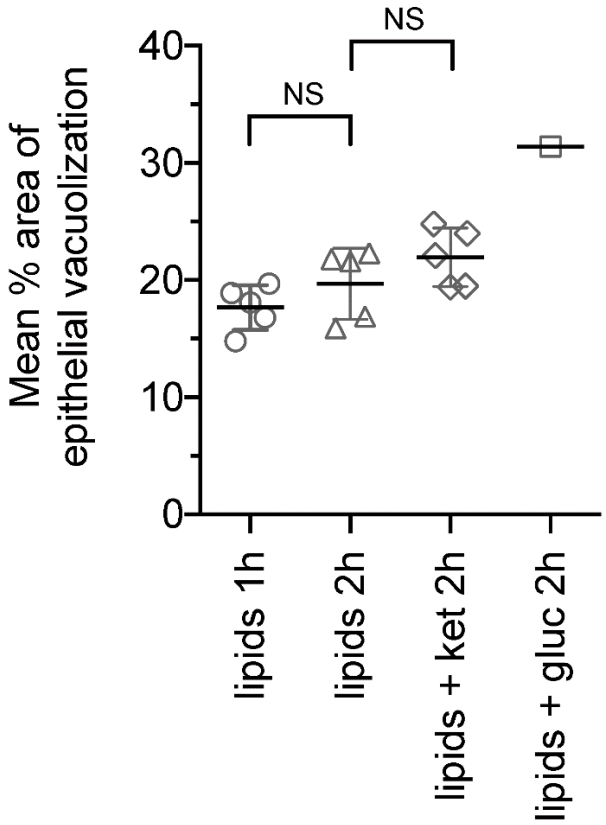

FIG. 7- *Scatterplot histogram summarizing the mean % area of renal tubular vacuolization between study groups. NS is not significant.*

CONCLUSIONS

Chapter 1: Clarification of terminology

1. Armanni-Ebstein lesions and basal vacuolizations represent two distinct histologic changes in renal tubular epithelial cells and are associated with different metabolic derangements which overlap in diabetic ketoacidosis.

Chapter 2: Histopathological studies to confirm or refute associations between histomorphology and pathologic conditions

2. Basal vacuolizations in renal tubular epithelial cells are caused by alcoholic ketoacidosis.
3. Basal vacuolizations in renal tubular epithelial cells are caused by septic ketoacidosis.
4. Basal lipid vacuolizations in renal tubular epithelial cells are caused by hyperlipidaemic conditions such as nephrotic syndrome, however, the vacuoles appear to be of a different morphology to those observed in ketoacidotic states.
5. Basal vacuolizations in renal tubular epithelial cells can be seen in deaths due to hypothermia, but the aetiology in some cases may be a manifestation of underlying metabolic derangements predisposing to the development of hypothermia, rather than to the hypothermia on its own.
6. Macroscopic renal cortical pallor is a useful marker for significant metabolic derangements and the underlying spectrum of histologic changes includes basal vacuolization, Armanni-Ebstein lesions, and osmotic nephrosis. Therefore, it's presence at autopsy should prompt measurement of vitreous humour glucose and β-hydroxybutyrate levels.

7. Formalin pigment deposition in a characteristic basal distribution is a useful surrogate marker for basal vacuolization, especially in cases where significant putrefaction or autolysis precludes accurate morphologic assessment of vacuoles.

Chapter 3: Determining incidence and further defining diagnostic utility

8. Armanni-Ebstein lesions are present in 38% of cases with terminal hyperglycaemia (≥11.1 mmol/l).
9. There is no significant difference in vitreous glucose levels between cases with and without Armanni-Ebstein lesions.
10. Cases with Armanni-Ebstein lesions have a significantly higher level of β-hydroxybutyrate compared to cases without, suggesting that ketoacidosis may facilitate the development of Armanni-Ebstein lesions.
11. Basal vacuolizations in renal tubular epithelial cells are present in 43% of cases with terminal ketoacidosis (>1mmol/l).
12. Basal vacuolizations are associated with ketoacidosis, and their incidence increases with the degree of ketone elevation.
13. Basal vacuolizations are not associated with hyperglycaemia.

Chapter 4: Establishing and utilizing animal models to facilitate further investigation

14. Basal vacuolizations in renal tubular epithelial cells are not associated with terminal hypothermia in an animal model.
15. Armanni-Ebstein lesions were not reproducible by simulating the osmotic effects of glucose, suggesting that these lesions likely result from the metabolic sequelae of hyperglycaemia.

16. The isolated perfused kidney model is a viable ex-vivo model for the investigation of renal tubular epithelial vacuolization.
17. Armanni-Ebstein lesions were not reproduced in the isolated perfused kidney model, suggesting that this change requires more time to develop or involves factors in addition to hyperglycaemia.
18. Isolated hypertriglyceridemia can cause basal lipid vacuolizations in an isolated perfused kidney model, but of a different morphology to those observed in ketoacidosis. The addition of ketones did not alter the morphology of the vacuoles, suggesting that other factors are involved in the pathogenesis of basal vacuoles in ketoacidosis.

APPENDIX

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PAPER

PATHOLOG/BIOLOGY

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Armanni–Ebstein Lesions: A Need for Clarification

ABSTRACT: Armanni–Ebstein lesions were first described by Luciano Armanni, a pathologist at the University of Naples, during autopsy studies undertaken in 1872, as a unique vacuolar nephropathy associated with poorly controlled diabetes that involves selective renal epithelial cell glycogen accumulation. However, within the last two decades, a broader range of vacuolar changes, including lipid deposition, have also been termed Armanni–Ebstein (AE) lesions, creating some confusion on possible etiology. We would suggest that the term AE phenomenon would be best reserved for the original clear cell change associated with glycogen deposition, and that this should be clearly distinguished from subnuclear lipid vacuolization ("basal vacuolization"). Although there is obvious inter-relation between these two types of vacuoles, they appear morphologically and biochemically distinct from each other. More precise classification may assist in clarifying the causal processes and possible diagnostic significance of different types of renal epithelial vacuolization at autopsy.

KEYWORDS: forensic science, Armanni–Ebstein, basal vacuolization, renal epithelial cell change, diabetes mellitus, ketoacidosis

Origins of the Armanni–Ebstein Lesion

In the late 1800s, Armanni observed that the morphology of epithelial cells in the proximal tubules of the deep cortex and outer medulla of the kidneys had altered; the cells had lost their normal polarized cuboidal shapes and were swollen, rounded, and transparent, with small dark nuclei that were often displaced to the periphery, rather than in their normal central position. This observation was presented to medical students in 1872 and first appeared in Italian in Cantani's textbook *Patologia e Terapia del Ricambio Materiale* in 1875, with the large polygonal cells described on page 257 and illustrated by a sketch in Fig. 6 (reproduced here as Fig. 1) (1). Armanni's initial study was later published in German in 1877 (2). Giordano's translation of Armanni's description of large polygonal epithelial cells filled with hyaline material appeared in the English literature in 1987 (3). Ten years after Armanni's initial observations, Ebstein reported similar lesions in the German literature in 1882, describing edematous cells with remnants of cytoplasm (4).

Contemporary Literature

One of the first analyses of Armanni Ebstein (AE) lesions in the English literature in more recent times was in a study by Ritchie and Waugh in 1957 (5). These authors described the lesions as occurring rarely in poorly controlled diabetic states after the advent of insulin therapy and appearing as markedly swollen epithelial cells with a normal appearing central nucleus but with "virtually total conversion of the cytoplasm into a single large vacuole." Affected cells bulged into and sometimes occluded the tubular lumen. This was consistent with the original descriptions by Armanni and Ebstein, as illustrated in their article in Figs 2 and 4 (5). The lesions principally affected the terminal straight portion of the proximal convoluted tubule, with a consistent localization at the corticomedullary junction. The affected area involved mainly the outer medulla with some extension into the inner cortex but did not affect tubules in the middle or outer cortices. Vacuoles in affected cells stained positively for glycogen with no stainable lipids (5). Of interest, the authors also mentioned that fatty vacuolization found in some of their cases was usually basal and present only in the upper proximal tubule regions, not in the areas typical of AE (5).

In 1978, AE lesions were described in Meadows' textbook, *Renal Histopathology* (6) as a rare tubular disorder principally affecting long-term diabetics. The lesions appeared as swollen cells that often occluded tubular lumina with vacuoles containing abundant glycogen (6) (Figs 2 and 3). Rasch (7) described AE lesions in streptozotocin-induced diabetic rats in 1984. In agreement with the findings of Ritchie and colleagues, the lesions were confined to the cortex and outer medulla with abnormal cells appearing "empty" or full of periodic acid-Schiff (PAS)-positive material but with an intact nucleus. Electron microscopy showed the cytoplasm of these cells to be filled with glycogen (7). In 1986, Bendon and Hug (8) described typical AE lesions in five patients with Fanconi syndrome and commented that the common feature of Fanconi syndrome and diabetes mellitus was glycosuria. The lesions were localized in tubular epithelial cells at the corticomedullary junction, which were abnormal in being filled with glycogen but which retained distinct cell membranes, clear cytoplasm and normal nuclei.

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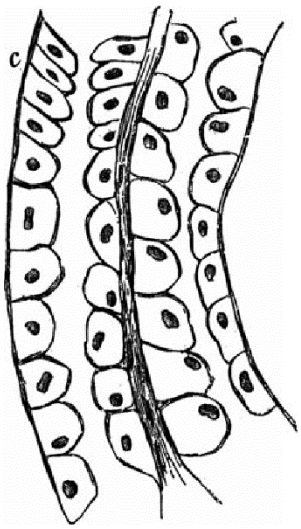

FIG. 1—*A sketch showing typical Armanni–Ebstein vacuolization of renal tubular epithelial cells, depicted by Luciano Armanni in Cantani's 1875 textbook* Patologia e Terapia del Ricambio Materiale, *showing swollen polygonal cells with normal nuclei.*

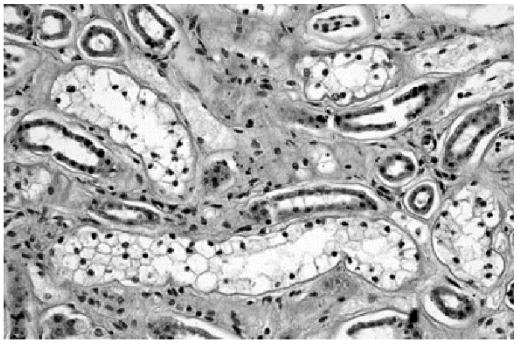

FIG. 2—*Classical Armanni–Ebstein changes in the proximal convoluted tubule, at the corticomedullary junction showing markedly swollen epithelial cells with almost total conversion of the cytoplasm of individual epithelial cells into a single large vacuole with normal appearing nuclei. The affected cells bulge into the tubular lumen.*

Animal Models

Ishizaki et al. noted AE lesions in the ascending limbs of the loop of Henle in all 20 of their spontaneously diabetic rats, with affected cells characterized by the presence of vacuoles filled with glycogen (9). In the same year, 1987, Orloff et al. (10) monitored the presence of AE lesions, characterized by vacuolization and distension of renal tubular cells due to glycogen accumulation, in alloxan-treated diabetic rats receiving pancreatic transplants. In their study, AE lesions appeared 1–3 months after the induction of diabetes with alloxan and progressively increased over the next 24 months.

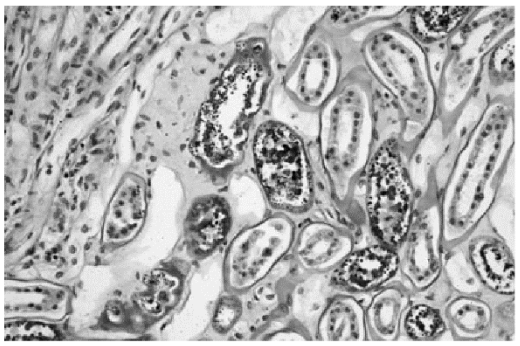

FIG. 3—*Special staining for glycogen in cases of classical Armanni–Ebstein is positive (PAS ×200).*

In 1990, Reyes et al. (11) found that cholesterol administration in streptozotocin-induced diabetic rats prevented the development of AE lesions despite persistent hyperglycemia. As with previous studies, the observed lesions were described as swelling of tubular epithelial cells with clear cytoplasm, which were shown by electron microscopy to contain diffusely distributed granules characteristic of glycogen. A study by Dobashi et al. in 1991 on streptozotocin-induced diabetic rats further refined the timeline for the development of AE lesions by showing that the lesions were not present at 2 weeks after treatment, but had appeared by 8 weeks (12).

Due to the consistent demonstration of glycogen within vacuoles, AE lesions have also been referred to as "glycogen nephrosis" (13–16), in keeping with the original work in the field linking the condition to hyperglycemia associated with diabetes. In a study of alloxan-diabetic rats, Curtis et al. concluded that the appearance of these lesions was solely dependent upon the terminal blood glucose level, with AE changes invariably present with levels above 350 mg/100 mL and consistently absent below 300 mg/100 mL (13).

Broadening of the Concept

The situation changed somewhat in the mid 1990s with work by Kock and Vestergaard expanding the range of lesion phenotypes. They found eight of 47 cases of insulin-dependent diabetics had tubular epithelial changes that were interpreted as AE lesions with two morphological classes of lesions described (14). One type of lesion consisted of tubular cells that had nuclei "completely surrounded by the vacuolation" that was glycogen-positive based on Best's carmine staining. Affected cells were located in the outer zone of the medulla, consistent with previous descriptions of AE lesions. The second type of lesion was composed of cells that had basally located vacuoles with luminally displaced nuclei, not characteristic of the classic AE phenomenon. PAS staining for glycogen was negative (14) (Fig. 4). A drawing of these basally located, nonglycogenic lesions is shown in Fig. 5 and contrasts with the illustration of the original AE findings shown in Fig. 1. Another contrasting feature of the second category was that vacuolization was observed in all regions of the cortex as well as in the outer zone of the medulla, although the middle and outer cortices had been previously described as being AE "immune" (5). With mixed data on the presence of glycogen and two morphologically

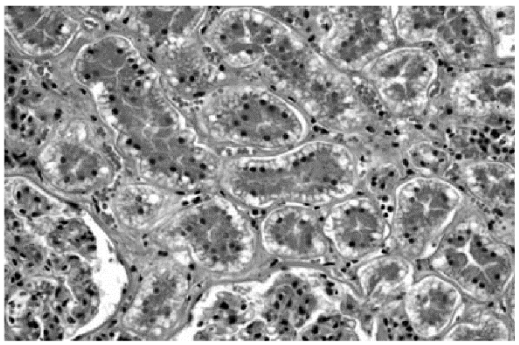

FIG. 4—*A lesion that has more recently also been called Armanni–Ebstein with orderly basal vacuolization of tubular epithelial cells and luminal displacement of nuclei. Special staining for glycogen in these cases is negative (hematoxylin & eosin ×200).*

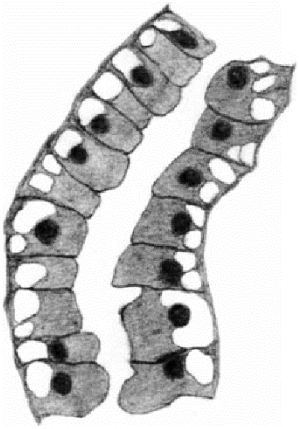

FIG. 5—*A sketch depicting vacuolization of the basal portions of renal tubular epithelial cells with luminal displacement of nuclei, a change that has been referred to in recent times as typical of Armanni–Ebstein.*

distinct patterns of vacuolization, the authors concluded that "a combination of glycogen nephrosis and hydropic vacuolization might well have caused the widespread changes of the tubular epithelium, which seemed to be more extensive than described in the characteristic AE lesions in the literature" (14). These observations effectively expanded the morphological spectrum of AE lesions to include PAS-negative tubular epithelial cells with basal vacuolization that were located in the middle and outer cortex.

Several years later, Thomsen and Hansen described a 47-year-old insulin-dependent diabetic woman with AE phenomenon where the epithelial vacuoles (which were located mainly in the proximal tubules) were PAS-negative but stained strongly for neutral lipid (15) (Fig. 6). This led to a new hypothesis that the AE phenomenon was attributed to the accumulation of triglycerides. It was noted that some tubules astride the corticomedullary junction did stain positively for glycogen (15), suggesting in retrospect that more than one class of pathology was present.

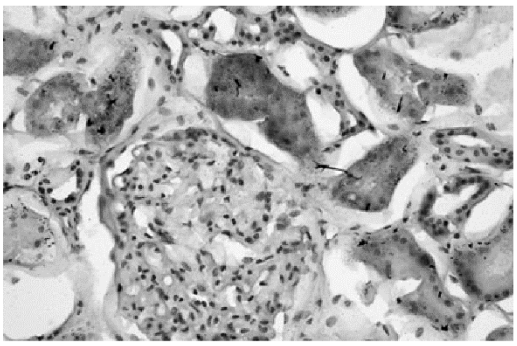

FIG. 6—*Positive staining of basal vacuoles in tubular epithelial cells for neutral lipid (oil-red-O ×200).*

Nielsen et al. (16) further described the AE phenomenon occurring in vacuolated tubular epithelial cells within the renal cortex as being "seen as a line of empty spaces in contact with the peritubular basement membrane." This again diverged from the original descriptions of cellular ballooning with a clear cell change. These authors also reported a tripling of renal cortical lipid due to a 60–100 fold increase in triglycerides which suggested that these lesions were caused by accumulated lipids. This supported the proposal of Kock and Vestergaard (14) that the AE phenomenon encompassed two types of lesions: one in the cortex associated with triglyceride accumulation and the other in the outer medulla due to glycogen deposition (16) (Fig. 7). In a further study by Thomsen et al. (17), the role of the original "glycogen nephrosis" was downplayed further with the statement that "in all probability the AE phenomenon is caused by the accumulation of lipids in the proximal tubules." The authors suggested that a more appropriate name would be "fatty kidney in diabetics."

Possible Association with Hypothermia

In 2004, Preuss et al. noted similar basal lipid vacuoles in 87% of cases of fatal hypothermia, suggesting that these lesions

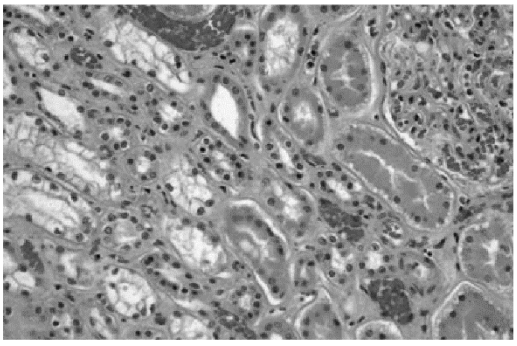

FIG. 7—*The corticomedullary junction showing tubules with distinctive basal vacuolization next to a glomerulus, with Armanni Ebstein vacuolization in tubules deeper within the medulla (hematoxylin & eosin ×200).*

were "a very reliable histologic diagnostic criterium in cases of hypothermia," comparable to Wischnewski spots (18). Wischnewski spots are superficial gastric lesions characterized histologically by necrosis of the mucosa with hematin formation (19), reportedly present in 40–90% of fatal hypothermia cases (20). This newly attributed significance of basal lipid vacuolization has subsequently been incorporated into forensic texts (19).

Recent Findings

In our investigations (21–24), we have found evidence of glycogen nephrosis consistent with the classic AE phenomenon in the tubules of the outer medulla but have concentrated on cases of basal vacuolization of the renal tubular epithelium, which we also termed AE phenomena. In a retrospective review of deaths with terminal hyperglycemia, we observed basal vacuolization in deaths caused by diabetic ketoacidosis and determined that there was no correlation between the extent of these lesions and the degree of terminal hyperglycemia (21). Similar lesions were observed in 33% of our hypothermic cases; however, given the significant number of diabetics in this group, it was suggested that these changes might have arisen because of underlying metabolic disturbances related to diabetes rather than to hypothermia per se (22). Renal cortical pallor was found to be a useful macroscopic marker for the presence of basal vacuolization at autopsy. Histologically, numerous subnuclear epithelial cell vacuoles were identified that contained lipid on electron microscopy and did not stain with PAS, consistent with the results of other work (20). Finally, we analyzed subnuclear vacuolization in deaths due to alcoholic ketoacidosis and found this to be a feature of nondiabetic ketoacidosis in the setting of hypo- or normoglycemia (23). Similar findings, again described as AE lesions, were recently reported in a child who died of ketoacidosis and starvation in the absence of hyperglycemia, further strengthening the association between basal (subnuclear) vacuolization and ketoacidosis (25).

Clarification

It appears, therefore, that there has been broadening of the definition of AE lesions in the more recent literature. Whereas classical studies described specific pathological swelling of cells, loss of shape, clearing of cytoplasm, and accumulation of cytoplasmic glycogen that was directly linked to hyperglycemia and subsequent glucosuria, the more recent literature includes other conditions of basal vacuolization with preservation of cellular integrity, luminal displacement of nuclei, accumulation of lipids and triglycerides within vacuoles, and an association with ketoacidosis.

How is this disparity best dealt with? We considered that as the terminology used to describe renal tubular epithelial vacuolization has altered over time with changing of original definitions, a review of early morphologic descriptions was timely. Having obtained translations of the original 19th century texts by Armanni and Ebstein and extensively reviewed the literature, we suggest that the term AE phenomenon would be best reserved for the original clear cell change described in their original work (Figs 1 and 3). This is not to suggest that subnuclear lipid vacuolization of renal tubular epithelial cells (Figs 2 and 4) is not an important finding at autopsy, as it indicates that there has been a significant metabolic derangement. These vacuoles, however, appear morphologically and biochemically distinct from the lesions described by Armanni and Ebstein and might be best referred to as "basal vacuolization."

Conclusion

Nielsen et al. (16) and Kock and Vestergaard (14) were correct in noting that tubular epithelial lesions in diabetic ketoacidosis are of two kinds. For purposes of clarity, it would seem logical to refine the nomenclature to distinguish these conditions and to restore the classical definition of AE to avoid any confusion that might have inadvertently arisen (16,17,20,24). The benefit of clarifying terminology and separating these two lesions (i.e., AE and basal vacuolization) will be in creating greater consistency in the reporting of findings, which will allow better linking of specific morphological findings at autopsy to unique, although possibly related, underlying pathophysiological processes, and diagnoses. The elucidation of different types of renal vacuolar change with more precise classification should help to further clarify the composition, causal processes, and possible diagnostic significance of particular types of renal epithelial vacuolization at autopsy. This may then assist in determining possible lethal mechanisms and metabolic derangements in forensic cases.

Acknowledgments

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PAPER

PATHOLOGY/BIOLOGY

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Basal Renal Tubular Epithelial Cell Vacuolization and Alcoholic Ketoacidosis

ABSTRACT: Subnuclear renal tubular epithelial cell vacuolization is a marker for diabetic ketoacidosis. Whether it is because of hyperglycemia or of ketoacidosis is unclear. To examine the effect of ketoacidosis on renal cells in isolation, five cases of lethal alcoholic ketoacidosis without hyperglycemia were examined (vitreous humor β-hydroxybutyrate: 6.42–8.75 mM, mean 7.66 mM; and glucose: 0.1–4.2 mM, mean 1.46 mM). Microscopic examination of the kidneys revealed basal vacuoles in three cases (60%). Seven control cases with acute alcohol toxicity without ketoacidosis (blood alcohol: 0.18–0.43%, mean 0.31%; and β-hydroxybutyrate: 0.12–0.42 mM, mean 0.21 mM) did not have these changes. In this study, basal epithelial vacuolization was found only in cases with significant ketoacidosis. Although the numbers are small, the finding of basal renal tubular epithelial vacuolization in normoglycemic cases with elevated β-hydroxybutyrate levels provide further evidence that disordered lipid metabolism may be involved in the pathogenesis of this phenomenon.

KEYWORDS: forensic science, alcoholic ketoacidosis, intoxication, Armanni–Ebstein phenomenon, diabetes mellitus, alcoholism

The term "Armanni–Ebstein phenomenon," first described in 1877 by Luciano Armanni, has been used to refer to subnuclear vacuolization of renal tubular epithelial cells, which when severe enough may present macroscopically as renal cortical pallor. First recognized in poorly controlled diabetic states, it was initially thought to be a direct effect of hyperglycemia causing cytoplasmic glycogen accumulation (1–5). However, there is now evidence that the intracellular vacuoles contain lipid (6–8). A recent study also failed to demonstrate a significant correlation between the degree of hyperglycemia and these morphological changes, suggesting a more complicated etiology possibly involving the effect of ketoacidosis or lipiduria on tubular cells (9). Although this subnuclear vacuolization has been reported in cases of lethal hypothermia, it appears that in at least some of these cases it may be related to underlying diabetic ketoacidosis, that is, basal epithelial vacuolization in these cases may be merely a marker of diabetic metabolic derangement rather than to hypothermia *per se* (10,11). To examine the effect of ketoacidosis on renal cells in isolation, without concomitant hyperglycemia, a series of deaths because of alcoholic ketoacidosis were reviewed. A control group consisted of cases with significantly elevated blood alcohol levels, but no evidence of ketoacidosis.

Materials and Methods

Case files over a 7-year period from 2004 to 2010 at Forensic Science SA, Adelaide, South Australia, were retrospectively reviewed for all cases of nontraumatic deaths where alcohol was listed as a direct cause of death. Cases where deaths were attributed to alcoholic ketoacidosis or where acute alcohol intoxication had played a role were then selected. All cases had full coronial and police investigations with complete forensic autopsies. Alcoholic ketoacidosis was diagnosed when the vitreous humor β-hydroxybutyrate was ≥5 mM with normoglycemia or hypoglycemia (vitreous glucose ≤ 11 mM), in conjunction with a history of alcohol abuse and/or autopsy findings suggestive of chronic alcoholism. Alcohol toxicity was taken as a blood alcohol level of >0.15%. Cases where vitreous humor biochemistry and full toxicology had not been performed were excluded. Case files were summarized and all available microscopic slides of the kidneys were then blindly reviewed for basal epithelial vacuolization; there were no histologic sampling differences between the alcoholic ketoacidosis and the acute alcohol toxicity group, with one section per kidney being reviewed. Cases where the kidneys were too autolysed for accurate assessment were also excluded.

Results

A total of 26 cases were identified, consisting of 12 deaths because of alcoholic ketoacidosis, and 14 because of acute alcohol toxicity. Of the 12 cases of alcoholic ketoacidosis, six were excluded because of incomplete vitreous humor biochemical evaluations, and one was excluded because of insufficiently raised β-hydroxybutyrate (<5 mM). Of the 14 deaths where alcohol toxicity had contributed to the lethal episode, five were excluded because of lack of vitreous biochemistry, and two were excluded because of insufficiently raised blood alcohol concentrations (<0.15%).

All five cases where death was because of alcoholic ketoacidosis had a history of chronic alcohol abuse, two with hepatomegaly, four with steatosis and periportal fibrosis, and one with splenomegaly. The age range was from 51 to 72 years (mean 61 years) and all were men. Vitreous humor β-hydroxybutyrate levels ranged from 6.42 to 8.75 mM (mean 7.66 mM) with no elevation in glucose levels (range 0.10–4.20 mM; mean 1.46 mM). In one case,

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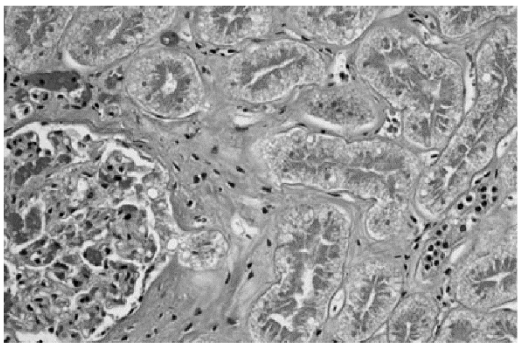

FIG. 1—*Typical vacuolization in the basal portions of renal tubular epithelial cells in a case of normoglycemic alcoholic ketoacidosis. Mild autolytic change is present (hematoxylin and eosin × 280).*

the blood alcohol concentration was 0.20%, and alcohol was not detected in the others. The postmortem interval in which samples were drawn ranged from 4 to 8 days, with a mean of 5.6 days. No cases showed evidence of significant putrefaction. Microscopic examination of the kidneys revealed basal epithelial vacuolization in three cases (Fig. 1). There was no history of diabetes mellitus in any of these cases.

A total of seven cases with acute alcohol toxicity without ketoacidosis were studied; six had a history of chronic alcohol abuse with hepatomegaly at autopsy in four cases, marked cirrhosis in one case, early cirrhotic changes in four cases, mild steatosis in one case, and splenomegaly in four cases. The ages ranged from 37 to 68 years (mean 51.1 years). The male to female ratio was 3:4. In one case, there was a medical history of insulin-dependent diabetes mellitus. Blood alcohol concentrations ranged from 0.18 to 0.43% (mean 0.31%). β-hydroxybutyrate levels ranged from 0.12 to 0.42 mM (mean 0.21 mM). Vitreous glucose was tested in three cases and ranged from 0.1 to 1.3 mM (mean 0.5 mM). The postmortem interval in which samples were withdrawn ranged from 2 to 6 days, with a mean of 3.9 days. No cases showed evidence of significant putrefaction. None of the cases exhibited basal epithelial vacuolization in the kidneys (Fig. 2).

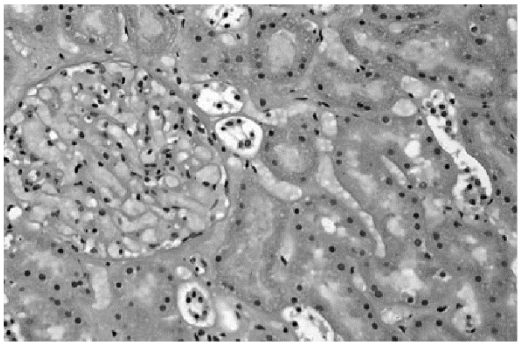

FIG. 2—*Normal renal tubular epithelial cells in a case of acute alcoholic toxicity (blood alcohol = 0.43%) with no evidence of vacuolization (hematoxylin and eosin × 280).*

Discussion

Ethanol is a commonly used substance, the excessive consumption of which can have a variety of effects on the body with forensic repercussions. Ethanol readily crosses the blood–brain barrier into the cerebral extracellular fluid and has a direct depressant effect on neurons. Chronic excessive alcohol intake is associated with many significant conditions including malnutrition, cirrhosis with liver failure, esophageal varices, chronic pancreatitis, and cardiomyopathy (12,13). Alcohol is also frequently an indirect cause of death with excessive alcohol consumption associated with aspiration of gastric contents and a variety of unnatural deaths that include drowning, falls, burns, and other forms of trauma (13).

Alcoholic ketoacidosis has been reported in 7–10% of alcoholic patients suffering sudden death (14). β-hydroxybutyrate levels >2.5 mM are considered lethal (15), and a typical episode involves binge drinking with subsequent reduced caloric intake (16). Ethanol levels are frequently low or absent (<10 mg/dL) at autopsy, as the victims have often stopped drinking after the onset of symptoms (17,18). Alcoholic ketoacidosis accounts for up to 25% of cases of ketoacidosis (19), occurring once for every four cases of diabetic ketoacidosis (17). It results from low protein and carbohydrate stores, and/or alcoholic liver disease with subsequent hepatic glycogen depletion, a situation that causes a fall in blood glucose concentrations with suppression of insulin secretion (14,19). Volume depletion caused by vomiting, decreased fluid intake, and/or diaphoresis can lead to hypotension and a sympathetic response, further decreasing insulin production and increasing catecholamines, cortisol, growth hormone, and glucagon levels (14,18). Starvation and hypothermia may also lead to an elevation in these hormone levels, which in conjunction with the direct affect of ethanol, promotes lipolysis, and increases the supply of fatty acids to the liver (14). When this exceeds the rate of oxidation, a surplus of acetyl-CoA results and is converted to acetoacetate (20). In addition, ketogenesis is favored by the inhibition of gluconeogenesis and decreased pyruvate levels. Subsequent accumulation results, as clearance of ketoacids is also impaired by volume depletion and low plasma insulin (17).

In the present series, typical basal epithelial vacuolization in renal tubular epithelial cells was found in three of the five cases (60%) of alcoholic ketoacidosis that fulfilled the criteria for the study. In addition, these so-called Armanni–Ebstein changes were present only in cases where there was significant ketoacidosis and were not related to blood ethanol levels. While vitreous glucose levels decline after death, in our experience markedly elevated levels can still be detected for many days after death (10), and thus, the glucose levels in the three cases with basal vacuolizations were not considered to have been significantly raised prior to death. In addition, there was no history of diabetes mellitus in any of these victims. Although the numbers are small, the detection of renal tubular epithelial vacuolization in cases where β-hydroxybutyrate levels were elevated in the absence of hyperglycemia may provide further evidence for the role of disordered lipid metabolism in the pathogenesis of this phenomenon.

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PAPER

PATHOLOGY AND BIOLOGY

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Septic Ketoacidosis—A Potentially Lethal Entity with Renal Tubular Epithelial Vacuolization

ABSTRACT: Fatal ketoacidosis due to diabetes mellitus, alcoholism, and starvation may produce characteristic basal vacuolization of renal tubular epithelial cells (RTEC). Septic ketoacidosis has recently been recognized clinically as a distinct condition in which septicemia can lead to elevation of ketones and various anions unrelated to diabetes mellitus, alcoholism, or caloric deprivation. We report four lethal cases with significantly elevated vitreous ketones secondary to sepsis and/or severe localized infection in individuals with no history of diabetes mellitus, alcoholism, or starvation. Three of four cases exhibited typical basal vacuolization of RTEC. We suggest that septic ketoacidosis is an appropriate cause of death in the forensic setting where sepsis or severe localized infection is found with significant ketoacidosis (β-hydroxybutyrate > 5 mmol/L)—in the absence of diabetes mellitus, alcoholism, starvation, or other states associated with accelerated ketogenesis. The finding of basal vacuolization of RTEC in such cases provides morphological support for the underlying metabolic derangement.

KEYWORDS: forensic science, ketoacidosis, sepsis, basal vacuolization, subnuclear vacuolization, diabetes mellitus, alcoholism, starvation

Ketoacidosis can lead to death via many mechanisms including severe extracellular volume contraction, electrolyte disturbances such as hypokalemia, cerebral edema, and sudden cardiac arrest (1,2). The cause of ketoacidosis is most commonly diabetes mellitus, but it may also be related to alcohol abuse, starvation, non-insulin mediated hypoglycemia (including inherent errors of fatty acid metabolism), and drugs (e.g., those that inhibit insulin release or acetyl-CoA carboxylase activity) (1). Septic ketoacidosis has recently been recognized in clinical settings as a separate pathological condition which can lead to marked ketoacidosis independent of diabetes mellitus, alcoholism, starvation, and drugs (3); it is therefore logical that sepsis or severe localized infections causing ketoacidosis could be a cause of death in forensic cases.

The postmortem diagnosis of ketoacidosis relies heavily on detecting elevations in vitreous β-hydroxybutyrate and acetoacetate, as often there is no gross pathology present at autopsy. Basal vacuolizations in renal tubular epithelial cells and characteristic formalin pigment deposition in the region of the basal vacuoles are, however, useful histological observations supporting significant ketoacidosis (4). These vacuoles have been reported in ketoacidosis due to diabetes mellitus, alcoholism, and starvation. We report characteristic basal vacuolizations found at autopsy in three of four cases of lethal septic ketoacidosis.

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Materials and Methods

Case files over a 5-year-period from 2010 to 2014 at Forensic Science SA, Adelaide, South Australia, were retrospectively reviewed for all cases of sepsis and/or where death had been attributed to severe infection. All cases had full coronial and police investigations with complete forensic autopsies. Cases with a history of diabetes mellitus, alcoholism, recent starvation, or autopsy findings supporting any of these conditions were excluded. Septic ketoacidosis was diagnosed when vitreous humor β-hydroxybutyrate ≥ 5 mmol/L with normoglycemia or hypoglycemia (vitreous glucose ≤ 11 mmol/L), in conjunction with positive bacterial cultures and autopsy findings of localized infection. Cases with insufficiently raised β-hydroxybutyrate levels, elevated glucose levels (>11 mmol/L), or where vitreous humor biochemistry had not been performed were excluded. Case files were summarized, and microscopic slides of the kidneys were then blindly reviewed for basal vacuolization of renal tubular epithelial cells. Cases where autolysis precluded accurate histological assessment were also excluded.

Case Reports

Case 1

A 41-year-old Caucasian male with a history of human immunodeficiency virus (HIV) and hepatitis C positivity was found dead lying face down outdoors. There were reports of him suffering from poor concentration and a change in behavior leading up to death. He had no history of diabetes mellitus, alcoholism, or recent starvation. At autopsy, he appeared adequately nourished with a BMI of 22; bilateral pneumonia was found in

the lungs with *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Penicillium*, and *Mycobacterium heckeshornense* identified on culture. The heart was unremarkable with no significant coronary artery disease, and the liver was also normal with no steatosis or cirrhosis. Subnuclear vacuolization of renal tubular epithelial cells was present in the kidneys, with no evidence of diabetic nephropathy. Vitreous biochemistry showed significant ketoacidosis with a β-hydroxybutyrate concentration of 13.32 mmol/L and lactate of 7.63 mmol/L. There was no evidence of hyperglycemia (vitreous glucose < 0.6 mmol/L). Toxicology detected acetone in the blood but was otherwise unremarkable. *Staphylococcus aureus* was also cultured from the spleen; thus, death was attributed to *Staphylococcus aureus* sepsis arising from pneumonia, with HIV as a likely significant underlying factor predisposing to infection.

Case 2

A 78-year-old Caucasian male (BMI = 21.5) with a past medical history of epilepsy, dementia, and mobility problems was found dead lying supine on a concrete floor at his home address. His medications include lamotrigine, aspirin, and ostelin. There was no history of diabetes mellitus, alcoholism, or starvation. At autopsy, extensive acute bilateral confluent bronchopneumonia was present on a background of chronic obstructive pulmonary disease. Postmortem bronchial swab and lung tissue showed a mixed growth of *Klebsiella oxytoca*, *Citrobacter sp.*, *Hemophilus influenzae*, and *Streptococcus pneumoniae*. Examination of the heart revealed severe triple vessel coronary artery disease with left ventricular hypertrophy and myocardial scarring; no acute changes were identified. Sections of the kidneys showed mild-to-moderate generalized nephrosclerosis and subnuclear vacuolization of renal tubular epithelial cells. Scattered mucosal erosions were noted in the lower stomach consistent with hypothermic changes. Neuropathological changes of Alzheimer's disease were present in the brain, with no evidence of acute trauma or ischemic injury. Multiple minor areas of bruising, abrasions, and underlying right rib fractures (8th, 9th, and 10th) were present, likely relating to a fall around the time of death. Vitreous biochemistry showed significant ketoacidosis with a β-hydroxybutyrate level of 8.13 mmol/L and no hyperglycemia (glucose < 0.6 mmol/L). Blood toxicology was unremarkable. Death was attributed to multi-organ failure, ketoacidosis, hypothermia and dehydration due to ischemic heart disease and bilateral lower lobe pneumonia.

Case 3

A 78-year-old Caucasian male (BMI = 22) was found dead on the floor of his residence. He had no history of diabetes mellitus, alcoholism, or starvation. Acute bronchopneumonia was found at autopsy, and postmortem lung swabs grew mixed organisms including *Hemophilus influenzae*. The heart was unremarkable with no significant coronary artery disease. Wischnewsky-type mucosal hemorrhages were present in the stomach and proximal small intestine. The liver showed moderate steatosis. There was no evidence of diabetic nephropathy or subnuclear vacuolization in the kidneys. There was a fractured left humerus with extensive bruising around the left shoulder most likely due to a fall associated with the terminal episode. There was no other evidence of trauma. Vitreous biochemistry showed a raised β-hydroxybutyrate level of 8.07 mmol/L with normoglycemia (1.4 mmol/L). Acetone was found in the blood on toxicology. Death was attributed to acute bronchopneumonia complicated by hypothermia with ketosis and fracture of the left humerus.

Case 4

A 72-year-old underweight Caucasian female (BMI = 14) was found dead on the floor of her lounge room. It appeared that she had collapsed striking her chin. She had a history of breast carcinoma 20 years previously, arthritis, and possible overuse of analgesics. She did not have a history of diabetes mellitus, alcoholism, or starvation. At autopsy, florid suppurative meningitis was found with chronic inflammatory cells suggesting that the process had been present for some time. *Streptococcus agalactiae* was cultured from the brain. Bacterial endocarditis involved the mitral valve, and abrasions were present on her chin and limbs consistent with terminal collapse. Examination of the heart showed mild (up to 30%) stenosis of the epicardial coronary arteries but no evidence of recent or old ischemic changes. The liver was unremarkable with no steatosis or cirrhosis. Histopathological examination of the kidneys showed bacterial overgrowth and focal collections of chronic inflammatory cells, as well as subnuclear vacuolization of renal tubular epithelial cells (Fig. 1); there was no evidence of diabetic nephropathy. Vitreous biochemistry showed ketoacidosis (β-hydroxybutyrate 6.26 mmol/L) but no hyperglycemia (glucose < 0.6 mmol/L). Blood toxicology was unremarkable. Death was attributed to *Streptococcus agalactiae* meningitis, and endocarditis.

Discussion

Ketoacidosis is a form of metabolic acidosis which is known to be a complication in diabetics, alcoholics, and those with prolonged starvation. Recently, septic ketoacidosis has been suggested as a separate pathological condition in the clinical setting by Nakamura and colleagues who reported a case of significant ketoacidosis (β-hydroxybutyrate 9.86 mmol/L, acetoacetate 2.55 mmol/L) caused by *Klebsiella pneumonia* sepsis due to acute obstructive cholangitis in a nondiabetic and nonalcoholic

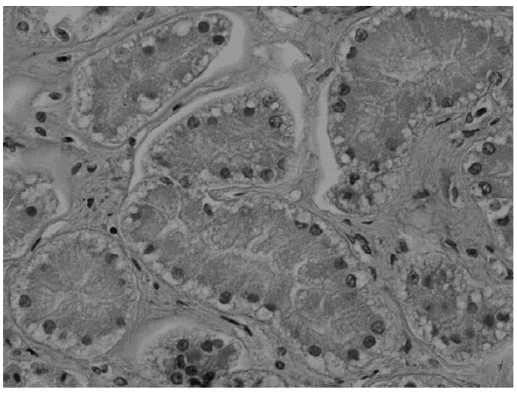

FIG. 1—*Characteristic basal vacuolizations of renal tubular epithelial cells in a 72 year-old woman with septic ketoacidosis (hematoxylin and eosin ×400).*

patient with no history of starvation (3). Previous studies have also shown that the septic state is associated with a decreased arterial ketone body ratio (acetoacetate/hydroxybutyrate)(5), as is seen in diabetic and alcoholic ketoacidosis (6,7). Although postmortem blood cultures were not undertaken in our cases, the pathological findings, in conjunction with the available microbiological results in Case 1 and 4, would be in keeping with disseminated sepsis. In cases 2 and 3, it was clear that a severe burden of infection was present as both were complicated by hypothermia and ketoacidosis, and Case 2 was complicated by multi-organ failure; however, as only the postmortem lung tissue or lung swab was submitted for microbiology, disseminated sepsis was not confirmed.

The mechanism by which sepsis causes ketoacidosis is likely multifactorial. Sepsis is associated with a relative increase in the level of glucagon and impairs the actions of insulin on endogenous glucose production and utilization, thus causing a functional insulin deficiency. (3,8). Sepsis can also lead to ketogenesis through acetic acid overload from bacterial metabolism in the gastrointestinal tract, and inhibition of acetyl-CoA carboxylase by adrenalin which is elevated during sepsis. The latter can lead to ketogenesis even without relative or absolute insulin deficiency (1,9). Furthermore, sepsis, particularly in its early stages, is associated with a hypermetabolic state; thus, there is a relative increase in carbohydrate demands compared to normal individuals (10,11). This can contribute to and exaggerate ketogenesis by inducing a relative state of starvation with increased lipolysis, increased circulating free fatty acids presented to the liver, and subsequent increase in hepatic ketone production (12). Approximately half of the ketoacids produced are removed by oxidation in the brain and one quarter by oxidation in the kidneys (1). Other mechanisms of ketone removal include urinary excretion, conversion to acetone, and oxidation in the gastrointestinal tract (1). Sepsis is a known cause and precipitant of multi-organ failure, as in Case 2, and can therefore also significantly impair the removal of circulating ketones by affected organs.

On retrospective review of autopsy reports, the cause of ketoacidosis in all four cases had been presumed to be dehydration and inadequate food intake in the preterminal period of the infective illnesses. However, although fasting may result in mild-to-moderate ketosis, it is not known to cause a marked elevation of ketoacids to the levels seen in diabetics and alcoholics. This may be because although low, plasma insulin levels are still consistently detectable in the fasted state (12), whereas diabetic ketoacidosis is associated with absolute insulin deficiency. Diets with over 100 g of carbohydrates a day are sufficient to prevent ketosis (β-hydroxybutyrate < 0.1 mmol/L), and an intake of approximately 20–40 g of glucose per day is associated with a β-hydroxybutyrate level of around 1 mmol/L (13). The average normal-weight adult only reaches a β-hydroxybutyrate level of 2.5–4.5 mmol/L after 5–7 days of starvation (12), and levels between 4–7 mmol/L after 2 weeks of fasting (13). There have, however, been two case reports of starvation ketoacidosis after approximately 3 days, with β-hydroxybutyrate levels of 4.5 and 5.21 mmol/L, respectively (1,14). In comparison, obese individuals take longer (10–14 days) to reach a β-hydroxybutyrate level of 2.5–4.5 mmol/L (12). Similarly, only mild ketosis has been reported in animal studies with small rodents achieving a β-hydroxybutyrate level of 2–3 mmol/L with starvation, and most rodents having levels <1 mmol/L (13,15). Starvation ketoacidosis with β-hydroxybutyrate levels of >5 mmol/L may be seen in children, possibly related to the increased rate of carbohydrate utilization by extrahepatic tissues and lower glycogen stores compared to adults (16).

As there is also accelerated hepatic ketogenesis in pregnancy and lactation, (pregnancy is a diabetogenic state associated with relative insulin resistance, increased lipolysis, and elevated free fatty acids), a shorter period of starvation may precipitate ketoacidosis (17). For example, a report of a 22-year-old woman at 32 weeks gestation revealed a blood ketone level of 4.0 mmol/L after only 24 h of starvation (18); the highest reported β-hydroxybutyrate level of 7.1 mmol/L associated with starvation ketoacidosis was in a 32-year-old lactating woman 10 days after commencing a low carbohydrate, high-fat diet, with an estimated carbohydrate intake of less than 20 g per day (19). The only case report of starvation ketoacidosis in a forensic setting was of a 3-year-old girl who had been starved by her parents for over 2 days with a vitreous β-hydroxybutyrate level of 3.97 mmol/L (20). Thus, starvation rarely causes ketoacidosis of >5 mmol/L in adults, particularly in the absence of pregnancy and/or lactation. Furthermore, as even small amounts of glucose, such as 7.5 g, can decrease ketone production (13), it may be incorrect to attribute significant ketoacidosis to starvation in the absence of known absolute or prolonged fasting, or conditions that predispose to accelerated ketogenesis.

In our cases, the β-hydroxybutyrate levels ranged from 6.26 to 13.32 mmol/L (mean = 8.95 mmol/L). None of these cases had a history of diabetes mellitus, alcohol abuse, prolonged fasting, or were pregnant/lactating. Vitreous glucose was not elevated in any case. Three cases had normal BMIs of 21.5–22 and only Case 4 having a low BMI of 14. Although the decedent in Case 4 was underweight, a comment was made in the autopsy report that "she appeared thin but in good general condition and well kept". While starvation as a cause of ketoacidosis is an important differential diagnosis to consider, the observed degree of ketoacidosis in these cases is beyond that which can be accounted for by fasting alone. In contrast, sepsis in the absence of fasting has been reported to show severe ketoacidosis with β-hydroxybutyrate of 9.86 mmol/L (3). Cases 2 and 3 also had evidence of hypothermia in the form of Wischnewsky ulcers (21,22). This could have been contributory to ketogenesis, as ketoacidosis can be aggravated by hypothermia (23), and hypothermia is a known complication of sepsis and severe infections associated with increased mortality (24–26). Therefore, the combination of an absence of documented fasting and the presence of sepsis or a severe burden of infection at autopsy makes septic ketoacidosis a more likely diagnosis in the reported cases.

Postmortem diagnosis of ketoacidosis can be a challenge as there are often little or no significant findings at autopsy. The diagnosis has traditionally relied upon detection of raised β-hydroxybutyrate levels on vitreous biochemistry, although vitreous humor may not always be available for analysis. Histological examination of the kidneys may also help in diagnosing ketoacidosis when characteristic basal vacuolizations in renal tubular epithelial cells are identified. These vacuoles contain lipid and appear as a single row beneath the nucleus in contact with the basement membrane, with possible luminal displacement of nuclei. These vacuoles have recently been identified as a separate phenomenon from the Armanni–Ebstein lesion (27), to which they have previously been referred (28). Their presence has been reported in diabetic, alcoholic, and starvation ketoacidosis (20,29,30).

In the current study, we observed characteristic basal vacuolization (Fig. 1) in 3 of 4 cases, which is in keeping with the association between these vacuoles and ketoacidotic states.

Importantly, however to the best of our knowledge, this is the first report of renal epithelial tubular basal vacuolization occurring in the setting of lethal septic ketoacidosis. It may therefore be a useful marker in the autopsy assessment of such cases.

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PAPER

PATHOLOGY/BIOLOGY

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Renal Tubular Epithelial Vacuoles—A Marker for Both Hyperlipidemia and Ketoacidosis at Autopsy

ABSTRACT: Review of 15 cases of nephrotic syndrome found that eight had significant hyperlipidemia with serum cholesterol levels ranging between 10.59 and 18.60 mmol/L (mean 12.88) and serum triglyceride levels between 2.30 and 9.92 mmol/L (mean 4.58); all of these cases displayed basal lipid vacuolization. Seven of the 15 study cases had normal–mild hyperlipidemia with serum cholesterol levels ranging between 4.71 and 7.54 mmol/L (mean 6.02) and serum triglyceride levels between 0.65 and 4.1 mmol/L (mean 1.57). Six of the seven cases had basal lipid vacuoles (86%). Of these, five cases were hyperlipidemic and one case had borderline hyperlipidemia with a serum cholesterol level of 4.71 mmol/L. Although hyperlipidemia was associated with renal tubular epithelial vacuolization, the vacuoles appeared morphologically different to those found in ketoacidosis. This study has shown that while hyperlipidemia in isolation may result in basal lipid vacuolization within renal tubular epithelial cells, the phenotype differs from that observed in ketoacidosis.

KEYWORDS: forensic science, hyperlipidemia, renal tubular epithelial vacuoles, ketoacidosis, Armanni–Ebstein phenomenon, diabetes mellitus, alcoholism

A variety of metabolic derangements cause pathological changes in renal tubular epithelial cells. Very recently, Armanni–Ebstein phenomenon and basal vacuolization have been distinguished from each other due to different morphologies and probable distinct etiologies (1). While the pathogenesis of Armanni–Ebstein lesions has been clearly attributed to hyperglycemia (2,3), the exact cause of basal vacuolization remains unclear; basal lipid vacuolization has been reported in ketoacidosis due to diabetes, alcoholism, and starvation (4–7). These vacuoles stain positively with Oil-red O, contain lipids on electron microscopy (8,9), and demonstrate formalin pigment artifact deposition (10). In addition, their pathogenesis appears to be independent of glucose levels (4–7), suggesting that their etiology instead involves elevation of serum lipid levels (8,9). This study reviewed renal biopsies in cases of nephrotic syndrome to test the hypothesis that hyperlipidemia, in the absence of hyperglycemia and ketoacidosis, could result in the basal vacuolization similar to that observed in ketoacidotic states.

Materials and Methods

A search of renal biopsy reports was retrospectively conducted over an 8-year period from 2005 to 2012 at the Department of Histopathology at the Women's and Children's Hospital, Adelaide, South Australia, where the clinical summary contained the keyword "nephrotic". Patients who did not have lipid studies, 24-h urine collection, serum biochemistry, or an acid–base profile within 1 month from the date of renal biopsy were excluded from the study.

Hospital case notes and blood/urine test results were then reviewed. Inclusion criteria involved a clinical presentation consistent with nephrotic syndrome (i.e., peripheral edema, weight gain, pleural effusion, ascites, etc.), proteinuria (total urinary protein >0.16 g/L, protein/creatinine ratio >20, and albumin/creatinine ratio >3), hypoalbuminemia (<35 g/L), and hyperlipidemia as defined by the American Academy of Pediatrics (borderline ≥4.40 mmol/L, high ≥5.17 mmol/L) (11). Cases that did not satisfy these criteria were excluded from the study. Ketoacidosis was excluded based on an absence of suggestive clinical signs and symptoms (e.g., history of diabetes, polyuria/polydipsia, alcohol intake, altered mental state, coma, Kussmaul breathing), the absence of urinary glucose and ketones, and normal acid–base profile.

Study cases were then divided into two groups: those with significant hyperlipidemia (serum cholesterol ≥6.5 mmol/L and hypertriglyceridemia ≥2.3 mmol/L) and those with normal–mild elevation in lipid profiles (serum cholesterol <6.5 mmol/L or triglycerides <2.3 mmol/L).

Renal biopsy slides stained with hematoxylin and eosin were then reviewed for vacuolization of tubular epithelial cells and graded "mild–moderate", where vacuoles were limited to a basal involvement, and "marked" if one or more tubules had epithelial cells completely effaced by vacuoles. Lipid was confirmed within the vacuoles by Oil-Red O staining when frozen sections of renal biopsies were available, or by electron

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microscopy in the other cases. The severity of lipid vacuolization was then plotted against the level of hyperlipidemia and hypertriglyceridemia to determine whether a relationship existed.

Approval was obtained from the Women's and Children's Hospital Human Research Ethics Committee prior to commencement of this study.

Results

A total of 15 cases of nephrotic syndrome fulfilling the above criteria were found. The age range was 2–15 years (mean 8.3 years) with a male to female ratio of 8:7. The interval between the date of renal biopsy and lipid studies ranged from 0 to 29 days (mean 9.9 days), 24-h urine collection ranged from 0 to 12 days (mean 2.7 days), and serum biochemistry including acid–base profile ranged from 0 to 12 days (mean 3.9 days).

Eight of the 15 study cases had significant hyperlipidemia. The age range was 2–14 years (mean 7.1) with a male to female ratio of 1:1. Serum cholesterol levels ranged from 10.59 to 18.6 mmol/L (mean 12.88) and serum triglyceride levels from 2.30 to 9.92 mmol/L (mean 4.58). The patients were all clinically edematous, severely proteinuric with a total urinary protein of 2.60–31.18 g/L (mean 9.16), protein/creatinine ratio of 295–2006 (mean 1191.6), albumin/creatinine ratio of 15.1–1863.7 (mean 986.7), and hypoalbuminemic with a serum albumin range of 13–30 g/L (mean 19.8). Three patients had no treatment prior to biopsy; three patients were treated with prednisolone, one with tacrolimus, and one with tacrolimus and prednisolone. Basal epithelial cell vacuolization was present in all cases including three graded as mild moderate and five graded as severe. Basal vacuolization ranged from clusters of basally located vacuoles (Fig. 1*A*) to cases where vacuoles occupied virtually the entire cytoplasm of the affected cells (Fig. 1*B*). Oil-red O staining was undertaken in five cases, all of which showed positive staining for lipids (Fig. 2). Three cases were reviewed by electron microscopy, and intravacuolar lipid was identified in all of these (Fig. 3).

Seven of the 15 study cases had normal–mild hyperlipidemia. The age range was 5–15 years (mean 9.7) with a male to female ratio of 4:3. Serum cholesterol ranged from 4.71 to 7.54 mmol/L (mean 6.02) and serum triglycerides from 0.65 to 4.1 mmol/L (mean 1.57). One case had an acute presentation with peripheral edema, and two biopsies were done for incomplete remission, two for relapse after initial remission, and the other two to monitor response to treatment and drug toxicity. Total urinary protein in these cases ranged from 0.06 to 27.93 g/L (mean 7.02), protein/creatinine ratio ranged from 50 to 1961 (mean 715.4), albumin/creatinine ratio ranged from 25.8 to 1693.3 (mean 514.2), and serum albumin ranged from 15 to 36 g/L (mean 24.6). One patient was not on treatment, two were on prednisolone, two were on tacrolimus, one was on prednisolone and tacrolimus, and one was on a combination of prednisolone, tacrolimus, and mycophenolate. Basal epithelial cell vacuolization was absent in one case and present in six cases; two were graded as mild–moderate and four graded as severe. Of these six cases, five cases were hyperlipidemic and one case had borderline hyperlipidemia with a serum cholesterol level of 4.71 mmol/L 10 days after the date of renal biopsy. Oil-red O staining was performed on one case which stained positively for lipids. Electron microscopy demonstrated intravacuolar lipid in all three cases where material was available.

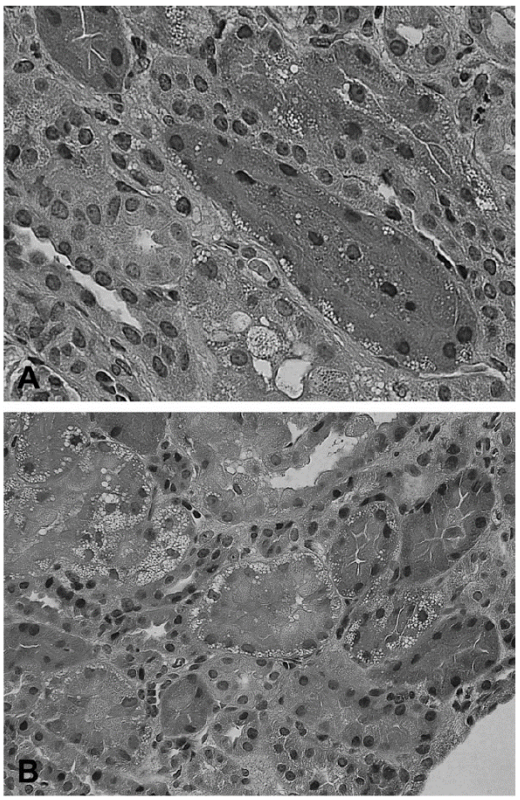

FIG. 1—*Vacuolization of renal tubular epithelial cells in a case of nephrotic syndrome with hypercholesterolemia and hypertriglyceridemia demonstrating multiple basally oriented vacuoles (A). Vacuolization in a case of nephrotic syndrome with severe hypercholesterolemia and hypertriglyceridemia demonstrating the progression of vacuoles from a basal location to involvement of the entire cell (B). (Hematoxylin and Eosin [H&E] ×100).*

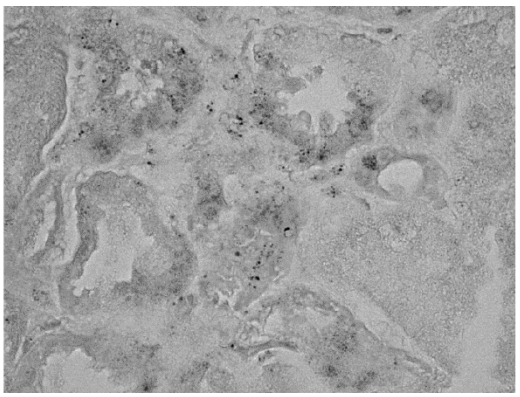

FIG. 2—*Positive lipid staining with Oil-Red O in hyperlipidemic basal vacuoles of nephrotic syndrome (black dots) (Oil-Red O ×100).*

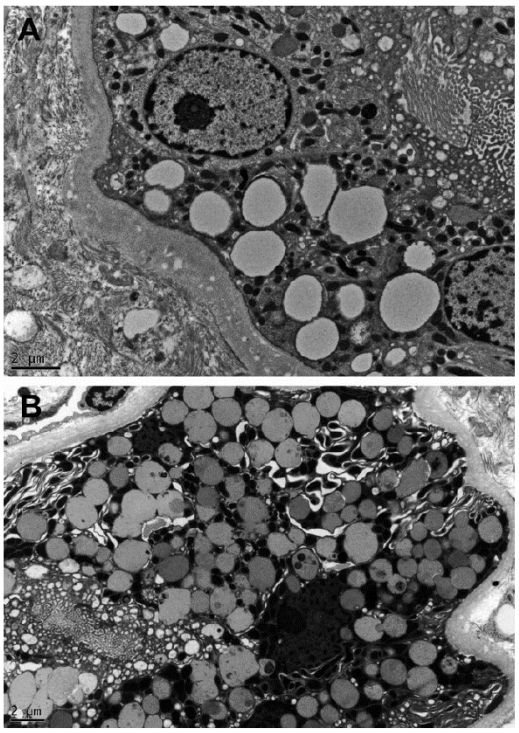

FIG. 3—*Electron microscopy of basal vacuoles in nephrotic syndrome demonstrating intravacuolar lipid close to, but separate from the basement membrane (A). Filling of the entire cell with vacuoles (B) (Magnifications ×8000, ×6000).*

Plotting the degree of hypercholesterolemia and hypertriglyceridemia against the severity of lipid vacuolization revealed no significant relationship.

Discussion

Renal tubular epithelial cells display varying patterns of vacuolization in response to different metabolic stimuli. Although basal vacuolization has been referred to as "Armanni–Ebstein" lesions due to the association with diabetic ketoacidosis, the two entities should be separated as they most likely have different constituents and etiology (1). The pathogenesis of Armanni–Ebstein lesions due to hyperglycemia has been clearly established in animal models (12); however, the pathogenesis of basal vacuolization remains unclear. Typically, basal vacuolization occurs with ketoacidosis in diabetes, alcoholism, and starvation, independent of blood glucose levels. The vacuoles stain positively with Oil-Red O, and intravacuolar lipids have been demonstrated on electron microscopy (13). Because of this observation, Thomsen et al. (9) have suggested that hyperlipidemia, lipiduria, and subsequent reabsorption in the renal tubules are the causes of these vacuoles. The association between ketoacidosis and hyperlipidemia, which has also been demonstrated in previous studies (14–17), lends support to this hypothesis. In the current study, patients with nephrotic syndrome were chosen for evaluation as

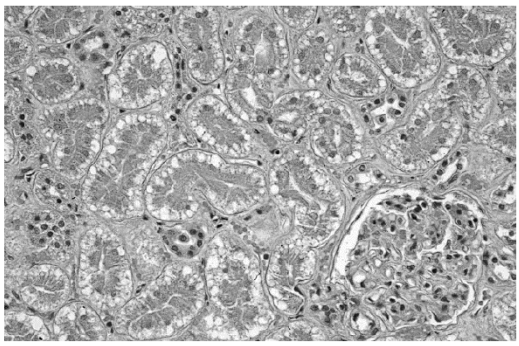

FIG. 4—*Typical subnuclear vacuoles in a case of diabetic ketoacidosis for comparison.*

hyperlipidemia occurs without a concurrent rise in serum ketones or glucose thus, enabling determination as to whether hyperlipidemia, independent of hyperglycemia and ketoacidosis, could result in basal lipid vacuolization. A limitation of this study was the time interval between lipid studies and the date of renal biopsy, and so a dose–response relationship could not be confidently established.

Hyperlipidemia is a feature of nephrotic syndrome with elevated concentrations of serum cholesterol, triglycerides, and phospholipids confined to lipoproteins that contain apoprotein B (18). One study of 207 adults with nephrotic syndrome due to nondiabetic renal disease showed a mean total cholesterol concentration of 203 mg/dL (7.8 mmol/L) (19), and another study with 100 patients revealed total serum cholesterol concentrations >200 mg/dL (5.2 mmol/L) in 87%, 300 mg/dL (7.8 mmol/L) in 53%, and 400 mg/dL (10.3 mmol/L) in 25% (20). The pathogenesis of hyperlipidemia is directly related to low plasma oncotic pressure secondary to proteinuria and hypoalbuminemia. There is an inverse relationship between serum albumin concentration, plasma oncotic pressure, and serum lipid concentrations (18,21). Furthermore, it has also been demonstrated that an increase in oncotic pressure secondary to albumin or dextran reverses these changes *in vitro* and decreases lipid levels (22). Decreased oncotic pressure leads to a compensatory increase in hepatic lipoprotein synthesis (23,24) through stimulation of hepatic apoprotein B gene transcription (22). Clearance of lipoproteins from the circulation may also be reduced in nephrotic syndrome as hypoalbuminemia causes decreased removal of free fatty acids and lysolecthin, which in turn leads to reduced activity of lipoprotein lipase and lectothin acyltransferase, respectively (24).

Although intracellular lipid vacuoles were observed in the patients with nephrotic syndrome, they appeared morphologically different to the typical lipid vacuoles found in ketoacidosis. Aggregated lipid in hyperlipidemia appeared as collections of circular vacuoles within the cytoplasm with well-defined rims that began basally and then spread to involve the entire cell (Fig. 1), whereas those in ketoacidosis tended to be purely basally located (Fig. 4) as a single large vacuole under the nucleus against the basement membrane. The numerous small subnuclear vacuoles in hyperlipidemia were also not necessarily in contact with the basement membrane. On light microscopy, vacuoles in ketoacidosis appeared more opaque than the translucent vacuoles in pure hyperlipidemia.

As basal lipid vacuolization was found in fourteen of the 15 study cases, it can be concluded that hyperlipidemia in the absence of hyperglycemia and ketoacidosis can be related to this type of change in renal tubular epithelial cells. However, as the vacuoles appeared morphologically different to those found in ketoacidosis, future studies will be needed to determine whether hyperlipidemia, independent of increased permeability in nephrotic syndrome, can result in lipid vacuoles that are similar structurally to those associated with ketoacidosis, or whether the synergistic effect of elevated serum ketones and lower pH is required to cause this particular phenotype. Despite these disparities, the differential diagnosis of renal tubular epithelial cell vacuolization detected in postmortem sections should include the possibility of hyperlipidemic states, rather than just ketoacidosis.

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Rapid communication

Armanni-Ebstein phenomenon and hypothermia

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ABSTRACT

Retrospective review was undertaken of 46 cases of lethal hypothermia for the presence of subnuclear vacuolization of renal tubular epithelial cells. Fifteen of the 46 cases (33%) had renal tubular vacuolization typical of the Armanni-Ebstein phenomenon. The age range was 30–87 years (average 59 years) with a male to female ratio of 6:9. Nine of the 15 cases with Armanni-Ebstein changes (60%) had a history of diabetes mellitus, and in seven of these vitreous humour biochemical analyses were performed, all of which revealed diabetic ketoacidosis (vitreous glucose levels = 32.9–85.3 mmol/L; β-hydroxybutyrate = 7.4–20 mmol/L). This study has confirmed the association between hypothermia and renal tubular epithelial vacuolization, but in addition raises the prospect that this may be contributed to in some cases by underlying diabetic ketoacidosis. Hypothermic deaths should, therefore, raise the possibility of diabetes mellitus and initiate postmortem biochemical measurement of vitreous humor glucose and β-hydroxybutyrate levels.

1. Introduction

Armanni-Ebstein is the term used to describe subnuclear vacuolization of renal tubular epithelial cells that is most often seen in poorly controlled diabetic states. Debate has occurred as to whether the accumulated material consists of glycogen or lipid, with recent data supporting the latter [1–3]. In addition to diabetes mellitus, renal tubular vacuolization has also been reported in cases of lethal hypothermia. The following study was undertaken to further investigate this association.

2. Materials and methods

Case files from Forensic Science SA, Adelaide, Australia were retrospectively reviewed over a six-year period from April 2004 to March 2010 for cases where deaths were due to, or contributed to, by hypothermia. All cases had undergone full police and coronial investigations with complete autopsy examinations being performed. Hypothermia had been diagnosed when there was a recorded antemortem core temperature less than 30 °C, or if erosive gastritis with so-called Wischnewsky spots was found at autopsy in an individual who had been alone for some time in a cool environment.

Autopsy reports were reviewed and the age, gender and pathological findings were summarized. Specific details of diabetic status, blood alcohol concentration and the results of vitreous biochemistry, if performed, were recorded. Histological sections of the kidneys were then examined for the presence of Armanni-Ebstein lesions. Cases where putrefaction and autolysis precluded accurate histological assessment were excluded from the series.

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3. Results

A total of 62 cases with terminal hypothermia were identified, 16 of which were excluded due to poor renal preservation. Of the remaining 46 cases, Wischnewsky spots were present in 44 (96%) and antemortem core temperatures were taken in 7 cases (ranging from 22.5 to 29.7 °C). Blood alcohol levels were measured in 31 cases ranging from 0 to 0.19%, with a mean of 0.02%. Fifteen of the 46 cases (33%) had renal tubular vacuolization typical of the Armanni-Ebstein phenomenon (Fig. 1). PAS/PAS-D staining did not reveal glycogen, although oil-red O staining and electron microscopy (Fig. 1 inset and Fig. 2) demonstrated lipid droplets. The age range was 30–87 years (average 59 years) with a male to female ratio of 6:9. Blood alcohol levels were measured in five of these cases and were negative. Nine of the 15 cases with Armanni-Ebstein changes (60%) had a documented medical history of diabetes mellitus; in the remaining six cases the clinical history was unknown and vitreous humour biochemical analyses had not been performed. Of the nine cases with a known history of diabetes mellitus, five had nodular glomerulosclerosis and seven had vitreous humour biochemical analyses performed, all of which revealed diabetic ketoacidosis (vitreous glucose levels = 32.9–85.3 mmol/L; β-hydroxybutyrate = 7.4–20 mmol/L); this was not tested for in the remaining two cases (Fig. 3). The postmortem interval when vitreous humor was withdrawn ranged from 2 to 6 days, mean 5.2 days.

A total of 31 of the 46 cases (67%) did not have Armanni-Ebstein lesions. The ages ranged from 38 to 89 years (average 69.23 years) with a male to female ratio of 13:18. A history of diabetes mellitus had been documented in four cases (13%) and was unknown in the

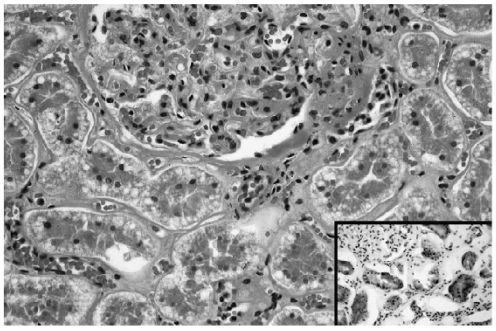

Fig. 1. Characteristic subnuclear vacuolization of renal tubular epithelial cells in a case of hypothermia and ketoacidosis (hematoxylin & eosin ×100). Inset shows patchy oil-red O staining of lipid droplets within tubular epithelial cells (×60).

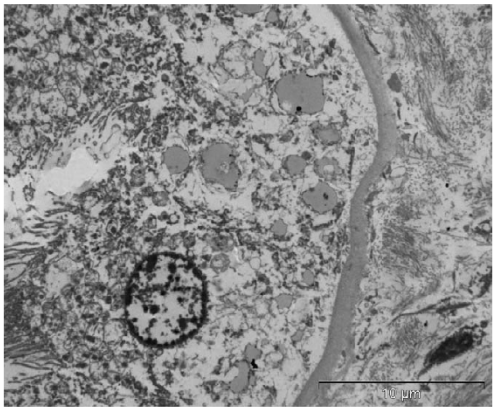

Fig. 2. Electron microscopy of the case shown in Fig. 1 demonstrating intraepithelial lipid accumulation.

remaining 27 cases. Diabetic ketoacidosis had not been tested for at autopsy.

Only one of the victims who had antemortem core temperatures taken (indicating survival for some time) had Armanni-Ebstein changes, and this was a woman who had died of diabetic ketoacidosis with significantly elevated glucose (47.6 mmol/L) and β-hydroxybutyrate (8.87 mmol/L) levels, and no detectable blood alcohol (Brief details of this case have been previously reported [4]). Deaths in the remaining six cases were due to: ischemic heart disease and dementia, ischemic heart disease, subdural hemorrhage, epilepsy and cardiomegaly, and bronchopneumonia ($N = 2$).

4. Discussion

Hypothermia results from the body core temperature falling to less than 35 °C and occurs when counter-regulatory mechanisms such as vasoconstriction and heat production are exceeded by heat loss to the environment [5,6]. Hypothermia is a significant event and has been associated with greater than 70% mortality when the core temperature drops to 30 °C, and 90% at 26 °C. Lethal mechanisms include ventricular fibrillation or asystole, contributed to by myocardial ischemia, hypoxia, electrolyte abnormalities, and elevated catecholamine levels [5].

The most common cause of severe hypothermia is accidental exposure to low ambient temperatures associated with a number of exacerbating factors such as damp conditions, air movement, inadequate or wet clothing, low muscle mass, alcohol ingestion and alcoholism, certain medications and drugs, trauma, open injuries, immobility, neurological, endocrine and cardiovascular disorders, and psychiatric illness [7]. Children and the elderly are at highest risk [5].

Erosive gastritis or Wischnewsky spots are present in 40–90% of fatal hypothermia cases, but this may be artificially high if this finding has been used as a diagnostic parameter, as in the current series. The lesions consist of superficial gastric erosions characterized histologically by necrosis of the mucosa with acid hematin formation. Acute pancreatitis with hemorrhage and surrounding fat necrosis may also rarely be present [5,6].

Fatty changes on routine histology have been associated with fatal hypothermia, with vacuolization of hepatocytes and cardiac myocytes occasionally being documented [6]. In terms of renal tubules, Preuβ et al. demonstrated basal vacuolization of tubular epithelial cells in 87% of cases of terminal hypothermia leading to the conclusion that these changes represented "a very reliable histologic diagnostic criterium in cases of hypothermia, comparable to the significance of Wischnewsky ulcers" [8]. The vacuoles stain positively for lipids and are morphologically similar to Armanni-Ebstein lesions that are strongly associated with poorly controlled diabetic states and deaths due to diabetic ketoacidosis [9,10]. While the vacuoles in Armanni-Ebstein lesions complicating diabetes were initially thought to contain to glycogen [9–13], recent studies have demonstrated positive staining for lipids, suggesting that the vacuoles consist of accumulated triglycerides [1–3]. Occasionally the vacuolization may be so profound that it can be observed macroscopically as cortical pallor [4]. In our series, a case of hypothermia and diabetic ketoacidosis demonstrated lipid on both histological staining and on electron microscopy (Figs 1 and 2).

Although it has been asserted that "fatty degeneration of renal tubules cannot be regarded as a sign of diabetes" [8], it is unclear from this study what the levels of glucose and β-hydroxybutyrate were. Certainly while many individuals with a history of diabetes mellitus do not manifest Armanni-Ebstein phenomenon, due to lack of terminal ketoacidosis, others do [1–3,9–14]. Thus, as the renal tubular vacuoles in hypothermia and in diabetic ketoacidosis appear similar in morphology and composition, the current study

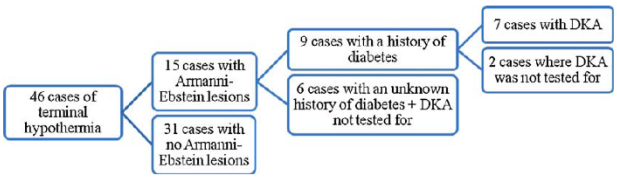

Fig. 3. An analysis of 46 cases with hypothermia showing the number of cases with Armanni-Ebstein phenomenon and the relationship to diabetes mellitus and ketoacidosis (DKA) on vitreous humor testing.

was undertaken to ascertain whether there might be a relationship between cases of hypothermia with Armanni-Ebstein phenomenon, and diabetes mellitus, as has been suggested in a case report that was not included in the present series [15].

The diagnosis of Armanni-Ebstein phenomenon at autopsy may be difficult if there has been significant putrefaction and/or autolysis, as this may cause nonspecific cytoplasmic vacuolization and artefactual separation of proximal tubular epithelial cells from the basement membrane, features that resemble Armanni-Ebstein lesions. This may be predisposed to in hypothermic deaths when there has been social isolation and delay in finding the victim, as this may enhance putrefactive changes [16].

The present study has shown that more than half of the cases of terminal hypothermia where Armanni-Ebstein change was observed (60%) had an established history of diabetes mellitus. This may well be a significant underestimation, as a history of diabetes mellitus may not have been volunteered in the remaining cases, and so vitreous biochemical testing was not routinely undertaken. Significantly, of the nine known diabetic victims with Armanni-Ebstein lesions, all who were tested ($N = 7$) had biochemical evidence of diabetic ketoacidosis. This suggests that renal tubular epithelial vacuolization in cases of hypothermia may have a significant association with diabetic ketoacidosis.

In turning to the literature, there is certainly a recognized association between diabetes mellitus and hypothermia, with diabetic individuals being more susceptible to dropping their core temperature [7] resulting in hospital admissions for hypothermia being more common among diabetic patients compared to the general population [17,18]. This may be influenced by the greater frequency of pathological conditions that are associated with an increased risk of hypothermia among the diabetic population, such as other endocrinopathies [18]. Autonomic neuropathy, which is a common complication of diabetes mellitus, also places individuals at increased risk of developing hypothermia due to impaired physiologic thermoregulatory mechanisms, including peripheral vasoconstriction [17,19,20].

Metabolic complications of diabetes mellitus can cause secondary hypothermia. For example, hypoglycaemia may result in increased heat loss from sweating, inhibition of shivering, and peripheral vasodilation [17–19]. Hypothermia may also be a cause and a complication of diabetic ketoacidosis [21] with Gale and Tattersall reporting diabetic ketoacidosis as the basis for 11.8% of all hospital admissions for severe hypothermia; a rate higher than that for hypothyroidism (8%) [22]. Acidosis can interfere with thermoregulation and cause peripheral vasodilation with a reduced ability to maintain core body temperature when exposed to cold ambient temperatures [23,24]. Insulin deficiency in diabetic ketoacidosis also decreases the uptake of glucose into muscles and adipose tissue, leading to decreased substrate availability for heat production and impaired chemical thermogenesis [7,21,23].

A reverse relationship between hypothermia and diabetes mellitus also exists as primary hypothermia can worsen a decompensated diabetic state. When the core temperature decreases below 32 °C, insulin activity and release are both markedly reduced, resistance to exogenous insulin develops, and peripheral utilization of glucose declines. Additionally, there is also increased secretion of catecholamines and cortisol during hypothermic states, which exacerbate diabetic ketoacidosis [7,22,24]. Consequently, concurrent hypothermia and diabetic ketoacidosis may initiate a vicious metabolic cycle associated with a high mortality rate exceeding 30% [25].

Although in the current study, Armanni-Ebstein lesions were found in only approximately a third of cases with terminal hypothermia, considerably less than other studies [8], the association between renal tubular epithelial vacuolization and hypothermia was confirmed. While the numbers in the study are relatively small and the review was retrospective, a history of diabetes mellitus was recorded in autopsy files in over a half of those with Armanni-Ebstein changes, and all of those from this group who were tested exhibited biochemical evidence of diabetic ketoacidosis. Thus, we concur with other authors that Armanni-Ebstein lesions are a feature of hypothermic deaths [5,6,8], but would also suggest that in some cases this may occur due to an association with diabetic ketoacidosis, rather than as a result of a significant reduction in body temperature *per se*. Thus when hypothermia is suspected at autopsy, specific enquiry should be made regarding a possible history of diabetes mellitus and vitreous humour should be tested for elevated glucose, β-hydroxybutyrate and lactate to determine whether there is biochemical evidence of underlying diabetic ketoacidosis.

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PAPER

PATHOLOGY/BIOLOGY

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Renal Cortical Pallor—A Useful Macroscopic Marker for Metabolic Derangements at Autopsy

ABSTRACT: Renal cortical pallor was studied as a potential marker at autopsy of diabetic ketoacidosis in 23 cases, hyperglycemic nonketotic coma in eight cases, and alcoholic ketoacidosis in five cases (vitreous humor glucose level $\geq$11.1 mM; β-hydroxybutyrate level $\geq$5 mM). Renal cortical pallor was noted on macroscopic examination in 10 of 23 cases of lethal diabetic ketoacidosis (43.5%), three of eight cases of fatal hyperglycemic nonketotic coma (37.5%), and in two of five cases of alcoholic ketoacidosis (40%). Histologic examination revealed basal vacuolization of renal tubular epithelial cells in 12 cases, Armanni–Ebstein lesions in 10, and osmotic nephrosis in three. Although renal cortical pallor did not appear to be a particularly sensitive marker for hyperglycemia or ketoacidosis, and did not correlate with the severity of these parameters, it may still represent a useful macroscopic marker for underlying metabolic conditions at autopsy and should therefore prompt measurement of vitreous humor glucose and β-hydroxybutyrate levels.

KEYWORDS: forensic science, renal cortical pallor, ketoacidosis, diabetes mellitus, alcoholism, hyperglycemia, epithelial vacuolization, Armanni–Ebstein

In previous studies, we have investigated the histologic manifestations of ketoacidosis and hyperglycemia in the tubular epithelium of the kidney, where it appears that at least two distinct changes may occur involving basal vacuolization and Armanni–Ebstein change (1,2). Although there has been some confusion in the literature regarding terminology, swelling of cells with loss of normal shape, clearing of cytoplasm, and accumulation of cytoplasmic glycogen represent the classical changes first described by Armanni and Ebstein due to glycogen nephrosis associated with hyperglycemia and glucosuria (3). In addition, basal vacuolization due to lipid accumulation with luminal displacement of nuclei may be present and linked to ketoacidosis (3). Osmotic nephrosis is another change that may occur in tubular epithelial cells exposed to high solute concentrations with diffuse intracytoplasmic vacuolization. One of the associated changes that we observed macroscopically in some of these cases was marked renal cortical pallor (4,5). The following study was undertaken to determine how useful this feature is at the time of autopsy dissection as a marker of underlying metabolic disturbance and whether it is of value in the assessment of lethal diabetic ketoacidosis, hyperglycemic nonketotic coma, and alcoholic ketoacidosis.

Materials and Methods

Case files over a 6-year period from 2004 to 2009 at Forensic Science SA, Adelaide, South Australia, were retrospectively reviewed for all cases in which the cause of death was listed as diabetic ketoacidosis, hyperglycemic nonketotic coma, or alcoholic ketoacidosis. All cases had full coronial and police investigations with complete forensic autopsies. Significant diabetic ketoacidosis was recorded when the vitreous humor glucose level was $\geq$11.1 mM and β-hydroxybutyrate level was $\geq$5 mM. Hyperglycemic coma was recorded when there was vitreous hyperglycemia ($\geq$11.1 mM) with no elevation of β-hydroxybutyrate levels. Significant alcoholic ketoacidosis was diagnosed when the vitreous humor β-hydroxybutyrate level was $\geq$5 mM with normo- or hypoglycemia (vitreous glucose level $\leq$11 mM) in conjunction with a history of alcohol abuse and/or autopsy findings suggestive of chronic alcoholism. Cases that did not meet these criteria, those with an incomplete vitreous humor biochemical screen, and those in which kidneys were autolysed to a level that prevented accurate histologic assessment were excluded from the series.

The autopsy reports of the study cases were then reviewed with special attention to the documentation of renal cortical pallor. When pallor was present, histologic slides of the kidneys were examined. Statistical analysis was conducted using unpaired Student's *t*-test, and statistical significance was defined as $p < 0.05$.

Results

A total of 36 study cases were identified with 23 deaths due to diabetic ketoacidosis, eight due to hyperglycemic nonketotic coma, and five due to alcoholic ketoacidosis.

The 23 cases of lethal diabetic ketoacidosis consisted of 13 men and 10 women (age range 21–80 years; mean 47.6 years). Ten had renal cortical pallor documented at autopsy (43.5%; age range 21–80 years; mean 43.4 years; M:F = 1:1; Fig. 1). In this

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group, vitreous humor glucose level ranged from 32.9 to 71.8 mM (mean 47.33 mM) and β-hydroxybutyrate level ranged from 8.85 to 20.40 mM (mean 13.15 mM). The remaining 13 cases did not have pallor of the kidneys documented. These consisted of eight men and five women, whose age ranged from 25 to 80 years (mean 50.8 years). Their vitreous glucose levels ranged from 22.2 to 91.9 mM (mean 54.73 mM) and β-hydroxybutyrate levels ranged from 6.3 to 20.0 mM (mean 12.06 mM). There was no statistically significant difference in the glucose ($p = 0.35$) and β-hydroxybutyrate levels ($p = 0.53$) between the cases with renal cortical pallor and those without. Seven cases with renal cortical pallor showed superficial gastric erosions or Wischnewsky spots, indicating that hypothermia played a role in the terminal episodes.

The eight cases of fatal hyperglycemic nonketotic coma were aged between 14 and 60 years (mean 43.1 years; M:F = 5:3). Three of these cases had pallor of the renal cortices (37.5%; age range 37–60 years; mean 50.3 years; all were men). The vitreous glucose levels ranged from 19.7 to 35.8 mM (mean 28.3 mM), with normal β-hydroxybutyrate levels (range 0.2–2.55 mM; mean 1.25). The remaining five cases that did not have noticeably pale renal cortices were aged from 14 to 52 years (mean 38.8 years; M:F = 2:3) with vitreous glucose levels ranging from 17.0 to 49.7 mM (mean 28.88 mM) and β-hydroxybutyrate levels ranging from 0.02 to 1.65 mM (mean 0.7 mM). There was no statistically significant difference in the glucose ($p = 0.95$) and β-hydroxybutyrate levels ($p = 0.41$) between the cases with and without renal cortical pallor.

All five cases where death was due to alcoholic ketoacidosis had a history of chronic alcohol abuse, two with hepatomegaly, three with steatosis and periportal fibrosis, and one each with macrovesicular steatosis and splenomegaly. The age ranged from 51 to 72 years (mean 61 years), and all were men. Two cases had renal cortical pallor (40%), both were men aged 63 and 51 years, respectively. Vitreous humor glucose levels were 2.6 and 4.2 mM and β-hydroxybutyrate levels were 7.40 and 6.42 mM, respectively. The remaining three cases without pallor of the kidneys were aged from 51 to 72 years (mean 63.7 years). There was no elevation in glucose levels (range 0.1–0.3 mM; mean 0.17), with elevated β-hydroxybutyrate levels ranging from 7.55 to 8.75 mM (mean 8.17). Given the low numbers of these cases, statistical evaluations were not performed. One case with renal cortical pallor had superficial gastric erosions (Wischnewsky spots) in keeping with hypothermia.

Histologic examination of slides from the cases with diabetic ketoacidosis and cortical pallor revealed basal vacuolization of tubular epithelial cells in all 10 cases (Fig. 2), with Armanni–Ebstein changes in seven (Fig. 3). In the three cases of hyperglycemic nonketotic coma and cortical pallor, there were two cases with Armanni–Ebstein change and no cases with basal vacuolization. Both cases of alcoholic ketoacidosis had basal vacuolization with one case having Armanni–Ebstein changes. In this case, there was demonstrable pancreatic fibrosis with a 4-day postmortem interval. Three cases had diffuse vacuolization and swelling typical of osmotic nephrosis.

Discussion

Pallor of the kidneys involving both the cortex and medulla may be observed in situations of acute blood loss. Differential pallor of the cortex compared with the medulla is, however, suggestive of a more specific and localized effect, which may be a marker for local cell necrosis. For example, pallor of the renal cortices is a feature of renal cortical necrosis, in which coagulative necrosis results in the development of patchy or diffuse sharply demarcated zones of pallor with hyperemic rims (6,7). It may also be seen in acute tubular necrosis (8,9) and has been

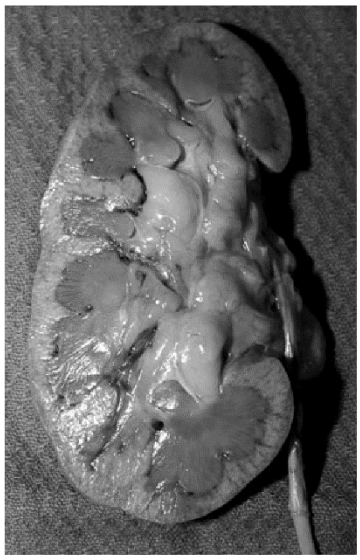

FIG. 1—*Typical pallor of the renal cortex relative to the medulla that may be observed in cases of diabetic ketoacidosis, hyperglycemic nonketotic coma, and alcoholic ketoacidosis.*

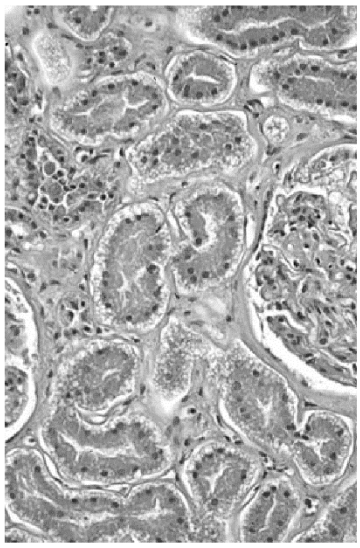

FIG. 2—*Diffuse basal submuclear vacuolization of renal tubular epithelial cells in a case of ketoacidosis with macroscopic renal cortical pallor (hematoxylin and eosin × 150).*

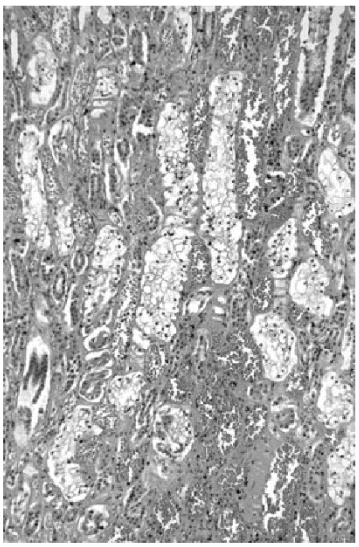

FIG. 3—*Armanni–Ebstein lesions of renal tubules with ballooning of epithelial cells in a case of diabetic ketoacidosis with macroscopic renal cortical pallor (hematoxylin and eosin × 100).*

reported in cases of severe asphyxia (10) and septicemia (11,12). Cortical pallor may also be a feature of acute renal failure, possibly due to the redistribution of blood flow away from cortical glomeruli (13). Renal cortical pallor may also be observed in cases where death involves diabetic ketoacidosis and hypothermia, due to underlying metabolic derangements that may merely alter the composition and structure of renal tubular epithelial cells, rather than inducing necrosis (4,5).

Three different metabolic disorders commonly seen in the forensic context were investigated in this study, including diabetic ketoacidosis, hyperglycemic nonketotic coma, and alcoholic ketoacidosis. Diabetic ketoacidosis arises when counterregulatory hormone excess in conjunction with insulin deficiency leads to increased gluconeogenesis, increased glycogenolysis, and decreased glyconeogenesis. Ketogenesis also occurs as oxaloacetate is directed to gluconeogenesis, resulting in a relative lack of oxaloacetate for use in the Krebs cycle, and a corresponding rise in acetyl-CoA, which would otherwise have been consumed. Consequently, acetyl-CoA is used for the production of ketones (14,15), and elevated levels of both glucose and β-hydroxybutyrate may be detected in the vitreous humor at autopsy.

In contrast, ketoacidosis does not occur in most type 2 diabetics with metabolic decompensation; instead, they progress to a hyperglycemic hyperosmolar nonketotic state. This is most likely due to the presence of residual pancreatic function with secretion of small amounts of insulin that depress the release of counterregulatory hormones and the mobilization of free fatty acids, thus inhibiting ketogenesis (16). The vitreous biochemistry profile in these cases is, therefore, one of elevated glucose levels in the absence of elevated β-hydroxybutyrate.

Alcoholic ketoacidosis tends to occur in malnourished, chronic alcoholics who have low protein and carbohydrate stores and/or alcoholic liver disease with subsequent hepatic glycogen depletion (17,18). This, in addition to volume depletion caused by vomiting, decreased fluid intake, and/or diaphoresis, leads to a fall in blood glucose levels, the suppression of insulin, and a sympathetic response causing increases in catecholamines, cortisol, growth hormone, and glucagon levels (17,19). These hormones, in conjunction with the direct effect of ethanol, promote the mobilization of free fatty acids to the liver (17). When the supply exceeds the rate of oxidation, a surplus of acetyl-CoA results, which is converted into acetoacetate (20). Additionally, alcohol dehydrogenase preferentially uses NAD^+ to convert ethanol to acetaldehyde, leading to less NAD^+ being available to convert lactate to pyruvate, with subsequent decreased production of oxaloacetate required for gluconeogenesis; thus, gluconeogenesis is inhibited. Alcoholic ketoacidosis can be identified on postmortem vitreous biochemistry by an elevated β-hydroxybutyrate levels in the setting of hypo- or normoglycemia.

Macroscopic pallor of the renal cortices was found in all three of these metabolic disorders, specifically in 43.5% of those with diabetic ketoacidosis, in 37.5% of those with hyperglycemic nonketotic coma, and in 40% of those with alcoholic ketoacidosis (Table 1). In the cases of diabetic ketoacidosis and those dying from hyperglycemic nonketotic coma, no statistically significant difference could be demonstrated between the cases that had renal cortical pallor and those that did not, in the glucose ($p = 0.35$ and 0.95, respectively) and β-hydroxybutyrate levels ($p = 0.53$ and 0.41, respectively). Thus, the presence of macroscopic pallor did not appear to correlate with the severity of the underlying metabolic disturbance.

Histologic investigation was also carried out in those cases with pale renal cortices to identify corresponding morphological abnormalities. Subnuclear vacuolization of tubular epithelial cells, as described by Thomsen et al. (20,21), was found in all of the cases of diabetic and alcoholic ketoacidosis ($N = 12$), in keeping with the effects of ketoacidosis. Characteristic Armanni Ebstein changes were seen in the tubules of 70% (7/10) of those who had died of diabetic ketoacidosis and in 67% (2/3) of those who had died from hyperglycemic nonketotic coma whose kidneys displayed renal cortical pallor. This would be in keeping with the effects of hyperglycemia. It is apparent, therefore, that renal cortical pallor may be caused by different subcellular morphological abnormalities.

An unusual finding was that of Armanni–Ebstein change in renal tubular epithelial cells in a case of alcoholic ketoacidosis without an elevated glucose level. However, in this case, it is possible that the glucose level was artifactually reduced following death as there was a postmortem interval of 4 days. The presence of pancreatic fibrosis could be further evidence to suggest that there may have been an underlying disturbance in pancreatic function.

A potential problem with this study is its retrospective nature, in that not all cases of renal cortical pallor may have been documented. However, alternatively, this also means that at the time of assessment of renal color, there was no skewing by potential observer bias.

TABLE 1—*Numbers and percentages of cases of diabetic ketoacidosis, hyperglycemic nonketotic coma, and alcoholic ketoacidosis with macroscopically noted renal cortical pallor at autopsy.*

	Renal Cortical Pallor		
	+	–	*N*
Diabetic ketoacidosis	10 (43.5%)	13	23
Hyperglycemic nonketotic coma	3 (37.5%)	5	8
Alcoholic ketoacidosis	2 (40%)	3	5

An interesting observation made during the study was that concurrent hypothermia was present in seven of the 10 individuals who died from diabetic ketoacidosis with renal cortical pallor. It was also present in the single case of alcoholic ketoacidosis with pallor. Although it has been proposed that one of the substrates of cortical pallor, renal epithelial subnuclear vacuolization, may be caused by hypothermia in isolation (22), we consider that it is more likely to involve a more complex interaction between both diabetic ketoacidosis and reduced body core temperature (23). The concurrence of the two in the present study may suggest that hypothermia may add to the underlying metabolic disturbance and therefore could contribute to the development of macroscopic pallor.

In conclusion, this study has shown that renal cortical pallor in cases of suspected ketoacidosis or hyperglycemia represents a marker for significant metabolic derangement in only 37.5–43.5% of cases. Once observed at autopsy, however, full biochemical screening may provide further useful information. The histologic basis for pallor was underlying glycogen nephrosis (Armanni–Ebstein phenomenon), subnuclear lipid vacuolization, and/or osmotic nephrosis. The presence of renal cortical pallor did not appear to be a reflection of the severity of the underlying metabolic derangement and may have been contributed to by hypothermia. The sensitivity and specificity of renal cortical pallor in the general autopsy population as a marker for metabolic disturbance remains to be determined.

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Original communication

Basal epithelial formalin pigment deposition in the kidneys – A useful marker for ketoacidosis at autopsy

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ABSTRACT

Basal vacuolization of renal epithelial cells occurs in diabetic and alcoholic ketoacidosis, hypothermia and starvation. The vacuoles contain triglycerides. Following a case where formalin pigment deposition within these vacuoles led to the identification of ketoacidosis, a retrospective review of a further 31 cases with ketoacidosis, was undertaken. There were 24 diabetics and 7 alcoholics (age range 21 80 yrs; mean 50.9 yrs; M:F ratio = 2:1. The post-mortem interval was 1–12 days (mean – 4.5 days). Characteristic basally-located pigment surrounding vacuoles was found in 16 cases (51.6%) (14 diabetic ketoacidosis; 2 alcoholic ketoacidosis). Fifteen cases had no formalin pigment deposition. No relationship could be found between the intensity of staining and the postmortem interval, degree of putrefaction, or level of vitreous humour β-hydroxybutyrate. No staining was demonstrated in control cases matched for postmortem interval. Although formalin pigment deposition occurred in only 51.6% of cases with proven ketoacidosis at autopsy, it appeared to be a highly specific phenomenon. As these deposits were identifiable after recognizable cellular morphology had been lost due to autolysis and putrefaction, this artefact of fixation may be of particular use in suggesting the possibility of ketoacidosis in decomposed bodies with compromised histology.

1. Introduction

The aetiology of basal vacuolization of epithelial cells of the proximal convoluted tubules of the kidney in the cortex and outer medulla remains unclear.[1] Cases have been reported in association with diabetic and alcoholic ketoacidosis, hypothermia and starvation,[2–12] and may be identified macroscopically at autopsy by renal cortical pallor.[13,14] Vacuoles have been shown to contain the neutral lipid, triglyceride.[3,4] As this could be the only indication of ketoacidosis at autopsy, this may be a very significant diagnostic finding. Unfortunately, cellular morphology is progressively lost after death; a process which may be exacerbated by the high glucose levels in diabetics,[15] resulting in almost complete loss of microscopic detail in badly decomposed bodies. One of the authors (JDG) has observed, however, that formalin pigment preferentially deposits in the areas of basal vacuolization. The results of the following case and study are reported to demonstrate the usefulness of this finding as a marker of basal vacuolization from ketoacidosis, particularly when tissue preservation is suboptimal.

2. Case report

A case of unexpected death is reported in an adult male who was found dead in bed at his home address. At autopsy there were moderate putrefactive changes. Toxicology did not reveal any lethal drugs or poisons. There were no injuries present. Although extensive putrefactive changes hindered accurate assessment of the histology (postmortem interval = 6 days), the pancreas showed patchy lobular atrophy and fibrosis and the liver focal severe steatosis, in keeping with alcohol related damage. In addition, deposits of birefringent formalin pigment were noted outlining subnuclear basal spaces in proximal convoluted tubular epithelial cells of the kidneys (Fig. 1). As this had been previously observed by one of the authors (JDG) in cases of ketoacidosis, biochemical analysis of vitreous humour was undertaken. This showed a markedly elevated glucose level of (51.3 mmol/L) and a raised level of

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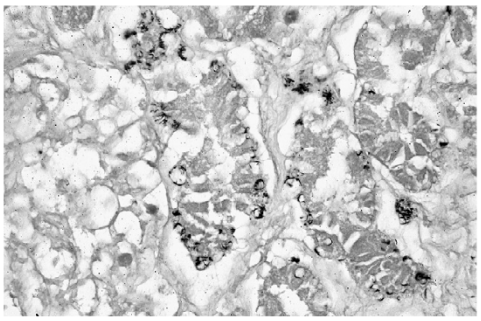

Fig. 1. Prominent formalin pigment deposition in the reported case with a prolonged postmortem interval (6 days) and marked loss of cellular detail demonstrating the usefulness of the finding in cases where cellular morphology is lacking (Haematoxylin and Eosin [H&E] × 100).

β-hydroxybutyrate (8.33 mmol/L). Death was therefore attributed to diabetic ketoacidosis. Subsequent information indicated that the deceased had been complaining of polyuria and polydipsia in the week preceding death and had a family history of diabetes mellitus.

3. Materials and methods

In view of these results, case files were retrospectively reviewed at Forensic Science SA, Adelaide, South Australia, over a six-year period from 2004 to 2009 for other cases of diabetic and alcoholic ketoacidosis. All cases had full coronial and police investigations with complete forensic autopsies. Ketoacidosis was diagnosed when the vitreous humour β-hydroxybutyrate was ≥5 mmol/L. Postmortem interval was noted. A control group matched for postmortem interval was randomly selected from case files from the same time period for comparison. All cases had renal tissue routinely sampled at autopsy and fixed in 10% buffered formalin solution for 24–48 h prior to processing for histology.

A single renal slide stained with haematoxylin and eosin from each case was evaluated blind for basal vacuolization and the presence of characteristic formalin pigment deposition: i.e. along the base of epithelial cells and outlining basal vacuoles within the proximal convoluted tubules of the kidney in the cortex and outer medulla. Cases were graded as 0 if there was no evidence of this type of basal tubular epithelial cell formalin pigment deposition, 1+ if there was mild focal deposition, 2+ if there was moderate-severe focal, or diffuse mild deposition, and 3+ if there was moderate to marked diffuse deposition. Cases were also graded from 0 to 3+ for autolytic/putrefactive changes with 0 being no autolysis or putrefaction, 1+ mild, 2+ moderate and 3− marked, the latter with complete loss of normal cellular detail.

The amount of formalin pigment deposition was compared between the cases with ketoacidosis and the controls, and was also plotted against postmortem interval, degree of autolysis/putrefaction and β-hydroxybutyrate levels.

4. Results

A total of 31 cases were found with ketoacidosis, consisting of 24 individuals with diabetes mellitus and seven with alcoholism. The age range was 21–80 years (mean 50.9 years) with a male to female ratio of 20:11. The post-mortem interval ranged from 1 to 12 days (mean − 4.5 days). A frequent feature of basal epithelial cell formalin pigment deposition was geographic variation, with no staining in some areas of the cortex contrasting with marked staining in other areas in the same case (Fig. 2). Formalin pigment was found in 16 cases (51.6%) (in 14 cases of diabetic ketoacidosis [58.3%] and in 2 cases of alcoholic ketoacidosis [28.6%] [Fig. 3]). The number of cases without formalin pigment deposition was 15. There were 13 cases with no signs of decomposition, 7 cases with 1+ decomposition, 11 cases with 2+ decomposition. Staining was intense (≥2+) in cases with short postmortem intervals and no autolysis/putrefaction (Fig. 4), and also in cases with long postmortem intervals and marked autolysis/putrefaction, as in the reported case. No staining was demonstrated in any of the 31 control cases.

Plotting the degree of formalin pigment deposition against postmortem interval revealed no significant relationship. Similarly, no relationship could be demonstrated between the degree of autolytic/putrefactive changes and degree of formalin pigment deposition, or β-hydroxybutyrate levels and formalin pigment deposition.

5. Discussion

A variety of different types of vacuoles occur in renal tubules. Clear cell change of the proximal tubular epithelium may be caused by hyperglycemia and is known as glycogen nephrosis or Armanni Ebstein change.[16–18] Ketoacidosis from a variety of causes may result in basal vacuolization of tubular cells, with displacement of nuclei towards the lumina.[2,3] The detection of these changes on postmortem histology should raise the suspicion of an underlying metabolic disturbance and initiate biochemical analysis of vitreous humour to test for this possibility.[14] However, renal tubular epithelium quickly degrades after death due to the combined effects of autolysis and putrefaction. Thus, basal vacuolization may be obscured by cytoplasmic degeneration and lifting of cells away from the basement membrane. In the absence of a history or other indications of underlying metabolic derangements, such as alcohol abuse or diabetes mellitus, these diagnoses may go undiscovered.

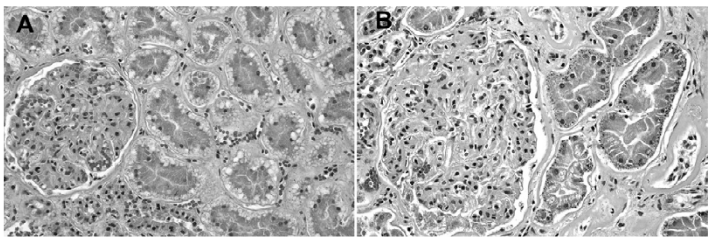

Fig. 2. Characteristic basal vacuolization of the epithelial cells of the proximal convoluted tubules of the kidney in the cortex in a case of diabetic ketoacidosis with no formalin pigment deposition (A), contrasting with adjacent areas where there was prominent deposition of pigment (B) (H&E × 100).

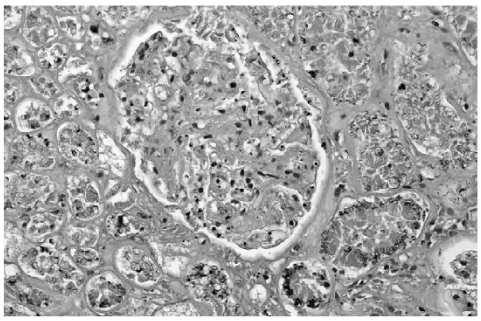

Fig. 3. Characteristic basal vacuolization of the epithelial cells of the proximal convoluted tubules of the kidney in the cortex in a case of alcoholic ketoacidosis with prominent formalin pigment deposition (H&E × 100).

Formaldehyde is used as a tissue preservative in most histology laboratories, and is commonly utilized in buffered preparations at 10% concentration. This fixative has been known to produce an artefact known as 'formalin pigment' or 'acid formaldehyde haematin', which appears as dark brown to black, finely granular, birefringent microcrystals.[19–21] These pigments are iron-free derivatives of haemoglobin, and are formed when blood-rich tissues are fixed with aqueous solutions of formaldehyde at pH less than 6.0.[20,22] Historically, these pigments have been deemed an artefact with no pathologic significance, and many techniques have been described for their prevention and removal.[19,23–25]

In 1971, Holmes reported an affinity of formalin pigment for fat, noting that it was often localized to cells which contained fat before processing of tissues in solvents. These included fatty vacuoles within cells of the liver, kidneys, and lungs.[21] The current study has demonstrated that formalin pigment also preferentially deposits in tissue sections of the kidney during fixation in areas of accumulated triglyceride. This striking aggregation of formalin pigment to the areas of triglycerides may occur because of the breakdown of triglycerides into component fatty acids, providing a localized area of reduced pH that provokes pigment deposition.

The observation that formalin pigment preferentially deposits in the vacuolated epithelial cells of the renal tubules in cases of ketoacidosis (either diabetic or alcoholic) may be significant as this may enhance the postmortem detection of metabolic disturbances. As pigment deposition may be very focal in nature, it may be useful to examine multiple areas of the cortex and outer medulla. The reason for the lack of correlation with postmortem interval and degree of decomposition is unclear, but may be related to factors that were not controlled for in this study such as minor differences in concentration of the formalin solution, amount of buffer, or time of fixation. While formalin deposition does not appear to be a particularly sensitive marker of ketoacidotic basal vacuolization (occurring in only 51.6% of cases), it does appear to be highly specific (100%). Given that these deposits are present even after autolysis and putrefaction have destroyed recognizable cellular morphology, this finding may therefore be of particular use in decomposed bodies, as was clearly demonstrated in the reported case.

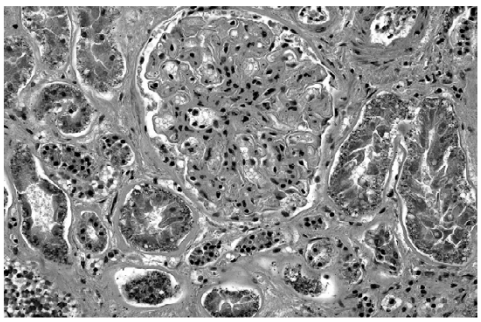

Fig. 4. Prominent formalin pigment deposition in a case with a short postmortem interval (1 day) and no autolysis or putrefaction (H&E × 100).

Ethical approval

Forensic Science South Australia.

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Conflict of interest

None.

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PAPER

PATHOLOGY/BIOLOGY

Chong Zhou,[1,2] *M.B.B.S.; Andrea J. Yool,*[3] *Ph.D.; and Roger W. Byard,*[2,3] *M.D.*

Armanni–Ebstein Lesions in Terminal Hyperglycemia

ABSTRACT: Armanni–Ebstein lesions (AEL) occur in deaths related to uncontrolled diabetes mellitus. To investigate the relationship between AEL and terminal hyperglycemia, we retrospectively reviewed 71 cases with vitreous glucose levels ≥11.1 mmol/L; 27 (38%) cases had AEL (vitreous glucose 14.0–77.3 mmol/L); and 44 cases (62%) did not (vitreous glucose 11.1–91.9 mmol/L). There was no significant difference ($p = 0.271$) in vitreous glucose levels between the cases with AEL (mean 39.2, SD 16.7 mmol/L) and those without (mean 34.2, SD 19.8 mmol/L). Similarly, there was no difference in the degree of dehydration, renal failure, or osmolality. However, there was a significantly higher level of β-hydroxybutyrate among the cases with AEL compared to those without ($p = 0.007$), suggesting that ketoacidosis may facilitate the development of AEL. Given the possible synergistic role of β-hydroxybutyrate, the correlation between AEL and terminal hyperglycemia in animal studies may not be applicable to humans. AEL may also possibly occur with sublethal elevations in glucose.

KEYWORDS: forensic science, Armanni–Ebstein phenomenon, glycogen nephrosis, diabetes mellitus, hyperglycemia, renal tubular vacuolization

The Armanni–Ebstein phenomenon refers to a pattern of renal tubular epithelial vacuolization which has been regarded as pathognomonic for hyperglycemia in diabetes mellitus (1). It is characterized by markedly swollen epithelial cells with clear cytoplasm, representing virtual total conversion of the cytoplasm into a single large vacuole filled with PAS-positive material (1). Microdissection studies have localized this lesion to the corticomedullary junction, mainly in the outer medulla, with the middle and outer cortex being spared (1). Electron microscopy has confirmed β-glycogen as the constituent of these vacuoles (2–4), hence the alternative name of glycogen nephrosis (5). Curtis et al. demonstrated that the presence of Armanni–Ebstein lesions in the kidneys in alloxan-induced diabetic rats depended solely upon the degree of terminal hyperglycemia, with lesions invariably present at levels above 350 mg/dL (19.4 mmol/L), and consistently absent at levels below 300 mg/dL (16.7 mmol/L) (5). No relationship was demonstrated between the presence of Armanni–Ebstein lesions and the initial blood glucose level after induction of diabetes, or with the maximum glucose level attained by the rats (5).

Although there have been numerous animal studies in diabetic rats (3,5–8), there are limited reports of Armanni–Ebstein lesions in a forensic context, and none that have investigated its correlation with terminal glucose levels. The phenomenon was originally observed in autopsy studies performed by Armanni in 1872 and appeared in Cantani's Textbook of Internal Medicine in 1875 only as an observation without correlations to glucose levels at death (9,10). Ritchie and Waugh conducted microdissection studies on the kidneys of five selected autopsy cases of diabetic decedents with Armanni–Ebstein lesions to localize these lesions, but the terminal blood glucose level was only reported in three of the five cases, ranging from 424 to 945 mg/dL (23.6–52.5 mmol/L). Smith and Glickman investigated diabetic vacuolization of the iris pigment in 57 postmortem cases and incidentally found 19 cases with terminal blood glucose levels >200 mg/100 mL (11.1 mmol/L) that displayed Armanni–Ebstein lesions in the kidneys; however, the degree of hyperglycemia in these cases was not reported (11). Armanni–Ebstein lesions have also been reported in five autopsy cases of severe Fanconi syndrome with glucosuria and death in infancy; however, again the blood glucose levels were not presented (2). Additionally, many of the more recent forensic studies and case reports of Armanni–Ebstein lesions refer to basal lipid vacuolization of renal tubular epithelial cells (12–18), which we now recognize as distinct from the Armanni–Ebstein phenomenon (19). Therefore, this study was undertaken to investigate the incidence of traditional Armanni–Ebstein lesions in terminal hyperglycemia, its correlation with vitreous glucose levels, and to identify potential factors which may have contributed to its pathogenesis.

Materials and Methods

All case files which included vitreous humor analysis over an 11-year period from 2004 to 2014 at Forensic Science SA, Adelaide, South Australia, were retrospectively reviewed for cases with terminal hyperglycemia, defined as vitreous glucose ≥11.1 mmol/L (199.8 mg/dL). All available microscopic slides of the kidneys in study cases were then blindly reviewed with respect to Armanni–Ebstein lesions, and cases where autolysis and putrefaction precluded accurate histological assessment were

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excluded from the study. All cases had full coronial and police investigations with complete forensic autopsies.

Cases were then classified into two groups based on the presence or absence of Armanni–Ebstein lesions. Vitreous glucose, β-hydroxybutyrate, urea, creatinine and sodium levels, and osmolality were then compared between the two groups to determine whether a statistically significant difference existed. This was done via F-tests for the equality of variances followed by the appropriate two-tailed t-test (with or without equal variance).

Results

A total of 71 cases with terminal hyperglycemia fulfilling the study criteria were identified. Their ages ranged from 14 to 80 (mean 47.5) years with a male to female ratio of 43:28. Vitreous glucose levels ranged from 11.1 to 91.9 mmol/L (199.8–1654.2 mg/dL) with a mean of 36.1 mmol/L (649.8 mg/dL) and β-hydroxybutyrate ranged from 0.1 to 29.35 mmol/L (1.04–305.7 mg/dL) with a mean of 9.09 mmol/L (94.7 mg/dL).

Twenty-seven of 71 cases (38%) had Armanni–Ebstein lesions of classic morphology (Fig. 1), and PAS-positive material was demonstrated within vacuoles in representative cases (Fig. 2). Their ages ranged from 17 to 63 (mean 39.9) years with a male to female ratio of 14:13. Postmortem interval ranged from 1 to 13 (mean 4.3) days. Vitreous glucose levels ranged from 14.0 to 77.3 mmol/L (252.0–1391.4 mg/dL) with a mean of 39.2 mmol/L (705.6 mg/dL) and β-hydroxybutyrate ranged from 0.1 to 23.18 mmol/L (1.04–241.5 mg/dL) with a mean of 12.33 mmol/L (128.4 mg/dL). Twenty-one of the 27 cases (77.8%) had elevated ketone levels of >5 mmol/L (52.1 mg/dL). Nineteen cases had vitreous sodium levels between 118 and 159 (mean 134.1) mmol/L (mEq/L), sixteen cases had urea levels ranging from 5.4 to 44.4 mmol/L (32.5–267.5 mg/dL) with a mean of 17.1 mmol/L (103.0 mg/dL), thirteen cases had creatinine levels ranging from 32 to 332 μmol/L (0.36–3.76 mg/dL) with a mean of 143.9 μmol/L (1.63 mg/dL), and ten cases had osmolality ranging from 319 to 495 (mean 391.1) mosmol/L. Causes of death were diabetic ketoacidosis (n = 21), ischemic heart disease (n = 3), cardiomegaly (n = 1), pulmonary thromboembolism (n = 1), and hanging (n = 1). Seventeen cases were known to be insulin-dependent diabetics (two known to be type 1), two cases had a history of diabetes, but it was unclear which type or whether they required insulin therapy, two cases were type 2 diabetics on oral hypoglycemic agents, and six cases had no known history of diabetes.

Forty-four of 71 cases (62%) did not have Armanni–Ebstein lesions. Their ages ranged from 14 to 80 (mean 52.3) years with a male to female ratio of 29:15. Postmortem interval ranged from 1 to 11 (mean 3.8) days. Vitreous glucose levels ranged from 11.1 to 91.9 mmol/L (199.8–1654.2 mg/dL) with a mean of 34.2 mmol/L (615.6 mg/dL), and β-hydroxybutyrate levels ranged from 0.1 to 29.35 mmol/L (1.04–305.7 mg/dL) with a mean of 7.09 mmol/L (73.85 mg/dL). Twenty-one of the 44 cases (47.7%) had elevated ketone levels of >5 mmol/L (52.1 mg/dL). Twenty-nine cases had vitreous sodium levels between 112 and 188 (mean 137.7) mmol/L (mEq/L), 27 cases had urea levels ranging from 5.1 to 45.3 mmol/L (30.7–272.9 mg/dL) with a mean of 17.6 mmol/L (106.0 mg/dL), 25 cases had creatinine levels ranging from 27 to 360 μmol/L (0.31–4.07 mg/dL) with a mean of 138.6 μmol/L (1.57 mg/dL), and twelve cases had osmolality ranging from 318 to 425 (mean 372) mosmol/L. Causes of death included diabetic ketoacidosis (n = 21), ischemic heart disease (n = 9), drug toxicity (n = 3), drowning (n = 2), multiple injuries (n = 2), congestive cardiac failure (n = 1), myocarditis (n = 1), pericarditis and hemopericardium complicating stab wounds (n = 1), dehydration and hyperglycemia (n = 1), hypothermia (n = 1), acute subdural hemorrhage (n = 1), and upper gastrointestinal hemorrhage (n = 1). Twenty-four decedents had been insulin-dependent diabetics (six known to be type 1), eight cases were type 2 diabetics, five cases had a history of diabetes recorded, but it was unclear which type, and seven cases had no previous history of diabetes.

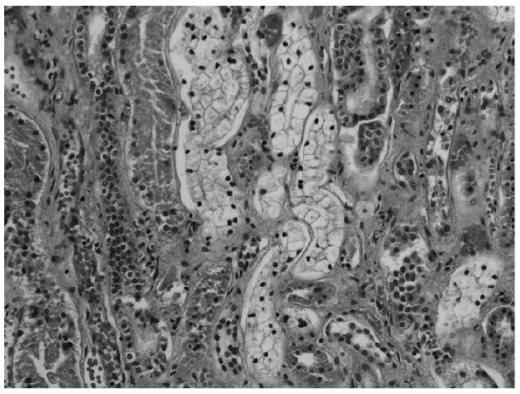

FIG. 1—*Typical Armanni–Ebstein vacuolization in a case of diabetic ketoacidosis (hematoxylin and eosin ×400).*

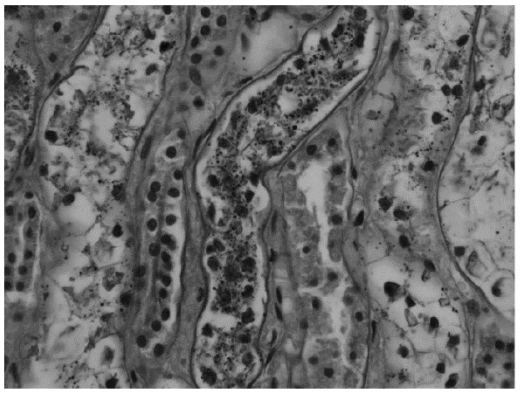

FIG. 2—*PAS-positive material within the vacuolated tubules (periodic acid–schiff ×400).*

F-tests indicated that the variances in the postmortem interval (F = 1.55, p = 0.100), vitreous glucose (F = 0.715, p = 0.183), β-hydroxybutyrate (F = 0.900, p = 0.395), sodium (F = 0.547, p = 0.092), urea (F = 1.080, p = 0.418), creatinine (F = 1.049, p = 0.440), and osmolality (F = 2.49, p = 0.078) of the cases with and without Armanni–Ebstein lesions were not different. There was also no statistically significant difference between the postmortem interval (p = 0.433), vitreous glucose (p = 0.271), sodium (p = 0.355), urea (p = 0.900), creatinine (p = 0.853), and osmolality (p = 0.359) of the cases with and without

Armanni Ebstein lesions. However, there were significantly higher levels of ketoacidosis in the cases with Armanni–Ebstein lesions compared to those without ($p = 0.007$). The quantitative comparison of the vitreous glucose and β-hydroxybutyrate levels are summarized in Figs 3 and 4, respectively.

Discussion

In the recent literature, the term Armanni–Ebstein phenomenon has been expanded to include basal vacuolization as well as the traditional cytoplasmic clear-cell change; however, a recent review by our group has suggested that basal vacuolization is a separate and distinct entity from the originally described Armanni–Ebstein lesion, based on morphology, constituents, and etiology (19). For example, in contrast to Armanni–Ebstein lesions, basal vacuoles contain lipids instead of glycogen and are associated with a variety of ketoacidotic states (19). It appears that basal vacuolizations were included under the umbrella of the "Armanni–Ebstein lesion" after a study by Ritchie and Waugh in 1957 which noted fine basal fat droplets in the cytoplasm of proximal tubular epithelial cells not involved by the Armanni–Ebstein lesion (1,19). Thus, many of the recent reports on the Armanni–Ebstein phenomenon in a forensic context have referred to this type of basal lipid vacuolization, rather than to the originally described clear-cell change (12–18,20). Similarly, the only prior study investigating the relationship between Armanni Ebstein lesions and hyperglycemia referred to basal vacuolizations rather than to cytoplasmic glycogen accumulation and concluded that no relationship existed between terminal hyperglycemia and basal vacuolization (12).

The etiology of the traditional Armanni Ebstein phenomenon is thought to involve hyperglycemia and glucosuria (1); however, results of the current study suggest that underlying mechanisms may be more complicated. To our knowledge, there has only been one study which investigated the relationship of Armanni–Ebstein lesions to terminal blood glucose levels which used alloxan to induce diabetes in 207 rats, of which 62 survived and met the study criteria (5). The authors observed that lesions were invariably present with glucose levels above 350 mg per cent (19.4 mmol/L) and that no lesions were present with levels below 300 mg per cent (16.7 mmol/L). However, the upper threshold level and a direct relationship with the level of hyperglycemia were not observed in our study, as only 31 of the 44 cases (70.5%) without Armanni–Ebstein lesions had a glucose level above 19.4 mmol/L (349.2 mg/dL). Additionally, there was no significant difference in the degree of terminal hyperglycemia between the cases with and without Armanni–Ebstein lesions ($p = 0.271$), and the case with the highest observed glucose level of 91.9 mmol/L (1654.2 mg/dL) did not have typical vacuoles. It is unclear why the direct relationship observed in rats was not observed in human subjects. A possible explanation may be that animal studies tend to be conducted on healthy rats of a selected breed, similar age, and with similar environmental parameters, with diabetes being induced in a uniform manner with a set dose of the same drug (i.e., alloxan or streptozotocin). In contrast, human cases in a forensic environment have more varied demographics, and often have other significant illnesses, impairments, and/or metabolic derangements. For example, 6 of the 27 cases (22.2%) with Armanni Ebstein lesions had hyperglycemia incidental to a cause of death other than decompensated diabetes. Therefore, the variable appearance of Armanni Ebstein lesions at similar glucose values may reflect other complex organic factors.

Ketoacidosis may, therefore, be contributory to the development of Armanni–Ebstein lesions. In this study, 21 of the 27 cases (77.8%) exhibiting Armanni–Ebstein lesions had concurrent ketoacidosis demonstrated by elevations in vitreous β-hydroxybutyrate. Although Armanni–Ebstein lesions also occurred in

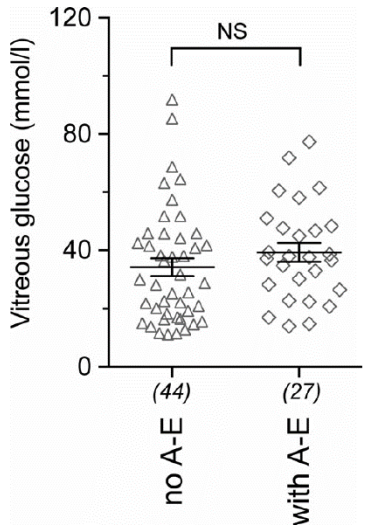

FIG. 3—*Scatterplot histogram summarizing the vitreous glucose levels in cases with and without Armanni–Ebstein lesions; NS is not significant.*

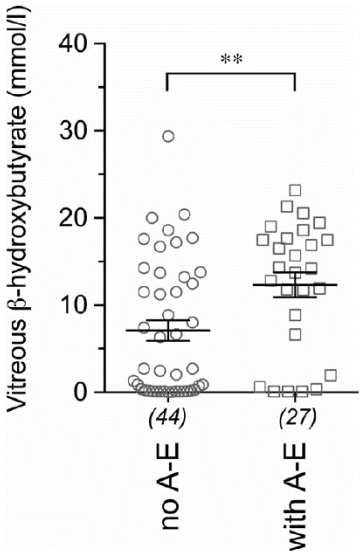

FIG. 4—*Scatterplot histogram summarizing the vitreous β-hydroxybutyrate in cases with and without Armanni–Ebstein lesions. Statistically significant differences are indicated as (**) for* p = *0.007.*

cases with hyperglycemia without elevations in ketones (n = 6), an overall significantly higher level of β-hydroxybutyrate was seen in the cases exhibiting Armanni–Ebstein lesions compared to those that did not (p = 0.007).

The exact mechanism for the development of Armanni–Ebstein lesions is unclear, but it is thought to represent excess reabsorption of glucose in the distal tubules secondary to hyperglycemia (3). However, injury to the pars recta has also been proposed as a mechanism for glycogen accumulation (2), as well as excessive glycogenesis or defective glycogenolysis as a result of cellular dysfunction (1). Ketoacidosis can contribute to this as it may cause renal tubular dysfunction and injury, evidenced by significantly increased urinary excretion of proximal tubular marker alpha 1-microglobulin and distal tubular marker Tamm–Horsfall protein compared to metabolically stable controls (21). The toxic effects of ketone bodies on renal tubular cells were also shown by Asami and colleagues with significantly elevated urinary beta-D-N-acetyl glucosamine and beta 2-microglobulin in patients with nondiabetic ketoacidosis (22). Thus, it is possible that the resultant tubular dysfunction in ketoacidosis may facilitate the intracellular accumulation of glycogen.

Higher levels of glucose in the current postmortem study were observed compared to previous animal studies. A number of diabetic rat models have demonstrated Armanni–Ebstein lesions in the renal tubules, some were spontaneously diabetic, and others were treated with alloxan or streptozotocin (3,5–8,23–29). Serum glucose levels reported in these rats ranged from 13.9 to approximately 40.0 mmol/L (250.2–720.0 mg/dL) (3,5–8,23–29) with only one study reporting a glucose level of 66.7 mmol/L (1200.6 mg/dL) in one rat (5). In contrast, the highest vitreous glucose level associated with Armanni–Ebstein lesions in the current study was 77.3 mmol/L (1391.4 mg/dL), and 10 of the 27 cases (37%) with Armanni–Ebstein lesions had a vitreous glucose level of >40 mmol/L (720 mg/dL). This may be because of more severe metabolic derangements in forensic cases.

A major limitation in attempting to correlate the presence of Armanni–Ebstein lesions with terminal glucose levels is the reduction in glucose after death due to continued consumption by cells. Therefore, the true terminal glucose level will be higher than the vitreous level obtained at the time of autopsy by an amount that depends on the postmortem interval. For example, in a series of 3076 cases by Zilg et al., the postmortem vitreous glucose level was observed to drop by approximately 3 mmol/L (54.0 mg/dL) in the very early postmortem period with levels remaining relatively stable for up to 4 days (30). This was attributed to initial survival of hyalocytes and inner retinal cells with gradual equilibration thereafter (30). Therefore, the observations made in this study are likely to be valid as the majority (65%) of our cases had a postmortem interval of 4 days or less, and there was no significant difference in postmortem interval between cases with and without Armanni–Ebstein lesions (p = 0.433). Furthermore, exclusion of the 25 cases (35%) with a postmortem interval of greater than 4 days leaves 18 cases with Armanni–Ebstein lesions [vitreous glucose mean 39.5 mmol/L (711.0 mg/dL), standard deviation 16.6 mmol/L (298.8 mg/dL)] and 28 cases without [vitreous glucose mean 31.6 mmol/L (568.8 mg/dL), standard deviation 15.6 mmol/L (280.8 mg/dL)]. Repeating the quantitative analysis similarly shows no statistically significant difference in vitreous glucose levels (p = 0.108) between these groups.

In summary, in the present series typical Armanni–Ebstein lesions were found in 38% of all cases with terminal hyperglycemia, and no significant differences in vitreous glucose levels could be demonstrated between cases with or without these lesions (p = 0.271). Additionally, the threshold glucose level for the invariable appearance of Armanni–Ebstein lesions established in animal studies did not correspond to the current human data, as 70.5% of cases without Armanni–Ebstein lesions had glucose levels exceeding 19.4 mmol/L (349.2 mg/dL). However, a significantly greater degree of ketoacidosis was present among cases with Armanni–Ebstein lesions (p = 0.007), suggesting that ketoacidosis may facilitate its development. Additional available biochemical parameters, including vitreous sodium, urea, and creatinine levels, and osmolality, were also compared between cases with and without Armanni–Ebstein lesions, and no significant differences were present. Although Armanni–Ebstein lesions can be used as a marker for terminal hyperglycemia at autopsy, its presence does not appear to correlate with the level of glucose as it can be seen with mild sublethal elevations. Conversely, its absence did not exclude significant hyperglycemia capable of inducing coma.

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PAPER

PATHOLOGY/BIOLOGY

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Basal Vacuolization in Renal Tubular Epithelial Cells at Autopsy and Their Relation to Ketoacidosis

ABSTRACT: Basal vacuolization of renal tubular epithelial cells is a useful postmortem marker for ketoacidosis. To investigate its incidence and relationship to the severity of ketoacidosis, 158 autopsy cases with elevated β-hydroxybutyrate (>1 mmol/L) over a 7-year-period were retrospectively reviewed. Sixty-eight cases (43%) exhibited basal vacuolizations (vitreous β-hydroxybutyrate: 1.16–29.35 mmol/L, mean 10.28 mmol/L), and 90 cases (57%) did not (vitreous β-hydroxybutyrate: 1.03 13.7 mmol/L, mean 2.84 mmol/L). Quantitative analysis revealed on average a fourfold elevation in β-hydroxybutyrate in cases with basal vacuolizations compared to those without; 10.3% of cases with β-hydroxybutyrate concentrations between 1.01 and 2.00 mmol/L had basal vacuolizations, and this incidence increased to 33.3% with concentrations between 4.01 and 6.00 mmol/L. A marked increase in incidence to >70% was observed with concentrations >6.00 mmol/L, and basal vacuoles were invariably present (100%) with concentrations >14.01 mmol/L. This study demonstrates that basal vacuolizations are a sensitive marker for significant ketoacidosis and reaffirms its use as an indicator for likely cases of fatal ketoacidosis at autopsy.

KEYWORDS: forensic science, basal vacuolization, diabetic ketoacidosis, starvation ketoacidosis, alcoholic ketoacidosis, septic ketoacidosis, diabetes mellitus

Basal vacuolizations of renal tubular epithelial cells appear as a row of clear spaces beneath the nucleus in close proximity to the basement membrane. Intravacuolar lipids have been demonstrated on Oil Red O staining and electron microscopy (1 3). Recently, this pattern of vacuolization has been referred to as the "Armanni–Ebstein phenomenon" (1,2,4–9), possibly after a report on autopsy cases of diabetics with Armanni Ebstein lesions by Ritchie and Waugh who mentioned fine basal fat droplets in the cytoplasm of proximal tubules not affected by the Armanni–Ebstein lesion in two of their five cases (10). However, a recent review of the literature showed that the originally reported Armanni–Ebstein lesion instead represented a clear-cell change with intracytoplasmic glycogen accumulation in hyperglycemic states, therefore suggesting that basal vacuolizations should be recognized as a distinct entity from the Armanni–Ebstein lesion on the basis of different morphology, constituents, and etiology (11).

Basal vacuolizations have been reported in deaths due to diabetic, alcoholic, starvation, and septic ketoacidosis (4,6,12,13). They have also been put forward as a marker for hypothermia (3,14), although a review of hypothermic deaths suggested that a significant proportion of cases had underlying metabolic derangements which may have been the cause of the basal vacuolizations rather than simply decreased core temperature in isolation (15). This concept was supported by an animal model inducing fatal hypothermia in rats where none of the cases demonstrated basal vacuolizations (16). However, to date there have been no studies investigating the overall incidence of basal vacuolizations in ketoacidosis or its relationship to the degree of ketosis.

Materials and Methods

All case files that included vitreous humor analysis over a 7-year-period from 2008 to 2014 at Forensic Science SA, Adelaide, South Australia, were retrospectively reviewed for cases featuring ketosis, defined as β-hydroxybutyrate ≥1 mmol/L (10.3 mg/dL); the cause of ketoacidosis was determined in each case. Cases were classified as diabetic ketoacidosis if concurrent hyperglycemia (vitreous glucose ≥11.1 mmol/L or 200 mg/dL) was present. Alcoholic ketoacidosis was diagnosed if there was a previous history combined with signs of chronic alcoholism at autopsy and normoglycemia. Starvation was considered the cause of ketosis when a documented history of decreased oral intake was present in the absence of elevated glucose concentrations or alcoholism. Septic ketoacidosis was diagnosed when proven sepsis or severe infection was present at autopsy in the absence of raised glucose concentrations or a history of alcoholism, diabetes, or starvation. The cause of ketosis in the remainder of cases was unclear and may have been due to a combination of the above factors.

All available microscopic slides of the kidneys were blindly screened for basal vacuolizations of renal tubular epithelial cells

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and/or formalin pigment deposition around basal vacuolar spaces (17). Cases exhibiting significant autolysis precluding accurate histological assessment were excluded from the series. All cases had full coronial and police investigations with complete forensic autopsies. Study cases were divided into two groups based on the presence or absence of characteristic basal vacuolizations. The vitreous β-hydroxybutyrate concentrations of the two groups, as well as other biochemical parameters such as glucose, urea, creatinine, and sodium levels, and osmolality, were compared to determine whether a statistically significant difference existed. This was carried out by *F*-tests for the equality of variances followed by the appropriate *t*-test (with or without equal variance). The incidence of basal vacuolizations at different β-hydroxybutyrate concentrations was also plotted to determine whether increasing ketosis correlated with an increased incidence of basal vacuolizations.

Results

A total of 158 cases fulfilling the study criteria were identified, including 44 cases of diabetic ketoacidosis, 17 cases of alcoholic ketoacidosis, five cases of starvation ketoacidosis, five cases of septic ketoacidosis, and 87 cases where the cause of ketosis was unclear and a single causative mechanism could not be determined.

Sixty-eight out of the 158 study cases (43%) displayed characteristic basal vacuolization of the renal tubular epithelial cells (Fig. 1). Their ages ranged from 15 to 92 (mean 54.2) years, and they comprised of 40 males and 28 females. The postmortem interval ranged from 1 to 13 (mean 4.7) days. The cause of ketosis was diabetic in 36 cases, alcoholic in 11 cases, septic in three cases, and starvation in one case, and a single cause of ketosis could not be identified in 17 cases. Vitreous β-hydroxybutyrate concentrations ranged from 1.16 to 29.35 (mean 10.28) mmol/L, and vitreous glucose concentrations ranged from 0.1 to 91.9 (mean 23.3) mmol/L. Thirty-two of the 68 cases (47.1%) also displayed characteristic basal formalin pigment deposition in the vicinity of the vacuoles (17).

Ninety out of the 158 study cases (57%) did not have characteristic basal vacuolization of renal tubular epithelial cells. Their ages ranged from 5 to 94 (mean 64.1) years, and they comprised of 58 males and 32 females. Postmortem intervals ranged from 1 to 12 (mean 4.3) days. The cause of the ketosis was diabetic in eight cases, alcoholic in six cases, starvation in four cases, and septic in two cases. A single cause for the elevation in ketones could not be determined in the remainder of the 70 cases. Vitreous β-hydroxybutyrate ranged from 1.03 to 13.7 (mean 2.84) mmol/L, and vitreous glucose concentrations ranged from 0.1 to 72.7 (mean 4.5) mmol/L. None of these cases displayed characteristic basal formalin pigment deposition.

Quantitative Analysis

The *F*-test indicated that the variances in the vitreous β-hydroxybutyrate concentrations ($F = 7.731$, $p < 0.001$) and postmortem interval ($F = 1.540$, $p = 0.034$) in cases exhibiting basal vacuolizations were not equivalent to cases without basal vacuolizations. Subsequent t-tests assuming unequal variances demonstrated a statistically significantly higher vitreous β-hydroxybutyrate concentration ($p < 0.001$) in cases with basal vacuolizations compared to those without, with no significant difference in the postmortem interval ($p = 0.22$). The quantitative comparisons of vitreous β-hydroxybutyrate concentrations between cases with and without basal vacuolizations are summarized in Fig. 2.

The incidence of characteristic basal vacuolizations observed at different β-hydroxybutyrate concentrations is summarized in Table 1 and Fig. 3. The incidence of basal vacuolizations markedly increased when β-hydroxybutyrate concentration exceeded 6.0 mmol/L from 33.3% to above 70%. Basal vacuolizations were seen in >80% of cases with β-hydroxybutyrate concentration >10.0 mmol/L and were invariably present (100%) with concentrations >14.0 mmol/L. The incidence of basal vacuolizations among different causes of ketoacidosis was 81.8% in diabetic, 64.7% in alcoholic, 60% in septic, and 20% in starvation ketoacidosis. Basal vacuolizations were present in 19.5% of the

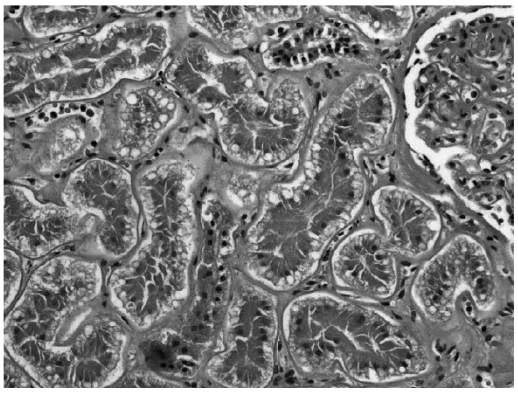

FIG. 1—*Characteristic basal vacuolizations in renal tubular epithelial cells in a case of diabetic ketoacidosis (Hematoxylin and eosin ×400).*

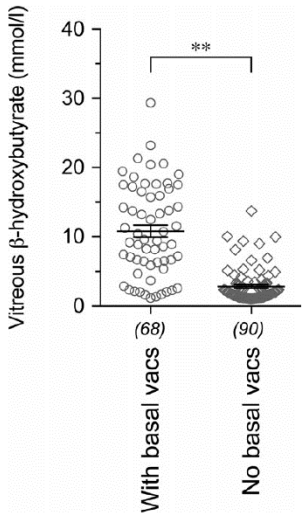

FIG. 2—*Scatterplot histogram summarizing the vitreous β-hydroxybutyrate in cases with and without basal vacuolizations. [Horizontal bars indicate mean ± standard error of the mean (SEM). Statistically significant differences are indicated as (**) for* $p < 0.001$*].*

cases in which the exact cause of ketoacidosis was unclear or multifactorial.

Data sets were each fitted with a Gaussian distribution function using GraphPad Prism software; plotting these data on a frequency histogram (Fig. 4) demonstrates that cases without basal vacuolization were tightly clustered at low concentrations of β-hydroxybutyrate, while cases with basal vacuolizations were widely distributed over a broad range. Additionally, cases exhibiting basal vacuoles had on average a fourfold elevation in β-hydroxybutyrate concentration (mean 5.91 ± 4.64 mmol/L) compared to cases lacking basal vacuoles (mean 1.43 ± 0.10 mmol/L).

Discussion

Lethal ketoacidosis encountered in the forensic context is most commonly due to decompensated diabetes mellitus, but elevations in ketones may also occur in alcohol abusers and in cases of starvation, sepsis, noninsulin mediated hypoglycemia, and drug abuse, or in cases involving a combination of these factors (18,19). The mechanism in which ketoacidosis may lead to death includes extracellular volume contraction, electrolyte disturbances (including hypokalemia), sudden cardiac arrest, and cerebral edema (18,20). In the current study, basal vacuolizations were seen in all categories of ketoacidosis, including diabetic, alcoholic, septic, starvation-induced, and in cases where the cause of ketoacidosis was unclear or multifactorial.

Most prior studies have reported on vitreous acetone rather than β-hydroxybutyrate concentrations. However, β-hydroxybutyrate is the major ketone body produced in ketosis accounting for approximately 78% of total ketones, with acetoacetate (20%) and acetone (2%) produced in much smaller amounts (21). The ratio of β-hydroxybutyrate to acetoacetate is normally 1:1; however, this ratio can increase to as high as 10:1 in diabetic and alcoholic ketoacidosis (22,23). Additionally, β-hydroxybutyrate concentrations have also been shown to correlate more closely than serum ketones with the degree of anion gap elevation in ketoacidotic states (21), therefore making it the ideal biochemical marker to reflect the severity of ketoacidosis. However, there has only been one previous retrospective review of basal vacuolizations in alcoholic ketoacidosis, which reported β-hydroxybutyrate concentrations ranging from 6.42 to 8.75 mmol/L (12), and one case report on starvation ketoacidosis, which had a vitreous β-hydroxybutyrate of 3.966 mmol/L (6). In the current study, basal vacuolizations were seen at a β-hydroxybutyrate level of 1.16 mmol/L with the highest level being 29.35 mmol/L, representing a wider range than previously reported. Three prior studies reported on vitreous acetone levels which ranged from 0.5 to 12.8 mmol/L in decedents with basal vacuolization

TABLE 1—*The incidence of basal vacuolizations in renal tubular epithelial cells at different vitreous β-hydroxybutyrate levels in 158 autopsy cases with ketosis (β-hydroxybutyrate >1.0 mmol/L).*

β-hydroxybutyrate Level (mmol/L)	Incidence of Basal Vacuolizations (%)
1.01–2.00	10.4
2.01–3.00	24.1
3.01–4.00	25.0
4.01–6.00	33.3
6.01–8.00	80.0
8.01–10.00	71.4
10.01–12.00	85.7
12.01–14.00	83.3
14.01–	100

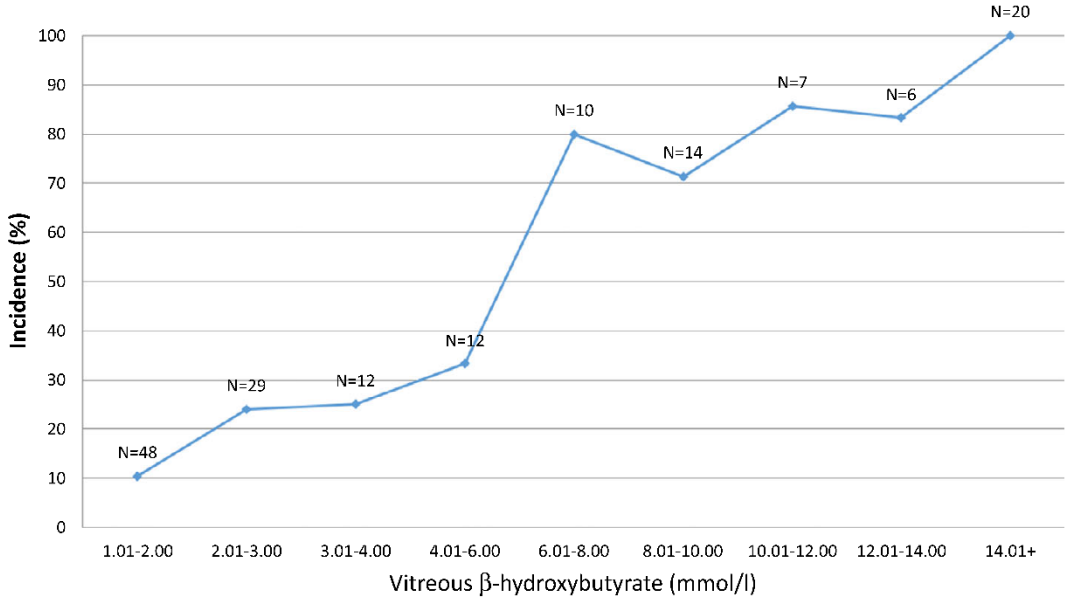

FIG. 3—*Plotting the incidence of basal vacuolizations in renal tubular epithelial cells against incremental increases in vitreous β-hydroxybutyrate levels in 158 autopsy cases with ketosis (β-hydroxybutyrate >1.0 mmol/L). [Color figure can be viewed at wileyonlinelibrary.com]*

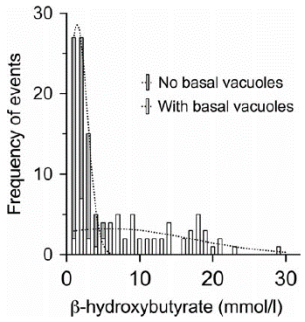

FIG. 4—*Frequency histogram summarizing vitreous β-hydroxybutyrate in cases with and without basal vacuolizations [Dotted line indicates the Gaussian distribution].*

who died of starvation and diabetic ketoacidosis (4,7,9). At Forensic Science SA, acetone can be detected in the blood on toxicological screening, but it does not appear as a quantitative measurement. An acetoacetate screen is also occasionally conducted on vitreous humor biochemistry which appears in a semi-quantitative manner as "small," "moderate," or "large." Therefore, these measurements were not reported in the current study.

Basal vacuolizations are a useful marker for lethal ketoacidosis, with the incidence increasing with the extent of metabolic derangement. A large retrospective review of 1795 forensic cases recommended that β-hydroxybutyrate levels <0.4 mmol/L should be interpreted as normal, 0.41–1.2 mmol/L to be slightly elevated and rarely (<1%) of concern, 1.21–2.0 mmol/L to be moderately elevated and less rarely (2.5%) of concern, 2.01–6.0 mmol/L to be significantly elevated and frequently (12–48%) of concern, and concentrations above 6.0 mmol/L to indicate life-threatening conditions (24). In this study, we divided the cases according to incremental increases in vitreous β-hydroxybutyrate levels and found that the lowest β-hydroxybutyrate concentration in which basal vacuolizations could be identified was 1.16 mmol/L. Above this concentration, and regardless of the cause of ketoacidosis, the incidence of basal vacuolizations increased with greater elevations in β-hydroxybutyrate (summarized in Table 1 and Fig. 3). Interestingly, 6.0 mmol/L also seemed to be a threshold concentration in this study, above which the incidence of basal vacuolizations more than doubled from 33.3% to over 70%. The incidence of basal vacuolizations was >80% with β-hydroxybutyrate concentrations of 10.0-14.0 mmol/L and 100% when levels exceeded 14.0 mmol/L. Therefore, basal vacuolizations were certainly found to be sensitive markers for fatal ketoacidosis.

In the current study, the incidence of basal vacuolizations in diabetic ketoacidosis was slightly lower than previously reported figures, whereas the incidence in alcoholic ketoacidosis is slightly higher. Basal vacuolizations have been reported in 30.8% of deaths in individuals with a history of diabetes (4) and 84–100% of deaths due to diabetic ketoacidosis (1,9), whereas it was noted in 81.8% in the present series. Two previous studies on alcoholic ketoacidosis reported basal vacuolizations in 47.8–60.0% of cases (7,12), whereas it was seen in 64.7% in the present study. There have been no previous reports on the incidence of basal vacuolizations in septic or starvation ketoacidosis, which, in this study, was found to be 60% and 20%, respectively. The higher incidences found in diabetic, alcoholic, and septic ketoacidosis are likely a reflection of the typically higher degrees of ketone elevation seen in these conditions compared to starvation (19,25).

Vitreous humor has been proven as a reliable alternative to blood for the determination of β-hydroxybutyrate levels (26–28). Additionally, studies suggest that decompositional changes are not associated with β-hydroxybutyrate production; therefore, the concentration detected at autopsy can be considered a reflection of concentrations at the time of death (29,30). This includes a study by Iten and Meier demonstrating no statistically significant increase in serum β-hydroxybutyrate concentration with increasing postmortem interval (30) and another study by Palmiere et al. (31) whom observed lower concentrations of serum β-hydroxybutyrate in 50 cases exhibiting advanced decomposition compared to 400 cases that did not. These observations are supported in the present study as no statistically significant difference in postmortem interval could be demonstrated between cases with and without basal vacuolizations ($p = 0.22$). The exact role that micro-organisms play in β-hydroxybutyrate metabolism after death is unclear; however, it seems that vitreous humor is a relatively protected environment from postmortem microbiological contamination (32) and has an inherent antibacterial capacity *in vitro* which does not support bacterial growth (33). Therefore, any potential alterations micro-organisms may have on β-hydroxybutyrate concentrations would be reduced in vitreous humor compared to blood.

The current study is limited by its retrospective nature. Although selection bias was eliminated by conducting a search for all cases containing keywords "vitreous humor" biochemistry and "vitreous biochemistry" rather than specific causes of ketosis, it is possible that a small number of cases may have been missed if these keywords were not used in a report. Cases with β-hydroxybutyrate concentrations <1.0 mmol/L were not reviewed in the present study. Review of these cases in the future may, however, be useful to establish whether basal vacuolization can occur with normal ketone concentrations, although there have been no reports of this in the literature to date. Review of larger numbers of cases would also assist in more accurately quantifying the incidence of basal vacuolization in septic and starvation ketoacidosis as the current study had relatively low case numbers due to the infrequent nature of these conditions leading to death.

In conclusion, this study has demonstrated that basal vacuolization in renal tubular epithelial cells may be found in autopsy material from all categories of ketoacidosis, including diabetic, alcoholic, septic, and starvation. It is also present in both minor and severe ketosis, with β-hydroxybutyrate concentrations ranging from 1.16 to 29.35 mmol/L. Additionally, the incidence is related to the degree of ketoacidosis (Table 1, Fig. 3), with a significant increase in incidence to >70% at β-hydroxybutyrate concentrations of >6.0 mmol/L, and vacuoles invariably (100%) present at concentrations >14.0 mmol/L. Therefore, basal vacuolization in renal tubular epithelial cells in autopsy tissues is a very useful marker for ketosis, even in putrefied and/or autolytic kidneys. Once it has been identified, it should initiate investigation into the quite diverse potential underlying causes, with full vitreous humor biochemistry (including quantitative β-hydroxybutyrate levels).

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Original communication

Lethal hypothermia in an animal model, not associated with basal renal epithelial vacuolization

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ABSTRACT

A rodent model was used to evaluate the association between hypothermia and basal vacuolization in renal tubular epithelial cells. 28 Sprague Dawley rats were anaesthetized in non-stressful conditions and placed two at a time into a cooling chamber. Body core temperatures dropped to a minimum of 7–10 °C, causing death under anaesthesia at times varying from 120 to 240 min. The animals were then subjected to necropsy; the kidneys were removed and placed in 10% buffered formalin. Examination of haematoxylin and eosin-stained renal sections failed to reveal basal vacuolization of renal tubular epithelial cells in any of the 28 animals. In this model, no evidence of subnuclear lipid vacuolization of renal tubular cells could be demonstrated despite significant and eventually lethal hypothermia. These results lend support to the hypothesis that the basal vacuolization in hypothermia may be a manifestation of a more complex pathophysiological pathway rather than being due simply to low body core temperatures.

1. Introduction

Deaths from hypothermia are encountered in forensic mortuaries in both temperate and cold climates.[1] Unfortunately the findings at autopsy, although characteristic, are not diagnostic, and their underlying pathophysiology is poorly understood.[2] Typical features include Wischnewsky spots of the gastric mucosa in 40–90% of cases, pinkish discolouration of the skin over larger joints, and acute pancreatitis with haemorrhage and surrounding fat necrosis.[3,4] Microscopic findings have included vacuolization of hepatocytes, cardiac myocytes and renal tubular epithelial cells.[4,5] It has been suggested that basal lipid vacuolization in renal epithelial cells could serve as a potential diagnostic tool for identifying hypothermic deaths, as it has an "equal value of diagnostic sensitivity compared to that of Wischnewski-ulcers".[5]

However, basal vacuolization of renal tubular epithelial cells may be also caused by underlying metabolic derangements, rather than by the direct effects of hypothermia.[6 9] A recent study by the authors demonstrating basal vacuolization in 15 out of 46 cases of hypothermia (33%), revealed that 9 of the 15 cases (60%) had a documented history of diabetes mellitus (which is known to be associated with basal vacuolization).[6] Vitreous humour biochemical analyses were performed in seven of these cases, all of which demonstrated diabetic ketoacidosis.[6] Based on these findings we suggested that diabetic ketoacidosis may be a more significant factor in the generation of basal vacuolization than hypothermia in isolation.[6] We also noted that hypothermia might, in fact, be both a cause and a complication of diabetic ketoacidosis, which accounts for 11.8% of hospital admissions of all patients with markedly low core temperatures.[6,10] The same may apply to alcoholic ketoacidosis, which is also known to be associated with the formation of renal basal vacuolizations[13,14]: e.g. a study of 51 fatal hypothermic deaths in Sweden revealed that in 47% of cases the subjects were long-term alcoholics, and that 65% had detectable levels of ethanol in the blood and/or urine.[11]

We chose to use a rat model to specifically test whether basal vacuolization of renal tubular epithelial cells could be caused by lethal hypothermia in isolation.

2. Materials and methods

A model using male Sprague Dawley rats was developed to assess the effects of hypothermia in anaesthetized, minimally-

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stressed animals.[12] Twenty-eight rats were given an acclimatization period of one week with free access to food, water, and social interaction to reduce psychological and physiological stress. They were then sequentially anaesthetized with isoflurane at a flow rate of 3–5% and an oxygen flow rate of 1–2 ml/min prior to placement into a cooling chamber. The isoflurane flow rate was decreased to 1–2% during the procedure to avoid significant respiratory depression whilst maintaining deep sleep. Constant monitoring of body core temperatures was achieved using a rectal temperature probe until death occurred. Core temperatures dropped over time to a minimum of 7–10 °C which resulted in death of the animals under anaesthesia at times ranging from 120 to 240 min after placement in the cooling chamber. The animals were then subjected to necropsy and the kidneys removed and immediately placed in 10% buffered formalin. Routine haematoxylin and eosin-stained slides were then prepared and examined. This study was fully approved by the Animal Ethics Committee at The University of Adelaide.

3. Results

Microscopic examination of coronal sections through each kidney from all 28 animals failed to reveal any evidence of basal vacuolization in the renal tubular epithelial cells. Although occasional sections displayed areas of non-specific, irregular cytoplasmic vacuolization, sections of the kidneys were generally unremarkable. (The latter vacuolization was thought to represent an artefact due to delayed fixation of deeper tissues.)

4. Discussion

Although lipid-containing vacuoles have been found in the basal portions of renal tubular epithelial cells in cases of hypothermia in humans[5,6] the pathophysiology remains uncertain. It appears likely that such deposits may occur when there has been a significant metabolic disruption resulting in lipid mobilization from tissue stores, as occurs with ketoacidosis arising from a number of different conditions including diabetes mellitus, alcoholism and starvation.[13–16] Over the last decade, the term “Armanni-Ebstein phenomenon” has been applied broadly to include descriptions of subnuclear lipid vacuolizations of renal tubular epithelial cells in the outer medulla; however, a recent review has clarified the precise use of the Armanni-Ebstein term, indicating that this classification should refer specifically to clear cell changes of swollen, rounded and transparent cells having peripherally displaced nuclei associated with hyperglycemia.[17]

In the present study there was no evidence of Armanni-Ebstein changes, nor of subnuclear lipid vacuolization of tubular cells, despite the imposition of significant and eventually lethal hypothermia. The rats used in this study had been subject to minimal psychological and physiological stress, and had been in good physical condition, therefore creating a suitable model to study the effect of hypothermia in isolation.[12]

A possible limitation of this study is that the rat model might not develop the subnuclear vacuolization phenotype. However, studies have shown that rats exposed to hypothermic conditions under stress do demonstrate other features associated with hypothermia such as Wischnewsky spots of the gastric mucosa.[18–22] Sprague Dawley rats have also been successfully used in previous studies to manifest other renal histological changes such as Armanni-Ebstein lesions.[23–26] These studies suggest the rat is a valid animal model for exploring renal sequelae of pathophysiological conditions. A second possible limitation is that the time of cold exposure may have been too short to allow for the development of subnuclear vacuolization; however some animals did survive for hours with extremely low core temperatures. Of note, the rats in this study also did not develop Wischnewsky spots, which could be directly related to the role that stress may have in the pathological manifestations of hypothermia.[12]

This study suggests that basal renal tubular cell vacuolization may not be caused solely by lethal hypothermia. In cases where renal pathology is observed following significant hypothermia, additional studies testing for underlying metabolic disturbances, perhaps involving lipid mobilization due to ketoacidosis, will be required to determine the potential role of disturbances of glucose metabolism associated with lowered core temperatures, in creating this phenotype.

Ethical approval

The University of Adelaide Animal Ethics Committee.

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None.

Conflict of interest

None.

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TECHNICAL NOTE

PATHOLOGY/BIOLOGY

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Hyperosmolarity Induces Armanni-Ebstein-like Renal Tubular Epithelial Swelling and Cytoplasmic Vacuolization

ABSTRACT: Armanni–Ebstein lesions have been considered pathognomonic for diabetes mellitus and appear as markedly swollen renal tubular epithelial cells with cytoplasmic clearing and glycogen accumulation. However, the extent to which hyperosmolarity contributes to the Armanni–Ebstein phenotype is unclear. Ten sheep were injected intravenously with 20% mannitol at 11 mOsm/kg, and subsequent histological evaluation of the kidneys showed variable degrees of osmotic nephrosis and cytoplasmic clearing of renal tubular epithelial cells similar to that seen with Armanni–Ebstein lesions. However, although morphological changes similar to Armanni–Ebstein lesions could be produced, no intracytoplasmic glycogen was demonstrated with periodic Acid–Schiff (PAS) stain. This suggests that while hyperosmolarity may contribute to the development of an Armanni–Ebstein phenotype, glycogen accumulation may result from the more complex metabolic effects of glucose on renal tubular epithelial cells. Thus, when Armanni/Ebstein-like vacuolizations are seen at autopsy, a confirmatory PAS stain is recommended because of the potential effect of hyperosmolar states.

KEYWORDS: forensic science, Armanni–Ebstein phenomenon, diabetes mellitus, mannitol, osmotic nephrosis

The Armanni–Ebstein phenomenon refers to markedly swollen renal tubular epithelial cells with small dark nuclei that are often peripherally displaced and almost complete conversion of the cytoplasm into a single large vacuole filled with glycogen (1,2). These lesions occur in poorly controlled diabetic states such as diabetic ketoacidosis. Although their pathogenesis has been consistently attributed to hyperglycemia and subsequent glycosuria in both autopsy and animal studies (2–5), it is unclear whether the vacuoles occur secondary to the osmotic effect exerted by glucose, or whether the pathogenesis is specific to the chemical properties of glucose molecules. To investigate this, serum hyperosmolarity was induced in a sheep model using mannitol, a structurally similar osmotic agent to glucose, with subsequent histological analysis of the kidneys to look for the presence of Armanni–Ebstein lesions.

Materials and Methods

This project used scavenged sheep kidneys from a study approved by the local animal ethics committees of SA Pathology and The University of Adelaide, South Australia (6). Animal studies were performed according to the guidelines established by the National Health and Medical Research Council (NHMRC).

As part of a neurotrauma study (6), a subgroup of ten anesthetized male merino sheep were intravenously administered 20% mannitol (11 mOsm/kg) over a 10- to 15-min period and euthanized by barbiturate overdose 4 hours later for tissue analysis. Animals were initially anesthetized by intravenous injection of thiopentone before intubation and ventilation (4 L/min) with oxygen enriched (30–35%) air containing 2.5% isoflurane. Ventilation parameters were adjusted as necessary on the basis of arterial blood gases sampled at regular intervals. Isoflurane was then lowered to 1.0–1.5% subsequent to insertion of a femoral catheter, and an intravenous infusion of ketamine (4 mg/kg/h) was initiated for ongoing anesthesia. Mean arterial blood pressure was continuously monitored via a femoral arterial catheter using a MacLab data acquisition system, while core body temperature was maintained using a thermostatically controlled heating pad.

After 4 h, animals were euthanized by barbiturate overdose. Sheep were then intravenously perfused with 10% buffered formalin for 15 min before the kidneys were surgically removed and immediately immersed in 10% formalin for fixation. Representative sections of the kidneys were taken and routinely processed, embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E). Periodic Acid–Schiff (PAS) stain for glycogen was done in representative cases.

Results

Histological examination of the kidneys showed variable degrees of osmotic nephrosis with a uniform degree of nonspecific vacuolization of renal tubular epithelial cells (Fig. 1). There was also an associated variable degree of cytoplasmic clearing of tubular epithelial cells in all cases ranging from incomplete

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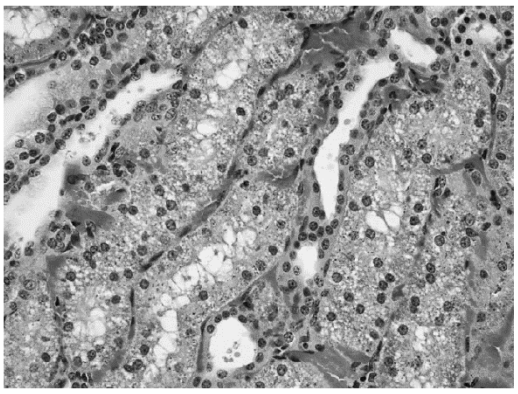

FIG. 1—*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). There is nonspecific uniform vacuolization of renal tubular epithelial cells consistent with osmotic nephrosis (hematoxylin & eosin, H&E × 40).*

clearing with undisplaced central and normal appearing nuclei (Fig. 2), to complete cytoplasmic clearing with small dark peripherally displaced nuclei (Fig. 3) suggestive of Armanni–Ebstein lesions. PAS staining in two cases with Armanni/Ebstein-like lesions did not show any PAS-positive material within the affected tubules (Fig. 4).

Discussion

The Armanni Ebstein phenomenon refers to vacuolated renal tubular epithelial cells and was first described by Luciano Armanni in 1877 (7). Microscopically, these cells show a loss of their normal cuboidal shapes, are markedly swollen with small dark nuclei that were often peripherally displaced, and have almost complete conversion of the cytoplasm into a single large vacuole (1,2). Affected cells often bulge into and occlude the

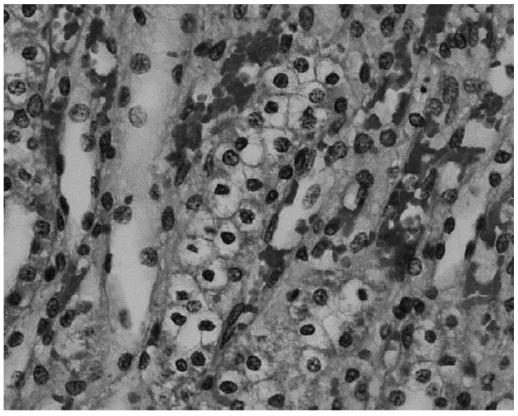

FIG. 2—*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). There is incomplete cytoplasmic clearing with undisplaced normal appearing nuclei (hematoxylin & eosin, H&E × 40).*

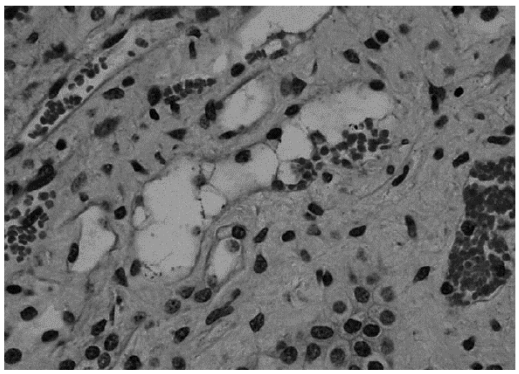

FIG. 3—*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). There is complete cytoplasmic clearing with peripherally displaced small dark nuclei resembling the Armanni Ebstein lesion (hematoxylin & eosin, H&E × 40).*

tubular lumen (2). Intravacuolar glycogen accumulation can be demonstrated on periodic Acid–Schiff (PAS) staining and on electron microscopy (3). Microdissection studies have shown that Armanni–Ebstein lesions were consistently localized astride the corticomedullary junction and principally affect the terminal straight portion of the proximal convoluted tubule (2). These lesions have been reported in poorly controlled diabetic states such as diabetic ketoacidosis (2,8), with a demonstrated correlation with hyperglycemia in animal studies (3–5,9). Thus, their presence has been deemed pathognomonic for diabetes (2). It is proposed that vacuolizations reflect marked glycosuria and the excessive reabsorption of glucose (8); however, the extent to which hyperosmolarity contributes to the development of these lesions is unclear.

The current study used scavenged sheep kidneys from a neurotrauma study investigating the effects of substance P antagonists as agents to reduce intracranial pressure (ICP) following traumatic brain injury (6). Four study groups were involved including uninjured controls and injured animals that were administered intravenous normal saline, 20% mannitol

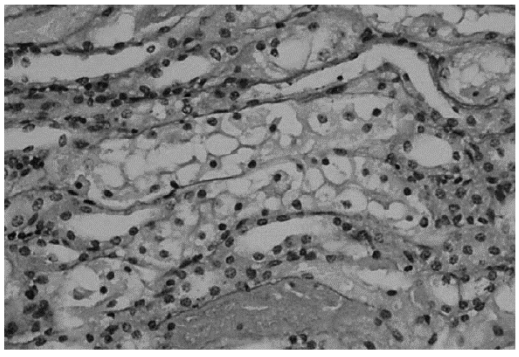

FIG. 4—*A section of kidney from an anesthetized sheep that was injected with 20% mannitol (11 mOsm/kg). No glycogen can be demonstrated within the vacuolated tubular cells (periodic Acid–Schiff, PAS ×40).*

(11 mOsm/kg), or 2.5 mg/kg n-acetyl-tryptophan (substance P antagonists). ICP monitoring revealed significantly increased ICP ($p < 0.05$) to approximately 20 mmHg by 30 min after traumatic brain injury compared to the uninjured controls (ICP 7 ± 2 mmHg). The authors found a continued increase in ICP in saline-treated animals and a reduction in ICP following administration of mannitol or substance P antagonist, concluding that substance P antagonists may offer a novel therapeutic approach to the treatment of acute brain injury (6). The kidneys from the group administered with 20% intravenous mannitol were analyzed in this study.

This study is subject to limitations as a result of the scavenged nature of the tissues. Kidneys from the uninjured control group or the group administered with saline were not obtained at the time of the experiments; therefore, there is an absence of a control group to compare the histological changes induced by mannitol. In hindsight, this would have been greatly beneficial to the study. Serum osmolarity was not measured in the sheep postadministration of mannitol as it was not deemed relevant to the initial study from which the kidneys were scavenged (6). In the absence of this, a surrogate marker for increased serum osmolarity can be drawn from the significantly reduced ICP at 3 ($p < 0.05$) and 4 h ($p < 0.01$) in mannitol-treated animals compared to saline-treated animals (6), although ideally, the availability of serum osmolarity as a direct correlate would be better. Animals were also given other medications as part of the anesthetics including intravenous thiopentone and ketamine, and inhalation of isoflurane. The histological effects of these agents on the renal tubular epithelium, if any, are not well documented in the literature so it is not known whether they could have impacted on the observed pathology. The current study is also limited by low numbers, and further studies are warranted.

Mannitol was used as a substitute for glucose to induce hyperosmolarity and investigate whether the phenotypic features of Armanni–Ebstein lesions could be induced by an osmotic effect. Mannitol ($C_6H_8(OH)_6$) is a six-carbon polyol (sugar alcohol) of the sugar mannose with a molecular mass of 182 and a chemical structure very similar to that of glucose and sorbitol (10,11). After intravenous injection, however, mannitol is metabolically inert, distributes almost entirely in the extracellular space, is filtered at the glomerulus, and then excreted unchanged in the urine (10). Studies have shown that the elimination half-life after a single dose of 0.5–0.71 g/kg of mannitol varies widely in humans from 39 to 103 min with a mean of 71 min (12). Due to its osmotic properties, intravenous mannitol has been used clinically to reduce intracranial and intraocular pressures, as a diuretic agent, for renal protection in transplant surgery, and for the prevention of nephrotoxic renal failure caused by radiocontrast, myoglobin, and cisplatin (10,11,13).

The glucose molecule ($C_6H_{12}O_6$) is structurally similar to mannitol, and it is a key fuel for mammalian cells, as well as being an important metabolic substrate (14). In contrast to mannitol, approximately 90% of filtered glucose is reabsorbed in the proximal convoluted tubules via a low-affinity, high-capacity sodium glucose transporter, SGLT2, located on the apical membrane. Residual glucose is then absorbed by the high-affinity and low-capacity SGLT1 located in the proximal straight tubules (14,15). Two basolateral membrane glucose transporters, the low-affinity GLUT2 and high-affinity GLUT1, then facilitate transcellular glucose transport (14,15). There is also evidence to suggest that there is increased expression of renal glucose transporters in diabetics (15), with hyperglycemia in diabetes leading to enhanced proximal tubular glucose reabsorption (16). Glycosuria only develops once the filtered glucose exceeds the renal threshold for reabsorption. Additionally, glucose molecules are metabolically active and induce a range of effects on proximal tubular cells, including increase synthesis and secretion of vasoactive hormones (e.g., angiotensin II), cytokines (e.g., transforming growth factor-β), and extracellular matrix proteins (16). Hyperglycemia has also been shown to increase the formation and accumulation of intracellular sorbitol via metabolism of glucose by aldose reductase (16).

In the present study, intravenous injection of 20% mannitol in sheep induced vacuolization of renal tubular epithelial cells in all ten cases with variable degrees of osmotic nephrosis and some tubules exhibiting cytoplasmic clearing and Armanni/Ebstein-like changes. Compared to the classical Armanni–Ebstein lesion, the membranes of the individual swollen and vacuolated epithelial cells were, however, less distinct, with variable nuclear changes ranging from normal appearing undisplaced nuclei to more characteristic peripherally displaced small dark nuclei. Mannitol-induced cytoplasmic clearing was also more commonly observed as a field effect with many adjacent tubules also affected; this is in contrast to traditional Armanni–Ebstein lesions in which the affected tubule is surrounded by normal unaffected tubules. PAS staining of affected tubules did not demonstrate any intravacuolar glycogen presumably because filtered mannitol is excreted and remains metabolically inert.

This study has demonstrated that the histological appearance of Armanni–Ebstein lesions can be reproduced by mannitol infusion, although no intravacuolar glycogen was present. This suggests that hyperosmolarity may contribute to the formation of Armanni–Ebstein lesions, but that the accumulation of glycogen is more specific to the metabolic effects of the glucose molecule on renal tubular epithelial cells. Thus, when Armanni/Ebstein-like tubules are seen on routine sections of the kidneys, a subsequent PAS stain should be conducted to demonstrate glycogen because of the possibility of these changes being induced by nondiabetic hyperosmolar conditions. Further controlled studies are warranted as the current study was subject to limitations due to the scavenged nature of the kidneys.

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PAPER

PATHOLOGY AND BIOLOGY

Chong Zhou,[1,2] *M.B.B.S.; Andrea J. Yool*,[1] *Ph.D.; and Roger W. Byard*,[1,2] *M.D.*

An Isolated Perfused Rat Kidney Model for the Evaluation of the Effect of Glucose on Renal Tubular Epithelial Morphology

ABSTRACT: An isolated perfused kidney model was used to evaluate the effect of hyperglycemia on renal tubular epithelial cell morphology. Ten Sprague–Dawley rat kidneys were perfused with Krebs–Henseleit buffer containing 70 mmol/L of glucose (five for 1 h and five for 2 h). Two control groups consisted of 10 kidneys perfused with Krebs–Henseleit buffer without hyperglycemia (five for 1 h and five for 2 h), and 10 nonperfused contralateral kidneys placed in the same environment for the same duration. The hyperglycemia group had significantly increased renal tubular vacuolization ($p < 0.001$) compared to both control groups at 1 and 2 h. The isolated perfused kidney model recapitulates the renal tubular vacuolization phenotype found in hyperglycemia and may be a potential tool for the investigation into causal factors in renal histology. The full pattern of the Armanni–Ebstein phenomenon was not, however, reproduced, suggesting that this change requires more time or involves more complex factors.

KEYWORDS: forensic science, Armanni–Ebstein phenomenon, nephrotic syndrome, isolated perfused kidney, diabetes mellitus, hyperglycemia

Identifying histological changes in renal tubular cells is useful in a variety of clinical situations, such as in the diagnosis of calcineurin inhibitor nephrotoxicity (1) and acute tubular necrosis. In addition, it has been shown to be useful in a forensic context in the diagnosis of diabetic, alcoholic, and starvation ketoacidosis (2–4). Studies have reported the morphology, constituents, and associated metabolic conditions of various types of renal tubular epithelial changes, such as in Armanni–Ebstein lesions and basal lipid vacuolization; however, the pathogenesis of such lesions remains unclear.

The Armanni–Ebstein lesion appears as swollen epithelial cells with almost complete conversion of the cytoplasm into a single large vacuole filled with glycogen (2,5). Although these lesions appear in hyperglycemic states (2,6,7), it has remained uncertain whether elevated glucose levels alone can directly cause this change. Renal biopsies are not routinely undertaken in metabolic states such as diabetic ketoacidosis and hyperglycemic hyperosmolar nonketotic coma, because the outcomes would not affect patient management, thus making controlled studies difficult. Previous investigations have focused solely on *in vivo* or postmortem studies; thus, the histological consequence of hyperglycemia in isolation on renal tubular epithelial cells is unknown.

This study utilized an *in vitro* isolated perfused rat kidney model to directly control the duration and magnitude of hyperglycemia, in the absence of other confounding metabolic derangements (such as hyperlipidemia and ketoacidosis), to determine whether a viable model could be established for linking physiological conditions to renal histological outcomes and to evaluate whether Armanni–Ebstein lesions could be reproduced by high glucose exposure alone.

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Materials and Methods

The use of scavenged Sprague–Dawley rats for this study was approved by the local University Animal Ethics Committees at both SA Pathology and The University of Adelaide, South Australia. Animal studies were performed with approved protocols, in accordance with the guidelines established by the National Health and Medical Research Council (NHMRC).

Dextran (MW = 48,000–90,000), bovine serum albumin (MW = 66,000), sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), monopotassium phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), L-cysteine ($C_3H_7NO_2S$), glycine ($C_2H_5NO_2$), L-glutamic acid ($C_5H_9NO_4$), D-glucose ($C_6H_{12}O_6$), and heparin sodium salt (50 mg/mL) were purchased from Sigma-Aldrich (St. Louis, MO). The isolated perfused kidney system and perfusion medium parameters were based on previously described methods (8). The perfusate for the control treatment group ($n = 10$) consisted of Krebs–Henseleit buffer (pH 7.4) containing sodium chloride (118 mmol/L), potassium chloride (4.7 mmol/L), calcium chloride (1.25 mmol/L), magnesium sulfate (1.24 mmol/L), monopotassium phosphate (1.2 mmol/L), sodium carbonate (25 mmol/L), D-glucose (5 mmol/L), L-cysteine (0.5 mmol/L), glycine (2.3 mmol/L), L-glutamic acid (0.5 mmol/L), bovine serum albumin (6.5 g/L), and dextran (36 g/L). The perfusate for the hyperglycemia treatment group ($n = 10$) contained the

above, with the addition of 70 mmol/L of D-glucose. 1 mg (200 units) of heparin sodium salt in 1 mL was added to the perfusates at the beginning of each experiment. The perfusion media were filtered through 1.2- and 0.45-μm filters (Millipore, Bedford, MA) prior to use and equilibrated with a mixture of moistened 95% oxygen and 5% carbon dioxide for 1 h before and during the kidney perfusion process. The nonperfused group (n = 10) consisted of the contralateral kidneys of the control group placed in the same environment for the same duration of time without being connected to the recirculating perfusion system.

Male and female Sprague–Dawley rats >200 g were euthanized individually using carbon dioxide (CO_2) inhalation. The abdominal cavities were opened, and the intestines and liver were ligated and removed. Retroperitoneal soft tissue was dissected to expose the vena cavae and abdominal aorta and to free the kidneys (Fig. 1). The renal artery was then cannulated through its ostia in the abdominal aorta with a 20-gauge intravenous cannula pre-infused with perfusate including heparin (Fig. 2). Both the cannulated kidney and the contralateral kidney were removed and immediately placed on a tray on ice for transportation to the isolated perfusion system.

The kidneys were placed into a thermostatically controlled cabinet at 34–37.5°C. The cannulated kidney was connected to the recirculating system containing 180 mL of perfusate, recycled using a peristaltic pump at approximately 32–38 mL/min with continuous adjustments as necessary to maintain renal artery perfusion pressure at 110 ± 20 mmHg, which was monitored using a manometer. The postmortem interval between death and the initiation of perfusion was recorded for each case. Perfusion of the kidney was monitored by the macroscopic color change in the parenchyma from dark maroon to yellow/tan and the change in the perfusate color from dark red to clear as the residual red blood cells were flushed out. The temperature, flow rate, renal arterial pressure, and macroscopic color changes were routinely recorded at five-minute intervals. After the designated perfusion time of 1 2 h, both the perfused and nonperfused kidneys were bisected and immediately placed in 10% buffered formalin for overnight fixation. Kidneys were then routinely processed, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) for histological assessment. In the perfused kidney, tissue areas that showed no remaining red blood cells within the vessels were considered to have been adequately perfused and were assessed histologically for vacuolization.

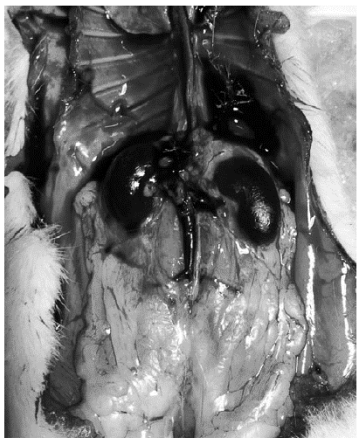

FIG. 1—*Dissection of Sprague Dawley rat to expose the aorta, vena cava, and bilateral kidneys.*

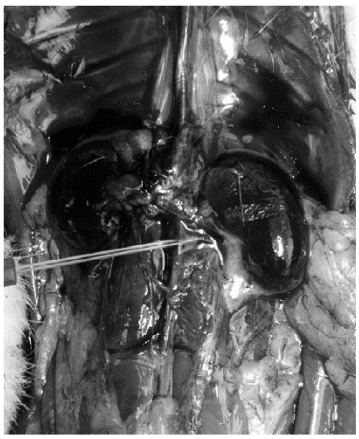

FIG. 2—*Cannulation of the left renal artery through its ostia in the abdominal aorta.*

Ten adjacent fields of each kidney were photographed at 400× magnification, and assessed for the degree of vacuolization using previously described methods (9) to determine the percentage area of unstained tissue. Differences between the high glucose concentration group and the two control groups were assessed by one-way ANOVA, followed by post hoc Tukey HSD test. To determine whether there were significant differences between the percentage area of vacuolization at 1 and 2 h within each group, initial tests for the equality of variances were conducted using an F-test followed by the appropriate t-test (with or without equal variance).

Results

Control Treatment Group

Ten Sprague Dawley rats, with a male-to-female ratio of 4:7, and whole animal weights ranging from 206 to 420 g (mean = 284.8 g) were used in the perfused control group, in which kidneys were perfused with Krebs–Henseleit buffer without added glucose. Kidneys from five rats (three male, two female; weight range from 226 to 350 g (mean 293.5 g)) were perfused for 1 h. Their postmortem interval ranged from 20 to 30 min (mean = 27 min). During the perfusion, the temperature was maintained between 34 and 37°C (mean = 36.1°C), with a flow rate of 34–36 mL/min (mean = 35.2 mL/min) required to maintain a renal arterial pressure of 100–122 mmHg (mean 110.4 mmHg). The time required for the efferent perfusate to become clear and for the majority of the visible surface of the kidney to change to a yellow/tan color ranged from 10 to 15 min (mean 14 min). Nonspecific tubular epithelial vacuolizations were observed histologically, and the unstained area per high power field ranged from 3.14 to 10.59% (mean 5.73%).

Kidneys from five female rats weighing from 206 to 320 g (mean = 255 g) and with a postmortem interval ranging from 20 to 40 min (mean 26.6 min) were perfused for 2 h. Their temperatures were maintained between 34 and 37.5°C (mean = 36.8°C), with a flow rate of 33.4–35.8 mL/min (mean = 35.1 mL/min) required to maintain a perfusion pressure of 100–128 mmHg (mean = 111.1 mmHg). The time taken for efferent perfusate to clear ranged from 10 to 15 min (mean = 11 min), and the majority of the kidneys changed color in 2–10 min (mean = 8.4 min). Nonspecific tubular vacuolizations (Fig. 3) and occasional tubules with cytoplasmic clearing and normal nuclei were observed, with an unstained area ranging from 12.33 to 33.75% (mean 22.15%).

Hyperglycemia Treatment Group

Kidneys from ten female Sprague–Dawley rats weighing between 202 and 260 g (mean = 234.7 g) were perfused with Krebs–Henseleit buffer with 70 mmol/L of D-glucose. Five kidneys were perfused for 1 h. In this group, whole animal weights ranged from 202 to 247 g (mean 226.4 g), and the postmortem intervals were 25–50 min (mean = 33 min). During perfusion, temperature was maintained between 34 and 37°C (mean = 36.4°C), with a flow rate between 34 and 36.2 mL/min (mean = 35.1 mL/min) to maintain a renal arterial pressure of 102–126 mmHg (mean 112.7 mmHg). The time for the efferent perfusate to clear ranged from 5 to 15 min (mean 10.2 min) and the time for the majority of the kidney to change to yellow/tan ranged from 5 to 25 min (mean = 14.2 min). Histologically, isometric fine vacuolizations of tubular epithelial cells consistent with osmotic nephrosis and occasional cytoplasmic clearing with normal-appearing nuclei were seen; however, PAS stain did not demonstrate any glycogen deposition. The percentage of unstained area per high power field ranged from 15.83 to 43.90% (mean 31.23%).

Kidneys from five female rats weighing from 210 to 246 g (mean = 243 g) with a postmortem interval ranging from 20 to 25 min (mean 21.6 min) were perfused for 2 h. Their temperatures were maintained between 34 and 37°C (mean = 36.6°C), with a flow rate of 34–36.2 mL/min (mean = 35.5 mL/min) to maintain a perfusion pressure of 102–122 mmHg (mean = 112.8 mmHg). The time taken for efferent perfusate to clear ranged from 3 to 30 min (mean = 14 min), and the majority of the kidney changed color in 3–35 min (mean = 15 min). Isometric vacuolizations of osmotic nephrosis were observed histologically (Fig. 3), along with numerous tubules demonstrating cytoplasmic clearing with normal-appearing nuclei surrounded by nonvacuolated tubules (Fig. 4). PAS staining in all cases did not demonstrate any accumulated glycogen (Fig. 5). The unstained areas ranged from 15.24 to 44.67% (mean 34.64%) per high power field.

Nonperfused Group

Ten contralateral kidneys from animals used in the control and hyperglycemia treatment groups were placed adjacent to their corresponding perfused kidney, in the same perfusion cabinet, with the same temperature and environment, for the same amount of time. Analyses of nonperfused kidneys in the one- and two-hour experiments showed a total cleared area ranging between 1.069 and 18.09% (mean 4.79%) and between 0.92 and 23.30% (mean 5.57%) per high power field, respectively. In both groups, occasional small nonspecific cytoplasmic vacuoles were observed; additionally, tubules with cytoplasmic clearing and pyknotic nuclei were observed in the two-hour group.

Quantitative Analyses

Analysis via one-way ANOVA revealed a statistically significant difference in renal tubular epithelial vacuolization in the group infused with 70 mmol/L of glucose compared with both control groups at 1 (F (2,147) = 697.47, p = <0.001) and 2 h (F (2,147) = 438.78, p = <0.001). Quantitative comparisons of epithelial cell vacuolization between the different study groups are summarized in Fig. 6.

The magnitude of the response correlated with glucose treatment. *Post hoc* comparisons using the Tukey HSD test revealed a significantly greater unstained area ($p < 0.001$) in the high glucose group (M = 31.23, SD = 5.91) compared to the perfused control group (M = 5.73, SD = 1.53). The high glucose group also showed a significantly greater clear area than the

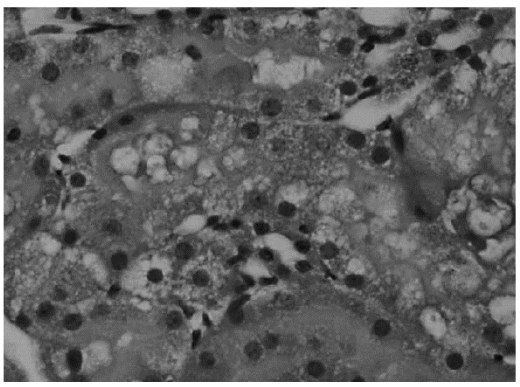

FIG. 3—*Osmotic nephrosis in a rat kidney perfused with Krebs Henseleit buffer and 70 mmol/L of glucose for 2 h (Hematoxylin and eosin ×400).*

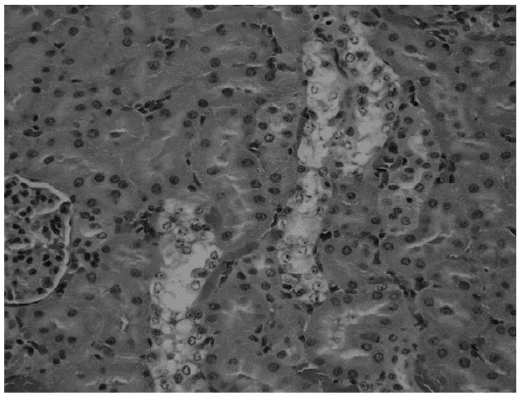

FIG. 4—*Swelling and cytoplasmic clearing of renal tubules in a rat kidney perfused with Krebs Henseleit buffer and 70 mmol/L of glucose for 2 h (Hematoxylin and eosin ×400).*

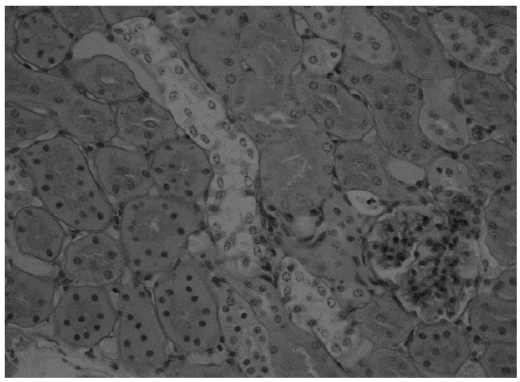

FIG. 5—*No glycogen within vacuolated tubular epithelial cells in a rat kidney perfused with Krebs–Henseleit buffer and 70 mmol/L of glucose for 2 h (Periodic Acid-Schiff ×400).*

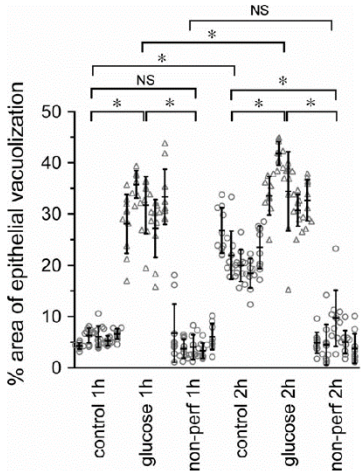

FIG. 6 *Scatter plot histogram summarizing the degree of renal tubular vacuolization between study groups. Statistically significant differences are indicated as (*) for* $p < 0.001$*; NS is not significant.*

nonperfused group (M = 4.79, SD = 3.34) after 1 h. Similarly, after 2 h, there was a significantly greater amount of cellular clearing in the high glucose group (M = 34.64, SD = 5.83), compared with the perfused control (M = 22.15, SD 4.75) and nonperfused control (M = 5.57, SD = 4.02) groups. There were no statistically significant differences between the perfused control treatment group and the nonperfused control groups at 1 h.

The *F*-tests indicated that the variances in the hyperglycemic groups ($F = 0.104$, $p < 0.001$) were not equivalent to those in the control groups ($F = 0.104$, $p < 0.001$). However, the variances for the one- and two-hour nonperfused kidneys ($F = 0.690$, $p = 0.099$) were not different.

The magnitude of the response in the hyperglycemic groups correlated with the time of exposure. The *F*-test indicated no significant difference in variances between the kidneys perfused for 1 h and those perfused for 2 h with hyperglycemic perfusate ($F = 1.026$, $p = 0.465$). Subsequent two-sample *t*-test assuming equal variances showed that the mean unstained area per high power field in the hyperglycemia treatment group at 1 h (M = 31.23, SD = 5.91) was significantly less than at 2 h (M = 34.64, SD = 5.83), t (98) = −2.910, $p = 0.002$.

Similarly, kidneys perfused with nonhyperglycemic Krebs–Henseleit buffer for 1 h (M = 5.73, SD = 1.53) also showed significantly less clearing of tubules compared to the two-hour group (M = 22.15, SD = 4.75), t (59) = −23.275, $p < 0.001$. There was no significant difference in vacuolization among the one-hour (M = 4.79, SD = 3.34) and two-hour (M = 5.57, SD = 4.02) nonperfused kidneys, t (98) = −1.053, $p = 0.147$.

Discussion

The *in vitro* isolated perfused rat kidney model has been extensively utilized for investigating renal drug handling (8,10–12) and the effects of drugs and various substances on renal vasculature (13,14), glomerular function (15,16), renal cellular metabolism (17), and gluconeogenesis (18). However, the model has not been tested for the investigation into etiologic factors causing changes in renal histology. Rodents are appropriate candidates for establishing a new model for renal histological studies as histological abnormalities noted in human kidneys, including the Armanni–Ebstein phenomenon, have been demonstrated previously with *in vivo* studies in rats (7,19,20).

The current study perfused kidneys with Krebs Henseleit buffer with and without 70 mmol/L of glucose. This concentration was chosen as it represents a 10-fold elevation in the threshold fasting glucose level of 7.0 mmol/L which is diagnostic for diabetes in humans (21). The degree of vacuolization after hyperglycemic treatment was significantly greater than that observed in two different control groups: (i) a perfused group without excess glucose which controlled for any histological changes induced by the perfusion system and (ii) a nonperfused group which controlled for nonspecific vacuolizations induced by postmortem autolysis. Results showed a statistically significant increase in renal tubular epithelial vacuolization in the group infused with 70 mmol/L of glucose compared to both control groups at one and 2 h. Additionally, there was a significant increase in vacuolization among the kidneys perfused with high glucose for 2 h compared to those perfused for 1 h. This suggests that the isolated perfused kidney system is capable of inducing renal tubular epithelial vacuolizations that increase with perfusion time. The absence of accumulated glycogen (as determined by PAS staining) in the hyperglycemic treatment group may suggest that the pathogenesis of the Armanni–Ebstein lesion requires factors more complicated than simple exposure to hyperglycemia alone or that cytoplasmic glycogen accumulation requires more than 2 h of exposure to high glucose concentrations to develop.

Limitations of the system are the need to prevent thrombosis and the limited duration of the treatment period that can be used to simulate a chronic disease condition (here not exceeding 2 h). Prevention of thrombosis was addressed by the addition of 1 mg (200 units) of heparin to the perfusate in each experiment. This dose was based on previous studies injecting 100 units of heparin into the penile vein of rats weighing between 346 and 459 g (8) or 200 units injected into the renal vein (18). With sporadic thrombi, individual regions and tubules of the kidneys might have been perfused for less than the specified time. The reason why thrombi developed in some cases and not in others

is unclear; perhaps more immediate delivery of heparin (e.g., antemortem) or a higher dose would facilitate better perfusion. While morphological changes that emulate human postmortem histology do develop in the *ex vivo* kidney model over time; the temporal limitation of this model is that the interpretation of vacuolization becomes increasingly more difficult with the onset of autolytic changes associated with increasing postmortem interval.

To optimize the model, this study used dextran as an oncotic agent to substitute for a large portion of bovine serum albumin (BSA) in a ratio that has been shown previously to confer higher functional viability of the perfused kidney as compared with BSA alone; this was demonstrated by the preservation of the tubular reabsorption of water, glucose, and sodium (8). Dextran is preferred over BSA due to the significantly lower cost and in pharmacokinetic investigations into drugs that are extensively bound to BSA (22). However, the histological consequences of this substitution are unclear.

The current study has demonstrated that the isolated perfused kidney model is a viable model for the study of renal tubular epithelial cell histology. A significantly greater degree of vacuolization was observed after treatment with high glucose compared to both control groups, which also correlated with the duration of exposure. The morphological features of Armanni–Ebstein lesions (i.e., swelling and cytoplasmic clearing of renal tubular epithelial cells) were observed in the hyperglycemia treatment group; however, intravacuolar glycogen, which is a defining feature of classical Armanni–Ebstein lesions, was not demonstrated. Therefore, further studies need to be conducted, perhaps with the addition of ketones and/or regulatory/counter-regulatory hormones (e.g., insulin and glucagon), and over longer periods of time to reproduce glycogen accumulation.

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PAPER

PATHOLOGY/BIOLOGY

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The Etiology of Basal Vacuolizations in Renal Tubular Epithelial Cells Evaluated in an Isolated Perfused Kidney Model

ABSTRACT: To determine whether basal lipid vacuolization characteristic of ketoacidosis could be induced with short-term hypertriglyceridemia, adult Sprague Dawley rat kidneys were perfused in an isolated perfused kidney model with, and without, 11.3 mM (10 g/L) of triglycerides in Krebs-Henseleit buffer, for 1 and 2 h (n = 5/group). Additional treatments included perfusion with triglycerides with 20 mM of β-hydroxybutyrate and 2 mM of acetoacetate (n = 5) and perfusion with triglycerides with 70 mM of glucose (n = 1). Basal vacuolization was produced in all groups, but differed in morphology to that reported in postmortem studies. There was no further increase in vacuolization after 2 h of perfusion compared to 1 h (p = 0.24), and the addition of ketones did not alter the morphology or extent of vacuolization. This study using an *ex vivo* model has confirmed that isolated hypertriglyceridemia is sufficient to cause basal lipid vacuolization in renal tubular epithelial cells, but with different morphology to vacuoles observed in lethal ketoacidosis at autopsy.

KEYWORDS: forensic science, basal vacuolization, subnuclear vacuolization, isolated perfused kidney, nephrotic syndrome, ketoacidosis, hyperlipidemia

Renal tubular epithelial cells respond to metabolic insults with a variety of distinctive patterns of vacuolization, as is seen in osmotic nephrosis secondary to hyperosmolarity (1), the Armanni–Ebstein phenomenon secondary to hyperglycemia (2), hypokalemia with coarse irregular vacuolization (3), and calcineurin-inhibitor toxicity with secondary isometric vacuolization (4). Basal vacuolization, characterized by a row of subnuclear vacuoles in close proximity to the basement membrane, has been reported in postmortem samples from subjects with diabetic (5,6), alcoholic (7), septic (8), and starvation (9) ketoacidosis. Basal vacuolization of a different morphology, appearing as numerous circular vacuoles which begins basally and then spreads to involve the entire cell, has also been observed in cases of pediatric nephrotic syndrome (10). A consistent feature in all these conditions has been the demonstration of intravacuolar lipids with Oil Red-O staining and by electron microscopy (5,9,10), suggesting that the etiology of the pathological phenotypes is linked to hyperlipidemia (5). However, all studies to date have involved postmortem tissues, which have made the determination of a direct association between basal vacuolization and hyperlipidemia in isolation challenging. The use of a perfused kidney model has allowed separation of possible causal factors, with minimization of other confounding metabolic abnormalities that are often present in these conditions *in vivo* (11).

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Materials and Methods

This use of scavenged Sprague Dawley rats for this study was approved by the Animal Ethics Committees of SA Pathology and The University of Adelaide, South Australia. Animal studies were performed according to the guidelines established by the National Health and Medical Research Council (NHMRC).

The isolated perfused kidney system and perfusion medium composition were based on previously described methods (12). The perfusate consisted of Krebs-Henseleit buffer (pH 7.4) containing 118 mM of sodium chloride (NaCl), 4.70 mM of potassium chloride (KCl), 1.25 mM of calcium chloride ($CaCl_2$), 1.24 mM of magnesium sulfate ($MgSO_4$), 1.20 mM of monopotassium phosphate (KH_2PO_4), 25 mM of sodium bicarbonate ($NaHCO_3$), 5 mM of D-glucose ($C_6H_{12}O_6$), 0.5 mM of L-cysteine ($C_3H_7NO_2S$), 2.3 mM of glycine ($C_2H_5NO_2$), 0.5 mM of L-glutamic acid ($C_5H_9NO_4$), 6.5 g/L of bovine serum albumin (MW = 66,000) and 36 g/L of dextran (MW = 48,000–90,000) (Sigma-Aldrich, St. Louis, MO). Intralipid 20% (Fresenius Kabi AB, Sweden), which contains triglyceride 20%, phospholipid 1.2% and glycerol 2.2%, was added to the perfusate medium to achieve a triglyceride concentration of 11.3 mM (10 g/L) (n = 10). An additional treatment group (n = 5), used to simulate concurrent ketoacidosis, was exposed to perfusate containing 20 mM of β-hydroxybutyric acid ($C_4H_8O_3$) and 2 mM methyl acetoacetate ($C_5H_8O_3$). A pilot treatment assay (n = 1) used to simulate concurrent hyperglycemia contained 70 mM of D-glucose instead of the standard 5 mM in the perfusate medium. 1 mg (200 units) of heparin sodium salt in 1 mL was added to the perfusate solutions at the beginning of each experiment. The perfusion media were successively filtered through 1.2 and 0.45 μm filters (Millipore, Bedford, MA) prior to use and

equilibrated with a gaseous mixture of moistened 95% oxygen and 5% carbon dioxide for 1 h before and throughout the kidney perfusion.

Adult Sprague Dawley rats (>200 g each) were euthanized using carbon dioxide (CO_2) inhalation. The kidneys were subsequently cannulated through the renal artery via the previously described dissection techniques (11) and placed into a thermostatically controlled cabinet at 34–37.5°C. The cannulated kidney was connected to the recirculating system containing 180 mL of perfusate pumped using a peristaltic pump at approximately 32–38 mL/min with continuous adjustments as necessary to maintain renal artery perfusion pressure at 110 ± 20 mmHg. The postmortem interval between time of death and the initiation of perfusion was recorded for each case. Renal perfusion was monitored via macroscopic color change from dark maroon to yellow/tan and a change in the efferent perfusate from dark red to clear as red blood cells within the kidney were flushed. The temperature, flow rate, renal arterial pressure, and macroscopic color changes were routinely recorded at 5-min intervals. After perfusion for the designated time of 1–2 h, a small portion of the kidney was excised and immediately placed in glutaraldehyde for fixation. The remainder of the kidney was bisected and placed in 10% buffered formalin for overnight fixation. Kidneys were then routinely processed, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin (H&E) for histological assessment of basal vacuolization. Only perfused areas of the kidney (those with a microscopic absence of red blood cells) were assessed for vacuolization area and morphology.

The average area of vacuolization per high power field was assessed by photographing ten consecutive fields of each kidney at 40× magnification and calculating the percentage of negative staining in ImageJ using previously described methods (13). Analysis of statistically significant differences between the means of all the treatment groups began with a preliminary test for the equality of variances (*F*-test) and followed by the appropriate two-tailed *t*-test which either assumes or does not assume equal variances.

Results

Controls

The control group was taken from a previous study using the same perfusion system with the same concentration of constituents making up the Krebs-Henseleit buffer (11). This consisted of ten Sprague Dawley rats, with body weights ranging from 206 to 420 g (mean 284.8 g) which were perfused for 1 and 2 h with Krebs-Henseleit buffer in the absence of added triglycerides or ketones. Nonspecific tubular epithelial vacuolizations consistent with osmotic nephrosis were observed in all cases, with the unstained area per high power field ranging from 3.14 to 10.59% (mean 5.73%) in the kidneys perfused for 1 h, and 12.33–33.75% (mean 22.15%) in the kidneys perfused for 2 h. Separation of tubules from the basement membrane was focally observed in some cases, but no discrete subnuclear vacuolizations were present (11).

Hypertriglyceridemia in Isolation

Ten Sprague Dawley rats, with body weights ranging from 210 to 342 g (mean 270.2 g), were used in the groups perfused with hypertriglyceridemia alone, at 1 and 2 h. Five rats weighing between 210 and 246 g (mean 225.2 g) were perfused for 1 h. Their postmortem interval ranged from 25 to 35 min (mean 30 min). Perfusion temperature was maintained between 34 and 37°C (mean 36.0°C), and a flow rate of 33.2–37.2 mL/min (mean 34.9 mL/min) was required to maintain a renal arterial pressure of 100–130 mmHg (mean 111.6 mmHg). The time for the efferent perfusate drops to become clear and for the majority of the kidney to change to a yellow/tan color ranged from 1 to 15 min (mean 11.2 min) and 1–20 min (mean 9.6 min), respectively. Subnuclear vacuolizations were observed histologically in all cases, and the mean unstained area ranged from 14.8 to 19.7% (mean 17.7%).

Five rats weighing from 292 to 342 g (mean 315.2 g) and with a postmortem interval ranging from 15 to 20 min (mean 18.4 min) were perfused for 2 h. Environmental temperature was maintained between 35 and 37.5°C (mean 36.8°C), and a flow rate of 33.6–36 mL/min (mean 35.3 mL/min) was required to maintain a perfusion pressure of 100–120 mmHg (mean 110.6 mmHg). The time taken for efferent perfusate to clear and the change in kidney color to occur ranged from 10 to 20 min with a mean of 17 and 15 min, respectively. Subnuclear vacuolizations of renal tubular epithelial cells were observed in all cases, with a mean unstained area ranging from 15.9 to 22.3% (mean 19.7%).

Hypertriglyceridemia with Concurrent Ketoacidosis

Kidneys from five Sprague Dawley rats weighing between 280 and 380 g (mean 323 g) were perfused with added triglycerides and ketones for 2 h. Their postmortem interval ranged from 15 to 30 min (mean 19.6 min), and the perfusion temperature was maintained between 34 and 37°C (mean 36.7°C). A flow rate of 32–36 mL/min (mean 34.9 mL/min) was required to maintain a renal arterial pressure of 100–120 mmHg (mean 110.4 mmHg). The time for the efferent perfusate drops to clear and the time for the majority of the kidneys to change to yellow/tan ranged from 10 to 20 min with a mean of 17 and 15 min, respectively. Histologically, subnuclear vacuolizations of similar morphology to the ones observed in the hypertriglyceridemia in isolation group were seen in all cases. The mean percentage of unstained area ranged from 19.4 to 24.8% (mean 22.0%).

Hypertriglyceridemia with Concurrent Hyperglycemia

One kidney from a Sprague Dawley rat with a weight of 316 g and a postmortem interval of 11 min was perfused for 2 h with added triglycerides and glucose. The perfusion temperature was maintained between 35 and 37°C (mean 36.8°C), and a flow rate of 34.8–35.6 mL/min (mean 35.3 mL/min) was required to maintain renal arterial pressure between 104 and 117 mmHg (mean 111.2 mmHg). Efferent perfusate drops cleared in 20 min, and the vast majority of the kidney changed color to yellow/tan in 15 min. Subnuclear vacuolization of the same morphology as the other two treatment groups was noted.

Histological Observations

Subnuclear vacuolizations of similar morphology were observed in all study groups (Figs 1 and 2). The dominant pattern of vacuolization appeared as a single vacuole in contact with and beneath normal appearing nuclei but not in contact with the basement membrane (Fig. 1). As these vacuoles enlarged, they displaced nuclei luminally, and also engulfed the

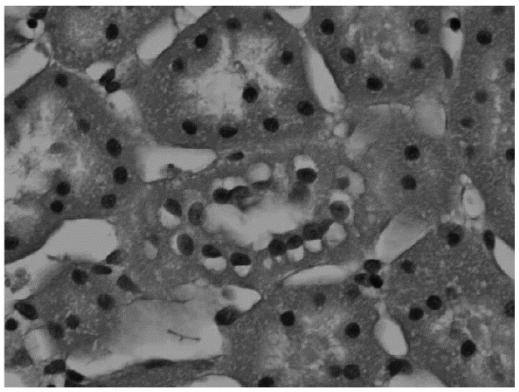

FIG. 1—*Subnuclear vacuolization in a kidney perfused with 11.3 mM triglycerides for 2 h (Hematoxylin and eosin ×400).*

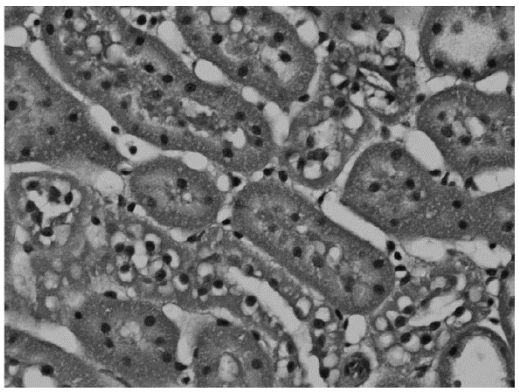

FIG. 2—*Subnuclear vacuolization in a kidney perfused with 11.3 mM triglycerides, 20 mM β-hydroxybutyrate and 2 mM acetoacetate for 2 h (Hematoxylin and eosin ×400).*

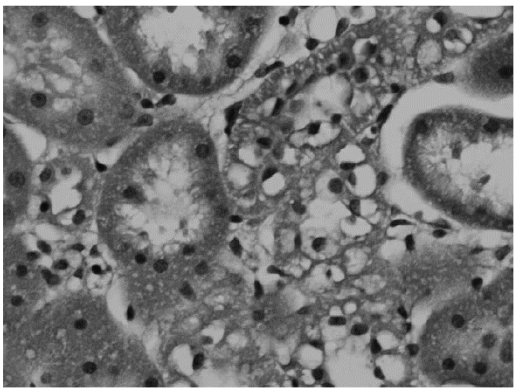

FIG. 3—*Subnuclear vacuolization with focal expansion of vacuoles to involve entire cells in a kidney perfused with 11.3 mM triglycerides, 20 mM β-hydroxybutyrate and 2 mM acetoacetate for 2 h (Hematoxylin and eosin ×400).*

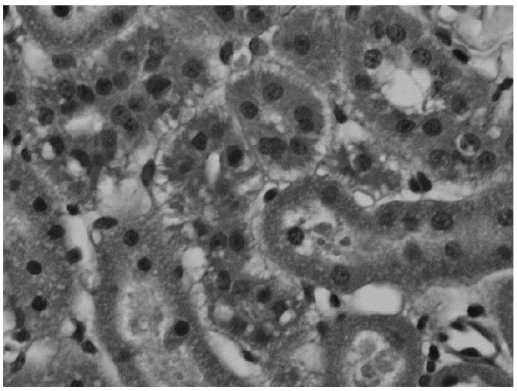

FIG. 4—*Basal vacuolization with vacuoles in contact with the basement membrane in a kidney perfused with 11.3 mmol/triglycerides for 1 h (Hematoxylin and eosin ×400).*

nucleus and involved the entire cell, giving the appearance of cellular cytoplasmic clearing (Fig. 3). Occasional focal subnuclear vacuoles in contact with the basement membranes were also observed (Fig. 4), and in a few cases, these vacuoles were seen to simultaneously affect the same tubules as the other subnuclear vacuoles (Fig. 5). The addition of ketones into the perfusate did not change the overall morphology of the basal vacuolization. Electron microscopy was undertaken on one kidney from the hypertriglyceridemia in isolation group perfused for 1 h and one kidney from the hypertriglyceridemia with concurrent ketoacidosis group. Both kidneys showed intracytoplasmic lipids within vacuoles which were in contact with the nucleus but not with the basement membrane (Fig. 6).

Quantitative Analyses

F-tests for the equality of variances showed no statistically significant difference in variances between the 1- and 2-h hyperlipidemia in isolation group ($F = 0.39$, $p = 0.19$), nor between the 2-h hyperlipidemia and hyperlipidemia with concurrent ketoacidosis groups ($F = 1.49$, $p = 0.35$). Quantitative comparisons of areas of epithelial vacuolization in the different treatment conditions are summarized in Fig. 7. *T*-tests assuming equal variances showed no statistically significant differences in the mean unstained areas of the kidneys perfused with added triglycerides for 1 h ($M = 17.65$, SD = 1.92) and 2 h ($M = 19.69$, SD = 3.06), $t(8) = -1.26$, $p = 0.24$. Similarly, the degree of vacuolization in the kidneys perfused for 2 h with elevated triglycerides alone ($M = 19.69$, SD = 3.06) as compared to those perfused with elevated triglycerides and ketones ($M = 21.95$, SD = 2.51) was not statistically different [$t(8) = -1.28$, $p = 0.24$].

Discussion

An *ex vivo* isolated perfused rat kidney model was used in this study to investigate the etiology of basal vacuolizations as it

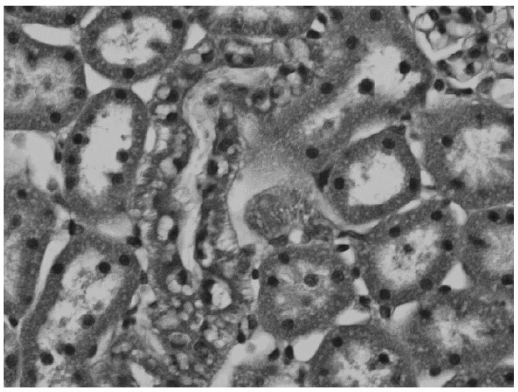

FIG. 5—*Two concurrent patterns of subnuclear vacuolization in a kidney perfused with 11.3 mM triglycerides for 1 h (Hematoxylin and eosin ×400).*

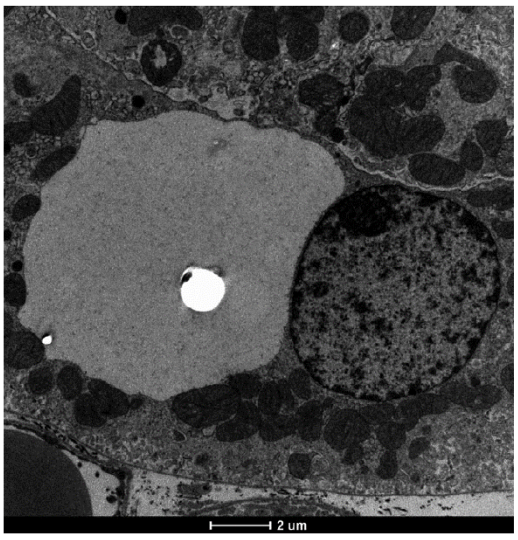

FIG. 6—*Intravacuolar lipid on electron microscopy within the cytoplasm of a renal tubular epithelial cell perfused with 11.3 mM triglycerides for 1 h.*

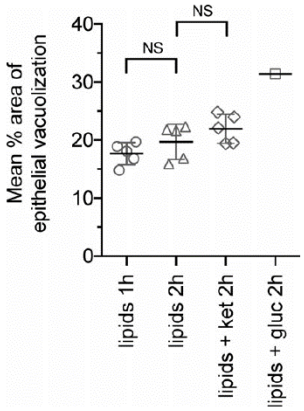

FIG. 7—*Scatterplot histogram summarizing the mean % area of renal tubular vacuolization between study groups. NS is not significant.*

allows for control of a single metabolic derangement (e.g., hypertriglyceridemia in isolation), and the addition of other known derangements (e.g., ketosis). This is difficult to investigate with postmortem material as individuals with ketoacidosis can often have other known or unknown co-occurring metabolic abnormalities. This model has previously been validated for the investigation of drug effects on glomerular function (14,15), renal vasculature (16,17), cellular metabolism (18) and gluconeogenesis (19). A recent study has also shown that it is a viable model for the investigation of renal tubular epithelial histology, showing the induction of osmotic nephrosis subsequent to hyperglycemic perfusate with a statistically significant increase in vacuolization in the hyperglycemia treatment as compared with control perfused kidneys (11).

In the current study, hyperlipidemia was induced by the addition of triglycerides, following on studies by Nielsen et al. that demonstrated triglycerides as the major constituent of the basal vacuolizations in diabetic ketoacidosis (20). Hypertriglyceridemia is also a feature of nephrotic syndrome; however, the hyperlipidemia seen in nephrotic syndrome usually consists of both elevated cholesterol as well as triglycerides (21). The dual effect is thought to result from proteinuria and subsequent hypoalbuminemia, leading to decreased plasma oncotic pressure, and stimulation of hepatic apoprotein B gene transcription that in turn mediates a compensatory increase in hepatic lipoprotein synthesis (22). The absence of hypercholesterolemia within the perfusate may, therefore, explain the difference in morphology of the subnuclear vacuoles that were observed compared to basal vacuoles in nephrotic syndrome.

Hypertriglyceridemia commonly occurs in diabetic ketoacidosis. For example, a study of 46 patients hospitalized with diabetic ketosis and ketoacidosis showed that 64% had moderate hypertriglyceridemia with levels above the 95th percentile; 28% had severe hypertriglyceridemia (>5.65 mM) including six patients (13%) with triglyceride levels >11.29 mM (23). Treatment of the ketoacidosis was also usually associated with a rapid decrease in plasma triglyceride levels. Hypercholesterolemia was less frequent, with 36% of patients having values above the 95th percentile (23). In a study of plasma endogenous triglycerides in 74 patients with different types of diabetes, the highest rates of triglyceride synthesis were recorded in those with ketoacidotic diabetes (24). Hypertriglyceridemia in diabetic ketoacidosis occurs because insulin deficiency activates lipolysis in adipose tissue, thus releasing free fatty acids (FFA) which stimulate hepatic production of very low-density lipoprotein (VLDL) (24,25). Insulin deficiency also increases the esterification of FFAs and impairs removal of triglycerides from the circulation by inhibiting lipoprotein lipase activity in adipose tissue (24,26). It has been suggested that coexisting genetic abnormalities, such as mutations on the lipoprotein lipase gene, may play a role in ketoacidotic hypertriglyceridemia (26); however, a study of 15 patients with ketosis and severe hypertriglyceridemia of >11.3 mM showed that at least 10 and perhaps 12 of these

patients did not have an underlying genetic abnormality (27). There are no reports in the current literature on lipid levels in alcoholic, starvation, or septic ketoacidosis, but Fulop and Eder reported that only 11 of their 15 patients with ketosis and severe hypertriglyceridemia (>11.3 mM) had definite or probable insulin-dependent diabetes mellitus (27), implying that other causes of ketoacidosis can also be associated with significant derangements in serum triglycerides.

The current study used a hyperlipidemic perfusate with the addition of 11.3 mM (10 g/L) of triglycerides. This concentration was chosen as it represents double the threshold concentration for the highest tier of hypertriglyceridemia ("Very high"; 500 mg/dL) as set by the American Heart Association guidelines (28). β-hydroxybutyrate and acetoacetate are the main ketones in ketoacidosis, and they have a ratio of 1:1 in normal circulating blood. In diabetic ketoacidosis, this ketone body ratio is initially 3:1 or greater, but can be as high as 10:1 as a result of the highly reduced state of hepatic mitochondria (29). Similarly, a high ratio (7:1–10:1) of β-hydroxybutyrate to acetoacetate is seen in alcoholic ketoacidosis (30). The concentration of 20 mM of β-hydroxybutyrate was chosen as this reflected the highest observed range seen in autopsy studies (31), and 2 mM of acetoacetate was chosen to reflect a 10:1 ratio of ketone derangement seen in severe ketoacidosis.

The predominant pattern of basal vacuolization observed in this study differed from that seen in autopsy cases of ketoacidosis, as vacuoles did not abut the basement membrane unless they had expanded to involve the entire cell. Additionally, basal vacuolization was only seen as a focal effect, with vacuolated tubules surrounded by normal unaffected tubules. In contrast, subnuclear vacuoles in postmortem cases of ketoacidosis were often observed as a diffuse effect which could occur with β-hydroxybutyrate levels less than 20 mM (7). A possible shortcoming of this study is that the concentration of triglycerides was insufficient to cause diffuse basal vacuolization; however, more marked basal vacuolization with clusters of tubules affected has been noted in nephrotic syndrome cases with lower levels of hypertriglyceridemia (<9.92 mM) (10). This may suggest that a perfusion time of >2 h is required for more widespread changes to develop. It may also suggest that additional factors or other concurrent metabolic derangements, such as hypercholesterolemia, might be required to cause the morphological changes and extent of basal vacuolization seen in ketoacidosis. The basal vacuoles which were in contact with the basement membrane (Fig. 5) also appeared morphologically different to basal vacuoles in ketoacidosis in that they were elongated perpendicular to the basement membrane and were frequently confluent, as opposed to discrete and circular. No demonstrable lipids were present within these vacuoles on electron microscopy; therefore, it is likely that these vacuoles may represent a nonspecific early autolytic change with separation from the basement membrane.

The study is also limited by the low numbers of animals in each treatment group. To maximize utility and preserve animal usage, experiments in the hypertriglyceridemia treatment group were limited to ten kidneys, and the hypertriglyceridemia with ketosis treatment group was limited to five kidneys as they all demonstrated the same pattern of subnuclear vacuolization (Fig. 1), and it was thought further experiments in the same group were unlikely to yield different results. However, larger studies would add more statistical significance to these findings. The single kidney perfused with added glucose was intended to be a pilot study, which did not demonstrate a difference in the pattern of vacuolization; however, further trials in this group are clearly needed.

The current study has demonstrated that hypertriglyceridemia in isolation is able to cause subnuclear lipid vacuolization in renal tubular epithelial cells; however, the morphology of these vacuoles differs from those observed in postmortem tissues that have been exposed to ketoacidosis *in vivo* (2,7–9). This may suggest that a longer duration of exposure (>2 h) or metabolic derangements other than hypertriglyceridemia, ketosis, and hyperglycemia are involved in the pathogenesis of ketoacidotic basal vacuoles. Further studies will be required to determine the precise contribution of each metabolic derangement, in isolation and also synergistically, to the renal phenotype characteristically observed in autopsy tissues from cases of lethal ketoacidosis.

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ADDITIONAL FIGURES OF ISOLATED PERFUSED KIDNEY MODEL

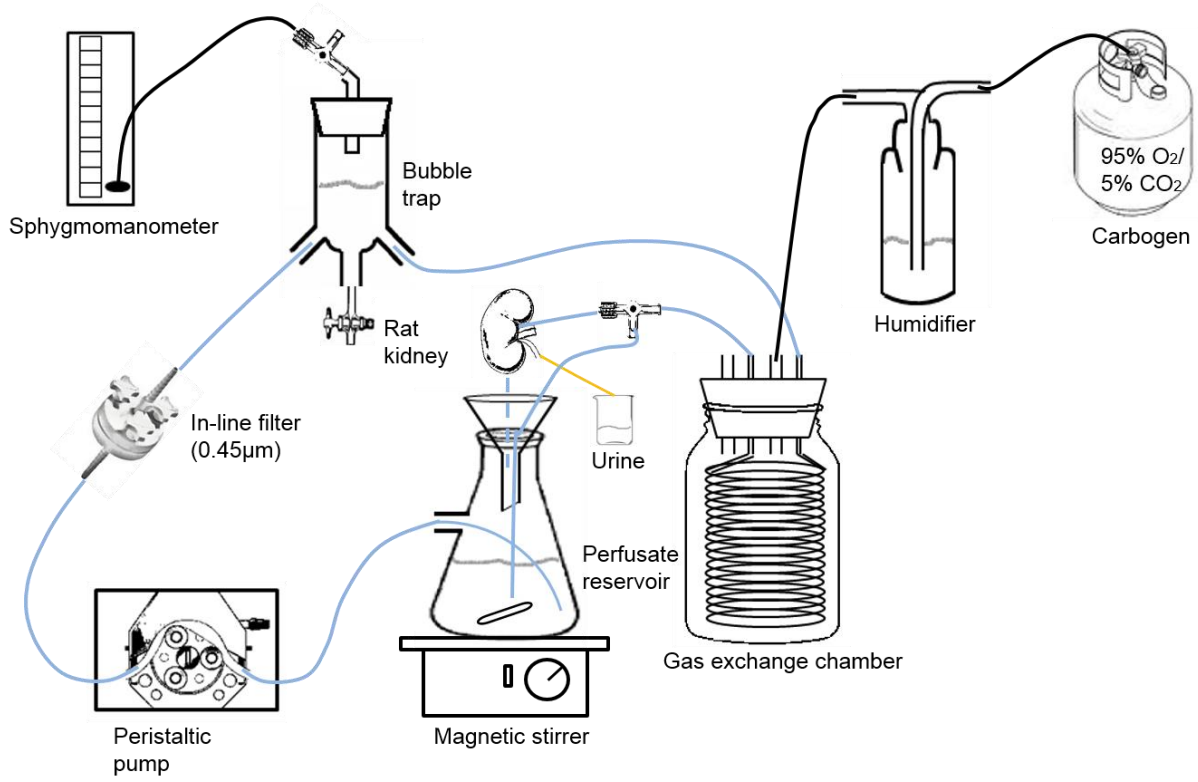

FIG. 1-*Schematic design of the isolated perfused kidney model.*

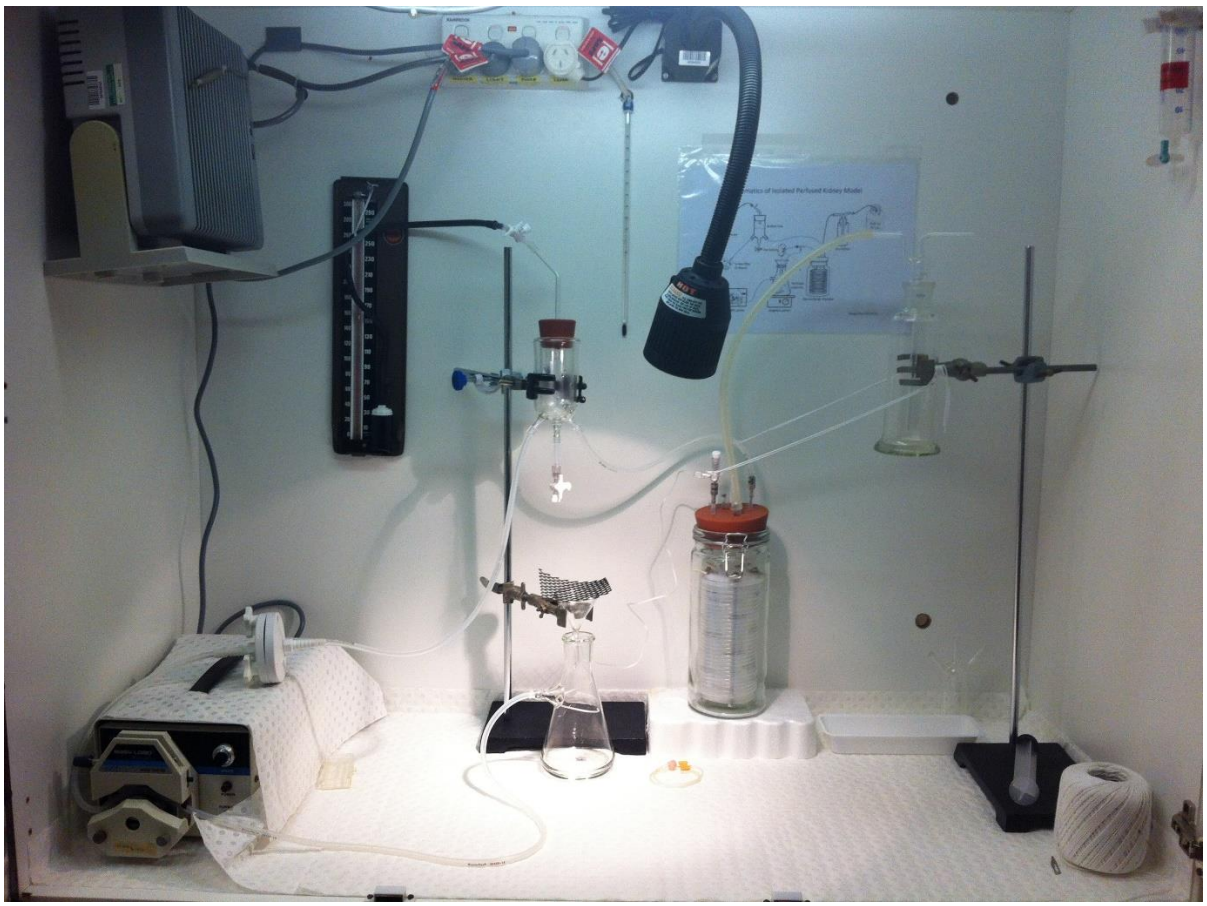

FIG. 2-*Photograph inside the thermostatically controlled perfusion cabinet used in the isolated perfused kidney model (magnetic stirrer is not pictured).*

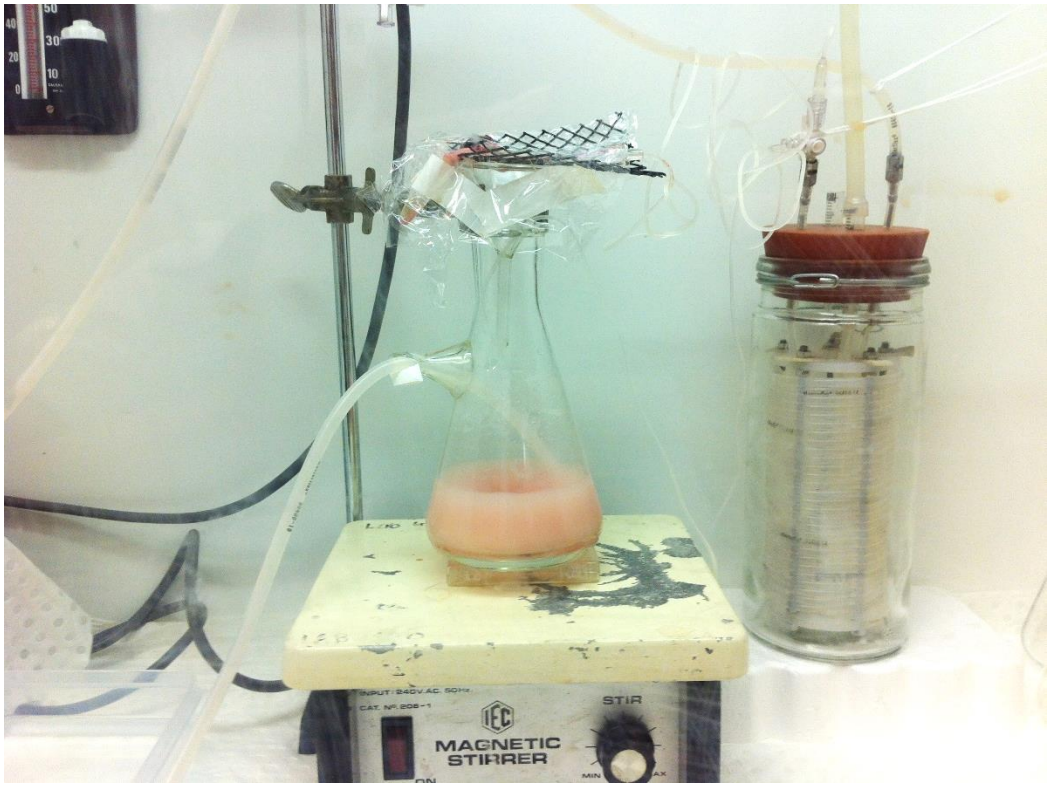

FIG. 3-*Rat kidney being perfused in the isolated perfused kidney system. The kidney sits on top of the mesh platform and is perfused with filtered and oxygenated perfusate. After passing through the kidney, efferent perfusate drops into the reservoir below and is recirculated.*

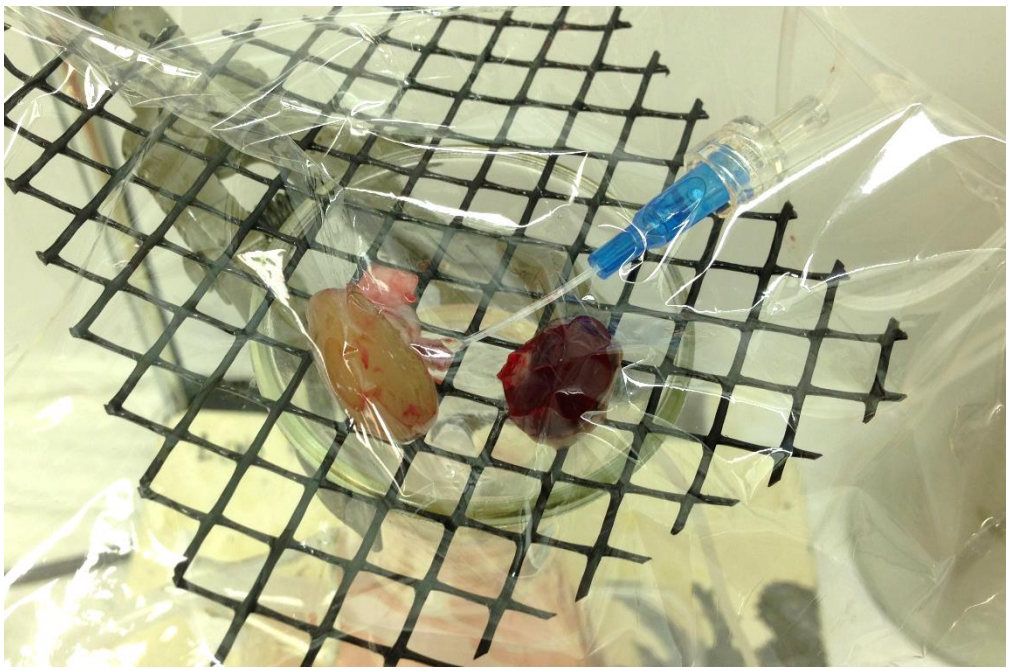

FIG. 4-*Sprague Dawley rat kidney being perfused via a cannula inserted through the renal artery. The non-perfused contralateral kidney is placed adjacent to the perfused kidney as a control. Kidneys are covered with thin plastic wrap to prevent surface dehydration.*

FIG. 5-*Same experiment as in Fig. 4 with plastic covering wrap removed.*

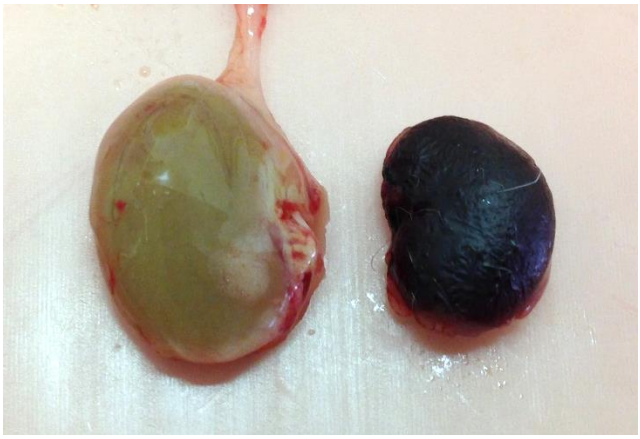

FIG. 6-*Paired perfused (left) and non-perfused (right) kidneys of the same rat after cessation of the experiment. The perfused kidney is homogenously yellow/tan, and appears plump and moist. In contrast, the non-perfused kidney is dark maroon, and appears smaller and shrunken with crinkling and drying of the renal capsule*.

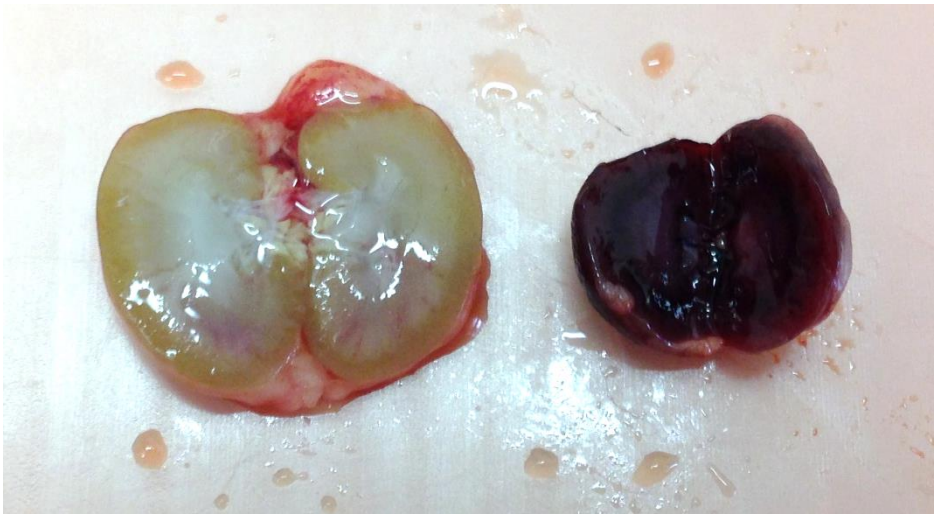

FIG. 7-*Coronal section through both kidneys demonstrate a lighter colour of the perfused kidney (left) due to an absence of red blood cells within the parenchyma following successful perfusion. The non-perfused kidney appears dark maroon due to the presence of red blood cells.*