EFFECTS OF FREEZING ON RAM AND BOAR SPERM

a thesis submitted by

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Declaration

I declare that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any University; and that to the best of my knowledge it does not contain any material previously published or written by another person except where due reference is made in the text.

Betty Y.M.Cheng (nee Ju)

論文摘要

程朱友梅

觀查冷凍精子存活的最重要因素，是在冷凍及解凍之後，測定其細胞內酵素的遺失量。本文資料解釋，不同的稀釋液，溫度，甘油的含量，及蛋黃的添加量對GOT酵素遺失的影响。上述的影响是與稀釋液中的滲透壓，離子傳導性及酸鹼度有相關性的。GOT酵素的重要性是測知細胞破損的程度，以及在30分鐘內有效的鑑定冷凍精子的品質。

膠質電泳法在本文用來探測蛋白質的遺漏，在冷凍的精液中顯示有特別加深的蛋白質帶。由此可見蛋白質會從受傷的精子遺漏出來。

電子顯微鏡的照片上也表示出在冷凍之後，豬的精虫有非常嚴重的細胞膜破損現象。

由實驗結果可以綜合結論，最適合羊和豬精子冷凍的情況是：

	溫度	稀釋液	酸鹼度(7.4)
豬	37°c	Tris 緩衝液加果糖	

	5°C	TES 緩衝液加蛋黃及甘油
	解凍 40°C	Tris 緩衝液加果糖
	冷凍 -196°C	不適合
羊	37°C	精子林格液加果糖
	解凍 40°C	精子林格液加果糖
	冷凍 -196°C	檸檬酸緩衝液加葡萄糖

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Summary

Sperm survival in frozen semen appears to be determined largely by the amount of intracellular enzymes during freezing and thawing. Data are presented to show the effects of different diluents, temperature and glycerol as well as egg yolk concentrations, on glutamic-oxaloacetic-transaminase (GOT) release. The influence of different diluents has been related to their osmolality, ionic conductivity and pH. The importance of the enzyme GOT is that it indicates the degree of cell damage, and also efficiently shows the quality of frozen semen in a 30 minute test.

Polyacrylamide gel electrophoresis has been used to detect extra dense protein bands which appear in frozen samples of semen. It seems that the protein leaks from damaged cells.

Electron microscopy of boar and ram spermatozoa yields a picture of severe disruption of the frozen cell membrane.

It is concluded that the optimal conditions for freezing ram and boar spermatozoa are:

Animal	Temperature	pH	Diluents
Boar	37°C	7.4	Tris buffer fructose
	5°C	7.4	Tes buffer with egg yolk and glycerol
	-196°C	7.4	Tes buffer with egg yolk and glycerol or BF3 extender
	Thawing 40°C	7.4	Tris buffer with fructose
Ram	37°C	7.4	Sperm Ringer phosphate with fructose
	-196°C	7.4	Sperm Ringer phosphate with fructose
	Thawing 40°C	7.4	Citrate glucose

These diluents which minimize GOT release.

Introduction

Recently intensive breeding programs for domestic animals have been developing in all countries. In the small farming systems of Asian countries, there is a need to use artificial insemination(AI) since maintenance of high quality sires is too difficult for small operations.There are two requisities for successful AI. One is optimal media and storage conditions for spermatozoa, the second is the acquisition of skill in insemination and detection of oestrus.

Artificial insemination is important for genetic improvement in the quality of livestock, and also for cutting down the costs of breed stock. Following the advent of deep freeze and other methods of prolonged storage of semen, it has been possible to obtain the benefits of long distance transport of semen. There is now a general export of cattle semen. Economically the purchase of semen is cheaper than buying a male animal. Moreover, there is no risk of sickness or sterility due to enviromental change infection, or because of transportation.

Storage of sheep spermatozoa

Lightfoot& Salamon (1970 ; 1971) reported that ram spermatozoa, pellet frozen and stored for several weeks, showed relatively satisfactory fertility. Salamon (1972) obtained similar results using ram semen diluted with egg yolk-raffinose-citrate medium. The semen was frozen on dry ice in pellet form (0.4 ml) and stored in liquid nitrogen for 3 years. Of 91 ewes inseminated intravaginally with the semen, 52.9% lambed. Ewes inseminated by the same method but with fresh semen, produced 76.5% lambing.

The low temperature storage of ram spermatozoa was first described by Emmens & Blackshaw (1950).

The addition of glycerol with slow freezing as described by Polge (1949), and Smith & Polge (1950) for semen of other species such as cattle gives only poor revival of ram spermatozoa. Some good revivals were obtained by rapid freezing with diluent containing sugars, however the best appearing to be 40% sucrose in 0.9% sodium chloride. Martin

& Emmens(1958) have shown that fructose can be used to replace arabinose with bull semen. Blackshaw (1955) found that the revival of ram and bull spermatozoa was not significantly affected by the temperature of mixing with glycerol as long as it was between 5°C and 15°C ; but results were better with thawing at 40°C rather than at 5°C.

Ram semen revived from -79°C very well , with pronounced swirling motion if frozen in a simple diluent containing 3 % sodium citrate adjusted to pH 7.1 with 0.1 M phosphate buffer, in a final concentration of 7.5% to 10% glycerol and 1.25% arabinose.

A total of 13 alcohols and 11 sugars, plus urea and inositol, tried in various combinations, did not yield better results (Polge et al 1949).

Before Salamon in 1972 obtained a 52.9% lambing rate, the deep freezing of ram semen did not allow effective revival of ram spermatozoa.

Emmens & Blackshaw (1955) in tests of deep freezing of ram semen obtained no better than 5% fertile insemination in a total of 779 ewes. Smirnov (1951) stated that in 1949 he obtained 11 normal lambs from 19 ewes inseminated with semen that had been deep frozen, but no other comparable claim had been made.

Galkin in (1951-1959), worked out a method for storing ram semen at -8°C to -10°C in a salt-ice freezing mixture, with the slow introduction of glycerol by dialysis, and final storage at -10°C to -20°C. However, he did not claim better than 19% fertility for semen stored for 20 days. Again Galkin (1954) found that ram semen preserved at 0°C or -8°C for several days gave fair fertility rates, the diluent being yolk-citrate -glucose- glycerol. Fresh semen in Galkin's series gave an 86% lambing rate; semen stored for 2 days at 0°C, a rate of 30%. Semen stored for 5 days at -8°C yielded 45% lambed, and for 10 days had 43%. All the groups were of 100 ewes. Insemination details and dilution rates were not given. However, the fertility rate depends upon other variables than semen, such as the detection of oestrus and the skill of insemination.

Experiments with pig spermatozoa

Pursel & Johnson(1975) used BF5 extender which has basically the same components as Tes-egg yolk-glycerol buffer (Crabo, Brown, Graham 1972) for storing boar spermatozoa. In an experiment using frozen semen stored in BF5 extender, 26 gilts were inseminated intracervically with frozen homospermic semen from an individual boar. Among 22 gilts, 87% ova were fertilized and developed normally. A great number of diluents has been tried with boar sperm, including yolk diluents with citrate or phosphate, milk in its various forms, glucose saline, glycine, saccharose, and various combinations of salts.The literature is full of conflicting claims as to the value of various diluents, but it is becoming obvious that farrowing rates are not influenced by variation in these diluents.

Crabo, Brown & Graham 1972 made very detailed studies of hydrogen ion buffers on boar spermatozoa. The motility was highest in Tris [Tris (hydroxymethyl) aminomethane as base], Hepes[N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid], Tes [N-tris (hydroxymethyl)methyl 2 aminoethanesulfonic acid], also Mops and Bes with glucose and egg yolk after cold storage and freezing. These buffers have low ionic strength. Phosphate and citrate have high ionic strength, and in them motility of boar sperm was low after cold storage and freezing. Crabo et al 1972 measured GOT release after treating the boar semen with these buffers. Among all the buffers, the loss of GOT was lowest when sperm were stored in Hepes buffer. However, GOT leak from sperm stored in Hepes was not significantly different from that when sperm were stored in Bes, Tes and Pipes. These buffers seemed to prevent loss of GOT from the boar spermatozoa, especially during the cold storage phase and during freezing.

The investigation also showed that the release of GOT during storage at 37°C was not significantly different from the initial value. The GOT values obtained after storage at 5°c for 24 hours, freezing and plunging the semen into liquid nitrogen were all significantly different from each other.

Therefore, the temperature of 5°C was chosen for cold storage although better survival of spermatozoa could be obtained at 15°C (Niva,1959).

The conclusion of the experiments of Crabo et al (1972) was that the percentage of motile spermatozoa in various buffers was inversely related to the ionic strength of the buffers at both 5°C and after freezing, perhaps because of protein that precipitated at low temperature in the seminal plasma (Boursnell, Nelson & Cole 1966). This particular protein is believed to be associated with zinc (Boursnell, 1976).

Pursel & Johnson (1975) reported that 87% of fertilized ova developed normally from frozen boar semen extended in BF5 mixture which similar to Tes buffer. This is encouraging but the possibility of using frozen boar semen still needs to be examined in the field.

Freezing, storage and thawing injury

In the CIBA Foundation symposium on cellular injury (DeReuck & Knight, 1964) it is suggested that as the cells were injured, membranes were inactivated or destroyed and cellular material was lost. Flipse (1960) demonstrated that glutamic oxaloacetic trans-aminase (GOT) and glutamic pyruvic transminase (GPT) can be found in bull seminal plasma. He also reported a high correlation between the transaminase activity and the number of spermatozoa present. Presumedly therefore, GOT and GPT release can be related to the number of spermatozoa which have been damaged. Graham & Pace (1967) reported that (GOT) and lactic dehydrogenase (LDH) enzymes increased in the seminal plasma subsequent to plunging semen into liquid nitrogen. Likewise, they confirmed the work of Flipse, and in 1965 Roussel and Stallcup found that GOT concentration is highly correlated with bovine sperm cell numbers, compared with GPT which shows less correlation. Pace & Graham (1970) confirmed that most of the GOT is found in the bovine sperm cell initially and that the amount of enzyme left in the cell after freezing is important to fertility. The results of the experiment showed that the correlation between the total amount of GOT released from the cell and fertility was

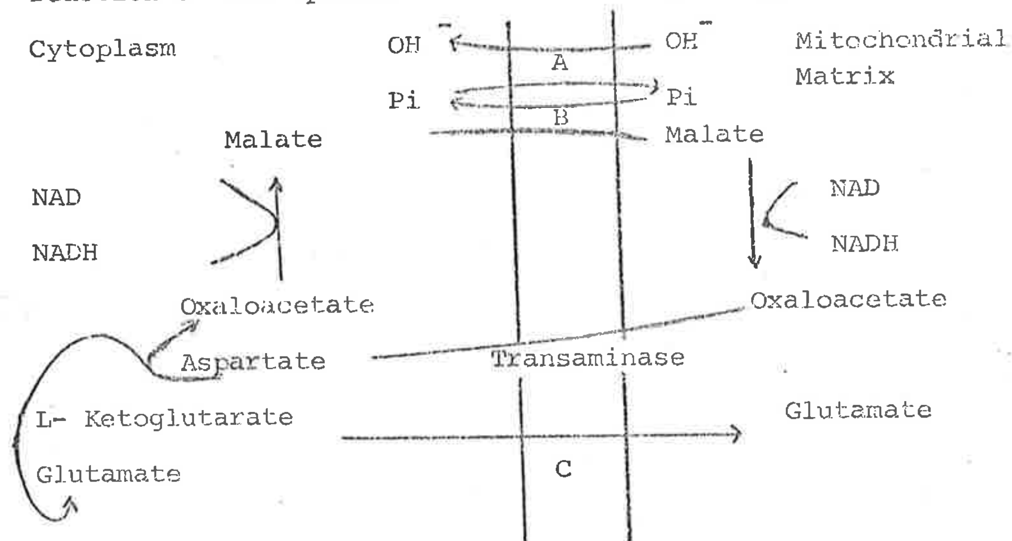
significant (P≤0.5). This indicated that the sperm cells containing higher amounts of GOT have a higher fertilizing capacity..

The biochemical function of GOT

The function of GOT is specifically to catalize the reaction:

L- Aspartate + L- Ketoglutarate ⇄ Oxaloacetate + L- Glutamate

Transminases are found in mitochondria and in the cytosol. In mammals the exchange of amino groups from other amino acids takes place in the cytosol, catalyzed by the cytosol form of glutamic oxaloacetate transaminase (aspartate transaminase), with formation of glutamate. The glutamate so formed then enters the mitochondrial matrix via a specific membrane transport system. In the mitochondrial matrix, glutamate is either directly deaminated or becomes the amino group donor for oxaloacetate by the action of mitochondrial aspartate transaminase to yield aspartate, one of the immediate amino group donors in the formation of urea (Lehninger). There is an intermediate reaction of the specific membrane transport system (Fig 1)



A,B,C, represent specific anion protons or exchange diffusion carriers

Presumably, therefore the GOT release affects mitochondrial respiration and metabolism, and so indirectly affects the fertility.

The release of GOT and GPT from ram and boar spermatozoa

In 1974 Bonadonna & Roychoudhury studied the release of glutamic oxaloacetic transaminase and GPT from boar and ram spermatozoa in diluents which containing glycerol. In the sample of ram spermatozoa GOT concentration at the second hour of conservation at 37 °C was found

to be increased by 37.3 $\mu mol/10^9/min$ which was statistically highly significant (P≥0.01) while the GPT was not significantly increased. For the boar spermatozoa GOT has increased 80 $\mu mol/10^9/min$ after 4 h incubation, while in the same condition no GPT release was observed. From this experiment it may be presumed that the GOT method is more sensitive than the GPT method of predicting cell damage.

Protein leakage from frozen semen

Freezing and thawing induce a number of changes in spermatozoa apart from the intracellular enzyme leakage, such as decreased motility, increased membrane permeability (Quinn & White, 1969) the leakage from the spermatozoa of protein (Harris & Sweeney, 1971) lipid (Pickett & Komarek, 1970).

Boursnell & Mustill (1975) showed a loss of zinc precipitable nitrogen when boar seminal plasma was repeatedly absorbed at 4°C with homologous spermatozoa. Also Boursnell et al (1975) found that the material precipitated by zinc from seminal plasma is a complex mixture of proteins containing material other than haemagglutinin and the zinc precipitated protein, after gel filtration. However, the role of proteins in reactions at low temperature is still unknown.

Lavon & Boursnell (1971) found 11 protein bands from boar seminal plasma. Alumot & Lenskey & Schindler (1971) found 17 bands in ram seminal plasma by the polyacrylamide gel disc electrophoresis. Each method has a different resolving power in separating proteins.

The protein tests of the effects of freeze and thaw still need further study. Since however, gel electrophoresis shows only the position of the leakage protein bands, identification of the protein bands requires other methods.

Electron micrograph description of ram and boar frozen sperm

Quinn & White (1969) have shown morphological changes in the acrosome after freeze-thaw. The acrosome is a Golgi-derived body at the anterior tip of the sperm, partly covering the nuclear material. It contains a trypsin-like enzyme and hyaluronidase, which are required for fertilization processes (Polakoski 1972). From the

possibility of using electron microscopy to reveal the fine structure of the cell, a useful approach can be made to locating the details of damage to spermatozoa due to freezing and thawing.

Since there are some biologically characteristic differences between the semen of the ram and the boar, it seemed possible that these could provide clues to the differential survival of sperm in the frozen state.

General characteristics of ram and boar semen

Semen characteristics are usually assessed in practice in terms of (a) semen volume (b) concentration of spermatozoa (c) proportion of live and dead sperm (d) motility of the spermatozoa (e) chemical constituents of semen plasma.

A. Boar semen

Constituents of semen

Mammalian semen is composed of two parts, the spermatozoa and seminal plasma, the first being produced in the seminiferous tubules of the testes, while the seminal plasma is produced from the male accessory organs. The male accessory organs comprise the epididymis, seminal duct, prostate gland, seminal vesicle and urethral gland. The male reproductive tract arises primarily from the Wolffian ducts, which in turn, differentiate from the genital ridges of the early embryo. The sex organs of the cattle embryo are discernible by about 30 to 45 days of gestation, but development of the reproductive tract continues and the relationship of the various parts changes through foetal and early postnatal life until puberty is reached. However, the secretion of the prostate gland is thick and rich in proteins and salts. It is alkaline, and it has a charateristic odor. Cowper's gland (Bulbo-urethral gland) produces an alkaline secretion for the purpose of neutralizing or cleaning the urethra prior to the passage of semen. The seminal vesicle secretes a fluid which provides a medium of transport of spermatozoa. Boar semen consists of a seminal plasma, spermatozoa, and a large amount of gelatinous material (Niwa 1969). The colour of the semen alters according to the concentration of spermatozoa, in that the higher the concentration of spermatozoa,

The whiter the colour of the semen. So the plasma is yellowish in colour when there is a high volume of seminal fluid. The quantity of boar semen is the largest found among domestic animals. However, the semen quantity and number of spermatozoa are influenced by the breed, age, season, and condition of the animal at the time of collection. Generally, the quantity of semen is about 200-250 ml and 20% of it is gelatinous material.

The number of spermatozoa is approximately 1-2.5 x 10^8/ml and the total number of spermatozoa per ejaculate is about 44-70 x 10^9.

Gelatinous material

The gelatinous material in semen is secreted by Cowper's gland and immediately after semen collection should be filtered out, to prevent it from blocking the injector which is used to introduce semen into the cervix uteri. The semen injector is made to prevent a backward flow of semen and leakage, therefore, it is important that the injector is not blocked.

Chemical components sperm numbers and semen properties

Table 1 (Mann 1964, Niwa 1969)

	Boar	Ram	Sperm Ringer Phosphate (Mann)
1. Quantity of ejaculated semen	200-250 ml	0.7-2 ml	
2. Sperm concentration	1.2-2.5x10^8/ml	2-5x10^9/ml	
3. Physical properties of fresh semen			
pH	6.6-7.9	6.8-7.3	7.2
Osmotic pressure	320 mosmol/l	320 mosmol/l	300 mosmol/l
Electrical conductivity	8.5± 0.25 mmho	3.0±0.5 mmho	13±0.5 mmho
4. Chemical composition			
Total nitrogen	10.27 mg/10^9	3 mg/10^9	
Protein	64.17 mg/10^9	18.79 mg/10^9	
Fructose	126 µg/ml	2470 µg/ml	
Pyruvate		160 µg/ml	
Lactate	210±30 µg/ml	360 µg/ml	
Citric acid	1.29 mg/ml	1.74 mg/ml	

Na	6.4 mg/ml	1.03 mg/ml
K	2.4 mg/ml	0.7 mg/ml
Ca	0.05 mg/ml	0.09 mg/ml
Fructolysis aerobic 37°C		1.74 mg/10^9/h
Respiration 37°C	120 µlO_2/10^9/h	175-225 µlO_2/10^9/h
Inositol	382-607 mg/100ml	10-15 mg/100ml
Choline	235 mg/100ml	1770 mg/100ml

The main chemical components of boar semen are an electrolyte medium of Na, K, Ca, HCO_3, PO_4, N, etc. containing protein, ergothioneine, citric acid, inositol and a comparatively low content of fructose (Mann, 1964). They are all secreted by the accessory male sex organs, and are the natural components of the seminal plasma. They help sustain the sperm motility acquired in the epididymis, and they assist sperm transportation in the female genital tract.

(a) Choline - phosphorycholine and glycerylphosphorylcholine in semen are compounds which are specifically linked with the metabolism of phospholipids which occurs in either the accessory male organs or in the spermatozoa. The level of glycerylphosphorylcholine depends on androgenic stimulation, and is effectively suppressed by castration, and strongly stimulated by testosterone.

(b) Ergothioneine

Ergothioneine exerts a specific physiological role in boar and stallion semen mediated by its reducing sulphydryl groups, resulting in a protective influence upon the spermatozoa. The effect of testosterone on ergothioneine secretion is not very marked.

(c) Lipid in the seminal plasma

The lipids of plasma originate chiefly from the prostatic fluids. In normal prostatic secretions, there are three clearly discernible electrophoretic components. Two have the same rate of migration as the "b" and "r" serum globulins and behave similar ly to "b" and "r" lipoproteins. The third group do not move at all, and are termed chylomicrons or similar " lipid bodies ". Among the lipid-soluble

substances which occur in the seminal plasma are ProstaglandinsE and F. Both are fatty acids with 2 carbon chains, the highest concentrations of prostaglandins are found in human (25 µg/ml) and in ram (300 µg/ml) sperm. A number of functions have been suggested, such as affect platelet function, ovarian function, lipolysis in fat cells. They also mimic the actions of certain hormones (ACTH, LH) or inhibit actions (epinephrine, vasopressin) hormonal activities, possibly by an action on cyclic AMP. However, none of these function have been proved.

(d) Fructose

The formation of fructose in accessory male organs is a hormone-dependent process. The first experiment demonstrating the stimulating influence of testosterone in fructose production and secretion was carried out on rabbits (Mann, 1947). The experiment showed that fructose almost completely disappears from the ejaculate within 2 weeks after castration. However, this post-castrate decrease in the level of fructose can be prevented or restored by the implantation of testosterone.

Insulin and blood glucose exert an influence upon the level of fructose in semen.A decrease in blood glucose results in a reduction in the fructose level of semen (Mann, 1954).

Mammalian spermatozoa rely on carbohydrate metabolism as an energy source, as is seen under the conditions of semen storage for artificial insemination. The dependance is on an extracellular source of carbohydrate in the form of seminal sugar, fructose.

(e) Inositol and Sorbitol

Inositol occurs as a major chemical constituent in boar semen. The function of inositol appears mainly to be associated with maintenance of the osmotic equilibrium of the seminal plasma since the seminal vesicle secretion unlike other body fluids of the pig, is almost completely devoid of NaCl (Mann, 1953;1954).

Another polyol present is sorbitol. The occurance of sorbitol in seminal plasma was first reported by King, Isherwood & Mann (1958). The level of sobitol in fresh semen has a definite relation to the concentration of fructose and to the activity of two enzymes, aldose

reductase and sorbitol dehydrogenase (ketose reductase) in accessory organs (Samuel 1962). The bull and ram seminal vesicles, which are efficient fructose producers also possess the highest aldose reductase and sorbitol dehydrogenase activity, which means that these glands have an equal capacity to reduce glucose to sorbitol and to oxidize sorbitol to fructose. The boar seminal vesicles are different in producing less fructose, and although they have some aldose reductase there is hardly any sorbitol dehydrogenase. They cannot be expected therefore to oxidize sorbitol to fructose as efficiently as the glands of bull and ram.

(f) Citric acid

Most higher mammals, possess a high concentration of citric acid in the semen. In the bull, ram, boar and stallion it is derived mainly from seminal vesicle secretion, but in humans the citric acid originates in the prostate. The function of citric acid is linked with the calcium-binding ability of seminal plasma. A number of theories have been advanced to explain the mode of action of citric acid.

(1) The citric acid in combination with potassium and sodium ions, may play a part in maintaining osmotic equilibrium in semen (Mann 1954a).

(2) Fructose synthesis influences citrate oxidation in the secondary male sex organs with low NADH -cytochrome C-reductase activity.

(3) The physiological role of citric acid in semen and the beneficial effect of citrate on sperm motility, is not due to direct utilization by sperm, because the citric acid is connected with the coagulation and liquefaction of semen and with the calcium-binding capacity of seminal plasma.

(4) Lundquist (1947) believes that the citrate may act as an activator of the prostatic "acid" phosphatase.

(g) Protein components

Eleven protein bands have been obtained from boar seminal plasma on a gel disc electrophoresis system at pH 8.6. The seminal plasma protein has three major protein components, designated as A, B, and H. These were identified and studied by Boursnell (1966) and Boursnell& Briggs(1969). These components represent a mixture of various proteins.

The main proteins in the seminal plasma and vesicular secretion, are basic in character and appear to comprise 80% to 90% of the total amount of protein.

The function of the seminal amino acids are:

1. Amino acids enable the spermatozoa to make fuller use of their endogenous substrate.
2. Detoxicating effects by removal of heavy metals which are commonly present in sea-water or diluents.
3. Amino acids and proteins may act on the dilute sperm suspension by virtue of their buffering capacity.

B. Ram semen

(1) Grading ram semen

The methods of Gunn et al (1942) are used to count the number of spermatozoa in semen. A scale similar to Gunn's is used in the U.S.S.R. for grading (Maule 1962 C.A.B.).Gunn defined the grades of ram semen as follows:

Type of semen	Approx sperm count in 10^8/ml
Very thick, creamy	30
Thick, creamy	25
Creamy	20
Thin, creamy	15
Thick, milky	10
milky	5
Cloudy	1
less then cloudy	1

(2) Quantity of semen and number of spermatozoa

The semen quantity and number of spermatozoa are affected by season, breed, management, disease, enviromental temperature, nutrition, frequency of collection etc. Generally, the quantity of semen is between 0.7 and 2 ml for mature rams.

The number of ram spermatozoa is about 3×10^9/ml. According to Salamon,(1964) a ram may ejaculate 20-30 times a day, but the number of spermatozoa per ejaculate decreases from 46×10^8 to 2×10^8/ml, on

the first day, and from 2.5×10^8 to 0.6×10^8 on the second day.

There is little change in motility as density of sperm decreases.

(2) Chemical composition and metabolism

The chemical composition of ram semen is different from that of the boar, a comparison is given at table 1.

Fructose and citric acid are present in ram semen in large amounts. Motile ram spermatozoa are able to convert the fructose to lactic acid, producing a certain amount of heat. Using a new sensitive calorimetric method, which is capable of detecting a heat output of 10 µcal/min (Rothchild 1960) dertermined the heat which is produced by bull and human spermatozoa under anaerobic as well as aerobic conditions. Ram spermatozoa were capable of maintaining their fructolysis under both aerobic and anaerobic conditions.

There are other substrates which the spermatozoa are capable of oxidizing such as acetate, glycerol, and sorbitol.

Ram and bull spermatozoa metabolize fructose anaerobically at a rate of 1.5-2 mg fructose/10^9 sperm/h at 37°C.

(3) Protein constituents of ram semen

Proteins of ram epididymal fluid, seminal plasma and accessory gland fluid were fractionated by gel disc electrophoresis by Eugenia Alumot et al (1971). They found 19 bands present in each sample, bands 14 and 18 are contributed to the seminal plasma mainly from the accessory gland fluid. The function and variations of these proteins need further study.

Sperm motility and evaluation of semen quality

A. Evaluation of semen quality

i. General methods

Walton (1952) explained that fully motile spermatozoa are the sperm groups that have good wave motion under the microscope. The characteristic of the wave is dependent on both the activity of individual spermatozoa and their concentration. When the sample is suspended in a small volume, as on a slide, the movement is in one direction. These spermatozoa create local disturbances of equilibrium in the medium

and cause " wave motion ".

Hence the method of visual estimation of motility is totally subjective. Rothchild (1949,1950b) suggested the use of impedance change frequency, since active samples of bull and ram semen have been shown to exhibit well defined changes in electrical impedance, the frequency of these being related to visual estimations of motility. Measurement of impedance change frequency is a more objective test of sperm motility.

There are other methods available for the evaluation of semen quality, including histological, physio-chemical and biochemical methods.

ii. Histological methods

(a) Determination of the concentration of motile spermatozoa (Tampion & Gibbon 1963).

(b) Electron microscope examination (Jones, 1973).

(c) Differential count of abnormal forms of spermatozoa (Williams 1950, Bretcheider,1955 Hancock, 1953;1955).

(d) Determination of the incidence of dead spermatozoa by " live-dead staining" methods (Bodnar, 1969 Hancock,1952).

(e) Filtration method (Hancock & Shaw, 1960).

(f) Sperm smear (Gunn, 1942 Starke, 1949).

iii. Chemical and biochemical methods

(a) Light scattering and light absorption power.

(b) Electrical conductivity and impedance change frequency.

(c) pH, buffering capacity, sperm respiratory rate, fructolysis index and methylene-blue reduction rate.

(d) Specific gravity, osmotic pressure and resistance to cold shock etc.

(e) DNA study of aging spermatozoa (Lodge & Salisbury, 1972).

(f) Enzyme study of sperm quality (Graham & Pace 1967).

In 1954 Bishop et al , found that there was no evidence to suggest that " initial motility " could be closely correlated with metabolic activity, which is a measure of the number of living spermatozoa contributing their metabolic activity. Metabolic tests indicate the functional activity of sperm cells.

B. Factors which affect sperm motility *in vivo*

1. Management

Once a male animal had been selected for artificial insemination, it is important to obtain the maximun number of sperm cells with optimum motility. The animal must be trained to serve an artificial vagina, and also be free of Pavlovian type conditioned reflexes derived from incidental aspects of the environment that may decrease the libido after a period of time. Injury, pain, fear, or cruel treatment may reduce sexual drive.

Maintenance of the maximum libido of each male, will be reflected in the quality of the semen obtained.

2. Temperature and light

De Alba & Riera (1966) held 10 bull calves in a hot chamber at 35-36°C for 8 h daily from 26 weeks of age to the age of puberty (41-45 weeks for semen collection with bulls). Another group was held at 27°C. Both chambers had relative humidities of 80-90%. The results are compared in Table for average ejaculates

Temperature	35°C	27°C
Motility	4.1%	41.5%
Sperm number	92×10^6	928×10^6

However, the heat stress did not inhibit libido. Cupps (1960) found semen quality to be low in summer even if the fructose concentration in the seminal plasma remained high.

Yeates (1949) demonstrated in Suffolk sheep that the sexual season can be completely reversed by artificially providing additional amounts of light during the winter, and by decreasing the amount of light during the summer. The effects of day length on sperm motility are not yet clear, but sperm numbers are not influenced. Low fertility is probably caused by endocrine disturbance.

3. Nutrition

Flipse (1963) found that feed protein in the range of 10-20% gave semen of normal quality. Davids, Mann & Rowson (1957) selected twin calves, fed high and low planes of nutrition and noted the effect on body weight and on semen production.

Table2 Effects of high and low planes of nutrition on sperm numbers and semen plasma metabolites

	High nutrition		Low nutrition	
Age	5 months	10 months	5 months	10 months
Body weight	180 kg	350 kg	70 kg	250 kg
Sperm density	1.5×10^8		0.35×10^8	
Fructose	140 mg/100ml		130 mg/100ml	
Citric acid	140 mg/100ml		90 mg/100ml	

Sperm motility *in vitro*

1. pH

Generally, pH 7.0 gives the optimum survival rates. The buffering capacity of semen was reviewed by Anderson (1945,1946) who showed that whole semen was more completely buffered on the acid than the alkaline side of neutrality and pH change in whole semen was related to duration of motility. Mann (1954) considered that the pH changes were due to lactic acid production. Salisbury & Kinney (1957) reported on bull semen diluted in a phosphate buffer containing Na, K, Mg, and Cl ions, where he found that the higher the pH, the higher was the lactic acid production. There was little variation of pH in successive individual ejaculates. Cragle & Salisbury (1959) measured the oxygen uptake at the different pH values of 4, 5, 6, 7, and 8. They found the oxygen uptake was small at pH 4, increasing to a maximum in 5.5-6.5 and decreasing again at the highest pH.

2. Diluents

Current knowledge indicates that the features of a satisfactory diluents are:

(1) Osmotic pressure is isotonic with blood and capable of maintaining that approximate pressure during storage.

(2) Presence of sperm cell nutrients for both aerobic and anaerobic metabolic processes.

(3) Provision of a proper balance of mineral elements essential to the life of spermatozoa.

(4) Provision of chemical means for buffering toxic products and to maintain good buffering capacity.

(5) Absence of bacterial products or infectious organisms harmful to spermatozoa.

For the purpose of freezing sperm, protective substances should be added to semen.

However, the most important considerations in diluent studies are the length of storage of diluted semen prior to its use for insemination, and the actual semen dilution rates.

Recent developments of artificial insemination of ram and boar semen diluents are:

1) Sperm Ringer fructose phosphate- mainly used for biochemical studies, modified from Krebs Ringer by Mann (1954). Ram spermatozoa are well preserved in this medium, but not boar spermatozoa.

2) Raffinose- citrate diluents (Salamon,1971). He suggested that this diluents successfully preserved ram spermatozoa frozen for 3 years.

3) Tris buffer extender- Tris base extender has been used by numbers of workers mainly for frozen boar semen (Salamon,1974 Crabo , Brown & Graham, 1972)

4) Tes-glucose- egg yolk buffer. Crabo, Brown, & Graham (1972) found that Tes buffer provided boar spermatozoa with the best buffering capacity and good performance after freezing and thawing.

5) BF3 extender (Pursel, 1969). The extender is compounded of lactose, casein, tris, citric acid and fructose. This extender has been studied in the freezing of boar spermatozoa.

6) BF5 extender (Pursel & Johnson 1975). This extender has the same components and proportions as Tes-glucose-egg yolk buffer, and is reported to yield 85% fertilized ova from frozen boar semen.

3. Temperature and storage

The temperature of spermatozoa determines the rate of metabolism, since the rate of chemical reactions varies directly with the temperature.

Both motility and metabolic activity (essential for fertilization) may be considered optimum at body temperature. The rate of these activities is increased outside the male body as the temperature is raised above normal, and the total life expectancy of the cells is reduced proportionally. There are upper extremes of temperature which cause irreversible changes in the protoplasmic complex of living cells, resulting in their death. Irreversibility of enzyme action takes place by heat denaturation of the proteins or other causes, then the sperm cells die. This upper temperature for bull and ram spermatozoa is about 50°C (Salisbury & Vandemark 1961).

As the temperature is lowered, metabolism and the motility of the cells decrease but not necessarily at the same rate. At about 7°C all visible motility ceases. There are abundant observations of persistent motility at 5°C (Salisbury & Vandemark 1961). At temperatures only slightly above freezing the motility ceases, but the sperm cell continues to carry on functions vital for life, at a rate which limits the time period over which the fertilizing capacity can be maintained.

Aslanjan (1950) has reported on transport of ram semen at 0°C for as far as 1200 km, by plane in up to 168 h, with a 93% conception rate. Results were best with 1 : 1 dilution. After transport up to 7000 km conception rates of 75-80% were obtained with semen two to three days old, and 33% with seven days old semen from Rambouillet rams (Aslanjan, 1954) . Korotkov (1951) experimented with the storage of ram semen at 0°C for 1-2 days. Undiluted fresh semen gave 83% lambing, with yolk-glucose-citrate-glycine aerobically 72% lambing was obtained. Yolk-glucose-citrate anaerobically gave 71% lambing, but yolk-citrate without glucose yielded only 55% of lambs. Salamon (1971) reported a 51-54.9% lambing rate from ram spermatozoa frozen and stored for 3 years. He suggested that the cell concentration of samples should be 1.5-1.6 x 10^9/ml.

For boar semen Ito (1948) recommended 0.7% sodium chloride or 5-6% glucose as a diluent, with warming and shaking following storage to reactivate the spermatozoa. He recommended a storage temperature of 15-20°C for whole semen, and 3-10°C for fractionated semen. He also suggested (1948) that for storage times of less than 24 h, 5 x 10^8/ml spermatozoa were required, and for longer storage 7 x 10^8/ml were necessary.

Consistently successful fertilizations with intracervical insemination of frozen boar spermatozoa were first reported in 1971 (Crabo & Einarsson,1971; Graham et al 1971 a,b; Pursel & Johnson 1971 a,b,c,). Since then, other successful freezing procedures have been reported by Crabo et al 1972 ; Salamon & Visser,1974 and Wilmut & Polge,1972.

A number of these freezing procedures appears to give conception

rates and litter sizes approaching those required for use by swine producers.

Pursel and Johnson (1975) reported 26 gilts inseminated twice with 6×10^8 spermatozoa per dose. Twenty-two of 26 gilts (85%) produced fertilized ova and 87% of the ova from 22 gilts were fertilized and developed normally.

4. Osmotic pressure

The osmotic pressure of seminal plasma is similar to that of other physiological fluids. Rothschild (1960) has investigated the effect of incubation (37°C) on the freezing point of human seminal plasma. He showed in sample after 4 h incubation, that the freezing point was depressed. Presumably the change is caused by the hydrolytic processes in the seminal plasma during incubation (such as the breakdown of proteins to peptides and amino acids or splitting of phosphorycholine to choline and inorganic phosphate).

However, osmotic shock produces a bending of sperm tails and circular swimming patterns as well as high mortality under the microscope, particularly during freezing in hyperosmotic solution (high in electrolytes).

Sensitive indications of tonicity of the medium can be measured by changes in cell size shown by electron microscope (Drevius & Eriksson, 1966).

5. Electrolytes

Cragle & Salisbury (1959) reported that a low concentration of potassium is necessary for optimum metabolism of spermatozoa. Also, their experiments showed that, in the presence of calcium, high potassium levels do not have any measurable effects on sperm cell metabolism. Mcgrady, Nelson & Ireland (1974) made a very detailed study of the effects of varying ion concentration on motility and membrane potential of bull sperm. They reported that the major ionic conditions leading to a depression of bull sperm motility were:

(1) Lack of potassium (K^+ free).

(2) Deficient chloride (below 100 mequiv/l).

(3) Potassium concentration higher than 6 mequiv/l .

(4) Calcium chloride concentration in excess of 1 mM.

(5) Sodium content lower than 100 mequiv/l.

Ion imbalance in semen or in the female reproductive tract fluids could seriously interfere with fertility.

6. Female genital tract fluid

Grotjan, Say & Mayer (1975) studied respiration and motility of washed porcine spermatozoa in the presence of bovine follicular fluid. Whole bovine follicular fluid (as 5, 10, or 20 % of the suspension medium) had a detrimental effect on respiration rates and motility as well as causing head to head agglutination.
Dialyzed or heated bovine follicular fluid was less toxic to porcine spermatozoa than whole bovine follicular fluid.

7. Toxin

There are potentially harmful effects of the metabolic products from sperm metabolic processes, such as lactic acid and hydrogen peroxide. These may be toxic to spermatozoa.

The influence of lactic acid and hydrogen peroxide may be minimized by using the proper buffering solution, refrigeration, and exclusion of oxygen.

Some chemical and pharmacological products have toxic actions on spermatozoa. 1-Chlorhydrin has been used in antifertility experiments. The mechanism of action in rats is depression of glycolysis (Coppola, 1969).1-Chlorhydrin causes infertility in rams (Kreider & Dutt,1970) , boars (Johnson & Pursel, 1973)rats(Ericsson & Baker,1970), but the mechanisms may be different in various species.

Some cryophylactic agents like methyl pyrolidone, diethylene glycol, propylene glycol, tetramethyl acetamide, erythritol, dimethyl sulphoxide, dimethyl acetamide etc. also produce toxic effects in over-dosage.

8. Nutrients

Mammalian spermatozoa rely on carbohydrate metabolism as the chief source of energy. Under anaerobic conditions they must depend on extracellular sources of carbohydrate such as fructose (Mann, 1964).

The formation of fructose in the male accessory gland is a hormone-dependent process (Mann & Parsons,1949). It was first demostrated in the rabbit that fructose disappears from the ejaculate almost completely, within two weeks after castration, but that the postcastration decrease

in the level of fructose can be prevented or restored by the implantation of testosterone.

Material and method

(1). Animals

Rams

All rams selected were from 2-4 years old. The rams were trained in the pen which would hold the animals through the whole period of training and collection.

One oophorectomised ewe used as a teaser one week after operation, was injected with 17-α-oestradiol (3 μg/ml/kg) for 3 days. The hormone was continued for 3 weeks at 1.5 μg/ml/kg/day.

All the rams and the ewe had been hand fed by the trainer for 2-3 weeks.

Before the training, the ewe was tied in one corner of the pen then 2 or 3 rams were let into the pen. When a dominant ram attempted to mount, he was allowed to complete copulation, since this increases his sexual drive and engages the interest of the other rams through visual stimulation.

The orientation and exploratory phase may last 1 to 1.5 h. Rams which do not show interest after this time were removed from the teaser ewe.

It is necessary to have some previous knowledge of the temperament and behaviour of the rams, for useful semen collection.

Boars

Boar semen was collected from a 10 month old boar trained by the Department of Agriculture (S.A.) Pig Reaearch Unit, where it served several oestrous sows every day. A dummy sow was made of metal stand. It's height was 70 cm and length 130 cm with 20 cm width.

(2) Semen collection

Rams

After the rams had overcome their initial fear or inhibitions, and had become accustomed to the presence of the operator handling the sheath, rams rapidly served the teaser ewe. However, there were individual aspects of behaviour to which the operator adjusted.

An artificial vagina was made of hard plastic casing, 20 x 5 cm. The liner was 30 x 5 cm latex rubber. A plastic funnel connected the lower end to a graduated centrifuge tube was attached at one side of the vagina. The temperature of water maintained between the plastic casing and latex

rubber was 37°C-40°C.

Boar

When boars were allowed near the dummy, a hand massage of sheath was necessary for stimulation. After the boar mounted the dummy, a rubber gloved hand was used for collection. The best conditions for ejaculation should be with no external inhibition or force. The duration of ejaculation was about 10 minutes in 2 waves.

(3) Semen evaluation steps

1. The volume was read directly from the graduated collection tube.
2. The pH was determined by pH paper (6-8 range).
3. Sperm numbers were estimated using a Neubauer haemocytometer counting chamber. Semen was diluted with formol saline (10%) 1000 times for fresh ram semen; 10 times for fresh boar semen.

The counting chamber was flooded from the side towards the centre, rejecting the first few drops from the pipette.

The chamber was placed on a microscope stage and allowed to settle for 5 minutes. Sperm were counted by using sperm heads only. In the count sperm heads touching the top or right hand lines of the large square were included.

(4) Live-dead staining to examine the percentage of viable spermatozoa in semen samples.

The preparation was stained using 5 g water soluble eosin Y dissolved in 300 ml of 10% nigrosin solution made up in distilled water.

Classification of spermatozoa under the microscope.

In "live" non-staining sperm, the outline was clearly defined with a bright refractive head in sharp contrast to the dark background. The boundary zone was clear between anterior and posterior poles.

"dead" sperm stained a pinkish colour. Estimates of the percentage of living spermatozoa were obtained from counting.

(5) Motility

a) Wave motion

1) The sample was kept in a warm container at about 30°C-37°C.
2) It was transferred to a slide previously warmed to 30°C on a warm microscope stage and examined with low power magnification and reduced light.
3) The semen sample was suspended in a very small volume of seminal

plasma and sperm mixture and the wave motion was graded 1 to 5 scale.

b) Oxygen consumption

After examination of the wave motion, 3 ml (about 10^9 spermatozoa) of semen were diluted in sperm Ringer solution and washed 3 times. Oxygen consumption was measured at 37°C in a Warburg manometer. Sperm Ringer phosphate fructose was used for dilution. The shaking rate was 120 cyc/min. Carbon dioxide was absorbed in 0.2 ml 10% KOH in the center tube of each flask. A base line reading was taken after an equilibration period of 10 min with the manometer tap open. Subsequent readings were taken every 15-30 min for 3 h. Samples were measured in duplicate.

The washing steps were:

1) Dilution of ram semen with diluent 1:6.
2) Centrifugation at 1000 rpm for 15 min .
3) The pellet was then suspended in fresh medium.
4) The sample was spun again at 1000 rpm for 10 min.
5) Ram semen was washed 3 times.
6) With boar semen the seminal plasma was centrifuged before washing in the medium.

(6) Freezing and thawing procedures and media

a) Ram semen

Semen was collected from 6 rams with the artificial vagina. Ejaculates from these rams were examined and those with good wave motion were pooled, then diluted with the various media prepared, such as citrate raffinose solution and sperm Ringer phosphate fructose. The components are shown in the Appendix.

Semen diluted was 1:4 at 30°C then cooled to 5°C over 1.5 h.

The semen was then dropped into liquid nitrogen.

Thawing solution

Citrate 72.8 mM

Glucose 44.4 mM at 37°C

Thawing dilution 1:3 (pellet : thawing solution V/V)

b) Boar semen

1) Boar semen was allowed to cool to 20°C over 1 to 1.5 h.
2) It was diluted 1:1 at 20°C with the different media (see Appendix).

If the medium contained egg yolk, it was centrifuged at 3,200 g for

10 min before use.

3) Diluted semen was cooled to 5°C over 3 - 3.5 h in a refrigerator at 5°C.

4) Immediately after 5°C was reached semen was frozen in pellets by dropping the tube into liquid nitrogen.

Thawing was performed in tris buffer with fructose, in a 60°C water bath, holding the thawing solution temperature at 35°C throughout the thawing procedure.

5) Sperm motility after thawing was graded.

(7) Osmolality and conductivity measurements

Before the semen was mixed with the diluents, the osmotic pressure , pH, ionic conductivity and ionic strength were measured and calculated.

pH - A radiometer type PHM 27 meter was used, at 22°C.

Ionic conductivity - A radiometer type CDM 2 with platinumelectrodes, was employed.

Osmolality - A Fiske osmometer was used, the operative steps are given in the Appendix.

(8) Glutamic-oxaloacetic-transaminase analysis by the method of Babson et al 1962 and Crabo, Graham 1972.

Spermatozoa:

Sperm cells were removed from the extracellular medium by filtering through glass fibres and cellulose powder.

1. Before semen was stressed, one sample was filtered and identified as " pre-treatment ".

2. Another sample was plunged into liquid nitrogen and identified as " maximum releasable GOT ".

3. Semen was frozen after putting the semen sample in 450 ml water at 37°C, then it was cooled to 5°C in a cold room, as" normal freezing".

4. Frozen samples were thawed in water at 5°C for 1.5 h before analysis, and then low speed centrifuged to remove the spermatozoa.

The colo rimetric reaction for determination of GOT required four solutions: substrate, diazonium salt, oxaloacetate standards and control buffer.

To 1.0 ml of substrate at 37°C, 0.2 ml of semen was added and incubated

for exactly 20 min. 1.0 ml of diazonium salt solution was added to the reaction mixture. 10 min later the tube was removed from the water bath and 10 ml of water was added and mixed, immediately the optical density at 530 mμ was read.

The transaminase activity was determined by reference to the standard curve. A graph was constructed relating transaminase units to optical density (Table 5). The oxaloacetic acid formed is a measure of the units of glutamic-oxaloacetic-transaminase present:

umol of oxaloacetate used per assay	GOT enzyme units/l
0	0
0.1	25
0.2	52
0.3	82
0.4	116
0.5	157
0.6	209
0.7	272
0.8	367

(9) Polyacrylamide gel electrophoresis

Semen separation of ram seminal plasma proteins.

1. Semen was diluted with Ringer solution, then centrifuged at 1000g. Ram semen was diluted 1:5 with sperm Ringer solution, boar semen 1:1, with tris buffer fructose solution.
2. The sample was stored at $-20^{\circ}C$ if necessary.
3. For electrophoresis, 150 μl to 200 μl of ram semen; 150 μl to 250 μl of boar semen were employed.
4. Triplicate aliquots of 0.15 ml to 0.25 ml per tube were analysed.
5. 2 mA were applied per tube until the marker had left the upper large pore gel, and then changed to 3.5 mA per tube in the small pore gel.
6. For each gel, 3 μl of tracking dye (0.05% bromophenol blue in water) was added.
7. After completion of electrophoresis, the gels were removed from the

tubes by rimming under water. The water lubricates the gel surface and prevents mechanical damage to the gel by the rimming needle.

8. After the gel was removed from the tube it was put in a numbered rack made from plastic net, to await further staining procedures.

9. Stain:

The gel was stained in 10% of TCA with 0.01% Coomassie blue for 20 h. then rinsed twice in 10% TCA.

10. Deataining undertaken with 10% acetic acid.

(10) Electron microscopy of spermatozoa

This was using the methods of Jones,(1971b; 1973).

a) Ram and boar spermatozoa were washed in sperm Ringer solution and tris buffer solution for 10 min and this was repeated twice.

b) Spermatozoa were fixed in buffered glutaraldehyde 2.5% for 4 h, in the refrigerator.

c) Sperm were then rinsed in cold buffered glucose.

d) Post-fixation was carried out with osmium tetroxide for 1 h. The time depended on the size of the pellet.

e) Dehydration was carried out in ascending concentrations of acetone, starting with 30% and progressing to 95% in 3 or 4 steps of 15 min each.

f) After dehydration the sperm were embedded in epoxy resins, preferably the hard block which hardened in a rubber mould.

g) Sectioning was undertaken using an LKB microtome with glass knife. Each knife edge was examined by low power microscope and parts of the edge free of nicks or fine lines were used for cutting. A suitable water trough was attached to the knife to catch the sections as they were cut. The sections were then picked up on prepared grids.

h) The sections (supported by the grids) were then stained in lead citrate solution 5% for 4-5 min. After staining the grids were washed in 0.02 N NaOH.

Double staining with lead citrate increased the contrast. Uranyl acetate 5% followed by lead staining was useful for the fibrous proteins.

i) After fixation and preparation of spermatozoa for examination with the transmission electron microscope, electron micrographs were taken at different magnifications and printed.

Results

The respiratory activity of spermatozoa is usually correlated both with their motility and concentration. It is mostly expressed in terms of ZO_2, to denote the amount of oxygen taken up by 10^8 sperm cells during 1 h at 37°C, a convention introduced by Redenz(1933).

ZO_2 of ram and boar sperm

1. Effects of diluent solution

The oxygen consumption of ram spermatozoa diluted with sperm Ringer phosphate frutose was compared with that when citrate raffinose solution was used. Spermatozoa were counted and diluted to 10^9 in 3 ml. The initial motility was scored at 4.5. Semen was washed 3 times in a diluent and the motility score was maintained about 4-4.5. The results of triplicate experiments are shown in Fig 1.

Citrate raffinose solution had a lower ZO_2, differing by 100 µl at 1 h and by 200 µl at 2 h from SRPF. Compared with control samples, (which were maintained without adding fructose in sperm Ringer phosphate) the respiratory rate declined after 2.5 h. There was a higher ZO_2 in control samples than in citrate raffinose samples of the same sperm. The osmotic pressure of this citrate raffinose solution was 260 mosm/l compared with 330 mosm/l for the sperm Ringer phosphate fructose. To determine whether osmotic differences altered ZO_2,a comparative study was made of the effects of osmotic pressures on the oxygen consumption of ram spermatozoa Fig 2.

The highest ZO_2 of ram spermatozoa was obtained with a 300 mosm/l solution (Fig 2). At 380 mosm/l, 180 µl of oxygen was taken up by 10^9 cells in an hour, compared with 220 µl in a 300 mosm/l solution. In the 100 mosm/l and 140 mosm/l media sperm used about 100 µl oxygen per hour. With 200 mosm/l to 240 mosm/l solutions the ZO_2 were around 130 ul to 150 µl/10^9/h. Therefore, it appears that, 300 mosm/l maintained the best motility and the highest oxygen uptake of spermatozoa.

The reason why the citrate-raffinose solution was not as good as SRPF for spermatozoa respiration, at 37°C is indicated in Figs 1 and 2. The shortage of exogenous substrate (fructose) appears to bring about a decline of the respiratory rate after 2.5 h. The control samples which

had been adjusted to 300 mosm/l without adding fructose also had somewhat higher oxygen consumption rates than the citrate raffinose solution.

Boar spermatozoa were incubated at 37°C with different diluents in a similar fashion to ram sperm. But tris fructose buffer and Tes egg yolk media were compared, as well as BF3 extender and SRPF. Semen was washed in a diluent and the motility score was about 4-4.5. The results are shown in Fig 3. Visual examination under the microscope revealed little difference of motility between these samples. However, from the results shown tris fructose buffer supported 100 µl O_2 uptake higher than SRPF at 1 h, and 250 µl O_2 uptake after 3 h. Tes and BF3 extender were lower than tris fructose, but there was only 20 µl O_2 uptake between these two solution. The standard deviations were ± 6.68 in BF3 extender at 3 h incubation, with an S.D. ± 5.62 in Tes egg yolk solution. Tris buffer solution had ±15.77 S.D. and ± 11.24 in SRPF.

Table 1

The osmotic pressures of the boar sperm diluents are listed in Table 1, as well as the conductivity, and ionic strength of the diverse media.

Diluent	Osmotic pressure	pH	Ionic conductivity	Ionic strength
Tris fructose buffer	300 mosm/l	7.6	4.5 mmho	0.027-0.031
Tes egg yolk buffer	385 mosm/l	6.8	6.5 mmho	0.068-0.081
BF3 extender	346 mosm/l	7.3	2.0 mmho	0.015
SRPF	300 mosm/l	7.4	13±0.5 mmho	0.239

From the Table 1 shown the Tes egg yolk buffer had the highest osmolality and Tris fructose is the lowest, while both allowed good sperm respiration, the differences of the oxygen uptake for boar spermatozoa were obviously not due to the osmotic pressure of the diluents, although this is the suggestion of Crabo et al. (1972).

The low ionic strength solution (Tes egg yolk) significantly increased motility. Phosphate and citrate buffers had high ionic strength, and the motility in these buffers was low both after cold storage and after freezing. The effects of different osmolalities on the oxygen consumption of the boar spermatozoa were studied in triplicate experiments as shown in Fig 4.

From Fig 4 a hyposmotic solution had similar effects to the hyperosmotic solution. A 300 mosm/l solution gave the best respiratory rate.

In Fig 4 is shown the oxygen uptake increasing as the osmotic pressure increased, but a severe decline occured with the hyposmotic samples.

Fig 1

Comparison of ram spermatozoa diluted with sperm Ringer phosphate fructose solution (SRPF) and with citrate raffinose solution (CR).

The control sample was diluted with sperm Ringer phosphate without adding fructose. The oxygen consumption was higher in SRPF than in the others. Each 3 ml flask had 10^9 spermatozoa incubated at 37°C. The sperm Ringer phosphate fructose solution allowed oxygen consumption at 225 $\mu l/10^9/h$, compared with 165 $\mu l/10^9/h$ in citrate raffinose solution. The control sperm used 210 $\mu l/10^9/h$ for the first hour of the incubation. However, the oxygen consumption rate was highest in SRPF and lowest in citrate raffinose solution at 37°C. The respiratory rate declined in control sample after 2.5 h incubation.

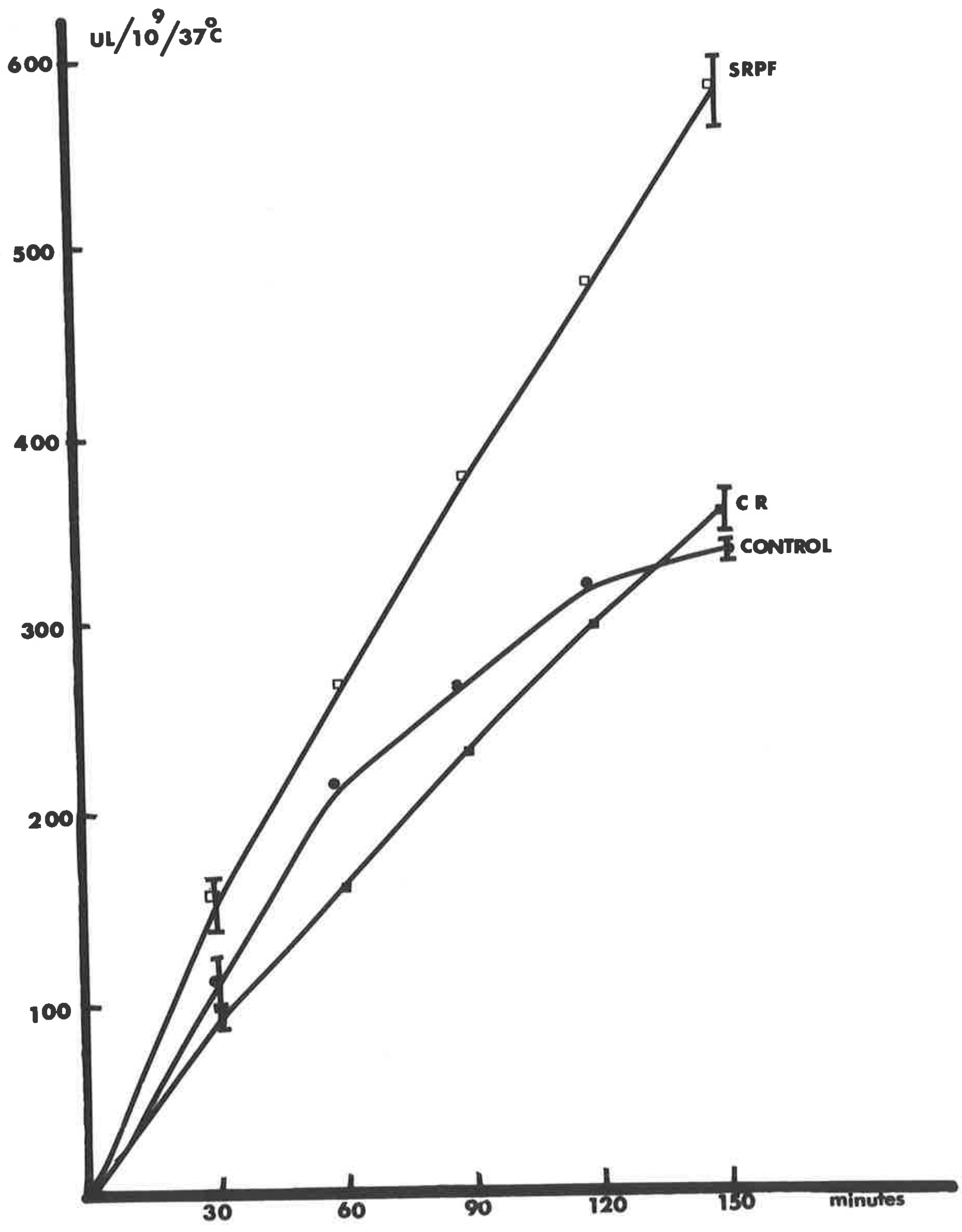

UL/10^9/37°C
600
500
400
300
200
100
SRPF
C R
CONTROL
30
60
90
120
150
minutes

Fig 2

Ram spermatozoa oxygen uptake from 10^9 cells at $37^{o}C$, in different osmolalities of suspending solution. The basic components of the media were similar to the Krebs Ringer solution. Differentces of osmolality were obtained by alteration of NaCl concentration in the Ringer solution.

0.06 M NaCl	100 mosm/l
0.077 M NaCl	140 mosm/l
0.096 M NaCl	200 mosm/l
0.130 M NaCl	240 mosm/l
0.154 M NaCl	300 mosm/l
0.191 M NaCl	380 mosm/l
0.200 M NaCl	400 mosm/l

The highest ZO_2 of ram spermatozoa was obtained with a 300 mosm/l solution. The lowest ZO_2 was gained from 100 mosm/l solution, which was not much different from 140 mosm/l, 200 mosm/l and 240 mosm/l solution, ZO_2 were around 130 μl to 150 $\mu l/10^9/h$. At 380 mosm/l, only 180 μl of oxygen were taken up by 10^9 cells in an hour.

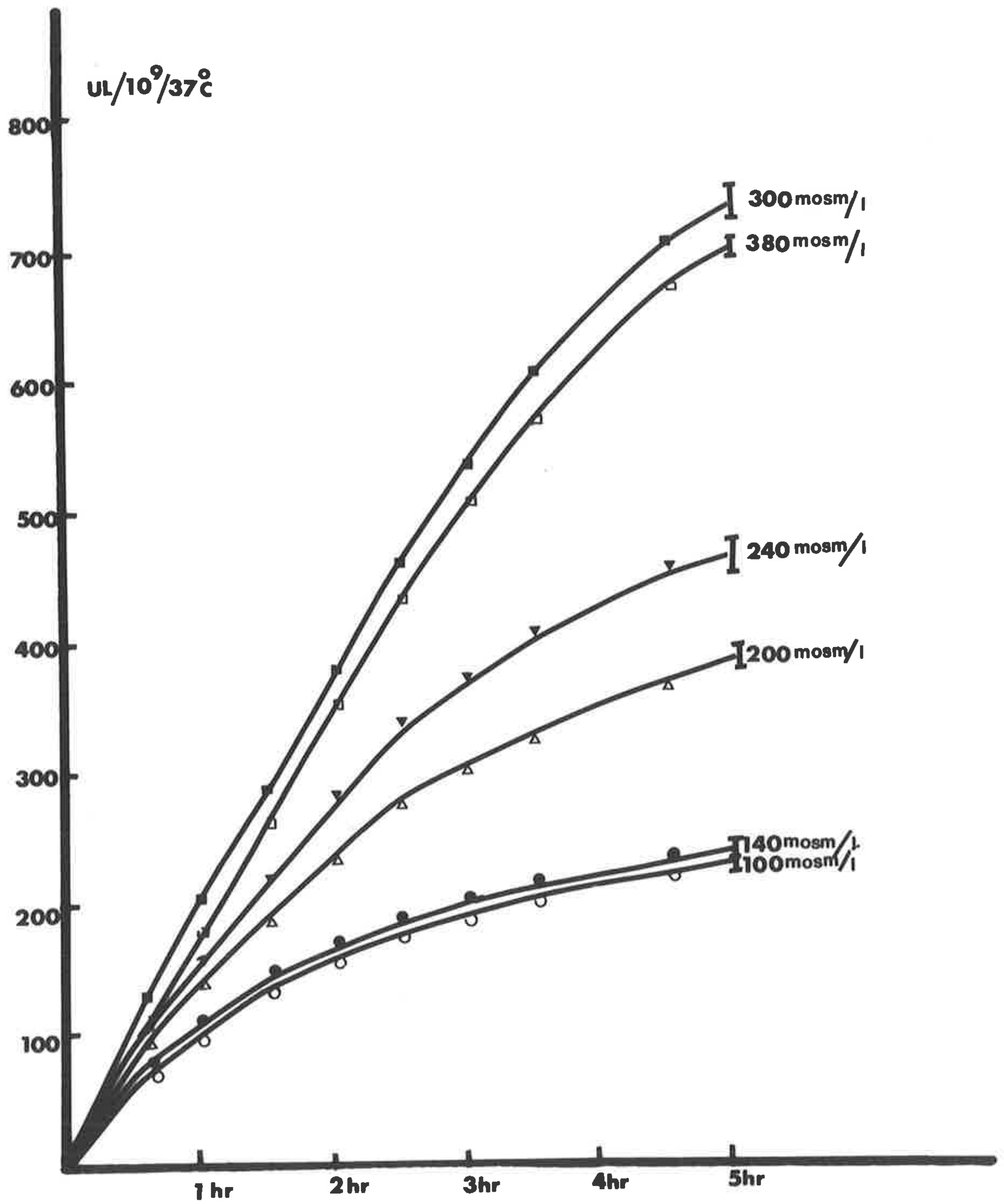


Fig 2

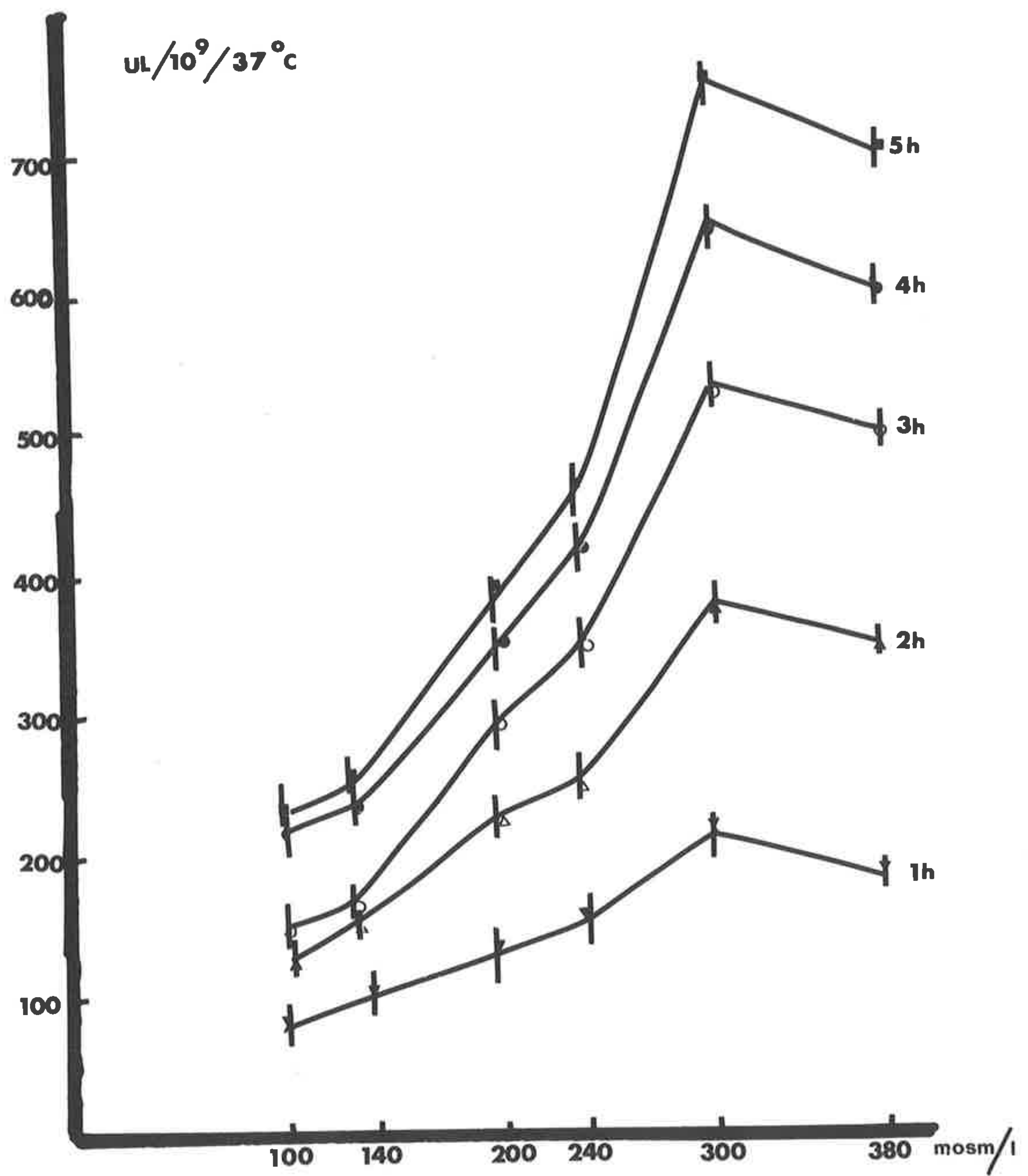


Fig 2

Fig 3

Boar spermatozoa oxygen consumption in different diluents, with 10^9 cells at 37°C.

TRF- Tris fructose buffer

TES- Tes buffer with egg yolk

BF3- This extender is compounded of lactose, casein, tris, citric acid, and fructose

SRPF - Sperm Ringer phosphate fructose

TRF at all times gave the highest oxygen uptakes, while SRPF allowed only about half the oxygen consumption of TRF.

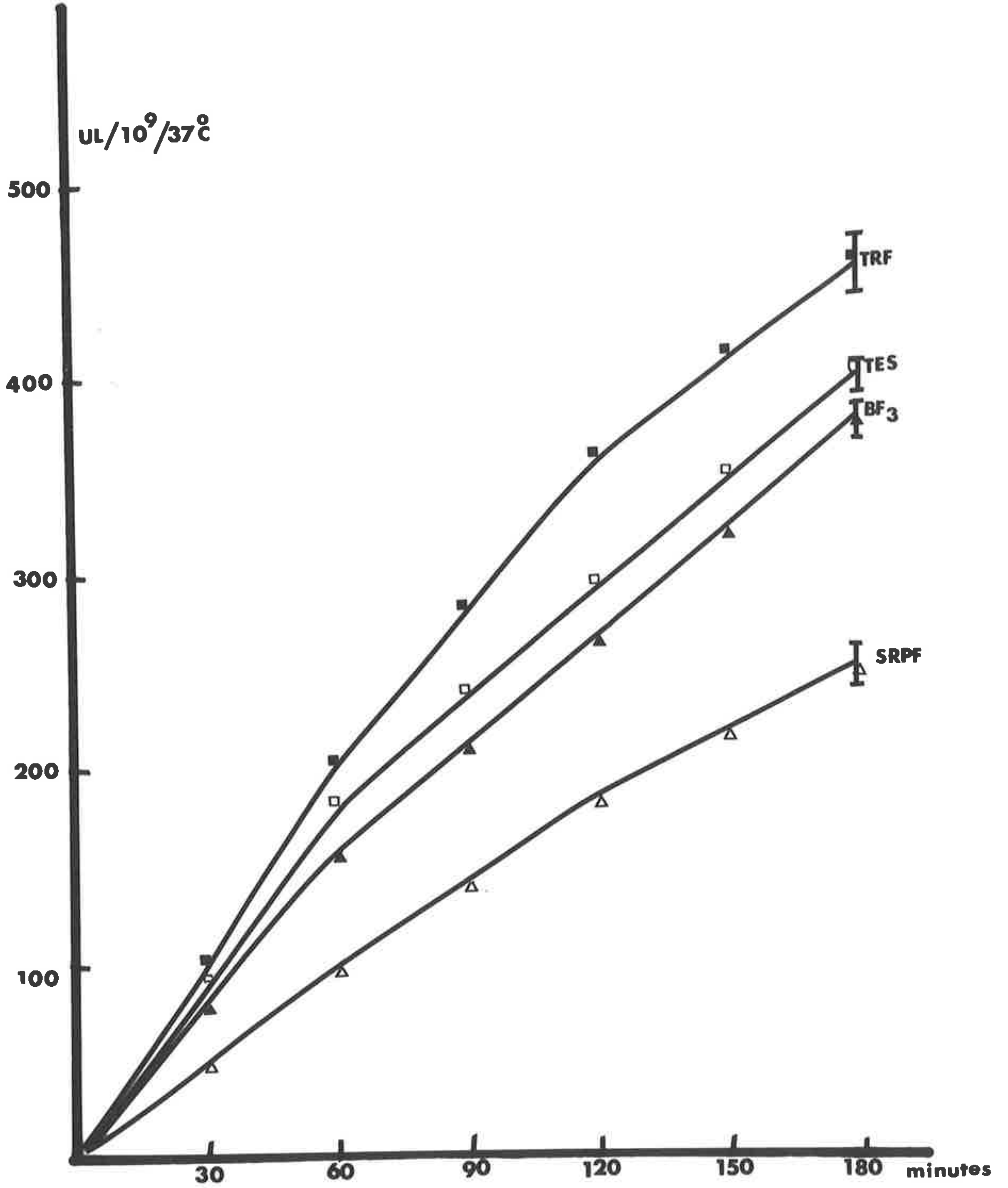

UL/10^9/37°C
500
400
300
200
100
30
60
90
120
150
180
minutes
TRF
TES
BF$_3$
SRPF

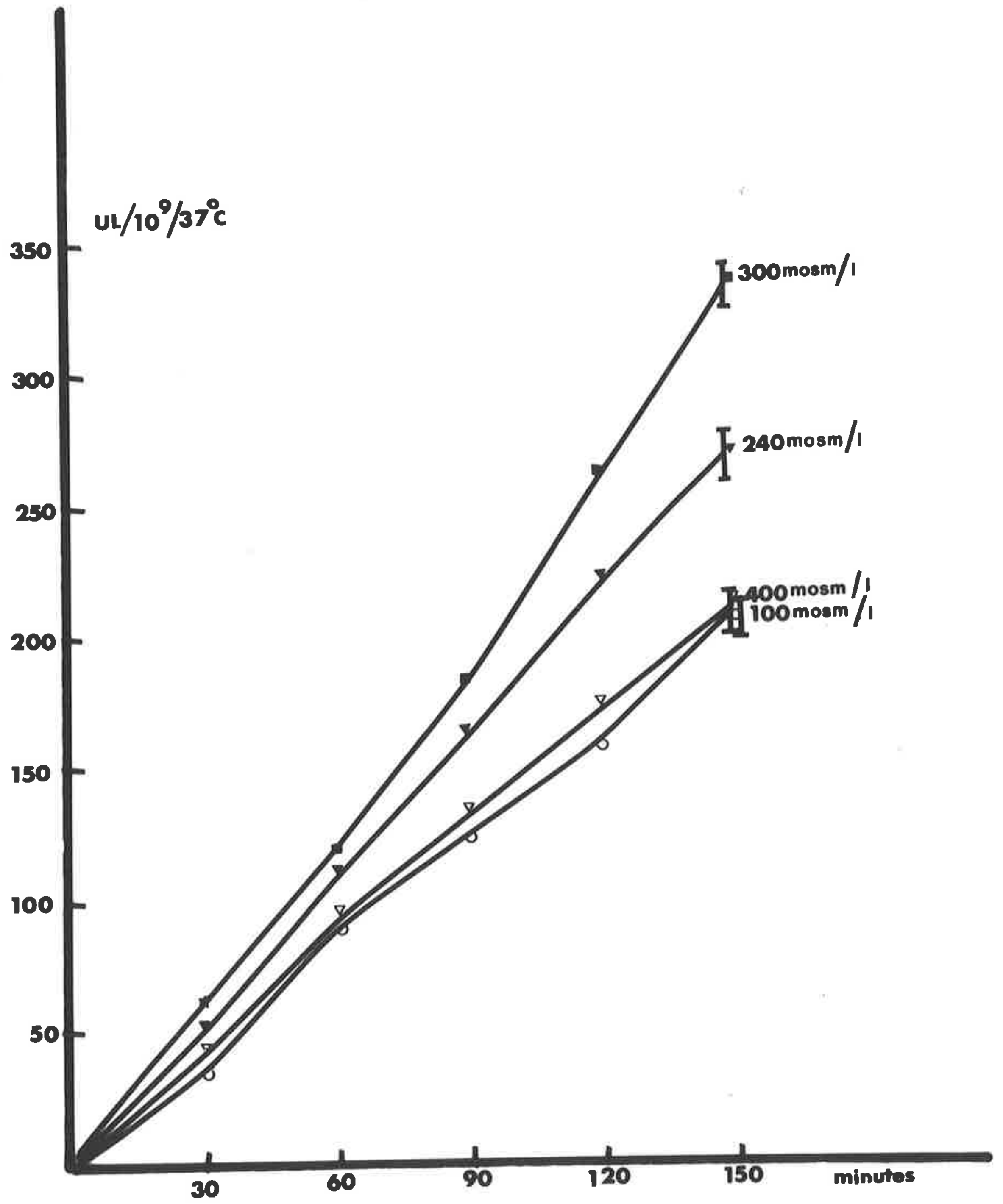
Fig 4

Boar spermatozoa oxygen uptake from 10^9 cells in $37^\circ C$ with solutions of different osmolality.

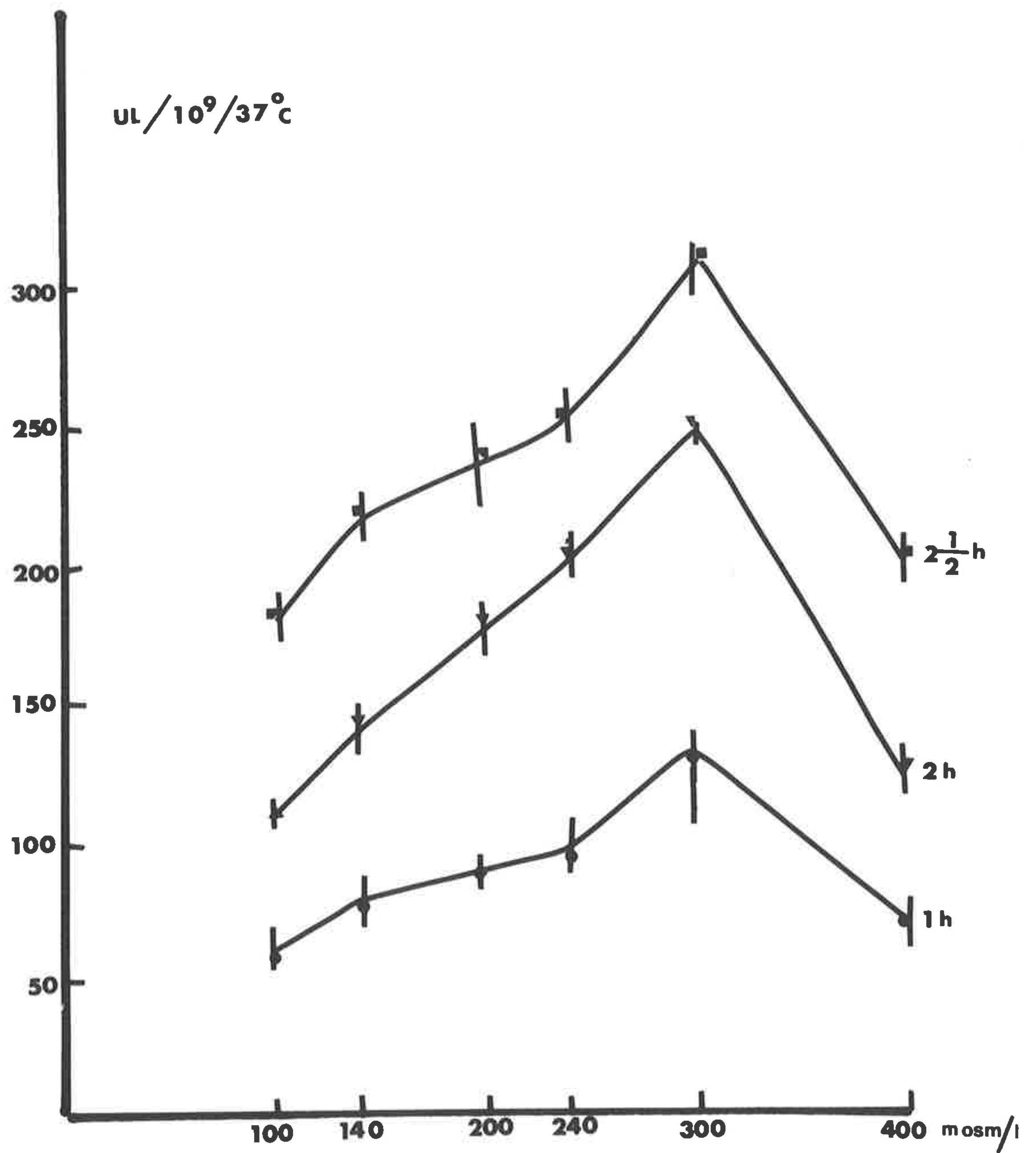
The basic components of the solution were similar to the Krebs Ringer solution, and the different osmolalities were obtained by alteration of NaCl concentration in the Ringer solution.

100 mosm/kg H_2O	3.089 g NaCl
200 mosm/kg H_2O	6.27 g NaCl
300 mosm/kg H_2O	9.457 g NaCl
400 mosm/kg H_2O	12.70 g NaCl

The oxygen uptake increased as the osmotic pressure increased up to 300 mosm/l, but a severe decline occured in hyperosmotic media.



UL/10⁹/37°C
350
300
250
200
150
100
50
30
60
90
120
150
minutes
300mosm/l
240mosm/l
400mosm/l
100mosm/l


UL / 10⁹ / 37°C
300
250
200
150
100
50
100
140
200
240
300
400
mosm/l
2 1/2 h
2 h
1 h

The effects of different diluents on GOT release were studied on sperm removed from seminal plasma by filtering through glass fibres and cellulose powder.

Before sperm were stressed, a sample was filtered and used as a control for pre-treatment release of GOT. Another sample was plunged into liquid nitrogen to provide the maximum releasable GOT sample. Frozen samples were thawed in 40°C water for 20 min before analysis and then filtered to remove the sperm cells. The ZO_2 of these spermatozoa was measured as in the diluent studies.

Table 2

Ram spermatozoa : GOT release after freezing and thawing in different diluents.

Diluent	GOT release
Citrate-raffinose solution	160 $\pm$ 8 units/10^9/min
Sperm Ringer phosphate fructose	62 $\pm$10 units/10^9/min
Pretreat (fresh semen)	55 $\pm$ 5 units/10^9/min
Maximum (without diluent)	232 $\pm$ 8 units/10^9/min

SRPF caused very little GOT release in ram spermatozoa after freezing and thawing. But citrate-raffinose released three times the amount of GOTwhen compared with the pretreatment sample. The highest GOT release was from the sample of sperm which were plunged into liquid nitrogen without any diluents.

Table 3

The effects on GOT release of addition of egg yolk and glycerol to the different media.

Diluent	GOT release
Citrate raffinose + egg yolk + glycerol	160 $\pm$ 16 units/10^9/min
Sperm Ringer phosphate fructose + egg yolk +glycerol	180 $\pm$ 20 units/10^9/min
Pretreat (fresh semen)	55 $\pm$ 5 units/10^9/min
Maximum (without diluent)	232 $\pm$ 8 units/10^9/min

From Table 3 it appears that citrate raffinose and sperm Ringer phosphate fructose solution each with 5% glycerol and 10% egg yolk added, were identical in GOT release. Therefore, an experiment was designed to investigate whether addition of egg yolk and glycerol to the diluents separately altered GOT release.

Table 4

Effects of 5% glycerol & 10% egg yolk on GOT release from ram sperm

Diluents	GOT release
Citrate raffinose + 5% glycerol	192 $\pm$ 8 units/10^9/min
Citrate raffinose + 10% egg yolk	128 $\pm$ 5 " " "
SRPF + 5% glycerol	185 $\pm$ 5 " " "
SRPF + 10% egg yolk	86 $\pm$ 6 " " "
Pretreat	55 $\pm$ 5 " " "
Maximum	232 $\pm$ 8 " " "

As the result of adding 5% glycerol to both C.R. and SRPF or with 10% egg yolk . There was very high GOT release, citrate raffinose solution brought little improvement. For SRPF 86 $\pm$ 6 GOT units/10^9/min were measured compared with 55 $\pm$ 5 units/10^9/min in the pretreatment sample. Combining the results in Table 2 with Table 4, the SRPF on its own released only 62 $\pm$ 10 units/10^9/min, therefore presumably the egg yolk and glycerol are not necessary for ram spermatozoa diluted with SRPF. If they were diluted with citrate raffinose solution, there addition of 10% egg yolk gave lower GOT release levels compared with citrate raffinose solution alone or with 5% glycerol added to this solution.

Fig 5

GOT release from ram spermatozoa in different diluents after freezing and thawing.

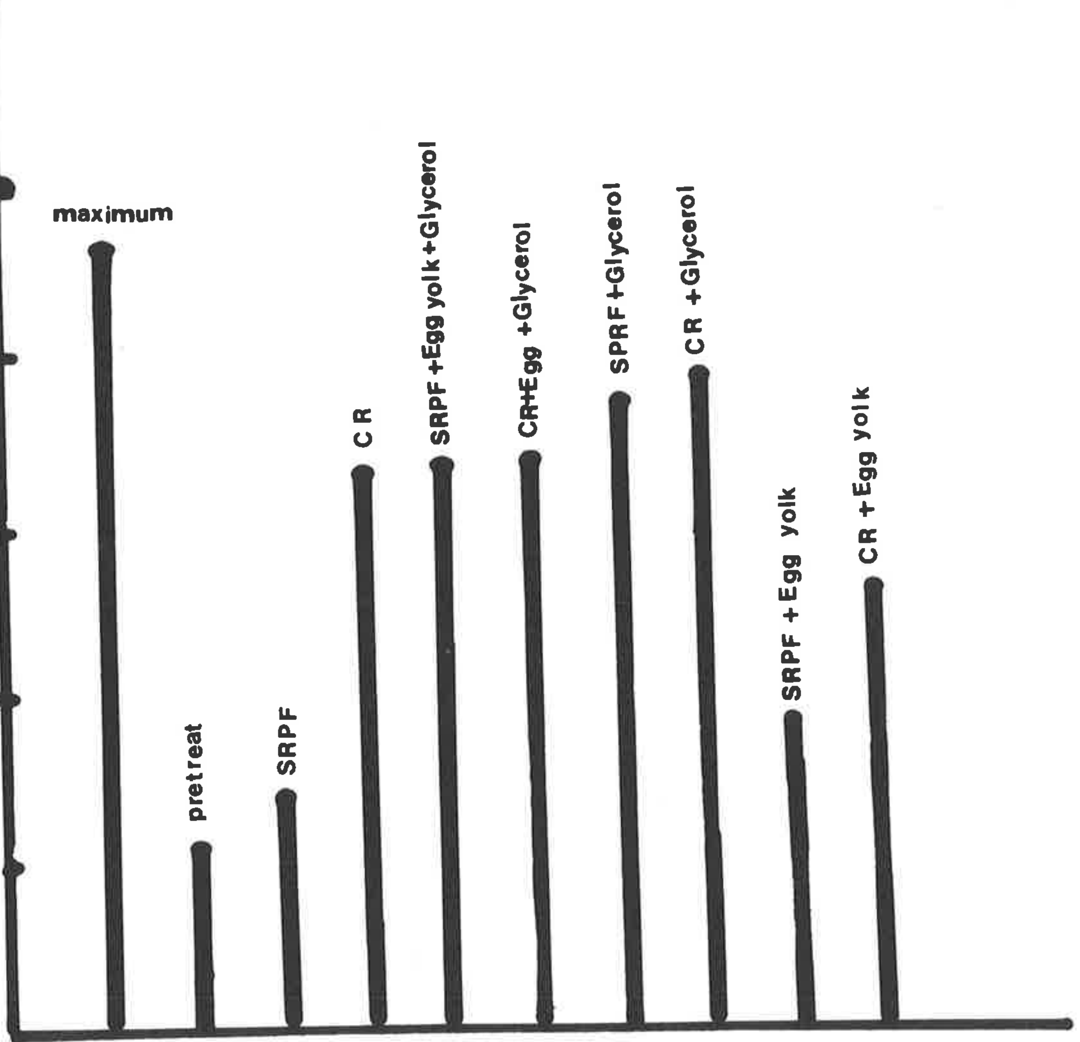
Maximum- sample plunged into liquid nitrogen without diluent

Pretreatment- the spermatozoa were separated from the seminal plasma, and measurement took place from the seminal plasma

SRPF- Sperm Ringer phosphate fructose

CR- Citrate raffinose

The GOT release from ram spermatozoa in different diluent is summarised. The lowest GOT release from ram spermatozoa was on the pretreatment sample, and the SRPF gave comparably low GOT release rates.



GOT release
unit/10^9/min
250
200
150
100
50
maximum
pretreat
SRPF
C R
SRPF +Egg yolk +Glycerol
CR+Egg +Glycerol
SPRF +Glycerol
CR +Glycerol
SRPF +Egg yolk
CR +Egg yolk

Boar spermatozoa GOT release after freezing and thawing in different diluents

Boar semen after collection was concentrated to 10^9 cells per ml and diluted 1:1 with different diluents before freezing. freezing was carried out in liquid nitrogen. A pretreatment release sample was measured from the fresh seminal plasma. The maximum sample was measured from the sample of 10^9 cell/ml plunged into liquid nitrogen. Frozen samples were thawed in water at 40°C for 20 min before analysis and then filtered to remove the sperm cells.
The results shown in Table 5 compare the effects of different diluents in modifying the damage due to quick freezing and thawing.

Table 5

Boar sperm GOT release in different media

Diluent	GOT release units/10^9/min
Pretreatment	14 ± 1.62
Maximum	110 ± 10
Tris - fructose	70 ± 2.5
Tris + egg yolk + glycerol	80 ± 5
Tes + egg yolk + glycerol	60 ± 10
BF3 extender	53 ± 14
SRPF	66 ± 1.8
SRPF + 1% DMSO	74 ± 1

BF3 gave the best protection, then SRPF and Tes with egg yolk and glycerol. There was, however, little difference between treatments.

For a long time it has been suggested that slow freezing and longer equilibration time may decrease the " cold shock " effects on spermatozoa. Therefore, a slow freezing test on GOT release was undertaken (Table 6).

Table 6

Slow freezing and diverse diluents in GOT release from boar spermatozoa

Diluents	Slow freezing 5 C/min, GOT release
Pretreat	14 ± 1.6 units/10^9/min
Maximum	40 ± 5 "
Tris fructose	75 ± 5 "
Tris egg yolk + glycerol	75 ± 5 "
Tes egg yolk + glycerol	50 ± 0 "
BF3 extender	41 ± 0 "
SRPF	47 ± 2.5 "
SRPF + 1% DMSO	62 ± 0.5 "

slow freezing was of significant benefit only to the maximum treatment sample, though diluents Tes and BF3 extender were satisfactory.

But compared with the pretreatment sample, there was still 50% -75% damage to cells after the spermatozoa were frozen and thawed. So, another experiment on boar spermatozoa cooled at 5°C for 2 weeks was undertaken (Table 7).

Table 7

GOT release from boar sperm stored for 2 weeks at 5°C

Diluents	GOT release
Pretreat	14 ± 1.6 units/10^9/min
Maximum	32.5 ± 2.5 "
Tris fructose	23.5 ± 2.5 "
Tris egg yolk + glycerol	12.5 ± 1.5 "
Tes egg yolk + glycerol	16.5 ± 1.5 "
BF3 extender	12.5 ± 1.5 "
SRPF	32.5 ± 2.5 "
SRPF + 1% DMSO	32.5 ± 2.5 "

Table 7 shows that tris +egg yolk + glycerol and BF3 extender had similar effects on GOT release, Tes egg yolk and glycerol released 4 units/10^9/min GOT more than the other two diluents. However, SRPF and SRPF + 1% DMSO did not differ from the maximum damage.

Disc Electrophoresis

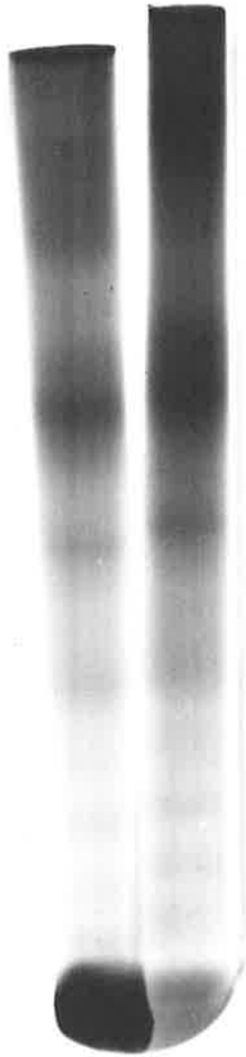
Fresh spermatozoa yielded 9 identifiable protein bands in the electrophoresis system.

There were four bands from the frozen boar spermatozoa (Nos 1, 2, 5, 6) which after freezing increased in density relative to those from unfrozen sperm. Presumably these proteins leaked from the frozen cells. In Fig 6 the arrows indicate the protein bands which increased in density after treatment. Specific identification of the proteins was not undertaken.

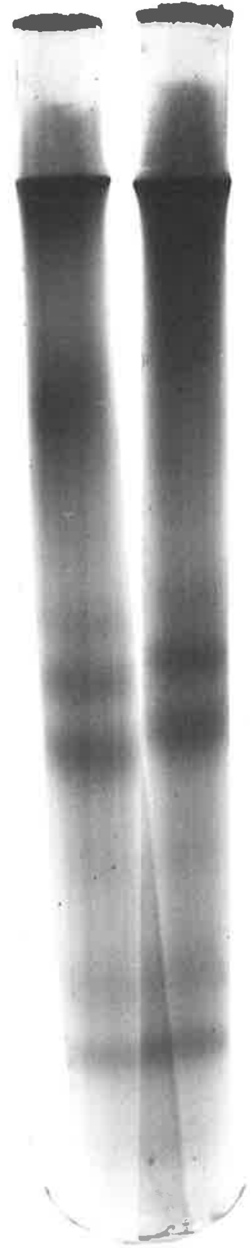
Fig 6

Electrophoresis of fresh and frozen boar spermatozoa on polyacrylamide gel.

FRESH



FROZEN



Ram semen does not show any significant band which increased in density or changed position after freezing. All samples were in sperm Ringer phosphate fructose solution, which gives nearly the same GOT release as the pretreatment sample after freezing. Freezing, however, did not cause much damage to ram spermatozoa in sperm Ringer phosphate fructose. The polyacrylamide gel was run in pH 8.6 buffer.

Fig 7

Electrophoresis of fresh and frozen sperm from ram.

Semen obtained from 6 rams was mixed then run electrophoretically. The frozen sperm had relatively lighter bands because staining was less intense overall. Similar bands were obtained from both samples.

FROZEN

FRESH

8
7
6
5
4
3
2
1

8
7
6
5
4
3
2
1

8
7
6
5
4
3
2
1

8
7
6
5
4
3
2
1

Electron microscopy

Washed semen was examined before and after freezing.Even in fresh control preparations the plasma membrane of the sperm head often stripped away from it (Plate 1). It is presumed, but by no means proved, that this was an artifact. The membrane changes in the acrosomal region were much more pronounced in frozen spermatozoa. The swelling of the acrosome is marked, presumedly there is a loss of acrosome contents. (Plates 4&5).

No differences could be observed in the plasma membrane covering the middle piece or mitochondrial sections, between control and frozen spermatozoa (Plate 2,3,4,5,).

The boar sperm head plasma membrane was disrupted but remained intact, and the acrosome nucleus was normal in the fresh sample (Plate 6).

The frozen boar sperm head plasma membrane was very severely disrupture as was the acrosomal exterior membrane. There is debris in the damaged cell (Plate 7). Also acrosomal vacuoles formed, and the post-nuclear cap became detached (Plate 8).

Plate 1

Fresh ram spermatozoa x 3000

Stain : lead citrate + uranyl acetate

Sperm head and longitudinal section of mitochondria. Note the plasma membrane, the acrosome and terminal part of acrosome in the sperm head. The plasma membrane covering the acrosomal region is detached but intact.

Abbreviations

pf - peripheral fibrils

af - axial filaments

mc - mitochondria

at - terminal part of acrosome

ac - acrosome

pm - plasma membrane

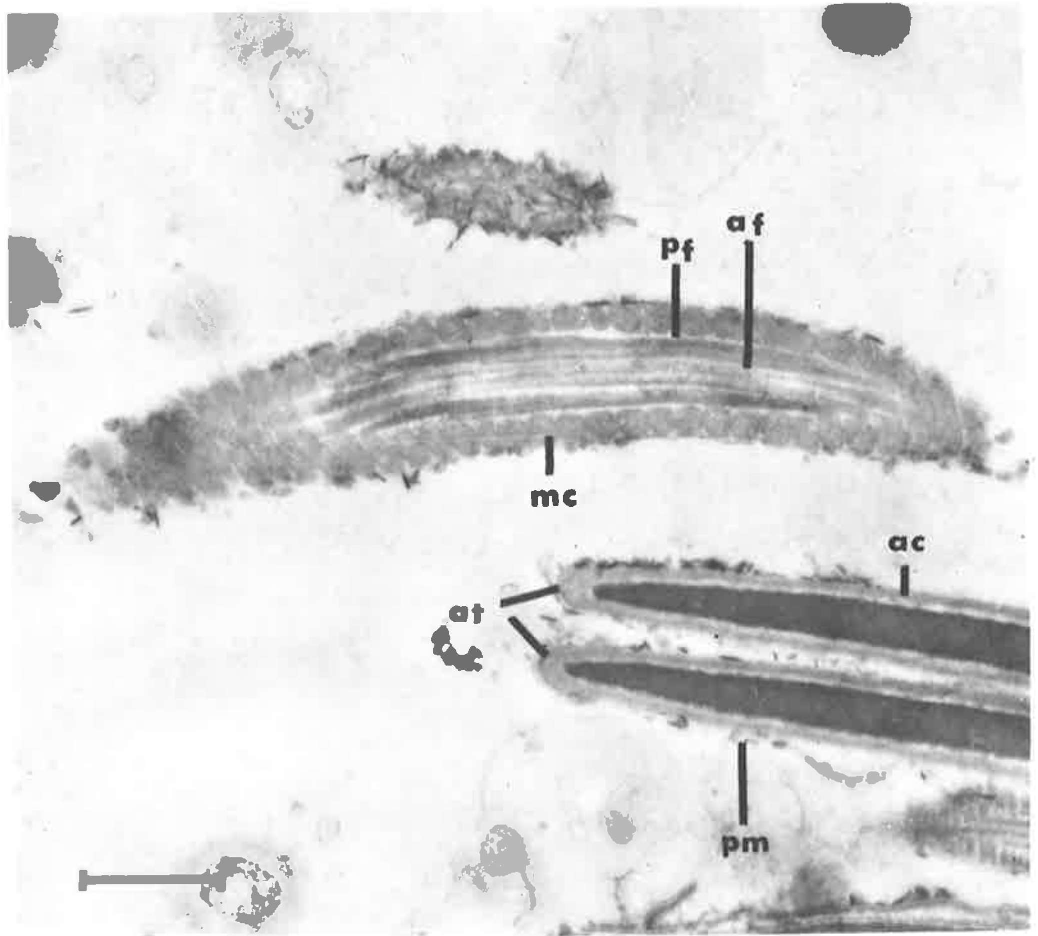

pf
af
mc
ac
at
pm

Plate 2

Fresh ram spermatozoa x 4000

Stain : lead citrate and uranyl acetate

Sperm head and cross section of mitochondria

Note that the plasma membrane is disrupted but the mitochondrial cross section is normal.

Abbreviations

af - axial filaments

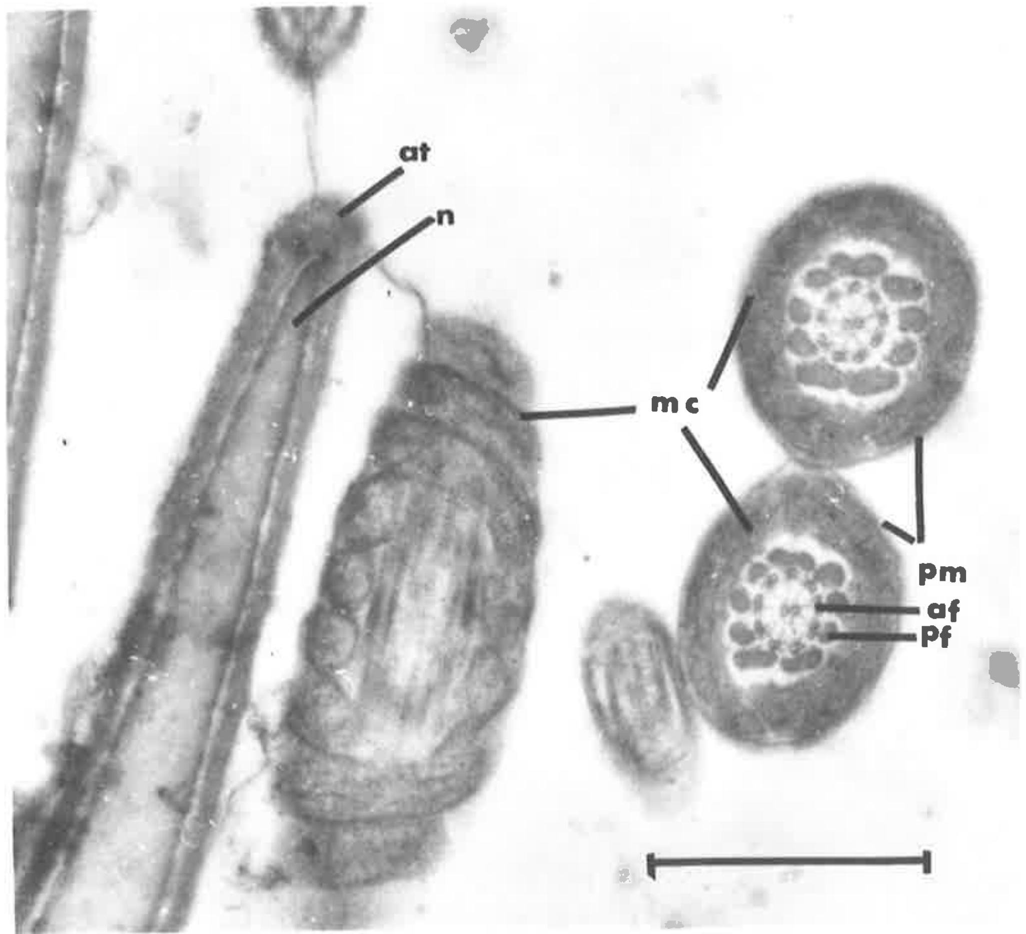
at - terminal part of acrosome

n - nucleus

mc - mitochondria

pf - peripheral fibrils

pm - plasma membrane



at
n
mc
pm
af
pf

Plate 3

Fresh ram spermatozoa x 3000

Stain : lead citrate and uranyl acetate

Note longitudinal section of mitochondria showing normal structure.

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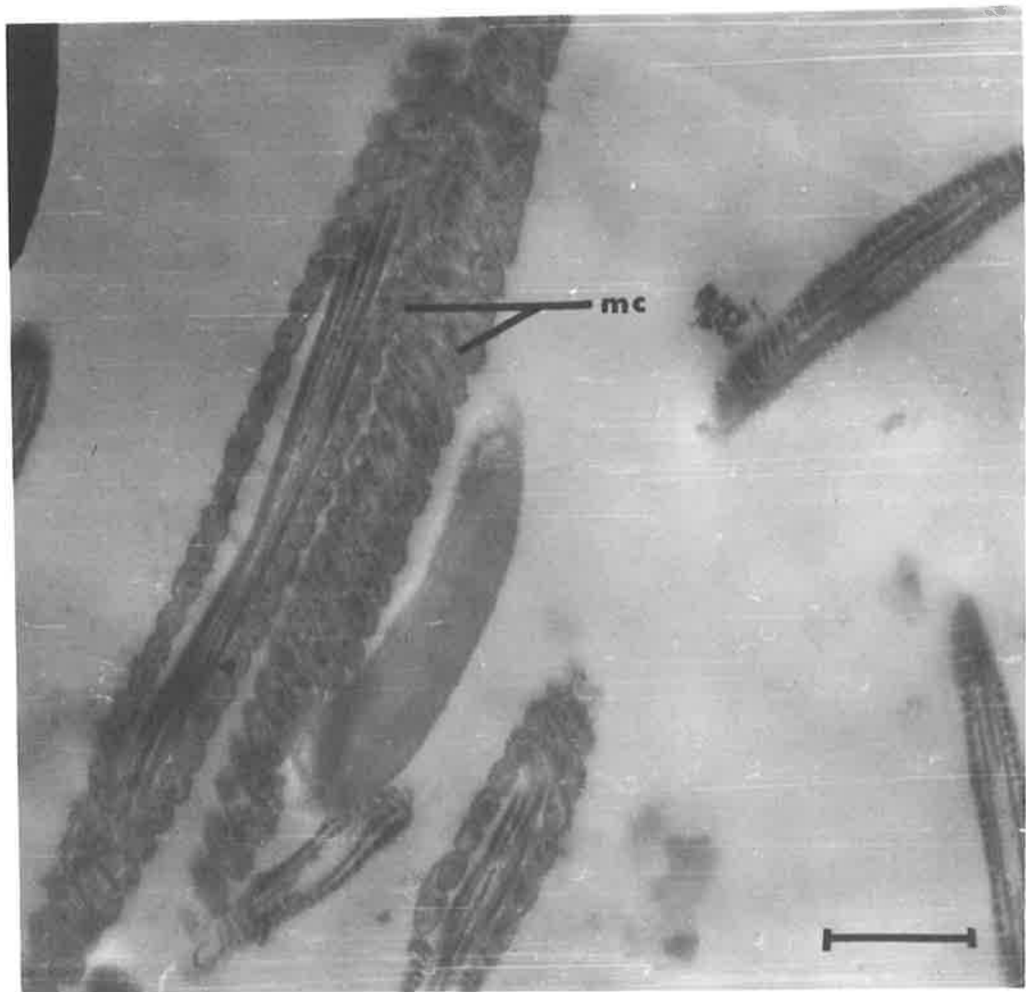


Plate 4

Frozen ram spermatozoa x 3500

Stain : lead citrate and uranyl acetate

The sperm heads have plasma membrane broken and acrosomal exterior membrane disrupted but intact. Mitochondria remain normal

Abbreviations

pm - plasma membrane

mc - mitochondria

aom - acrosomal exterior membrane

aim - acrosomal interior membrane

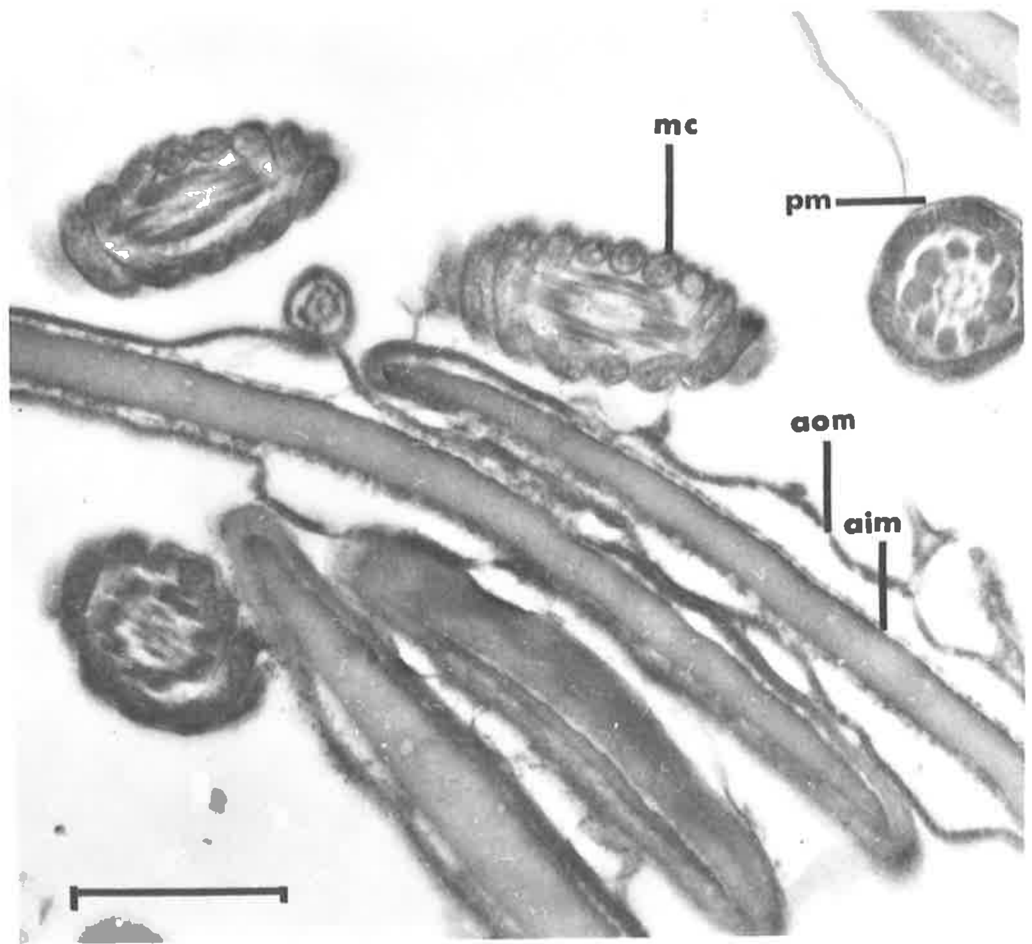

mc
pm
aom
aim

Plate 5

Frozen ram spermatozoa x 5000

Stain : lead citrate and uranyl acetate

The sperm head show the plasma membrane broken, while only the acrosome outer membrane and interior membrane can be seen. Acrosome debris is present. No significant changes in mitochondria and nucleus have occured.

Abbreviations

ne - nuclear envelope

aim - acrosomal interior membrane

aom - acrosomal exterior membrane

ac debris - the debris which is released from acrosomes after cell injury

pf - peripheral fibrils

af - axial filaments

mc - mitochondria

pm - plasma membrane

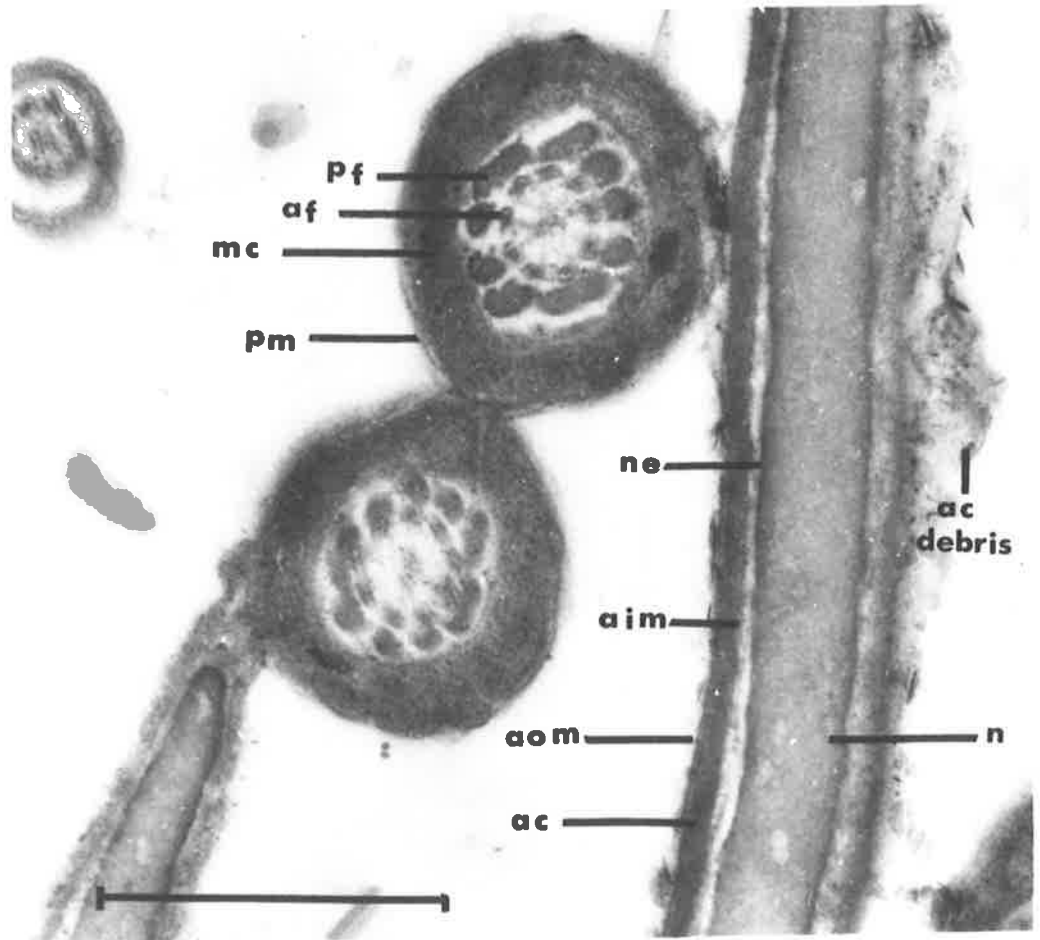

Pf
af
mc
pm
ne
ac
debris
aim
aom
n
ac

Plate 6

Fresh boar spermatozoa x 1000

Stain : lead citrate and uranyl acetate

The sperm head plasma membrane is loosened but intact, acrosome and nucleus normal. Mitochondria not very clear because of the cutting.

Abbreviations

pm - plasma membrane

at - terminal part of acrosome

aom - acrosome exterior membrane

aim - acrosome interior membrane

ne - nuclar envelope

n - nucleus

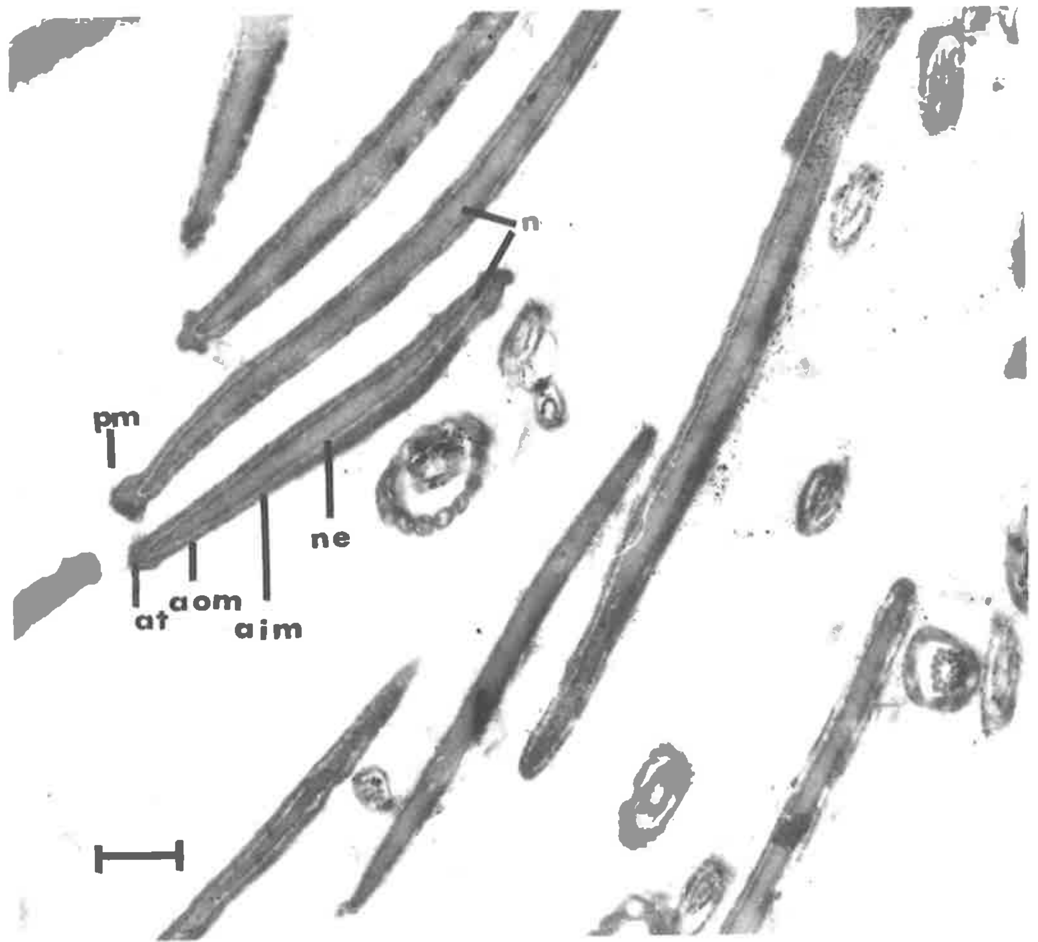

n
pm
ne
at
aom
aim

Plate 7

Boar frozen spermatozoa x 1000

Stain : lead citrate and uranyl acetate

The sperm head plasma membrane is severely disrupted but still intact. The acrosomal exterior membrane is loosened. There are acrosomal fragments in the damaged cell.

Abbreviations

pm - plasma membrane

aim - acrosomal interior membrane

aom - acrosomal exterior membrane

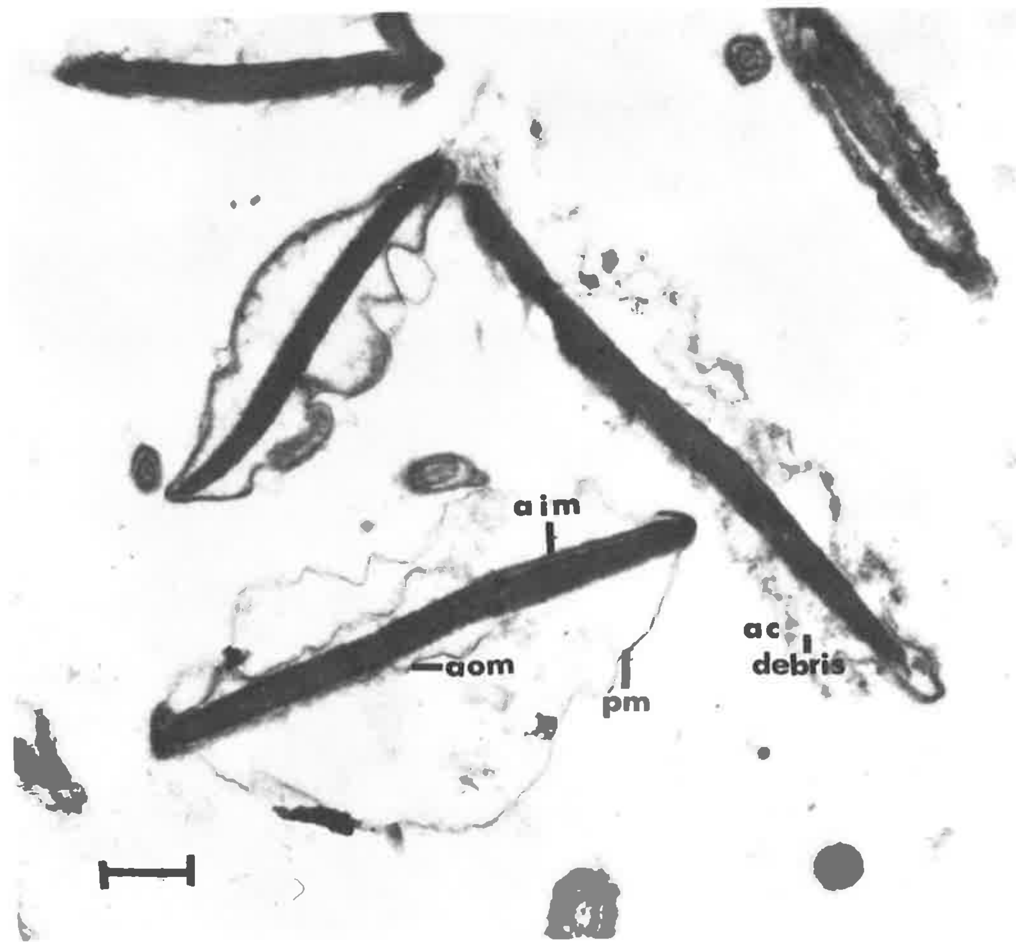

aim
aom
pm
ac
debris

Plate 8

Boar frozen spermatozoa x 2000

Stain : lead citrate and uranyl acetate

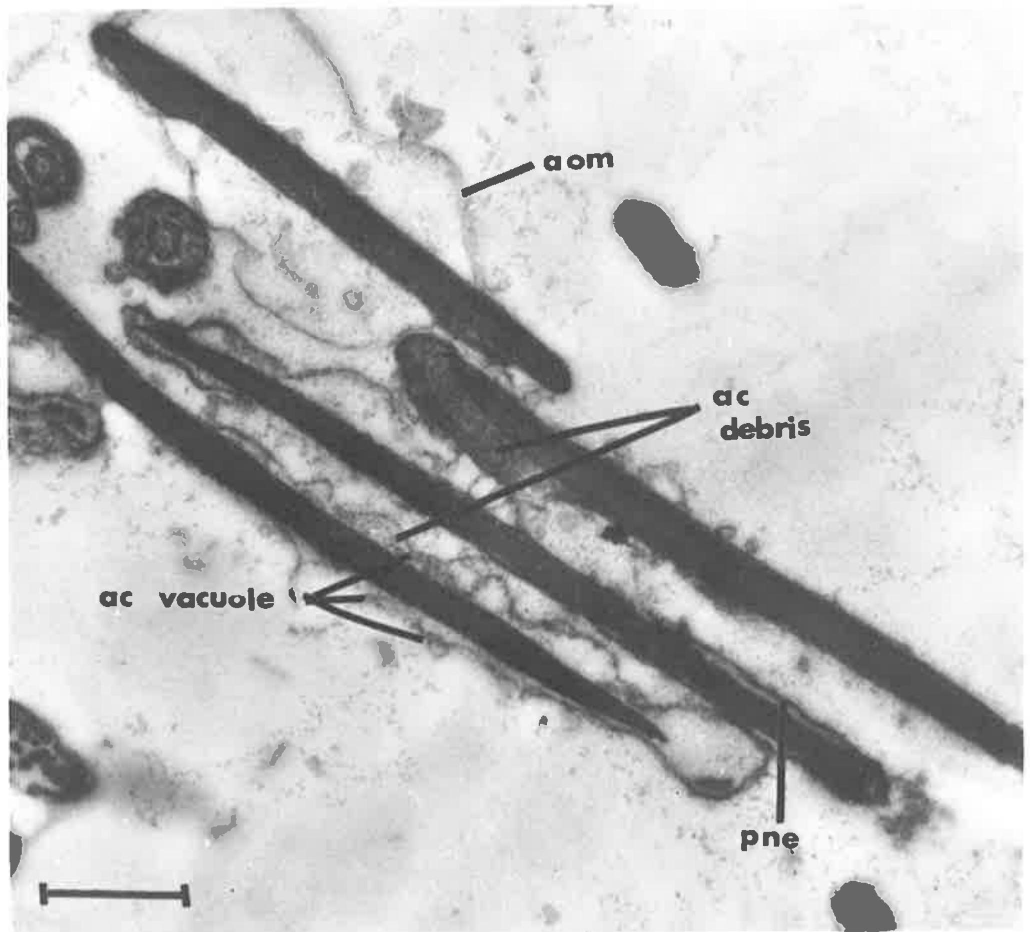
Sperm head with acrosomal exterior membrane disrupted and acrosome de'bris.

Vacuoles also have formed in the acrosome. Post nuclear cap is detached.

Abbreviations

aom - acrosome exterior membrane

pne - post nuclear cap


aom
ac
debris
ac vacuole
pne

Discussion

Much information is available about frozen semen diluents and frozen semen evaluation by GOT enzyme methods in cattle (Brown, Crabo et al 1971; Crabo & Erinasson 1971; Graham & Pace 1967; Lightfoot & Salamon 1970, 1971; Maule 1962; Pursel 1973, 1975; Salamon 1972). But less is known about ram and boar semen. Only one experiment is reported on the release of glutamic-oxaloacetic-transaminase from ram and boar spermatozoa (Bonadonna & Roychudhury 1970). They incubated ram spermatozoa in 2.9% citrate solution for 2 h at 37°C. The GOT release increased from 78.6 unit/10^9/min to 115.8 unit/10^9/min.

Boar semen in BF3 extender with 5% glycerol was also measured for GOT output. There is no detailed work on how the different diluents affect GOT release from ram and boar semen. Table 2 gives the results of release of GOT from ram sperm after freezing in citrate raffinose solution and sperm Ringer phosphate fructose. The reason for choosing these two solutions was the suggestion that citrate raffinose offered very good preservation of ram spermatozoa (Salamon 1972). Sperm Ringer phosphate fructose also maintained good motility of ram spermatozoa (Mann 1964).

In terms of the GOT release index of damage, citrate raffinose solution was the least satisfactory after freezing. After adding egg yolk and glycerol (Table 3) there was no reduction of GOT release. The amount of egg yolk and glycerol used was that suggested by Salamon (1972). It appears, however, from the results in Table 3 and 4, that there is no need to add egg yolk and glycerol when freezing ram spermatozoa. But from the studies by Mann & White (1957) on glycerol metabolism in ram spermatozoa, it appears that the rate of utilization of glycerol is 0.5 - 1 mg/10^9/h. Throughout the period of 3 h, the utilization of glycerol was accompanied by an increase in oxygen uptake of the order of 100 - 250 µl/10^9/h. From Fig 1, the O_2 uptake of ram spermatozoa without glycerol in sperm Ringer

phosphate fructose was 275 $\mu l/10^9/h$, compared with only 175 $\mu l/10^9/h$ in citrate raffinose solution. From the studies of the effects of pH, ionic strength and osmotic pressure, it seems likely that the finding of a lower osmotic pressure in citrate raffinose solution,may be the factor which affects the oxygen uptake. From the tests it seems that solutions of different osmolality affect the respiratory rate. The osmolality of citrate raffinose solution was 265 mosm/l, ZO_2 was 175 $\mu l/10^9/h$, but a 240 mosm/l solution reduced ram spermatozoan metabolism to ZO_2 of 150 $\mu l/10^9/h$. Presumably the citrate raffinose solution was associated with lower oxygen uptake due to the lower osmotic pressure.

From the polyacrylamide gel electrophoresis study there appeared to be no extra protein leak into the ram seminal plasma, before or after freezing, because the samples were in sperm Ringer phosphate fructose solution, which gives very low GOT release, nearly the same as the pretreatment sample after freezing. Therefore it may be that freezing did not cause much damage to ram spermatozoa after being frozen in sperm Ringer phosphate fructose.

The electron micrographs show good preservation of sperm acrosomal plasma membrane and inner membrane in fresh sperm. Only the plasma membrane covering the acrosomal region is detached but it ramains intact, whereas in the frozen sample the cell is swollen and acrosomal de'bris can be seem. Quinn (1969) studied cold shock and the effects of freezing on the ultrastructure of ram semen. He made similar observations. Jones & Martin (1973) distinguished condensed mitochondria and large intracristal spaces and considered that the later irreversible damage, could be due to the different position of the cutting.

Crabo, Brown & Graham (1971) regard motility as a measure of the effects of buffers and other treatment on the metabolic system of the spermatozoa; while the release of GOT they thought of as a measure of sperm membrane permeability. An increased membrane permeability, related to elevated extracellular GOT concentration, could probably arise from membrane distintegration.

Also (Crabo, 1971) observed that large amounts of GOT in the extracellular fluid after freezing were partly due to the addition of glycerol, which increases the release of GOT from boar spermatozoa. He suggested that this release could be caused by osmotic disintegration of the sperm membrane.

Graham et al, (1972) found higher motility of boar spermatozoa after dilution with lower ionic strength buffers both at 5 C and after freezing. They also found depression of motility in the phosphate and tris-HCl buffers during warm storage. They thought that this might be due to differences in the metal binding properties of the buffers used (Good et al, 1966; Graham 1972).

Boursnell, Nelson & Cole (1966) observed that there are proteins which precipitate at low temperature in the seminal plasma of the boar. Also Boursnell(1975) showed that there is a complex mixture of proteins which is precipitated by zinc from seminal plasma. However, Mann (1964) suggested that amino acids and proteins may act on the dilute sperm suspension by virtue of their buffering capacity. Therefore a further study of these zinc precipitated seminal proteins could be productive. From Fig 4 and Table 5,6 it appears that tris fructose buffer sustains very good O_2 uptake at 37°C, but it led to high rates of release of GOT on freezing. Sperm Ringer with phosphate, allowed only a very low motility rate and also caused a large GOT release on freezing. However, Table 7 shows that tris fructose with egg yolk and glycerol and Tes buffer with egg yolk and glycerol were associated with low GOT release during 5°C storage for 2 weeks. Glycerol addition is still of benefit during the 5°C storage.

Electron microscopy showed that freezing has very serious effects on boar spermatozoa. Apart from plasma membrane rupture, the exterior acrosomal membrane was swollen. There is a large number of cytoplamic microtubules and filaments associated with this sheath. Presumedly there are some lysosome residual bodies - acrosome de'bris (AcD) left by the freezing trauma.

Jones (1973) has shown that the ultrastructure of ram spermatozoa

cooled to 5°C reveals a severly swollen and vacuolated acrosome.

Williamson (1974) examined the fine structure of ejaculated ram spermatozoa following scrotal heating. He found pronounced swelling of the anterior acrosome. The acrosomal membrane was broken and acrosome de'bris lay beneath the convoluted outer acrosome membrane. Presumably all these phenomena indicated spermatozoan death.

From the polyacrylamide gel electrophoresis it appears that there is a leakage of proteins from boar spermatozoa, which increases the density of No. 1, 2, 5, 6, bands. No other information disproves this observation. So, the frozen boar spermatozoa probably do suffer protein leakage.

Conclusion

Graham & Pace (1967) investigated possible methods of determining the degree of cellular damage caused by freezing and found that the GOT release from sperm cells correlated significantly with cell damage. Numerous studies have since been undertaken on the effects which different diluents have on GOT release from ram and boar spermatozoa. Results to date suggest that ram spermatozoa survive best in the physiological diluent, Sperm Ringer phosphate fructose solution (pH 7.4) and boar spermatozoa are most successfully preserved in Tes buffer or BF3 extender (pH 7.6). The results of GOT release, metabolic activity, polyacrylamide gel electrophoresis and electron microscopy, provide good evidence to assess the quality of ram and boar semen before and after freezing.

Table (2) shows that ram spermatozoa have a lower GOT release in SRPF when compared with citrate raffinose solution. There is less cell damage in the SRPF solution. The addition of 10% egg yolk and 5% glycerol together (Table 3), did not increase protection against cell damage.

Table 4 shows that sperm in a solution of SRPF + 10% egg yolk lost GOT at a rate of 86 units/10^9/min. It can be concluded, therefore, that egg yolk and glycerol are not necessary to protect ram spermatozoa in the freezing procedure. The measurement of the metabolic activity of ram spermatozoa in SRPF and citrate raffinose solution by the Warburg manometer support the above conclusion. In SRPF solution , the rate of oxygen uptake was 275 μl/10^9/h at 37°C. In the citrate raffinose solution it was 160 μl/10^9/h at 37°C. The best explanation of the differences appears to relate to osmolality. Citrate raffinose has an osmotic pressure of 260 mosm/l compared with 330 mosm/l for SRPF.

In the polyacrylamide electrophoreses of frozen sperm there were no significant bands in different positions on the gel, possibly because both fresh and frozen samples are not affected by the diluent and by temperature. Even in the GOT results,

fresh samples contained 55 units/10^9/min compared with 62 units/10^9/min in the frozen sample.

In electron microscopy there are problems of fixation and dehydration which confound morphological changes due to freezing. By electron microscopy morphological changes due to freezing are very difficult to distinguish from those arising from fixation and dehydration processes.

The GOT results from the boar spermatozoa diluted with different media were measured. There was nearly 50% cell damage caused by freezing. The procedure of slow freezing did not improve survival very much, possibly because of difficulties with the thawing solution and the thawing rate.

Cool storage of boar semen at 5°C for 2 weeks, has been successful when boar spermatozoa were diluted 1:1 with tris buffer fructose, Tes buffer egg yolk or BF3 extender. Only the results of storage in SRPF were not satisfatory.

A possible explanation is that the ionic conductivity of SRPF was 13 $\pm$ 0.5 mmho compared with 2 mmho of Tes buffer and 4 mmho of tris buffer. The conductivity of boar semen seminal plasma was 8.5 $\pm$ 0.25 mmho. It seems clear that lower ionic strengh solutions were more favorable to the boar spermatozoa.

To study the characteristic protein changes in semen, after it being frozen and thawed, polyacrylamide gel electrophoresis has been used. Increase of the protein bands from frozen boar semen was observed. Presumedly these protein bands represent leakage from the cell due to freezing.

From the electrophoresis studies it seems that boar semen has very significant bands No. 1, 2, 5, 6, for frozen samples. These protein bands may have leaked from the cell after freezing. Further comparative studies of different diluents may make clear the properties of the optimal medium for the storage of ram and boar semen at low temperature.

Appendix

1. Citrate raffinose pH 7.4

   166.5 mM raffinose

   68.mM sodium citrate

2. Sperm Ringer phosphate fructose pH 7.4

   119.3 mM NaCl

   4.8 mM KCl

   1.2 mM KH_2PO_4

   1.2 mM $MgSO_4$

   2.4 mM $NaHCO_3$

   41.7 mM sodium phosphate

   0.5 g fructose in 100 ml solution

3. Tris buffer with fructose

   2.822 g tris

   1.354 g citrate

   1.4 g fructose in 100 ml distilled water

4. Tes glucose egg yolk buffer

   1.2 g Tes

   0.2 g tris

   3.5 g glucose

   20 ml egg yolk in 100 ml distilled water

5. BF3 extender

   4.0 g lactose

   2.0 g casein

   2.0 g tris

   1.0 g citric acid

   0.5gfructose in 100 ml distilled water

Polyacrylamide gel electrophoresis stock solutions:

(a) 1N HCl 48 ml pH8.9
Tris 36.6 g
TEMED 0.23 ml water to 100 ml

(b) 1 N HCl 48 ml pH 6.7
Tris 5.98 g
TEMED 0.46 ml water to 100 ml

(c) Acrylamide 28 g
Bis 0.735 g
Water 100 ml

(d) Acrylamide 10 g
Bis 2.5 g
Water 100 ml

(e) Riboflavin 4 mg
Water 100 ml

(f) Sucrose 40 g
Water 100 ml

Small pore solution #1 and #2

Solution #1	solution #2
1 part (a)	Amoniun Persulfate
2 part (c)	0.14 g to 100 ml water
1 part water	

1 #1 : 1 #2 pH 8.9

Large pore solution pH 6.7
1 part (b)
2 parts (c)
1 part (e)
4 part (f)

Stock buffer solution pH 8.3
Tris 6.0 g
Glycine 28.8 g
Water 1 l.

Fiske osmometer operation :

1. Into special test tubes 2ml of solution (sample or standard) was Pipetted.
2. The samples were precooled in an ice bath.
3. The tube with a sample was put into the probe holder.
4. Dials A,B and extender were set on the expected range.
5. The middle milliosmols dial was set at the predicted answer.
6. The sample was placed in the cooling bath, and the control changed from 0 off to 1 cool.
7. The stirring knob vibrated.
8. The sensitivity switch was turned to low, for a second.
9. The galvanometer swung to the right. If it swung slowly to the right, it was on scale, and then it moved slowly to the left.
10. When the spot reached 25-30 then turned onto freeze.
11. The spot then swung towards zero.
12. The milliosmols knob was turned until the spot was on zero.
13. With the sensitivity switch on high the milliosmols knob was adjusted until the spot was on zero, and stable.

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