"THE EFFECT OF LOCAL ANAESTHETIC CONTAINING ADRENALIN ON THE HEALING OF CRYOSURGICAL WOUNDS"

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PRECIS

Control of tissue temperature by vascular homeostasis is a basic physiological principle and one that relates directly to the use of cryosurgery. This is because the biological events that occur during cryosurgery are temperature dependant changes resulting from the rapid removal of heat from the tissues.

Although the literature contains much information on general wound healing, there is comparatively little on the healing of cryosurgical wounds and even less on the effects of vascular ischaemia on the healing of such wounds.

This paucity of information prompted the present study which was initiated:-

1. To design an animal model suitable for a comparative study of cryosurgical wounds created with and without local ischaemia.

2. To carry out macroscopic and microscopic studies of the wounds created using this model.

3. To draw conclusions as to the effect of initial ischaemia on the healing of cryosurgery wounds.

The cryosurgical wounds studied, were created on the dorsal skin of female Sprague - Dawley rats, with local ischaemia produced by using 0.5 ml 2% lignocaine with 1/80,000 adrenalin prior to the cryosurgery.

The experimental wound was created after pretreatment with the local anaesthetic whereas the control wound was created after pre-treatment with 0.5 ml normal saline.

All wounds were examined after 15 minutes, 1 day, 2 days, 4 days, 6 days, 11 days, 14 days, 18 days, 21 days and 25 days.

At the time of cryosurgery and prior to sacrifice, examination and assessment included comparisons of ice ball temperature, size and thaw time, together with gross wound area. The degree of inflammation and histopathology, were assessed after sacrifice.

From the outset the experimental wounds were larger and more severe than the controls as demonstrated by gross area and histologic assessment. The experimental wounds also showed a more severe acute inflammatory reaction and a more prolonged chronic reaction than the controls.

The initial ice ball diameter was not significantly different between the two groups.

Despite the difference in wound severity both wounds decreased in size at approximately the same rate although the control wound healed slightly faster.

It would, therefore, appear that injection of local anaesthetic containing a vasoconstrictor prior to cryosurgery produces more severe tissue destruction over a greater area.

However, this increased destruction does not unduly affect the healing rate and apart from more obvious muscle scarring the amount of resultant fibrous connective tissue formation is comparable.

DECLARATION

This thesis is submitted in partial fulfilment of the requirements for the Degree of Master of Dental Surgery in the University of Adelaide. Candidature for the degree was satisfied by obtaining the Honours Degree of Bachelor of Science in Dentistry in 1981.

This thesis contains no material which, except where due mention is made, has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

A.H. ANKER

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CHAPTER ONE

INTRODUCTION

Cryosurgery or surgery employing the physical properties of very low temperatures is a discipline of unknown age. For although many authors quote the analgesic and haemostatic properties of extreme cold as being first employed for limb amputation during the Franco-Prussian War, few agree as to its exact origins.

Gill et al., (1970) attribute the concept to James Arnott, who in 1851 used a salt ice mixture to treat breast neoplasms.

Jolly (1976) confers the honour on Campbell White (1899) a dermatologist, while Passey et al., (1971) regard it's modern use as beginning with a neurosurgeon, Cooper in 1963.

The principle of tissue destruction by cryosurgery is the rapid removal of heat from the tissues. The replacement of this heat, is largely dependant on a continuous blood supply. Any decrease in this blood supply during cryosurgery, would suggest the possibility of enhanced tissue destruction.

Cryosurgery is currently a mode of treatment in the fields of vascular and pigmented naevae, granulomatous lesions, some leukoplakias, and many benign, malignant, vascular and neoplastic diseases. Bradley and Fisher (1975); Chapin and Burkes (1973); Cooper (1963); Holden (1972); Leopard (1975).

Any adjunctive treatment that enables a larger, more consistent area of tissue death would result in more effective cryosurgery.

Local ischaemia at the site of cryosurgery would seem a simple effective method of achieving this aim, but the occlusion of large local feeding vessels as proposed by Neel et al., (1971a), is not routinely possible.

A simpler method and one that the present study proposes to demonstrate as effective, is the use of local anaesthetic containing adrenalin to control local blood flow through the area to be frozen. This should lower tissue temperature, and enable greater tissue destruction with the advantage of local analgesia.

Although current biological literature abounds with detailed histologic and histochemical studies on general wound healing there is comparatively little available information on the healing of cryosurgical wounds. Few studies in the literature to date, have dealt with the effects of local ischaemia on the healing of cryosurgical wounds (Neel et al., 1971a,b, 1973; Myers and Donovan 1981; Passey et al., 1971).

The paucity of available information on the effects of local ischaemia on cryosurgical wounds prompted the present study, which aimed to demonstrate some of the qualitative and quantitative differences in healing of cryosurgical wounds created with and without local ischaemia.

To do this, experimental cryosurgical wounds were created using local ischaemia on the dorsal skin of white rats and were studied

macroscopically and microscopically to determine any healing differences between these and control cryosurgical wounds created without ischaemia.

For the purposes of this study, local ischaemia was produced using the vasoconstrictor adrenalin in a proprietary local anaesthetic solution, while control wounds were created with saline.

By using local anaesthetic solution, which is often used clinically to relieve the pain and discomfort of cryosurgery, it was possible to extrapolate the proposed experimental protocol to the clinical situation.

The ability of vasoconstrictors such as adrenalin to decrease the blood supply to a surgical site for even a short time, would suggest the possibility of enhanced tissue destruction if used in conjunction with cryosurgery.

To date there have been few reports on the use of local anaesthetic containing vasoconstrictor prior to cryosurgery, other than passing remarks on specific case reports - Chapin and Burkes (1973). Consequently, there is only scanty data available on the resultant tissue effects. Myers and Donovan (1981).

The importance of wound healing as an integral part of this study necessitated a brief review of the general features and stages of wound healing, together with reference to wound contraction as part of the healing process. This is done in Chapter Two.

Chapter Three is devoted to a brief review of the principles behind cryosurgical equipment and its biological effects, especially with respect to wound healing.

The types, structure, and systemic effects of local anaesthetics, together with a review of the most common vasoconstrictors found in them is covered in Chapter Four.

The experimental protocol designed to demonstrate comparative differences between the wounds created with and without local anaesthetic is explained in Chapter Five, with the results presented in Chapter Six.

The ability of local anaesthetic with adrenalin to produce both the necessary regional analgesia with the added possibility of greater tissue destruction is an inviting concept.

If proven so, this concept has far reaching implications for the clinician involved in the treatment of benign and malignant pathology.

CHAPTER 2

WOUND HEALING

Although would healing as a biological phenomenon is not restricted to stages, for convenience, its features will be discussed here under the classical headings of:-

1. GENERAL FEATURES

a) Initial hemorrhage and clot formation

b) Acute inflammation

c) Granulation

d) Epithelialisation

2. CONTRACTION

These stages will be illustrated diagramatically.

2.1 GENERAL FEATURES

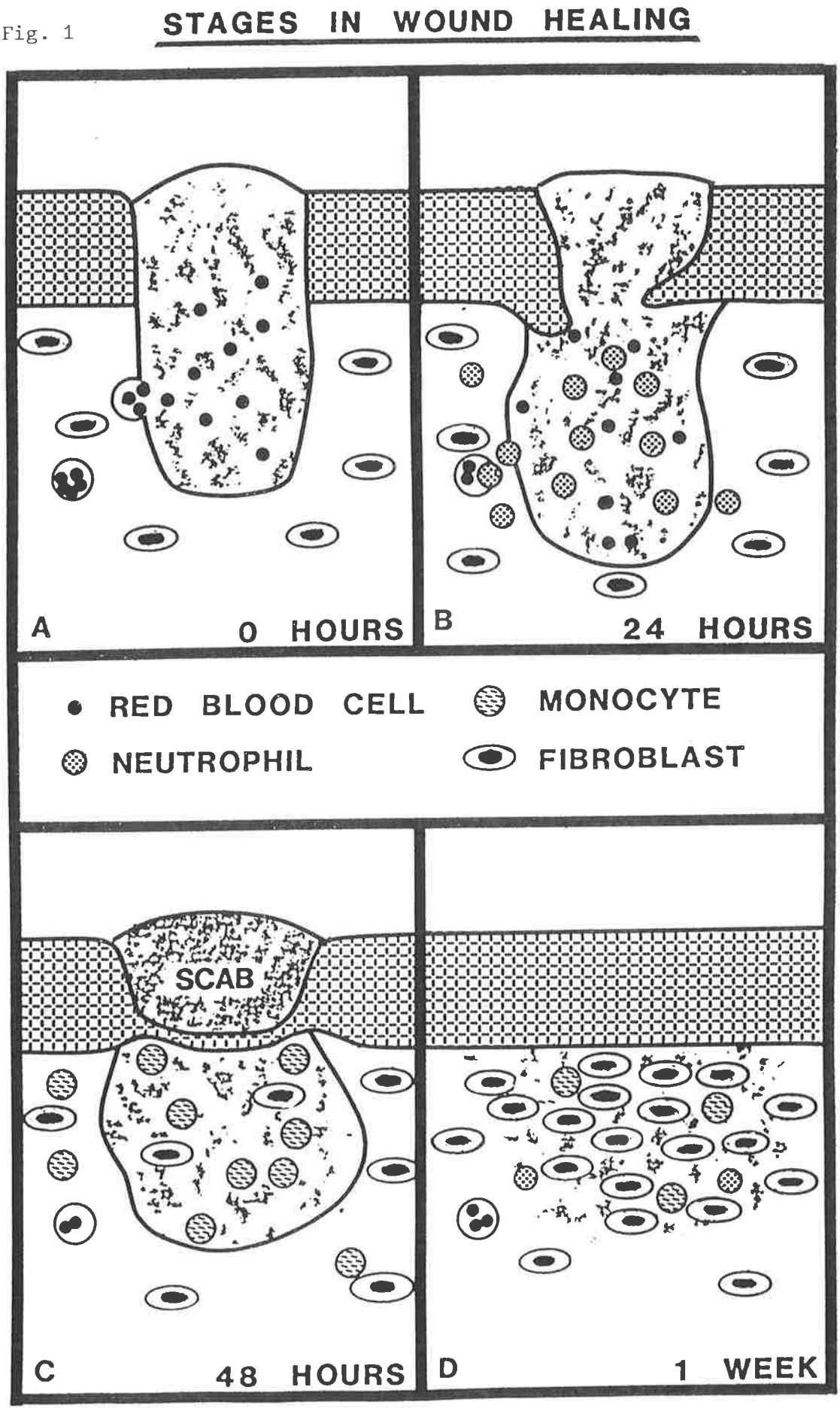
a) Initial Hemorrhage and Clot Formation.

A wound is initially bridged from edge to edge with a blood mass that clots - due in part to the normal clotting mechanism aided by functional characteristics of collagen, platelets and oxygen. Howes and Hoopes (1977); Wilner et al., (1968); Segrest and Cunningham (1970).

The initial clot dehydrates and a scab unites the wound edges. Ross (1969).

This stage is represented diagramatically in Fig 1A.

Fig. 1


STAGES IN WOUND HEALING
A
0 HOURS
B
24 HOURS
RED BLOOD CELL
MONOCYTE
NEUTROPHIL
FIBROBLAST
SCAB
C
48 HOURS
D
1 WEEK

b) Acute Inflammation

The vascular phase of the initial acute inflammatory response is characterised by changes in blood flow, vessel calibre and permeability Ryan & Majno (1977) and leads to the high specific gravity, high protein content oedema that follows as - seen in Fig 1B.

Polymorphonuclear leucocyte (P.M.L.), (Polymorph) emigration marks the onset of the later, cellular phase of acute inflammation that occurs about 6 hours after wounding and is pathognomonic of acute inflammation.

Mechanisms for this emigration that follows pavementing of polymorphs, range from insinuation between endothelial cells to endothelial damage as a result of pavementation. Hurley (1963); Stewart et al., (1974).

After emigration, polymorphs are guided to the injury site by chemotaxis though there is considerable debate over the mechanism involved. Zigmond and Hirsch (1973); Ward (1974).

The function of the guided polymorphs is the phagocytosis of foreign particles employing opsinisation and ingestion. Stossel (1974).

Within twelve hours of initial wound formation the polymorphs are joined by the longer lived, protein producing macrophages derived from blood monocytes. Ross (1968).

c) Granulation

During the granulation phase the connective tissue elements proliferate to form capillary loops and so mark the more vascular period of granulation tissue formation, the role of which is not fully understood. Hadfield (1963a, b); Meyers and Wolf (1974).

Fibroblasts are present very early in the wound area. Hadfield (1963a, b); Grillo (1964); Allgower and Hulliger (1960). From 24 hours onward they increase in number and size in readiness for collagen formation.

By the fifth day no appreciable increase in wound strength has occurred. This lag phase ends as the new fibroblasts reach maximum size and numbers, prior to producing the first fine collagen fibres that thereafter thicken and lengthen.

In the mucopolysaccharide matrix, with the onset of this fibroplastic phase, devascularisation proceeds and the granulation tissue takes on all the characteristics of young collagenous connective tissue.

d) Epithelialisation

The epithelial migration stage of wound healing begins within hours of injury. Devito (1965); Ross (1968, 1969).

Whether or not mitotic figures are present at this early stage is still debated. Bullough and Lawrence (1957, 1960); Viziam (1964).

The epithelial cells mobilise, proliferate and differentiate as they travel between the blood clot and granulation tissue ingesting protein and fibrin in their path and secreting a fibrinolytic enzyme. Ross (1968); (1969).

Mitotic response occurs one to two days after epithelial continuity is restored, Bullough and Lawrence (1957, 1960).

The epithelium then matures reproducing itself except for specialised structures like rete pegs and hair follicles. Figs 1C, 1D.

2.2 WOUND CONTRACTION

Wound contraction is a feature of wound healing that occurs concomitantly with the other phases. While often described separately as is here for convenience, it should not be regarded as a separate entity, but rather as a feature of the overall process.

Van Winkle (1967b) described contraction as "the diminution of the size of an open wound which is the result of the centripetal movement of the whole thickness of the surrounding skin".

The possible mechanisms involved in wound contraction have been the subject of much controversy.

Carrel (1910) was one of the first to realize the role of contraction in wound healing, since then many theories have been proposed on the possible mechanisms involved.

Watts et al., (1958); Grillo et al., (1958) proposed the picture-frame theory implicating the outer most granulation tissue of a wound, as the site of contraction.

Abercrombie et al., (1954) was the first to implicate wound fibroblasts in his 'Pull Theory' - that was later modified by Van Den Brenk (1956).

Zahir (1964) showed that wound contraction was due to changes to wound contents, possibly by evaporation rather than changes at the wound edges.

Gabbiani et al., (1972) proposed that fibroblasts in granulation tissue assumed ultrastructural, chemical, immunological and functional characteristics that made them responsible for wound contraction.

Later, the same authors proposed the principle of the myofibroblast. Montandon et al., (1973).

Montandon et al., (1977) having described the ultra structure of the myofibroblast, confirmed the multipotential role of myoblasts and fibroblasts from Ross' (1968) work, and claimed that the myofibroblast was the responsible element for wound contraction.

The theory of the myofibroblast being an element in the phenomenon of contraction is quite feasible on the current available evidence; however, it will undoubtably remain the subject of further study. Rosin et al., (1976).

HEALING BY PRIMARY AND SECONDARY INTENTION

In the past, a distinction has been made between these two types of healing, especially at a clinical level.

Healing by primary intention occurs when wound edges are approximated - such as in a surgically sutured wound.

Healing by secondary intention - or by granulation, refers to wounds that have widely separated edges. Healing must occur from the base of the wound through the granulation tissue.

The biological changes occurring during wound healing are constant - the difference between healing by primary intention or secondary intention is purely quantitative not qualitative.

CHAPTER 3

CRYOSURGERY

Experimental investigation has shown that cryosurgery or surgery using equipment capable of achieving extreme low temperatures, can give a reproducible area of cell death provided the temperature and duration of application are constant. Gill et al., (1968).

This controlled destruction of living tissue by utilising the mechanical application of extreme cold is now an established surgical technique, that has quite distinct advantages. Gill et al., (1970).

These advantages may be broadly discussed as advantages of:-

1. Tissue Destruction,
2. Manipulation,
3. Healing. Leopard (1975).

1. Tissue Destruction

The tissue destruction characteristic of cryosurgery can range from non-specific tissue selectivity as in the treatment of most localised lesions within the oral cavity, to highly specific selectivity as in the treatment of extrapyramidal lesions responsible for Parkinsonism. Leopard (1975).

2. Manipulation

This range of selectivity enables a variety of manipulative uses, from the treatment of very vascular lesions, without the fear of great

blood loss, to the extraction of ophthalmic lenses using the property of physical adhesion of the frozen tissue to the cryoprobe.

3. Healing

The healing of cryosurgical wounds is said to be characterised by absence of infection, decreased scarring and less wound contraction. Holden (1972). This latter feature may well prove to be of benefit in the future treatment of burn injuries.

A full understanding of these advantages necessitates explanation of the biological effects, equipment used, and the healing of cryosurgical wounds with special reference to the role that temperature plays in the production, extent, and healing of these wounds.

All these aspects are convened in the following chapter to enable a fuller understanding of the experimental procedure discussed in materials and methods.

3.1 BIOLOGICAL EFFECTS OF CRYOSURGERY

The biological aim of cryosurgery is to destroy cells either selectively or non-selectively by subjecting them to very low temperatures.

Cell death occurs by a combination of mechanisms broadly classified as being due to either direct or indirect effects.

a) Direct Effects

1. Intra-cellular and extra-cellular ice crystal formation Meryman (1957); Whittaker (1974a, b).
2. Cell dehydration and electrolyte disturbance. Leopard (1975)
3. Denaturation of lipo-protein complexes. Jolly (1976).
4. Inhibition of enzymes. Leopard (1975).

b) Indirect Effects

1. Vascular. Gill et al., (1970); Whittaker (1972)
2. Immunological. Neel et al., (1971a, b); Holden (1972)

DIRECT EFFECTS

1. INTRA-CELLULAR AND EXTRA-CELLULAR ICE-CRYSTAL FORMATION

Until the experiments of Whittaker (1974), the concept that ice crystal formation, either intra or extra-cellular, was responsible for cell death following cryosurgery, was only a theory and one based solely on the demonstration of ice crystals in cornea frozen cryosurgically. Whittakers experiments (1974), demonstrated both intra and extra-cellular ice crystal formation. Moreover, he showed that these crystals were commonly intra-cellular in the depths of the frozen area and extra-cellular at the periphery. The site of crystal formation is a product of rate of freezing. Faster freezing produces larger and intra cellular crystals.

Destruction is thought to be due to mechanical distraction of cell constituents - specifically in cell membranes, due to their different co-efficients of thermal contraction. Apart from this mechanical destruction, ice-crystal formation is directly relevant to cell dehydration and electrolyte disturbance.

2. CELL DEHYDRATION AND ELECTROLYTE DISTURBANCE

Because of extra-cellular ice-crystal formation, due to the slower freezing rate at the periphery, the cell membrane acts as a barrier to crystal formation. Ionic concentrations of extra-cellular electrolytes, increase in the crystal, - causing an outflow of water from within the cells, to correct the imbalance. The cell becomes dehydrated and shrinks. This results in an increased concentration of electrolytes, which reach toxic levels, causing irreversible cell damage. Leopard (1975).

3. DENATURATION OF LIPO-PROTEIN COMPLEXES

In many ways this is another consequence of competition between the cell membrane and the growing ice crystals for extra-cellular water. Many cellular macromolecules maintain their spatial relationship only in the presence of water. Loss of this water allows abnormal contact between them with resultant chemical and physical changes. (Jolly 1976).

4. INHIBITION OF ENZYMES

As each cellular enzyme system acts optimally over a narrow temperature range, sudden cooling, upsets the equilibrium constant of the system causing enzyme inhibition. Leopard (1975).

Freezing and thawing has also been shown to reduce the activity of certain enzymes. Jolly (1976).

b) INDIRECT EFFECTS

1. VASCULAR EFFECTS

On freezing, intra-cellular damage can be demonstrated within a minute of thawing. Whittaker (1972); However, despite the complete vascular stasis evident during freezing, normal blood flow returns within 10 minutes of thawing. Because of this, it would seem that vascular stasis is not the primary cause of cell death and assumes the more secondary role of causing death by delayed changes summarised by Gill et al., (1970) as 'ischaemic infarction'. These effects are evident as stasis, microthrombus formation and ischaemic necrosis.

2. IMMUNOLOGICAL EFFECTS.

The concept that cryonecrosis is accompanied by an immunologic response is based on the observation that cryosurgery is followed by a greater degree of tissue destruction than would be expected from the effects of cold alone.

Holden (1972), has demonstrated a non-specific increase in lymphocyte activity following cryosurgery. Other experiments, have shown an increase in tissue destruction when the afferent blood supply was compromised, that far exceeded the expected result. Neel et al., (1971a, b).

Whether the postulated mechanism of antigenicity is due to release of antigen, or molecular change due to freezing that induces antigenicity, is not yet known.

3.2 CRYOSURGICAL EQUIPMENT

Cryosurgical apparatus is designed to conduct heat rapidly away from the tissues. The available apparatus can be classified into 'Open' and 'Closed' systems of delivery.

(i) Open Systems

These involve direct application of the cold source to the tissues and include carbon dioxide "snow", used in the form of moulded "dry ice", or as a 'slush' where acetone is added; Both forms can achieve temperature differentials of around $-80^{o}C$. Jolly (1976).

Other open systems include the use of liquid Nitrogen allowing a temperature drop of $-196^{o}C$ or liquid Freon sustaining temperatures of $-65^{o}C$. Hill (1968). With such systems the temperature drop is profound, as the latent heat of vaporisation is extracted from the tissues.

(ii) Closed Systems

Closed systems are more indirect, conducting the heat away with the use of an instrument. While these systems offer a greater degree of control, it is at the expense of more complex equipment and often, less profound temperatures. There are 3 main types of closed system:

(a) Thermo-Electric

This system is cumbersome and inefficient. Operating by the Peltier effect, the apparatus uses direct current electrics and water cooling.

(b) Evaporative

Very similar to an open system this involves the use of controlled evaporation of liquid gas either Nitrogen or Freon. It can produce a profound drop in temperture of approximately ($-190^{o}C$).

(c) Joule-Thomsom Apparatus

In this system the rapid expansion of a compressed gas - usually nitrous oxide through a small orifice produces a rapid drop in temperature of approximately (-20 to $-40^{o}C$) as heat is extracted from the environment. Jolly (1976); Poswillo (1971).

3.3 TEMPERATURE AND CRYOSURGERY

After a cryoprobe has been applied to the tissues for 1-2 minutes the iceball formed reaches an equilibrium situation. Because there is a temperature differential between the edge of the iceball and its centre, the temperature of the cells affected, depends on their position in the iceball.

Although there is no specific critical temperature that is invariably lethal to animal cells it has been shown that effective

cryosurgery depends on the rapid removal of heat from the tissues rather than the accomplishment of very low temperatures. Jolly (1976); Poswillo (1971).

Farrant (1972) has shown the effect of different cooling rates on the survival of three different cell types (red blood cells, hamster cells, marrow cells) following thawing from -196^{o}C.

Farrant's (1972) results show several important features:

1. The absolute survival or damage is different for different cell types.

2. Each cell type is greatly affected by rate of cooling.

3. There is a cooling rate that gives optimal survival or damage as the case maybe.

Farrant (1972) found that the slower the rate of thawing, the greater the cryo-destruction. The optimal temperature range for cryo-destruction remains a subject of debate. Lovelock (1954) has noted that some cells can be destroyed at temperatures above freezing, yet he advocates a critical temperature range of (-3^{o}C to -40^{o}C).

Poswillo (1971) gives a figure of -20^{o}C for necrosis of "virtually all biological tissues even though a few individual cells might survive procedures that are lethal to their neighbours". Poswillo (1973).

It would appear that because of the individuality of biological tissues no specific temperature can be stated.

Some authors, Gill et al., (1970) state that this problem may be overcome by multiple freezes because no cell can survive "ischaemic infarction", regardless of its sensitivity to cold.

The concept of repetitive freezing was examined by Gill et al., (1968) who conducted repeated freeze-thaw experiments on rat livers. They found in principle, that for any cryo-probe temperature, a series of maximum freezing effects was possible:- those obtained by single application and those from repeated cycles.

Macroscopically the tissue changes resulting from repetitive freezing were more intense but basically similar to a single freeze; and the histologic features were almost identical between the two. Gill et al., (1968).

3.4 HEALING OF CRYOSURGICAL WOUNDS

3.4.1 Gross Features

As the temperature of the cryoprobe decreases, an iceball forms within the area being frozen. This iceball gradualy thaws after removal of the cryo-probe. In cases of repetitive freezing the size of the iceball may increase until an ultimate maximum diameter is reached.

A diagramatic representation of the iceball and the gross form of the resultant lesion can be seen in Fig 2. Whittaker (1972), speaks of two main zones of the cryo-lesion. A central part of the lesion and a peripheral zone separating this from the surrounding normal tissue.

Integral to the production and subsequent healing of cryosurgical wounds, is the effect of cryosurgery on the surrounding blood vessels.

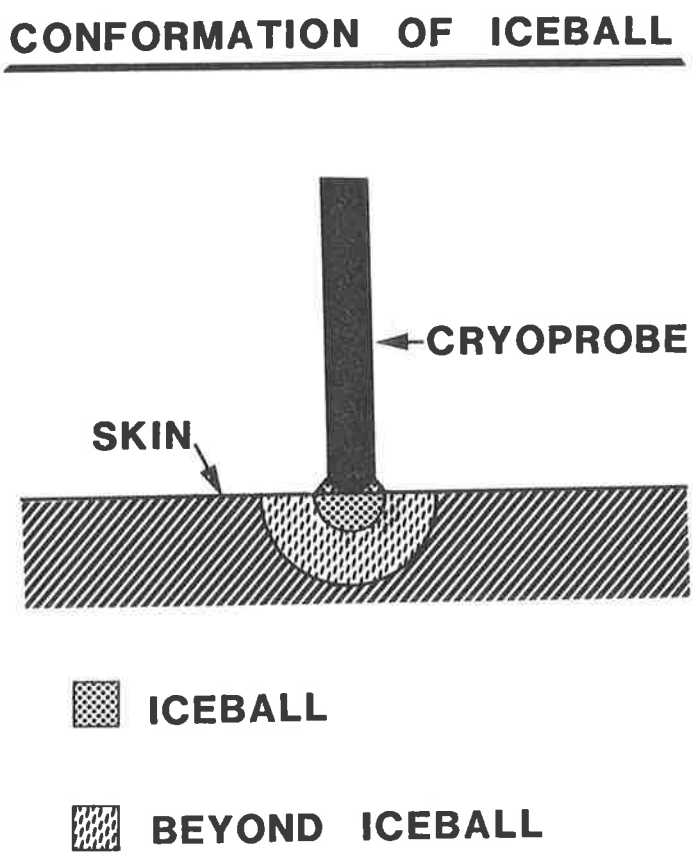
Whittaker (1972) has shown with vascular permeability studies, that following cryosurgery to the oral mucosa - normal blood flow is restored within a few minutes; however, this is later followed by a delayed type increase in permeability and an increasingly severe stasis that he regards as a secondary cause of cell damage.

Neel et al., (1971a,b, 1973) in their studies on cryosurgery with and without induced ischaemia, found that the cryosurgical iceball produced without ischaemia, closely approximated the histological size of the lesion, whereas the histological size of lesions produced with ischaemia exceeded the iceball size.

Cryosurgical wounds once created, heal by secondary intention, the frozen area undergoes necrosis and sloughs off. Marciani and Trodahl (1975). They then heal in three stages, 1. Ulceration, 2. Granulation tissue proliferation, 3. Cicatrix formation. Passey et al., (1971).

The macroscopic features of the wound vary with the vascularity of the tissue being frozen. Very vascular tissues such as spleen or liver, after thawing, produce the dark red appearance of an hemorrhagic infarct, while less vascular tissues such as skin appear pink. Gill et al., (1970).

Fig 2. A diagramatic representation of the iceball and the gross form of the resultant lesion.



Immediately after freezing the area is swollen, and pale, but petechiae, mild hyperaemia and oedema, appear within an half hour of thawing. Neel et al., (1973).

By 12-24 hours swelling and discolouration begin and by 3 days necrosis and ulceration spread to involve all of the frozen area. Poswillo (1973). Epithelial wounds become covered by a surface scab of fibrin debris and tissue detritus - Passey et al., (1971).

The oedema and ulceration lasts for about 4 weeks when initial clinical healing becomes evident in the form of tissue granulation and epithelialisation. Passey et al., (1971).

The granulation tissue matures and by approximately one month, a small fibrous scar has formed. Fraser and Gill (1967).

3.4.2 Microscopic Features

The histological features after cryosurgery are evident almost immediately and become more marked over 2 hours. The two zones spoken of by Whittaker (1972) are reflected microscopically. Specifically the central area of the lesion contains cells displaying pyknotic nuclei, vacuolated cytoplasm, and a disrupted reticulin network of the surrounding connective tissue. Gill et al., (1970); Fraser and Gill (1967). This area corresponds to the area of intra-cellular ice crystal formation which probably constitutes the main cause of death. Whittaker (1974a, b).

Surrounding this area is a band of cells of variable depth, showing eosinophilic staining characteristics, basophilic nuclei, and coarse cytoplasmic granularity. Gill et al., (1970); Fraser and Gill (1967). This area is the boundary zone where extra-cellular ice crystals have produced gross cell distortion without evident penetration of cell membranes.

During the first 24 hours epithelial necrosis occurs and dermal fibroblasts take on a pyknotic appearance, as they lie within a desmo plastic, polychromatic, fibro-connective tissue matrix. Passey et al., (1971). A cellular infiltrate of polymorphs with a few plasma cells is evident near the margins of the wound, together with vascular congestion, thrombus formation, changes in vessel walls and areas of eosinophilic necrosis. Neel et al., (1973). During this period capillaries become dilated and congested, and arterioles and venules lose the muscle layer of their walls as well as their endothelial lining. Buch et al., (1979); Li et al., (1980).

After 3-4 days necrosis and ulceration is evident, and the connective tissue lying below the fibrinous exudate is markedly oedematous with polymorphs, lymphocytes, plasma cells and areas of hemorrhage. Poswillo (1971); Hurt et al., (1972). A feature of unknown significance noted in superficial cryo wounds of the oral cavity is the presence of multinucleated epithelial cells. Hurt et al., (1972).

One week after freezing there is partial resurfacing of the defect which appears as a central epithelial ulcer containing neutrophils, fibrin debris and nuclear detritus. Passey et al., (1971). The new epithelium appears as an acanthotic, parakeratotic, spongiotic

stratified squamous epithelium - depending on the nature of the previous tissue. Passey et al., (1971).

By the second week the epithelium which by this stage is not as acanthotic nor spongiotic, is generally intact and the slightly hyalinised underlying connective tissue stroma, features capillary sprouts, lymphocytes, histiocytes and plasma cells.

This underlying bed of connective tissue gradually matures to form scar tissue. The duration of healing and the amount of scar tissue formed depends on the area frozen and the extent of freezing. Marciani and Trodahl (1975).

These features vary in sequence and timing from tissue to tissue. Natiella et al., (1979) one week after cryosurgery to the submandibular glands of rhesus monkeys found marked interstitial hemorrhage, and ghosting of cell outlines. He further commented on squamoid and hyperchromatic changes to the epithelial cells, small vessel thrombosis and an infiltrate of polymorphonuclear leucocytes. Tissue effects of the cryosurgery were still evident four months later. As capsular thickening, vessel thrombosis, and connective tissue scarring.

Mayers et al., (1971) in superficial wounds to human gingiva found little histologic evidence of injury after only 48 hours. Other authors have found healing times that range from between 4-6 weeks in other tissues. Gill et al., (1970); Passey et al., (1971); Poswillo (1973); Fraser and Gill (1967).

The response of bone, to cryosurgery, for example, is said to have three principle phases according to Bradley and Fisher (1975). These are the necrotic, osteogenic and remodelling phases.

After cryosurgery there is almost immediate loss of osteocytes, nutrient vessels and endosteal soft tissue. It take several weeks before subperiosteal and endosteal bone formation takes place, together with an ingrowth of fibrous connective tissue into marrow spaces. The completion of healing is the replacement of non-vital lamellar bone with vital lamellar bone.

Thermal wounds show similar features of inflammation, granulation tissue formation; and epithelialisation to cryosurgical wounds, however, the freeze injury does not show the dramatic contraction characteristics demonstrated in other wounds. Li et al., (1980).

The reason postulated for this is the apparent retention of the connective tissue matrix in cryogenic wounds. Studies by Li et al., (1980) have shown that the decrease in hydroxyproline content in wound areas after cryosurgery is much less than in comparable burn wounds.

This would seem to indicate a lesser need for connective tissue replacement during healing, and so account for the decrease in contraction, by cryogenic effect on the myofibroblasts.

3.4.3 Ultrastructural Features

The changes within the nucleus seen through the election microscope include loss of cell fluid, irregular elevation of the nuclear membrane

and the presence of heavily stained heterochromatin.

Within the cytoplasm the alpha-cyto membranes of the endoplasmic reticulum dilate, there is separation of ribosomes and the mitochondria show pleomorphism with loss of their staining characteristics, and the integrity of their cristae.

All these features are consistent with the ultrastructural changes seen in a spectrum of degenerative changes. Gill et al., (1968).

CHAPTER 4

4.1 LOCAL ANAESTHETICS AND VASOCONSTRICTORS

Local anaesthesia, (Regional anaesthesia/analgesia) is widely used by the medical and dental professions. Local anaesthetic offers regional anaesthesia with ease of administration, minimum physiological side effects and can be used for many outpatient surgical procedures.

The majority of clinical cryosurgery is carried out as an outpatient procedure often in combination with a local anaesthetic.

The use of local anaesthetic in these situations is, more often, primarily for pain control.

In the present study local aneasthetic was used as a convenient vehicle for a chemical vasoconstrictor - Adrenalin. Such an experimental model provided both the necessary vasoconstriction and a directly relevant use, to that in clinical cryosurgery.

Because local anaesthetics and vasoconstrictors of themselves produce a number of local and systemic effects a review of these effects, and in particular, the real or potential effects on wound healing, are reviewed.

4.1.1 LOCAL ANAESTHETICS

A local anaesthetic substance is a drug that can produce the temporary loss of all sensation modalities to a limited region of the body, without loss of consciousness - Roberts and Sowray (1970).

A local anaesthetic can, therefore, halt the neural traffic along a nerve axon in a predictable and reversible manner, leaving the nerve none-the-worse for its brief period of rest. De Jong (1977).

4.1.2 STRUCTURE AND TYPES OF LOCAL ANAESTHETICS

Most local anaesthetics are tertiary amines with an intermediate ester or amide (anilide) linkage separating the hydrophilic hydrocarbon tail and the lipophilic aromatic head. Fig. 3.

The alkaline anaesthetic amine is lipid soluble, but water insoluble and unstable. Salts of the anaesthetic base are water soluble and stable, but lipid insoluble.

For this reason the local anaesthetic consists of an alkaline analgesic radical combined with a strong acid radical to form a water soluble salt.

In aqueous solution the salt ionises to form a cation (in the form of a positively charged quarternary amine) and an anion, that is:

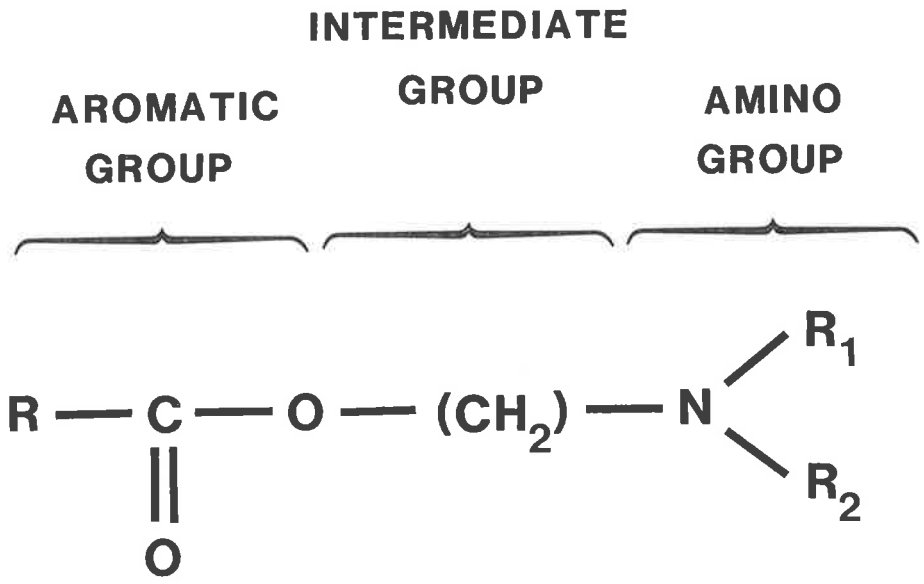
$$X = NHCl \rightleftharpoons X = NH^{+} + Cl^{-}$$

In the tissues, the salt is hydrolysed (by alkaline tissue fluid) to form the basic radical, that is:

$$X = NH^{+} + Cl^{-} + NaOH \rightleftharpoons X = N + NaCl + H_2O$$

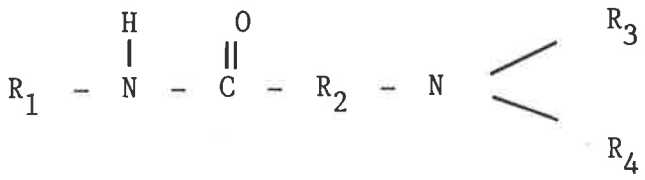
where X = N is the active alkaline portion of the local anaesthetic.

Figure 3 General formula for a local anaesthetic

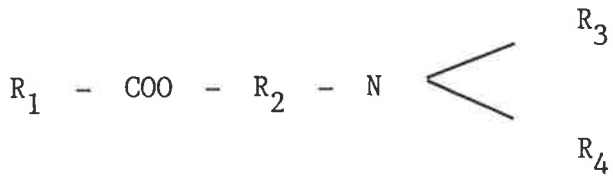


The equilibrium constant of the reaction is governed by ph and the PKa (dissociation constant) of the drug.

The basic formula for the amide type local anaesthetics can be represented by:



whereas the ester-type local anaesthetics can be represented by



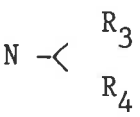
thus, both satisfy the basic formula for a local anaesthetic.

Aromatic Portion - Intermediate Chain - Amine Portion

where R_1 = Aromatic Portion - Lipophilic properties

$NHCO-R_2$ = Intermediate Chain - Amide

$COO-R_2$ = Intermediate Chain - Ester

 = Amine Group - hydrophilic properties

The aromatic - intermedite chain linkage affects the metabolism of the drug; being metabolism by either Liver Amidases or Pseudocholinesterase. Garfield (1976).

4.1.3 SYSTEMIC EFFECTS

Local anaesthetics exert a generalised action on the excitable tissues of the body but specifically the cardiovascular (C.V.S) and central nervous systems (C.N.S.).

a) Cardiovascular System

At non toxic levels local anaesthetics cause:

(i) Abolition of the slow phase of depolarisation in Purkinje fibres during diastole. Covino (1976)

(ii) A decrease in the Action Potential. Covino (1976)

(iii) A decrease in the duration of the refractory period. Davis and Tempe (1969); Bigger and Mandel (1970).

Although these changes are minimal, as toxic levels are approached, there is vasodilatation and a decrease in myocardial contractility, that results in systemic hypotension. At truly toxic levels there is depressed cardiac conduction, with the possibility of atrio-ventricular dissociation and ultimately, cardiac arrest. Davis and Tempe (1969); Bigger and Mandel (1970).

b) Central Nervous System

At non-toxic levels there are little or no C.N.S. effects. However, as toxic levels are approached there is a generalised excitatory phase

with tremours, shivering and often convulsions. At toxic levels generalised C.N.S. depression causes respiratory depression and finally respiratory arrest.

The reasons for the initial excitatory phase are largely unknown. A recent theory proposes that local anaesthetics initially produce a selective blockade of inhibitory cortical synapses thus these facilitating neurones can function unopposed, producing excitation and finally convulsions. Tanaka and Yamasaki (1966); De Jong (1969).

4.2 LIGNOCAINE

a) Chemistry

Lignocaine is a moderate duration, non-ester, amide type local anaesthetic and its formula is shown in Fig. 4.

It is a white crystalline powder with a melting point of $60^{o}C$ and is used as the hydrochloride salt. Covino (1976).

As a local analgesic it was first synthesised by Nils Lofgren in 1943, then tested by Bengt Lunquist.

b) Absorbtion and Distribution

Lignocaine readily diffuses through the tissues and the nerve sheath. A 5% solution in ointment is used for topical analgesia with or without the addition of enzymes such as hyaluronidase. Lignocaine is

not, however, easily absorbed by skin surfaces. Sowray and Roberts (1970).

Being a non-ester local anaesthetic, lignocaine is only slightly hydrolysed in the plasma before being shunted to other tissues.

Radio-isotope distribution studies by Keenaghan and Boyes (1972) have shown that lignocaine is rapidly absorbed by the intravenous or oral routes - more slowly by the intra-muscular route - then distributed to stomach intestine, brain, liver, spleen, heart, lung, kidney, and throughout the body muscle.

Maximum tissue distribution occurs within 30 minutes due mainly to the metabolites of lignocaine.

After absorbtion the lignocaine is rapidly distributed to those tissues which activate fast equilibration. Following this there is redistribution to other tissues with slow equilibration (Fig. 5, after Scott et al., (1972).

c) Metabolism

Amide local anaesthetics are generally degraded in the liver with up to 70% being removed on first circulation through this organ.

The structures of lignocaine and its metabolites identified in the urine of cats, guinea pigs, dogs and man after administration is shown here in Fig. 6 Keenaghan and Boyes (1972). The arrows indicate the most likely pathways in view of current thoughts.

Figure 4

LIGNOCAINE STRUCTURE

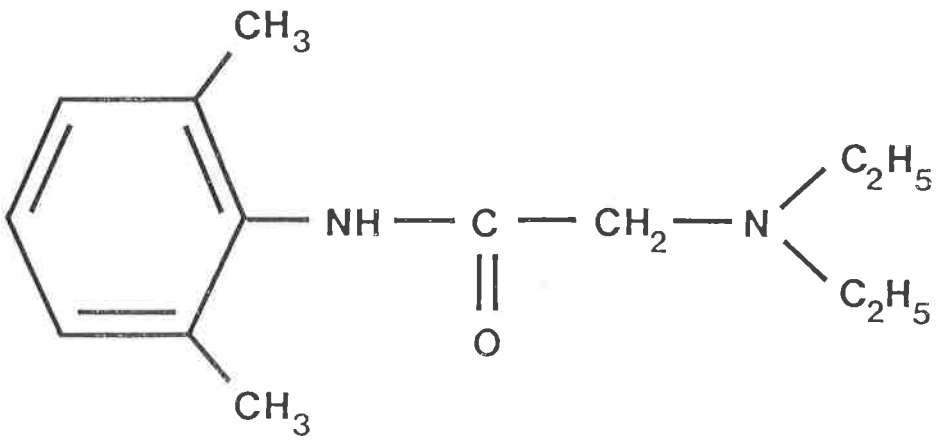


Figure 5

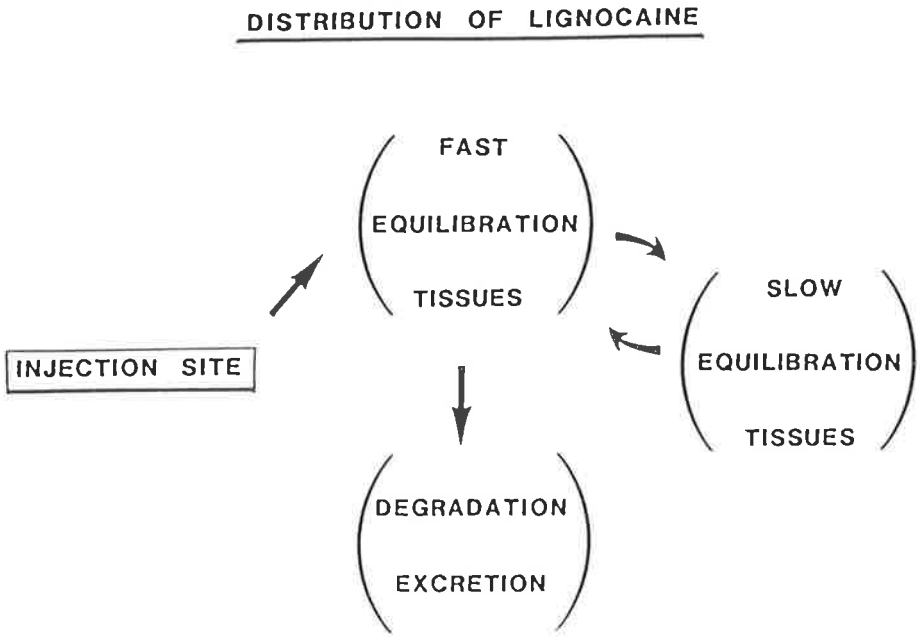
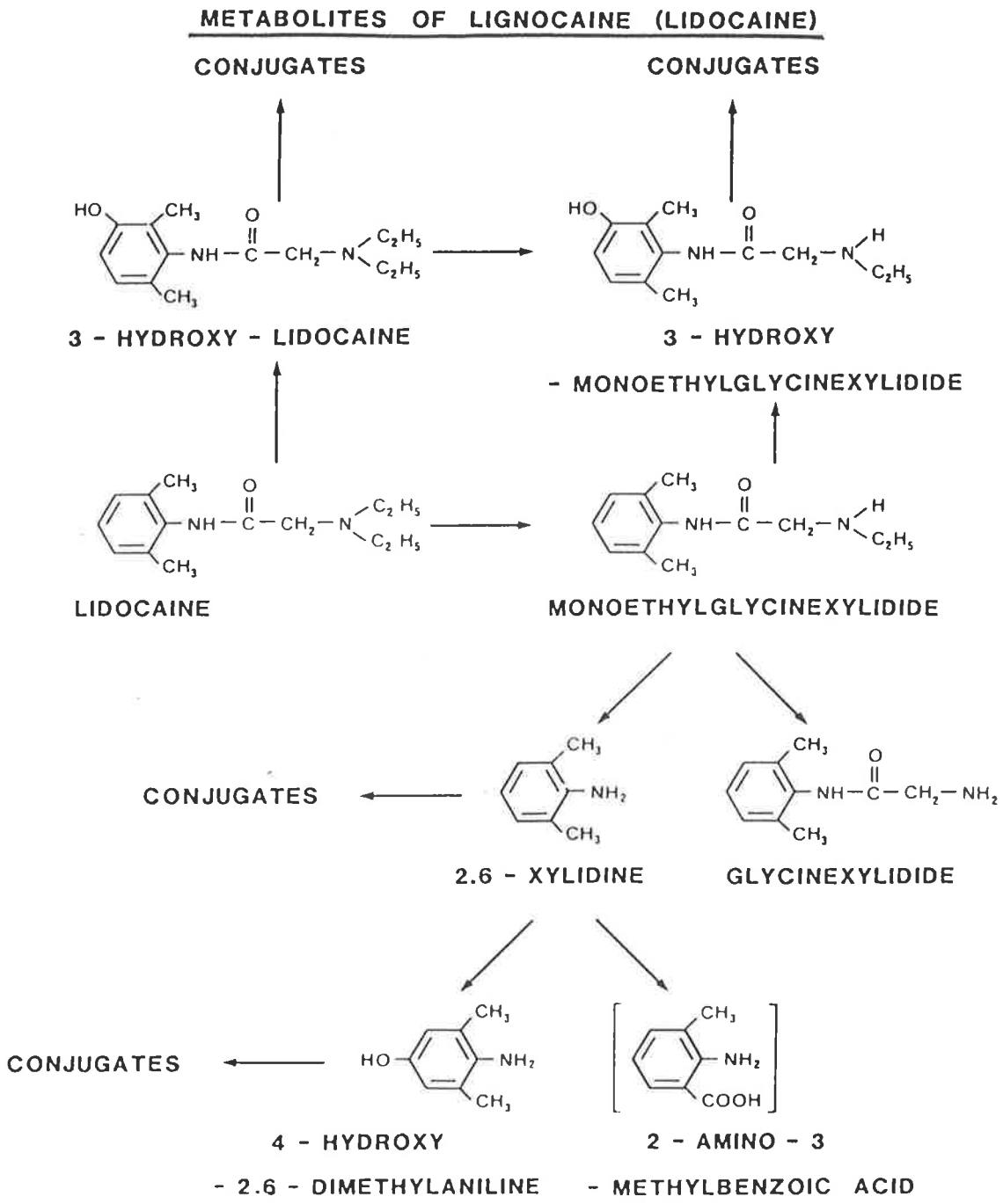

DISTRIBUTION OF LIGNOCAINE
FAST
EQUILIBRATION
TISSUES
SLOW
EQUILIBRATION
TISSUES
INJECTION SITE
DEGRADATION
EXCRETION

Figure 6


METABOLITES OF LIGNOCAINE (LIDOCAINE)
CONJUGATES
CONJUGATES
3 - HYDROXY - LIDOCAINE
3 - HYDROXY
- MONOETHYLGLYCINEXYLIDIDE
LIDOCAINE
MONOETHYLGLYCINEXYLIDIDE
CONJUGATES
2.6 - XYLIDINE
GLYCINEXYLIDIDE
CONJUGATES
4 - HYDROXY
- 2.6 - DIMETHYLANILINE
2 - AMINO - 3
- METHYLBENZOIC ACID

c) Excretion

Lignocaine being an amide exhibits both a urinary and biliary excretion pathway.

Radio-isotope studies indicate that although there are minor species differences, the biliary metabolites of lignocaine are mostly excreted in the urine. This indicates that the lignocaine metabolites excreted in the bile, must be reabsorbed from the gastrointestinal tract; necessitating a very rapid initial metabolism in the liver. Keenaghan and Boyes (1972).

4.3 VASOCONSTRICTORS IN LOCAL ANAESTHETICS

A vasoconstrictor is any substance that causes contraction of the smooth muscle within blood vessels, thereby, decreasing the diameter of the blood vessel and reducing the blood flow through the vessel.

The vasoconstrictors reviewed in this study will be those commonly found in local anaesthetics - such as adrenalin, noradrenalin and octapressin (PLV-2). The most commonly used of these is adrenalin although octapressin with its wide margin of safety is becoming increasingly popular. Less commonly used vasoconstrictors such as phenylephrine and nordephrine will not be discussed.

The addition of a vasoconstrictor to a local anaesthetic decreases the circulation at the site of administration. This increases the

efficiency and duration of anaesthesia as well as its rate of metabolism Klingenstrom and Westermark (1963) Klingenstrom et al., (1967).

4.3.1 TYPES OF VASOCONSTRICTOR

The commonly used vasoconstrictors can be grouped as:

(i) Adrenal medullary derivatives

- Adrenalin
- Noradrenalin

(ii) Posterior Pituitary Hormones

- Felypressin (Octapressin/PLV-2)

(i) Adrenalin

An active principle of the adrenal medulla this vasoconstrictor is found in natural and synthetic forms, is stable in acid solution and is used in local anaesthetic in concentrations ranging from 1/50,000 - 1/300,000. Roberts and Sowray (1970).

Adrenalin acts on the effector cells of the autonomic nervous system - the alpha and beta receptors.

Stimulation of the alpha receptors causes excitatory effects, producing peripheral vasoconstriction and pupil dilation, while stimulation of the beta receptors causes inhibitory effects, producing vasodilatation of skeletal muscle vessels and broncho-dilation.

The nett effect on total peripheral resistance depends on which of the two systems predominates. Foldes et al., (1965).

(ii) Noradrenalin

Noradrenalin is produced in the adrenal medulla and is released by stimulation of post ganglionic adrenergic nerve fibres.

As a vasoconstrictor it is almost entirely alpha receptor stimulating, and its excitatory effects, therefore, cause central and peripheral vasoconstriction.

(iii) Octapressin

Octapressin is a synthetic posterior pituitary hormone that resembles vasopressin. It's formula is:

2 - Phenylalanine - 8 Lysine - Vasopressin (P.L.V.)

and while it's 'pressor' effects are less than those of adrenalin, they are of longer duration. Roberts and Sowray (1970).

As a vasoconstrictor it is noted for its low local and general toxicity, less pronounced effect on the circulatory system, metabolism, and its local ischaemic effects. Klingenstrom et al., (1967).

4.3.2 SYSTEMIC EFFECTS OF VASOCONSTRICTORS

(a) Adrenalin and Noradrenalin. Ganong (1971); Roberts and Sowray (1970)

Adrenalin has two principle effects:

1. It causes an increase in heart rate and contractility which causes an increase in cardiac output with a subsequent rise in systolic blood pressure.

2. Adrenalin also causes vasodilatation in skeletal muscle resulting in a decrease in peripheral resistance and consequent drop in diastolic blood pressure.

Noradrenalin on the other hand has no effect on cardiac output, with its major effect being to: cause central and peripheral vasoconstriction, resulting in a rise in both systolic and diastolic blood pressure - and so a resultant drop in heart rate.

(b) Octapressin

The cardiovascular effects of octapressin are generally considered to be minimal; Katz (1965). However, in some studies under similar operative conditions - octapressin caused a fall in heart rate on initial administration and a rise in blood pressure that were considered independant of the operative conditions. Shanks (1963).

4.4 EFFECT OF LOCAL ANAESTHETICS AND VASOCONSTRICTORS ON WOUND HEALING

The introduction of what we refer to as modern methods of local anaesthesia has been attributed Karl Koller in 1884 who used topical cocaine in the field of Ophthalmology.

Since that time due to the untoward effects of local anaesthetics and their vasoconstrictor constituents they have been the subject of a number of studies reviewing both their local and systemic toxicity. Davis and Tempe (1969); Bigger and Mandel (1970); Tanaka and Yamasaki (1966); De Jong (1969).

The very fact that local anaesthetics have an effect on normal tissue would seem to presuppose an effect on wound healing.

Meecker (1925), showed that serious tissue changes including necrosis and pus formation could follow the administration of a local anaesthetic, especially one containing a vasoconstrictor

Topical local anaesthetics have been shown to inhibit mitosis in rat corneal tissue. Smelser and Ozaniks (1945).

Later literature abounds with the systemic toxic effects of local anaesthetics. Covino (1971); Fink (1973). Corssen (1973) regards local anaesthetics as protoplasmic poisons.

In other experiments by Benoit and Belt (1970) and Benoit (1978b), substantial microscopic damage followed by micro-scarring was found in rat thigh muscle exposed to 2% lignocaine plus 1/50,000 adrenalin; the

scarring was not present in $^{1}/50,000$ adrenalin controls. A feature contradicted by the experiments of Myers and Rightor (1974) who found muscle scarring after using adrenalin on wounds.

Bodvall and Rais (1962) showed that the injection of local anaesthetic into healing wounds led to a decrease in the rate of healing by primary intention, wound rupture and that these features were more marked when a vasoconstrictor was used. Covino (1976) states that the local tissue toxicity of regional anaesthetics is limited to skeletal muscle and is spontaneously reversible.

Gothman (1962) found an increase in fracture strength in those fractures treated with local anaesthetic. This was in contradistinction to Flatmark (1967) who found that the treatment of fractures using local anaesthetic did nothing to promote fracture healing.

Reinsch and Meyers (1974) found that lignocaine alone had no influence on the viability of either delayed or non-delayed flaps. However, the addition of 1/200,000 adrenalin, while it did not affect non-delayed flaps it greatly reduced the survival of delayed flaps. However, Myers and Rightor (1974) found that wound strength was augmented by pre-treatment with adrenalin. Morris and Tracey (1977) have shown that lignocaine does of itself retard healing and that adrenalin merely potentiates this effect.

Many mechanisms have been proposed to support the apparent retardation of healing caused by local anaesthetics.

Giddon and Lindhe (1972) showed in vivo suppression of leucocyte adherence to vessel walls by local anaesthetic. Others have shown in vitro inhibition of phagocytosis and leucocyte metabolism by local anaesthetic. Cullen and Haschke (1974).

Schmidt and Rosenkranz (1970) suggest that local anaesthetics actually promote healing by antimicrobial activity, a proposal that found many antagonists. Chvapil et al., (1979); Eichorn and Peterkofsky (1979).

The role of vasoconstrictors alone has been examined by Klingenstrom et al., (1967) who found that octapressin produced little or not cyanosis as opposed to adrenalin. It's haemostatic ability was equal to adrenalin and more-over that skin flaps raised using octapressin rather than adrenalin had a better survival rate.

Thus, review of the literature is confusing and leaves the impression that local anaesthetic may or may not upset the healing process depending on location, volume, concentration and the presence or absence of a vasoconstrictor.

CHAPTER 5

MATERIALS AND METHOD

INTRODUCTION

The purpose of this series of experiments was:

1. To develop an animal model with which to study the comparative differences in the healing of experimental and control cryosurgical wounds.

2. To attempt to quantitate the differences in healing of cryosurgical wounds created with and without local anaesthetic containing adrenalin.

Cryosurgical wounds were created on the dorsal skin of white rats following pre-treatment with local anaesthetic containing adrenalin (L/A) or control wounds pretreated with normal saline (SAL).

The wounds so produced were assessed at specific time intervals both macroscopically and microscopically.

Preliminary anatomical and histologic studies were performed on unoperated animals to determine the morphologic features characteristic of this dorsal tissue skin.

5.1 ANIMAL MODEL

A total of fifty pathogen free Sprague-Dawley rats were used in this study.

These animals were housed four to a cage in a standardised, temperature controlled environment, and fed a diet of balanced rat pellets and water ad libitum (see appendix)

All animals were weighed prior to operation and sacrifice and checked for signs of ill health.

5.2 MATERIALS

5.2.1 CRYOGENIC APPARATUS

The cryosurgical wound was created using a Spembly Cryosurgical Unit fed by a CS size nitrous oxide cylinder capable of a pressure maximum of 700 lb/in^2 (Fig. 7)

Serial needle thermocouple readings were made at 2 points equidistant and 0.5 cm from the central location of the cryoprobe to ensure a uniformity of temperature differential. This was done using rapid response Copper Constantan thermocouples linked in-line with a Comark electronic thermometer type 1624. (Fig 8). Cylinder pressure markedly affects probe tip temperature. Therefore, subsequently no cylinder with a pressure reading of less than 600 lb/in^2 was used in the experiment.

5.2.2 LOCAL ANAESTHETIC AGENT

The local anaesthetic used in the experiments was 2% lignocaine with a concentration of 1/80,000 adrenalin.* This was drawn up from an ampule into a sterile luer-lok syringe and delivered via a sterile 25 guage needle. 0.5 ml of the local anaesthetic was administered to each wound site.

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* ASTRA PHARMACEUTICAL CO.

5.3 ANAESTHESIA

All animals were anaesthetised in a standardised and reproducible manner. Gaseous induction was carried out using an animal anaesthetic machine delivering a nitrous oxide, oxygen, halothane mixture. The induction normally lasted 3 minutes. Fig 9. Following induction, anaesthesia was maintained for the duration of the operation using intra peritoneal injection of chloral hydrate mixed to a formula concentration of 1mg/ml and a dosage of 5mls/kg body weight. Animals displayed the first signs of recovery after one hour. After induction of anaesthesia the animals were placed in the supine position and all limbs stretched and fixed with elastic ties. This position enabled good access for preparing, draping and operation.

5.4 SURGICAL TECHNIQUE

All animals were coarse shaved using hair clippers and subsequently close shaved with a safety razor and bland soap. The skin was shaved from the region of the scapula to lower lumbar level and was then washed with sterile water and prepared with a 1% chlorhexidine solution in water, then dried after a second wash with sterile distilled water. Fig 10. The local anaesthetic was delivered to the proposed wound site by an injection perpendicular to the epidermis in the proposed wound site to a depth of 2mm. This depth is sufficient in the experimental animal to just penetrate the muscularis (paniculus) carnosus. Using a 24 guage needle on the luer-lok syringe 0.5 ml of either local anaesthetic or normal saline was deposited. Fig 11.

5.5 EXPERIMENTAL WOUND AND RECOVERY

A total of two paravertebral wounds were created, one with local anaesthetic and one control. The wounds were distributed on right and left positions in a random manner to avoid a location bias.

The wound was made by application of the cryoprobe seven minutes after injection to allow permeation of the local anaesthetic or saline. The cryoprobe was placed on the skin using a contact jelly to ensure close apposition. Three consecutive freezes of two minutes duration were carried out with 4 minute intervals between first, second and third applications intervening each freeze. Fig 12.

Recovery was carried out by placing the animals on their side in a slight head down position to ensure adequate drainage of respiratory secretions. The first signs of recovery, - namely movement, increased respiration and heart rate, were apparent approximately one hour post induction.

All animals were fully recovered within 24 hours, without obvious distress or suffering.

Fig 7. Cryosurgical Unit - Spembly.

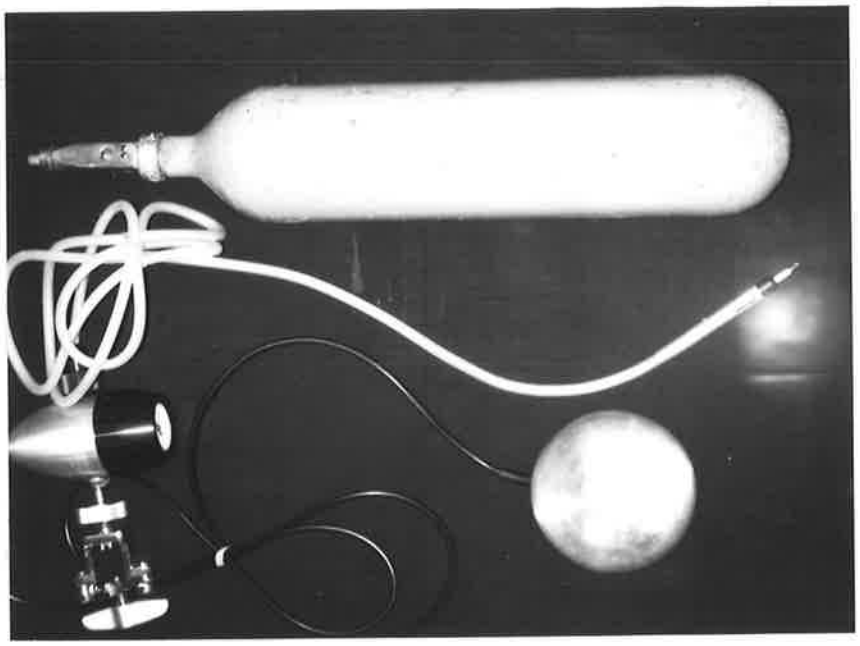


Fig 8. Comark Electronic Thermometer.

Fig 9. Animal - Anaesthetic Machine.

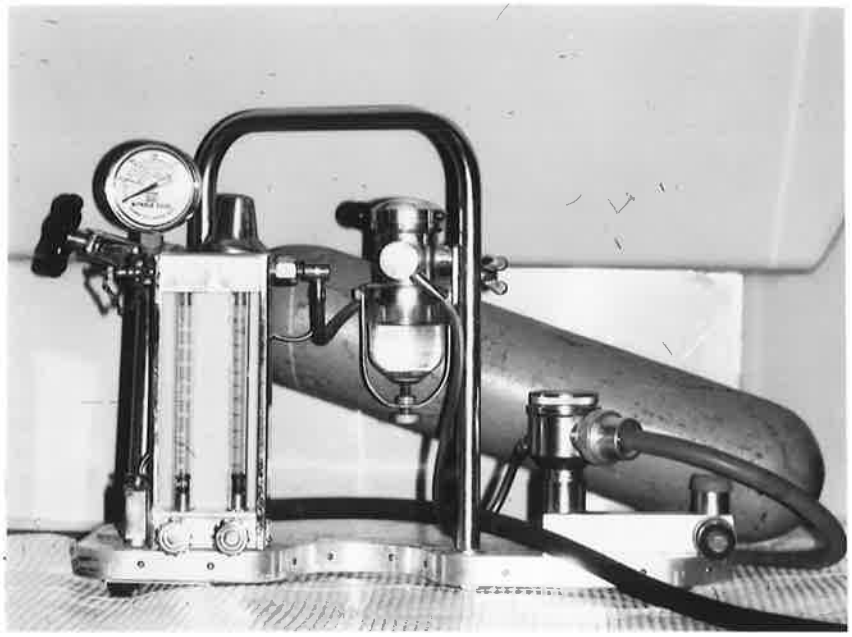


Fig 10. Animal Prepared for Surgery.

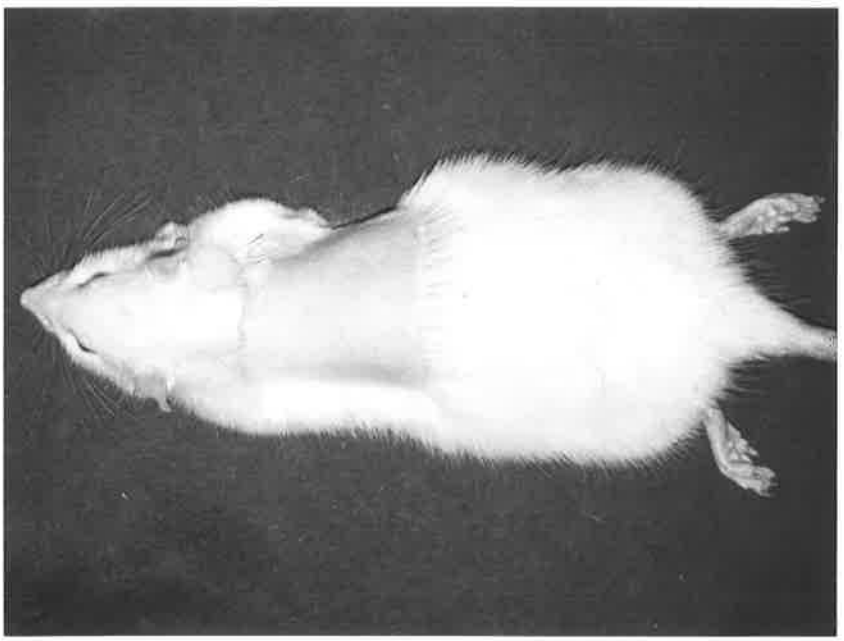


Fig 11. Administration of Local Anaesthetic or Saline.

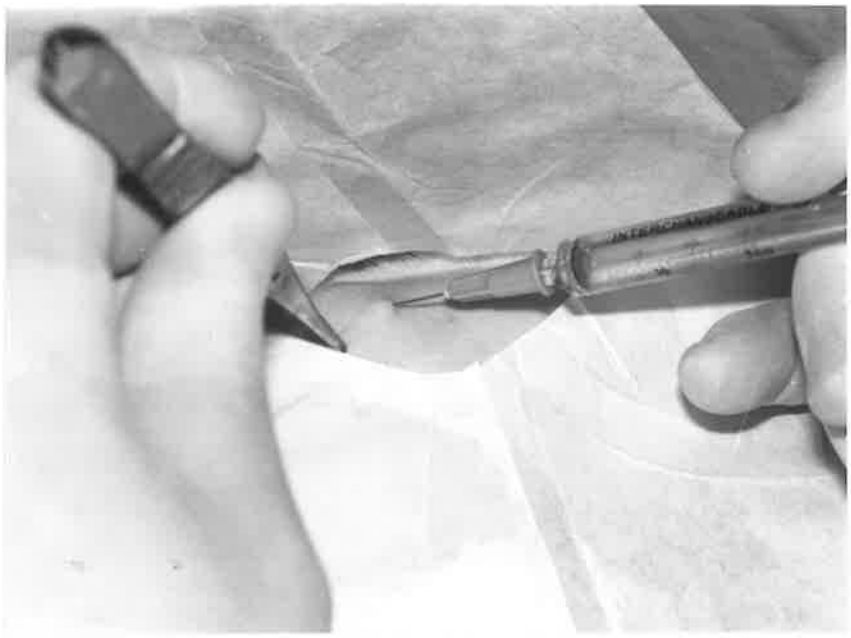
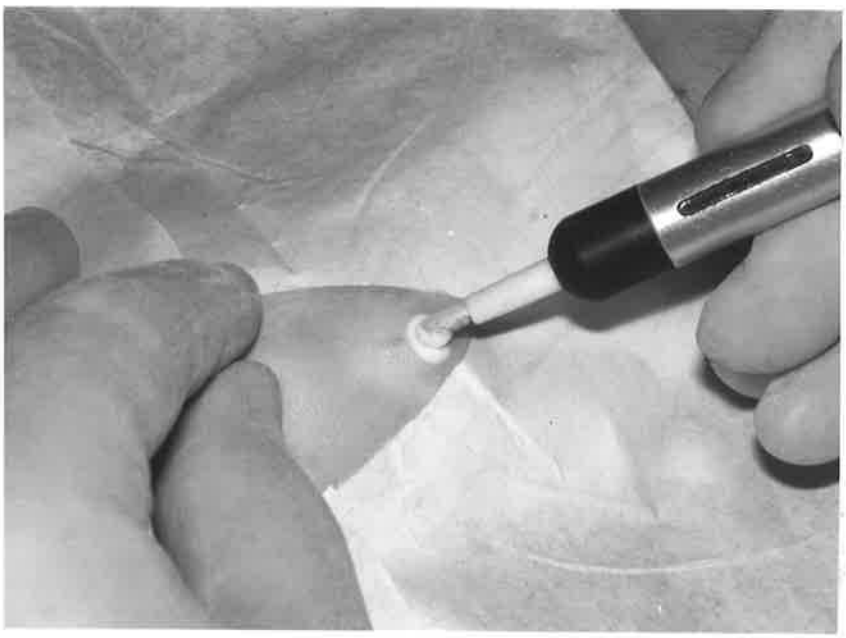


Fig 12. Cryosurgical Wound Production.



5.6 TIMING

The time intervals of sacrifice chosed for this study can be seen in table 1. Five animals were chosen for each time period and each was sacrificed at 15 mins, 24 hours, 2 days, 4 days, 6 days, 11 days, 14 days, 18 days, 21 days, 25 days. The time intervals were chosen to observe :

1. The 'initial effects' spoken of by Gill et al., (1970) and Fraser and Gill (1967)
2. The inflammatory infiltrate, vascular changes, necrosis and ulceration that follows a few days later (Poswillo 1971) and,
3. The resurfacing of the defect and the subsequent scar tissue formation within the dermis and muscle layers after three weeks. (Poswillo 1971, Passey et al., 1971)

The time periods were designed to catch any appreciable differences in tissue destruction, repair, regeneration, and scar tissue formed by the experimental and control models.

At the appropriate intervals the animals were re-anaesthetised and were sacrificed.

5.7 MACROSCOPIC INVESTIGATION

Macroscopic investigation was carried out at the time of operation and immediately pre mortem.

The macroscopic investigation at operation consisted of:-

1. Measurement of maximum diameter of the iceball achieved at each of the 3 consecutive freezes in both experimental and control animals.

2. Measurement of the time taken for the iceball to thaw after the 1st, 2nd, and 3rd freezes in experimental and control animals.

3. Serial thermocouple readings at the perimeter of the iceball.

Macroscopic measurements done pre mortem consisted of:-

1. Tracing of the gross wound area.
2. Photography of gross wounds.

TABLE 1

Time Period	Number of Animals
15 mins	5 animals
24 hours (1 Day)	5 animals
48 hours (2 Days)	5 animals
4 days	5 animals
6 days	5 animals
11 days	5 animals
14 days	5 animals
18 days	5 animals
21 days	5 animals
25 days	5 animals
Total	50 animals

PEROPERATIVE MEASUREMENTS

1. MAXIMUM ICEBALL MEASUREMENT

Initial measurements showed that the iceball achieved got no bigger after 1.5 minutes, therefore in this experiment the iceball was measured solely at 2 minutes. A set of Wild - Heerburg dividers was used to measure the distance between the points of greatest diameter across the iceball. This measurement was then converted to mm. using a Vernier caliper with computerised electronic display. Measurements were made from 1.5 minutes onwards and at each consecutive freeze.

2. THAW TIME MEASUREMENT

From the finish of freezing the determination of the 'point of thawing' for the purpose of this study was when there was no visible frost after probe removal. (This followed a method described by Neel, Ketcham and Hammond 1971a). The time of thaw was taken as the time from ceasing the power to the cryoprobe, and not its removal from the skin, to the thaw point.

The number of animals and times chosen for these experiments can be seen in table 2a.

Animals were then recovered and were not re-anaesthetised until the time of sacrifice.

3. THERMOCOUPLE MEASUREMENTS

Five animals from different time periods were chosen to carry out serial temperature measurements in both control and experimental wounds. Table 2a. Two Copper Constantan wire couples were introduced at the assumed iceball periphery, as judged by previous experimental animals within the study, using 18 gauge needles. The needles were withdrawn and the wire coupling connected in-line with a Comark electronic thermometer type 1624 - see Figs 8, 13. The reading was in degrees celcius. A baseline animal temperature was taken pre freeze and after either local anaesthetic or saline was injected. Serial temperature recordings were made every 30 seconds for the total of 2 minutes at each of three freeze times.

TABLE 2a

MEASUREMENT OF THAW-TIME AND THERMOCOUPLE TEMPERATURE

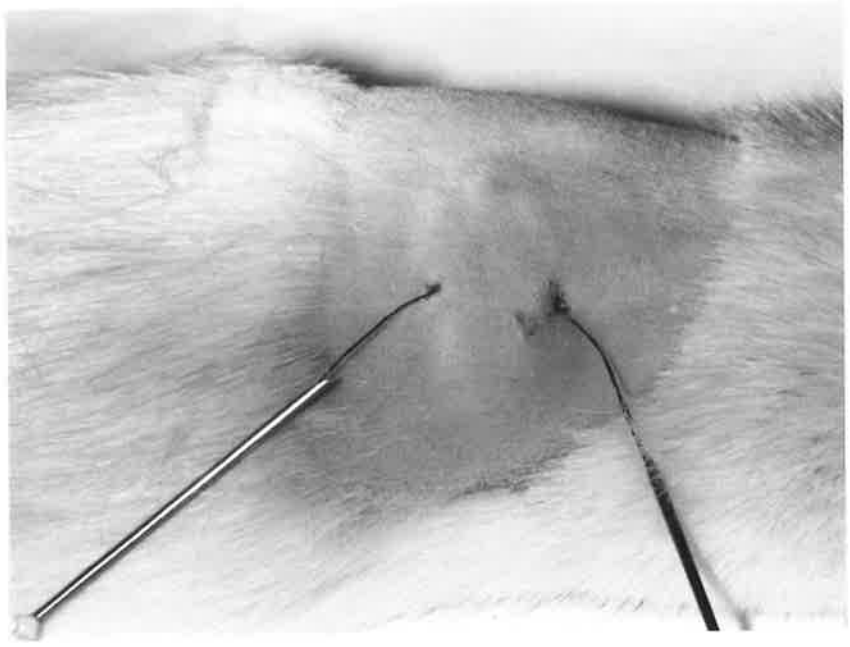
Time Period	Number of Animals
15 Minutes	1
48 Hours (2 Days)	1
6 Days	1
14 Days	1
21 Days	1
Total	5

TABLE 2b

MEASUREMENT OF ICEBALL DIAMETER

Time Period	Number of Animals
24 Hours	1
4 Days	1
11 Days	1
18 Days	1
25 Days	1
Total	5

Fig 13 Insertion of Thermocouples to Proposed Surgical Site.



A lapse time of 4 minutes was allowed between freezes to allow equilibration of temperature and to comply with the determined protocol carried ou allowed between freezes to allow equilibration of temperature and to comply with the determined protocol carried out during the remaining experiments.

MACROSCOPIC INVESTIGATION

PRE MORTEM MEASUREMENTS

Macroscopic investigation was also carried out prior to sacrifice when after being reanaesthetised the animals were repositioned on the operation table in the standardised fashion.

1. WOUND AREA MEASUREMENT

The dorsal skin wounds were slightly compressed beneath a glass slide and traced on clear acetate sheets, following a written description of each wound.

Using a Koizumi compensating polar planimeter type KP-27 the tracings of the wound peripheries were used to calculate the areas within each irregular circle. Fig 14. This was done by projecting the original tracing on an overhead projector to enlarge the area by a factor of three, and retracing the enlarged area. The increase in size enabled simpler use of the planimeter and each wound was measured 5 times non-consecutively, and a mean computation figure for the given area was achieved. These figures were then converted to cm^2 and reduced by a factor of 3, using a Hewlett-Packard 9815A computer.

2. PHOTOGRAPHY

The animals' wounds were then photographed beneath a Wild Makroskop using a standardised focal distance of 16 cms. Following photography the animals were weighed and checked for any signs of gross pathology. (see Appendix).

All tissue specimens were taken in vivo by sharp dissection as a piece of epidermis and underlying connective tissue down to an including the Paniculus Carnosus. Animals were then sacrificed by an overdose of Chloral Hydrate delivered intraperitoneally, at a concentration of 10mg/ml and a dosage of 5mls/kg body weight.

5.8 HISTOPATHOLOGIC TECHNIQUE

After sacrifice all specimens were fixed in 10% formol/buffered saline for a minimum of 24 hours. The specimens were then trimmed, and cut in half through the point of largest wound diameter. All half pieces were oriented and marked prior to a standardised technique of processing, wax embedding and blocking. The finished blocks were sectioned by microtome at 7 micrometres. One in five sections was mounted on glass slides for staining according to a standardised Haematoxylin - Eosin technique. (See Appendix).

5.9 MICROSCOPIC INVESTIGATION

Microscopic investigation was carried out using an Olympus EH light microscope at powers, Plan 4, 10/0.25, 20/0.42, and 40/0/.65. Each slide was assessed both qualitatively and quantitatively. The quantitative

assessment was by histologic description and the qualitative assessment was by histologic scoring, according to the proforma in the appendix .

The basis of all assessments was the division of wound healing into three primary phases.

1. Tissue necrosis
2. Inflammation
3. Tissue repair

a) QUALITATIVE ASSESSMENT

All wounds were considered to have 3 zones apart from the epithelial covering. These were:

1. The dermis, (excluding adipose zone)
2. The adipose zone,
3. The muscle zone.

Together with the epithelium, each was described for effects of injury on tissue morphology, zones of tissue damage, tissue and cellular regeneration and resultant tissue response.

b) QUANTITATIVE ASSESSMENT

The quantitative assessment consisted of cell counts and determinations of stage of epithelialisation.

Fig 14. Compensating Polar Planimeter.

1. CELL COUNTS

Cell counts were taken at nine designated positions across the wound area. These positions can be seen in Fig 15. Six lateral positions and three central positions were chosen as a range both across the wound and within each tissue zone of dermis, adipose or muscle.

Cell counts were confined to what were regarded as either acute or chronic inflammatory cells. Acute cells were restricted to polymorphonuclear leucocytes recognised by their characteristic histologic morphology. The term chronic inflammatory cells would normally include lymphocytes and plasma cells, however, for the purposes of this study the term 'round cell' was used to refer to perfectly round chronic inflammatory cells with darkly staining nuclei and without evident cytoplasm. Cell counts were done under light microscopy at magnification plan 4, 40/0.65 to cover as large an area as possible but retaining a magnification suitable to identify particular cell morphology. Cells were counted using a standardised Olympus optical eyepiece micrometer (260 micrometres x 260 micrometres) with squares. Only cells within the grid were counted.

Wound margins were determined arbitrarily within each tissue zone, and for marginal (lateral) counts, the wound margin was placed centrally within the grid. Tissue zones of greater area than one grid size received mean cell counts determined by the total count divided by the number of grids within the region.

Total cell counts were expressed as an average number of cells in the grid for a high power view of a particular site within the wound

area. These means were then converted to an inflammation index of

0 - Nil inflammation

1 - Mild inflammation

2 - Moderate inflammation

3 - Severe inflammation

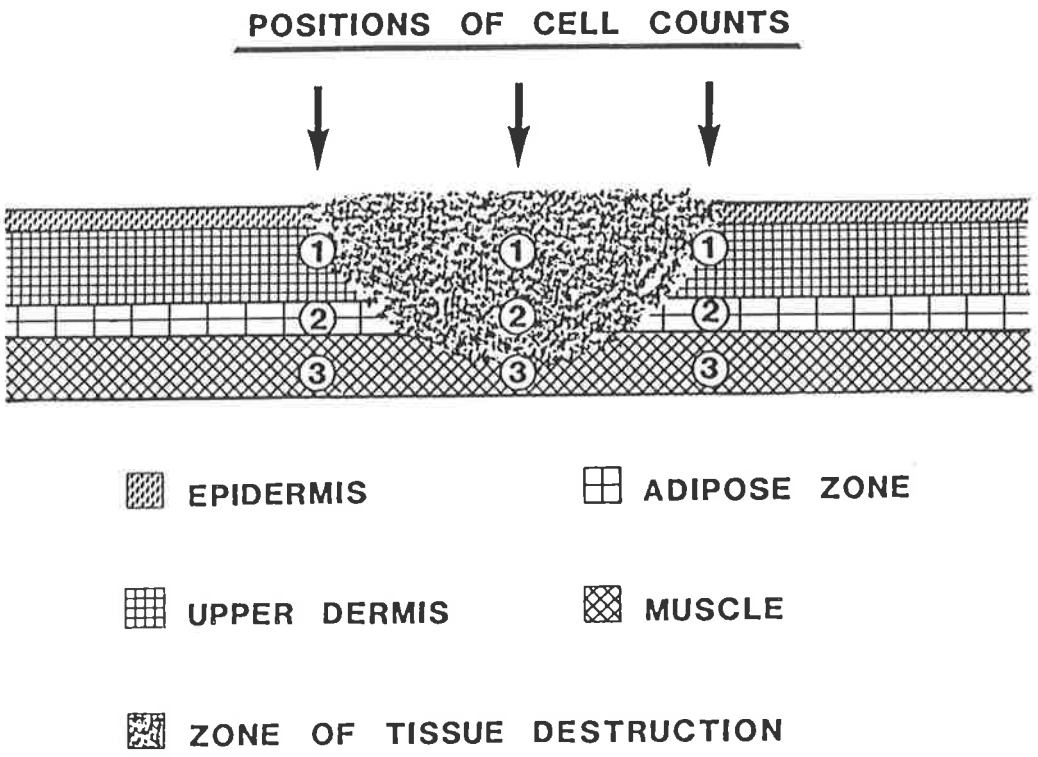
The scoring proforma for acute and chronic cells in both experimental and control groups, is presented in Tables 3 and 4.

Standardisation of results was achieved by:

1. Using the same microscopic power

2. Double determination techniques. (10% of all section remeasured)

Fig 15 Positions of Cell Counts



2) RATE OF EPITHELIALISATION

The amount of epithelial migration was assessed from slides sectioned within 35 micrometres of the apparent wound centre. The first five slides from each block were assessed under light microscopy at power plan 4, 10/0.25. Scoring of migration was, as seen below. The site of initial migration was arbitrarily considered to be where the epithelium initially took on the appearance of "new" epithelium; having the feature of hyperplasia and hyperchromatism. Photographic scoring of the results was as follows:

Score	Degree of Epithelial Migration
0	No Migration
1	Initial migration, less than or equal to one third of the wound diamter.
2	Moderate migration, greater than one third but less than or equal to two thirds of the wound diameter.
3	Almost complete migration, greater than two thirds of the wound diameter.

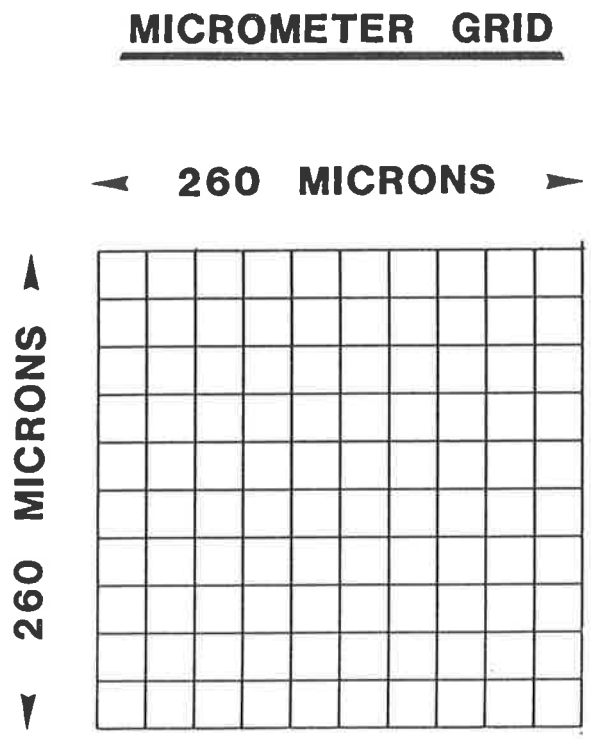


Fig 16 Graticule for Cell Counts.

TABLE 3

ACUTE CELL SCORING PROFORMA

No. of cells within grid	Histologic Index
0	0
0 - 5	I
6 - 20	II
> 20	III

TABLE 4

CHRONIC CELL SCORING PROFORMA

No. of cells within grid	Histologic Index
0	0
0 - 1.5	I
1.5 - 3.0	II
> 3.0	III

RESULTS

6.1 NORMAL RAT SKIN

6.1.1 ANATOMY

The skin or integument of the rat covers all the exposed external areas of its body. It functions as a protective layer and a containing external anatomical entity. In gross form, it is covered by dense hair except in several modified areas such as nasal, scrotal, tail, palmar and plantar regions.

It combines functionally with the underlying cutaneous muscle layer the paniculus carnosus to form a unit approximately 2mm thick that slides freely on the underlying loose areolar connective tissue.

The integument is composed of three principal layers:-

1. Epidermis
2. Dermis
3. Hypodermis

Specialised structures - namely hair follicles, bristles and glands are contained within these layers. Fig 17.

6.1.2 HISTOLOGY

EPIDERMIS

The skin of the white rat is very hairy and the epidermis is composed of four basic layers.

a) STRATUM BASALE

This layer is composed of regular, columnar shaped cells with darkly staining nuclei.

b) STRATUM SPINOSUM

This layer is also referred to as the prickle cell layer and together with the Stratum Basale forms the Stratum Germinativum, a layer in which the cells have their long axis perpendicular to the basement membrane.

Mitotic figures usually occur within these layers, though they may not be confined to them. The cells of the so-called spinous layer are attached to one another by well developed desmosomes.

c) STRATUM GRANULOSUM

This consists of three to five sub-layers of flattened cells with conspicuous, irregularly shaped granules that stain deeply with basic dyes. These granules are Keratohyalin granules intimately associated with keratin formation.

d) STRATUM CORNEUM

This layer consists of many sublayers of flattened cornified cells without a nucleus and with keratin replacing the cytoplasm, the cells are loosely packed without obvious intercellular spaces.

There is no evident Stratum Lucium in rat epithelium and in very young animals the Stratum Granulosum may be absent. The thickness of the epithelium varies between the sexes and the different stages of the female menstrual cycle Spain (1915).

DERMIS

Dermis, also called the Corium. In very young animals, as a layer it is extremely cellular. Fig 18.

In the Stratum Papillare the collagen fibres are delicate, run parallel to the epidermis and mingle with the highly branched elastic fibres.

In the deeper reticular layer the collagen fibres are unoriented, coarser and it is here that the elastic fibres assume an orientation parallel to the epidermis.

The dermis of dorsal and ventral skin is different. Dorsal skin dermis has a higher water content, lower lipid content and it's fibres are less dense that the corresponding ventral skin dermis. Ebling and Hale (1966).

Throughout the dermis and hypodermis there are numerous mast cells.

The thickness of the dermis is hard to determine as it passes over into the subcutaneous layer without a sharp boundary. Spain (1915).

At various levels of the dermis there can be found hair follicles, sweat and sebacious glands which are merely epidermal derived structures extending into the dermis.

There are also numerous blood vessels, nerves and many nerve endings.

HYPODERMIS

The subcutaneous layer consists of loose connective tissue and is a deeper continuation of the dermis. It's collagenous and elastic fibres run in all directions. Fig 18.

Where the skin is flexible and freely moving these fibres are few, but where it is closely attached to the underlying parts as in palmar and plantar regions they are thick and numerous.

The hypodermis contains moderate deposits of adipose tissue in the form of white unilocular fat though in some regions (Axilla, Neck) multilocular deposits of brown fat are found.

SPECIALISED STRUCTURES WITHIN THE SKIN

These comprise hairs, hair follicles and glands.

1. HAIRS

Noback (1950) broadly classifies the hairs into three groups:

a) Guard Hairs

b) Under Hairs

c) Tactile or Sinus hairs

2. HAIR FOLLICLES

Hair follicles are arranged in groups around a central follicle, these groups average about three to nine follicles. The central follicle contains a large bristle hair and awn hairs while the others contain fur hairs.

Compound follicles contain several hairs with follicles arranged in rows and oriented perpendicularly to the long axis of the body and limbs.

Hair follicles are tubular invaginations of the epidermis. The active follicle has a bulbous terminal expansion with a concavity below - occupied by connective tissue.

3. GLANDS

(i) SEBACIOUS GLANDS

Scattered over the surface of the skin these lie in the dermis and their excretory ducts open into the necks of hair follicles.

The secretory portions open into a duct from grape-like clusters of rounded alveoli which are lined by a single layer of thin cells with rounded nuclei. Munger and Brusilow (1971).

Secretion is the result of epithelial cell destruction - its oily substance resulting from breakdown of the cells to form a fatty detritus. This is followed by epithelial cell regeneration.

(ii) SWEAT GLANDS

These are simple coiled tubular glands with short tortuous secretory ducts rising from a secretory organ deep within the dermis or hypodermis.

In the rat, sweat glands are only found on the feet.

The walls of the secretory portion rest on a thick basement membrane and are unique in that they consist of only one glandular cell type. Munger and Brusilow (1971)

Fig 17 Low power (x10) view of rat skin showing Epidermis (E), Dermis (D), Adipose (A) and muscle zones (M). There are evident Hair Follicles (HF) and Sebacious Glands (SG).

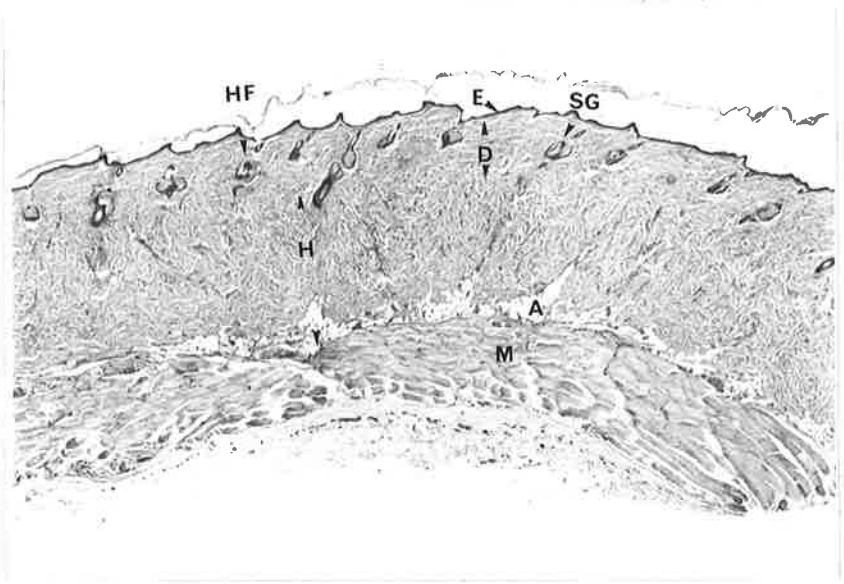
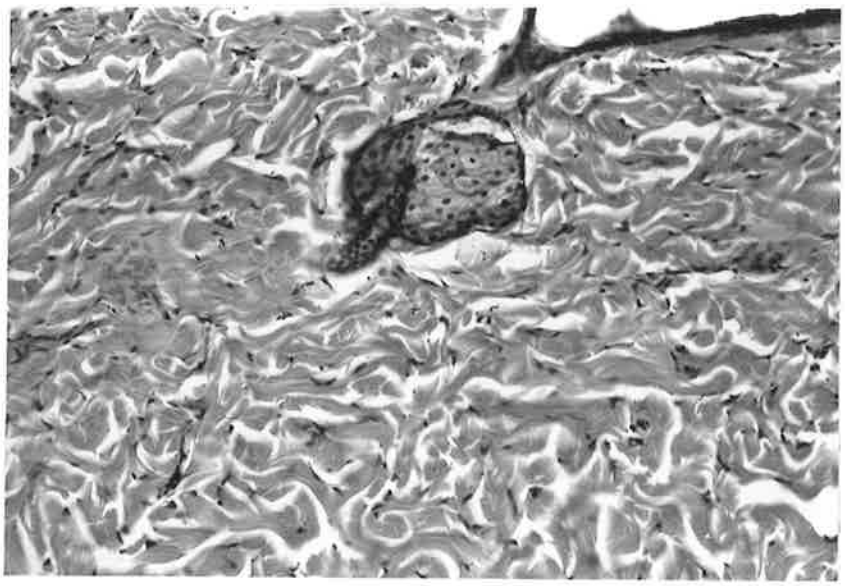


Fig 18 Medium power (x25) view of rat skin showing the Collagenous structure within the Dermis.



6.2 MORTALITY

During the course of the experiment there were two animal deaths. One of the deaths occurred pre-operatively within 5 minutes of the administration of Chloral Hydrate. This death was presumably due to a relative anaesthetic overdose and an idiosyncratic reaction. The one other death occurred post-operatively and was due to a bowel stricture - requiring the animal to be sacrificed pre-term.

Animal mortality can be seen expressed in Table 5. A total of fifty animals were sacrificed and the total animal mortality amounted to two animals.

TABLE 5

MORTALITY

TIME PERIOD	NO. OF ANIMALS	DEATHS
15 minutes	5	
24 hours	5	
48 hours	5	1
4 days	5	
6 days	5	
11 days	5	1
14 days	5	
18 days	5	
21 days	5	
25 days	5	
	50	2

6.3 GROSS OBSERVATIONS

As the wounds thawed they became raised and oedematous. A clear serous-type exudate covering the wound was visible as the skin changed from frosty white to pink and slightly hemorrhagic. Four to five minutes after freezing the experimental wounds had a distinctly pale ischaemic ring of skin surrounding the actual wound site that was not present in the controls.

By 15 minutes the pink hemorrhagic area had deepened in colour in both wounds, but more so in the controls, which retained a more hemorrhagic appearance centrally.

The experimental wounds appeared more definite than the controls due to the halo effect of surrounding ischaemia. Figure 19

By 24 hours the differences between the two wounds were quite marked. The experimental wounds appeared larger and more irregularly shaped than the controls which did not have a covering area of scab, and retained a roughly circular shape with several dark hemorrhagic spots within. Figure 20

The wounds at 48 hours were again dissimilar, the experimental wounds retained their irregular shape and showed initial signs of healing while the circular control wounds were indefinite and had changed little from Day 1 (24 hours). The scab covering the experimental wound appeared to be lifting slightly at the margins with a ring of pale new epithelium evident. Figure 21

From 48 hours to 6 days the wounds began to approximate one another in size and shape. As they grew smaller and more mature, the experimental wound was roughly circular in shape with a covering scab that was curling at the margins as the new epithelium grew beneath. The control wounds were roughly the same shape though they appeared smaller. Figures 22, 23

By 11 days the control wound again appeared to be smaller than the experimental wound, and its shape was changing from very circular to a more irregular form. Both wounds continued to retain a dark scab that was lifting peripherally. Figure 24

From day 14-21 both control and experimental wounds were grossly very similar, the covering scab remained until day 18 in both groups and although the size and shape of the underlying scar were different they healed at the same rate.

The differences in size and shape of the scar were retained up to and including day 25. Figures 25, 26, 27

By day 25 the control scar was a more regular, approximately round shape, while the experimental scar had a semilunar shape - both were becoming increasingly difficult to see, as they had decreased markedly in size. Both wounds presented as hairless areas with indefinite margins, although the visible area of scar tissue appeared larger in the wounds treated with local anaesthetic. Figure 28

From the outset both series showed progressively healing wounds that after early differences showed grossly similar healing characteristics. The impression gained was that the experimental wounds were slightly larger, and took longer to heal than the controls.

Fig 19a Experimental Wound - 15 minutes

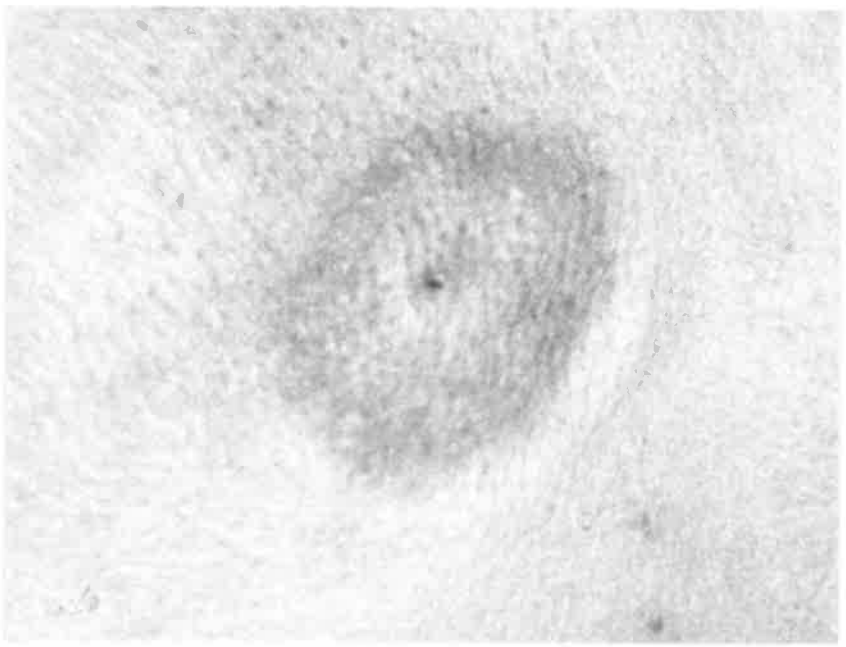


Fig 19b Control Wound - 15 minutes

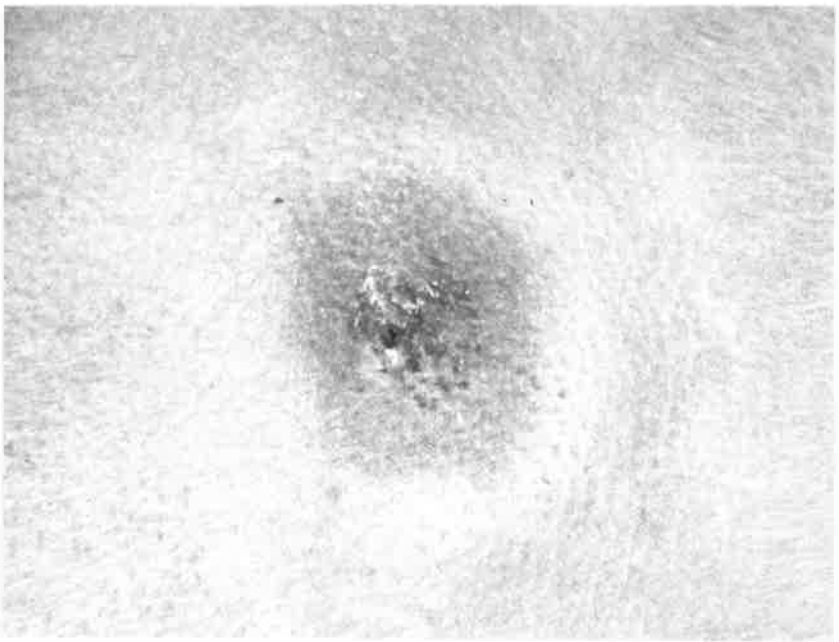


Fig 20a Experimental Wound - 24 hours

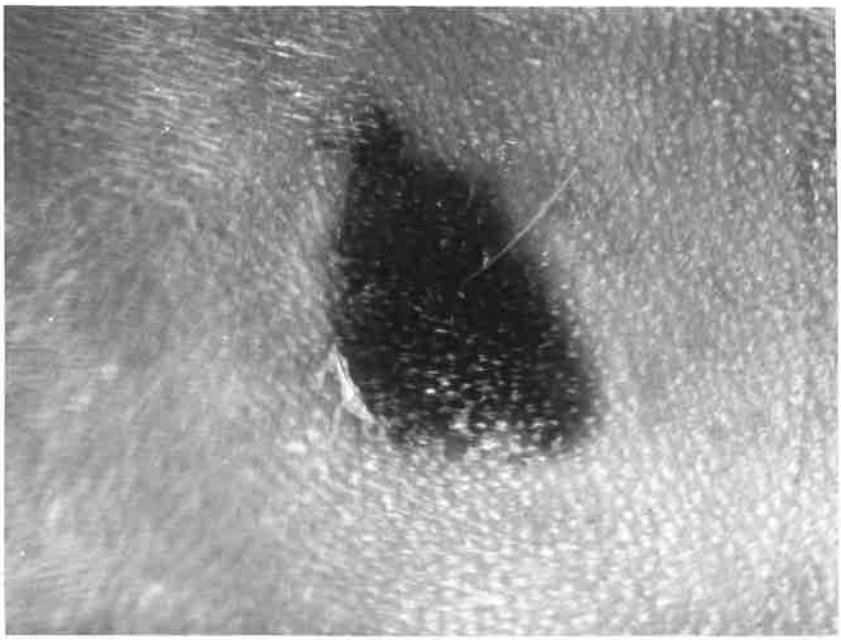


Fig 20b Control Wound - 24 hours

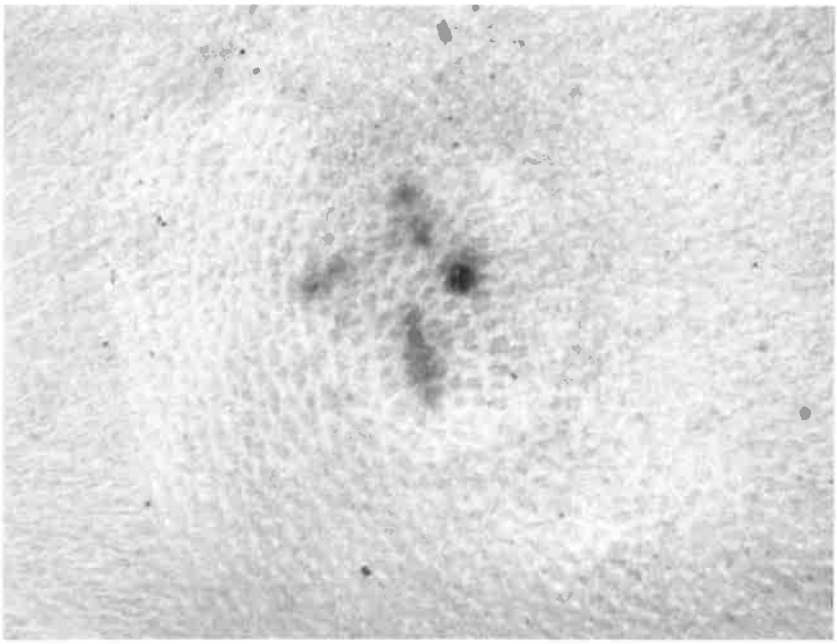


Fig 21a Experimental Wound - 2 days

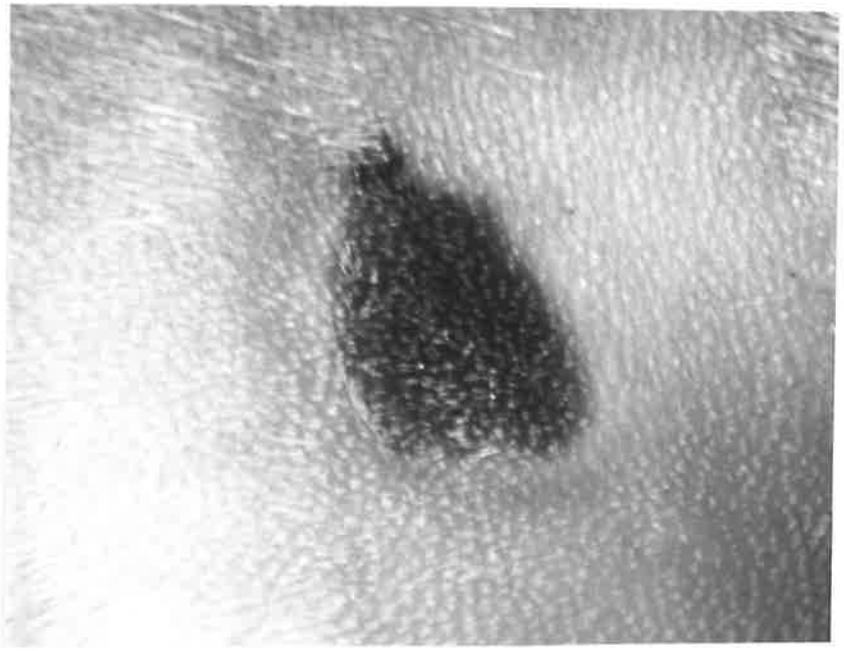


Fig 21b Control Wound - 2 days

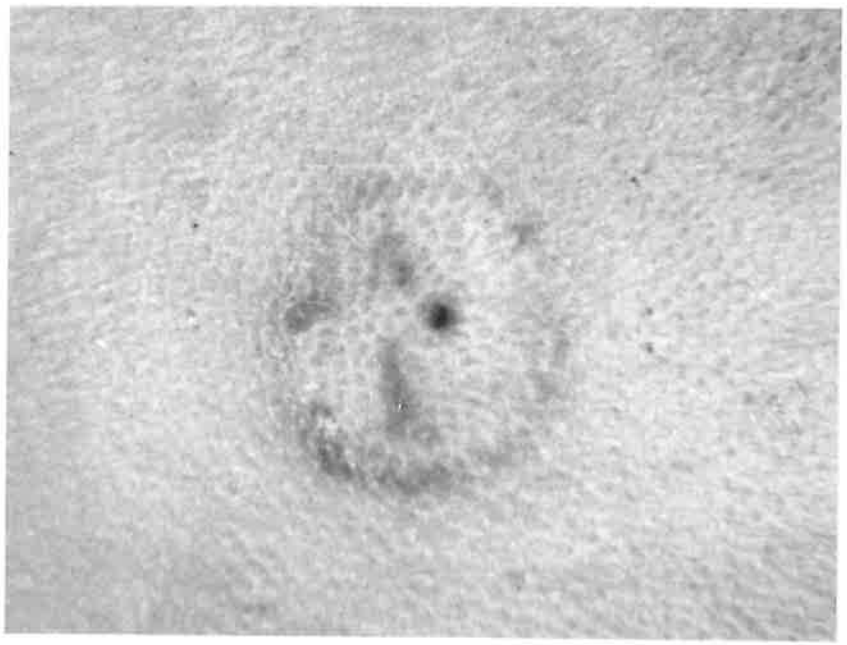


Fig 22a Experimental Wound - 4 days

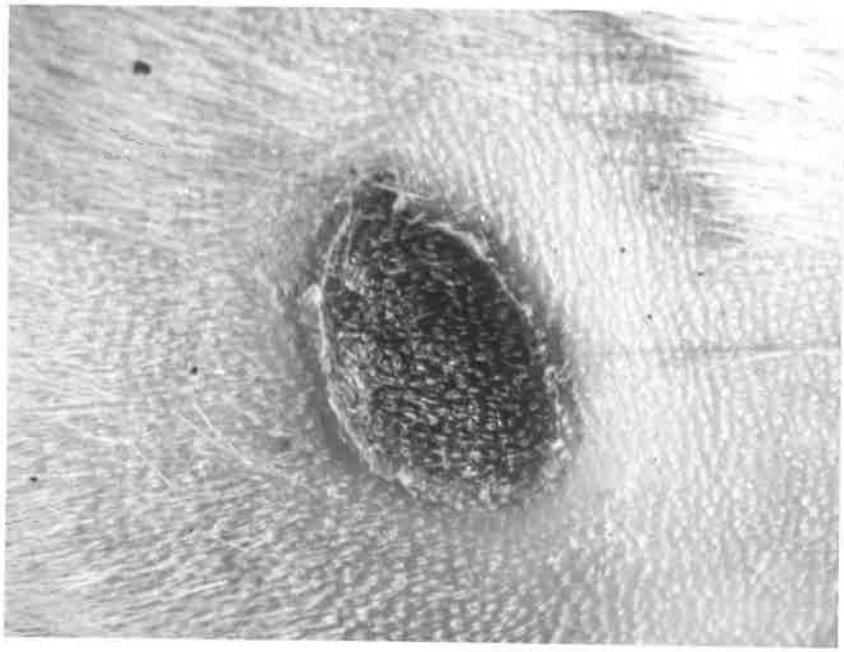


Fig 22b Control Wound - 4 days

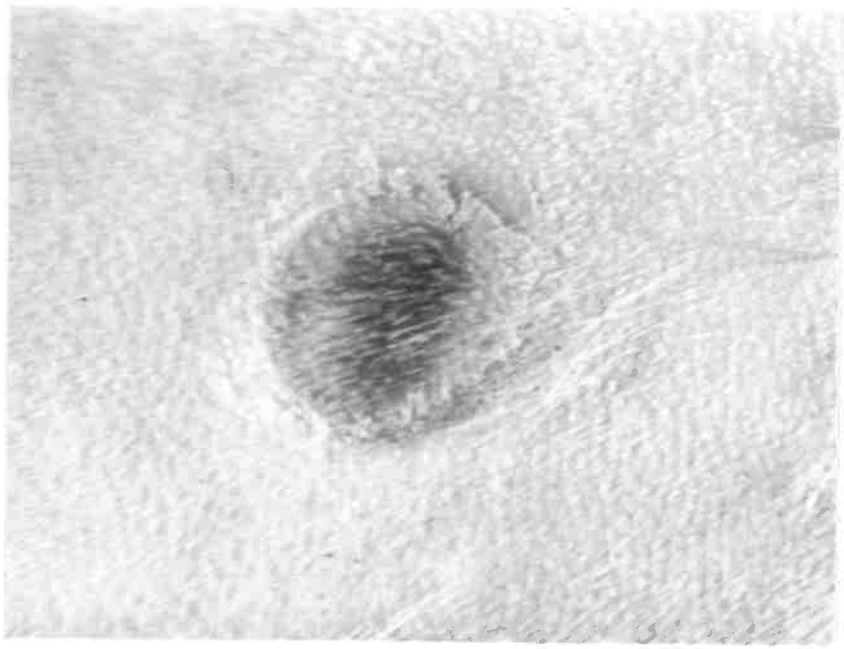


Fig 23a Experimental Wound - 6 days

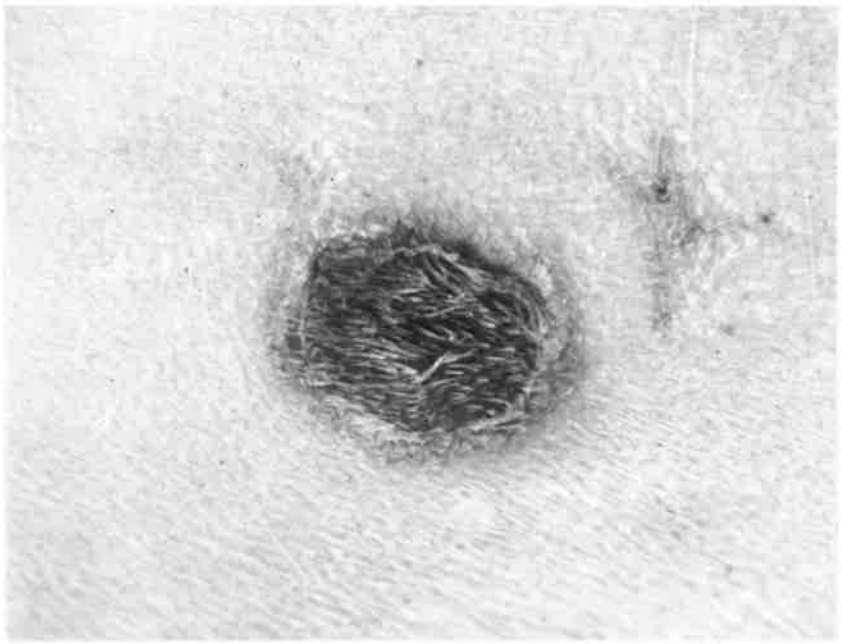


Fig 23b Control Wound - 6 days

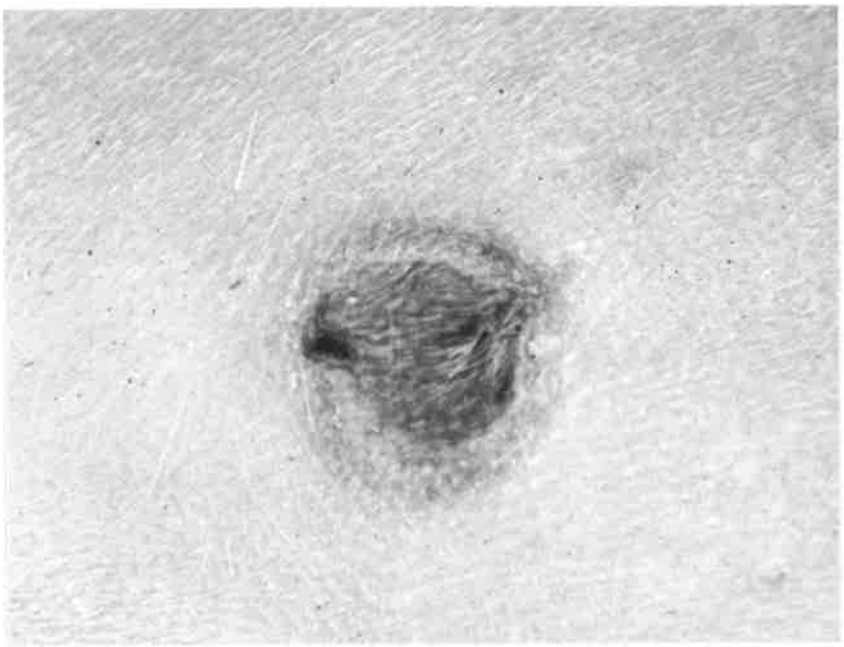


Fig 24a Experimental Wound - 11 days

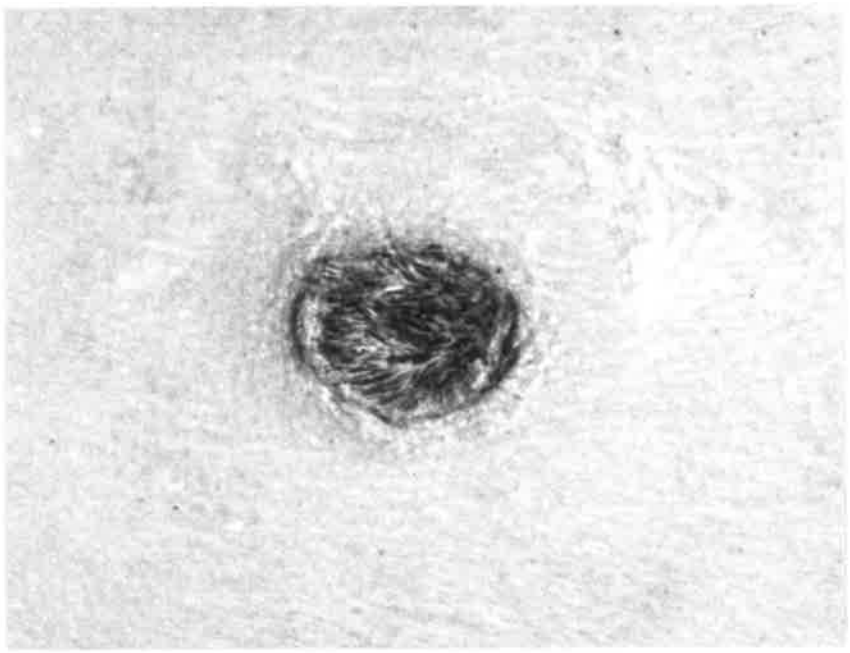


Fig 24b Control Wound - 11 days

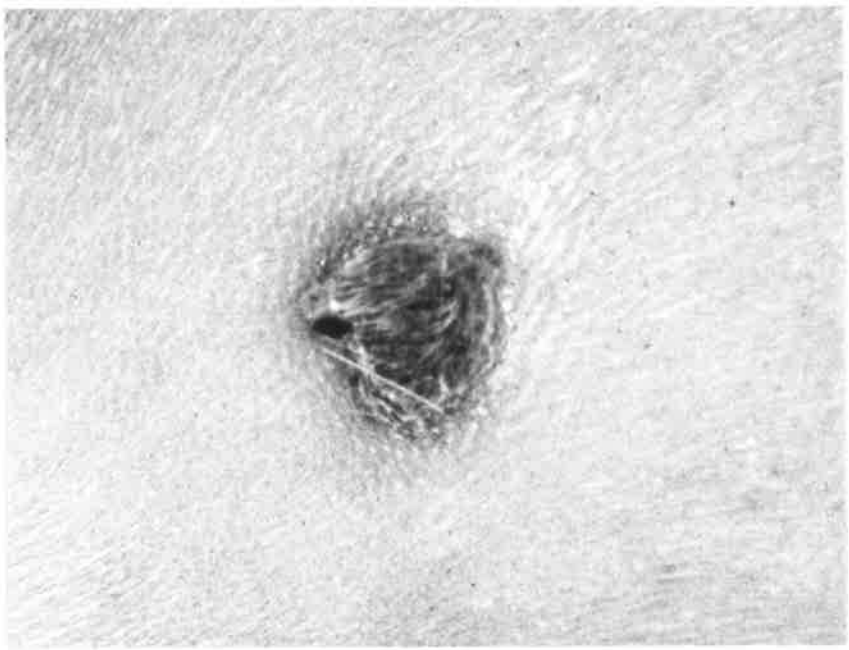


Fig 25a Experimental Wound - 14 days

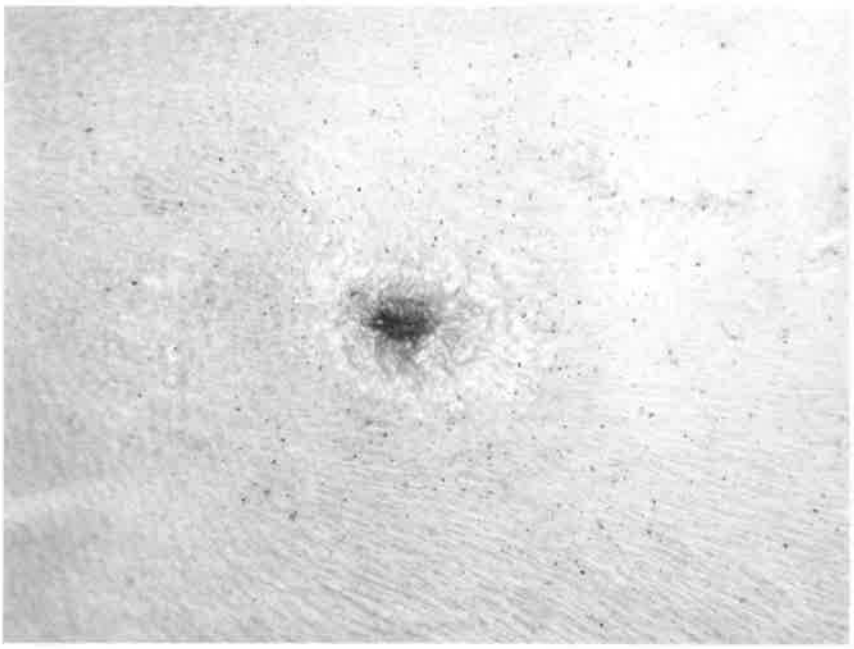


Fig 25b Control Wound - 14 days

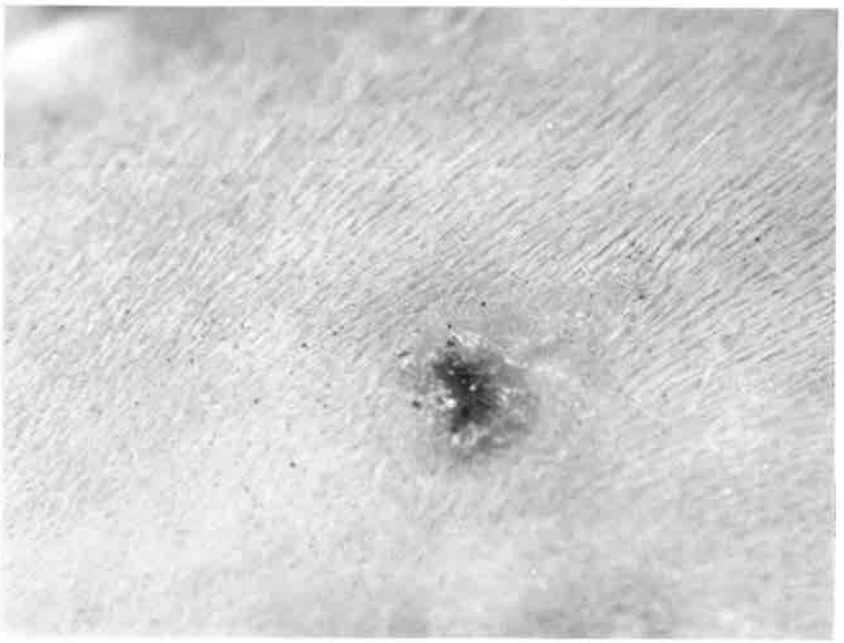


Fig 26a Experimental Wound - 18 days

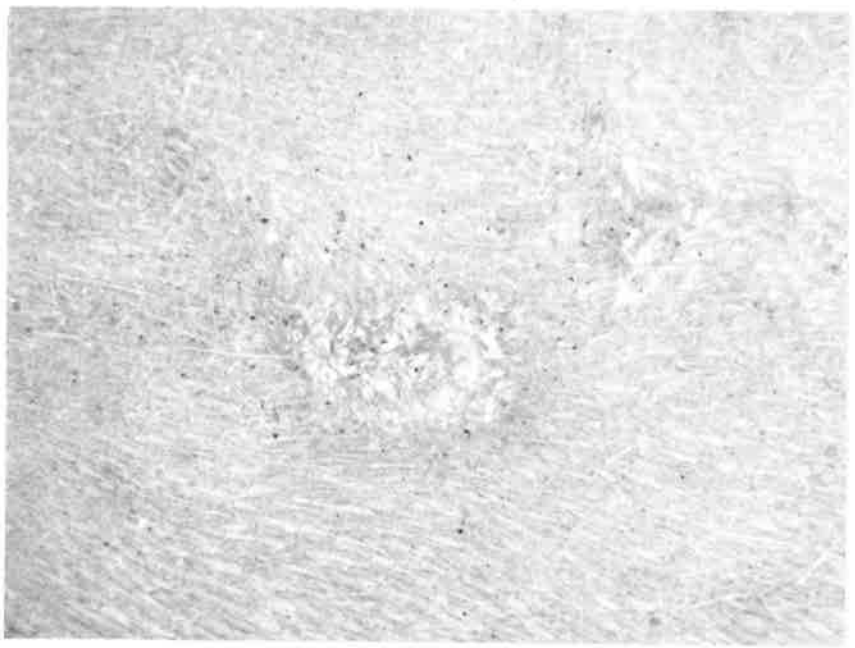


Fig 26b Control Wound - 18 days

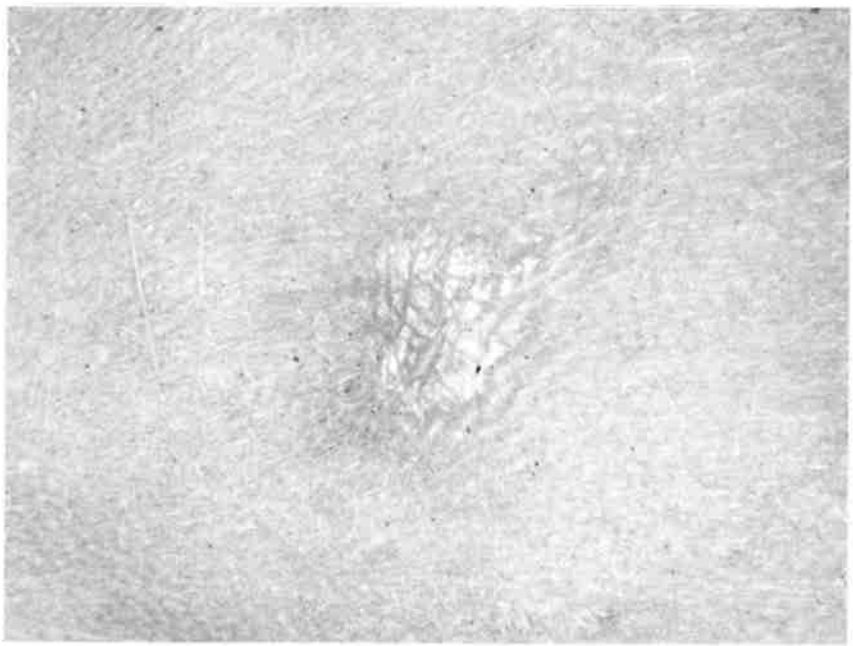


Fig 27a Experimental Wound - 21 days

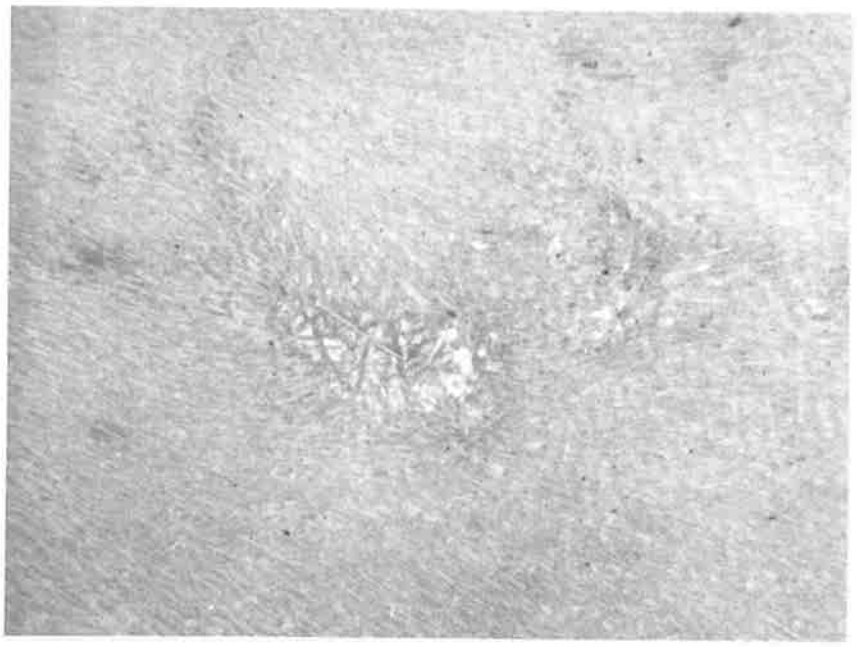


Fig 27b Control Wound - 21 days

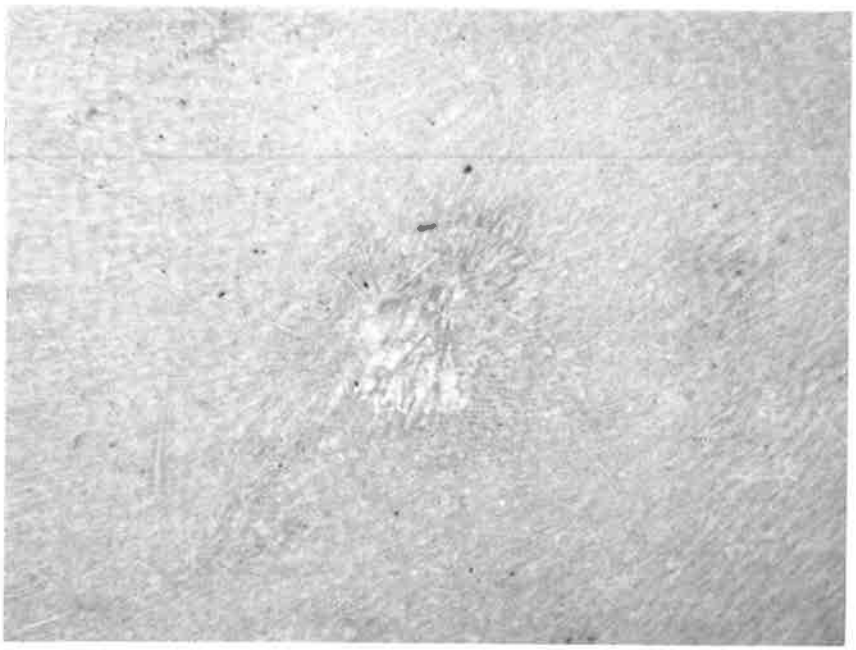


Fig 28a Experimental Wound - 25 days

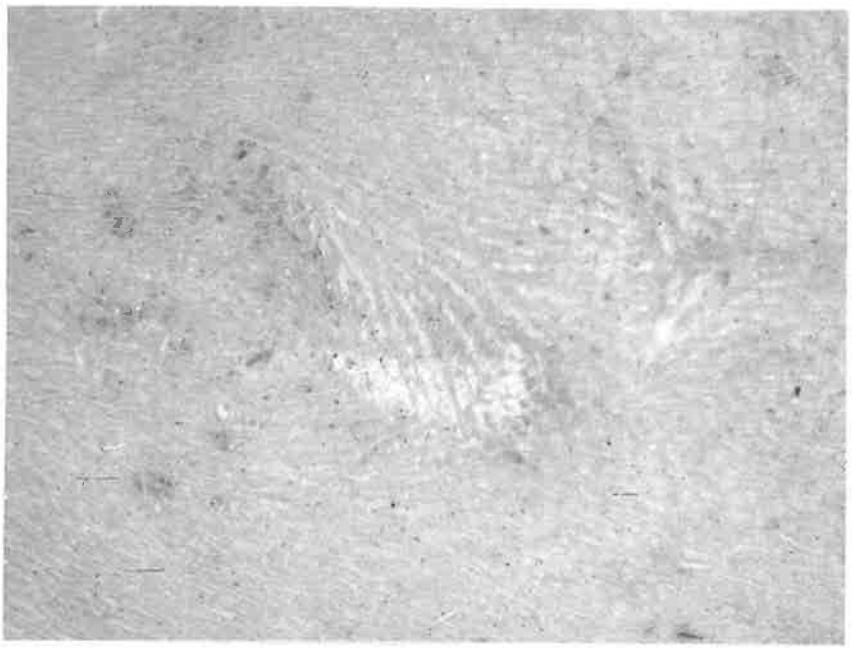
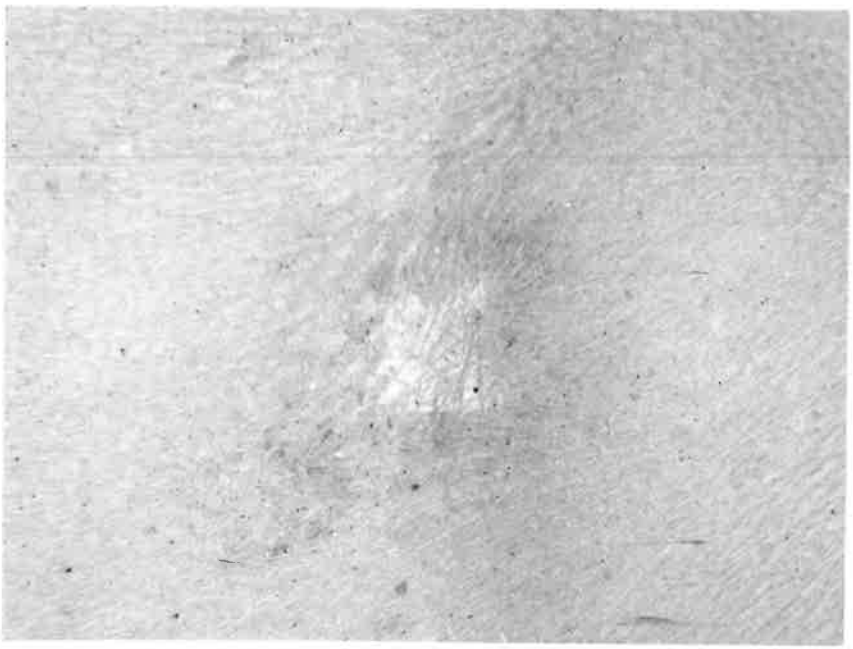


Fig 28b Control Wound - 25 days



6.4 HISTOLOGIC OBSERVATIONS (Figs. 29-48)

EXPERIMENTAL WOUND 15 minutes

The surface epithelium is degenerating. The cells are losing polarity and staining characteristics as they become less basophilic and display smaller hyperchromatic nuclei.

Evident tissue damage within the dermis is difficult to determine. The epithelial cells of the hair follicles and sebacious glands, within the adipose zone show a smaller nucleo-cytoplasmic ratio with eosinophilic, vacuolated cytoplasm and pyknotic, hyperchromatic nuclei.

There is sludging of small blood vessels and the deeper layers of the dermis show the presence of a diffuse acute inflammatory infiltrate of cells. There would appear to be little adipose tissue damage, at this stage.

The muscle layer is broadly oedematous with separation of the muscle bundles, sludging of small veins and extravascular aggregations of acute inflammatory cells.

CONTROL WOUND 15 minutes

The surface epithelium although intact shows distinctive changes in staining characteristics, over the wound centre, the cells show pleomorphic, hyperchromatic nuclei in a slightly eosinophilic cytoplasm. The basal layer is disrupted.

The epithelial cells of hair follicles and sebacious glands show similar features. Laterally the damaged epithelium blends imperceptibly into normal epithelium at the wound margins.

Apart from evident changes to the hair follicles and sebacious glands the dermis as a whole is without obvious gross damage, other than the sludging of capillaries, venules and small capillaries containing entrapped polymorphonuclear leucocytes. The adipose layer is largely unaffected and apart from vessel sludging and a diffuse infiltrate of polymorphs, it's general morphology is quite normal.

The muscle layer however shows quite marked changes in structure, with central oedema causing wide separation of the muscle bundles and the presence of a very mild acute inflammatory cell infiltrate.

EXPERIMENTAL WOUND 24 hours

The epithelium over the wound area has been replaced by a coagulum of, 1) flattened necrotic epithelial cells with featureless cytoplasm and hyperchromatic nuclei and 2) structureless collagen containing degenerated hair follicles and sebacious glands.

The epithelium bordering the immediate wound margins has lost polarity with the cells having pale granular cytoplasm with flocculent density. This epithelium gradually blends into more normal epithelium which at this point shows increased mitotic activity but is without evident cell migration. Centrally, the upper 1/3 of the dermis has been markedly affected, and is isolated from the underlying tissues by a dense band of acute inflammatory cells. The tissue beneath, including

the adipose layer, is the area of future regeneration and shows gross oedema, hemorrhage, with sludged small blood vessels and a diffuse inflammatory infiltrate.

The lateral region forming the peripheral margin of the wound has a diffuse inflammatory infiltrate of both acute and chronic inflammatory cells and in this area the return of hair follicles and sebacious glands roughly marks the lateral wound periphery.

The deepest area of damage is in the muscle layer which shows quite marked degeneration of the widely separated and oedematous muscle bundles and surrounding nerves. Even here the peripheries are demarcated by a round cell infiltrate and obvious sludging of small blood vessels.

CONTROL WOUND 24 hours

The epithelium covering the wound area is thin, pale and the cells show small pyknotic hyperchromatic nuclei. Laterally the evident damage is less, and increased mitotic activity, prepares the epithelium for migration.

The wound area is hard to determine in the dermis, the degenerating epithelial cells of hair follicles and sebacious glands act as indicators of the wound margins. The collagen within the upper level of the dermis appears somewhat hyalinised and vessel sludging is apparent.

The adipose layer is markedly oedematous with a heavy acute inflammatory infiltrate that increases just above the muscle.

In the central area of muscle the bundles are separated and fragmented. The bundles are less eosinophilic than normal and have a diffuse inflammatory infiltrate that extends beyond the margins as depicted by the epithelium and seems to emanate from large unsludged blood vessels.

EXPERIMENTAL WOUND 2 days

The lost epithelium and connective tissue has been replaced by a surface coagulum bordered by a pale staining epithelium that has begun to migrate beneath it and along the line of polymorphs which form a band above the remaining dermis.

Centrally within the saucer shaped wound, the dermis has been lost down to the level of the adipose layer, while laterally, the damage is less evident and involves only the upper part of the dermis. This difference, is reflected by the dramatic reappearance of hair follicles and sebacious glands at the periphery, together with aggregations of chronic inflammatory cells.

The adipose layer is grossly oedematous and subsequently occupies a more prominent position as the central fluid oedema gives way to a markedly more cellular oedema laterally, with mixed inflammatory cells both acute and chronic evident.

Sludging of the blood vessels is a feature of the dermis and muscle, with the trapped blood cells undergoing lysis. Immediately

beneath the centre of the wound area, the muscle is oedematous, pale staining and acellular with the widely separated muscle bundles showing early degeneration. At the periphery, the muscle is less oedematous although vessel sludging and degeneration of the muscle cells is still a feature. The muscle is much more cellular at the outer edges - a featured shared at this time with the adipose layer. This increase in cellularity is due to the presence of a mixed inflammatory cell infiltrate together with initial influx of fibroblasts.

CONTROL WOUND 2 days

The epithelium over the wound area has been replaced by a thin surface slough of dead epithelial and connective tissue remnants. At the edges, small, pale staining tongues of epithelium, are beginning to migrate beneath the coagulum and the superficial, localised aggregation of polymorphs. This migration demarcates the dermis that is to be retained, after removal of the slough.

The upper part of the dermis has been lost and replaced by the coagulum. The tissue beneath this area, in the wound centre, is more eosinophilic than normal and contains degenerating epithelial components. Small blood vessels within the dermis show sludging, with evident intravascular red blood cell breakdown. Although there are still isolated areas of acute inflammatory cells still able to aggregate, a mixed infiltrate emanates from the fat and muscle layers. At the wound perpiphery initial fibroblast aggregation is occurring.

Centrally there is marked oedema and disruption of the upper level of the adipose layer with a mild acute inflammatory infiltrate that

intensifies laterally, and includes a number of round cells. The blood vessels producing this inflammation laterally are patent vessels closely associated with the oedematous muscle capsule.

Beneath the capsule, there is marked separation of the muscle bundles but no obvious inflammatory infiltrate.

The forthcoming muscle cell degeneration is heralded by initial fibroblast and macrophage activity that intensifies at the periphery, where muscle cell degeneration has begun and thickening of some blood vessel walls can be seen. There are still many sludged blood vessels with intravascular degeneration of red blood cells.

EXPERIMENTAL WOUND 4 days

The mitotic activity and migration of the epithelium present at day 2 continues with increase vigour at the wound margins. The new epithelium bridging the defect, is slightly thicker than normal and is more basophilic. Beyond the tip of the advancing epithelium, the layer is well differentiated with prominent keratin formation.

Centrally the connective tissue framework of the dermis, is almost totally disrupted to the level of the adipose layer and is covered by the intense band of acute inflammatory cells that separates it from the surface coagulum. At the peripheries the wound area is demarcated by an intense cellular infiltrate of fibroblasts, macrophages and chronic inflammatory cells that lie within an area of initial granulation tissue formation. The remaining collagen in this area is very pale staining.

The adipose layer of the dermis is centrally very oedematous with sludged blood vessels still prominent and the appearance of new fine capillaries. Peripherally, the adipose layer is much less oedematous, although markedly more cellular, with intense aggregations of fibroblasts and macrophages, together with chronic inflammatory cells. These features are reflected again in the muscle which centrally is very eosinophilic and oedematous, while laterally, the margins, which extend further than the boundaries reflected in the dermis, are prominent, with a heavy infiltrate of fibroblasts, macrophages and round cells.

The muscle layer in many slides has been reduced to a very thin band. Initial granulation tissue can be seen at the lateral margins of the wound.

CONTROL WOUND 4 days

The coagulum, composed of dead and dying epithelial and connective tissue components and their ghost-like remains, lies over the wound area. At the edges there is an increased mitotic activity within the epithelium and migration at the edges produces a hyperplastic, darkly staining, well differentiated, parakeratinised epithelium edging its way across the wound.

The upper dermis centrally, has been replaced by the coagulum and it is sharply demarcated from the underlying dermis by an intense band of polymorphs that cover the upper part of the adipose tissue. Throughout the dermis there is evident intravascular red blood cell breakdown within the sludged blood vessels.

The lateral wound peripheries are indistinct. However, the absence of viable hair follicles and sebacious glands and aggregation of fibroblasts and round cells is a guiding feature to the main wound area which is saucer shaped.

The coagulum in the centre of the wound, is demarcated from the adipose layer, by an intense band of acute imflammatory cells that involve not only the upper level of the dermis, but also the upper part of the fatty hypodermis.

The deeper levels of the fat are without viable hair follicles and sebacious glands and show a diffuse inflammatory infiltrate with sludged blood vessels standing out within the marked oedema of this layer.

The peripheries of the adipose layer are intensely cellular, with aggregations of mixed inflammatory cells, macrophages and fibroblasts. There is a dramatic loss of muscle morphology and the connective tissue capsule of the muscle is very oedematous. Generally there is intense cellularity of the muscle layer due to aggregations of fibroblasts and macrophages around the degenerating muscle bundles. This cellularity decreases rapidly at the periphery which blends gradually to more normal muscle.

EXPERIMENTAL WOUND 6 days

At the peripheries, initial migration of the epithelium has produced a tongue of epithelium insinuating its way between the acute inflammatory reaction overlying the dermis and the underlying connective tissue. The new epithelium is somewhat hyperplastic in some areas,

shows maturing differentiation with conspicuous keratohyalin granules and a light parakeratinisation.

Centrally, the upper part of the dermis has been destroyed except for a very thin degenerating layer above the adipose tissue. Sludged blood vessels exhibiting red blood cell breakdown, can be seen within the dermis, which more laterally, beneath the advancing epithelium, shows granulation tissue formation with prominent dilated blood vessels. The wound area within the dermis, is still demarcated by the loss of specialised epithelial components such as hair follicles and sebacious glands. Chronic inflammatory cells can be seen aggregating at the wound margins rather than across the whole wound.

The adipose layer is still grossly oedematous and forms the floor of the primary wound area. Within this area a few sludged blood vessels remain visible but new blood vessels can also be seen across the wound at this level. The fat layer laterally is very cellular and fibroblasts, round cells and macrophages can be seen within the network of new blood vessels of the granulation tissue.

The fascial layer above the muscle is grossly inflamed laterally, and the wound margins show many chronic inflammatory cells.

The muscle at the centre has an infarcted appearance - virtually free of inflammatory cells and the muscle fibres are eosinophilic, oedematous and fragmenting. The margins show an intense cellular infiltrate of fibroblasts and round cells amongst dilated blood vessels some of which display a degree of fibrosis. The muscle gradually returns to a more normal morphology but at a point well past the margins indicated at the level of the epithelium.

CONTROL WOUND 6 days

There is evident epithelial migration at the wound edges. The new epithelium is hyperplastic, and well differentiated, with an evident basal layer and prominent keratohyalin granules and is lightly parakeratinised. The very tip of the ingrowth is pale-staining, one cell thick and has an indistinct nuclear outline.

Centrally little remains of the dermis and here, only the fatty hypodermis separates the coagulum from the muscle.

Laterally, the dermis is less effected although the collagen seems somewhat hyalinised. Beneath the advancing epithelium the more superficial part of the dermis contains small blood vessels, hemorrhage, plump fibroblasts and a diffuse round cell infiltrate that is indicative of young granulation tissue.

With the dermis all but gone, the adipose tissue, which is grossly oedematous with separation of the individual fat cells, forms the roof of the wound, separated from the coagulum by the intense acute inflammatory cell infiltrate.

The fatty hypodermis is less inflamed and oedematous laterally and initial granulation tissue can be seen as the layer thins to more normal dimensions.

The muscle layer which has a mild inflammatory infiltrate is fragmented and thinner. Enclosed in an oedematous capsule the individual bundles are degenerating and surrounded by an intense

aggregation of fibroblasts and hemosiderin filled macrophages. The central area of muscle is undergoing progressive fibrosis.

EXPERIMENTAL WOUND 11 days

The new epithelium remains as a tapering tongue of well differentiated though darkly staining epithelium 1-2 cells thick at its tip and having a thin parakeratinised surface.

The dermis centrally, is still absent, down to the adipose layer which is covered by the surface band of acute inflammatory cells.

The wound peripheries in the dermis, are still indistinct, though a range of healing features helps to outline the area of tissue involved.

The granulation tissue laterally is now forming mature scar tissue, with many of the fibroblasts oriented parallel to the surface. The young proliferative granulation tissue of the dermis is located immediately beneath the epithelial edge.

The adipose tissue in the centre is markely oedematous, and covered by a band of acute inflammatory cells. Beneath, it is supported by granulation tissue with prominent blood vessels and a cellular infiltrate down to what remains of the muscle layer. The granulation tissue has a mixture of macrophages and chronic inflammatory cells with young plump fibroblasts producing new collagen.

Laterally the adipose tissue with its inflammatory cells and granulation tissue thins to become almost non-existent - before gradually returning to normal.

The muscle layer in the centre remains pale staining and oedematous, with marked separation of the muscle bundles and a less cellular inflammatory infiltrate than the periphery. A young granulation tissue is evident amongst the damaged muscle bundles. Laterally the muscle damage extends past the margins depicted within the upper dermis and fibrosis of small blood vessels is apparent.

CONTROL WOUND 11 days

The covering epithelium is hyperplastic, darkly staining and moderately well differentiated, with some intraepithelial oedema and inflammation present in some cases. The more lateral epithelium thins and matures to normal depth and morphology.

A subepithelial area of granulation tissue persists centrally with many small blood vessels and plump fibroblasts that in many cases are beginning to orient parallel to the surface epithelium.

The edges of the wound area have a more mature granulation tissue component with initial peripheral fibrosis, that gradually blends into the less cellular, and more mature undamaged dermis.

The fatty hypodermis is an ill defined layer with disrupted morphology. It is oedematous with interwoven granulation tissue through out which is a diffuse round cell infiltrate.

The separated fat cells are surrounded by aggregations of fibroblasts and macrophages, while at the edges, there is fibrosis and loss of the adipose tissue.

The underlying muscle retains the heavy infiltration of fibroblasts and continuing fibrosis to the margins, with remnants of granulation tissue.

EXPERIMENTAL WOUND 14 days

The new epithelium growing across the wound is slightly more hyperplastic and hyperchromatic than normal apart from the centre where it is well differentiated with prominent keratohyalin granules and a well developed basal lamina.

Centrally the whole of the dermis has been damaged and replaced with a very cellular basophilic immature fibrous connective tissue that retains many of the features of maturing granulation tissue with many fibroblasts and round cells. These plump young fibroblasts are producing new collagen that in some slides has an orientation parallel to the surface epithelium. Beneath this the collagen assumes the more characteristic three dimensional orientation, which blends into the adipose layer all of which remains is a few encased fat cells.

Laterally, the area of scar tissue immediately below the surface epithelium, is less vascular and less inflamed and blends into normal dermis containing hair follicles and sebacious glands, which are the ultimate boundary of the wound.

In most specimens, the adipose layer has all but gone, however, others show it almost 'exploding' to the surface. The immature fibrous connective tissue that has replaced the adipose layer is very cellular and has a granulation tissue component in the form of some large blood

vessels. The adipose layer returns sharply to normal at the wound margins and a line of demarcation is obvious.

In some slides, the central area of muscle is not readily apparent as a separate layer, and is without distinct boundaries. Only a few small spindle shaped cells remain within a cellular fibrous connective tissue stroma, with many fibroblasts oriented parallel to the surface. In other slides the superficial muscle layer is grossly thickened and scarred with a diffuse round cell infiltrate. This central muscle damage gradually diminishes as the muscle layer thickens laterally and returns to normal morphology.

CONTROL WOUND 14 days

The covering epithelium is hyperplastic, well differentiated and parakeratinised with what appears to be attempts at rete peg formation.

The underlying dermis is basophilic and cellular with many small blood vessels and a mild round cell infiltrate among the maturing fibroblasts producing the collagen characteristic of the future scar tissue. There are no hair follicles or sebacious glands developing within the wound area and their presence laterally, helps depict the wound margins.

Laterally, the scar tissue is more mature with the fibroblasts and the collagen oriented parallel to the surface.

The adipose tissue is totally disrupted in the wound area, with what few cells remain being harhazardly arranged, and surrounded by many fibroblasts and immature collagen in a mild round cell infiltrate.

Although there are still many thin walled blood vessels the area is undergoing fibrosis. The upper level of the muscle layer is also fibrotic with an oedematous capsule containing aggregated fibroblasts producing collagen oriented parallel to the surface. Fibrosis of small blood vessels is also a feature within this layer.

EXPERIMENTAL WOUND 18 days

The wound centre is covered by a thickened parakeratotic epithelium that is without an evident basal lamina, but shows attempts at rete peg formation. The prickle cell layer is quite oedematous. Laterally, the epithelium covering the more mature dermis remains thickened with a well defined polarity and parakeratin formation. The maturity of the overlying epithelium in the centre determines the nature of the underlying dermis in this area. Well epithelialised wounds show an immature scar tissue whereas recently, or wounds just epithelialised, display a mature granulation tissue. Beneath this lies a mature area of fibrous scar tissue, which is very basophilic and cellular with many plump young fibroblasts producing collagen that orients parallel to the surface.

The peripheries of the dermal scar tissue blend gradually into less basophilic normal dermis, with the collagen oriented haphazardly, and the return of hair follicles and sebacious glands.

In the centre of the wound area only remnants of the adipose tissue remain, interspersed in either a very cellular densely collagenous region of scar tissue or maturing granulation tissue. In the more mature wounds the central area of scar tissue occupying the region of the fatty

layer, displays many plump fibroblasts, foamy macrophages, with the fibroblasts and collagen in some slide oriented parallel to the surface. Epithelium this area thins out laterally as the adipose layer abruptly returns to normal at the wound margins. Peripherally the scar tissue is less basophilic with fibroblasts and collagen less densely aggregated but still often oriented parallel to the surface.

The underlying muscle is present centrally as a thin remnant and has been incorporated into the central area of scar tissue that is undergoing complete fibrosis. The muscle remnants are surrounded by many fibroblasts and macrophages, together with a diffuse round cell infiltrate.

The muscle fibrosis decreases laterally but marked fibroblastic activity is still present, but diminishing, as unaffected muscle is encountered.

CONTROL WOUND 18 days

A well differentiated, hyperplastic, parakeratinised epithelium covers the wound centre. The junction betwen new and old eipthelium is gradual with the thinning at the periphery into a paler well differentiated epithelium associated with prominent hair follicles and sebacious glands.

The central area of the dermis is composed of densely basophilic though vascular scar tissue with both collagen and fibroblasts oriented parallel to the surface.

Laterally, the line of demarcation between the area of scar tissue and bordering normal dermis is abrupt, with dramatic differences in collagen appearance and orientation and the re-appearance of the specialised epithelial components.

The adipose layer is almost non-existent in the centre of the specimens at 18 days and the isolated remnants are surrounded by dense scar tissue, in which the fibroblasts and collagen are oriented again parallel to the surface. This remains so even laterally to the wound margin, though in this position, the area of what was the fatty hypodermis is even more cellular with densely packed fibroblasts.

Centrally, the muscle layer is slightly thinner and more cellular, with the individual bundles appearing smaller and paler staining. The upper part of the muscle has undergone fibrosis, while the deeper layers, while not badly fibrosed, are more cellular with many fibroblasts and a mild round cell infiltrate that diminishes gradually to the periphery.

EXPERIMENTAL WOUND 21 days

The covering epithelium is slightly hyperplastic well differentiated and has a thin layer of parakeratin. This new epithelium blends gradually at the lateral margins with the old undamaged epithelium.

The dermis in the centre of the specimen, is very cellular, avascular and basophilic with plump young fibroblasts producing collagen oriented parallel to the surface in the upper region. Beneath this, the

orientation of the fibroblasts and collagen is more haphazard, as the connective tissue takes on a more mature appearance. The superficial area laterally, is mainly scar tissue and it's basophilic cellularity is sharply demarcated from the adjacent normal dermis which has prominent hair follicles and sebacious glands. The lateral area of scar tissue extends past the major wound area. The adipose layer in the centre of the wound is non-existent and has been replaced by avascular mature scar tissue with the fibroblasts and collagen oriented parallel to the surface. The scar tissue extends laterally to normal adipose tissue outside the wound.

Changes to the muscle are variable, ranging from superficial fibrosis, to damage of the whole depth of muscle, destroying the layer as a functional unit. A mild to marked inflammatory infiltrate is present and thick walled blood vessels are evident through the layer. In the lateral areas the muscle damage blends gradually into normal muscle but at a point beyond the obvious dermal boundaries of the wound.

CONTROL WOUND 21 days

The covering epithelium is darkly staining, well differentiated, regular, parakeratinised epithelium that is losing its hyperplastic appearance.

The upper part of the dermis is made up of densely cellular, basophilic scar tissue with parallel orientation of fibroblasts and collagen. Beneath this the scar tissue is more mature with small blood vessels and the collagen assuming a more normal appearance with whorls and thickening of the fibres.

The wound margins in the dermis are well defined laterally and the cellularity diminishes as does the orientation of the collagen within the scar tissue. The adipose layer returns abruptly, with reappearance of hair follicles and sebacious glands.

The adipose layer centrally, has been replaced by dense scar tissue, with a marked round cell infiltrate. Apart from a few trapped fat cells this scar tissue extends to the periphery.

The muscle morphology centrally has been disrupted, and it is thinner, more cellular with evident fibrosis of the upper portion and a marked round cell infiltrate. The muscle laterally returns to normal morphology with a less basophilic, acellular appearance and less fibrosis.

EXPERIMENTAL WOUND 25 days

The slightly hyperplastic, parakeratinised epithelium over the centre of the wound is not completely differentiated but does show good keratin formation with an evident basal layer.

Beneath this the basophilic scar tissue occupying the upper part of the dermis is heavily infiltrated with plump young fibroblasts laying down collagen parallel to the surface epithelium. This tissue is still reasonably vascular with fine capillaries and venules visible. Beneath the upper part of the dermis, is a less dense region of scar tissue which is a markedly more vascular area with more mature fibroblasts and collagen with a normal orientation. Laterally, this area blends into more normal dermis, a change that at low power is quite abrupt. The adipose layer is notably absent with only a few cells remaining; replaced by densely collagenous mature scar tissue.

Above the muscle, lies a diffuse band of hemosiderin filled macrophages that extends in part into the upper part of the muscle. The muscle has thinned out and become fibrotic with widely separated spindle shaped muscle cells within a fibroplastic stroma. This remains so to the wound margins where normal morphology gradually returns.

CONTROL WOUND 25 days

The epithelium covering the wound area is darker staining, remains slightly hyperplastic, is moderately well differentiated and parakeratinised.

The central area of scar tissue within the upper dermis is composed of densely cellular basophilic scar tissue with collagen orientated parallel to the surface. This primary area of scar tissue has a dense population of plump fibroblasts, numerous new blood vessels, and retains a mild chronic inflammatory cell infiltrate. Hemosiderin filled macrophages can be seen scattered through the area.

Beneath this zone the collagen replacing the fatty hypodermis has assumed a more normal hap-hazard arrangement as both the fibroblasts and collagen mature. Areas of intense but localised aggregations of round cells can be seen above the muscle. Laterally, the change at the periphery is abrupt with the return of the adipose layer and its incumbent hair follicles and sebacious glands.

The upper zone of muscle has undergone fibrosis, with a diffuse infiltrate of fibroblasts among the separated muscle bundles. The wound area has generally thinned and the area of damage is depicted by the evident cellularity.

6.4.1 COMPARATIVE HISTOPATHOLOGY

Time	Control	Experimental
15 mins.	An indistinct wound showing initial degenerative changes to epithelial cells, connective tissue oedema, but little inflammatory cellular infiltrate, has escaped from the many small sludged blood vessels.	An indistinct wound showing degenerative changes to epithelial cells, and connective tissue including cellular inflammation of muscle and adipose layers, wherein vessel sludging is apparent.
24 hrs.	A well defined wound still covered by epithelium but showing marked degenerative changes of epithelium, dermis and muscle amidst a generalised acute inflammatory infiltrate.	A well defined wound with a covering coagulum of degenerated epithelial and connective tissue, with damage down to and including the muscle, which demonstrates peripheral round infiltration cell.
2 days	A well defined wound covered by a coagulum of degenerated epithelial and connective tissue components and demonstrating underlying connective tissue oedema, degeneration and peripheral fibroblast and round cell infiltration.	A coagulum covered wound showing gross tissue loss to the adipose layer and showing initial epithelial ingrowth and peripheral round cell and fibroblast infiltration.
4 days	A coagulum covered wound showing initial epithelial migration with evident chronic inflammation and initial fibroblast acitivity peripherally.	A coagulum covered wound showing epithelial migration, central tissue loss and oedema and peripheral granulation and cellular infiltration with round cells and fibroblasts.
6 days	Epithelial migration covers peripheral granulation tissue formation within the dermis. The central area of muscle is undergoing fibrosis.	Epithelial migration covers peripheral granulation tissue within the dermis. The lateral area of the muscle is undergoing maturation of granulation tissue.
11 days	Wound is almost completely epithelialised over a central area of remaining granulation tissue around which	Epithelium is continuing to migrate across a granulating base extending through what remains of the dermis.

	peripheral fibrosis of the dermis is evident including the underlying muscle.	The peripheral granulation tissue is maturing to form early scar tissue. The underlying muscle is undergoing initial fibrosis.
14 days	A parakeratinised hyperplastic epithelium covers a well defined area of maturing scar tissue within the underlying dermis and muscle.	A well defined wound area with a parakeratinised, hyperplastic epithelium covering maturing scar tissue within the dermis, and muscle, which is grossly fibrotic.
18 days to 25 days	The covering epithelium matures to a regular parakeratinised stratified squamous epithelium that apart from the absence of rete pegs hair follicles and sebacious glands is very much like the original epithelium. The dermis underlying this epithelium gradually matures to become densely collagenous scar tissue with the fibroblasts and collagen oriented parallel to the surface. The adipose layer is almost nonexistent and the ultimate boundary of the scar area is determined by the return of hair follicles and sebacious glands. The muscle layer immediately beneath - is slightly thinner and atrophic in the upper zone with smaller paler muscle bundles and evident fibrosis.	The covering epithelium matures to a regular, parakeratinised, stratified squamous epithelium without rete pegs, hair follicles or sebacious glands.The underlying dermis matures to a well defined zone of densely collagenous scar tissue with collagen fibres oriented parallel to the surface. Only remnants of the adipose layer remain within the wound area. The muscle layer beneath has become very atrophic and fibrotic with widely separated spindle shaped remnants of muscle bundles within a fibroblastic stroma.

Fig 29a 15 minute experimental specimen.

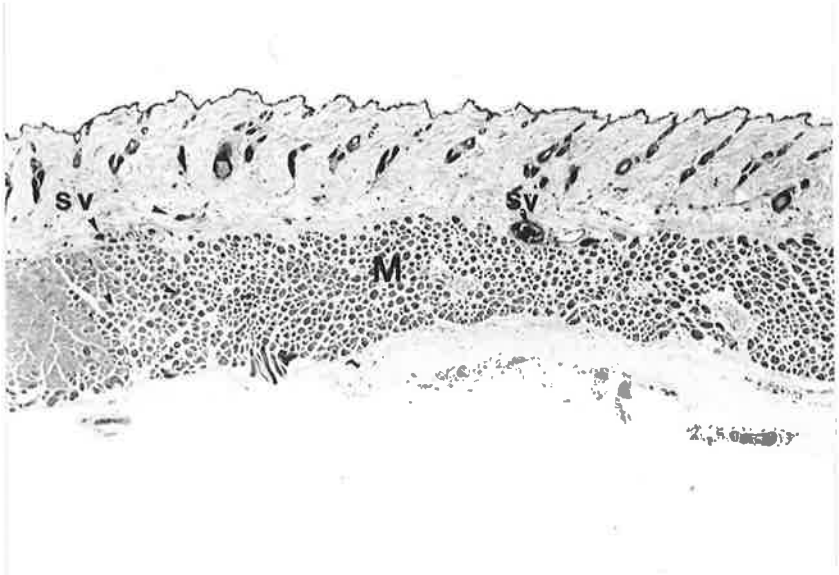


Fig 29b 15 minute control specimen.

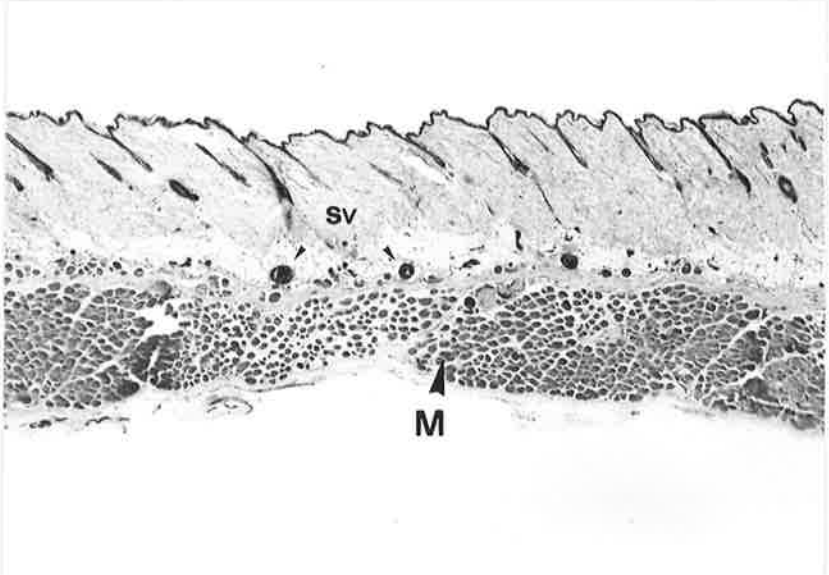


Fig 29a 15 minute experimental specimen

This low power (x10) section shows only an indistinct wound, with obvious sludging of blood vessels (S.V.) and oedema of of the muscle zone (M).

Fig 29b 15 minute control specimen

This low power (x10) section shows an indistinct wound area with obvious vessel sludging (S.V.) and oedema of the muscle zone (M).

Fig 30a 15 minute experimental specimen.

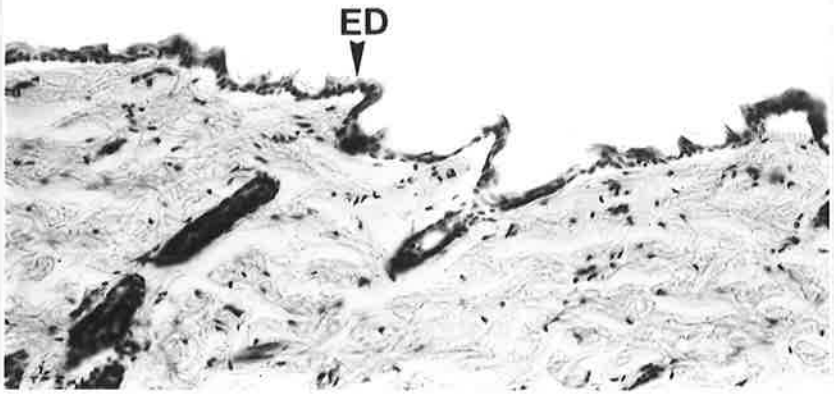


Fig 30b. 15 minute control specimen.

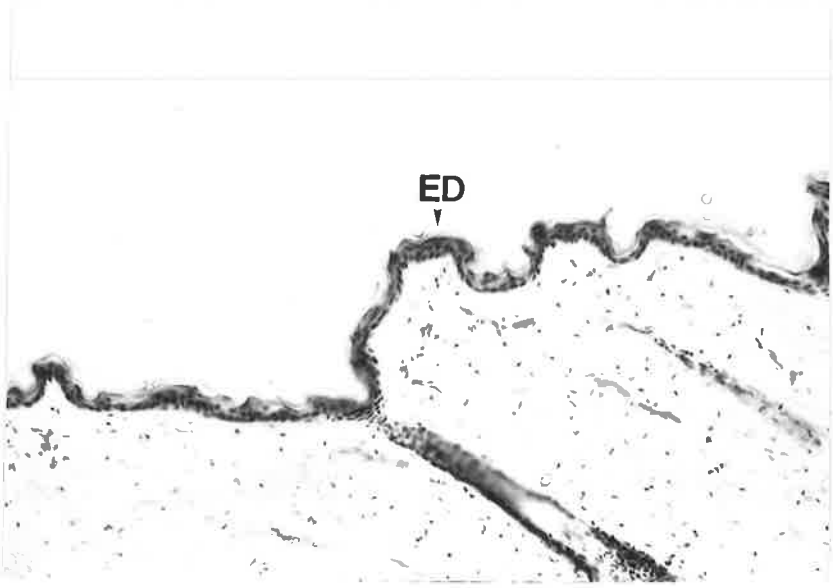


Fig 30a 15 minute experimental specimen

High power (x40) view showing initial epithelial degeneration (E.D.)

Fig 30b 15 minute control specimen

High power (x40) section showing initial epithelial degeneration (E.D.)

Fig 31a 24 hour experimental specimen.

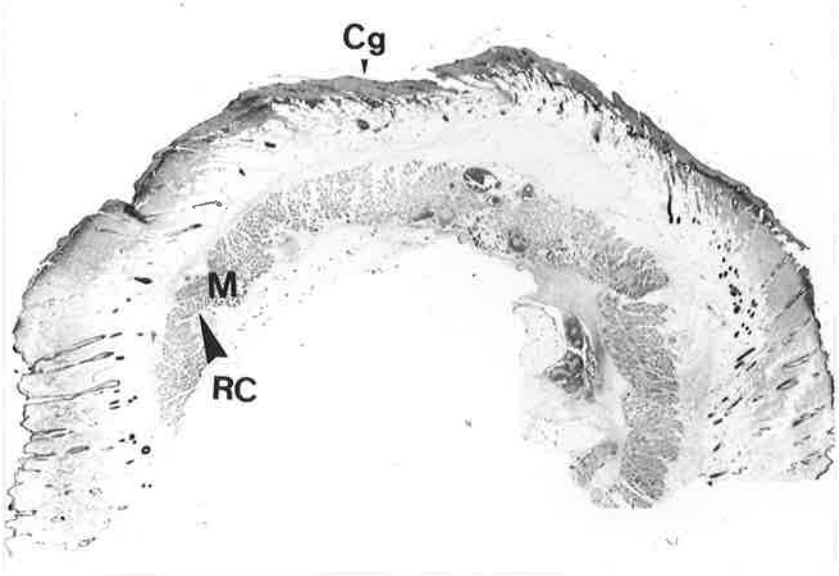


Fig 31b. 24 hour control specimen.

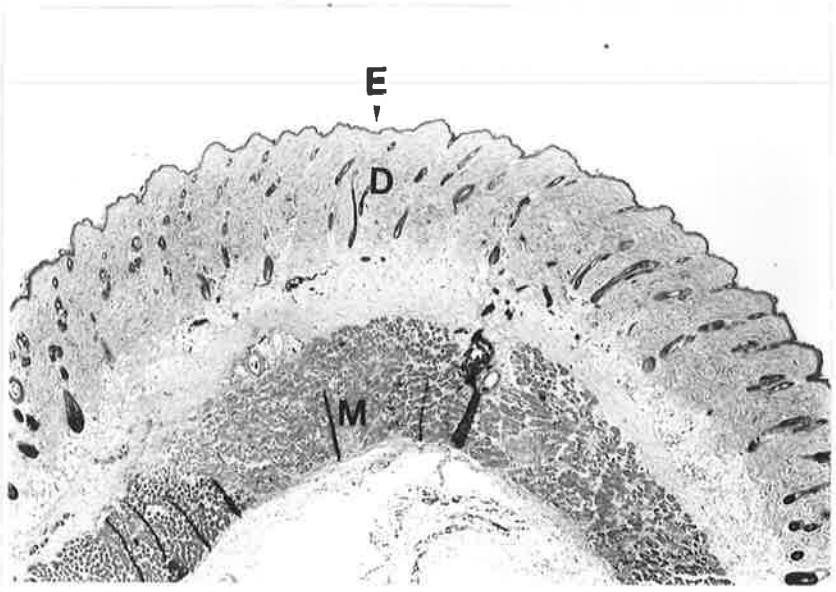


Fig 31a 24 hour experimental specimen

This low power (x10) section shows a well defined wound with a covering coagulum (Cg) of degenerated epithelial cells and connective tissue components with evident tissue damage down to and including the muscle (M), which demonstrates peripheral round cell (R.C.) infiltration

Fig 31b 24 hour control specimen

A low power (E x10) section showing a well defined wound still covered by epithelium but displaying marked degenerative changes of the epithelium (E), dermis (D) and muscle (M) amidst a generalized inflammatory infiltrate.

Fig 32a 24 hour experimental specimen.

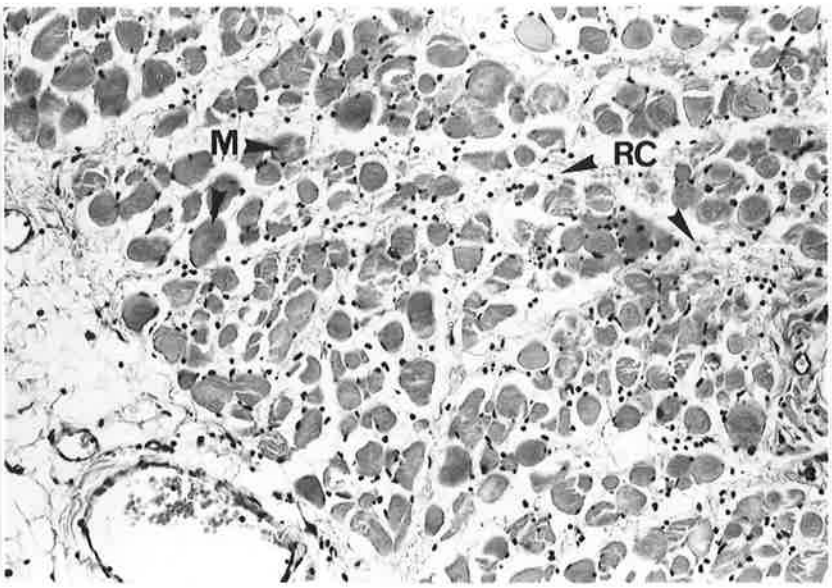


Fig 32b. 24 hour control specimen.

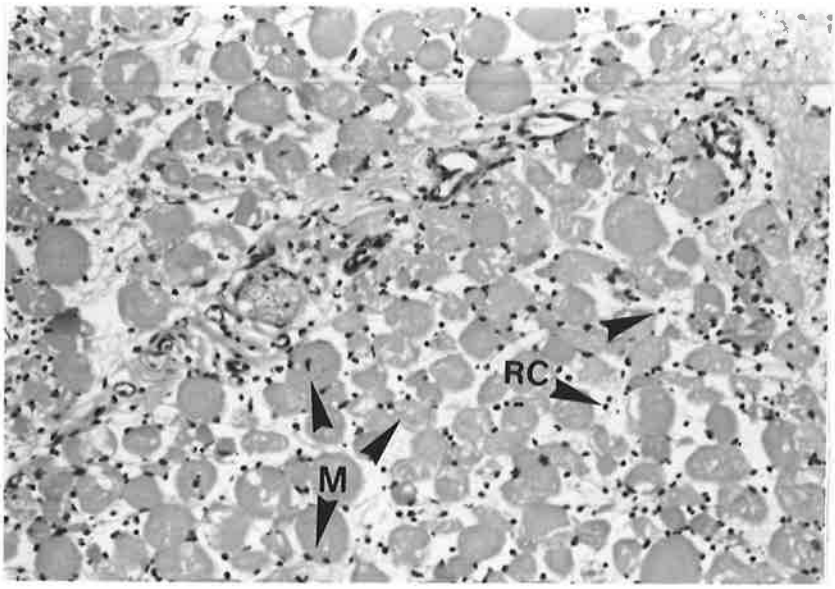


Fig 32a 24 hour experimental specimen

High power (x40) view showing round cell (R.C.) infiltration muscle (M)

Fig 32b 24 hour control specimen

High power (x40) view showing muscle bundle (M) separation and round cell infiltration (R.C)

Fig 33a 2 day experimental specimen.

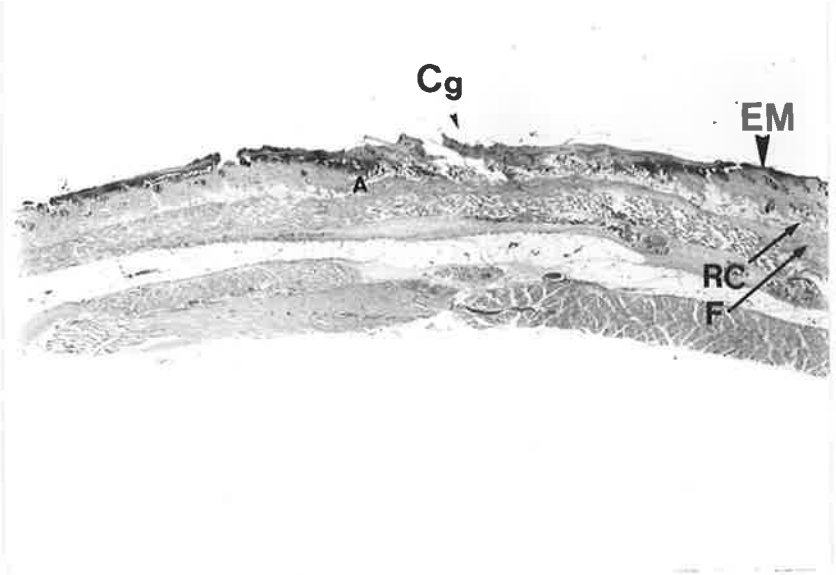


Fig 33b. 2 day control specimen.

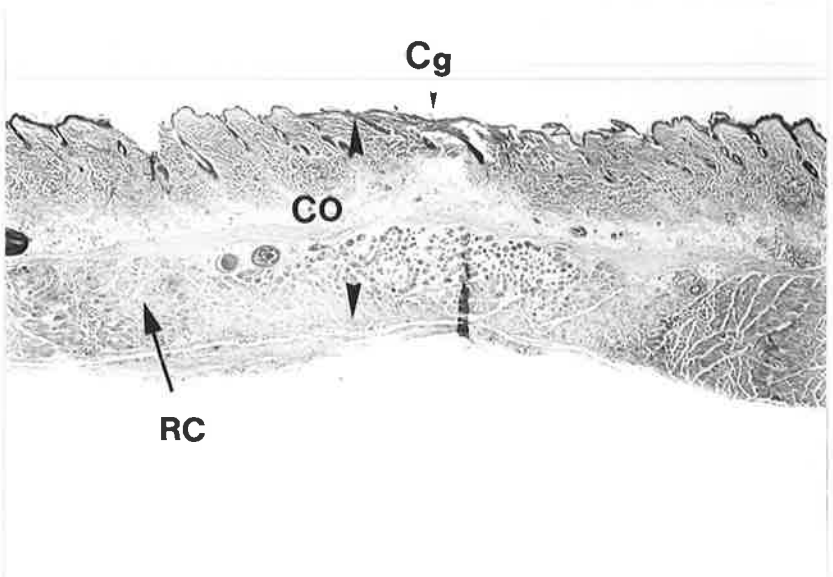


Fig 33a 2 day experimental specimen

This low power (x10) section shows a coagulum (Cg) covered wound with gross tissue loss to the adipose zone (A) and showing initial epithelial ingrowth, (E.M.) periphelial round cell (R.C.) and fibroblast infiltration.

Fig 33b 2 day control specimen

A low power (x10) section showing an obvious defect covered by a coagulum of degenerated epithelial and connective tissue components (Cg) and demonstrating underlying connective tissue oedema (C.O.) degeneration and a peripheral round cell (R.C.) infiltrate.

Fig 34a 2 day experimental specimen.

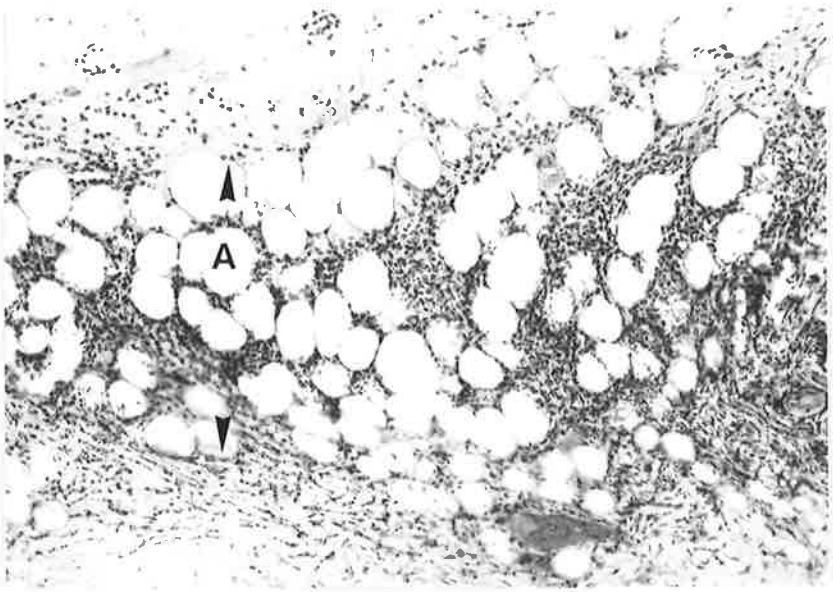


Fig 34b. 2 day control specimen.

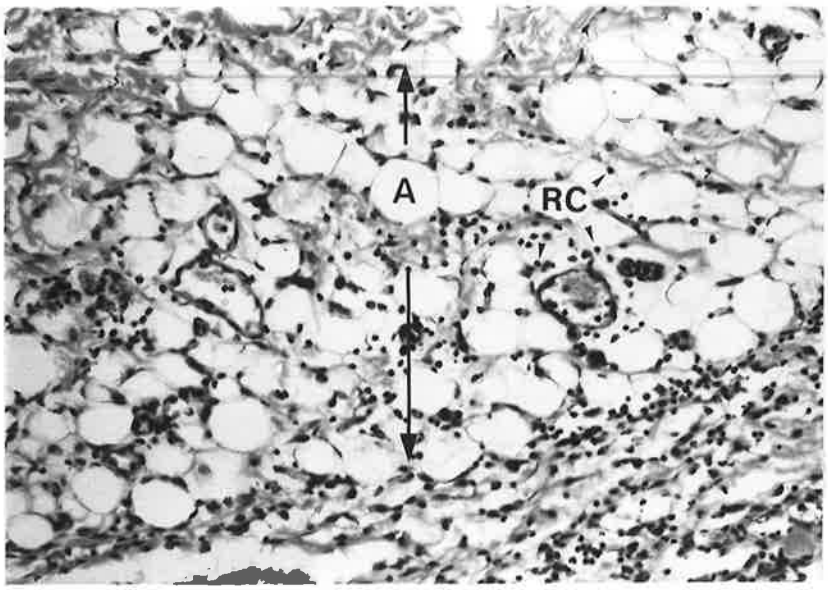


Fig 34a 2 Day experimental specimen

High power (x40) view oedema of the adipose zone (A)

Fig 34b 2 day control specimen

High power (x40) view of round cell (R.C.) infiltration of adipose (A)

Fig 35a 4 day experimental specimen.

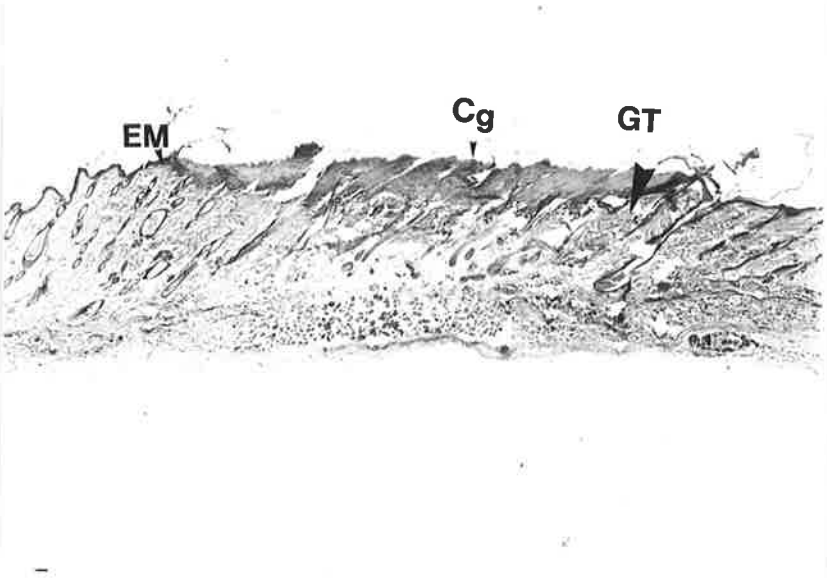


Fig 35b. 4 day control specimen.

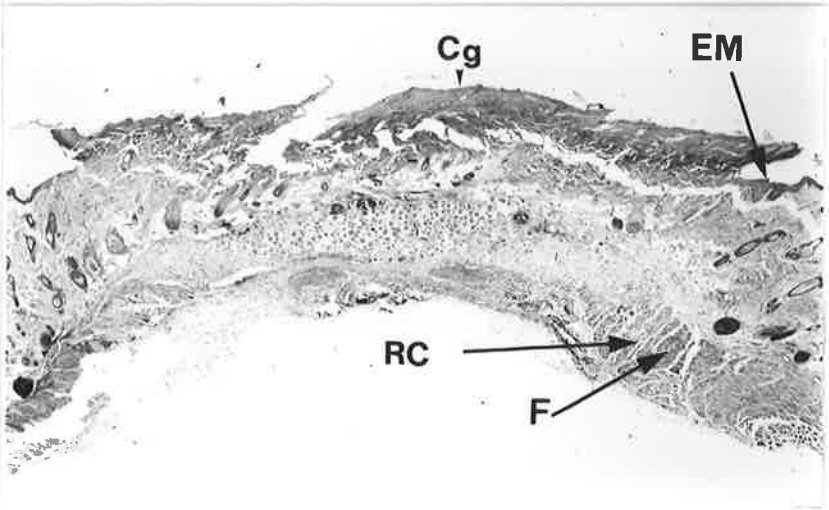


Fig 35a 4 day experimental specimen

This low power (x10) shows again the coagulum (Cg) covering the central tissue loss and oedema. Epithelial migration (E.M.) continues over areas of granulation tissue (G.T.), cellular infiltration with round cells and fibroblasts.

Fig 35b 4 day control specimen

This low power (x10) section shows a coagulum (Cg) covered wound with initial epithelial migration (E.M.), the presence of a round cell (R.C.) and fibroblast (F) infiltrate peripherally.

Fig 36a 4 day experimental specimen.

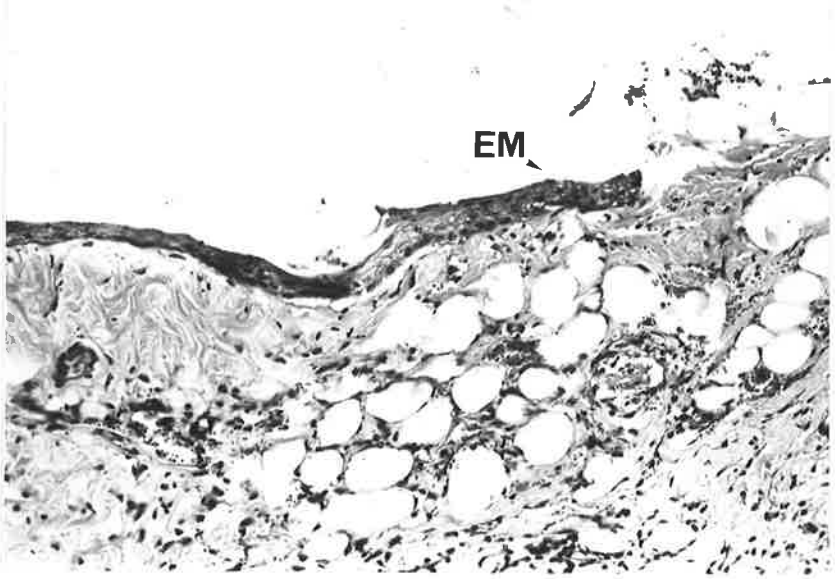


Fig 36b. 4 day control specimen.

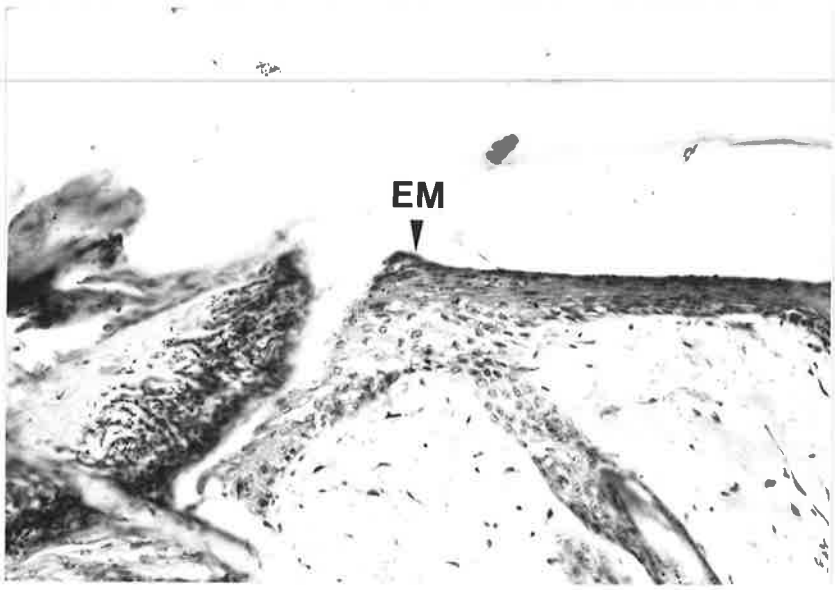


Fig 36a 4 day experimental specimen

High power (x40) view showing epithelial migration (E.M.)

Fig 36b 4 day control specimen

High power (H and E x40) view of epithelial migration (E.M.)

Fig 37a 6 day experimental specimen.

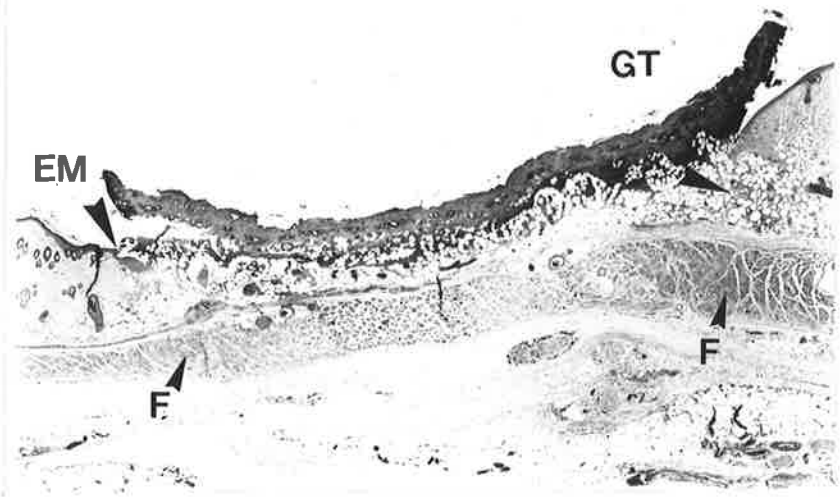


Fig 37b. 6 day control specimen.

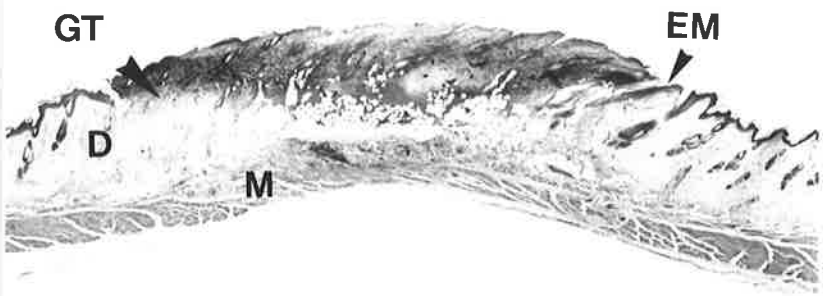


Fig 37a 6 day experimental specimen

This low power (x10) shows epithelial migration (E.M.) covering the lateral areas of granulation tissue (G.T.) within the dermis. The lateral area of muscle is heavily infiltrated with fibroblasts (F).

Fig 37b 6 day control specimen

In this low power (x10) section epithelial migration (E.M.) covers the peripheral granulation tissue (G.T.) within the dermis (D).
The central area of muscle (M) is undergoing fibrosis.

Fig 38a 6 day experimental specimen.

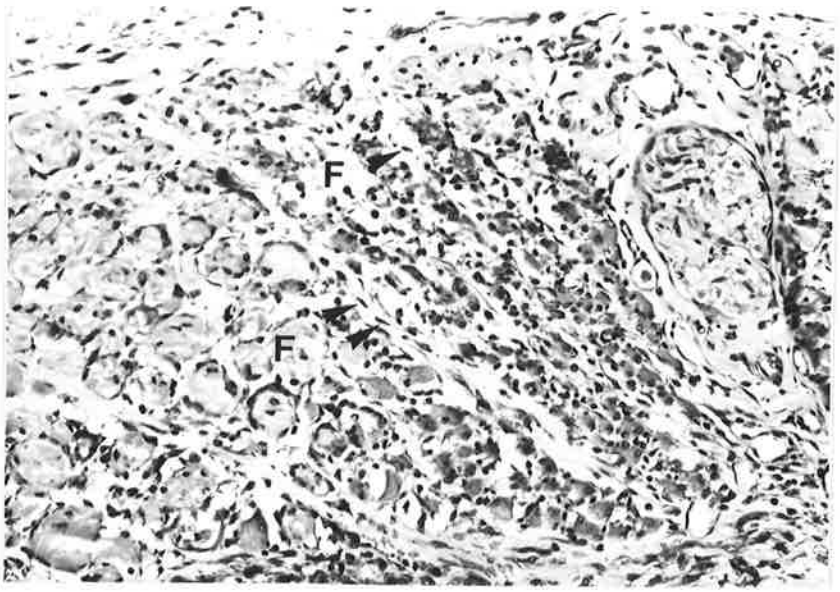


Fig 38b. 6 day control specimen.

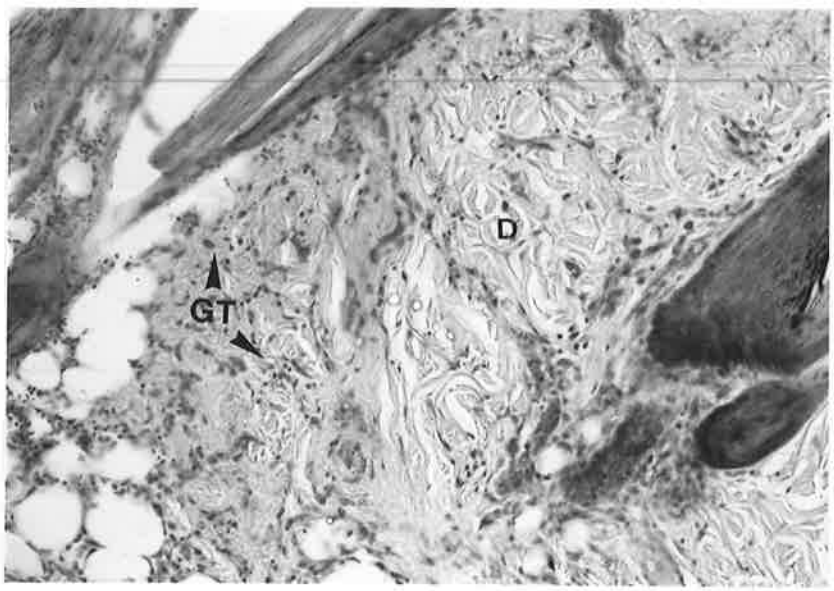


Fig 38a 6 day experimental specimen

High power (x40) view showing aggregation of fibroblasts (F) within the muscle.

Fig 38b 6 day control specimen

High power (x40) view showing peripheral granulation tissue (G.T.) in the dermis (D).

Fig 39a 11 day experimental specimen.

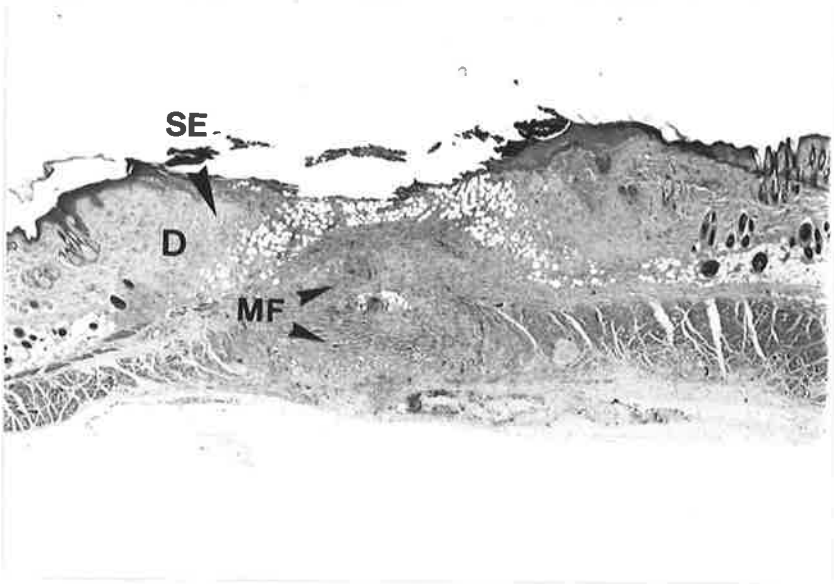


Fig 39b. 11 day control specimen.

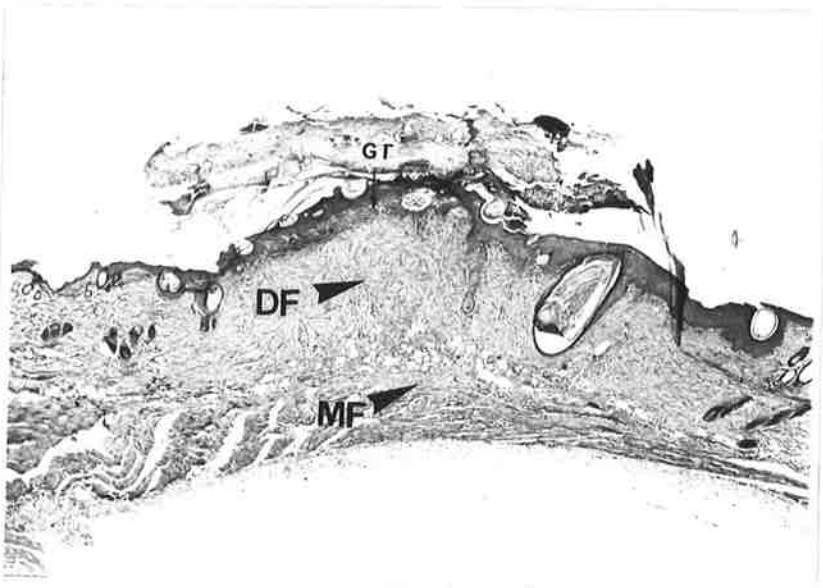


Fig 39a 11 day experimental specimen

In this low power (x10) specimen epithelium continues to migrate across a granulating base extending through what remains of the dermis (D). The peripheral granulation tissue (G.T.) is maturing to form early scar tissue (S.E.). the underlying muscle is undergoing initial fibrosis (M.F.)

Fig 39b 11 day control specimen

In this low power (x10) section the wound is almost completely epithelialised over a central area of remaining granulation tissue (G.T.) around which peripheral fibrosis of the dermis (D.F.) is evident including the underlying muscle (M.F.)

Fig 40a 11 day experimental specimen.

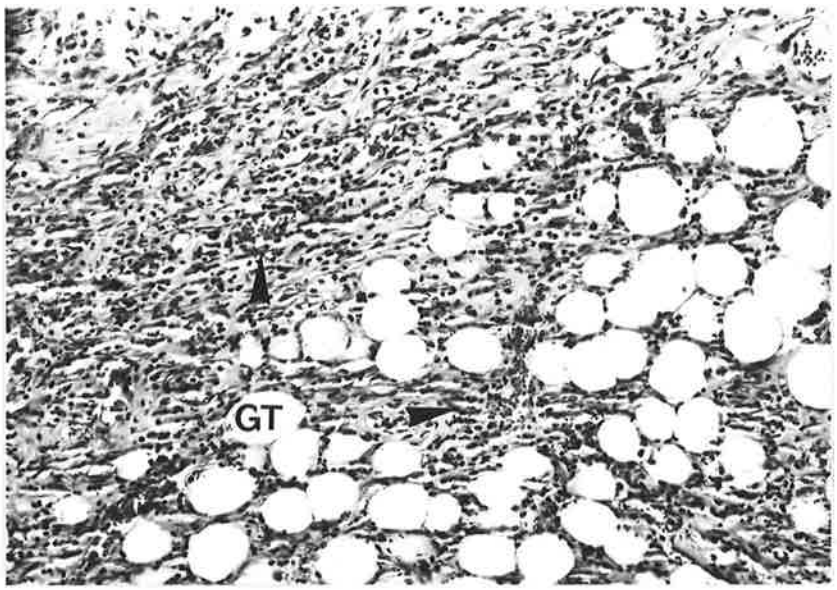


Fig 40b. 11 day control specimen.

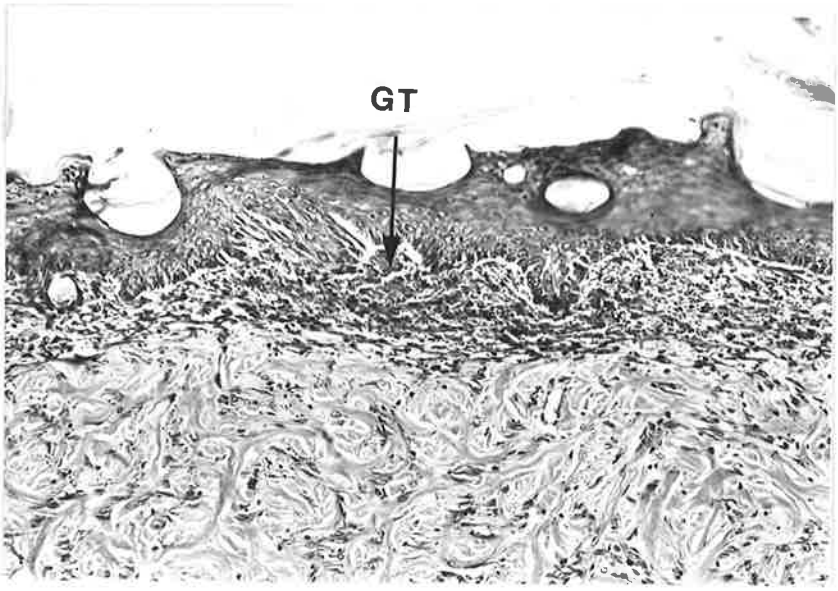


Fig 40a 11 day experimental specimen

High power (x40) view showing granulation tissue in the adipose zone (G.T.)

Fig 40b 11 day control specimen

High power (x40) view showing the central area of granulation tissue (G.T.)

Fig 41a 14 day experimental specimen.

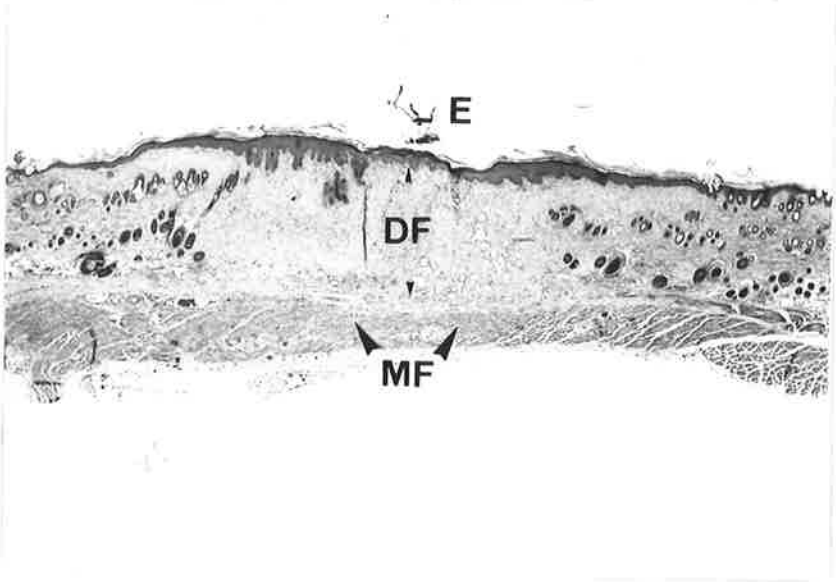


Fig 41b. 14 day control specimen.

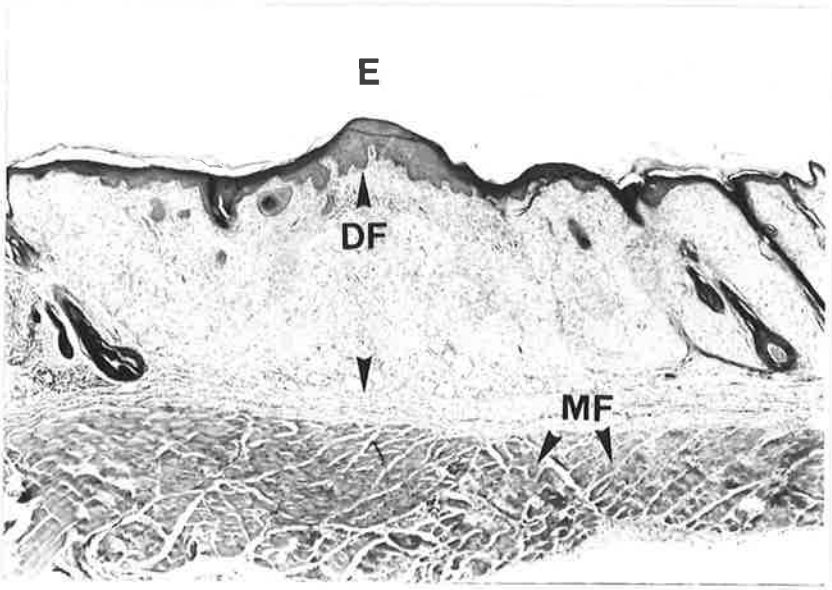


Fig 41a 14 day experimental specimen

A low power (x10) view of a well demarcated wound area with a parakeratinised, hyperplastic epithelium (E), covering maturing scar tissue (DF) within the dermis and fibrotic muscle (MF)

Fig 41b 14 day control specimen

This (x10) section shows a parakeratinised hyperplastic epithelium (E) covering a well defined area of maturing scar tissue within the underlying dermis (DF) and muscle (MF).

Fig 42a 14 day experimental specimen.

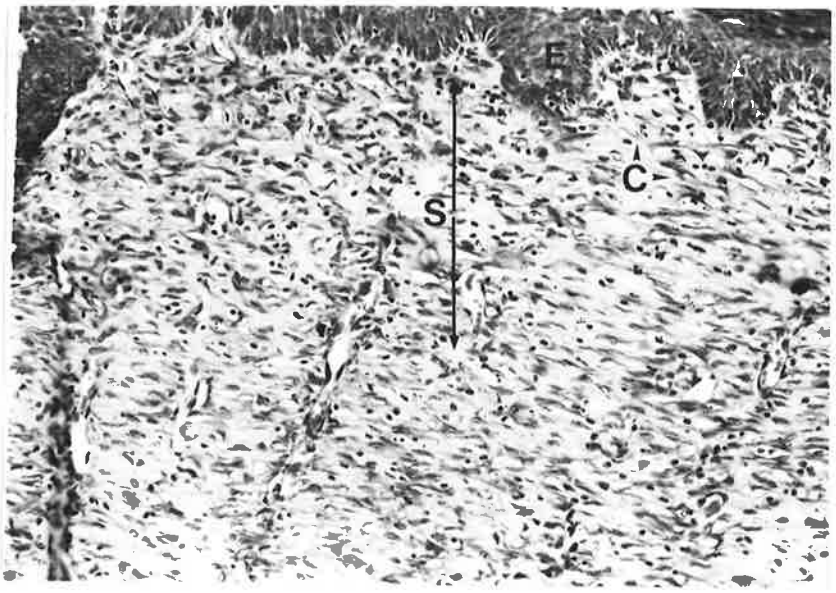


Fig 42b. 14 day control specimen.

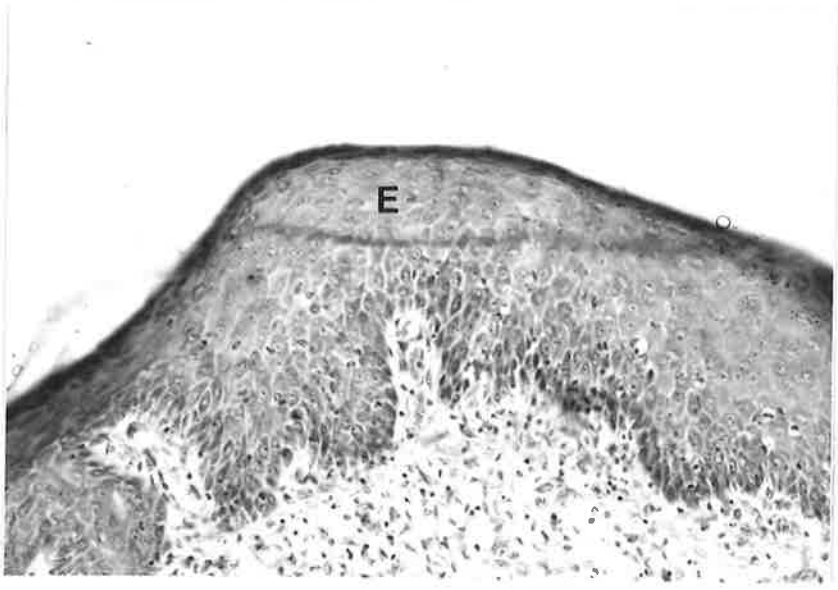


Fig 42a 14 day experimental specimen

A high power (x40) view of the maturing scar tissue (S) with collagen (C) oriented parallel to the surface epithelium (E)

Fig 42b 14 day control specimen

High power (x40) view of maturing epithelium over the wound (E)

Fig 43a 18 day experimental specimen.

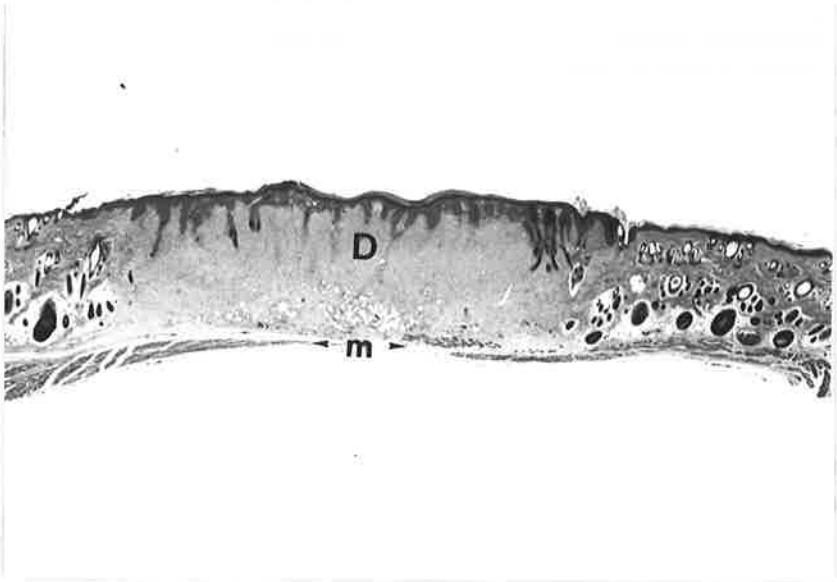


Fig 43b. 18 day control specimen.

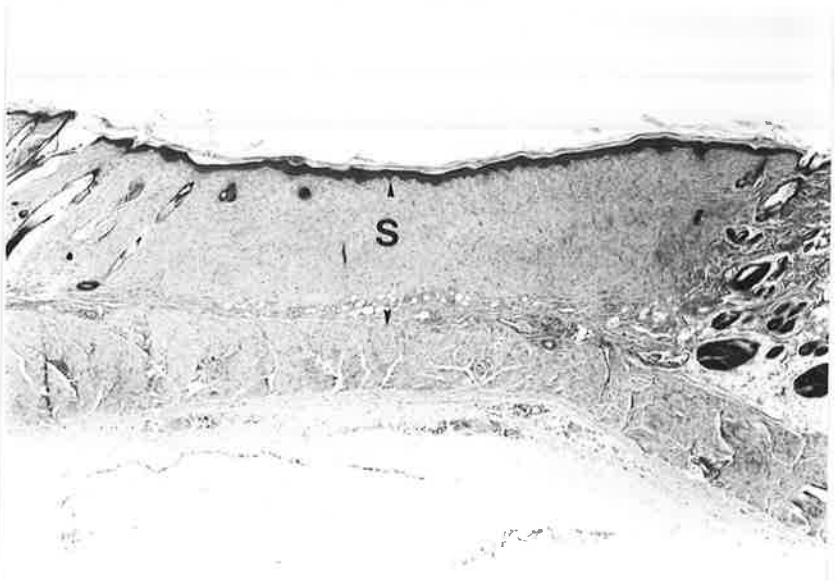


Fig 43a 18 day experimental specimen

This low power (x10) section shows a fully epithelialised wound displaying an area of basophilic scar tissue through the dermis (D) down to and including the underlying muscle (M)

Fig 43b 18 day control specimen

This low power (x10) section shows a well defined fully epithelialised area of scar tissue (S) extending through the dermis and involving the upper layers of the muscle.

Fig 44a 18 day experimental specimen.

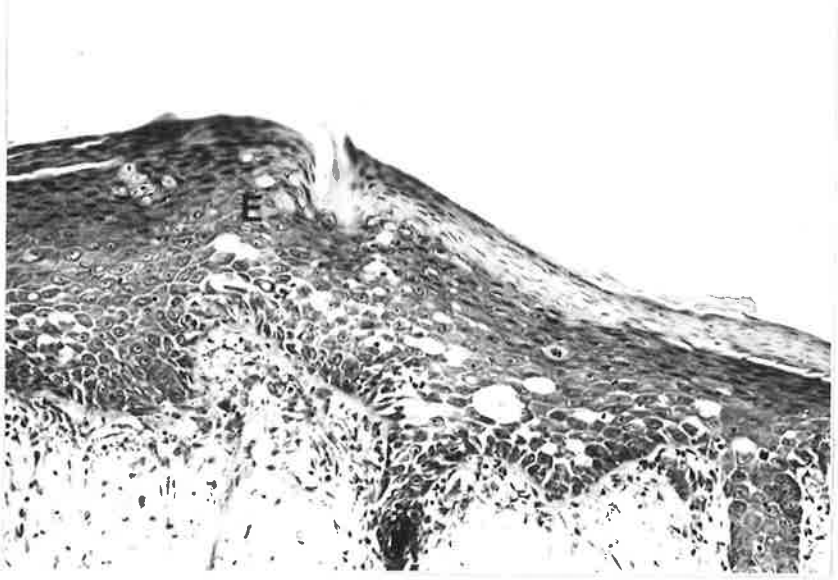


Fig 44b. 18 day control specimen.

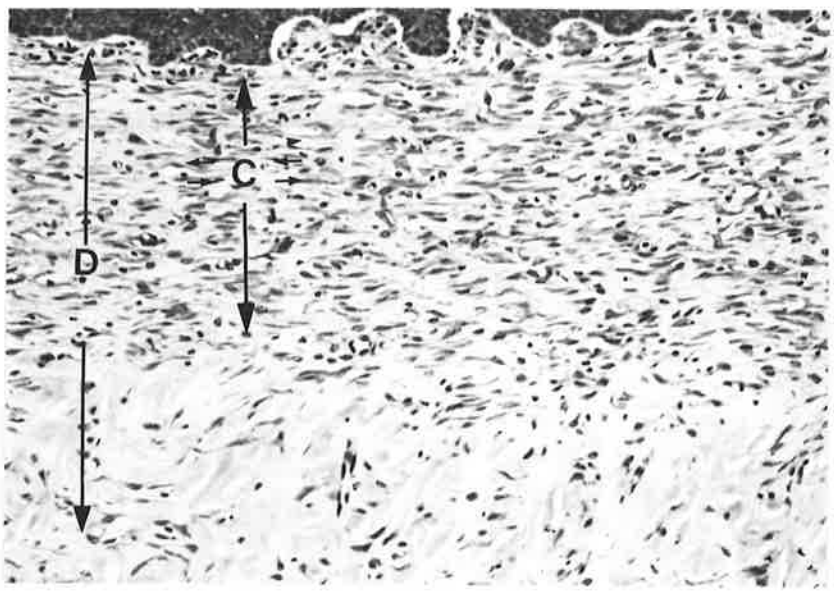


Fig 44a 18 day experimental specimen

High power (x40) view showing maturing epithelium covering the wound area.

Fig 44b 18 day control specimen

High power (x40) view showing maturing collagen (C) orientation within the dermis (D)

Fig 45a 21 day experimental specimen.

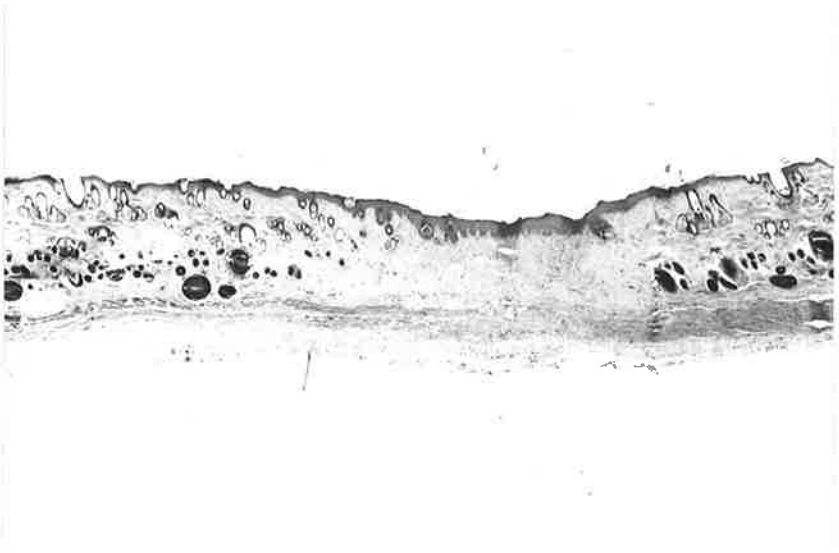


Fig 45b. 21 day control specimen.

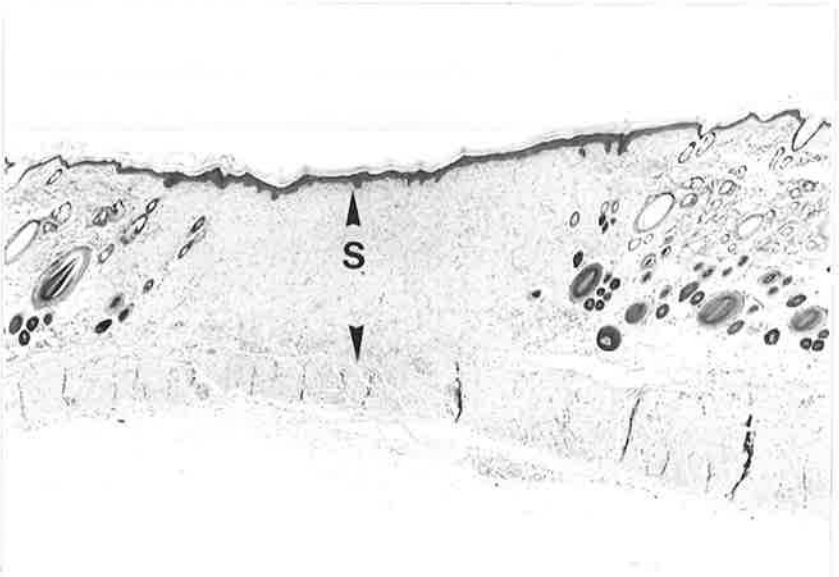


Fig 45a 21 day experimental specimen

This low power section (x10) shows the same features as at day number 18.

Fig 45b 21 day control specimen

This low power (x10) section is basically the same as at 18 days with an area of scar tissue (S) extending through the dermis and muscle.

Fig 46a 21 day experimental specimen.

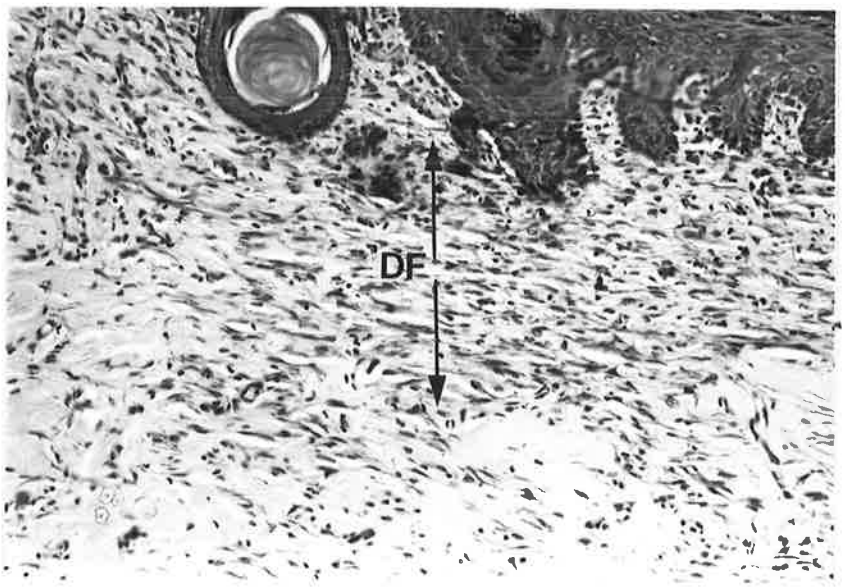


Fig 46b. 21 day control specimen.

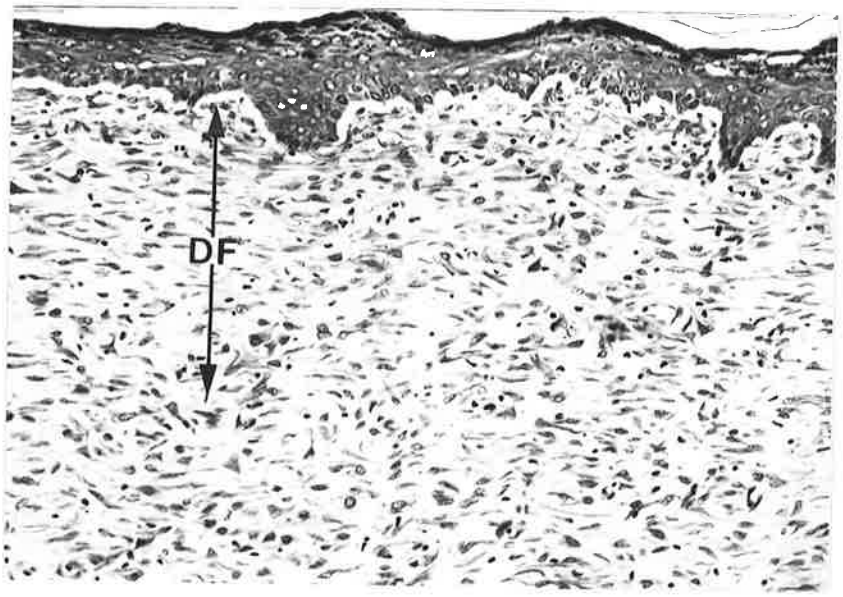


Fig 46a 21 day experimental specimen

High power (x40) view of maturing scar tissue in the dermis (D.F.) and orientation of collagen fibres.

Fig 46b 21 day control specimen

High power (x40) view showing maturing scar tissue within the dermis (D.F.)

Fig 47a 25 day experimental specimen.

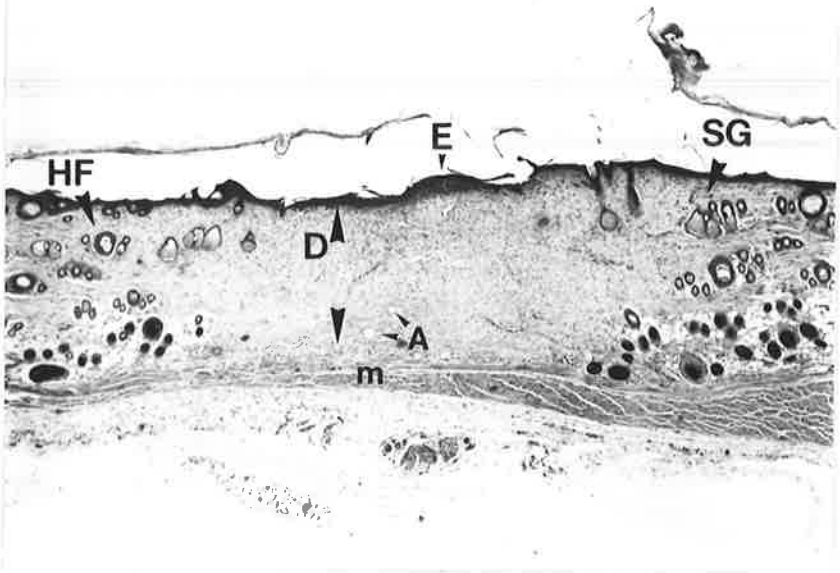


Fig 47b. 25 day control specimen.

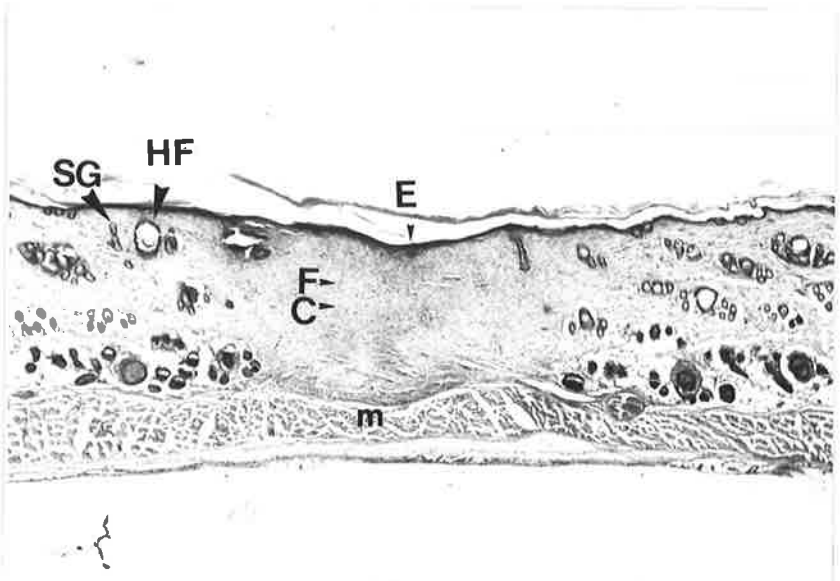


Fig 47a 25 day experimental specimen

In this low power (x10) section the covering epithelium (E) is regular, parakeratinised and stratified squamous in type. There are no rete pegs, hair follicles or sebacious glands in the wound area. The dermis (D) is densely collagenous with collagen fibres (C) oriented parallel to the wound surface. Only remnants remain of the adipose layer (A) overlying the muscle layer (M) which is very atrophic with widely separated spindle shaped remnants of muscle fibres.

Fig 47b 25 day control specimen

This low power (x10) section shows a regular parakeratinised stratified squamous epithelium (E) that apart from the absence of rete pegs, hair follicles (H.F) and sebacious glands (S.G) is very much like the original. The underlying dermis is densely collagenous with fibroblasts (F) and collagen (C) oriented parallel to the surface. The adipose layer is non-existent and the underlying muscle (M) is slightly thinner and atrophic in its upper zone.

Fig 48a 25 day experimental specimen.

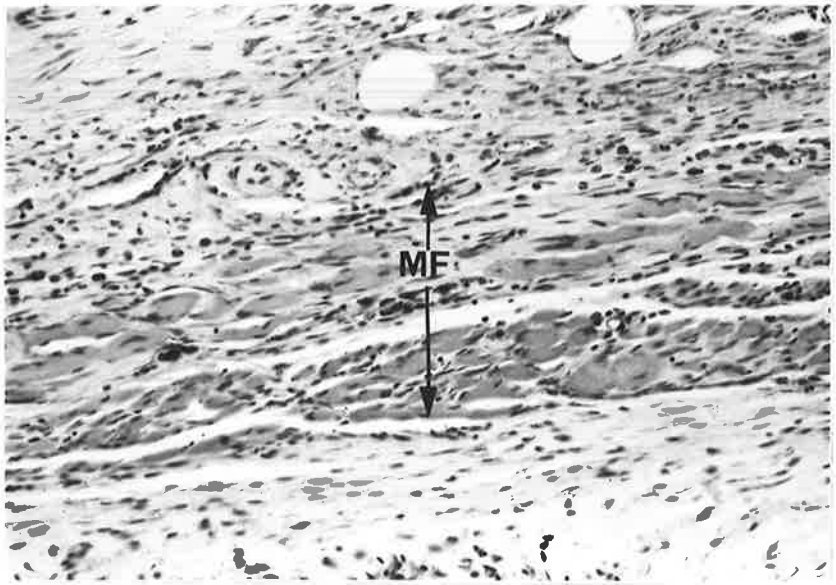


Fig 48b. 25 day control specimen.

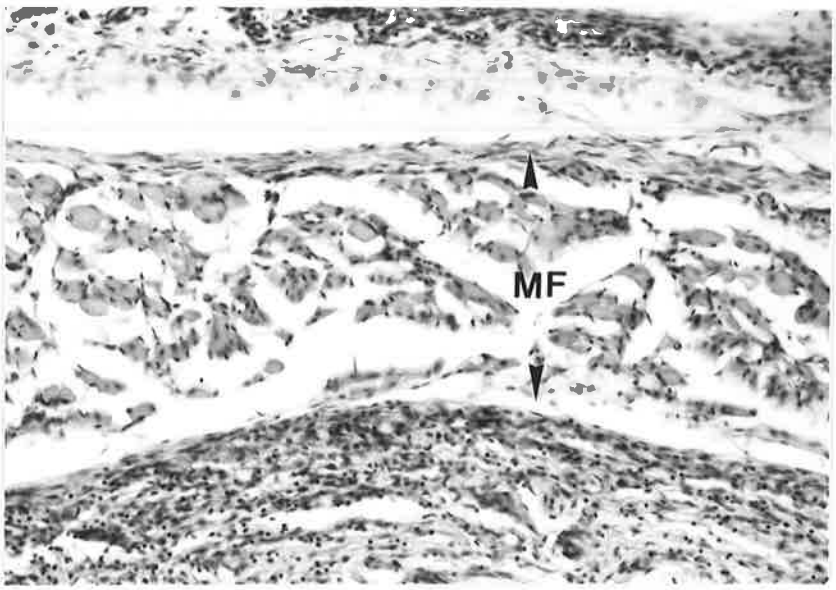


Fig 48a 25 day experimental specimen

A high power (x40) view showing residual muscle fibrosis (M.F.)

Fig 48b 25 day control specimen

High power (x40) view showing residual muscle fibrosis (M.F.)

6.5 DATA ANALYSIS

6.5.1 DATA ANALYSIS - MACROSCOPIC

(a) Iceball Diameter

From both experimental and control series, five animals were chosen to carry out iceball measurements as described in chapter 5.

The time periods chosen were 24 hours, 4 days, 11 days, 18 days and 25 days as four representative time periods. The sites chosen were at random to avoid a time or location bias in iceball creation. Table 2b.

The results of these measurements can be seen depicted graphically in Figure 49.

The mean values between experimental and control groups were compared at different time periods by means of a student 't' test. See Appendix.

There was found to be no significant difference between the size of the iceball at any of the 1st, 2nd, or 3rd freeze times.

The results show that the iceball diameter between the 2nd and 3rd freeze times in both the experimental and control groups did not change significantly. The final cryo freeze had little or no effect on subsequent iceball size.

(b) Thaw Time

The determined values for thaw time after consecutive freezes are presented in graphic form in Figure 50.

In both experimental and control groups the thaw time rose to a peak by the end of the second freeze, then dropped by the third freeze.

The mean thaw times in both experimental and control groups were compared by means of a students 't' test.

The experimental group demonstrated a longer thaw time only after the initial freeze, thereafter, the control group had consistently longer thaw times.

The results show a significant difference at the $p \leqq 0.05$ level for each freeze.

(c) Thermocouple Readings

Serial thermocouple readings were made at the iceball periphery in both the experimental and control series, at 15 min., 48 hrs., 6 days, 14 days, 21 days.

The results of these readings are depicted in Figure 51.

Fig 49.

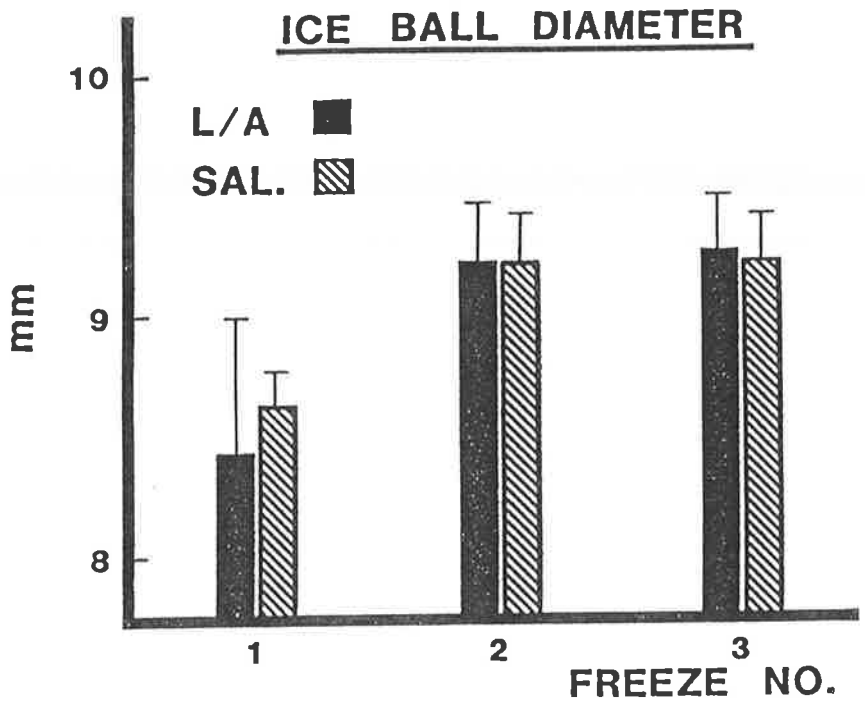

ICE BALL DIAMETER
10
9
8
mm
L/A
SAL.
1
2
3
FREEZE NO.

Fig 50

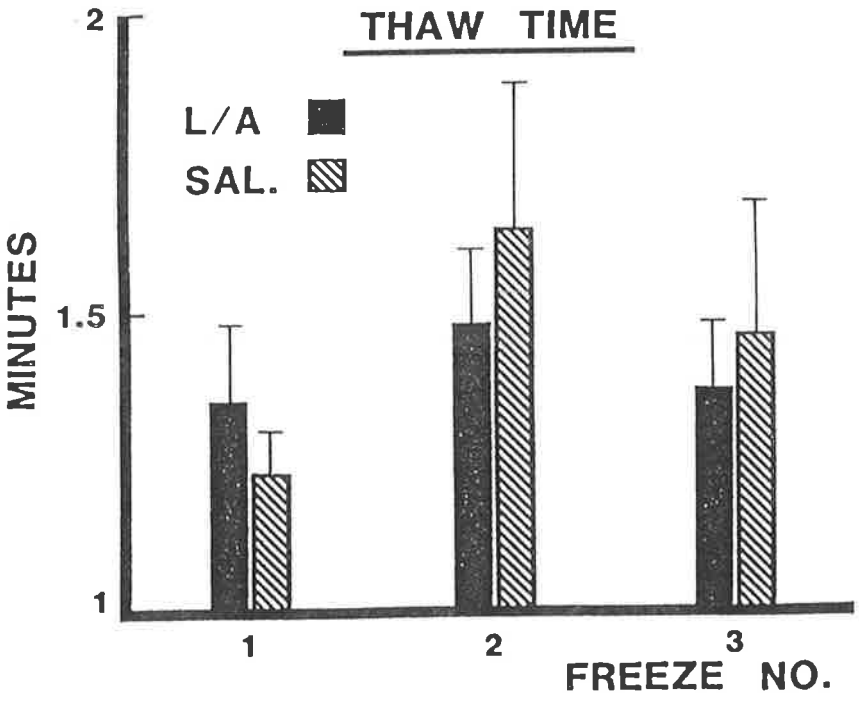

THAW TIME
2
1.5
1
MINUTES
L/A
SAL.
1
2
3
FREEZE NO.

Fig 51.

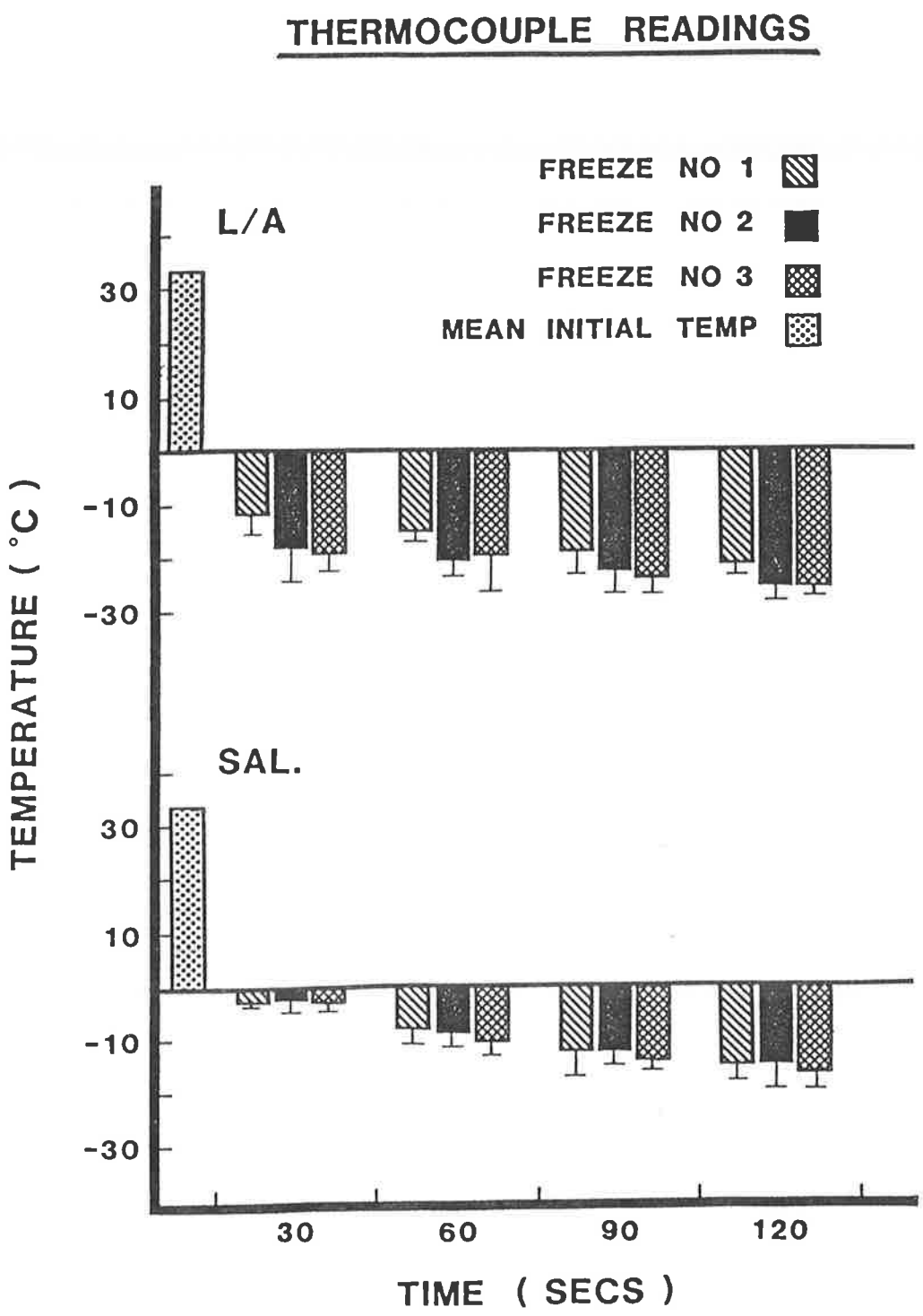
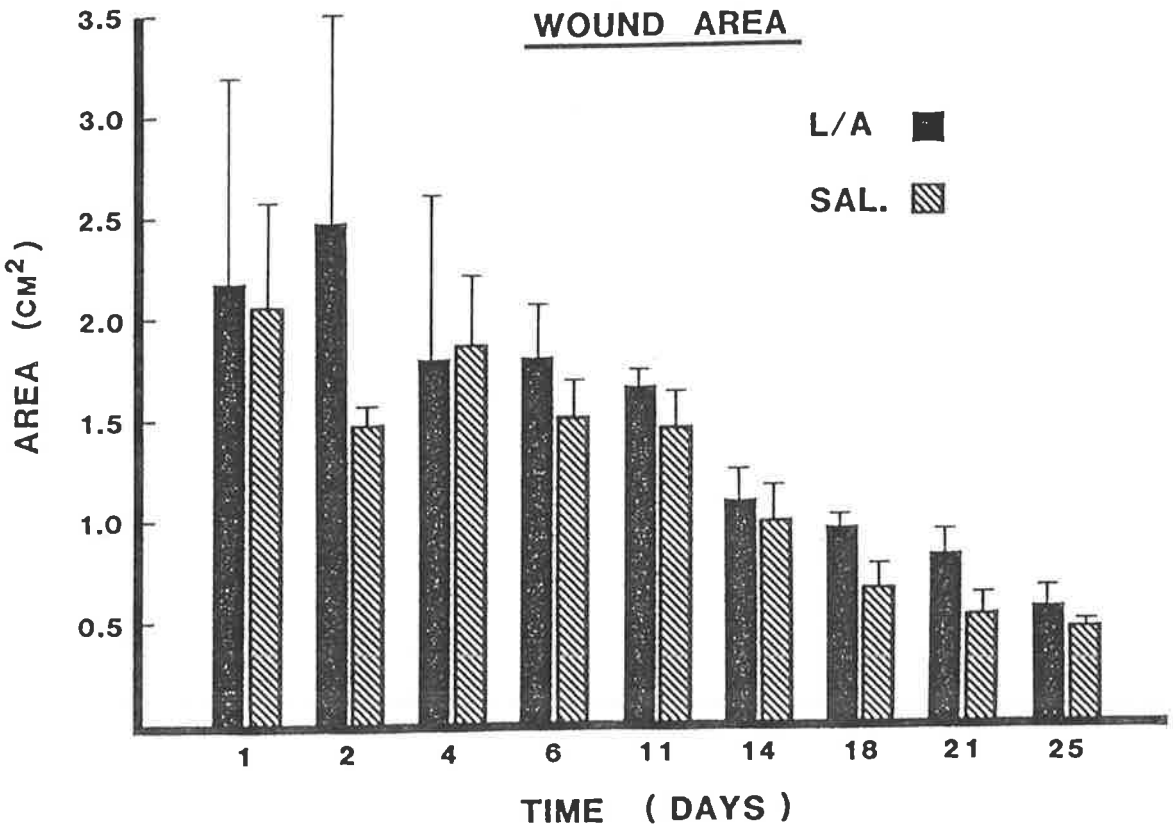

THERMOCOUPLE READINGS
FREEZE NO 1
FREEZE NO 2
FREEZE NO 3
MEAN INITIAL TEMP
L/A
SAL.
30
10
-10
-30
TEMPERATURE (°C)
30
60
90
120
TIME (SECS)

Fig 52.


WOUND AREA
L/A
SAL.
AREA (CM2)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1
2
4
6
11
14
18
21
25
TIME (DAYS)

(d) Wound Area

The mean gross wound area at each time interval for both experimental and control groups is depicted in Fig 52.

At all times the wounds created using local aneasthetic with cryo surgery are consistently larger and remain so until healing is complete.

The rate of change of wound area is, however, approximately the same in both groups.

6.5.2 DATA ANALYSIS - MICROSCOPIC

Inflammation

Cell counts were taken to express both acute and chronic inflammation across the wound area at the different levels within the wound.

The cell count results were then converted to scores as detailed in Chapter 5 and the proforma in Tables 3 and 4.

The scores were tabulated for:

1. Total Inflammation.
2. Inflammation of the Dermis.
3. Inflammation of the Adipose Tissue.
4. Inflammation of the Muscle.

The values so determined can be seen expressed as histograms in Figures 53 to 60 inclusive.

1. Total Inflammation Figs 53, 54.

Important differences can be seen to exist between the experimental and control groups when the total inflammation across the wounds is compared.

(i) The experimental animals have a faster acute reaction, which persists longer than the controls.

(ii) The chronic reaction in the experimental animals is more intense than the controls.

(iii) Acute and chronic reactions in experimental animal re-peak over the course of wound healing.

(iv) Both the acute and chronic reaction in the control group did not agree in general with what would be regarded as a 'normal' time/occurrence process of wound healing. Though this can be both tissue and wound dependant. Hurley et al., (1966).

2. Inflammation within the Dermis Figs 55, 56.

The acute response in the experimental group was more rapid and persisted longer than in the control group.

Comparison of the chronic response between the two groups showed unusual differences.

(i) The chronic response in the control group showed a peak at 2 days that was greater than the response in the experimental group. This was due to an intense localised chronic response in one animal and and not reflect accurately the events as a whole.

(ii) The chronic response in the experimental series peaked unusually at day 18, again due to unusually intense cell clumping in one animal.

3. Inflammation of the Adipose Tissue Figs 57, 58.

Inflammation within the adipose zone fluctuated as the chronic inflammatory cells moved from a lateral to medial aggregation within the wound. This is shown in Figure 58 where both experimental and control groups display another peak around 11-14 days post-op. Although the chronic response was faster in the experimental group they presented a comparable overall picture.

The acute response was more immediate and sustained in the experimental group.

4. Inflammation of the Muscle Figs 59, 60.

Acute inflammatory events were rapid and short-lived in the muscle layer. The acute response was more severe in the experimental animals

and was maintained for a longer period than the controls; which showed a loss of reaction by day 6. The chronic response fluctuated in both series but the experimental animals retained a moderate chronic activity throughout the term of the experiment.

Epithelial Migration

The rate of epithelial migration was assessed, and the results of this assessment are depicted graphically in Figure 61.

In the control group the rate of epithelial migration was consistently faster up to the completion of epithelialisation of the wounds.

The control wounds were fully epithelialised by day 11 whereas the experimental wounds continued to close until day 14.

Fig. 53

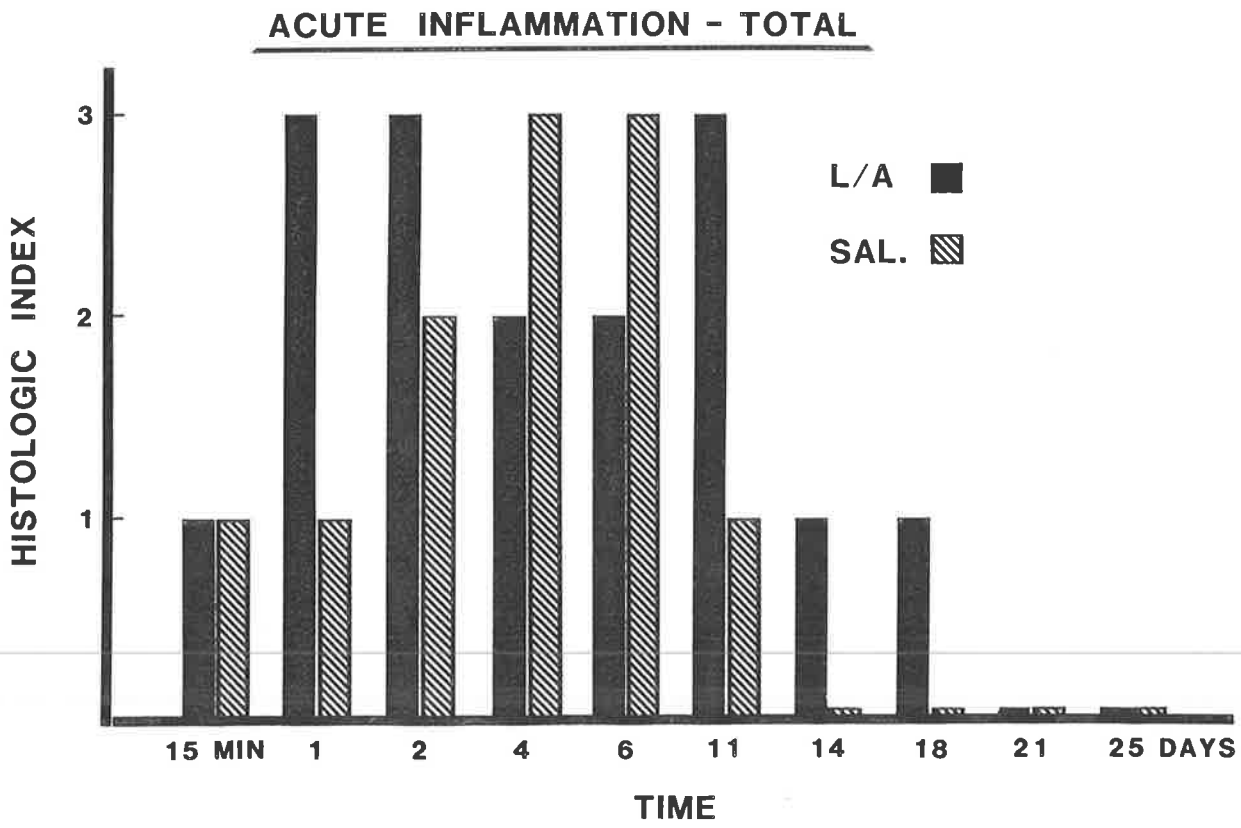

ACUTE INFLAMMATION - TOTAL
HISTOLOGIC INDEX
3
2
1
L/A
SAL.
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 54

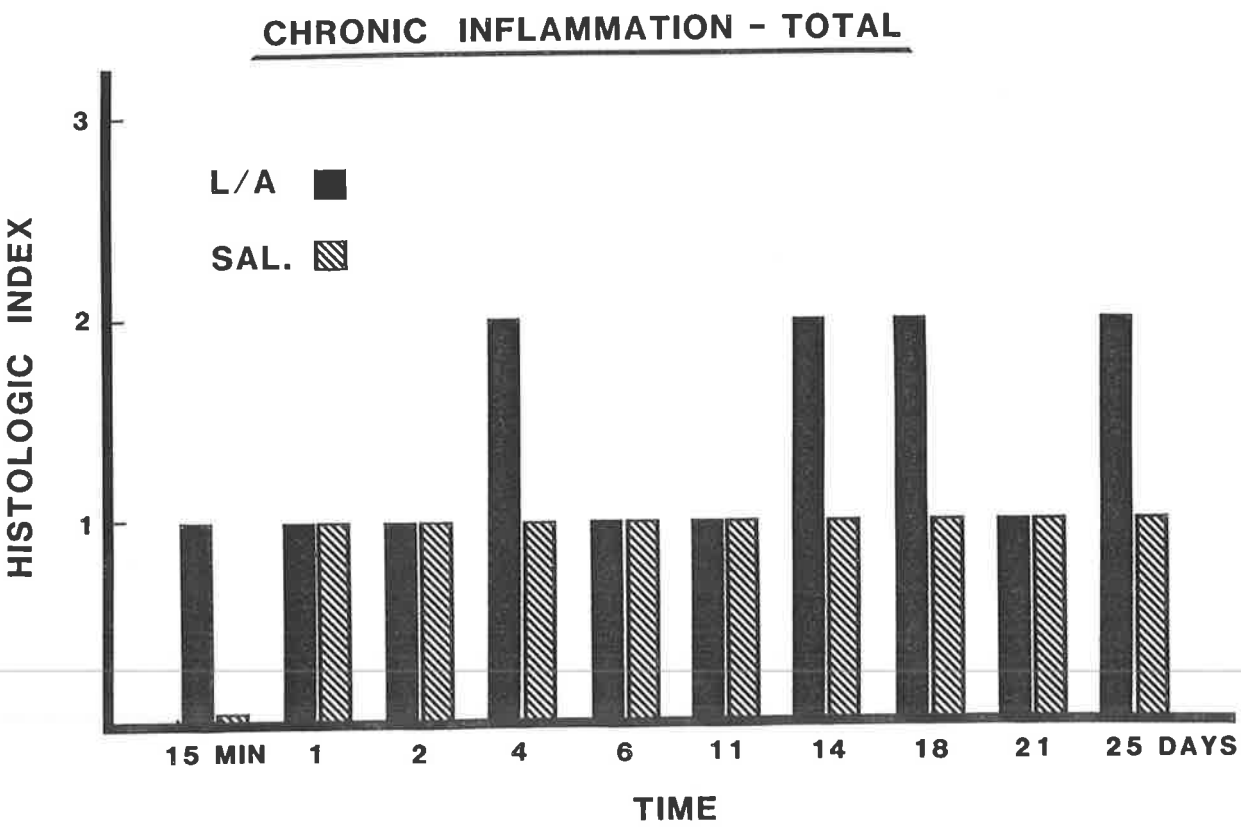

CHRONIC INFLAMMATION - TOTAL
HISTOLOGIC INDEX
3
2
1
L/A
SAL.
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 55

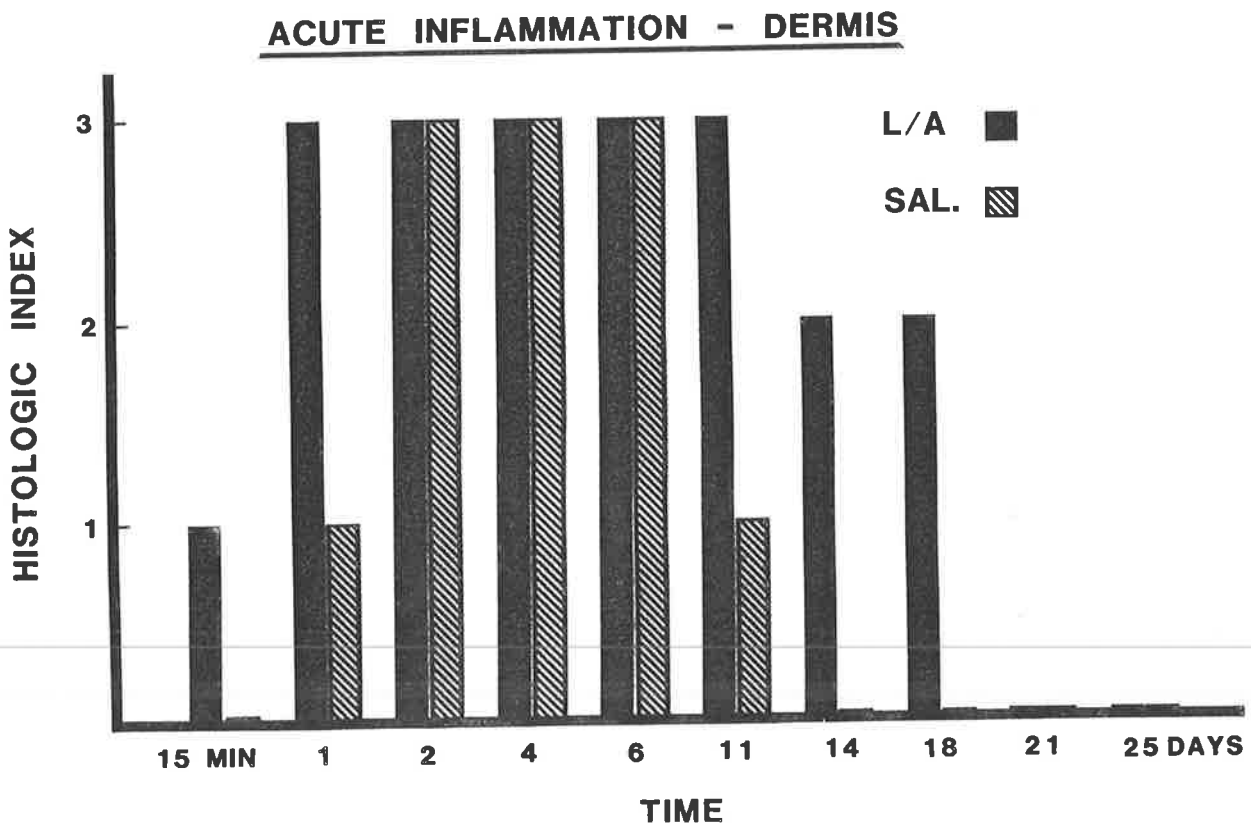

ACUTE INFLAMMATION - DERMIS
HISTOLOGIC INDEX
3
2
1
L/A
SAL.
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 56

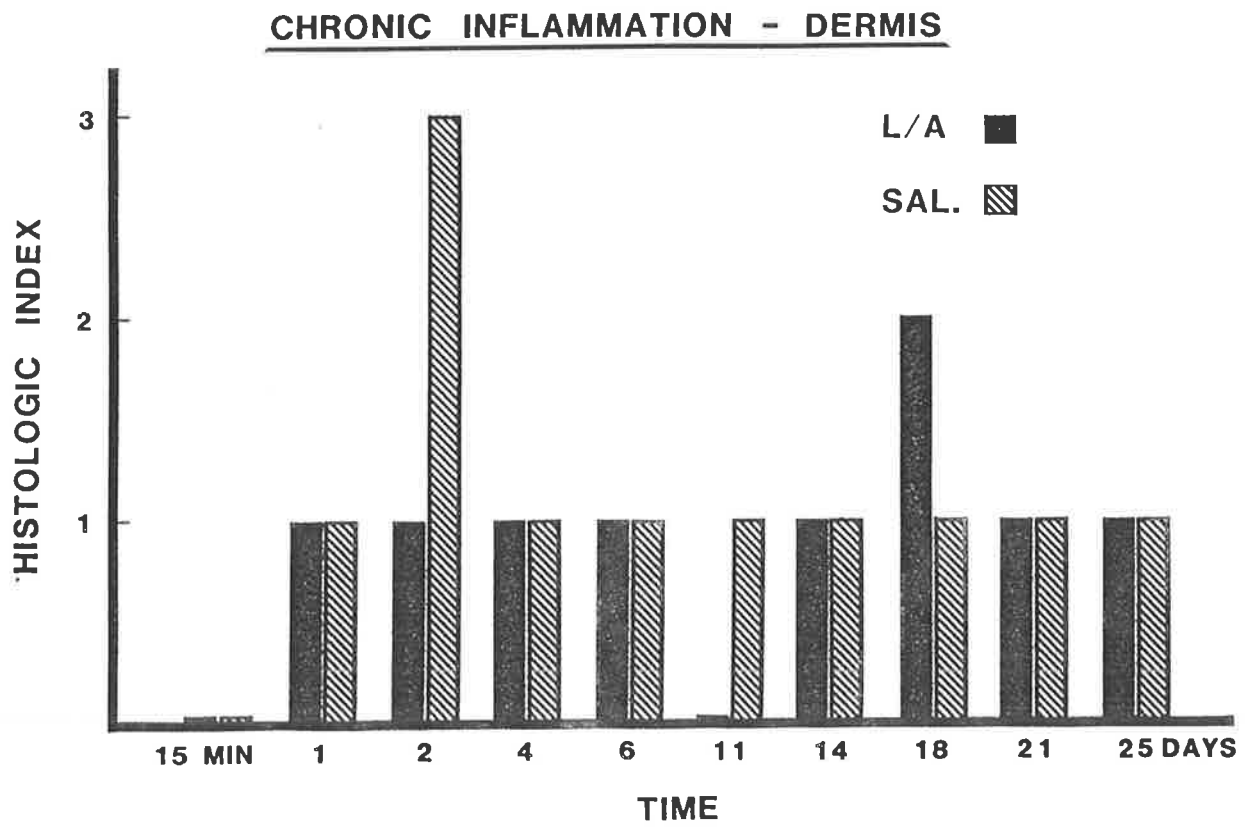

CHRONIC INFLAMMATION - DERMIS
L/A
SAL.
HISTOLOGIC INDEX
3
2
1
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 57

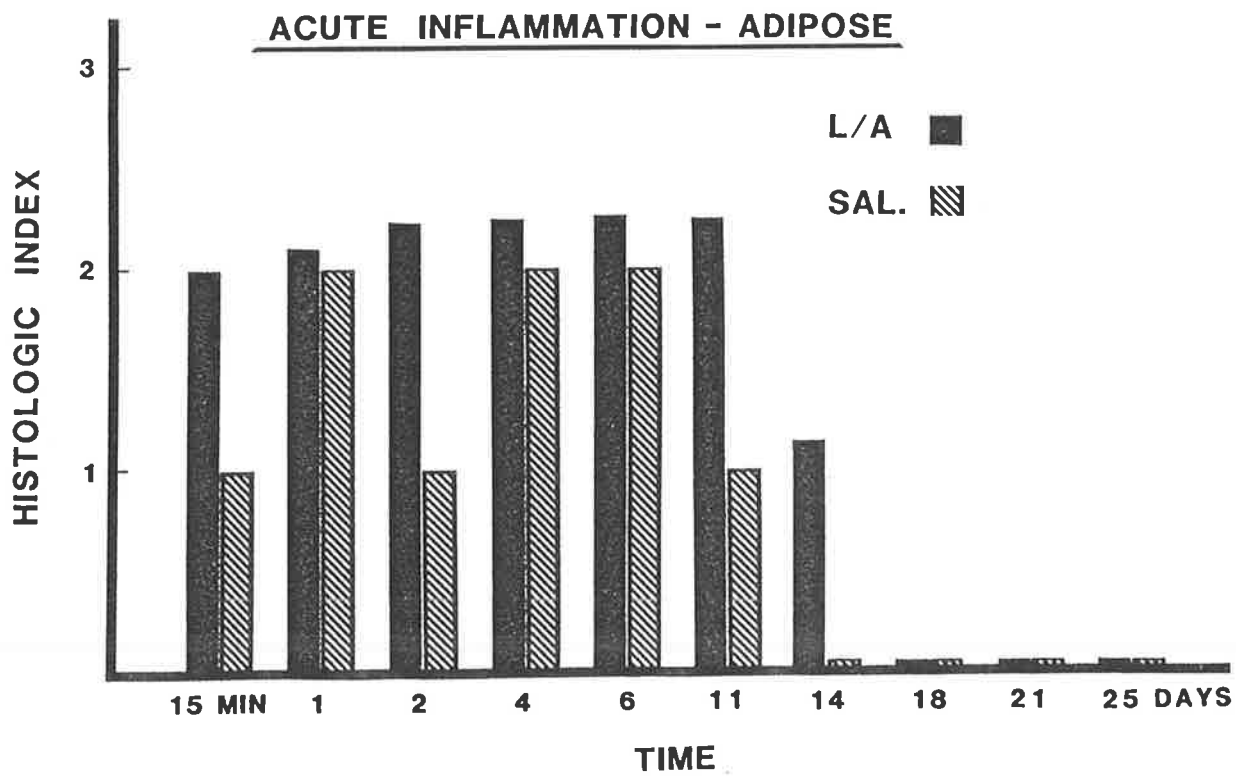

ACUTE INFLAMMATION - ADIPOSE
L/A
SAL.
HISTOLOGIC INDEX
3
2
1
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 58

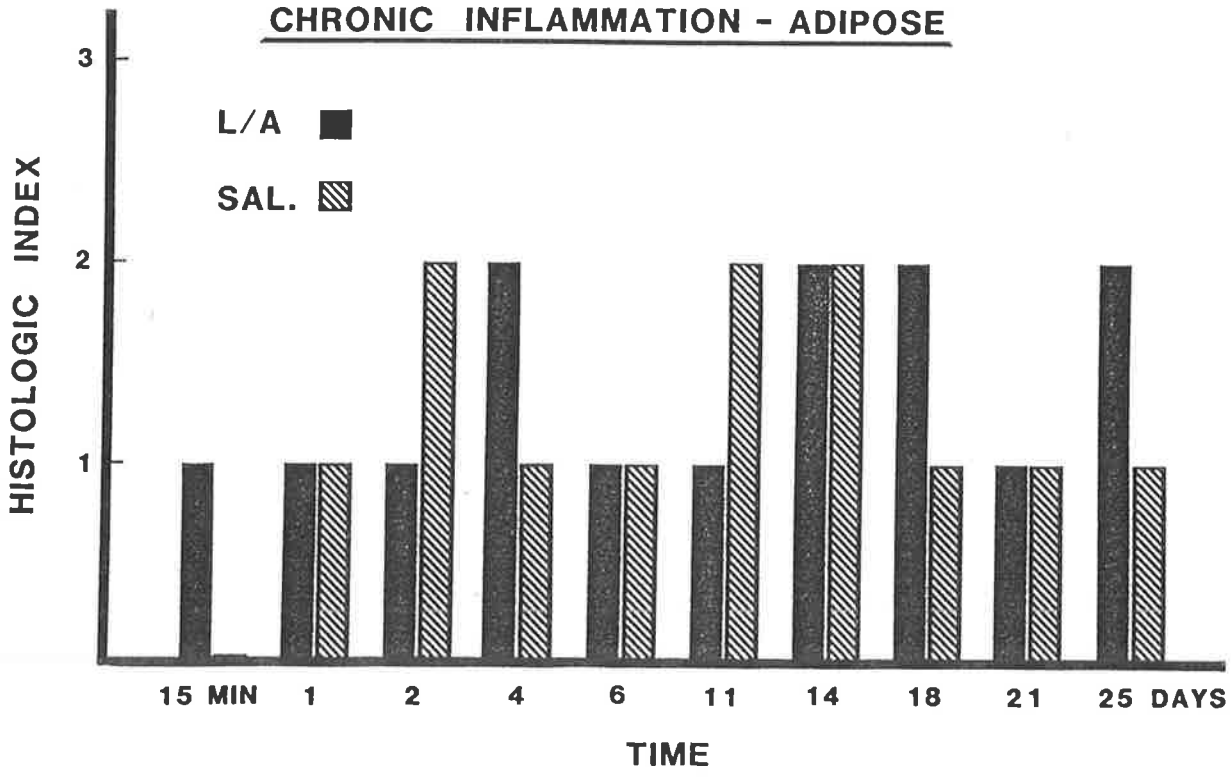

CHRONIC INFLAMMATION - ADIPOSE
L/A
SAL.
HISTOLOGIC INDEX
3
2
1
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 59

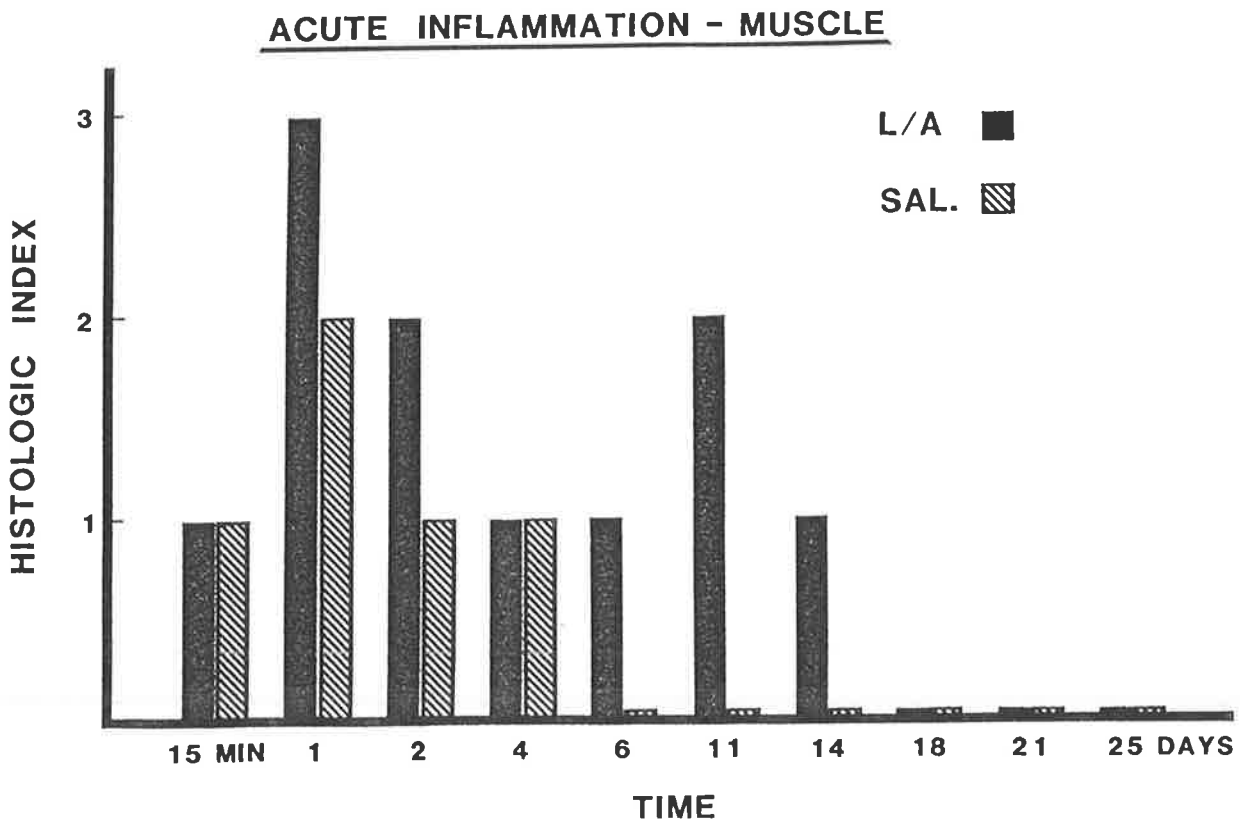

ACUTE INFLAMMATION - MUSCLE
L/A
SAL.
HISTOLOGIC INDEX
3
2
1
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 60

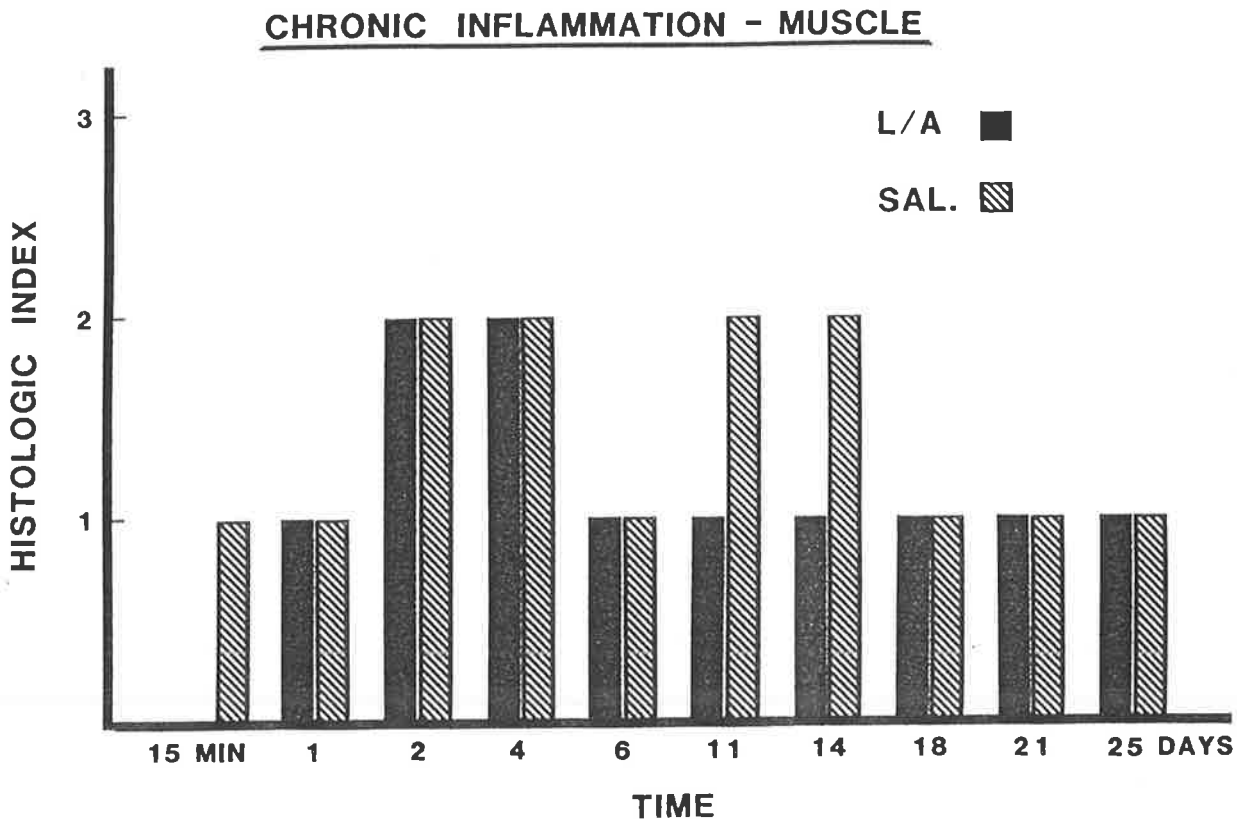
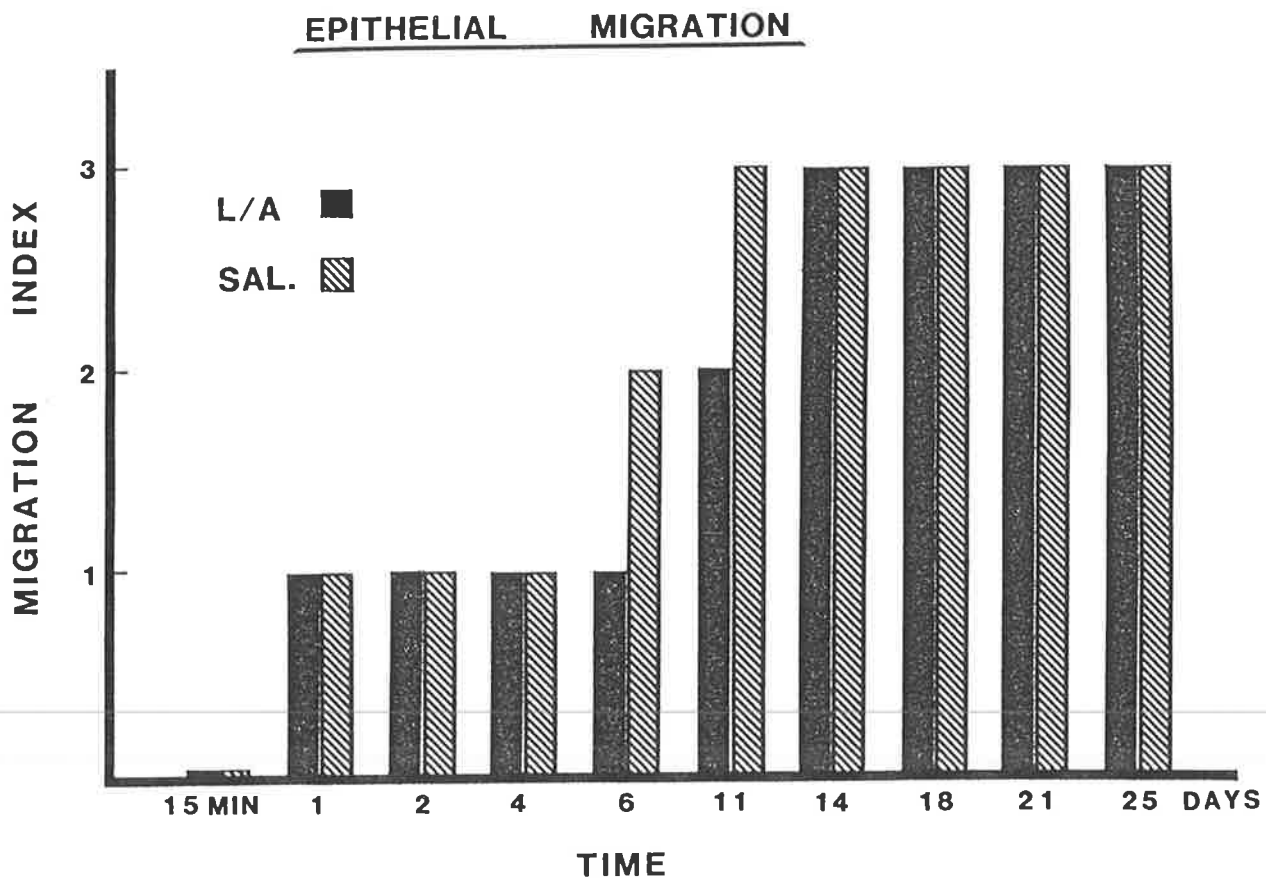

CHRONIC INFLAMMATION - MUSCLE
L/A
SAL.
HISTOLOGIC INDEX
3
2
1
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

Fig. 61


EPITHELIAL MIGRATION
MIGRATION INDEX
3
2
1
L/A
SAL.
15 MIN
1
2
4
6
11
14
18
21
25 DAYS
TIME

CHAPTER 7

DISCUSSION

This study showed that there were small but important differences in both tissue destruction and subsequent healing between the cryo surgical wounds created following pre-treatment with local anaesthetic containing adrenalin (L/A), and pre-treatment with normal saline. (SAL)

These findings will be discussed under the headings of:

1. Animal Model
2. Local Anaesthetic
3. Wound Production
4. Methods of Data Collation
5. Results

7.1 Animal Model

The animal model used in this study was the female Sprague-Dawley white rat. Pathogen-free laboratory rats have been used in many studies of a similar nature. (Hadfield 1963a, b; Levenson et al. 1965; Dann et al., 1941; Abercrombie et al., 1954; Cuthbertson 1959; Bjorlin 1954).

These animals are an excellent model for the production and study of skin wounds produced using cryosurgery and local anaesthetic containing adrenalin. (Bjorlin B. 1954; Neel et al., 1971a).

The general anaesthetic used in this study was Chloral Hydrate and the animals responded well to its intra peritoneal use, with only one death directly attributable to its use. Chloral Hydrate is not a known teratogen in mammals (Shepard 1973).

From the surgical viewpoint the dorsal skin of the white rat produced a reliably reproducible cryogenic wound. A feature also found by Li et al, (1980). At the end of the experimental period there was little difficulty in removing the skin down to and including the underlying muscle. The paniculus carnosus has no anatomic connections with the underlying subcutaneous fascia other than minute branches of the subcutaneous plexus which reunite to form the dermal plexus. (Hadfield 1963).

7.2 LOCAL ANAESTHETIC AGENT

The mechanisms of cell death within the iceball have been discussed in Chapter 3. From these mechanisms it would seem probable that in repetitive freezes the larger the iceball the greater the tissue necrosis.

The maintenance of low cryoprobe temperatures is primarily due to constant withdrawl of heat from the tissues or by blocking the heat entry into the area of freezing (Neel et al., 1971a). Local anaesthetic containing adrenalin should decrease the blood inflow to the area; thereby, increasing the size of the iceball and subsequent area of necrosis.

In the present study local anaesthetic containing adrenalin was used because of its widespread use in clinical cryosurgery and because it was a convenient vehicle for an adrenalin solution.

Although local anaesthetic containing adrenalin has been an advocated clinical adjuvant to cryosurgery, there are few studies reflecting this in the literature. While Myers and Donovan, (1981) studied the effects of 1% lignocaine and 1/100,000 adrenalin on cryosurgical wounds on the dorsal skin of farm pigs, most other authors, have delivered the vasoconstrictor in saline or distilled water. (Neel et al, 1973; Passey et al., 1971). In the present study a number of manipulative factors emerged with the use of local anaesthetic.

An experimental trial determined that the amount of local anaesthetic needed to disperse further than the iceball was 0.5 (ml).

This was to be deposited immediately beneath the muscle layer of the skin. However, this area forms part of a free fascial plane and great care was needed to produce a localised 'bleb' of the local anaesthetic. Penetration of the thick rat skin also proved to be a problem. The smallest possible guage needle to effect this without bending was 25 guage.

The effective delivery of the local anaesthetic to the proposed cryosurgical site was routinely possible.

7.3 WOUND PRODUCTION

Cryosurgical wounds have been created in a variety of experimental animals including hamsters, rats, dogs and pigs, (Whittaker 1974b; Bradley and Fisher 1975; Fraser & Gill 1967; Buch et al 1979; Li et al 1980, Myers and Donovan 1981, Passey et al., 1971).

The methods of producing the experimental wounds have ranged from liquid nitrogen probes (Neel et al., 1973; Myers and Donovan 1981) to liquid nitrogen sprays (Natiella et al 1979) or metal discs equilibrated to the temperature of liquid nitrogen (Li et al 1980).

The present study was carried out using a liquid nitrogen probe, producing a wound similar to that described by Li et al (1980).

The method of wound production proved to be consistent, however, close attention was needed to standardize the pressure of application of the tip of the cryoprobe to the skin.

The greater the depth of ingress the larger the iceball and the lower the temperature. Care taken to hold the proble just in contact with the skin enabled acceptable standardisation in this respect. Fig. 12.

7.4 METHODS OF DATA COLLATION

MEASUREMENT OF MACROSCOPIC WOUND AREA

Many methods of measuring the area of healing wounds have been described. Carrel (1910) studied the healing rate of superficial skin wounds of different geometric form, in dogs. He made successive measurements of skin edges either marked with indian ink or determined arbitrarily.

Spain and Loeb (1916) made direct serial measurements of healing skin wounds in the guinea pig by measuring both the gross wound length and the length of the tongues of regenerating epithelium at the wound margins. They differentiated between the phenomena of wound contraction and epithelialisation. Later literature, dealing with comparative changes in gross wound size, deals directly with the problem of wound contraction. (Abercrombie et al, 1954, 1960); (Cuthbertson 1959); (Zahir 1964).

Any measurement of gross wound size must make allowances for the combined effect of wound contraction and epithelialisation.

An accepted method of achieving this distinction is tattooing of skin lines or points at known distances around the wound. (Carrel 1910); (Cuthbertson 1959); (Abercrombie et al., 1954, 1960). These points are then joined and the enclosed area determined by multiple length and breadth measurements of the resultant shape.

More recently the area of cryosurgical wounds has been assessed by injecting the experimental animal with vital dyes post operatively and measuring the unstained area of subsequent post mortem sections of the wound (Myers and Donovan 1981).

In the present study contraction and epithelialisation were considered as separate entities.

Wound area tracings were done pre mortem under high power magnification of the gross wound. The margins determined were those of the covering scab until the junction of the new and old epithelium was visible. This was then a measure of wound contraction.

Similar techniques have been used as long ago as Carrel (1910) and in the present study, a modification of techniques used by Dann et al 1941; Zahir 1964, Li et al 1980; and Montandon et al., 1977 was employed.

The technique of tattooing the wound margins as described by other authors was found to be traumatic, inaccurate and cumbersome in the hands of the author; and a photograph of the result can be seen in Fig. 62.

The small calibre of available peripheral veins excluded other techniques involving the injection of intravenous fluorescein, in this animal model.

MEASUREMENT OF ICEBALL SIZE & THAW TIME

The literature contains few accounts of quantitative measurements of iceball size or thaw time in rat skin or comparative animal models (Neel et al 1971a,b, 1973).

The method of assessing these parameters in this study, was a modification of a technique used by (Neel et al, 1971a,b; Neel et al, 1973) who studied the effects of vascular control with inflow occlusion and vasoconstriction, on iceball size and thaw time in rat liver and on mouse skin. They compared these results with the ultimate histological size of the lesion.

In this study the use of local anaesthetic with adrenalin paralleled the experiments of Neel et al (1973) who found that compared to single

probe applications, repetitive freezing consistently increased the rate and depth of freezing. They further discovered that the size of the histologic lesions of wounds produced by freezing with ischaemia, exceeded the size of the visible iceball.

In the present study the injection of the operating site with lignocaine + 1/80,000 adrenalin was a simple and effective method of producing ischaemia, as evidenced by the immediate and late pallor of the area.

Measurement of the iceball by dividers and vernier calipers was not found to be prone to noticeable variation. Any problems in this respect were overcome by multiple measurements and only measuring the maximum diameter of the iceball.

The maximum iceball was always achieved within the 2 minute time span of freezing.

The estimation of thaw time after Neel et al., (1971) was fraught with problems because of its inherently empirical nature. Problems encountered with thermocouples which will be discussed later in this chapter excluded their use to determine thaw time. For this reason the thaw time was always considered to be the point of disappearance of all visible frost from the wound site.

TEMPERATURE MEASUREMENT - THERMOCOUPLE

Five animals were chosen for serial thermocouple measurements of iceball temperature of both experimental and control wounds.

Although carried out along similar lines to experiments by Neel et al (1971a,b, 1973), because of the small number of animals used in the present study no attempt to compare thermocouple readings with the resultant histologic size of the lesion or macroscopic area.

Accurate placement of thermocouples proved to be more difficult than expected. The dorsal skin of the white rat is tough and resistant to the introduction of any needle smaller than a 25 guage needle. For this reason 30 guage copper-constantan thermocouples were introduced using 18 guage needles. Despite difficulty introducing the thermo couples, the results achieved, however, indicated at least comparable success with the literature.

However, firm comparative conclusions must be guarded, because of the small number of animals involved in this aspect of the study.

INFLAMMATION

The sequence of events in a healing wound have been discussed in Chapter 2.

Integral to the healing process is the sequence and degree of inflammation and the literature abounds with studies on the inflammatory process. Ryan and Majno (1977).

In the present study the cellular inflammatory infiltrate was regarded along the classic lines of being either acute or chronic.

Cell counts along the lines used in the present study and their conversion to an histologic index have been used before, to quantitate the extent of the inflammatory progress. Seymour et al., (1981, 1983).

However, raw cell counts of their own must be qualified by histologic technique, staining, cell identification methods, and legitimate statistical analysis.

The present study has examined the infiltration of polymorpho nuclear leucocytes and round cells (plasma cells and lymphocytes) into the wound area.

Problems encountered, included cell identification under H & E and an alternative stain such as Methyl Green Pyronin (MGP) (Johnson and Hopps, 1975) may well have been a more effective technique to identify plasma cells and lymphocytes.

EPITHELIALISATION

As the wounds healed the epithelium at the margins proliferated and produced a tongue of new epithelium that grew across the defect.

Quantitative assessment of this new growth at microscopic level has been made by measuring the distance from the junction of the old and new epithelium to the tip of the tongue (Im and Hoopes, 1973; Spain and Loeb, 1916). This measurement was, however, independant of wound contraction.

An alternative method, carried out by Dann (1941) was to measure the distance between both old epithelial edges and both new epithelial edges. However, this was a relative measurement only and included the inevitable closure of the wound by contraction.

The problem of contraction may be overcome by specific wound location and morphology according to Bullough and Lawrence (1957), who created radial, through and through wounds on mice ears because such wounds were less prone to contraction.

Abercrombie et al., (1960), overcame the problem of wound contraction as a parameter of wound closure, by splinting the wound margins, at operation.

The present study accepted the occurrence of wound contraction, and employed a modification of Dann's (1941) method of measurement, expressing epithelialisation as a ratio of length of new epithelium to the apparent old wound width.

These ratios were then converted to an epithelial index and plotted against time.

Direct measurements of the length of epithelial tongues do not take into account differing epithelial regeneration rates at different sites in the same wound, so epithelialisation was calculated from the first five mounted sections from each block. Because the gross specimens were bisected through the point of greatest diameter the resulting sections gave an indication of the epithelialisation at this point.

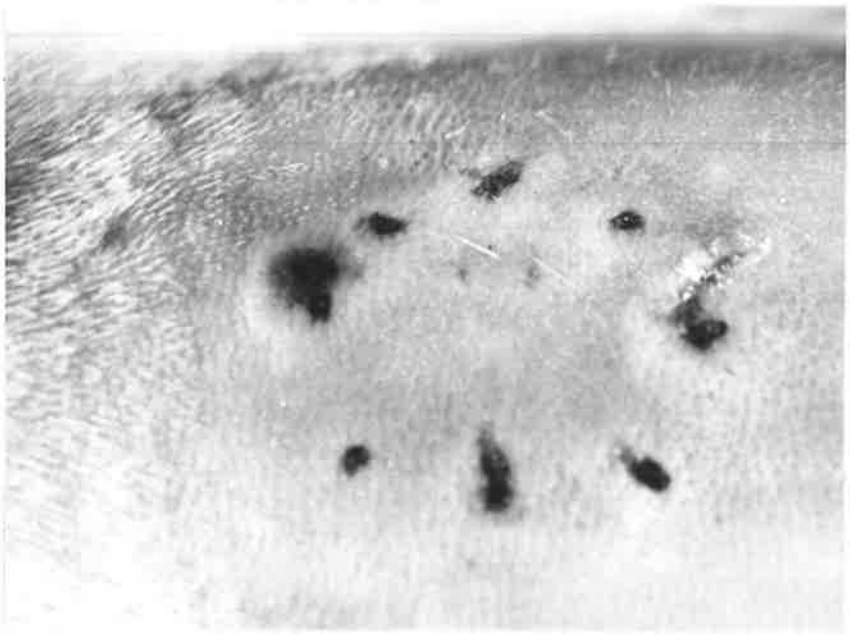


Fig. 62 Result of skin tattooing with Indian Ink

7.5 RESULTS

7.5.1 Macroscopic Results

(a) Iceball Diameter

The results of measurements of iceball diameter presented in Chapter 6, would indicate that although there was a progressive increase in iceball diameter after 3 freezes in both the experimental and control series, there was no significant difference in iceball size between the two groups. This finding is in accordance with the results of Neel et al (1971a, 1971b), and would seem to indicate that the use of a vasoconstrictor prior to cryofreeze has little or no effect on the size of the resultant iceball.

While there was a noticeable difference between the size of the iceball after the first freeze, and compared to the 3rd freeze, there was, however, no significant difference between the 2nd and 3rd freezes. Repetitive freezing has been postulated for many years as the method of inducing a surer area of tissue necrosis Gill et al (1970). If this was the case, then perhaps iceball diameter is not an accurate measure of area of kill, an idea also proposed by Neel et al (1971a). However, Gill et al., (1968, 1970), believe that, the reason for the apparent increased efficiency of repetitive freeze-thaw cycles is that they only extend the boundary of freezing. They feel that the macroscopic changes after repetitive freezing are more intense but basically the same as after a single freeze.

It would appear that for the purposes of this experiment, iceball size was independant of the use of Local Anaesthetic with adrenalin and that only two of the freeze-thaw cycles were necessary. Reasons for this may well involve the observed changes in thermal conductivity after breakdown of dense cellular tissue made by Fraser and Gill (1967).

(b) Thaw Time Cycles

The thaw time of the experimental iceball was longer than the control after the initial freeze. Succeeding thaw times of the control group were consistently longer than the experimental group.

Although thaw times increased to the end of the second freeze in both groups they also dropped off after the second freeze. These findings are not consistent with those in the literature Neel et al, (1971b). The author could find no reason for these results, other than

(1) Experimental error; Logically this may well have occurred, owing to the very arbitrarily determined thaw point - as 'the point at which all visible frost disappears'.

or,

(2) A reactive vasodilation after vasoconstriction. Future experiments of this kind may well need to utilise a thermocouple determined, "thaw point".

At the outset of this experiment the author felt that iceball diameter and thaw times could be very directly related to subsequent healing of cryosurgical wounds.

Similar experiments carried out by other authors Neel et al., (1971b) indicated that such was not the case. However, these experiments had not been carried out on the skin of the white rat, and the experimental tissue used had been very vascular. A feature that may have precluded optimal results utilising ischaemia as a parameter.

(c) Thermocouple Readings

While local anaesthetic with adrenalin had no observably profound effect on iceball size, the serial thermocouple reading indicated that the edge of the iceball was consistently and significantly colder at the end of each freeze than in the controls.

While these results agree with those of Neel et al (1971a) the concept of iceballs of similar size having significantly different peripheral temperatures is difficult to accept, unless, beyond the ice-point of maximum diameter, the withdrawl of heat from the tissues has continued to occur.

The results of the thermocouple readings agreed not only with the literature but also the assumption that vasoconstriction would increase the depth of freezing.

(d) Gross Wound Area

The results of gross wound measurement indicated that from day one the experiment wound was significantly larger than the control.

Exceptions to this trend occurred at 4 days, 14 days and 25 days when the wound areas were not significantly different.

The unusual results obtained at days two and four are difficult to assess - wherein the control wound after decreasing in size, rapidly increases to become larger than the experimental wound at day four. Although the difference is difficult to account for, it is not significant at day four.

Gross wound contraction prior to the full effects of cryosurgery is an inviting though unlikely conclusion, because no effective granulation tissue was present at this stage.

These unusual results may well be due to experimental error.

More important, however, was the finding that both wounds decreased in size at approximately the same rate. The significance of which is related to the method of gross wound measurement.

The measurement of wound area as used in this study, and taken from the junction of the new and old epithelium, was a measure of wound contraction. The rate of change of area was a measure of contraction rate.

Some authors believe that cryosurgical wounds do not contract (Li et al, 1980). From the presented data it would appear that such is not the case and that cryosurgical wounds do indeed contract though local anaesthetic with adrenalin has little effect on the rate of contraction.

Comparative assessment of contraction rates of cryosurgical wounds to other wounds eg. burns was not possible in the present study.

7.5.2 MICROSCOPIC RESULTS

(a) Inflammation

Inflammation represents the response of living tissue to injury - Ryan and Majno (1977). As such, the inflammatory profile must in someway relate to the healing process. Cell counts were carried out to

1. Measure the immediate tissue response and
2. Measure the ongoing tissue response

as parameters of wound healing.

The acute inflammatory response in the experimental group was generally faster, more intense and more prolonged in comparison to the control group.

The reasons for this would seem to be intimately related to the greater degree of tissue destruction exhibited by these wounds as verified by the histologic and gross wound data. No literature could be found at the time of writing that compared the degree of inflammation and subsequent healing in a similar cryosurgical wound.

The chronic inflammatory reaction as evidenced by the round cell histologic index showed that the experimental group peaked sooner and maintained the level for a longer period than the controls. The round cell count in the experimental group repeaked at about 11 days.

This observed repeaking may have been indicative of an immune-type response in the treated tissue.

Cryotherapy has been implicated in a non-specific increase in lymphocyte activity that apparently is further increased when the afferent blood supply is compromised. (Holden 1972).

Neel et al (1971b) spoke of a tumour-specific immune response to subsequent tumour challenge, after cryosurgery, lending further support to this concept.

If such is the case, then the round cell response in the experimental group could be explained on these grounds, however, such a conclusion can only be advanced with caution as the evidence is very indirect.

It would be of considerable interest to carry out a differential round cell assessment using Methyl Green Pyronin (M.G.P.) (Johnson and Hopps, 1975) with subsequent immunofluorescence to determine T-cell numbers and so draw a more positive conclusion, as to immune-type responses.

(b) Histology

In the present study the microscopic wound healing followed the classic stages as described by Ross (1968) and confirmed the features of cryosurgical wounds as seen by Gill et al (1970), Passey et al (1971), Fraser and Gill (1967) and Hurt et al (1972).

Both experimental and control wounds had healed from initial defect to small fibrous scars by day twenty five.

The experimental wounds specifically, showed earlier vessel sludging, extravascular inflammation and more severe muscle changes than the equivalent control wounds.

Such results are in agreement with the literature as represented by Myers and Donovan (1981) who found that the addition of lignocaine with adrenalin to a cryosurgical lesion significantly increased the killing effectiveness. Passey et al (1971) found that epinephrine without local anaesthetic significantly enhanced tissue necrosis in combination with cryosurgery.

The two wounds by day four are basically comparable, with evident epithelial migration and granulation tissue formation.

From day four on, when the stages of repair and regeneration began the control wounds began to heal faster and were fully epithelialised by day eleven whereas the experimental wounds took until day fourteen to achieve coverage.

It would appear that these results agree with the findings of Bodvall and Rais (1962) who found the decrease in healing rate following the use of local anaesthetic was more marked when a vasoconstrictor was used.

Smelser and Ozaniks (1945) found that local anaesthetics inhibited cell mitosis. A fact that may well account for the differential epithelial mitotic activity and resultant slower migration shown by the experimental wounds at this stage.

After completion of epithelial continuity, the two wounds were again histologically comparable and from day fourteen exibited little difference other than in degree of muscle fibrosis and scarring.

The wounds treated with local anaesthetic exibited much greater scarring than the controls. A feature also mentioned by Benoit (1978a, b) who produced similar results in rat thigh muscle using local anaesthetic and adrenalin. He claimed that the scarring was due entirely to the local anaesthetic, not the adrenalin, because his adrenalin controls had no evident scarring.

By day twenty five healing was complete and the only gross difference between the experimental and control groups was in the retained fibrosis and scarring of the muscle in the experimental wound.

From the histology it would appear that a number of factors accounted for differences seen between the two wounds.

The vasoconstriction due to the adrenalin although transient does contribute to the initial severity of the experimental wound.

The experimental wounds respond more rapidly to the cryotherapy than do the controls.

This initial response is, however, transitional and the control wounds heal faster thereafter, possibly due to a delayed effect of Local Anaesthetic on the healing process.

The wounds when healed are generally comparable except for the damage to the underlying muscle probably caused by the local anaesthetic or vasoconstrictor.

CHAPTER 8

CONCLUSION

Despite the increase in clinical use of cryosurgery the biological effects, including the effect of local ischaemia on the healing of cryosurgical wounds, has received scant attention in the literature.

A satisfactory model was established with which cryosurgical wounds could be examined to determine the effect of different local factors on wound healing.

The present study compared experimental cryosurgical wounds created using local anaesthetic containing adrenalin (L/A), with control cryosurgical wounds created using normal saline (SAL).

From the experiments conducted certain conclusions may be drawn.

A. At the time of freezing:

1. There was no significant difference in the size of the iceball between the two groups. This finding is consistent with previous reports.

2. Thermocouple readings at the iceball periphery indicated that in the experimental series, wounds created using L/A were consistently colder. This finding is consistent with the literature and is a predictable effect of vasoconstriction.

3. The iceball thaw-time in the control group (SAL), was longer than the experimental group (L/A). This finding is contrary to literature and the reasons for this are discussed in Chapter 7.

B. At the times of subsequent healing:

1. The experimental wounds were consistently larger than the controls throughout the course of the experiment and were signficiantly so on Days 2, 6, 18, 21 and 25.

2. Both groups underwent considerable wound contraction from day 14 to Day 25. It has been suggested in the literature that cryosurgical wounds undergo only limited contracture. Thus this finding is contrary to the literature.

3. Histologically the experimental wounds were more severe and took longer to heal than the corresponding controls. This feature was reflected in the different relative inflammatory response between the two groups.

4. The acute inflammatory reaction was generally faster, more intense, and prolonged in the experimental (L/A) wounds while the chronic response peaked sooner and lasted longer in these animals. This would seem a direct consequence of the more pronounced tissue damage in the experimental wounds.

Thus, it would appear that the pretreatment of cryosurgical wound sites with a local anaesthetic containing adrenalin increases the volume

and degree of tissue damage without greatly delaying the rate of healing. This provides experimental evidence to support the commonly held clinical belief that for maximum effect from cryosurgery a local anaesthetic containing a vasoconstrictor should be used.

APPENDIX

1. HOUSING OF EXPERIMENTAL ANIMALS

All experimental animals were housed in RB 3 polypropylene cages with stainless steel tops. The dimensions of the cages were 45 cm x 28 cm x 22 cm. A total of four rats were placed in each cage.

DIET

All animals were fed on a standard diet with the following composition.

Diet:

Ground wheat	40.0%
Ground barley	18.0%
Bran and pollard	12.0%
Meat and bone meal	9.6%
Extract soya meal	6.2%
Fish meal	6.2%
Milk powder	3.0%
Brewer's yeast	1.0%
Salt	1.0%
Molasses	3.0%

Vitamin Supplement/Kg. of feed:

Vit. A_3	3928 I.U.
Vit. D_2	928 I.U.
Vit. B_6	1.5 mg
Vit. B_{12}	0.2 mg
Vit. B	3.4 mc gms
Vit. E	1.2 mg
Vit. K	0.5 mg

2. PHOTOGRAPHIC FORMAT

(1) GROSS MORPHOLOGIC OBSERVATIONS

The rat specimens experimental and control, were positioned in a right side lateral view on a maroon felt background. A constant light source was provided. The specimens were then photographed under constant magnification employing the WILD Photomakroskop M400 system.

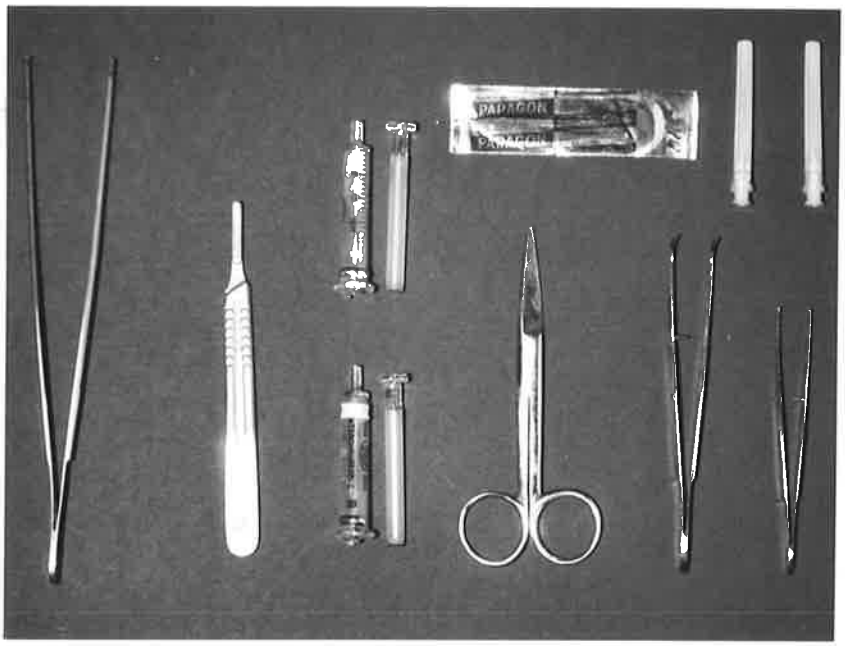
(2) HISTOLOGIC SPECIMENS

The histologic sections were photographed employing the M400 system using a 1:5 Makrozoom lens and WILD Photoautomat M.P.S. 55 Exposure System.

APPENDIX

3. SURGICAL INSTRUMENTS:

HAIR CLIPPERS
RAZOR
BLAND SOAP



TISSUE FORCEPS
SCALPEL HANDLE AND BLADE
GRADUATED LUER - LOK SYRINGES
SURGICAL SCISSORS
25 gu NEEDLES

APPENDIX

4. HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

(A) Tissue Fixation

All specimens were fixed in neutral buffered FORMALIN

Recipe:

Formalin	500 mls
Tap Water	4500 mls
Acid Sodium Phosphate Monohydrate	20 gr
Anhydrous Disodium Phosphate	32 gr

(B) Embedding Procedure

All tissue specimens went through the following reagents:

(1) Alcohol 70%	Overnight
(2) Absolute Alcohol	2 Hours
(3) Absolute Alcohol	2 Hours
(4) Absolute Alcohol	2 Hours
(5) Absolute Alcohol	2 Hours
(6) Methyl Salicylate and Absolute Alcohol (50:50)	Overnight
(7) Methyl Salicylate	8 Hours
(9) Methyl Salicylate and Celloidin 1%	2 Days
(10) Methyl Salicylate and Wax (50:50)	1 Hour
(11) Wax 100%	2 Hours
(12) Wax 100%	2 Hours
(13) Wax 100%	2 Hours

All specimens were placed in a vacuum chamber for 1 hour for the evacuation of air bubbles, and subsequently blocked in wax at $56^{o}C$.

Sections were cut in a rotary microtome set a 7 microns. Each 5th section was retained and of these, each 3rd section was stained with haematoxylin and eosin.

(C) Staining Procedure

Haematoxylin and Eosin Stain

(i) Method

(1) Xylol 2 to 5 minutes

(2) Xylol 2 to 5 minute

(3) Absolute alcohol 2 to 5 minutes

(4) Absolute alcohol 2 to 5 minutes

(5) Dip in tap water

(6) Harris' Haematoxylin 3 to 5 minutes

(7) Tap water 5 minutes

(8) Differentiate in 0.5 to 1.0% hydrochloric acid in 70% alcohol for 30 seconds

(9) Immerse in running tap water for 10 minutes

(10) Eosin 45 seconds

(11) Absolute alcohol 1 minute

(12) Absolute alcohol 1 minute

(13) Absolute Xylol 1 minute

(14) Xylol 1 minute

(15) Mount in Depex

(ii) Results

Nuclei	: Blue to blue black
Karyosomes	: Dark blue

(ii) Results (continued)

Basophil	: Purplish
Red blood cells, eosinophil granules, zymogen granules	: Bright orange red

APPENDIX

5. HISTOLOGIC PROFORMA

NECROSIS

Epithelium	- intact - broken
Vessel Sludging	- none - mild - moderate - severe
Inflammation	- present - absent
Polymorph Location	- dermis - fat - muscle
Polymorph Severity	- none - mild - moderate - severe
Round Cell Location	- dermis - fat - muscle
Round Cell Severity	- none - mild - mild - severe

RECONSTRUCTION

Epithelial Migration	- initial - moderate - almost complete to complete
Mitotic Activity	- none - equal to normal epithelium - greater than normal epithelium
Blood Vessel Ingrowth	- present - absent
New Blood Vessels	- none - upper half - lower half - both

RECONSTRUCTION (continued)

New Fibroblasts
- present
- absent

New Collagen
- present
- absent

Connective Tissue Fibrosis

- dermis
- fat
- muscle

- none
- mild
- severe

APPENDIX

6. STATISTICAL METHODS

1. STANDARD DEVIATION

"The standard deviation is a measure of the dispersion of values about their mean and is used to compare samples with one another"

Von Fraunhofer and Murray 1976.

Mathematically the standard deviation is the square root of the arithmetic average of the squares of the differences between the observations and their mean.

i.e. the standard deviation 'S'

$$S = \sqrt{\left[\frac{N\,\Sigma x^2 - (\Sigma x)^2}{N^2}\right]}$$

where: N = number of values
x^2 = sum of the squares of the values
$(x)^2$ = sum of the values squared

However, when the standard deviation is used as a population estimate then the following formula was used,

$$S = \sqrt{\left[\frac{N\Sigma x^2 - (\Sigma x)^2}{N(N-1)}\right]}$$

for the reason that as an estimate of a population, it has been found that $N(N-1)$ instead of N^2 gives a more accurate standard deviation of a

population. When the sample is very large, then N^2 is very nearly the same as N(N-1).

Test of Significance

The test of significance chosen was the 'student' "t" test.

The test of significance is used when two sets of data are compared without the use of a known standard value.

The 't' test compares the two sets of data to determine whether there is any real difference between the means. Thus, for two groups N_1 and N_2 with means and standard deviations x_1, S_1 and x_2, S_2 the 't' test,

$$t = \frac{x_1 - x_2}{\sqrt{(S^2 \frac{1}{N_1} + \frac{1}{N_2}}}$$

where S^2 = pooled variance and is calculated as,

$$S^2 = \frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}$$

the 't' value is then plotted using the distribution of 't' to determine the significance of any difference between the two means.

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