SEMINAL PLASMA AND FREEZE-THAW INJURY TO BOVINE SPERM

by

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF RELATED LITERATURE	3
Role of Seminal Plasma in Bovine Semen	3
Acrosomal Maintenance as a Laboratory Test for Spermatozoan Injury	8
EXPERIMENTAL PROCEDURE	11
Experiment I	13
Experiment II	13
Statistical Analysis	14
RESULTS	15
Experiment I	15
Experiment II	19
DISCUSSION	29
BIBLIOGRAPHY	34
VITA	38

LIST OF TABLES AND FIGURES

Table		Page
1	Analyses of variance of percent intact acrosomal caps and percent motile spermatozoa in Experiment I.	16
2	Effect of holding time in seminal plasma on the percent intact acrosomal caps of spermatozoa incubated at 37°C post-thaw (Experiment I).	17
3	Effect of holding time in seminal plasma on the percent intact acrosomal caps of frozen spermatozoa from fourteen ejaculates (Experiment I).	18
4	Effect of holding time in seminal plasma on the percent motility of spermatozoa incubated at 37°C post-thaw (Experiment I).	20
5	Analyses of variance of percent intact acrosomal caps and percent motile spermatozoa in Experiment II.	21
6	Effect of holding time in seminal plasma on the percent intact acrosomal caps of spermatozoa incubated at 37°C post-thaw (Experiment II).	23
7	Effect of holding time in seminal plasma on the percent intact acrosomal caps of frozen spermatozoa from eighteen ejaculates (Experiment II).	24
8	Effect of holding time in seminal plasma on the percent motility of spermatozoa incubated at 37°C post-thaw (Experiment II).	27

Figure		Page
1	Effect of holding time on the acrosomal maintenance of semen in regard to the interaction of holding time x ejaculates.	26
2	Effect of energy conservation by cooling on optimum exposure to seminal plasma.	28

INTRODUCTION

Artificial insemination (AI) as a means of genetic improvement in dairy cattle has been highly successful. Since the advent of artificial insemination, techniques involving collection, processing, evaluation, and insemination of semen have continually been refined to maximize conception rates. These advancements which have benefited the industry greatly are the result of costly and time consuming research involving fertility studies on large numbers of cattle. The major reason for such studies was the poor relationship of laboratory tests of semen quality and actual fertility. Recently, a laboratory test of semen quality, having a closer relationship to fertility than those previously used, has been reported (38). The test involves quantitative measurements of acrosomal alteration using differential interference contrast microscopy on unfixed semen smears. It, therefore seems appropriate to re-examine methods of processing semen for AI using this technique as a measure of cell integrity. One of the initial questions in preparation of semen for frozen storage involves the duration of sperm exposure to seminal plasma, pre-freeze. Based on current literature, the importance of this exposure, pre-freeze, to the post-thaw survival of spermatozoa is quite conflicting.

Conventionally, semen has been diluted immediately following collection to reduce duration of sperm contact with the plasma. Recent studies on boar (16, 17) and bull (31) semen have revealed that there may be some benefit from exposure of spermatozoa to seminal plasma prior to dilution for freezing.

Using acrosomal integrity as a criterion of cell injury, the present study was undertaken to determine if exposure of spermatozoa to seminal plasma was beneficial to post-thaw survival and to determine the optimum exposure time to seminal plasma.

REVIEW OF RELATED LITERATURE

Role of Seminal Plasma in Bovine Semen. The composition of seminal plasma has received much attention. While the role of many constituents have been established, others are quite obscure. Presently, there are conflicting reports relative to the influence of seminal plasma on spermatozoa.

The effect of seminal plasma on livability and freezability of sperm has been observed in several studies (4, 5, 13, 22, 29, 30, 41, 42). Shannon (41, 42) detected a toxic protein fraction in bovine semen which depresses the livability of extended semen during storage. This was observed when seminal plasma was added to diluted semen containing egg yolk and glycine, and stored at 5°C. The nature of this toxic factor was believed to result from its sulfhydryl binding capacity. In contrast, King and Macpherson (22), upon examining the freezability and viability of bovine semen in the absence of seminal vesicular secretions prior to dilution, found that post-thaw survivability was decreased. This was apparent when vital staining of spermatozoa following freeze-thawing revealed a greater loss of cells from seminal vesiculectomized males than from intact males. These workers postulated that removal of the seminal vesicles may have eliminated or reduced some factor(s) which protects spermatozoa during freezing and or thawing. A recent study by Faulkner et al (13) revealed that seminal vesiculectomy in the bovine does not improve or impair the fertilizing capacity of spermatozoa, suggesting that seminal plasma is of no value to the fertility of spermatozoa (the number of first services represented in these fertility studies were very low). On the other

hand, evaluation of semen quality in this study revealed that seminal vesiculectomy had depressing effects. A decrease in volume, motility, and percentage of live cells was noted upon removal of the seminal vesicles. Evaluation of abnormal cell content of the ejaculates in the study revealed that secondary morphological abnormalities increased following seminal vesiculectomy.

Pursel et al (29) have shown that the viability of boar semen can be enhanced by seminal plasma exposure. It was found that boar spermatozoa develop a cold shock resistance when incubated in seminal plasma for 2.5-4.5 hr following collection. Quinn et al (30) demonstrated a similar effect on ram spermatozoa held in seminal plasma for 30 min following collection. Studies involving epididymal spermatozoa in the bovine (4, 5) revealed that epididymal spermatozoa were more resistant to cold shock than ejaculated sperm. This resistance was exhibited by epididymal sperm in the presence as well as the absence of seminal plasma. In addition, the study revealed that bovine seminal plasma reduces the impedance change frequency (ICF) of epididymal spermatozoa. This was apparent when epididymal sperm diluted with seminal plasma resulted in a lower ICF than epididymal spermatozoa remaining undiluted or extended with a standard diluent.

Early studies, interchanging seminal plasma among consecutive ejaculates within bulls (46), among ejaculates of different bulls (19), and among different species (11) demonstrated that the superiority in freezability of one ejaculate over another was basically attributed to factors associated with the sperm, and not with the seminal plasma. Recently, however, studies (16, 17, 31) have shown that within

ejaculates spermatozoa can obtain an additional benefit in freezability and fertility from seminal plasma exposure prior to dilution. Graham et al (16, 17) have demonstrated this benefit in the boar. In this study, boar semen was held in seminal plasma for a period of two hr prior to dilution for freezing. This resulted in an increase in post-thaw survival of sperm, and was necessary for fertility of frozen boar semen. Prior to these experiments, freezing of boar semen had encountered little success. Another factor attributing to the success of freezing boar semen was the elimination of glycerol from the diluent.

Rajamannan et al (31) observed a benefit from exposing bovine spermatozoa to seminal plasma prior to dilution for freezing. They held the semen at 26°C for 0, 10, 20, 30, 40, 50, and 60 min. After each holding time, the semen was diluted and cooling to 5°C was initiated immediately (samples reached 5°C in 60 min). Laboratory evaluation, post-thaw, revealed no significant difference in motility among the different exposure times. However, an increase in what was termed abnormal cells appeared when holding times exceeded 30 min. A preliminary fertility trial was conducted with two bulls using holding times of 0 and 20 min. Twenty minutes exposure revealed a 5% and 10% increase in fertility of the two bulls, based on 60-90 day non-return to first service.

Although it has been demonstrated that seminal plasma imposes an additional benefit on the fertilizing capacity of frozen spermatozoa, the effect of seminal plasma on the uterine wall and mammalian egg in the female has been shown to be detrimental. Mather and Dale (27), working with endometrial respiration, found that seminal plasma decreases

endometrial respiration of the uterus. The factor(s) in seminal plasma responsible for this depression is unknown. The effect of seminal plasma exposure to bovine, goat, and rabbit eggs in vitro, caused the vitelline membrane of these eggs to be lysed within three minutes of initial contact (26). However, the mucin coat of the rabbit eggs and the zona pellucida of all eggs were not affected by the exposure.

Metabolic studies (14, 24) have shown sperm metabolism to be affected by seminal plasma. Lodge and Salisbury (24), studying the effect of metabolically produced CO_2 and added fructose on the aerobic metabolism of bovine spermatozoa, demonstrated that metabolically evolved CO_2 at levels produced by spermatozoa in diluted seminal plasma increased aerobic glycolysis and respiration. Results showed that metabolic CO_2 significantly stimulated the O_2 consumed by sperm cells in the presence of seminal plasma, but not in its absence. This suggested the presence of an interaction between CO_2 and some constituents of seminal plasma. However, Flipse (14) has observed a reduced uptake of glucose-C^{14} by spermatozoa in the presence of seminal plasma. The cause of this effect is unknown.

The source of the beneficial effect coming from seminal plasma is unknown. However, based on the previously described vesiculectomy studies of King and Macpherson (22), and Faulkner et al (13), it is possible that this beneficial effect could very likely originate from the seminal vesicles. On the other hand, studies involving the detrimental effects of seminal plasma to spermatozoa (4, 5, 41, 42) did not suggest the origin of this adverse effect.

Although the nature of the beneficial effect is unknown, an effect

related to a change in sperm and or seminal plasma composition could be important. The composition of bovine semen has been reviewed (25). Many components of seminal plasma have been shown to fluctuate widely, not only among consecutive ejaculates within bulls but among bulls as well (7, 8, 9, 12, 23, 33, 39, 40). Cragle et al (8, 9) demonstrated that sodium and potassium concentrations in seminal plasma were characteristic of this fluctuation, in that they varied widely from one ejaculate to another. Other constituents of seminal plasma such as fructose (7, 12, 23, 39) and glycerlphosphorylcholine (33, 39) have been influenced markedly in concentration by sexual preparation and frequency of semen collection. The effect of natural variation in concentration of seminal plasma components on spermatozoa has been dealt with on a speculative basis only.

Johnson et al (21) observed that phospholipid content of spermatozoa varies in different parts of the male tract. This preliminary study revealed that sperm phospholipid content had a tendency to decrease from the caput to the cauda epididymis and then increase after ejaculation. It appeared that boar sperm acquired additional phospholipid upon seminal plasma contact.

Several studies have revealed that spermatozoa absorb materials from the seminal plasma (3, 6, 15, 20, 28). Garner et al (15) have proposed that certain protein fractions present in the acrosome of the bovine sperm were originally components of the seminal plasma. Using discontinuous electrophoretic techniques, three protein fractions were found in acrosomal materials which apparently resulted from the contact of spermatozoa with seminal plasma. It was implied that these seminal

plasma proteins were presumably bound to or absorbed by the spermatozoa since density gradient centrifugation and washing did not eliminate them. It was suggested that these proteins could be bound to the spermatozoa at the time of or shortly after ejaculation.

Studies on sperm-coating antigens have been conducted (3, 20, 28). Hunter et al (20), working with sperm-coating antigens, described seven different antigens in ejaculated bovine spermatozoa. Five of these were found to be shared with seminal plasma. Matousek (28), working with specific antigens and common or cross-reacting antigens of spermatozoa and seminal plasma, found results similar to those of Hunter et al (20) and also observed that most of the sperm antigens were derived from the fluids of the accessory genital glands and the testes. Barker et al (3) found that coating antigens were more tightly bound to ejaculated spermatozoa which had come in contact with seminal plasma than to cauda epididymal spermatozoa brought in contact with seminal plasma. This suggests the presence of maturational changes in extra gonadal sperm that ultimately affect their antigen-binding capacity.

In the human, Boettcher (6) observed that ABO antigens of the blood were found only on spermatozoa exposed to seminal plasma. It was concluded that these antigens were absorbed from the seminal plasma. The actual influence of seminal plasma antigens or proteins that are bound to sperm is still uncertain; however it is conceivable that they may offer protection to the cell.

Acrosomal Maintenance as a Laboratory Test for Spermatozoan Injury. Since the advent of artificial insemination, the primary method for evaluating bovine semen viability or injury has been motility estimates.

Recently, Saacke and Marshall (35) have characterized the acrosomal cap alterations which accompany aging of bovine spermatozoa. This was accomplished using differential interference contrast microscopy on unfixed semen smears. The rate of acrosomal cap alteration was shown to be dependent upon bulls, ejaculates, and semen handling procedures (35, 36, 37). The sequential alterations of the acrosomal cap due to cell aging and or injury were as follows: (a) intact acrosomal cap characterized by a distinct apical ridge, (b) loss of apical ridge, (c) swelling of anterior acrosomal cap with formation of the equatorial segment, (d) continued swelling of anterior acrosomal cap, (e) deterioration of anterior acrosomal cap, leaving only the equatorial segment or posterior portion of acrosomal cap. The disappearance of the apical ridge, early in the alteration process, offered the most abrupt and precise change permitting quantification of the rate of acrosomal alteration. Other changes were very gradual in relation to time. Quantitative measurements based on possession of an apical ridge by spermatozoa has made it possible to more accurately evaluate cell injury of bovine sperm. Using this technique, replicated samples yielded extremely low variability (coefficient of variation (CV) = 6%) as compared to motility estimates (CV = 25%).

Saacke and White (38) have shown the significant, positive relationship of acrosomal maintenance with fertility. This study involved 156 ejaculates from sixteen Holstein bulls. Each ejaculate averaged 271 first services, and fertility was evaluated on the basis of 90 day non-return to first service. Laboratory evaluation included direct counts of intact acrosomes and estimates of percent motility. Results

from the field and laboratory study revealed that acrosomal maintenance was more highly correlated with fertility (r = .6) than motility (r = .46).

Differential interference contrast microscopy has made it possible to quantitatively measure rate of acrosomal alteration in live semen smears of the bovine, which in turn has helped to better understand differences observed in acrosomal characteristics of stained spermatozoan (18, 45). The use of differential interference contrast microscopy in evaluating the morphology of spermatozoa in other species such as human, monkey, and rabbit has been demonstrated by El-Minawi et al (10). Bajer and Allen (1, 2) with the aid of the differential interference contrast microscope have shown new details of mitotic spindle structures and cell plate formation in living tissue. Differential interference contrast microscopy has complemented other optical methods and has added another link in the chain of microscopic techniques (43). Recently, this system has become accepted as another important tool in biological research.

EXPERIMENTAL PROCEDURE

Two experiments were designed to determine the effect of exposing bovine spermatozoa to seminal plasma prior to dilution and freezing. Post-thaw acrosomal maintenance and percent motile sperm were the parameters used to evaluate this effect.

Semen used in the study was collected from Holstein bulls housed at Select Sires, Inc., Columbus, Ohio. The fertility of all bulls were within one standard deviation point of stud average. Semen was collected from each bull 4 x weekly. Ejaculates had at least the following characteristics: volume of 4 ml, sperm concentration of 1×10^9 cells/ml, and an initial motility of 60%.

Initial estimations of sperm motility were made with a Leitz phase contrast microscope equipped with a stage incubator adjusted to 37°C. Semen was diluted with 2.9% sodium citrate dihydrate and examined as a wet smear. Spermatozoan concentrations were determined by diluting raw semen 1:80 with 2.9% sodium citrate dihydrate and measuring light transmittancy using a Bausch and Lomb "Spectronic 20" photoelectric colorimeter.

Egg yolk-citrate-glycerol was used as the semen diluent. The buffer portion contained 2.9 g of sodium citrate dihydrate made up to 100 ml with double distilled water. Buffer and fresh egg yolk were combined to give an 80% buffer, 20% egg yolk mixture by volume. This basic diluter was then divided into two fractions (A and B). The glycerol portion of the diluter (Fraction "B") contained 14% glycerol by volume in egg yolk-citrate. The "A" fraction consisted of the basic egg yolk-citrate diluter. Antibiotics and α amylase were added to each

fraction at a rate of 500 µg of dihydro streptomycin sulfate, 500 units of potassium pencillin G, and .01 µg of α amylase per ml of diluent. Following the treatments outlined in Experiments I and II (p 13), semen was diluted in 25 ml aliquots of Fraction "A". Each aliquot of diluted semen was placed into a 225-ml water bath at 32°C which was in turn placed in a 5°C cold room. In this manner, the semen was cooled to 15°C in approximately one hr. The samples were removed from the water baths and permitted to air-cool to 5°C (15 min). The diluted semen was glycerolated at 5°C by adding an equal volume of Fraction "B" to Fraction "A". Fraction "B" was pipetted into the diluted semen in increments of 10%, 20%, 30%, and 40% by volume at ten-min intervals. The samples of diluted semen were then loaded into .5-ml French straws. Semen was equilibrated for 4 hr and frozen in N_2 vapor at -160°C for 9 min. Storage of the frozen semen was in liquid N_2.

Evaluation of semen was made within 15-35 days of storage. Cell injury was based on estimates of percent motility and direct counts of intact acrosomes. Estimates of percent motility were accomplished using a Leitz phase contrast microscope equipped with an incubator stage. Direct counts of intact acrosomes were achieved using a Zeiss differential interference contrast microscope. The latter method of evaluation which measures the presence of an apical ridge on the acrosomal cap of spermatozoa was carried out in accordance with the procedure outlined by Saacke and White (38). For evaluation, semen was thawed in a 5°C water bath for 4 min, pooled to give 6-ml volumes which were coded and incubated at 37°C in a water bath. Percent motility and percent intact acrosomes were recorded at 0, 2, 4, 8, and

10 hr of incubation.

Experiment I

Using a split-ejaculate technique, 14 ejaculates from 10 bulls were collected and held at 32°C for 0, 20, 40, 60, 120, and 240 min. After each holding time, a portion of each ejaculate was diluted in 25 ml of egg yolk citrate (Fraction "A") to a concentration of 56×10^6 cells/ml.*

The diluted semen was maintained at 32°C until the final holding time of 240 min, thus temperature remained constant for 240 min and only the duration of time that sperm spent in plasma vs diluter, differed. Once the final holding time was reached, the aliquots of diluted semen were cooled to 5°C.

Experiment II

Again using a split-ejaculate technique, 18 ejaculates from 10 bulls were collected, exposed to their own seminal plasma for 0, 20, 40, 60, 120, and 240 min followed by dilution in egg yolk-citrate (Fraction "A") to a concentration of 56×10^6 cells/ml. In this experiment however, each sample of diluted semen was cooled to 5°C beginning immediately following dilution of that sample; ie, temperature among treatments was not held constant throughout the pre-glycerolation period. This experiment was undertaken to determine if holding sperm in seminal plasma would still exert a beneficial effect on

*Due to a reduced concentration of cells obtained from two bulls, their ejaculates were diluted at a concentration of 50×10^6 cells/ml. Past studies have revealed no difference in rate of acrosomal maintenance among samples of diluted semen varying in concentration from 5×10^6 to 60×10^6 cells/ml (34).

motility and rate of acrosomal alteration post-thaw, if energy was conserved by lowering the temperature immediately following dilution.

Statistical Analysis

These experiments were designed as a 3 x 3 x 2 factorial arrangement of treatments in a mixed model, completely randomized design. All analyses were performed using analysis of variance and Duncan's multiple range test as described by Sokal and Rohlf (44).

RESULTS

Experiment I. This experiment was designed to study the effect of exposing bovine spermatozoa to their own seminal plasma for various periods of time prior to dilution and freezing. Post-thaw acrosomal maintenance and percent motile sperm were the parameters used to evaluate this effect.

The analyses of variance for this experiment (Table 1) revealed that exposure to seminal plasma did not have a significant effect on percent motility; however, maintenance of the acrosome was significantly ($P < 0.01$) affected by holding time in seminal plasma. As expected, ejaculate variation and hr of incubation varied significantly for both motility and acrosomal maintenance. First order interactions which were highly significant ($P < 0.01$) included holding time x ejaculates and ejaculates x hr of incubation for acrosomal maintenance. Only ejaculates x hr of incubation was significant for spermatozoan motility. Of importance are the highly significant effect ($P < 0.01$) of holding time and holding time x ejaculates interaction in relation to acrosomal maintenance. The origin of these effects may be noted in Tables 2 and 3, respectively.

The influence of seminal plasma on spermatozoa prior to dilution revealed no significant difference (Duncan's multiple range test) in holding times from 0 to 120 min on acrosomal cap maintenance. Exposure for 240 min resulted in significantly lower ($P < 0.05$) acrosomal maintenance post-thaw, as compared to exposure times of 20 to 120 min. Raw semen diluted immediately following collection was superior to 240 min holding time at 4, 8, and the mean of the 10 hr incubation.

Table 1. Analyses of variance of percent intact acrosomal caps and percent motile spermatozoa in Experiment I.

Source of variation	Degrees of freedom	Mean squares of intact acrosomal caps	Mean squares of motile spermatozoa
Holding time (A)	5	177.7260**	34.6667
Ejaculates (B)	13	2574.0331**	189.0842**
Hour (C)	4	23691.7032**	12355.5952**
A x B	65	26.1019**	12.5128
A x C	20	7.3908	13.5952
B x C	52	62.5187**	93.1593**
Residual	260	7.8391	9.7747

**Significant at $P < 0.01$

Table 2. Effect of holding time in seminal plasma on the percent intact acrosomal caps of spermatozoa incubated at 37°C post-thaw (Experiment I). (Mean percent of 14 ejaculates)

	Holding time (min)						
Hour of incubation	0	20	40	60	120	240	Sig.
0	69.74^{ac}	69.89^{ac}	71.21^{bc}	69.79^{ac}	72.43^{b}	68.29^{a}	*
2	45.51^{ab}	45.68^{a}	48.26^{a}	47.66^{a}	46.89^{a}	42.81^{b}	*
4	37.32^{a}	38.14^{a}	38.84^{a}	38.86^{a}	38.11^{a}	33.25^{b}	*
8	31.14^{a}	32.21^{a}	32.71^{a}	32.86^{a}	31.94^{a}	28.54^{b}	*
10	27.82^{ab}	28.68^{a}	28.82^{a}	29.79^{a}	28.96^{a}	25.82^{b}	*
$\bar{x}$	42.30^{a}	42.92^{a}	43.97^{a}	43.79^{a}	43.67^{a}	39.74^{b}	*

*Significant at $P < 0.05$

a, b, c, Means not followed in the horizontal line by the same letter are significantly different.

Table 3. Effect of holding time in seminal plasma on the percent intact acrosomal caps of frozen spermatozoa from fourteen ejaculates (Experiment I).
(Mean percent of the 5 incubation periods, 0, 2, 4, 8, and 10 hrs)

	Holding time (min)					
Ejaculate	0	20	40	60	120	240
1	41.86	44.20	46.50**	45.70	44.50	36.40
2	53.20	58.80**	55.60	56.92	54.80	54.66
3	42.60	41.70	43.00**	37.00	39.20	37.50
4	53.30	54.20	53.50	52.40	55.22**	49.80
5	43.70	43.00	48.10**	44.80	44.80	34.10
6	24.50	23.30	21.90	23.70	27.60**	22.00
7	44.40	40.20	43.80	43.60	44.40**	35.60
8	47.90	47.40	48.10	54.30**	53.30	52.10
9	43.50	45.80**	44.12	44.20	44.00	40.30
10	42.28	43.80**	40.90	41.60	39.10	39.10
11	46.72	48.40**	48.30	47.02	45.20	43.30
12	39.10	38.00	44.00**	43.42	41.30	37.20
13	23.10	23.20	26.10**	25.90	25.00	23.50
14	46.10	48.90	51.66	52.50	52.90**	50.80
$\bar{x}$	42.30	42.92	43.97**	43.79	43.67	39.74

**Highest mean percent of intact acrosomal caps of spermatozoa for the ejaculate.

Most important was the highly significant ($P < 0.01$) interaction of holding time x ejaculates (Table 1). Both the optimum holding time and the degree of benefit varied among ejaculates (Table 3). Optimum exposure time for acrosomal maintenance ranged from 20 to 120 min with four ejaculates responding favorably at 20 min, five ejaculates responding favorably at 40 min, one ejaculate responding favorably at 60 min, and four ejaculates responding favorably at 120 min. The degree of benefit among the ejaculates in relation to maintenance of the acrosomal cap varied from slight to a substantial increase in acrosomal retention over the incubation period (Table 3). Over the entire population of ejaculates studied, holding times of 0 to 120 min were equal in maintenance of the acrosomal cap. However, 20 to 120-min exposure time showed an insignificant tendency to increase acrosomal maintenance post-thaw, over no exposure time.

There was no significant difference in motility of spermatozoa over the exposure times studied (Table 4). However, a trend toward an increased motility was observed when spermatozoa were exposed to seminal plasma for 20 to 120 min.

Experiment II. This experiment was designed to study the effect of holding time in seminal plasma prior to dilution followed by immediate cooling to 5°C. Post-thaw acrosomal maintenance and percent motile sperm were the parameters used to evaluate this effect.

The analyses of variance for this experiment (Table 5) revealed highly significant ($P < 0.01$) differences in percent motile spermatozoa and percent intact acrosomes due to holding time, ejaculates, and hr of incubation. Also, all first order interactions were highly significant

Table 4. Effect of holding time in seminal plasma on the percent motility of spermatozoa incubated at 37°C post-thaw (Experiment I).
(Mean percent of 14 ejaculates)

Hour of incubation	Holding time (min)					
	0	20	40	60	120	240
0	25.00	28.57	28.57	29.29	29.29	27.14
2	2.14	7.14	4.28	5.71	3.57	2.86
4	0	0.71	0.71	0.71	0	0
$\bar{x}$	5.43	7.29	6.71	7.14	6.57	6.00

*Values of motility at 8 and 10 hours were zero and were not included in the Table.

Table 5. Analyses of variance of percent intact acrosomal caps and percent motile spermatozoa in Experiment II.

Source of variation	Degrees of freedom	Mean squares of intact acrosomal caps	Mean squares of motile spermatozoa
Holding time (A)	5	1224.5290**	272.0000**
Ejaculates (B)	17	2732.9386**	278.0392**
Hour (C)	4	34360.7369**	26578.2407**
A x B	85	37.9293**	21.0980**
A x C	20	27.6602**	86.9074**
B x C	68	79.5490**	96.4760**
Residual	340	9.5370	13.8486

**Significant at $P < 0.01$

($P < 0.01$). Of importance are the highly significant ($P < 0.01$) factors holding time and interaction of holding time x ejaculates in relation to acrosomal maintenance (Tables 6 and 7, respectively).

Considering values obtained at all hr of incubation, exposure of spermatozoa to seminal plasma for 20 min prior to dilution resulted in significantly greater ($P < 0.05$) acrosomal retention than holding times of 60, 120, and 240 min (Table 6). However, 20-min exposure was significantly greater ($P < 0.05$) than semen diluted immediately following collection at 0 and 4 hr of incubation as well as the mean of the five incubation periods. Twenty-minutes exposure was also significantly greater ($P < 0.05$) than a holding time of 40 min at 2 hr of incubation and the mean of the five incubation periods. The 20-min exposure resulted in a higher percent of intact acrosomal caps of spermatozoa at all hr of incubation as compared to the other holding times. Holding times of 0 and 40 min were significantly greater ($P < 0.05$) than 60, 120, and 240 min exposure only at specific hr of incubation.

The highly significant ($P < 0.01$) interaction of holding time x ejaculates in relation to maintenance of the acrosomal cap was again the most critical factor (Table 7). Unlike Experiment I, optimum holding time in this experiment was 20 min for fifteen ejaculates, 40 min for two ejaculates, and one ejaculate was not affected by any exposure time. The degree of benefit achieved by a holding time of 20 min varied considerably among ejaculates, thus accounting for a large portion of the variation due to the holding time x ejaculates interaction. Ejaculates varied from no response to 10% improvement in acrosomal retention from a holding time of 20 min prior to dilution

Table 6. Effect of holding time in seminal plasma on the percent intact acrosomal caps of spermatozoa incubated at 37°C post-thaw (Experiment II).
(Mean percent of 18 ejaculates)

	Holding time (min)						
Hour of incubation	0	20	40	60	120	240	Sig.
0	74.14^{ac}	76.50^{b}	75.58^{ab}	73.81^{ac}	72.69^{c}	69.94^{d}	*
2	53.36^{ab}	56.31^{a}	52.25^{bc}	49.36^{cd}	47.31^{d}	41.83^{e}	*
4	42.39^{ac}	45.19^{b}	42.98^{ab}	40.00^{cd}	38.56^{d}	32.85^{e}	*
8	34.92^{ab}	35.72^{a}	33.67^{abc}	32.17^{c}	32.47^{bc}	27.14^{d}	*
10	31.33^{ab}	33.75^{a}	31.50^{ab}	29.47^{b}	26.86^{c}	23.08^{d}	*
$\bar{x}$	47.23^{a}	49.49^{b}	47.20^{a}	44.96^{c}	43.58^{c}	38.97^{d}	*

*Significant at $P < 0.05$

a, b, c, d, e, Means not followed in the horizontal line by the same letter are significantly different.

Table 7. Effect of holding time in seminal plasma on the percent intact acrosomal caps of frozen spermatozoa from eighteen ejaculates (Experiment II).
(Mean percent of the 5 incubation periods, 0, 2, 4, 8, and 10 hrs)

	Holding time (min)					
Ejaculate	0	20	40	60	120	240
1	56.00	57.20**	51.00	52.00	49.80	44.76
2	60.40	65.30**	59.80	59.50	60.10	59.70
3	49.90	49.30	50.90**	39.80	40.00	38.10
4	60.90	63.20**	62.60	61.50	61.90	56.10
5	45.10	47.10**	46.40	38.00	33.50	33.80
6	33.00**	32.40	29.30	30.70	32.00	26.60
7	44.30	49.70**	48.80	44.50	37.90	33.50
8	55.90	57.70**	57.50	50.40	50.30	44.10
9	44.40	46.70**	45.00	46.20	45.10	37.40
10	42.20	44.70**	44.42	41.20	41.10	38.30
11	47.40	48.00**	47.90	46.70	47.60	45.70
12	45.70	48.20**	45.10	42.70	37.90	30.80
13	26.60	27.90**	25.90	24.10	22.40	21.80
14	59.90	59.60	61.50**	54.40	56.00	48.30
15	41.00	41.20**	38.80	40.50	40.20	33.00
16	55.30	65.50**	56.10	51.10	46.40	42.40
17	38.30	43.00**	38.70	42.80	41.50	33.50
18	43.80	44.20**	39.80	43.20	40.70	33.60
$\bar{x}$	47.23	49.49**	47.20	44.96	43.58	38.97

**Highest mean percent of intact acrosomal caps of spermatozoa for the ejaculate.

(Figure 1). The mean of the eighteen ejaculates revealed a 2.1% increase in rate of acrosomal retention from 20 min compared to 0-min exposure of spermatozoa to seminal plasma prior to dilution (Figure 1).

In this experiment, percent motility did not reflect any benefit from holding sperm in seminal plasma (Table 8). Exposure times of 0 and 20 min resulted in the highest percent motility as compared to the other holding times, but the values obtained were not significant. The 240-min exposure to seminal plasma resulted in a significant depression of post-thaw sperm motility. Differences in percent motility among ejaculates revealed a highly significant ($P < 0.01$) interaction of holding time x ejaculates, indicating that time and degree of benefit was again dependent upon the ejaculate.

Comparison of the two experiments in this thesis (Figure 2) revealed the influence of the conservation effect of cooling sperm post-dilution, on the benefit derived from exposure to seminal plasma. The effect of cooling is often expressed as conserving energy. In Experiment I, where temperature among treatments remained uniform during the pre-freeze period, exposures of spermatozoa to seminal plasma up to 120 min prior to dilution disclosed no harmful effect on rate of acrosomal maintenance post-thaw. In Experiment II, where diluted semen was cooled immediately following dilution, 20-min exposure appeared most optimum and overall maintenance of the acrosome was maximized. Experiment II indicated that despite the importance of cooling in preservation of cells, a benefit from holding sperm in seminal plasma is in effect up to 20 min.

Figure 1

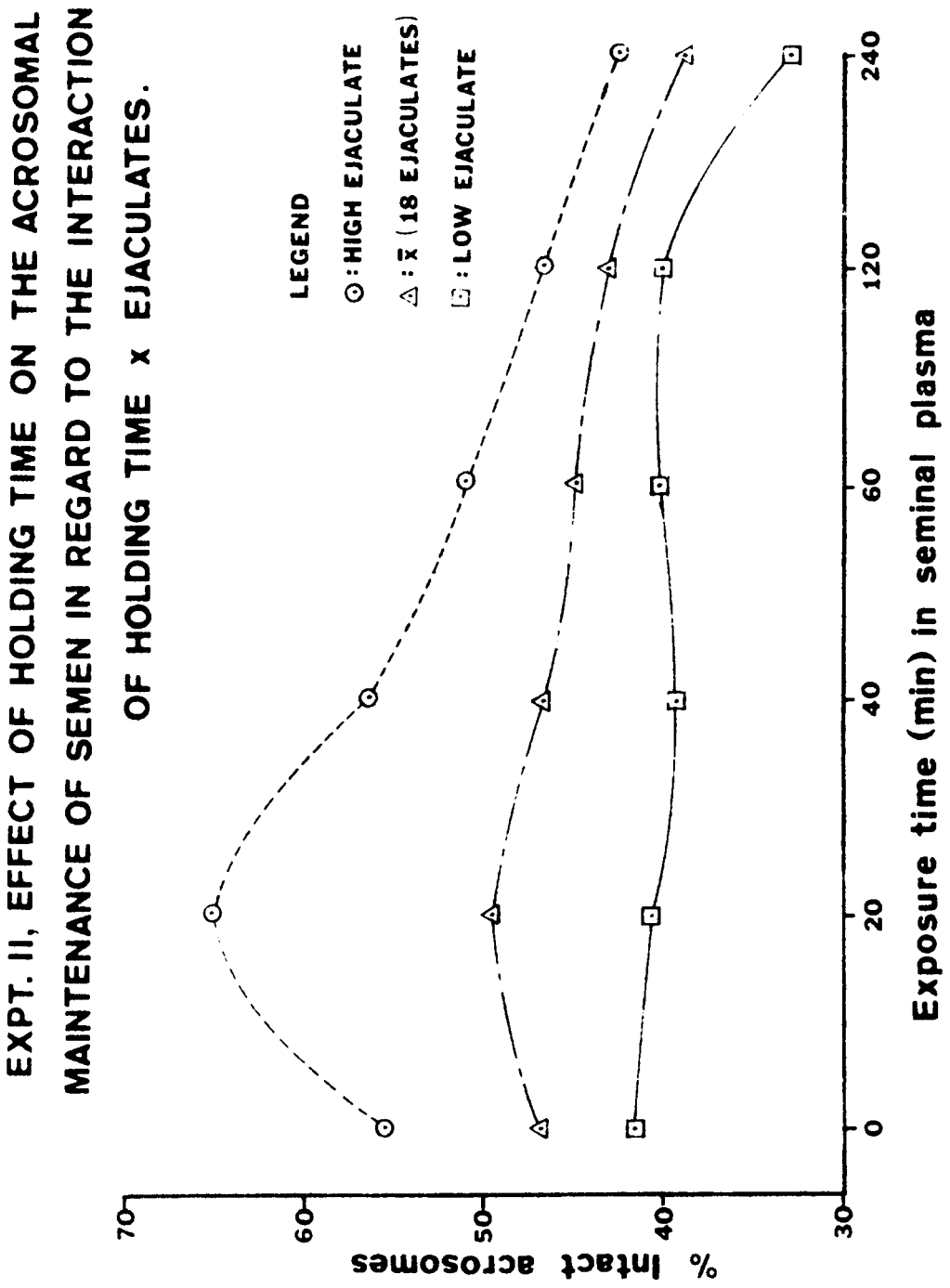

EXPT. II, EFFECT OF HOLDING TIME ON THE ACROSOMAL MAINTENANCE OF SEMEN IN REGARD TO THE INTERACTION OF HOLDING TIME x EJACULATES.
LEGEND
⊙: HIGH EJACULATE
△: x̄ (18 EJACULATES)
⊡: LOW EJACULATE
% Intact acrosomes
70
60
50
40
30
0
20
40
60
120
240
Exposure time (min) in seminal plasma

Table 8. Effect of holding time in seminal plasma on the percent motility of spermatozoa incubated at 37°C post-thaw (Experiment II).
(Mean percent of 18 ejaculates)

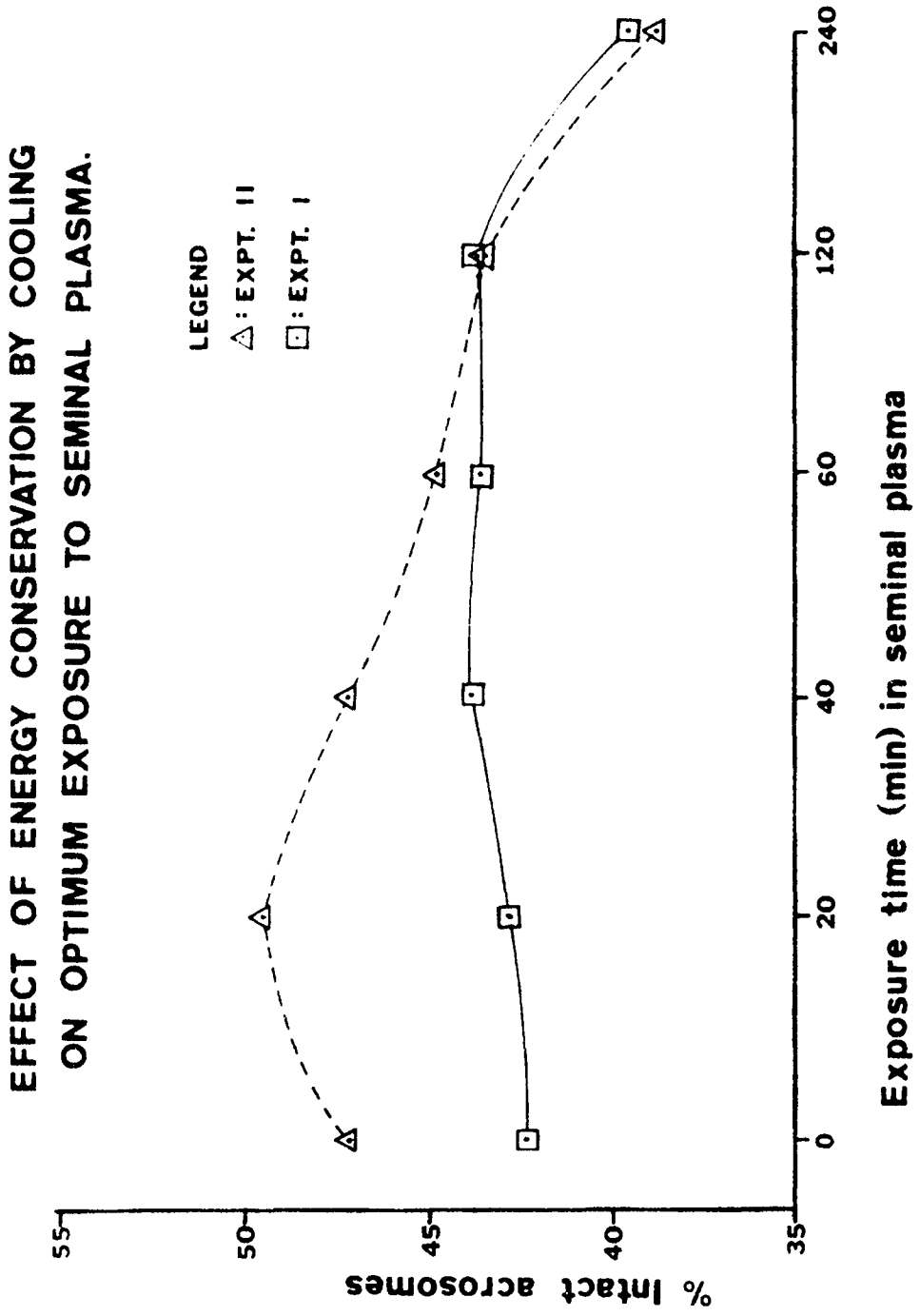
Hour of incubation	Holding time (min)						
	0	20	40	60	120	240	Sig.
0	40.00^{a}	38.89^{a}	36.67^{a}	37.78^{a}	36.67^{a}	30.56^{b}	*
2	15.00^{a}	15.56^{a}	14.44^{ab}	11.11^{bc}	8.89^{c}	3.33^{d}	*
4	2.22^{ab}	3.33^{a}	2.77^{a}	0.55^{b}	1.67^{ab}	0.56^{b}	*
$\bar{x}$	11.44^{a}	11.56^{a}	10.78^{a}	9.89^{a}	9.44^{a}	6.89^{b}	*

*Significant at $P < 0.05$

a, b, c, d, Means not followed in the horizontal line by the same letter are significantly different.

**Values of motility at 8 and 10 hours were zero and were not included in the Table.

Figure 2


EFFECT OF ENERGY CONSERVATION BY COOLING
ON OPTIMUM EXPOSURE TO SEMINAL PLASMA.
LEGEND
△: EXPT. II
□: EXPT. I
% Intact acrosomes
55
50
45
40
35
0
20
40
60
120
240
Exposure time (min) in seminal plasma

DISCUSSION

From this study, it is evident that exposure of bovine spermatozoa to seminal plasma for a period of time prior to dilution for freezing is not injurious to the survival of cells, post-thaw, and in most cases is beneficial.

When the thermal history of the semen was the same among treatments (Experiment I), exposure of spermatozoa to seminal plasma for 0 to 120 min prior to dilution for freezing revealed no significant difference in acrosomal maintenance, post-thaw (Table 2). However, exposure times of 20 to 120 min resulted in a slight, but insignificant increase in percent intact acrosomes as compared to semen diluted immediately following collection. From this table, it is apparent that over a population of ejaculates, exposure of spermatozoa to seminal plasma up to 120 min, pre-freeze, is not detrimental to acrosomal maintenance, post-thaw.

In this experiment, the highly significant ($P < 0.01$) interaction of holding time x ejaculates was of primary importance (Table 3). Individual ejaculates showed a marked variation in both the optimum holding time and the degree of benefit from that holding time with regard to acrosomal maintenance, post-thaw. Optimum exposure time ranged from 20 to 120 min. On this basis, it can be concluded that seminal plasma does benefit the survival of spermatozoa, post-thaw, with the optimum holding time dependent upon the ejaculate.

Motility estimates of spermatozoa in Experiment I revealed no significant difference among holding times (Table 4). However, exposure times of 20 to 120 min showed an insignificant trend to be slightly

superior to no holding time.

In Experiment II where thermal history was not the same among the various holding-time treatments (semen was cooled immediately following dilution), holding bovine semen for 20 min prior to dilution resulted in the highest percent of intact acrosomes, post-thaw (Table 6). Considering all hours of incubation, an exposure time of 20 min prior to dilution was distinctly superior ($P < 0.05$) in acrosomal maintenance to holding times of 60, 120, and 240 min. Twenty-minutes exposure was statistically more favorable in acrosomal maintenance to exposure times of 0 and 40 min at specific hours of post-thaw incubation.

As in Experiment I, the most practical factor which emerged from Experiment II was the highly significant ($P < 0.01$) interaction of holding time x ejaculates, for acrosomal maintenance (Table 7). In this experiment, 20-min exposure was most optimum for fifteen of the eighteen ejaculates, while two of the ejaculates responded best at 40 min and one ejaculate was not affected by any exposure time. The variation in degree of benefit was the primary cause for the interaction (Figure 1). Ejaculates varied from no effect to a substantial increase in percent of intact acrosomes from seminal plasma exposure. From a practical standpoint, it should be noted that although the degree of response to seminal plasma exposure time varied among ejaculates, 20-min exposure was not deleterious to any ejaculate. On this basis, a 20-min holding time at 32°C could be instituted as a practice across all bulls. Saacke and White (38) have shown that major differences obtained with acrosomal maintenance were due to variations from bull to bull rather than from ejaculate to ejaculate within a given

bull. Therefore, it is conceivable that a portion of the interaction, holding time x ejaculates, is due to significant bull to bull differences.

Results from motility estimates in this experiment revealed no significant differences among holding times of 0 to 120 min. However, there was a trend for a slight decline in motility following 20-min exposure. As shown in previous studies (36, 37), estimates of percent motility lack the sensitivity of acrosomal maintenance when evaluating cell injury.

Comparison of these two methods for evaluation of bovine semen in this study again indicated that acrosomal maintenance is a more sensitive test of cell injury, yielding a 5.4% coefficient of variation among samples while estimates of motility yielded a 33% coefficient of variation. The coefficient of variation for acrosomal maintenance in this study was in accordance with that of Saacke et al (38). However, the coefficient of variation for percent motility in this thesis was slightly higher as compared to the findings of Saacke et al (38).

The results of both Experiment I and Experiment II indicate that some type of protection is being imparted to the sperm by the seminal plasma at the time of or shortly after ejaculation. The nature of this protection is unknown. However, King et al (22) reported that vital staining of spermatozoa following freeze-thawing revealed a greater loss of cells from seminal vesiculectomized males than from intact males. These workers concluded that removal of the seminal vesicles may have eliminated or reduced some factor which protects spermatozoa during freezing and or thawing. From the results of their

study, it is therefore strongly suggested that the protective agent(s) originates in the seminal vesicles.

Early studies interchanging seminal plasma of consecutive ejaculates (46) of different bulls (19), and different species (11) have demonstrated that the superiority in freezability of one ejaculate over another is due to the factor(s) associated with the spermatozoa and not the seminal plasma. However, several studies (16, 17, 29, 30, 31) have shown that spermatozoa can obtain an additional benefit in viability and fertility from seminal plasma exposure. Pursel et al (29) have demonstrated this with boar spermatozoa. Sperm from this species becomes more resistant to cold shock from exposure to seminal plasma for 2.5-4.5 hr. Exposure of ram sperm to seminal plasma for .5 hr also increases resistance to cold shock (30). The results from these studies strongly indicate the presence of a protective factor in ram and boar seminal plasma. Graham et al (16, 17) further support the benefit of seminal plasma exposure to spermatozoa. They have shown that holding boar sperm in seminal plasma for 2 hr prior to dilution, results in an increase in post-thaw survival and is necessary for fertility in freeze-thawed semen.

The results from Experiment II in this thesis support those obtained by Rajamannan et al (31). These workers conducted a preliminary field trial which showed that 20-min holding time resulted in a 5-10% increase in fertility of frozen bull semen when compared with no holding time (dilution immediately following collection). However, it must be noted that this trial was a limited fertility trial, using two low fertility bulls and less than 350 first services for each holding time.

Maintenance of the acrosome and motility post-thaw was considerably greater in Experiment II than in Experiment I. This was obviously due to the conservation of sperm energy, pre-freeze, in Experiment II accomplished by cooling the semen to 5°C immediately following dilution. Despite the increased rate of cell aging at 32°C as compared to 5°C, Experiment II still indicated that exposure of sperm to seminal plasma at 32°C for 20 min resulted in less injury upon freeze-thawing.

Alterations of semen quality in the relatively new straw were important to this study. Due to the geometry of the straw vs the conventional ampule, the influence of different thaw rates on cell injury was of critical concern. Another study in this laboratory (32) observed the effect of thaw rates on maintenance of the acrosomal cap of sperm frozen in the straw. Frozen semen used in the study was from the 0, 20, and 40 min holding-time treatments of Experiment II. Results revealed that thaw rates of 5°C/4 min, 20°C/1 min, and 35°C/1/2 min differed significantly in rate of acrosomal alteration, however, the beneficial effect of seminal plasma exposure to spermatozoa was not altered.

The need for further information is evident. Fertility studies involving exposure times from 0 to 40 min should be undertaken to support the findings of this study. Further studies are needed to determine if the variation in response of ejaculates to seminal plasma exposure is due to the ejaculate itself or to the bull. The nature of the beneficial effect of seminal plasma is also of primary importance. Through characterization of this effect, the factor(s) involved may be intensified to provide even greater freezability of spermatozoa.

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SEMINAL PLASMA AND FREEZE-THAW INJURY OF BOVINE SPERM

by

Lawrence Edgar Gerber

(ABSTRACT)

Using a split-ejaculate technique, two experiments were conducted to determine the influence of sperm exposure to seminal plasma on freeze-thaw injury. In Experiment I, 14 ejaculates from 10 Holstein bulls were held at 32°C for either 0, 20, 40, 60, 120 or 240 min followed by dilution in egg yolk-citrate. All treatments were maintained at 32°C for 240 min post-collection, at which time semen was cooled to 5°C, glycerolated, and then frozen in .5-ml French Straws using N_2 vapor. Experiment II, using 18 ejaculates from 10 bulls, was conducted identical to Experiment I, except semen was cooled to 5°C immediately after each dilution. Semen was thawed at 5°C and incubated at 37°C. Direct counts of intact acrosomes and estimates of percent motility were recorded at 0, 2, 4, 8 and 10 hrs of incubation. In Experiment I, there was a highly significant interaction ($P < .01$) between holding time x ejaculates to seminal plasma with regard to acrosomal retention. Optimum exposure time ranged from 20 to 120 min and 240-min exposure was deleterious for all ejaculates ($P < .05$). Variation in motility was not significant among treatments. In Experiment II, holding time x ejaculates interaction was again the most significant factor ($P < .01$). However, 20-min exposure to seminal plasma resulted in optimum acrosomal retention post-thaw for 15 ejaculates, while 40-min exposure was optimum for 2 ejaculates and 1 ejaculate did not respond favorably to any exposure time. While degree of response to seminal plasma exposure

time varied among ejaculates, 20-min exposure was not deleterious to any ejaculate. Post-thaw motility was significantly ($P < .01$) reduced by 240-min exposure to seminal plasma.