Ife J. Agric 1 (2) 1979

An evaluation of damage to bovine spermatozoa during processing, freezing and thawing II. Effect on motility, percentage live and ultrastructure of spermatozoa

B. SOMADE*; G. J. KING AND J. W. MACPHERSON
Department of Animal Science, University of Guelph, Guelp, Ontario, Canada.

Abstract

Thirty semen samples from five bulls were processed and frozen. Each ejaculate was evaluated for percent percent progressive motility, percent live spermatozoa and percentage of heads, tails and mid-pieces with intact plasma membrane after collection, after extension and cooling just before freezing and after thawing.

The greatest decrease in both motility and percent live spermatozoa occurred before freezing. More spermatozoa heads were damaged at each phase of processing for freezing than mid-pieces and tails and there was also a greater number of damaged spermatozoa before freezing than after freezing and thawing.

Introduction

Numerous factors involving both inherent characteristics of the ejaculate and the techniques or prosses to which the ejaculate is subjected after collection affect the post-thaw survival of bovine spermatozoa. It has been reported that spermatozoa are sensitive to cold shock when cooled too rapidly (Bishop and Walton 1960; Mann, 1964; Harrison and White, 1972; Hammersted *et al.*, 1976). Spermatozoa can therefore be damaged even before freezing temperatures are reached. In order to minimize or prevent cold shock damage, spermatozoa require extension in a special physiological media which provide protection to the cells and do not interfere with their normal functional integrity prior to cooling and freezing. In addition the rate of cooling, the temperature reached on freezing and the rate of rewarming from the frozen state are all crucial to the survival of spermatozoa. Even when suitable freezing methods are employed, there is always some lost of spermatozoa during processing, freezing and thawing, probably due to variability in the tolerance of spermatozoa in the same ejaculate to the physical and

*Present Address: Department of Animal Science, University of Ife, Ile-Ife, Nigeria.

chemical effects of temperature changes.

It is desirable to have a quick and reliable method of evaluating semen. Two of the most common used methods are percentage progressive motility and percentage live spermatozoa. When the motility of ejaculated spermatozoa, equilibrated spermatozoa, and frozen-thawed spermatozoa were compared, it was reported that there was a significant reduction in motility from one stage to the other (Erickson and Graham 1959; Coulter and Foote 1973 and 1974). Since the use of methylene blue to differentiate live yeast cells from dead ones (Devereaz and Tarner, 1927) various combinations of stains have been used for the supravital staining of mammalian spermatozoa. Eosin b - aniline blue was found unsatisfactory for frozen semen diluted in sterile milk (Macpherson, 1970) but trypan blue gave satisfactory results (Hacket and Macpherson 1965). In general there appears to be no significant difference between eosin and congo red in estimates of live bovine spermatozoa although the colour contrast found with eosin was greater than with congo red. In addition, eosin tended to diffuse into previously unstained spermatozoa on standing (Emmens, 1947; Blackshaw, 1958). Ultrastructural changes are one of the inevitable consequences of processing and the freezing of bovine spermatozoa. One of the earliest reports on the effects of freezing on the ultrastructure of mamalian spermatozoa suggested that freezing and thawing do not cause plasma membrane damage to bovine spermatozoa and that preservation at low temperatures was thought to have little effect on the fertilizing capacity of such spermatozoa (Healy, 1969).

The distruptive changes observed were attributed to damage induced by freezing, but a critical examination of pre- and post- treatment electron micrographs suggested that the changes observed could have been caused by fixation or processing of the semen samples. The ultrastructure of both ejaculated and frozen spermatozoa from different species have been recently studied (Quinn, White and Cleland, 1969; Healy and Weir,1970; Pedersen and Lebech, 1971; Nath, 1972). These reports indicated that damage to the plasma membrane and the acrosome may be induced during phases of processing for freezing. Unfortunately, some of the distruptions of the plasma membrane can be noticed in the control micrographs as well as in the treated micrographs. Another study was however demonstrated that it is possible to use fixed and stained spermatozoa for

chemical effects of temperature changes.

It is desirable to have a quick and reliable method of evaluating semen. Two of the most common used methods are percentage progressive motility and percentage live spermatozoa. When the motility of ejaculated spermatozoa, equilibrated spermatozoa, and frozen-thawed spermatozoa were compared, it was reported that there was a significant reduction in motility from one stage to the other (Erickson and Graham 1959; Coulter and Foote 1973 and 1974). Since the use of methylene blue to differentiate live yeast cells from dead ones (Devereaz and Tarner, 1927) various combinations of stains have been used for the supravital staining of mammalian spermatozoa. Eosin b - aniline blue was found unsatisfactory for frozen semen diluted in sterile milk (Macpherson, 1970) but trypan blue gave satisfactory results (Hacket and Macpherson 1965). In general there appears to be no significant difference between eosin and congo red in estimates of live bovine spermatozoa although the colour contrast found with eosin was greater than with congo red. In addition, eosin tended to diffuse into previously unstained spermatozoa on standing (Emmens, 1947; Blackshaw, 1958). Ultrastructural changes are one of the inevitable consequences of processing and the freezing of bovine spermatozoa. One of the earliest reports on the effects of freezing on the ultrastructure of mamalian spermatozoa suggested that freezing and thawing do not cause plasma membrane damage to bovine spermatozoa and that preservation at low temperatures was thought to have little effect on the fertilizing capacity of such spermatozoa (Healy, 1969).

The distruptive changes observed were attributed to damage induced by freezing, but a critical examination of pre- and post- treatment electron micrographs suggested that the changes observed could have been caused by fixation or processing of the semen samples. The ultrastructure of both ejaculated and frozen spermatozoa from different species have been recently studied (Quinn, White and Cleland, 1969; Healy and Weir,1970; Pedersen and Lebech, 1971; Nath, 1972). These reports indicated that damage to the plasma membrane and the acrosome may be induced during phases of processing for freezing. Unfortunately, some of the distruptions of the plasma membrane can be noticed in the control micrographs as well as in the treated micrographs. Another study was however demonstrated that it is possible to use fixed and stained spermatozoa for

evaluating semen quality or damage to spermatozoa during storage for artificial insemination (Jones, 1971; 1973a, b, and c). It appears that an examination of the plasma membrane over the acrosome during processing and after thawing may indicate the type and the degree of damage to spermatozoa if a method which would ensure proper fixation and maintenance of the integrity of the plasma membrane was employed.

The purpose of the present experiment was to use percent progressive motility and percent live spermatozoa to evaluate the processing and freezing procedure employed in the freezing of bovine semen for artificial insemination and to determine the degree of ultrastructural change to bovine spermatozoa.

Materials and methods

Semen was collected from five bulls twice a week on Mondays and Thursdays, over a period of three weeks starting in the second week of January 1975. The bulls whose ages ranged from two to four years had been on a regular collection schedule prior to the start of the experiment. An initial evaluation of the quality of each collection (one ejaculate per collection) was made by estimating percent progressive motility and percent live spermatozoa. The semen samples at 37.5C were mixed with part A of the extender (Skim Milk 87% v/v, Egg yolk 10% v/v, D-Fructose 0.75% v/v, Glycerol 3% v/v and Antibiotics) at 35C to give a volume of 30 to 50cc depending on the volume of the ejaculate. This mixture cooled to 5C in one hour was further extended with part 'A' of the extender to give a concentration of 60 x 10^6 motile spermatozoa per ml and part 'B' of the extender added in one step (Skim Milk 81% v/v, Egg yolk 10% v/v, D-Fructose 0.75 w/v, Glycerol 9% v/v and Antibiotics) to give a final concentration of 30 x 10^6 motile spermatozoa per ml. Following the addition of the final fraction the extended semen was equilibrated for five hours. The semen was then packed in French straws, frozen in liquid nitrogen vapour and stored in liquid nitrogen.

Six straws were thawed in warm water at 37C and pooled prior to analysis. Estimates of percent live spermatozoa were also carried out on cooled samples before freezing and on frozen-thawed samples from each ejaculate.

Percent Progressive Motility

Percent motility was estimated by mixing one drop of semen with two drops of 2.9 percent sodium citrate dihydrate and one drop of the mixture was placed on a warm slide (37C) covered with a cover slip and examined with a compound microscope with a warm stage and a bright field condensor. Equilibrated semen and frozen-thawed semen were not diluted with sodium citrate dihydrate before evaluation.

Percent Live Spermatozoa

A drop of semen was mixed with two drops of eosin and four drops of nigrosin on a clean slide using a glass rod. This ratio of one drop of semen to six drops of stain, diluted the semen enough to allow easy examination and counting of the spermatozoa. Five smears were prepared for each sample and one hundred spermatozoa were counted per slide. The slides were examined under an oil immersion objective at a magnification of approximately X909. Spermatozoa heads completely or partially stained red were considered dead.

Electron Microscopy

Spermatozoa were fixed by the modification of a procedure previously described (Jones, 1973b). A small samples (0.2ml) of freshly ejaculated semen per collection from each of five bulls was initially fixed in picric acid formal-dehyde - glutaraldehyde cacodylate (1.0% w/v picric acid, 1.0 v/v formaldehyde, 1.25% v/v glutaraldehyde in 150mM sodium cacodylate) for twenty minutes. This was followed by two washing in sucrose cacodylate (105mM sucrose and 150mM sodium cacodylate). Hydrochloric acid was used to adjust pH of the fixatives and washers to 7.3. The samples were then concentrated by centrifugation at 700 xg for ten minutes and post fixed in osmium cacodylate (40 mM osmium/tetroxide, 75mM glucose and 150mM sodium cacodylate) for thirty minutes. The samples were once again concentrated by centrifugation, washed three times in sucrose cacodylate and suspended in agar. The solidified agar containing the spermatozoa were cut into blocks and stained in saturated uranyl acetate solution for two hours at 5C. The pellets were dehydrated in a graded series of ethanol and propylene oxide and embeded in Epon (Luft, 1961) 100 nm sections

were cut with an ultramicrotome and stained in uranyl acetate followed by Millonig's lead acetate (Millonig, 1961) and examined in a Phillips 600 electron microscope at 60K.V. Samples of extended semen cooled to 5C and samples frozen-thawed were fixed sectioned and stained as above.

The percentage of spermatozoa heads, mid-pieces and tails with intact plasma membrane were estimated by a procedure previously described (Somade, 1973).

All data were subjected to analysis of variance. The analysis was performed using differences between the original values at ejaculation and those recorded just prior to freezing as period one and the difference between values just prior to freezing and post-thawing as period two. This procedure considered bulls and collections to be random effects and periods as fixed effects. This method of analysis allows a direct comparison of losses in period one (i.e before freezing) with period two (i.e. during freezing and thawing). In addition, it overcame the problem of correlation between successive observations on the same sample. The statistical procedures described by John (1971) were followed in setting up the analysis of variance.

Results

The mean percent progressive motility estimates from the six first ejaculates for each of the five bulls as observed immediately after collection (ejaculated semen), after extension and cooling but just prior to freezing (equilibrated semen) and immediately after thawing (frozen thawed semen) along with the means for each stage are presented in table 1. Mean initial motility ranged from 57.50 to 69.17%. The reduction in motility from ejaculated to the end of equilibrated (Period 1) and from the end of equilibrated to freeze-thawing (Period 2) is presented in table 2. There was a marked reduction in motility by the end of the period one followed by a further reduction although not statistically significant in period two. The analysis of variance for the motility data when the two periods were regarded as fixed effects showed a significant difference in motility due to periods ($P < .05$). This difference indicates that the reduction in motility occurring in period one was greater than that occurring in period two.

	Bull[1]	Ejaculated Semen		Equilibrated Semen		Frozen-Thawed Semen	
		% Motility	% Live	% Motility	% Live	% Motility	% Live
A	Magician	69.17	77.67	56.67	53.00	54.17	40.67
B	Pure Gold	63.33	75.67	52.50	51.33	48.33	39.00
C	Roycidale	57.50	80.33	51.67	48.83	46.67	38.00
D	Pursuit	65.83	69.67	55.00	48.00	46.67	36.83
E	Astronaut	67.50	75.33	53.33	49.83	39.17	38.17
F	Mean + sd	64.7 ± 8.1[a]	75.7 ± 6.3[d]	53.8 ± 5.2[b]	50.2 ± 8.1[e]	49.0 ± 5.9[b]	38.6 ± 3. 7[f]

[1]Mean of six collections

Means with the same superscripts are not significant different (P .05).

TABLE 2

THE EFFECT OF COOLING FROM 37C TO 5C AND EQUILIBRATION (PERIOD 1) AND FREEZE-THAWING (PERIOD 2) BOVINE SEMEN ON PERCENT MOTILITY AND PERCENT LIVE SPERMATOZOA

	Decrease in percent progressive motility	Decrease in percent live spermatozoa
Period 1	10.9	25.5
Period 2	4.8	11.6

The percentage of live spermatozoa at various stages of processing along with the overall means for each stage are presented in table 1. The reduction in percentage live spermatozoa during one was substantial and greater than the loss during period two (P .05). There was also a period by collection interaction. The mean percentage of spermatozoa heads, mid-piece and tails with intact plasma membrane across ejaculates across bulb within each sampling time along are presented in table 3. There was a significant variation in the percentage of spermatozoa heads with intact plasma membranes between the three sampling times (P .05). There was also a significant difference between percentage intact plasma membranes on the mid-piece of ejaculated spermatozoa and frozen-thawed spermatozoa (P 0.05). The mean percentage intact plasma membrane on the tails of ejaculated spermatozoa was significantly different from both equilibrated and frozen-thawed spermatozoa (P 0.05).

The reduction in the percentage intact plasma membranes during periods one and two are presented in Table 4. An analysis of variance for the data showed a significantly greater reduction in intact plasma membranes on the head during period one. The differences observed on the tails were not significant.

TABLE 3

THE EFFECT OF PROCESSING, FREEZING AND THAWING BOVINE SEMEN ON PERCENT INTACT PLASMA MEMBRANE ON HEADS, MID-PIECES AND TAILS

Bull[1]	Ejaculated Semen			Equilibrated Semen			Frozen-Thawed Semen		
	Head	Mid piece	Tail	Head	Mid piece	Tail	Head	Mid piece	Tail
Magician	57.33	67.04	77.33	52.33	60.96	70.46	41.17	49.37	62.83
Pure gold	55.83	57.79	72.58	36.83	49.96	65.71	24.67	45.42	59.17
Roycidale	59.33	63.25	73.37	44.17	57.96	76.96	31.67	49.21	61.17
Pursuit	59.00	67.75	72.50	39.83	62.58	66.17	28.50	46.33	57.83
Astronaut	62.83	72.33	79.33	45.00	61.12	67.42	30.50	48.46	57.12
Mean ± Sd.	60.8 ±13.9[a]	65.6 ±11.1[d]	75.3 ±10.1[f]	43.6 +12.4[b]	58.5 +101.1[de]	66.8 +8.7[g]	31.3 +10.8[c]	49.8 +8.7[e]	59.8 +8.5[g]

[1]Mean of six collections

Means with the same superscripts are not significantly different ($P > .05$).

TABLE 4

THE REDUCTION IN PERCENTAGE INTACT PLASMA MEMBRANES ON THE HEADS MID-PIECE AND TAILS OF BOVINE SPERMATOZOA DUE TO COOLING FROM 37C TO 5C AND EQUILIBRATION (PERIOD 1) AND FREEZE-THAWING (PERIOD 2)

	Decrease in Percent intact plasma membrane		
	Head	Mid-Piece	Tail
Period 1	17.2	6.6	8.5
Period 2	12.3	8.7	7.0

DISUCSSION

Motility estimates have been used for evaluating semen since the advent of artificial insemination. Commercial laboratories have used motility estimates to evaluate both ejaculated spermatozoa and frozen-thawed spermatozoa. Decisions made as to whether or not a sample of semen is to be processed, frozen and kept or sold after freezing are based mainly on motility estimates, provided other minimal standards for concentration and the percentage abnormal spermatozoa are met. This makes motility estimates important to commercial artificial insemination.

Conflicting reports have been published on the correlation between fertility, as measured by 60 to 90 day non return rates and postthaw motility. An experiment conducted on 25 samples of bovine semen to compare the correlation between six conventional laboratory tests and fertility found a significant correlation, Y = .41 (P .05) between motility and fertility (Stewart *et al.*, 1972). However, it was pointed out by the authors that there was no apparent effect on non-return rates arising from the use of what would

normally be regarded as inferior semen for dairy bulls because of low postthaw motility (motility less than 35%). In another experiment a highly significant correlation, Y = .46 was reported between percent motility and percent 90 day non-return rate for 15 bulls. When the mean of all the ejaculates for each of the 15 bulls was considered the correlation rose to .56 (Saacke and White, 1973). In rabbits the correlation between motility and fertility was low (Howe, 1973). The greatest reduction in motility occurred in period one which would suggest that motility estimates obtained after equilibration may be a more valid evaluation of semen samples to be frozen and thawed than the initial motility. It has however been reported that differences in percentage motile spermatozoa were not significant during processing through 5C but were depressed by freezing and thawing (Coulter and Foote, 1973). Motility, as expected,was depressed by processing and freezing. However, the greater reduction in motility during cooling and equilibration was not expected.

Mean percent progressive motility of 57.5, 54.2 and 41.7 have been reported for fresh bovine semen at 37C, cooled and equilibrated semen at 5C and frozen-thawed semen respectively (Coulter and Foote, 1974). If the above percentages are expressed in terms of periods as in the present experiment, the reduction in period one would be 3.3 percent versus 12.5 percent in period two. This is a direct reversal of the results obtained in this experiment where average period one reduction in motility was 10.9 percent versus 4.8 percent for period two (Table 2). One reason for this large drop in motility during period one may be the rapid cooling of the partially extended semen from 37C to 5C in one hour. For optimal survival during freezing in straws, it has been recommended that at least two hours should be allowed for cooling to 5C (Berndtson *et al.*, 1975). For semen frozen in ampules a range of one and a half to two hours cooling time has been suggested (Van Demark, 1957).

The results of the statistical analysis of percent live spermatozoa data followed a trend similar to the motility results. More spermatozoa were damaged in period one (P .01).

Differential staining has not been extensively used for evaluating frozen bovine spermatozoa because of glycerol interference with the stain(Blackshaw, 1958). This interference was evident because

spermatozoa heads which were only partially stained red could be observed more frequently in extended and frozen-thawed samples. It is possible however that these were stressed spermatozoa showing the beginnings of membrane damage. The results presented in table 3 is in agreement with previous work in that the percent of spermatozoa with intact membranes will fall after extension of semen and cooling to 5C and a further reduction is expected after freezing and thawing (CIBA Foundation Symposium 1964). The decrease in percentage intact plasma membranes on the mid-pieces and tails were smaller than those observed for the head as evidenced by the fact that the percentage intact membranes on these areas after freeze-thawing was not significantly different from the percentage observed at the end of equilibration just before freezing. A similar observation was made by Jones, 1971 who showed that when the percentage of sperm heads with intact plasma membranes were compared with the percentage of mid-pieces and tails with intact plasma membrane the latter region showed considerably less damaged membranes.

An analysis of variance of the data on table 4 showed that there was a significant difference ($P < .05$) due to period on the percentage of spermatozoa heads with intact plasma membranes. More spermatozoa heads were damaged in period one than period two. This is not in agreement with other reports. Watson and Martin, 1972; Coulter and Foote, 1973 and 1974 all indicated that significantly fewer normal acrosomes were observed after freezing and thawing when compared to pre-freeze spermatozoa. Sehill and Wolff, 1974 also reported that human spermatozoa heads after glyceration before freezing did not significantly differ from control heads. However, in their micrographs both control and glycerolated spermatozoa had extensive plasma membrane damage. The authors however reported a complete loss of plasma membranes and acrosomes after freezing.

The greatest loss of spermatozoa actually occurred before freezing. This may indicate some deficiency in the particular laboratory where processing and freezing was carried out, particularly when one considers the fact that the partially extended semen was cooled from body temperature to 5C in one hour. However, if these findings can be sustantiated in other laboratories, more attention should be directed to this aspect of processing. The inclusion of butylated hydroxytoluene in the extender to possibly re-

duce cold shock during the initial cooling phase can be considered. It has been demonstrated that the cold shock associated with rapid cooling of spermatozoa from temperature to 5C can be reduced or prevented by the addition of butylated hydroxy toluene (Hammersted *et al.*, 1976). Perhaps artificial insemination units are creating problems by attempting to fit the entire processing into one normal working day.

References

Bishop, M.W.H. and Walton, A. 1960. Metabolism and motility of mammalian spermatozoa. In. A.S. Parkes (ed) Marshall *Physiology of Reproduction, 3rd ed. 1 (2): 264 Longmans Green, London.*

Berndtson, W.E., Ennen, B.D., Pickett, B.W., and Mortimes, R.G. 1975. Processing bovine semen for freezing in straws Abst. *67th Ann. Meeting A.S.A.J. Anim. Sci. 41: 343.*

Blackshaw, A.W. 1958. The effects of glycerol on the supravital staining of spermatozoa *Aust. Vet. J. 34: 71.*

Ciba Foundation Symposium (1964). **"Cellular injury"** A.V.S. de Reuck and J; Knight,(eds.) *Little Brown, Boston, Massachusetts.*

Coulter, G.H. and Foote, R.H. 1973. Sperm changes during processing in straws. Abstr. 65th Ann. Meeting A.S.A. *J. Anim. Sci., 73: 306.*

Coulter, G.H. and Foote, R.H. 1974. The motility, acrosomal morphology and oxygen uptake of bull spermatozoa during processing and after freezing in straws. *A Digst, 22: 12 - 15.*

Devereuz, E.D. and Turner, F.W. 1927. Observations on the growth of yeasts in pure nutrient solutions *J. Bact. 14: 317 - 322.*

Emmens, C.W. 1947. The motility and viability of rabbit spermatozoa at different hydrogen ion concentrations. *J. Physiol., 4: 471.*

Erickson, W.E. and Graham, E.F. 1959. Factors affecting the fertility of frozen bovine spermatozoa *J. Dairy Sci. 42: 520.*

Hackett, A.J. and Macpherson, J.W. 1965. A method for differential staining of bovine spermatozoa **after** extension in sterile milk *Can. Vet. J. 6 617 -620.*

Hammerstedt, A.H., Rupert, A.P., Rucinsky, T.,Morse, P.C., Lipock, J., Snipes, W. and Keith,.A.D. 1976. Use of spin labels and elctron spine resonance spectroscopy to Characterize membranes of bovine sperm. Effect of butylated hydroxytoluene and cold shock. *Biol. of Reprod. 14. 381.*

Harrison, R.A.A. and White, I.G. 1972. Glycolytic enzymes in the spermatozoa dnd cytoplasmic droplets of bull, boar and ram, and their leakage after cold sock. *J. Reprod. Fert. 30: 105.*

Howe, G.R. 1973. Efforts to relate spermatozoa motility to fertilizing capacity *Int. J. Fert. 18: 188 - 191.*

John, P.W.M. 1971. Statistical design and analysis of experiments. MacMillan. New York.

Jones, R.C. (1971). Ultrastructure of mammalian spermatozoa: the effects of buffer concentration in fixatives for boar spermatozoa. *Micron. 2: 350 - 355.*

Jones, R.C. (1973a). The plasma membrane of ram boar and bull spermatozoa. J. *Reprod. Fert.* **33:** *179 - 183.*

Jones, R.C. (1973b). Preparation of spermatozoa for light and electron microscopy. *J. Reprod. Fert. 33: 145 - 149.*

Jones, R.C. (1973c). Changes occurring in the head of board spermatozoa: Vesciculation of vacuolation of the acrosome. *J. Reprod. Fert. 33: 113-117.*

Luft, J. H. Improvements in epoxy resin embedding methods. *J. Biophys and Biochem. Cytol 9: 409-411.*

Macpherson, J.W. 1960. Sterile milk as a semen diluent *Can. Vet. J. 1: 551.*

Millonig, C. (1961). A modified procedure for lead staining of thin sections. *J. Biophys. Biochem. Cytol. 11: 735 - 738.*

Saacke, R.G. and White, J.M. 1972. Semen quality tests and their relationship to fertility. Proc. *4th Tech. Conf. A.I. and Reproduction. p. 22.*

Schill, W.B. and Wolff, H.H. (1974). Ultrastructure of human sperm acrosome and determination of acrosin activity under conditions of semen preservation. *Int. J. Fert. 42: 94-97.*

Somade, B., Macpherson, J.W. and King, G.J. (1974). Physical damage to the head of bovine spermatozoa induced by processing and freezing. *Proc. 69th Ann. Meeting A.D.S.A. P. 91.*

Stewart, D.L., O'Hagan, C. and Glover, F.A. 1972. The prediction of the fertility of bull semen from laboratory tests. *Proc. 7th Int. Congr. Anim. Reprod. and A.I., Munich 3: 1280.*

Van Demark, N.L., Muller, W.J., Kimmey, W.C., Rodriguez, C. and Friedman, M.E. 1957. Preservation of semen at sub-zero temperatures III. *Agric. Ext. Pt. Stat. Bull. 621: 33 - 42.*

Watson, P.F. and Martin, I.C.A. (1972). A comparison of changes in the acrosomes of deep-frozen and bull spermatozoa. *J. Reprod. Fert. 28: 99-101*