THE EFFECT OF EQUILIBRATION TIME ON BOVINE SPERMATOZOA FORZEN IN STRAWS

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1973

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
July, 1975

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ACKNOWLEDGEMENTS

The author wishes to express her sincere appreciation to Dr. Milton Wells, Associate Professor of Dairy Science, for his counsel, guidance, patience, and suggestions during the course of this study and in the preparation of this thesis.

The author is grateful to Dr. R. P. Wettemann and Dr. E. J. Turman for their suggestions and assistance in the preparation of this manuscrip.

The author is grateful to Jack Breuer and Ann Stotts for their assistance in the collection of the data.

Special appreciation is extended to the author's parents for their understanding and encouragement during the course of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The process of freezing bull semen for use in artifical insemination has been a wide spread practice for many years. Several methods of packaging frozen semen have been developed, with semen presently being frozen in ampules, pellets, and straws. The straw system was developed in Europe and has been extensively used there during the past decade. Only recently has the straw been investigated in the United States. Breeding organizations have shown increasing interest in packaging bull semen in plastic straws due to the fact that straws are more economical to store since they are smaller in volume, and fewer sperm are lost during the insemination process and straws have some advantage in conception rate when used properly.

There is little information on the optimum procedures for freezing bull semen in straws. It has been shown that some of the semen handling procedures used for ampules cannot be used on straws. Recently, some work has been published (Wiggin and Almquist, 1975; and Almquist and Wiggin, 1973a and 1973b) on rate of freeze, thawing rate and optimum glycerol level for straws, but little has been done to establish an optimum equilibration time (the length of time sperm cells are exposed to glycerol before freezing) or the effect of equilibration length on acrosome condition in straw-processed semen. It has been suggested that shortening equilibration time does not reduce fertility when using French

straws (Jondet, 1972). In recent years the acrosome has been studied for its role in fertilization. It is known that in the boar the integrity of the acrosome must be maintained for the sperm cells to be fertile (Graham et al., 1971, and Pursel and Johnson, 1971). The relationship of the acrosome state to fertility for bull spermatozoa has been obscure; however, Saacke and White (1972) indicate that sixty-five percent of the variation in fertility of bulls could be accounted for by intact acrosomes.

Freezing causes a loss of large numbers of motile sperm cells due to stress and relatively little work has been done on the effect of this stress upon the acrosome.

If the acrosome condition is influenced by length of equilibration, this could be an important factor in determining the optimum equilibration time for processing bull semen in straws. This study was conducted to evaluate the effect of a short equilibration and a long equilibration time and to study what effect these had upon cell motility, rate of movement, acrosome state and normality of the cell.

CHAPTER II

LITERATURE REVIEW

Glycerol Action

In 1949, Polge et al. discovered that glycerol was beneficial in protecting fowl spermatozoa from the harmful effects of freezing and thawing. The effects of glycerol during freezing were investigated by Smith and Polge (1950a) using horse, guinea pig and rabbit spermatozoa. Success post-freeze was obtained with the horse and rabbit spermatozoa. Bull spermatozoa was frozen successfully using glycerol by Smith and Polge (1950b). Various glycerol concentrations and freezing rates were tested. When samples containing five, ten, fifteen, and twenty percent glycerol were plunged directly into the freezing solution at -79 C, approximately ten percent motile cells were revived. Samples containing, five, ten, and fifteen percent glycerol were cooled slowly from 2 C to -79 C and the revival of cells ranged from 50 to 90 percent motile cells with the lower percent motile cells with the five percent glycerol concentration. Fertilizing capacity of the frozen bull spermatozoa was not measured in this study.

The results of Smith and Polge (1950b) indicated that glycerol modifies the process of ice crystal formation and dissolution in the medium, so that damage due to pressure and other mechanical effects was reduced. Crystal formation within the sperm was not seen by ultraviolet

photomicrography. In addition, glycerol appears to act as a salt buffer, minimizing the damage due to electrolyte shifts (Foote and Bredderman, 1969). Lovelock (1953a and 1953b) suggests that glycerol acts as a "salt buffer" preventing shifts in salt concentrations found at temperatures of -15 C to -40 C. Another proposed mechanism by which glycerol acts as stated by Saacke and Almquist (1961) is that glycerol assists in maintaining the integrity of the membrane, which is high in lipoprotein, during cooling and freezing. The mechanism by which glycerol acts is questionable (Sherman, 1963). Lovelock (1954) stated that glycerol must penetrate the cell in order to have a protective action; this is supported by the observations of Sloviter (1951) with red blood cells, by Polge and Lovelock (1952) for bull spermatozoa and is in agreement with White (1957) who feels that glycerol action is intracellular. Sherman (1963) disagrees with these ideas and suggests that the action of glycerol is extracellular; i.e., a coating mechanism.

Levels of glycerol which give optimum sperm survival after freezing for ampules have been well documented. Smith and Polge (1950a) found that glycerol levels of ten to fifteen percent were superior to five percent in sperm survival rates post-freeze. Polge and Rowson (1952) indicated that ten percent glycerol was more desirable than fifteen percent. Emmens and Blackshaw (1950) stated that best results were obtained when 7.5 to 10 percent glycerol and 1.25 percent pentose were used. Miller and VanDemark (1954) reported that six to eight percent glycerol resulted in optimum sperm survival post-freeze. Cragel et al. (1955) concluded optimum glycerol level to be 7.6 percent. Saroff and Mixner (1955) reported that an extender containing seven percent glycerol and twenty percent egg yolk gave the best post-freeze survival. The glycerol level

which is used by the industry generally ranges from four to eight percent and this is well within the range studied by researchers.

Effect of Equilibration Time

The recommendations concerning length of equilibration time in glycerol prior to freezing have been variable. It has been suggested that holding sperm cells in glycerol at 5 C for a time improves motility post-freeze. Polge and Rowson (1952) found that allowing bull sperm cells to equilibrate overnight (fifteen to twenty hours) resulted in a substantially increased rate of survival. Blackshaw (1955) investigated no equilibration time and an equilibration time of eighteen to twenty hours and found no difference in revival of bull spermatozoa (56 percent vs. 47 percent). Martin and Emmens (1961) found higher percent motility readings post-freeze with an eighteen hour equilibration time. Cragle et al. (1955) studied equilibration times of four to twenty-eight hours and found 14.9 hours to be the optimum. Jones and Martin (1965) investigated thirty minutes, four hour and five hour equilibration times in freezing ram semen and found the five hour equilibration time to be better ($p < .05$) for motility. Miller and VanDemark (1957) concluded that bull semen equilibrated for six hours gave higher ($p = .05$) sperm survival rates post-freeze than did either two or eighteen hour equilibration times. Similar results were obtained by Polge and Jakobsen (1959), who found a gradual increase in proportion of surviving bull spermatozoa for equilibration times of up to about seven hours, but survival declined if equilibrated beyond 24 hours. They suggested that increased resistance to freezing was a result of the length of time the

semen has been diluted and cooled before freezing rather than length of glycerol exposure time.

An increase in percentage of motile sperm as equilibration time increased was shown by Saroff and Mixner (1955). They also suggested that there may be a "tying up" effect of egg yolk on glycerol which might also be a factor in equilibration time. O'Dell and Almquist (1957) investigated the effects of diluters and equilibration time and found that with egg yolk-citrate diluter, an eighteen hour equilibration time resulted in a significantly higher percentage of motile sperm than did a 0.5 hour equilibration time. Earlier work by O'Dell and Almquist (1954) using heated skimmilk diluter showed no significant differences in sperm cell survival post-freeze when equilibration times of 0.5, four and eighteen hours were used. This is in contrast to the results obtained for a skimmilk diluter which showed that 0.5 and four hour equilibration times were superior to eighteen hour equilibration times (O'Dell and Almquist, 1957). This was in disagreement with the work of O'Dell and Hurst (1956) which showed that post-freeze motility was signiticantly ($p < .05$) higher for 0.5 hour equilibration time than for an eighteen hour equilibration time for both egg yolk-citrate and skimmilk diluters. They found significant bull x equilibration and ejaculate x equilibration interactions suggesting that when freezing bull semen, individual emphasis should be placed on each bull. Some bull differences were also observed by Graham et al. (1957), in addition to finding a significant difference ($p < .05$) in fertility rates for twelve hour vs. four hour equilibration times.

Shorter equilibration times have been investigated and have produced satisfactory results. Polge (1953) received excellent results with only

0.5 hour equilibration with bull spermatozoa. Survival rates of frozen spermatozoa of up to 80 percent were obtained in this study. Emmens and Blackshaw (1955) obtained excellent motility with non-equilibrated frozen semen but it had a lower fertility rate than did the semen equilibrated overnight (27 percent vs. 56 percent). Fertility rates from equilibrated semen equaled those obtained with fresh semen (56 percent vs. 60 percent).

Recently, Berndtson and Foote (1969) found that a very brief exposure to glycerol, ten seconds, significantly improved post-freeze motility when compared to equilibration times of thirty minutes and six hours. They suggest that part of the improvement in the ten second exposure is attributed to the slightly harmful effects of glycerol during longer exposure periods at 5 C prior to freezing.

The matter of length of equilibration time is still confusing. It appears that the protective action of glycerol is evident even with very short exposure times. However, most semen freezing laboratories utilize from six hours to overnight (approximately eighteen hours) equilibration which follows the early research findings rather than the more recent findings. The practicality of this is questionable, since the early equilibration research utilized slow freezing rates (approximately 2 C per minute) and various packaging techniques.

Most of this early research concerning freezing of bull spermatozoa dealt only with revival of motility and fertility results, no emphasis was placed on preserving acrosome integrity. Recent studies have shown that the acrosome plays an important role in fertilization and that it is advantageous to look at this aspect when freezing spermatozoa.

Morphology and Function of the Acrosome

The acrosomal cap is a membranous structure covering the anterior portion of the sperm head. The acrosome covers the anterior half of the sperm head in the bull and is a double membrane system enclosing a thicker moderately dense middle layer (Saacke and Almquist, 1964; Saacke and White, 1972; Blom and Birch-Anderson, 1965). This membrane system is external to the nuclear membrane and internal to the cell membrane. The apical ridge is an enlargement and backward reflecting portion of the acrosome along the apical edge of the sperm head and contains a small vacuole (Blom and Birch-Anderson, 1961). Saacke and White (1972) determined percent intact acrosomes by noting the presence of the apical ridge using a differential interference contrast microscope and found that acrosomal retention could be accurately (C. V. = six percent) measured this way.

The acrosome contains enzymes which aid in penetration of the sperm into the egg (Austin and Short, 1972). The exact mechanisms are still not clearly elucidated, but strong evidence does exist that the acrosome plays an important role in fertilization.

Acrosome Aging

Acrosome aging alterations were described by Saacke and Marshall (1968) using differential interference contrast microscopy. They stated that the aging sequence was a normal process which all sperm undergo. Further work (Saacke, 1970) showed the sequence of alterations due to aging or injury seems to be constant, but the rate of alteration depends upon the ejaculate as well as semen handling techniques or sperm environment. Salisbury and Flerchinger (1967) postulated that the capacity to

fertilize ova is gradually lost as spermatozoa age. They observed that seasonal variations in the aging of frozen spermatozoa were seen, with more rapid aging of spermatozoa occurring in the summer months than in the winter months. It was also noted that low temperature, -79 C to -88 C, storage of sperm slowed the aging process but did not prevent it.

Freezing of Bull Semen in Straws

Until 1970, bull semen was frozen principally in ampules with some being frozen in pellet form. In the 1960's European artificial insemination organization developed 0.25 and 0.5 ml. plastic straws in which to freeze bull semen (Cassou, 1968). This new approach to packaging semen has attracted considerable interest from breeding organizations in the United States.

Since straw packaging is relatively new, little work has been done to establish optimum processing methods for semen frozen in plastic straws. Many old and perhaps inappropriate processing methods which have been used for ampule freezing have been utilized. Recent work has shown that re-evaluation of processing and handling of straw methods are needed.

Coulter and Foote (1973 and 1974) investigated the effect of rate of freeze on acrosome morphology and motility using 0.5 ml. French straws. They found that a slow freezing rate of 8.4 C per minute supported a significantly ($p < .01$) higher percentage of normal acrosomes than did a faster freezing rate of 17.5 C per minute. No difference in motilities were seen between the two freezing rates. Robbins et al. (1973) indicated that freezing rate had little effect on acrosome retention. They also indicated that a freeze rate of 11.64 C per minute gave maximum sperm cell survival post-freeze. Almquist and Wiggin (1973a and 1973b)

looked at methods and rates of freeze on motility of sperm cells frozen in straws and found that highest post-thaw motility was obtained by freezing the straws singly in static nitrogen vapor (about -180 C) and cooling rates of 82 C per minute from 5 C to -15 C then at 43 C per minute from -15 C to -60 C. Saacke et al. (1974) estimated an optimum freeze rate of from -10 C to -80 C in 160 seconds in static nitrogen vapor.

Some work has been done to evaluate optimum glycerol level with straws and has suggested 8.5 percent glycerol (Saacke et al., 1974), 10.22 percent glycerol (Robbins et al., 1973), and 10.7 percent glycerol (Wiggin and Almquist, 1975) were optimum. These values were derived by using a three-dimentional central composite design, which will estimate the optimum for each of several factors combined in the model.

Thaw rate of spermatozoa frozen in straws has been given recent consideration by researchers. A rapid thawing rate in hot water (65 C to 92 C) for seven to fifteen seconds has been found to give best results on acrosome and motility (Almquist and Wiggin, 1973a and 1973b; Robbins et al., 1973; Saacke et al., 1974; and Wiggin and Almquist, 1975).

Almquist and Wiggin (1973a) compared semen frozen in straws with semen frozen in ampules. Post-thaw motilities obtained were 24.1 percent for straws and 33.5 percent for ampules. They attributed this lower motility in straws to a longer equilibration time (eighteen hours for straws vs. four hours for ampultes) and a slower freezing rate.

Wells and Hefley (1974) utilized four and eighteen hour equilibration times in freezing bull spermatozoa in straws. They evaluated motility and acrosome morphology and the eighteen hour equilibration time resulted in significantly better ($p < .05$) post-freeze motility (35.3 percent for four hours vs. 42.3 percent for eighteen hours). The longer equilibration

time resulted in a significantly higher ($p < .05$) percentage of normal cells with non-aged acrosomes (48.7 percent for four hours vs. 64.4 percent for eighteen hours) and a significantly reduced ($p < .05$) percentage of normal cells with aged acrosomes (36.6 percent for four hours vs. 23.7 percent for eighteen hours). No significant differences were seen among bulls in response to treatment. Wiggin and Almquist (1975) investigated the effect of 0.5 and two hour equilibration times on acrosomal maintenance and motility of bull semen frozen in straws. They found no significant differences between equilibration times for intact acrosomes or percent motile cells. A significant ($p < .01$) thaw rate x glycerol interaction for intact acrosomes was found. There was a significant increase ($p < .01$) in mean percentage of intact acrosomes for each increase in thaw rate (38.1, 17.7, 51.0, and 55.2 percent, respectively, for 35, 55, 75, and 95 C for seven to fifteen seconds). In another study by Siggin and Almquist (1975), two hour equilibration was predicted as the optimum by using a multiple regression model. Jondet (1972) found only small differences in post-thaw motility and fertility for semen frozen in straws after exposure to glycerol for either one minute, eight minutes or six hours. It was previously found (Jondet, 1967) that fertility of bull semen frozen in straws was not reduced by shortening the time it was held at 5 C in glycerol from twenty to six hours.

From the evidence presented here it is easy to see that the optimum equilibration time for sperm cells stored in straws needs more study. This study was initiated to investigate the effect of equilibration time on: 1) acrosome integrity, 2) sperm cell motility, and 3) cell morphology.

CHAPTER III

MATERIALS AND METHODS

This research explored the effect of two equilibration times, three and eighteen hours, on the characteristics of bull spermatozoa frozen in 0.5 ml. plastic straws. The criteria used to evaluate the effect of these equilibration times were:

1. percent motile sperm cells,
2. rate of movement,
3. acrosome characteristics, and
4. cell morphology.

Earlier work has failed to give a good measure of how equilibration time effects these characteristics when bull semen is frozen in straws.

Experimental Design and Statistical Analysis

Semen was collected from four bulls on five different collection days. The ejaculates were split into two parts and each was subjected to either a three or eighteen hour equilibration time. Differences among bulls, replicates, and equilibration time on percent motile sperm, percent abnormal cells, acrosome characteristics and rate of movement were subjected to an analysis of variance using a split plot design. An ejaculate from a given bull was considered the main plot and equilibration time the sub-plots. Least significant differences were used to test the means in this study. Bull x treatment, replicate x treatment, and

replicate x bull x treatment interactions were included in the analysis. If significant, these interactions will not allow unqualified statements about bull, replicate, or equilibration times. However, significant interactions will only allow the use of two-way interactions as error terms to use in testing the significance of bull, replicate and equilibration time. The analysis was done using the Statistical Analysis (SAS) computer program developed by Barr and Goodnight (1972) at North Carolina University.

Semen Collecting and Handling

The sperm cells in this experiment were obtained from four dairy bulls housed at the Oklahoma State University dairy facility. The bulls were housed in individual pens with adjacent runs at the dairy bull barn and were on a weekly ejaculation schedule.

Semen was collected on the day it was to be frozen. The bulls preputial area was cleaned with water and dried prior to collection. They were then led to the collection stall behind a teaser cow and restrained for four to five minutes. The semen was collected in an artificial vagina filled with 52 to 54 C water. The collection tube was collected by a plastic tube and these were inside an insulated jacket when the ambient temperature was below 20 C. Ejaculates were maintained at 35 to 37 C until intial evaluation and eventual extention was done. The intial ejaculate characteristics were determined using the following procedures:

1. Semen volume, to the nearest 0.1 ml., was obtained from the 15 ml. graduated collection tubes, to determine if adequate amounts were available for freezing.

2. Sperm concentration was measured spectrophotometrically by placing 0.05 ml. of fresh semen in 7.95 ml. of 2.9 percent sodium citrate solution and gently mixing it in a 17 mm. diameter absorption tube. Percent light transmittance readings were obtained with a "Spectronic 20" which had been standardized with hemocytometer counts. These were converted to concentration of spermatozoa per milliter of semen by reference to prepared tables.
3. Percent motile sperm was estimated by microscopic examinations to determine if semen was of desired quality for freezing. Samples with less than 75 percent motile cells were not used.
4. Rate of movement was determined by microscopic examination and assigned a value of 0 to 4.0 (in increments of 0.5) with the following rating:

   0 = No apparent movement

   1 = Sluggish or rocking motility showing no progressive movement

   2 = Forward progress slow and somewhat sluggish

   3 = Intermediate, fairly rapid progressive motility

   4 = Maximum progressive motility, very rapid and vigorous movement
5. Acrosome characteristics were determined by using the Wells-Awa acrosome stain (Wells and Awa, 197) which was prepared by combining one ml. of a one percent solution of eosin B, two ml. of a one percent solution of fast green FCF and 1.7 ml. of ethyl alcohol. Two drops of semen diluted 1:10 with 2.9 percent sodium citrate and four drops of stain were mixed, and allowed to stain for two to four minutes. One drop of sperm-stain mixture was

placed on a clean microscope slide, smeared, dried on a slide warmer at 37 to 38 C and a glass cover slip was applied using mounting media. Acrosome condition was classified as aged or non-aged within each cell type. The non-aged acrosomes were those which showed a uniformly smooth acrosome that was closely adherent to the nucleus. Aged acrosomes were those that exhibited any of the following morphological changes:

A. Elevated or thickened acrosome - characterized by a partial or complete swelling of the outer membrane of the acrosomal cap and thus an enlargement of the acrosomal size.

B. Ruffled acrosome cap - characterized by a wrinkled or irregular surface either over the anterior portion of the cap or spreading over the entire surface of the acrosome.

C. Enlarged equatorial segment - characterized by the typical half moon shape of the equatorial segment becoming exaggerated as the acrosome becomes loosened from the sperm cell head.

D. Loosened acrosome cap - the acrosome is in the process of loosening from the sperm head, eventually leading to the capless sperm state.

E. Disintegrating acrosome cap - characterized by fragmentation of the acrosome.

F. Capless sperm - characterized by the lack of the acrosome and is considered the terminal state of aging and/or deterioration sequence. The anterior portion of the nucleus stains a light pink instead of the green which is typical when the acrosome is present.

6. Percentages of normal cells were determined by disregarding acrosome characteristics and combining the two normal categories determined in the acrosome evaluations.

Dilution and Glycerolization

The ejaculates were diluted in twenty percent egg yolk - eighty percent citrate (2.9 percent) extender to give a final concentration of 25 million live sperm cells per 0.5 ml. straw. The semen was partially extended and cooled in a cold room for two to three hours to 5 C. Cooled glycerolated egg yolk-citrate was then added in increments of ten percent, twenty percent, thirty percent, and forty percent at ten minute intervals (Miller and VanDemark, 1957). Final glycerol level was six to seven percent by volume.

Straw Filling

The cooled extended semen with glycerol added was packaged into 0.5 ml. French Straws.

The straw filling procedure used is as follows and was described by Boese (1972). Ten straws were loaded into a clip, a manifold connected to a suction pump was placed over the closed end of the straw and extended semen was gently pulled into the straws. Excess semen was removed from the open ends of the straws using a bubbler to allow for expansion on freezing and to make room for the sealing powder. The filled straws were pressed firmly into poly-vinyl sealing powder resulting in about a 3/16 inch plug. The powder sealed straws were then placed in a 5 C water bath for one to two hours to allow the gelation of the powder plug. The above process was carried out in a cold room at 5 C.

Equilibration Treatments Studied

Two equilibration times were used in this study, a short equilibration time of 3.0 $\pm$ 0.5 hours and a long equilibration time of 18.0 $\pm$ 0.5 hours. Up until this point all straws were treated alike except that one-half of the straws from each bull were treated with one drop of one color of food coloring and the second half were marked with a second color of food coloring. This was done to aid in identification of treatment among bulls.

After 3.0 $\pm$ 0.5 hours of equilibration, straws of treatment one were removed from the 5 C water bath, dried, placed on racks and frozen as described in the freezing procedure. At this same time straws of treatment two were removed from the water bath, dried and replaced in the refigerator at 5 C to equilibrate overnight. After these straws had equilibrated for 18.0 $\pm$ 0.5 hours, there were frozen using the same procedure.

Freezing of Straws

Eight straws from each bull for each treatment were frozen simultaneously in an MVE Model CBF 21 vapor freezing chamber with approximately four inches of liquid nitrogen in the bottom. The straws were frozen on a rack suspended in static nitrogen vapor in the freezing chamber at the level where the static temperature was -180 C as indicated by a themocouple. The straws were held in the nitrogen vapor for seven minutes resulting in a rapid freeze of approximately 23 C per minute. They were then lowered into the liquid nitrogen and in a few minutes were transfered into the storage tank (LD 31) where they remained until they were thawed and evaluated.

Observation and Treatment of Straws Post-Freeze

Six straws for treatment replicate and bull were thawed on a slide warmer at 37 C and pooled and the following observations were made: percent motile sperm cells, rate of movement, and acrosome characteristics. The percent motile sperm cells and rate of movement were estimated by microscopic viewing while the cells were still in the egg yolk-citrate-glycerol extender. However, the acrosome characteristics were determined by staining procedures which required removal of sperm cells from the extender to secure desirably stained cells.

One-half ml. of the pooled semen was placed in glass centrifuge tubes and centrifuged for two minutes at slow speed (approximately 1700 x G). The supernatant was decanted and the sperm cell layer was resuspended in 0.5 ml. of 2.9 percent sodium citrate, and centrifuged for another two minutes. The supernatant was again discarded and sperm cells resuspended in 0.5 ml. of 2.9 percent sodium citrate and centrifuged for a final two minutes. The supernatant was discarded and 0.5 ml. of 2.9 percent sodium citrate added. The mixture was then used for staining. Two drops of the sperm cell suspension and four drops of acrosome stain were mixed and allowed to stand for four minutes. One drop of sperm-stain mixture was smeared on a glass slide and a coverslip was applied using mounting media.

Slide Evaluation

Two hundred sperm cells were examined on each slide to determine acrosome characteristics and morphological abnormalities as described previously in this section. This was done with a light microscope, at a

magnification of 430X, and using a blue filter. All slides were read by the same individual and coded to keep the slide reader completely unaware of what bull and treatment was being evaluated.

CHAPTER IV

RESULTS AND DISCUSSION

Five ejaculates from each of four bulls were utilized to study the effects of equilibrating extended semen for either three or eighteen hours in 0.5 ml. straws on post-freeze motility, morphology and the acrosome condition.

The results in this study were analyzed two ways. The first analysis included the initial ejaculate characteristics as a treatment; this was done so treatment effects could be assessed in relation to initial characteristics. In the second analysis intital characteristics were not included and only the two equilibration times were analyzed. All of the treatment comparisons in this chapter are from the second analysis.

Effects on Percent Motile Cells

Table I presents the mean effects of equilibration time by bull and Table II presents the statistical analysis of the means. The long equilibration time consistently resulted in a higher percentage of motile sperm for each bull and gave significantly ($p < .0001$) higher overall recovery of motile cells post-freeze (36.6 percent vs. 29.3 percent) than did the short equilibration time. Both treatments drastically reduced the percent motile cells from that in the initial ejaculate. The average percent kill was 59.5 percent which is within the realm of what is typically experienced in freezing of semen. It should be noted that the data

of this study suggest an average of 9.0 percent fewer cells will be lost in freezing when cells are equilibrated for eighteen hours vs. three hours.

TABLE I

OVERALL MEANS OF PERCENT MOTILE SPERM CELLS BY TREATMENT AND BULL

		Treatment	
Bull	Initial	3 hour Equilibration	18 hour Equilibration
1	83.0*	35.0	41.6
2	82.0	18.0	25.0
3	77.0	34.8	39.8
4	83.0	29.2	39.8
Mean	81.3^{a}	29.3^{b}	36.6^{c}

*5 observations/mean
S. E. treatment = $\pm$ 2.2
S. E. bulls = $\pm$ 4.6
Means with different superscripts in the same row are significantly different at the ($p < .01$) level.

TABLE II

ANALYSIS OF VARIANCE FOR PERCENT MOTILE SPERM CELLS

Source	DF	Mean Squares	F	p
Replicates	4	174.90	1.41	
Bull	3	603.47	4.86	$p < .05$
Replicate x Bull	12	124.05		
Treatment	1	532.90	36.72	$p < .0001$
Bull x Treatment	3	13.96	0.96	
Replicate x Treatment	4	34.53		
Replicate x Bull x Treatment	12	7.84		

Table II also shows that there was a significant difference among bulls ($p < .05$) in percent motile cells post-freeze. Examination of the means in Table I suggests that bull 2 was largely responsible for this difference. This is in agreement with general observations that some bulls' semen responds differently to treatment and freezing than do others, even though intial quality may be similar.

Differences among replicates (Table III) and interactions of treatment with either bull or replicates were not significant (Table II).

These data agree with that of Wells and Hefley (1974) who found that an eighteen hour equilibration time resulted in a higher percentage of motile cells post-freeze, but they did not observe a bull effect.

TABLE III

OVERALL MEANS FOR PERCENT MOTILE SPERM CELLS BY REPLICATE AND TREATMENT

		Treatment	
Replicate	Initial	3 hour Equilibration	18 hour Equilibration
1	82.5*	21.8	35.5
2	80.0	32.5	35.0
3	78.8	25.3	32.5
4	81.3	29.3	37.0
5	83.8	37.5	42.8
Mean	81.3^{a}	29.3^{b}	36.6^{b}

*4 observations/mean
S. E. Treatment = $\pm$ 2.5
S. E. Replicate = $\pm$ 5.1
Means with different superscripts in the same row are significantly different at the ($p < .01$) level.

Rate of Movement Evaluation

Tables IV and V present the means and statistical analysis for rate of movement. Differences between treatment means were not significantly different. However, there was a significant difference ($p < .05$) among bulls which indicates that different bulls respond differently to equilibration time. As seen in Table IV, the overall means for short and long equilibration times did not differ (2.8 vs. 2.7). However, there was a significant ($p < .05$) reduction in rate for both treatment with bull 2. The variation among bulls can clearly be seen in these means.

TABLE IV

OVERALL MEANS FOR RATE OF MOVEMENT BY BULL AND TREATMENT

		Treatment	
Bull	Initial	3 hour Equilibration	18 hour Equilibration
1	3.9*	2.8	3.0
2	4.0[a]	2.4[b]	2.3[b]
3	4.0	3.0	2.8
4	4.0	2.9	2.7
Mean	4.0	2.8	2.7

*5 observations/mean
S. E. Treatment = ± 0.04
S. E. Bull = ± 0.14
Means with different superscripts in the same row are significantly different at the ($p < .05$) level.

Differences among replications and interactions of treatment with bull and replication were not significant (Table V).

Acrosome Evaluation

Figure 1 diagramatically illustrates what can happen to a sperm cell population when it undergoes some type of stress such as freezing. The four types of cells represented here are usually present in any given sperm cell population. Theoretically, we would like a population of sperm cells with a large percentage of normal non-aged cells (it is assumed that these are the cells capable of fertilizing the ova) and a

small percentage of the other three cell types (assuming that these are the less desirable). The shift of cell types in this study for both treatments, as indicated in Table VI, was primarily a shift from normal non-aged cells to normal aged cells. Relatively little change was seen in the abnormal cell population.

TABLE V

ANALYSIS OF VARIANCE FOR RATE OF MOVEMENT

Source	DF	Mean Squares	F	p
Replicate	4	0.18	1.16	
Bull	3	0.69	4.50	$p < .05$
Replicate x Bull	12	0.15		
Treatment	1	0.06	0.86	
Bull x Treatment	3	0.09	1.36	
Replicate x Treatment	4	0.13		
Replicat x Bull x Treatment	12	0.04		

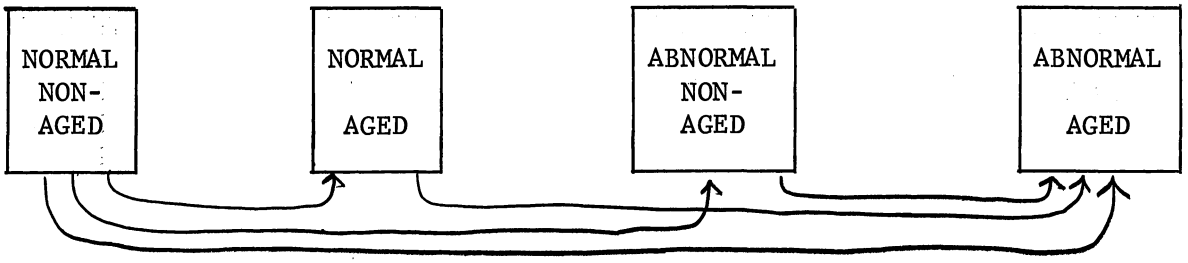

Figure 1. Shifts in Cell Type and Acrosome Characteristics in a Sperm Cell Population.

TABLE VI

MEANS OF SPERM CELL POPULATION BY TREATMENT, MORPHOLOGY AND ACROSOME CHARACTERISTIC

		Treatment	
Cell Type	Initial	3 hour Equilibration	18 hour Equilibration
	- - - - - - - - - - - - % - - - - - - - - - - - -		
Normal Non-aged	69.6	55.7	51.0
Normal Aged	18.0	35.7	36.2
Abnormal Non-aged	6.2	4.4	5.8
Abnormal Aged	6.2	4.2	7.0

The means for percent normal cells with non-aged acrosomes, by bull and treatment, are presented in Table VII. Both treatments significantly decreased ($p < .05$) the percent normal cells with non-aged acrosomes from the initial state. This was expected due to the stress the acrosome undergoes in freezing and thawing. The short equilibration showed a slight but non-significant advantage (4.2 percent) in maintaining acrosome condition over the long equilibration time. Work by Wells and Hefley (1974) with a limited number of ejaculates indicated that a long equilibration time (18 hours) significantly increased ($p < .05$) the percentage of normal cells with non-aged acrosomes. Bull 4 did not follow the trend shown by the other bulls (Table VII); he responded more favorably to the long equilibration time; however, this increase was only slight (1.9 percent).

The analysis of variance for percent normal cells with non-aged acrosomes is presented in Table VIII. No significant differences due to treatment, bull or replicates were observed. Likewise, no interactions were found to be significant.

Mean percentages of normal cells with non-aged acrosomes by treatment and replicates is shown in Table IX. These data suggest that circumstances unique to each population of sperm cells sampled during the different replicates may cause a different treatment response. This is in agreement with Hodson (1972) who found that acrosome conditions varied in response to rate of freeze in different collection periods and is also in agreement with the findings of Awa (1970) that the acrosome is highly sensitive to changes in season, temperature or animal status.

TABLE VII

OVERALL MEANS FOR PERCENT NORMAL CELLS WITH NON-AGED ACROSOMES BY BULL AND TREATMENT

		Treatment	
Bull	Initial	3 hour Equilibration	18 hour Equilibration
1	71.9*	61.8	50.8
2	68.0	51.1	50.3
3	72.8	57.4	48.5
4	65.7	52.6	54.5
Mean	69.6^{a}	55.7^{b}	51.0^{b}

*5 observations/mean
S. E. Treatment = $\pm$ 5.0
S. E. Bull = $\pm$ 3.9
Means with different superscripts in the same row are significantly different at the ($p < .05$) level.

TABLE VIII

ANALYSIS OF VARIANCE FOR PERCENT NORMAL CELLS WITH NON-AGED ACROSOMES

Source	DF	Mean Squares	F	p
Replicate	4	107.52	.97	
Bull	3	53.08	0.48	
Replicate x Bull	12	110.53		
Treatment	1	220.90	2.13	p = .16
Bull x Treatment	3	96.75	0.93	
Replicate x Treatment	4	45.23		
Replicate x Bull x Treatment	12	122.97		

TABLE IX

OVERALL MEANS FOR PERCENT NORMAL CELLS WITH NON-AGED ACROSOMES BY REPLICATE AND TREATMENT

		Treatment	
Replicate	Initial	3 hour Equilibration	18 hour Equilibration
1	66.8*	52.8	55.5
2	62.9	62.1	52.4
3	60.5^{a}	49.6^{b}	46.0^{b}
4	77.0^{a}	58.4^{b}	53.0^{b}
5	80.9^{a}	55.8^{b}	48.3^{b}
Mean	69.6^{a}	55.7^{b}	51.0^{b}

*4 observations/mean
S. E. Treatment = $\pm$ 5.6
S. E. Replicate = $\pm$ 4.4
Means with different superscripts in the same row are significantly different at the ($p < .05$) level.

The analysis of variance for percent normal cells with aged acrosomes is presented in Table X. The variance among bulls approached significant ($p < .10$) indicating that perhaps cells from some bulls age more upon freezing than others. No significant treatment differences or interactions were seen in this type of sperm.

Effect of treatment by bull means on percent normal cells with aged acrosomes is shown in Table XI. There were significant increases ($p < .05$ for bulls 1 and 4, $p < .01$ for bulls 2 and 3) in numbers of aged acrosomes from initial ejaculate for both equilibration times. This is

to be expected with the stress caused by freezing and thawing. The differences due to treatment were very slight, 35.7 percent for the short equilibration time and 36.2 percent for the long equilibration time. This is not in complete agreement with the findings of Wells and Hefley (1974) who found that an eighteen hour equilibration time gave a significantly lower ($p < .05$) level of normal cells with aged acrosomes.

TABLE X

ANALYSIS OF VARIANCE FOR PERCENT NORMAL CELLS WITH AGED ACROSOMES

Source	DF	Mean Squares	F	p
Replicate	4	32.18	0.28	
Bull	3	299.12	2.66	$p < .10$
Replicate x Bull	12	113.06		
Treatment	1	2.76	0.03	
Bull x Treatment	3	16.32	0.18	
Replicate x Treatment	4	65.66		
Replicate x Bull x Treatment	12	98.96		

TABLE XI

OVERALL MEANS FOR PERCENT NORMAL CELLS WITH AGED ACROSOMES BY BULL AND TREATMENT

		Treatment	
Bull	Initial	3 hour Equilibration	18 hour Equilibration
1	17.9^{a}*	30.1^{b}	30.3^{b}
2	16.8^{c}	39.3^{d}	41.1^{d}
3	11.2^{c}	30.9^{d}	33.9^{d}
4	$\underline{26.0^{a}}$	$\underline{42.5^{b}}$	$\underline{39.6^{b}}$
Mean	18.0^{c}	35.7^{d}	36.2^{d}

*5 observations/mean
S. E. Bull = $\pm$ 3.8
S. E. Treatment = $\pm$ 4.2
Means with different superscripts in the same row are significantly different at the $^{a}(p < .05)$ and $^{c}(p < .01)$ level.

Means of treatments for percent normal cells with aged acrosomes in replicates varied somewhat (Table XII) but not significantly. Similar variations were seen by Hodson (1972) when observing rate of freeze effects on acrosome aging, his findings showed that on different collection days acrosomes reacted differently to rates of freeze.

The analysis of variance for percent abnormal cells with non-aged acrosomes (Table XIII) shows variation among bulls and replicates to be significant ($p < .05$). This is to be expected because abnormal cell levels vary from collection to collection due to climate and animal status and also vary among bulls. The means for abnormal cells with

non-aged acrosomes showed little difference (Table XIV) for equilibration times, 4.4 percent vs. 5.8 percent for short and long equilibration. None of the possible interactions were significant for this cell type.

TABLE XII

OVERALL MEANS FOR PERCENT NORMAL CELLS WITH AGED ACROSOMES FOR REPLICATE AND TREATMENT

		Treatment	
Replicate	Initial	3 hour Equilibration	18 hour Equilibration
1	19.1^{a}*	38.4^{b}	37.9^{b}
2	27.5	32.4	39.8
3	11.6^{a}	41.3^{b}	33.3^{b}
4	16.6^{a}	33.6^{b}	37.4^{b}
5	15.0^{a}	32.9^{b}	32.9^{b}
Mean	18.0^{a}	35.7^{b}	36.2^{b}

*4 observations/mean
S. E. Replicate = $\pm$ 4.2
S. E. Treatment = $\pm$ 4.7
Means with different superscripts in the same row are significantly different at the ($p < .01$) level.

TABLE XIII

ANALYSIS OF VARIANCE FOR ABNORMAL CELLS WITH NON-AGED ACROSOMES

Source	DF	Mean Squares	F	p
Replicate	4	47.44	4.74	$p < .05$
Bull	3	41.37	4.13	$p < .05$
Replicate x Bull	12	10.01		
Treatment	1	21.03	2.64	$p = .12$
Bull x Treatment	3	11.44	1.44	
Replicate x Treatment	4	12.81		
Replicate x Bull x Treatment	12	6.35		

TABLE XIV

OVERALL MEANS FOR PERCENT ABNORMAL CELLS WITH NON-AGED ACROSOMES FOR BULL AND TREATMENT

		Treatment	
Bull	Initial	3 hour Equilibration	18 hour Equilibration
1	5.2*	4.4	8.1
2	7.3	4.8	3.8
3	8.6	5.8	8.5
4	3.7	2.4	2.8
Mean	6.2	4.4	5.8

*5 observations/mean
S. E. Treatment = $\pm$ 2.2
S. E. Bull = $\pm$ 1.2

Means for abnormal cells with aged acrosome showed a significant difference ($p < .05$) between treatments (Table XV) with the short equilibration time having 4.2 percent vs. 7.0 percent for the long equilibration time. The analysis (Table XVI) shows both bull and treatment differences to be significant ($p < .05$) with no replication or interaction significance.

Abnormal cell types showed acrosomal chances of a much smaller magnitude than did the normal cells. This agrees with the findings of Wells and Hefley (1974) who found that normal sperm cells in a population are the most likely cells to be damaged during freezing.

TABLE XV

OVERALL MEANS FOR PERCENT ABNORMAL CELLS WITH AGED ACROSOMES FOR BULL AND TREATMENT

		Treatment	
Bull	Initial	3 hour Equilibration	18 hour Equilibration
1	5.0*	3.7^{a}	10.8^{b}
2	7.9	4.8	4.8
3	7.4	5.9	9.1
4	4.6	2.5	3.4
Mean	6.2^{b}	4.2^{a}	7.0^{b}

*5 observations/mean
S. E. Treatment = $\pm$ 1.8
S. E. Bull = $\pm$ 1.9
Means with different superscripts are significantly different at the ($p < .05$) level.

TABLE XVI

ANALYSIS OF VARIANCE FOR ABNORMAL CELLS WITH AGED ACROSOMES

Source	DF	Mean Squares	F	p
Replicates	4	25.86	2.10	
Bull	3	46.64	3.78	$p < .05$
Replicate x Bull	12	12.33		
Treatment	1	78.40	4.72	$p < .05$
Bull x Treatment	3	25.08	1.51	
Replicate x Treatment	4	17.01		
Replicate x Bull x Treatment	12	16.48		

Effect of bull by treatment on percent total non-aged (the combination of normal and abnormal non-aged categories) is presented in Table XVII. Each bull showed similar response to treatment with the short equilibration having a slightly higher (3.1 percent) but non-significant percentage of total non-aged acrosomes than did the longer equilibration time.

Table XVIII shows the means by replicate and treatment on percent total non-aged acrosomes. Except for replicate 2 all replicates showed similar responses to treatment with the shorter equilibration time being slightly superior to the longer equilibration time.

TABLE XVII

OVERALL MEANS FOR PERCENT TOTAL NON-AGED ACROSOMES BY BULL AND TREATMENT

		Treatment	
Bull	Initial	3 hour Equilibration	18 hour Equilibration
1	77.1*	66.2	58.9
2	75.4	55.9	54.1
3	81.4	63.2	57.0
4	69.4	55.0	57.8
Mean	75.8	60.1	57.0

*5 observations/mean
S. E. Treatment = ± 4.7
S. E. Bull = ± 3.8

Percent motile sperm cells, rate of movement and acrosome evaluation were used in this study as indicators of how well sperm cells survived the stress of freezing and if a prolonged equilibration time aids in sperm cells withstanding this stress.

There appears to be little effect on the acrosome condition by a long equilibration time, but a significant ($p < .01$) increase in percent motile cells occurs with a long equilibration time.

Considerable variation among bulls was found in response to treatment dependent on cell characteristic measured. Differences among replicates were usually not an important source of variation in defining treatment effects.

TABLE XVIII

OVERALL MEANS FOR PERCENT TOTAL NON-AGED ACROSOMES BY REPLICATE AND TREATMENT

		Treatment	
Replicate	Initial	3 hour Equilibration	18 hour Equilibration
1	71.6*	56.5	68.1
2	65.9	64.9	55.3
3	79.5	54.3	55.0
4	79.9	62.5	58.0
5	82.3	62.3	58.4
Mean	75.8	60.1	57.0

*4 observations/mean
S. E. Treatment = $\pm$ 5.2
S. E. Replicate = $\pm$ 4.2

CHAPTER V

SUMMARY AND CONCLUSIONS

Five ejaculates from each of four bulls were used in this study to determine the effect of length of equilibration time on livability and acrosome characteristics of sperm cells processed in straws. Ejaculates were collected on five different days and a split-ejaculate study was conducted using two equilibration times, 3.0 $\pm$ 0.5 hours and 18.0 $\pm$ 0.5 hours.

Analysis of the data revealed a significant advantage ($p < .01$) for the eighteen hour equilibration time for percent motile sperm cells post-freeze. There were also significant differences ($p < .05$) among bulls for percent motile cells post-freeze, indicating that semen from different bulls responds differently to the two equilibration times. The stress of freezing was evident by the decrease in percent motile cells, the increase for percentage aged acrosomes and the reduced rate of movement in both equilibration treatments. Rate of movement was not significantly affected by treatment but there was a significant difference ($p < .05$) among bulls for rate. The initial ejaculates had greater ($p < .05$) of motile cells and cells with non-aged acrosomes than any of the frozen semen. There was no significant difference between treatments for normal cells with aged or non-aged acrosomes. Treatments did not influence percent abnormal cells with non-aged acrosomes; however, there were significant differences ($p < .05$) among bulls and replicates for

this characteristic. There were significant differences ($p < .05$) among bulls and treatments in percent abnormal cells with aged acrosomes. These abnormal cells are of little consequence since they make up only a small proportion of the population. This study indicates that the longer equilibration time resulted in better post-freeze motility and that there is little difference in acrosome condition due to either treatment.

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