FACTORS AFFECTING PRESERVATION OF LIQUID AND FROZEN RAM SPERMATOZOA

by

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Introduction

Large scale use of artificial insemination (AI) in sheep has been restricted to the U.S.S.R., other eastern and central European countries and some areas of South America. AI has not been a common practice in western Europe, United Kingdom, Australia or the U.S.A. (Emmens and Robinson, 1962). Low demand for AI of sheep in this country has limited studies on the preservation of ram spermatozoa. This low demand for AI reflects a general decline in sheep numbers and an increase in labor problems associated with handling and detection of heat in sheep. With improvements in estrus synchronization and the resulting simplification of heat detection, AI in sheep may become more practical to both pure-bred and commercial breeders. Consequently, there should soon be a demand for better techniques of ram semen preservation.

AI would permit the widespread use of fewer, but more superior, performance and progeny tested rams. With the use of fewer stud rams, there could be a greater control of reproductive diseases (e.g., epi-didymitis) through more intensive veterinary care and management. The reproductive potential would be increased through optimizing care and semen evaluation of stud rams.

Before AI can be used to its maximum advantage, better methods of semen preservation must be developed. The present study was under-taken in an attempt to improve the freezability of ram spermatozoa. The objectives of this study were: (1) to determine the effect of pH,

glycerol and Tris on acrosomal alteration and motile life of ram spermatozoa stored at 6 C, (2) to determine the effect of thaw rate and levels of glycerol and Tris on the motile life and retention of the acrosome of freeze-thawed ram spermatozoa and (3) to calculate an optimum combination of glycerol, Tris and thaw rate which will result in maximum recovery of frozen ram spermatozoa.

Review of Literature

The Effect of Extender pH on Spermatozoan Viability

As early as 1937, researchers were aware of the influence of pH on the viability of ram spermatozoa. McKenzie and Berliner (1937) noted that the semen of rams with high sperm concentrations exhibited a pH from 6.0 to 6.8. Also, a low sperm concentration and a high abnormal sperm content were associated with a pH shift toward alkalinity. Wiggins, Terrill and Emil (1953) found that the pH of ram semen averaged 6.59 with a range of 5.5 to 8.0. Earlier work by Blackshaw and Emmens (1951) showed that in room temperature storage, both ram and bull spermatozoa exhibited maximum motility when diluted in an extender with a pH range from 6.4 to 7.1; however, both species showed considerable tolerance to a wide pH range. Blackshaw (1960) observed that the post-thaw-motile life of frozen ram spermatozoa was maintained best at pH of 6.8. They further observed that the post-thaw motility of bull spermatozoa was preserved best at pH of 7.2.

A pH of 6.75 was more desirable than 6.50 or 7.0 for 5 C storage of bovine semen extended in a Tris-yolk-glycerol media (Davis *et al.*, 1963a); however, the differences were not significant. Foote (1970) found that pH 6.75 was superior to 7.00 or 6.50 in maintenance of motility of bull sperm in Tris extenders. Fertility of bull semen extended in Tris-buffered egg yolk at pH of 6.50 and 6.75 did not differ significantly; however, there was a trend toward increased fertility at the

lower pH. With Tris extended boar semen, Igboeli (1970) reported that pH of 6.50 was inferior to 6.75 or 7.00 ($P<.05$) in maintenance of motility after 3 days at 15 C.

An interaction between pH and Tris concentration was indicated by Steinback and Foote (1967) in their study of 5 C stored bull semen. At pH of 6.50, lower levels of Tris (.20 to .25 M) were optimal for maximum spermatozoan survival. But at pH of 7.00 a higher level of Tris (.25 to .35 M) resulted in maximum survival. Higher pH's (7.00 and 7.50) tended to be relatively more toxic at lower Tris concentrations (.10 and .15 M); however, at lower pH's (6.0 and 6.5), higher Tris concentrations (.35 and .40 M) were more toxic to sperm motility. It appears that bull, boar and ram spermatozoa survive storage best at a pH near 6.75.

The Effect of Tris Extenders on Spermatozoan Livability

The importance of a good buffering component in semen extenders has long been recognized. Commonly, sodium citrate has served in this capacity. More recently semen from different species has been extended in diluents buffered with tris(hydroxymethyl)aminomethane (Tris). Visser and Salamon (1973) found no significant difference in lambing rates when ram semen was pellet frozen in either Tris-glucose-yolk or raffinose-citrate-yolk extender. They reported lambing rates of 40% (28/70), 44.8% (30/67) and 65.7% (46/70), for Tris extended, raffinose extended, and unextended semen, respectively. Davis, Bratton and Foote (1963a) found Tris-yolk-glycerol extenders superior to both C.U.E. (Cornell University Extender) and citrate-yolk-glycerol for bull semen based on sperm motility during 8 days of 5 C storage. The mean motility values were 42% for Tris,

35% for C.U.E. and 18% for the citrate extender. With frozen bull semen, Davis, Bratton and Foote (1963b) indicated that .2 M and .25 M Tris-buffered extender was superior to the standard citrate-yolk-glycerol extender in percent post-thaw motility after 60 days of storage. Foote (1970) reported that the fertility of unfrozen bull semen stored in a Tris extender was equal to that of semen stored in C.U.E.

Studies with boar semen stored at 15 C for 4 days revealed that a Tris extender was better than citrate-glycine-yolk or skim milk ($P<.05$) in maintaining sperm motility (Igboeli, 1970).

Spermatozoa from different species have shown considerable tolerance to a range of Tris molarities, however, they did respond to the different tonicities. Davis et al. (1963a) stored bull semen up to 16 days at 5 C in .15, .20, and .25 M Tris-yolk-glycerol extender. The pH levels of the extenders were 6.50, 6.75 and 7.00. They reported motility percents of 27, 36, and 31 for .15, .20, and .25 M Tris extenders respectively. There was no significance due to Tris molarity. Yet, .15 M Tris produced poorest motility with most spermatozoa exhibiting bent tails in the mid-piece area suggesting osmotic shock. Tris at .25 M caused no signs of shock. Foote (1970) reported that .20 M Tris was better than .25 M at pH's of 6.50 and 6.75 for motility of bull semen stored at 5 C. Recently, Back (1974) obtained a 40.6% conception in mares inseminated with unfrozen stallion spermatozoa extended in a 2.4% Tris-buffered diluent compared to 15.4% of the mares inseminated with spermatozoa extended in .349% Tris extender.

With frozen bovine semen, Davis et al. (1963b) tested Tris molarities of .10, .15, .20, and .25 M in yolk-glycerol extenders and found

.2 M and .25 M Tris superior to .1 M and .15 M ($P<.01$). No difference was found in post-thaw motility between .2 M and .25 M Tris. Igboeli (1970) also found .15 M inferior in terms of sperm motility to .20 and .25 M Tris after the second day of 15 C storage of boar semen. The addition of glucose, glycine, and potassium chloride to Tris extenders produced no significant improvement in sperm motility. The author recommended an extender composed of .20 M Tris (3.028 g/liter) adjusted to pH of 7.0 with 1.516 g of citric acid.

The Effect of Glycerol Level on Spermatozoan Survival

The optimum glycerol level for survival of freeze-thawed sperm is influenced by the presence of other osmotically active compounds as well as thaw rate (Cragle et al., 1955; Salamon, 1968; Amann and Almquist, 1957; and Robbins, 1973). Cragle et al. (1955) reported a significant interaction between sodium citrate and glycerol on post-thaw motility of bull spermatozoa. As citrate levels increased, higher glycerol levels were also required for optimum recovery. Also, as the percent sodium citrate and percent glycerol increased or decreased beyond the point of maximum survival conditions, less decline in survival was associated with an increase or decrease in both variables simultaneously, rather than a change in only one at a time. Amann and Almquist (1957) noted a highly significant interaction between total milk solids level and glycerol level required for best post-thaw bull sperm motility. Optimum survival was observed when the glycerol level and the percentage of total solids (by weight) were equal at 9% or 11%. Steinback and Foote (1967) reported an interaction between glycerol and Tris molarity on the motility of

frozen bull spermatozoa. Also, a highly significant Tris x glycerol interaction on pellet frozen ram spermatozoa was reported by Salamon and Visser (1972). It was stated in each paper that higher glycerol levels 8.8% (Steinback and Foote, 1967) and 4% (Salamon and Visser, 1972) compared to lower glycerol levels improved frozen spermatozoan survival better at higher tonicities.

Thaw rate has been shown to interact with glycerol level on post-thaw recovery of bovine spermatozoa in yolk citrate extenders (Robbins, 1973). Robbins studied glycerol levels from 1% to 13% and thaw bath temperatures from 5 C to 65 C. He found that as the glycerol level was increased above 4%, faster thaw rates were required to minimize sperm damage based on percent intact acrosomes and motility estimates. Using a response surface design, optimum glycerol level for freezing bovine semen was calculated to be 10.22% with a thaw rate achieved by using a 92 C thaw water bath (Robbins, 1973).

Hill, Godley and Hurst (1959) found 7% glycerol superior to 3.5% and 14% glycerol for recovery of ram spermatozoan motility after ampule freezing, although 7% glycerol was not significantly different from 3.5%. The 14% glycerol level was toxic and reduced recovery. Salamon (1970) found no difference between 2 and 4% glycerol in pellet frozen ram semen extended in yolk-raffinose-citrate.

Studies by First et al. (1961) indicated that ram sperm in milk-glycerol extenders survived frozen storage better with 6 or 8% glycerol rather than 0, 2, 4, 12, or 15% glycerol. They stated that glycerol levels below 6%, or above 10%, resulted in inferior sperm survival (motility) when semen was thawed at 6 to 8 C. From these studies, it

appears that glycerol levels near 7% are optimum for ram and bull semen preservation.

The Effect of Thaw Rates on Spermatozoan Viability

Early reports covering rate of thaw of bull semen showed little difference relative to recovery of sperm motility (O'Dell and Almquist, 1954; Pickett et al., 1965). Robbins, Gerber and Saacke (1972) reported an increase ($P<.01$) in intact acrosomes when bull semen was thawed at 35 C or 75 C compared to semen thawed at 5 C and 20 C. Yet, no significant advantage was shown by increasing the thaw temperature above 35 C. Salamon (1970) observed no difference in survival of pellet-frozen ram semen thawed at 37 or 45 C.

Blackshaw (1955) noted that ampule frozen ram and bull semen thawed at 40 C produced greater sperm survival than that thawed at 5 C. The motility of unfrozen, 5 C-thawed and 40 C-thawed bull spermatozoa were 74, 24, and 45% respectively. For ram semen, the unfrozen control was 70% motile, while 5 C-thawed and 40 C-thawed spermatozoa resulted in post-thaw motility of 10 and 33%, respectively.

Pellet frozen ram semen responded favorably to faster thaw rates (Salamon, 1968). He thawed semen at temperatures of 5, 10, 15, 20, 30, 40, and 45 C and found 30, 40, and 45 C superior ($P<.01$) to the slower thaw rates in terms of post-thaw sperm motility. Aamdal and Anderson (1968) observed that ram semen frozen with 4 or 7% glycerol in synthetic straws responded favorably to higher thaw temperatures. They reported that semen thawed at 75 C exhibited 17% higher unstained (eosin-nigrosin stain) spermatozoa compared to the 35 C thaw temperature.

Robbins (1973) thawed bovine semen at temperatures of 5, 20, 35, 50, and 65 C. The glycerol level ranged from 1% to 13%. Spermatozoan motility and intact acrosomes were greater when thawed at higher temperatures. However, it was observed that an increase in glycerol level was required to obtain optimum recovery with the faster thaws. Thus, there was a significant ($P<.01$) interaction between glycerol and thaw temperature. It appears that ram and bull spermatozoa benefit from higher thaw temperatures following frozen storage.

Acrosome Deterioration as an Indicator of Spermatozoan Injury

Alterations of the acrosome of aging bovine spermatozoa have been observed and described by Saacke and Marshall (1968). They examined the acrosome with electron, brightfield, and differential interference-contrast microscopy. Garner et al. (1971) observed a similar acrosomal deterioration using Giemsa-stained smears and brightfield microscopy of ram spermatozoa after 96 hours of 4 C storage.

With the use of Giemsa stain and light microscopy, Watson and Martin (1972) observed the acrosomal alterations of bull and ram spermatozoa through liquid storage and after ampule freezing. Deterioration of the acrosomes of both species was highly significant after freezing. It appeared that ram spermatozoa were damaged more severely than bull spermatozoa by freezing. This observation was in agreement with those by Healey (1969) and Jones (1973) who studied the ultrastructure of freeze-thawed spermatozoa. After freezing, only 11.3% of ram spermatozoa exhibited no abnoramlities compared to 28.7% for bull spermatozoa. Watson and Martin (1972) also found that the proportional number of

spermatozoa damaged, as well as the severity of alteration of each damaged cell, constitute the difference between the two species in their ability to maintain viable acrosomal membrane systems. However, they stated that the differences in acrosomal damage were not sufficient to solely explain the difference in fertility of frozen semen from both species.

Reports by Healey (1969) and Jones (1973) strongly support the theory that the membranes of ram spermatozoa are more susceptible to damage after the freeze-thaw process than spermatozoa of other species. Healey (1969) noted that unlike bull spermatozoa, which appeared unaltered after freezing, the outer membrane and acrosome complex of ram spermatozoa was consistently damaged. Ultrastructural studies by Jones (1973) revealed that after osmotic shock with a .15 M cacodylate-buffered media, the mean percent intact plasma membranes were 70.2% for ram spermatozoa, 84.8% for boar spermatozoa and 92.8% for bull spermatozoa.

It seems logical that the acrosomal deterioration of ram spermatozoa would be an indicator of fertility since frozen ram spermatozoa have lower membrane tolerance and lower fertilizing capacity. Also, Saacke and White (1972) reported a correlation of .60 between percent intact acrosomes and fertility of bull semen. This acrosome-fertility correlation was the highest of any single semen quality test.

Experimental Procedure

General

Semen Collection and Handling. Experiment 1 was designed to study pH, glycerol level and Tris level in yolk-Tris-glycerol extenders on preservation of spermatozoa at 6C. Based on the results of Experiment 1, Experiment 2 was designed to study glycerol level, Tris level and thaw rate on survival of freeze-thawed spermatozoa. Cell injury was measured by the percent intact acrosomes and percent motile sperm post-thaw and after incubation at 37 C.

Pooled ejaculates of semen were collected per artificial vagina from 6 Finish x Landrace rams and 1 Dorset ram. Ejaculates were pooled to accommodate 15 treatments in each experiment. Sperm concentration of the pooled ejaculates was determined with a Bausch and Lomb photoelectric colorimeter (Spectrophotometer 20). The pooled ejaculates were discarded if sufficient sperm numbers were not obtained or if the initial sperm motility was below 50%.

Raw semen was held at 32 C during initial evaluation and dilution. Semen was diluted in a 32 C extender of 20% fresh egg yolk by volume and 80% Tris buffer in distilled water adjusted to the selected pH with citric acid monohydrate. Each extender was composed of two fractions. Both fractions contained equal amounts of yolk, Tris, and citric acid for the desired extender. In Fraction B, the volume of glycerol called for by the

experimental design displaced an equal volume of distilled water as represented in Fraction A of that same extender. Both fractions contained antibiotics at the rate of 1000 μg/ml of dihydrostreptomycin sulfate and 1000 IU/ml of potassium penicillin.

Semen was diluted in the A Fractions at the rate of 100×10^6 total cells/ml in Experiment 1 and 50×10^6 total cells/ml in Experiment 2. Tubes containing 10 ml of prediluted semen was positioned in a 2.7 liter water bath and cooled to 6 C. These conditions allowed cooling from 32 C to 6 C in 3.25 hours.

At this time, Fraction A diluted semen was further extended with an equal volume of the glycerol-containing Fraction B. Fraction B was added in a stepwise fashion of 10, 20, 30, and 40% by volume at intervals of 10 to 12 minutes. With the semen glycerolated and extended to its final concentration (half of the initial concentration), it was either held at 6 C until evaluated on days 1 and 3 of collection (Experiment 1) or immediately packed in ice and transported to Rocky Mount, Va., where it was frozen in French straws 3 to 4 hours later (Experiment 2).

Semen was evaluated by obtaining direct counts of intact acrosomes and estimating the percent motile sperm. The percent intact acrosomes were quantified by two direct sperm counts of 100 cells each from unfixed semen smears using a Zeiss differential interference-contrast microscope. This optical system enables observations of the acrosomal apical ridge since it produces a shadow cast image of the spermatozoa. A distinct apical ridge constituted an intact acrosome. Although this system has not been previously used for ram semen, it has been shown to be very useful in evaluating bull spermatozoa.

Motility estimates, based on the percent motile sperm (to the nearest 10%) were made at the same times the percent intact acrosomes were determined during the 37 C incubation. A Leitz phase contrast microscope was utilized in observing wet unfixed semen smears. Slides were warmed to 37 C on a stage incubator. All treatments were coded prior to evaluation to ensure objectivity.

Experimental Design. A three-dimensional central composite design was employed in Experiment 1 to determine the effects of and calculate optimum conditions of glycerol (%), pH and Tris (M). In Experiment 2 the same design was used to determine the effects of and calculate optimum conditions for glycerol (%), Tris (M) and thaw rate. Due to a number of interactions found in semen preservation studies of rams and bulls, this response surface design is a useful approach for establishing a set of optimal conditions for storage of spermatozoa. Also, this design has been effective in biological studies with the purpose of finding an optimum set of conditions (Cragle et al., 1955; Gardiner, Cragle and Chandler, 1967; Robbins, 1973).

With this composite design, 5 levels of each of three independent variables were incorporated into 15 treatments. The levels of each variable were coded -2, -1, 0, 1, or 2. These represented the level of each of the variables in the 15 treatments. The codes are graphically shown in Figure 1 as they are arranged in the 15 treatments. Some of the limitations of this design as well as advantages have been discussed by Cragle et al. (1955). This composite design estimates only the linear, quadratic and linear x linear interaction effects. The estimated response ($\hat{Y}$) to the three independent variables is calculated from the following multiple

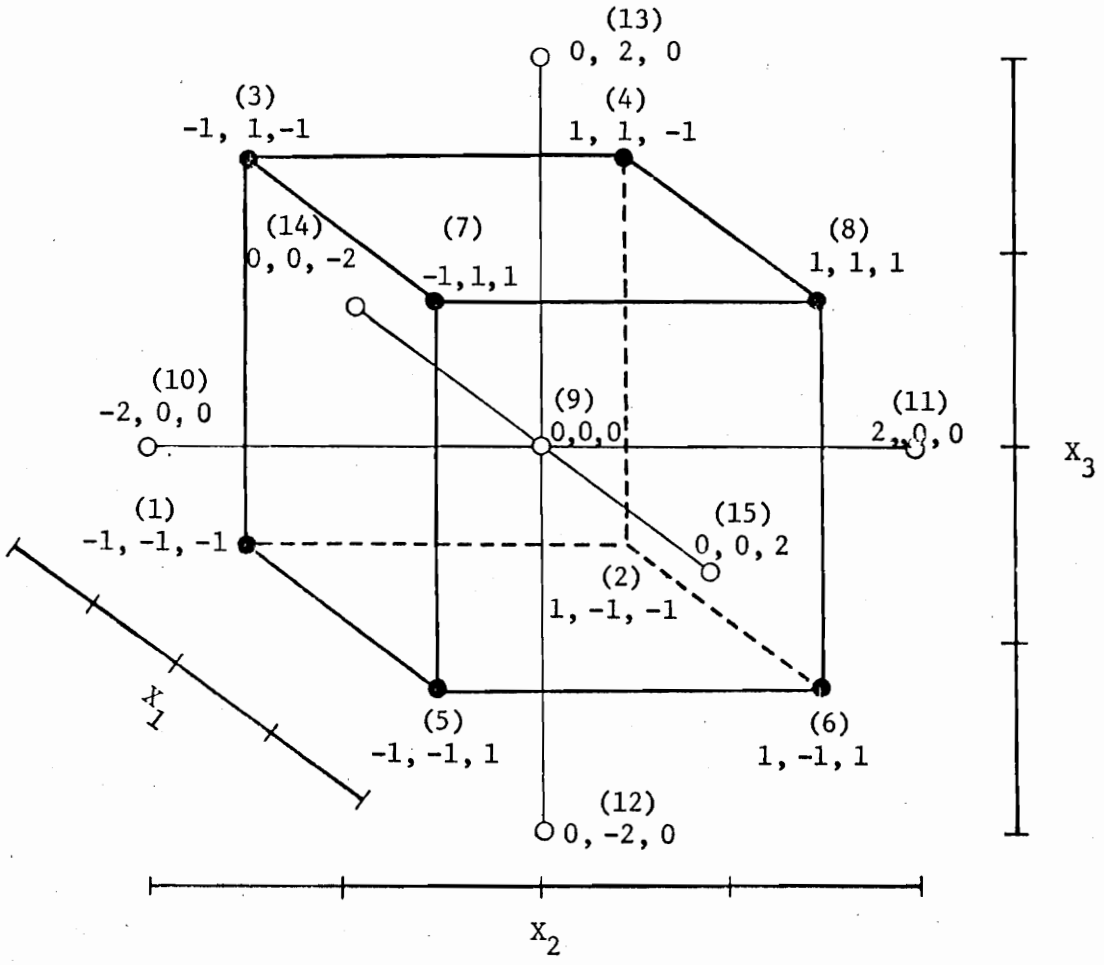

FIGURE 1. DIAGRAMATIC REPRESENTATION OF THE THREE-DIMENSIONAL CENTRAL COMPOSITE DESIGN

regression equation:

$$\hat{Y} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3.$$

The $\hat{Y}$ is the estimated response based on the b's (partial regression coefficients) of the 3 independent variables (X's). This multiple regression equation may be used in select ways. First, it may be used to calculate the dependent variable ($\hat{Y}$) or any one of the independent variables (X_1, X_2 or X_3) if three of the variables (dependent and/or independent) are given. The $\hat{Y}$ response is calculated by substituting the values for X_1, X_2 and X_3 which lie within the experimental limits. By calculating the $\hat{Y}$ response, enough estimated data may be obtained to allow plotting and observation of the effects of the independent variables. Also, any one of the independent variables may be calculated by setting Y at a desired response and substituting the experimental values for the remaining two independent variables. Since this multiple regression equation is a quadratic equation, there will be two estimated values for the independent variable sought. Therefore, the desired Y response may be obtained at any level between the two estimated values for that independent variable (area within the boundary). This relationship may be plotted and observed also. Another use for the multiple regression equation is to calculate the optimum combination of the three independent variables. From this regression equation, 3 equations may be drawn and solved simultaneously. The optimum is based on the maximum estimated response ($\hat{Y}$) calculated from the partial regression coefficients (b's).

The three equations are as follows:

$$0 = b_1 + 2b_{11}X_1 + b_{12}X_2 + b_{13}X_3$$

$$0 = b_2 + 2b_{22}X_2 + b_{12}X_1 + b_{23}X_3$$

$$0 = b_3 + 2b_{33}X_3 + b_{13}X_1 + b_{23}X_2.$$

All the graphs within this thesis are based on calculated data and must not be confused with actual observations.

Experiment 1

Seven ejaculates from 7 rams were split such that each accommodated 15 treatments representing 5 levels of glycerol, 5 pH levels and 5 Tris molarities. The experiment was centered on 7% glycerol, pH of 6.75 and .25 M Tris. These values have been shown to be productive in the maintenance of ram spermatozoan viability based on studies found in the literature. The levels and coded values of the 3 variables are shown in Table 1.

Glycerol levels were established by volume as a percentage of the buffer portion which constituted 80% of the extenders. Fraction B contained either 2, 8, 14, 20, or 26% glycerol depending upon the desired final concentration of 1, 4, 7, 10, or 13% respectively.

The pH of the extenders was adjusted by varying the level of citric acid monohydrate in relation to the given level of Tris present in the extenders. The amount of citric acid needed was calculated by multiplying a constant factor times the fixed Tris concentration (g/liter) in the extender. The constant factors for the pH levels studied (6.25, 6.50, 6.75, 7.00 and 7.25) were .6000, .5793, .5586, .5370, and .5154 respectively. The buffers were adjusted to a pH higher than desired to compensate for lower pH of the 20% yolk in the extenders. When the pH of the extenders

Table 1. LEVELS OF VARIABLES, CODED VALUES AND TREATMENT COMBINATIONS IN EXPERIMENT 1

Coded value	-2	-1	0	1	2
Glycerol[a] (X_1)	1.00	4.00	7.00	10.00	13.00
pH[b] (X_2)	6.25	6.50	6.75	7.00	7.25
Tris[c] (X_3)	.15	.20	.25	.30	.35

Treatment no.	Code	Glycerol	pH	Tris
1	-1,-1,-1	4.00	6.50	.20
2	1,-1,-1	10.00	6.50	.20
3	-1, 1,-1	4.00	7.00	.20
4	1, 1,-1	10.00	7.00	.20
5	-1,-1, 1	4.00	6.50	.30
6	1,-1, 1	10.00	6.50	.30
7	-1, 1, 1	4.00	7.00	.30
8	1, 1, 1	10.00	7.00	.30
9	0, 0, 0	7.00	6.75	.25
10	-2, 0, 0	1.00	6.75	.25
11	2, 0, 0	13.00	6.75	.25
12	0,-2, 0	7.00	6.25	.25
13	0, 2, 0	7.00	7.25	.25
14	0, 0,-2	7.00	6.75	.15
15	0, 0, 2	7.00	6.75	.35

[a]Percent glycerol by volume of buffer portion of final extender.

[b]pH of final extender.

[c]Tris molarity of buffer portion of final extender.

was slightly higher than desired it was adjusted down with a small amount of .5 M citric acid. No upward pH adjustment was made.

The 5 levels of Tris (tris(hydroxymethyl)aminomethane) studied were established in terms of molarity of the buffer. No pH adjustment was made with the addition of Tris above the given amount for that extender since a constant level was desired for each given extender.

The semen was diluted to a final concentration of 50 million sperm/ml and stored at 6 C until evaluated on day 1 and 3 after collection. Semen in all 15 treatments were pulled from the 6 C storage and evaluated at 0, 4, and 8 hr of incubation at 37 C.

Experiment 2

Ten ejaculates were collected from the same 7 rams used in Experiment 1. Each ejaculate was split to accommodate the 15 treatments representing 5 levels of glycerol, 5 Tris molarities, and 5 thawing temperatures. The levels of the three variables and coded values appear in Table 2. This experiment was centered on 7% glycerol, .29 M Tris and 35 C thaw temperature.

Glycerol levels were established as percent by volume of the final extender instead of expressed as percent of the buffer as in Experiment 1. Thus, the same percentage represented a larger (1.25X) amount of glycerol in the complete extenders in Experiment 2. However, the glycerol was handled the same in both experiments. Glycerol was added in Fraction B at levels of 2, 8, 14, 20, or 26% of the extender by volume, creating a final concentration of 1, 4, 7, 10, or 13% in the final extended semen.

Tris was added to distilled water at buffer levels of .09, .19,

Table 2. LEVELS OF VARIABLES, CODED VALUES AND TREATMENT COMBINATIONS IN EXPERIMENT 2

Coded value	-2	-1	0	1	2
Glycerol[2] (X_1)	1.00	4.00	7.00	10.00	13.00
Tris[b] (X_2)	0.09	0.19	0.29	0.39	0.49
Thaw[c] (X_3)	5.00	20.00	35.00	50.00	65.00

Treatment no.	Code	Glycerol	Tris	Thaw
1	-1,-1,-1	4.00	0.19	20.00
2	1,-1,-1	10.00	0.19	20.00
3	-1, 1,-1	4.00	0.39	20.00
4	1, 1,-1	10.00	0.39	20.00
5	-1,-1, 1	4.00	0.19	50.00
6	1,-1, 1	10.00	0.19	50.00
7	-1, 1, 1	4.00	0.39	50.00
8	1, 1, 1	10.00	0.39	50.00
9	0, 0, 0	7.00	0.29	35.00
10	-2, 0, 0	1.00	0.29	35.00
11	2, 0, 0	13.00	0.29	35.00
12	0,-2, 0	7.00	0.09	35.00
13	0, 2, 0	7.00	0.49	35.00
14	0, 0,-2	7.00	0.29	5.00
15	0, 0, 2	7.00	0.29	65.00

[a]Percent glycerol by volume of final extender.

[b]Tris molarity of buffer portion of final extender.

[c]Degrees C of thaw water bath.

.29, .39 or .49 M. The pH was adjusted to 6.50 by adding citric acid monohydrate in the amount of .5793 times the concentration of Tris (g). Both fractions (A & B) of the extenders contained the same amount of Tris and citric acid.

Thaw rates were obtained by plunging the frozen straws into water baths of 5 different temperatures and exposure times. The thaw rates studied were 5 C/2 min, 20 C/1 min, 35 C/30 sec, 50 C/15 sec, or 65 C/7.5 sec. These 5 thaw rates represent the lower conventional thaw temperatures and higher than conventional temperatures.

The semen was collected, diluted, and glycerolated as in Experiment 1. Once the semen was glycerolated, it was transported in ice to Rocky Mount, Va. At Rocky Mount it was loaded into .5-ml French straws at 5 C, which were plugged with PVC (polyvinyl chloride) powder. The loaded straws were placed in a water bath at the same temperature to solidify the PVC plug. Equilibration of about 3 hours was allowed during shipment and handling prior to freezing. Straws were placed in a 250-liter refrigerator at about 2.5 cm above the liquid nitrogen. After 8.5 min exposure to the N_2 vapor, the frozen straws were plunged into liquid nitrogen and stored until evaluation, 3 to 4 weeks later.

Six straws representing one ram and one treatment were thawed at the appropriate rate. The straws were then wiped dry and their contents were emptied into a 5-ml test tube. The tubes were stoppered and placed in a 37 C water bath for incubation. Semen was evaluated from unfixed smears based on percent intact acrosomes and percent motile sperm immediately post thaw and at 3, 6 and 9 hr of incubation.

Results

Experiment 1

The analysis of variance revealed that ejaculates, hours of incubation and treatments were all highly significant for percent intact acrosomes ($P<.01$). These main effects are shown in Table 3 along with the mean squares for these effects. At each hour of incubation, the effects of ejaculates and treatments were highly significant. The day effect was significant ($P<.05$) at the 8-hr incubation only. For the response of percent motility, all main effects (ejaculates, days of evaluation, hours and treatments) were highly significant (Table 3). For each hour of incubation, ejaculates, days and treatments were highly significant with the exception of the day effect at 0 hr, which contributed significantly ($P<.05$).

The variation accounted for by the multiple regression model (r^2) is shown in Table 4. The highest variation accounted for was at 0 hr for intact acrosome and at the 8-hr incubation for motility. With incubation, the (r^2) values decreased for intact acrosomes and increased for motility.

The actual and calculated (multiple regression model) response in intact acrosomes and motility for the 15 treatments are shown in Table 5. There was very little difference among treatments in intact acrosomes, with the exception of a poor response to Treatment 14, which contained the

Table 3. ANALYSIS OF VARIANCE OF PERCENT INTACT ACROSOMES AND PERCENT MOTILE SPERMATOZOA FOR ALL OBSERVATIONS IN EXPERIMENT 1

Source of variation	Degrees of freedom	Mean square for intact acrosomes	Mean square for motile spermatozoa
Ejaculates	6	.61838776**	1.79015212**
Days	1	.002202673	.73714683**
Hours	2	.114093297**	4.49086905**
Treatments	14	.044433059**	.58901531**
Residual	606	.002031785	.01863630

**P<.01

Table 4. VARIATION ACCOUNTED FOR BY THE MULTIPLE REGRESSION MODEL (R^2) FOR INTACT ACROSOMES AND MOTILITY FOR THE MEAN AND EACH HOUR OF INCUBATION
EXPERIMENT 1

	Hours of incubation at 37 C			
Dependent variable	0	4	8	$\bar{X}$
Intact acrosomes (r^2)	.82	.79	.76	.78
Motility (r^2)	.64	.72	.77	.71

Table 5. ACTUAL AND CALCULATED MEAN (7 EJACULATES) RESPONSES OF INTACT ACROSOMES AND MOTILITY TO EACH TREATMENT IN EXPERIMENT 1

Treatment no.	Treatment combination			% Intact acrosomes		% Motility	
				Actual response	Calculated response	Actual response	Calculated response
1	4[a]	6.50[b]	.20[c]	87.5	87.0	56.2	54.3
2	10	6.50	.20	86.0	85.2	51.4	49.3
3	4	7.00	.20	83.2	83.1	43.6	42.2
4	10	7.00	.20	84.9	84.3	39.3	36.6
5	4	6.50	.30	90.6	90.6	63.8	65.6
6	10	6.50	.30	90.2	89.5	66.7	66.6
7	4	7.00	.30	87.0	86.9	52.1	52.8
8	10	7.00	.30	89.1	88.8	52.6	53.2
9	7	6.75	.25	87.5	88.4	57.4	58.8
10	1	6.75	.25	89.1	89.1	61.0	60.6
11	13	6.75	.25	88.2	89.1	54.3	56.1
12	7	6.25	.25	89.5	90.1	65.7	66.1
13	7	7.25	.25	85.1	85.4	39.5	40.6
14	7	6.75	.15	77.8	78.5	22.3	25.9
15	7	6.75	.35	86.4	86.6	56.0	53.8

[a]Percent glycerol by volume of buffer portion of final extender.

[b]pH of final extender.

[c]Tris molarity of buffer portion of final extender.

lowest molarity of Tris. However, more variation among treatments was accounted for by motility. The greatest maintenance of motility occurred with Treatments 5, 6, 10, and 12. Treatment 14 produced inferior motility in addition to inferior acrosomal maintenance. The actual mean responses in intact acrosomes and motility and the responses for each hour of incubation at 37 C are shown in Appendix Tables 1 and 2. Both the percent intact acrosomes and motility decreased as incubation progressed. A greater decline was observed in motility than in intact acrosomes, especially noticeable in Treatment 14.

Partial regression coefficients were used to illustrate the linear, quadratic, and linear x linear interaction effects for the three variables tested. These coefficients for both intact acrosomes and motility over all ejaculates and incubations are shown in Table 6. Based on intact acrosomes, glycerol was highly significant as a linear component and in an interaction with pH. Tris level was significant as a linear component and highly significant as a quadratic. At 0 hr of incubation only, Tris as a quadratic was significant ($P<.01$) for intact acrosomes and motility. Incubation to 4 hours indicated that glycerol and Tris were significant as linear components, Tris as quadratic was highly significant and an interaction between glycerol and pH was significant for intact acrosomes. After the 8-hr incubation, only Tris as quadratic exhibited a significant ($P<.01$) effect on intact acrosomes. For motility, pH was significant as a linear and quadratic component and Tris exhibited highly significant linear and quadratic effects (Table 6). Also, a significant interaction between glycerol and Tris was observed. For motility at 4 hr of incubation, Tris was significant as a linear and highly

Table 6. PARTIAL REGRESSION COEFFICIENTS BASED ON INTACT ACROSOMES AND MOTILITY FOR LINEAR, QUADRATIC AND LINEAR X LINEAR EFFECTS (MEAN OF 0, 4 AND 8 HOURS OF INCUBATION)

	Partial regression coefficients ± s.e.	
Component	Intact acrosomes	Motility
Intercept		
b_0	0.170733±1.6118	-9.347352±4.8839
Linear		
b_1 (Glycerol)	-0.072348±0.0229**	-0.014347±0.0694
b_2 (pH)	0.209773±0.4570	2.754666±1.3846*
b_3 (Tris)	2.915987±1.4044*	11.110200±4.2553**
Quadratic		
b_{11} (Glycerol)	0.000204±0.0002	-0.000135±0.0007
b_{22} (pH)	-0.024989±0.0336	-0.219484±0.1018*
b_{33} (Tris)	-5.824715±0.8402**	-18.999008±2.5457**
Interactions		
b_{12} (Gly x pH)	0.009884±0.0033**	-0.001855±0.0100
b_{13} (Gly x Tris)	0.011371±0.0165	0.099842±0.0499*
b_{23} (pH x Tris)	0.047410±0.1978	-0.135230±0.5994

* P<.05
** P<.01

significant as a quadratic component. At the 8-hr incubation, motility was influenced by a significant Tris-linear and highly significant Tris-quadratic component.

The multiple regression equations were used to calculate the response of intact acrosomes and motility. These calculated responses were then plotted to illustrate the effects of the independent variables. Though these calculated responses are good estimates of the actual response, the variation accounted for by the multiple regression model must be considered.

As stated previously, there were very little treatment differences found in the maintenance of intact acrosomes with the exception of Treatment 14. Therefore, it seems that the acrosomal membrane of unfrozen ram spermatozoa has considerable tolerance to a wide range of pH, glycerol and Tris levels when stored at 6 C. Hypotonic extenders such as that represented in Treatment 14 were detrimental to acrosomal membrane maintenance. Higher glycerol levels were more beneficial than lower glycerol levels at higher pH's. In Figure 2, the pH was set at 6.50 and various responses were plotted for levels of glycerol and Tris. The multiple regression equation was used to calculate Tris molarities and glycerol level required to produce a given response for percent intact acrosomes (Y) when pH was fixed (Figure 2). Optimum conditions for maximum acrosomal maintenance of unfrozen spermatozoa appears to require low levels of glycerol and a Tris molarity of .25 to .30 M with pH of 6.50.

The effects of extender pH on the tolerance to varying levels of glycerol and Tris is shown in Figure 3. Conditions required to achieve a mean motility of 50% or better for the 0, 4, and 8 hr of incubation

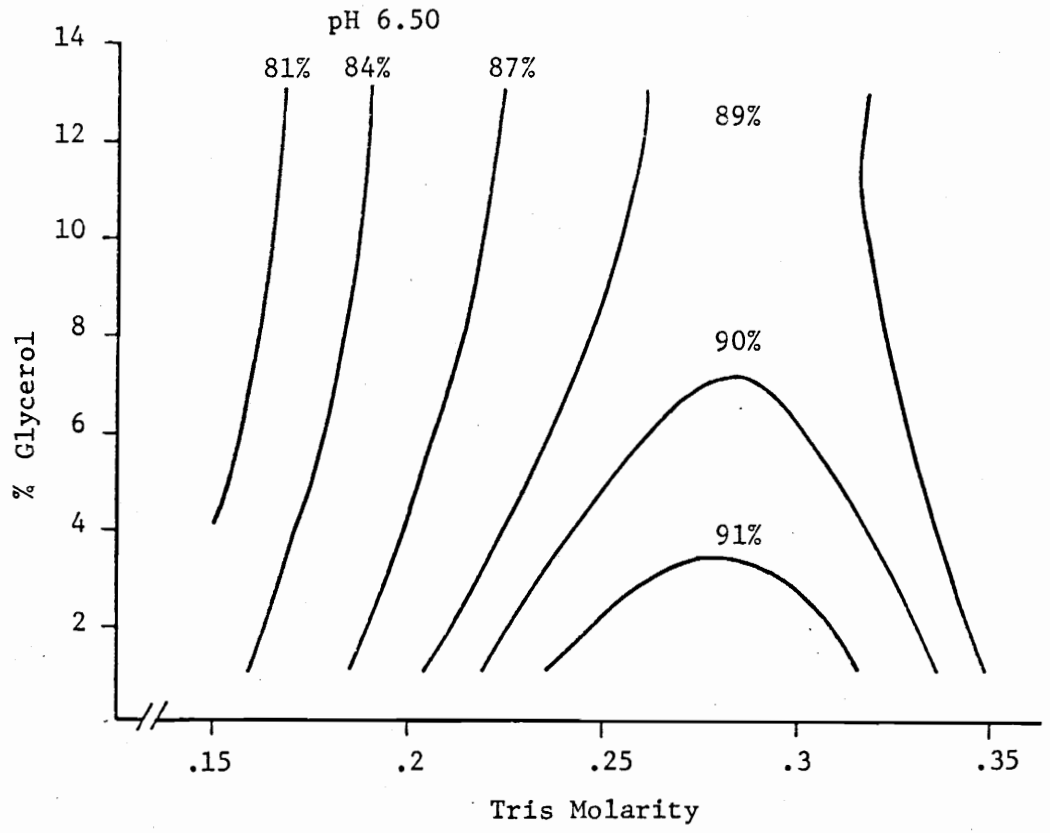

FIGURE 2. THE INFLUENCE OF TRIS MOLARITY AND GLYCEROL LEVEL ON ACROSOME RETENTION AT pH OF 6.50 (MEAN OF 0, 4 AND 8 HR OF INCUBATION)

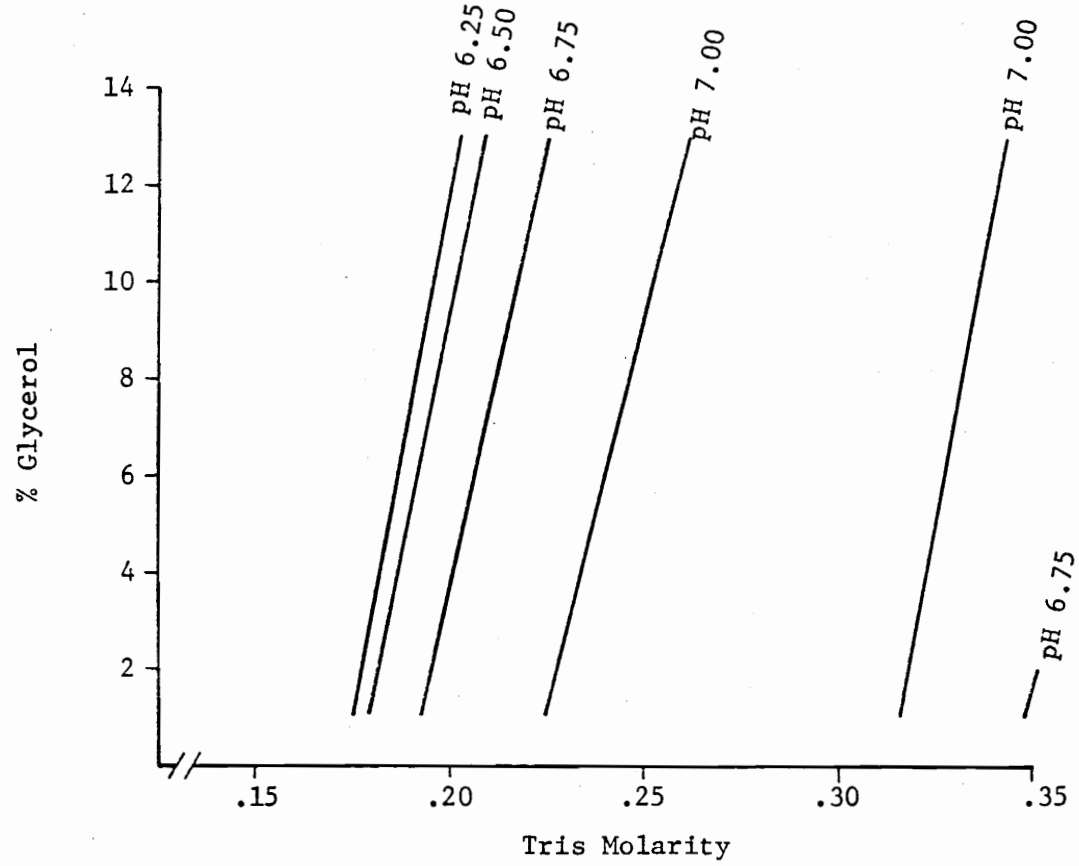

FIGURE 3. THE EFFECTS OF EXTENDER pH ON THE TOLERANCE TO LEVELS OF GLYCEROL AND TRIS TO PRODUCE 50% MOTILE SPERM OR BETTER OVER 0, 4 AND 8 HR OF INCUBATION (TOLERANCE INDICATED BY AREA BETWEEN THE SAME pH'S)

are represented by the area between the two lines labeled as the same pH. The upper limit of Tris molarity for pH's of 6.25, 6.50, and most of 6.75 were outside the experimental limits. For pH of 7.0, the two boundary lines fell within the experimental limits. However, 50% motility was not produced at pH of 7.25 at any glycerol or Tris level; therefore, pH 7.25 does not appear in Figure 3. Figure 3 also indicates that there was no effect of glycerol on motile sperm, stored at 6 C. Yet, there was a glycerol x Tris interaction as indicated by the slant of the lines which limit the area of tolerance to glycerol and Tris levels at a given pH to produce 50% or better motile cells. In this interaction, an increase in glycerol was accompanied with an increase in Tris molarity for maximum response. This figure indicates that in an environment of pH 6.50 there was a tolerance to glycerol levels from 1 to 13% as well as a tolerance to a wide upper range of Tris molarities.

Experiment 2

The analysis of variance revealed that ejaculates, hours of incubation, and treatments had highly significant effects on percent intact acrosomes and percent motility. The mean squares for these effects are shown in Table 7. The variation accounted for by the multiple regression model (r^2) is shown in Table 8. Although the variation accounted for by the regression model was quite adequate, there was a decline in this accountability with incubation. Since only 30% of the variation in motility was accounted for at the 9-hr incubation and all treatments exhibited essentially no motility, the 9-hr incubation was excluded in most of the analysis and interpretation.

Table 7. ANALYSIS OF VARIANCE OF PERCENT INTACT ACROSOMES AND PERCENT MOTILE SPERMATOZOA FOR ALL OBSERVATIONS IN EXPERIMENT 2

Source of variation	Degrees of freedom	Mean square for intact acrosomes	Mean square for motile spermatozoa
Ejaculates	9	.93461204**	.21293333**
Hours	3	1.06408088**	2.20135556**
Treatments	14	.76610553**	.57995714**
Residual	573	.00769037	.01307423

**P<.01

Table 8. VARIATION ACCOUNTED FOR BY THE MULTIPLE REGRESSION MODEL (R^2) FOR INTACT ACROSOMES AND MOTILITY FOR THE MEAN AND EACH HOUR OF INCUBATION EXPERIMENT 2

	Hours of incubation at 37 C post thaw				
Dependent variable	0	3	6	9	$\bar{X}$
Intact acrosomes (r^2)	.87	.82	.79	.78	.81
Motility (r^2)	.81	.69	.53	.30	.65

The actual mean responses and the responses calculated from the multiple regression model for intact acrosomes and motility for each treatment is shown in Table 9. Appendix Tables 3 and 4 show the actual responses (mean of 10 ejaculates) to the 15 treatments for intact acrosomes and motility.

The partial regression coefficients based on observations of intact acrosomes and motility immediately post thaw (0 hr) were calculated and appear in Table 10. Glycerol and Tris contributed highly significant ($P<.01$) linear and quadratic effects on both intact acrosomes and motility. Also thaw temperature affected intact acrosomes ($P<.01$) as a quadratic component. Based on intact acrosomes, glycerol x Tris and glycerol x thaw interactions were highly significant immediately post thaw. The glycerol x thaw interaction was observed only at the 0-hr incubation. No interactions were observed based on motility at any incubation period with the exception of a glycerol x Tris interaction ($P<.05$) at the 6-hr incubation.

Partial regression coefficients and the significance of the independent variables as different components of the model for the 3-hr and 6-hr incubation are shown in Appendix Tables 5 and 6. At the 9-hr incubation (Table 11), no treatment effects were observed based on motility and, therefore, dropped from most of the analyses. Thaw temperature as linear and quadratic components and glycerol as a quadratic component significant ($P<.05$) affected intact acrosomes. Tris did not contribute significantly as a linear or quadratic component. However, the glycerol x Tris interaction was consistently significant ($P<.01$) at all incubation periods.

Table 9. ACTUAL AND CALCULATED MEAN (10 EJACULATES) RESPONSE OF INTACT ACROSOMES AND MOTILITY TO EACH TREATMENT IN EXPERIMENT 2

Treatment no.	Treatment combination			% Intact acrosomes		% Motility	
				Actual response	Calculated response	Actual response	Calculated response
1	4[a]	.19[b]	20[c]	49.1[d]	44.8	15.8	11.9
2	10	.19	20	36.9	31.6	4.5	7.1
3	4	.39	20	50.2	46.7	22.8	15.8
4	10	.39	20	19.0	17.5	5.8	6.5
5	4	.19	50	57.6	54.4	22.5	20.7
6	10	.19	50	47.6	46.5	7.5	13.4
7	4	.39	50	57.9	58.6	32.3	28.6
8	10	.39	50	35.0	34.6	14.0	16.8
9	7	.29	35	46.0	50.9	29.0	30.1
10	1	.29	35	52.2	55.0	3.8	11.3
11	13	.29	35	16.0	17.8	1.3	-5.3
12	7	.09	35	37.2	41.8	0.3	-1.7
13	7	.49	35	31.7	31.8	2.5	5.5
14	7	.29	5	25.0	29.9	12.8	15.9
15	7	.29	65	57.0	56.7	37.0	34.9

[a]Percent glycerol by volume of final extender.

[b]Tris molarity of buffer portion of final extender.

[c]Degrees C of thaw water bath.

[d]Mean of 0, 3, 6 and 9 hr incubations at 37 C post thaw.

Table 10. PARTIAL REGRESSION COEFFICIENTS BASED ON INTACT ACROSOMES AND MOTILITY FOR LINEAR, QUADRATIC, AND LINEAR X LINEAR EFFECTS IMMEDIATELY POST THAW (0 HR)

	Partial regression coefficients ± s.e.	
Component	Intact acrosomes	Motility
Intercept		
b_0	-0.121138±.1571	-1.164410±.1838**
Linear		
b_1 (Glycerol)	0.060176±.0173**	0.148336±.0202**
b_2 (Tris)	4.423596±.5640**	7.449306±.6600**
b_3 (Thaw)	0.004600±.0035	0.004878±.0040
Quadratic		
b_{11} (Glycerol)	-0.005658±.0009**	-0.012423±.0010**
b_{22} (Tris)	-6.977500±.8024**	-12.430556±.9389**
b_{33} (Thaw)	-0.000096±.0000**	-0.000041±.0000
Interaction		
b_{12} (Gly x Tris)	-0.142708±.0315**	0.004167±.0368
b_{13} (Gly x Thaw)	0.000558±.0002**	0.000083±.0002
b_{23} (Tris x Thaw)	0.006925±.0063	0.025000±.0074

**P<.01

Table 11. PARTIAL REGRESSION COEFFICIENTS BASED ON INTACT ACROSOMES AND MOTILITY FOR LINEAR, QUADRATIC, AND LINEAR X LINEAR EFFECTS AT THE 9-HR INCUBATION

Component	Partial regression coefficients ± s.e.	
	Intact acrosomes	Motility
Intercept		
b_0	-0.030220±.1733	-0.083500±.1115
Linear		
b_1 (Glycerol)	0.036743±.0190	0.020324±.0122
b_2 (Tris)	1.201350±.6220	0.252917±.4002
b_3 (Thaw)	0.008571±.0038*	-0.000374±.0024
Quadratic		
b_{11} (Glycerol)	-0.002360±.0010*	-0.000648±.0006
b_{22} (Tris)	-1.161667±.8849	-0.583333±.5693
b_{33} (Thaw)	-0.000080±.0000*	0.000013±.0000
Interactions		
b_{12} (Gly x Tris)	-0.115417±.0347**	-0.020833±.0223
b_{13} (Gly x Thaw)	0.000092±.0002	-0.000250±.0001
b_{23} (Tris x Thaw)	0.003567±.0070	0.007500±.0045

*P<.05

**P<.01

Surprisingly, the interaction between glycerol and Tris for intact acrosomes changed with incubation (Figure 4). In this illustration thaw temperature was fixed at 50 C. Immediately post thaw, optimum responses were indicated for intermediate levels of Tris (.3 M) and 4% glycerol. However, after 9 hours of incubation, higher levels of Tris in conjunction with low levels of glycerol proved optimum.

Based on the mean response over all incubation periods, the partial regression coefficients for the two dependent variables were calculated (Table 12). Independently, glycerol, Tris and thaw temperature contributed highly significant effects on intact acrosomes as linear and quadratic components of the model. Interactions of glycerol x Tris ($P<.01$) and glycerol x thaw temperature ($P<.05$) were significant for intact acrosomes. Figure 5 illustrates the interaction between glycerol and Tris molarity on the percent intact acrosomes. For this illustration, the thaw temperature was fixed at 50 C, however, the response was representative of the other thaw temperatures. With the exception of 1% glycerol and very low Tris, the lower glycerol levels proved superior in maintaining intact acrosomes at all Tris molarities. In addition, as Tris increased, the demand for lower glycerol also increased to achieve maximum response.

Figure 6 illustrates the effect of thaw temperature and Tris molarity on acrosome retention. In this illustration, the glycerol level was set at 4%. An increase in thaw temperature had relatively little influence on the shape of the curve showing the relationship between intact acrosomes and Tris molarity. Faster thaws seemed to act only in increasing the acrosome maintenance at all Tris levels. No advantage was realized with a thaw temperature higher than 50C when the glycerol level

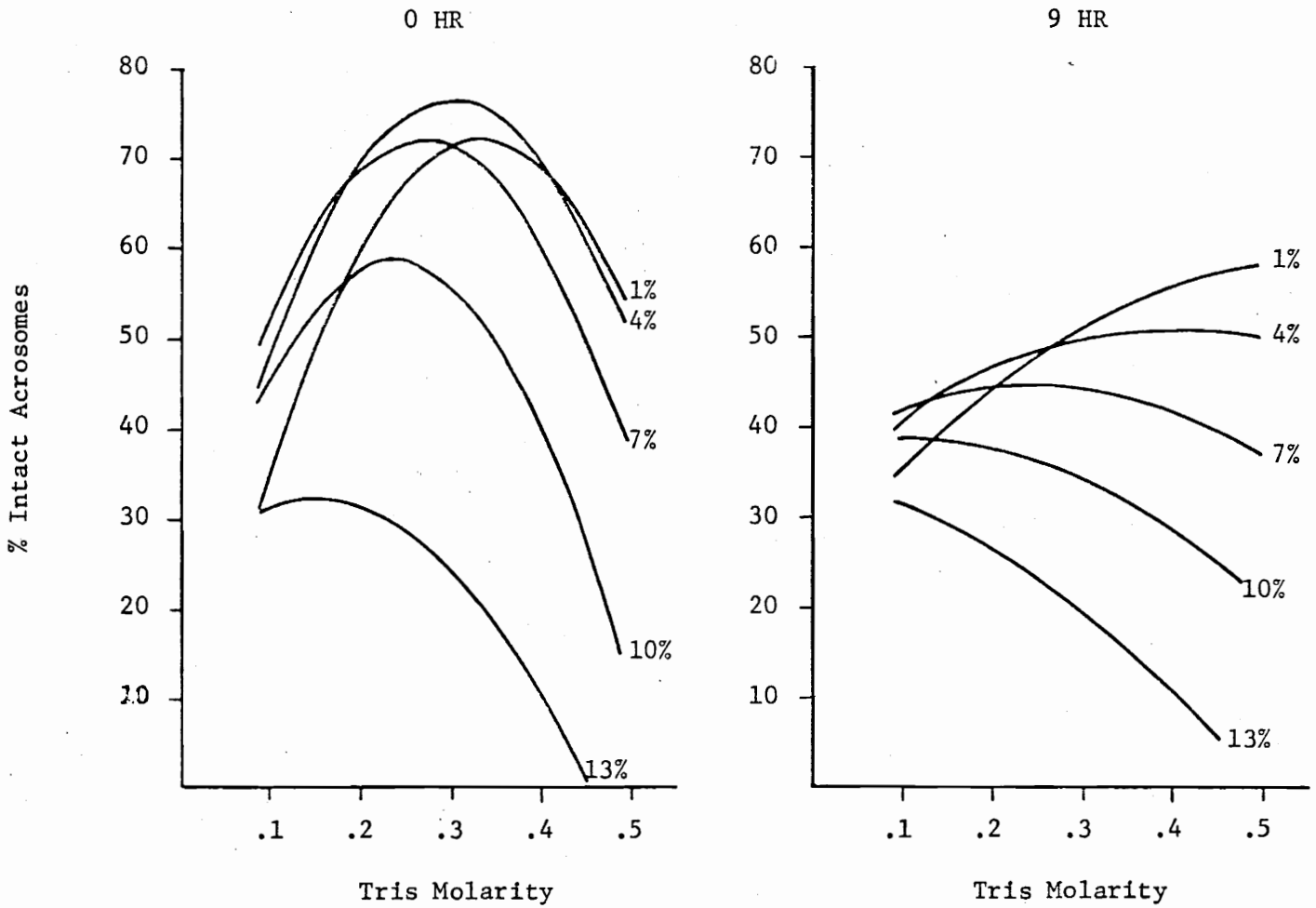

FIGURE 4. THE EFFECT OF GLYCEROL LEVEL AND TRIS MOLARITY ON ACROSOME RETENTION IMMEDIATELY POST THAW (0 HR) AND AT THE 9-HR INCUBATION (THAW TEMPERATURE FIXED AT 50 C)

Table 12. PARTIAL REGRESSION COEFFICIENTS BASED ON INTACT ACROSOMES AND MOTILITY FOR LINEAR, QUADRATIC, AND LINEAR X LINEAR EFFECTS (MEAN OF 0, 3, 6 and 9 HR OF INCUBATION)

	Partial regression coefficients ± s.e.	
Component	Intact acrosomes	Motility
Intercept		
b_0	-0.117681±.0866	-0.831467±.1129**
Linear		
b_1 (Glycerol)	0.054090±.0095**	0.106990±.0124**
b_2 (Tris)	2.601307±.3110**	4.292569±.4055**
b_3 (Thaw)	0.007245±.0019**	0.005824±.0025*
Quadratic		
b_{11} (Glycerol)	-0.004028±.0005**	-0.007508±.0006**
b_{22} (Tris)	-3.533333±.4424**	-7.038194±.5769**
B_{33} (Thaw)	-0.000084±.0000**	-0.000052±.0000*
Interaction		
b_{12} (Gly x Tris)	-0.133531±.0174**	-0.037500±.0226
b_{13} (Gly x Thaw)	0.000288±.0001*	-0.000139±.0002
b_{23} (Tris x Thaw)	0.003790±.0035	0.006667±.0045

*P<.05

**P<.01

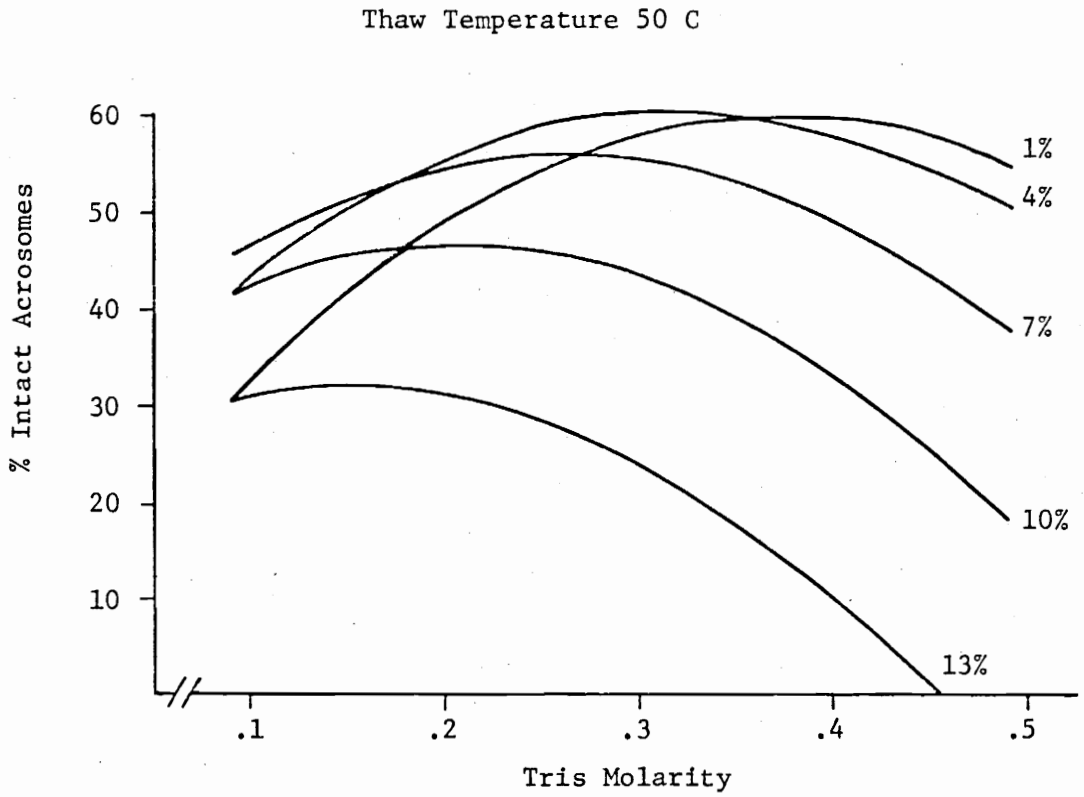

FIGURE 5. THE EFFECT OF GLYCEROL LEVEL AND TRIS MOLARITY ON ACROSOME RETENTION AT A THAW TEMPERATURE OF 50 C (MEAN OF 0, 3, 6 AND 9 HR OF INCUBATION)

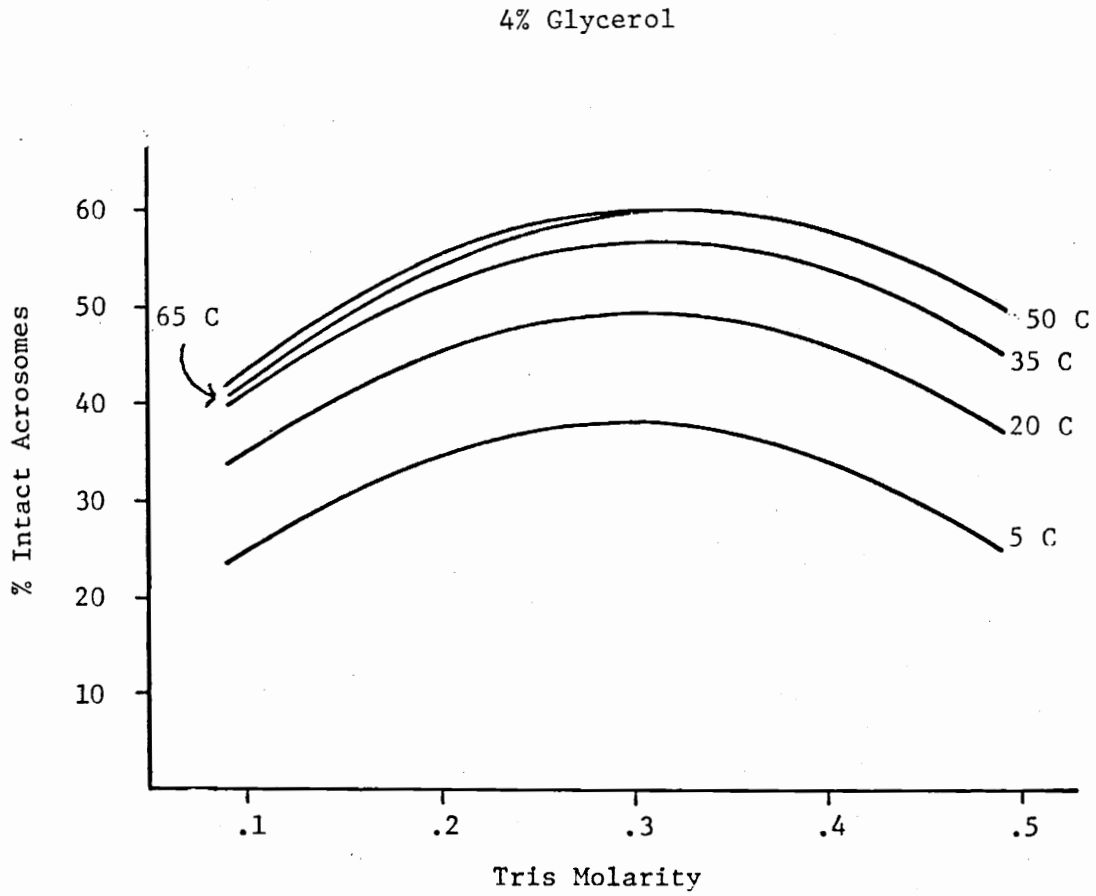

FIGURE 6. THE EFFECT OF THAW TEMPERATURE AND TRIS MOLARITY ON ACROSOME RETENTION AT 4% GLYCEROL (MEAN OF 0, 3, 6 AND 9 HR OF INCUBATION)

was fixed at 4% (Figure 6) or 35C at 1% glycerol. However, at 13% glycerol there was a trend toward better acrosomal maintenance with faster thaw rates.

The effect of glycerol and Tris molarity on intact acrosomes as influenced by thaw temperature is shown in Figure 7. Thaw temperature was fixed at 20 C or 50 C in this illustration. The Y response sought may be expected to be produced by all combinations of Tris and glycerol falling on that contour. The area within the contour represents a response higher than that sought. It may be noted that there were less restrictions on Tris and glycerol levels to obtain a given response (Y) with an increase in thaw temperature from 20 C to 50 C. If the desired response was 60% intact acrosomes, then 50 C thaw must have been used in conjunction with low glycerol and Tris between .3 and .4 M. Thus the optimum combination of glycerol and Tris fell within the experimental limits with at least a 50 C thaw temperature.

Figure 8 shows the influence of glycerol on the combination of Tris and thaw temperature for a given percent intact acrosomes. At 4% glycerol compared to 10%, lower thaw temperatures and a wider range of Tris molarities were tolerated to produce the same Y response. The optimum combination of thaw temperature and Tris molarity with 4% glycerol fell within the experimental limits. This optimum lies within the boundary labeled 60% intact acrosomes with 4% glycerol.

The percent motility was affected ($P<.01$) by glycerol and Tris as linear and quadratic components (Table 12). Also, thaw temperature contributed significantly to linear and quadratic effects on motility. Interactions of the independent variables were not observed in percent

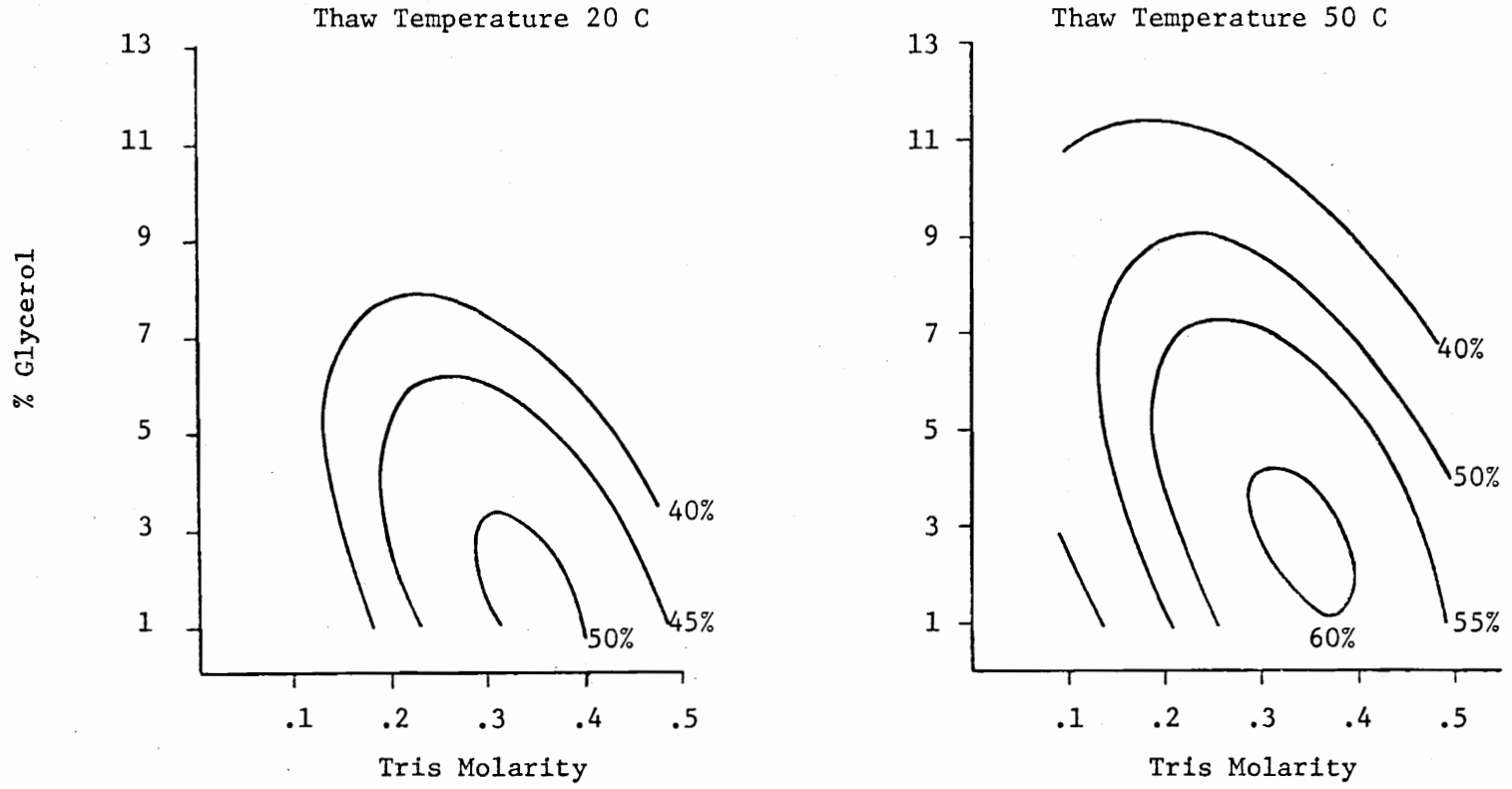

FIGURE 7. THE EFFECTS OF 20 C AND 50 C THAW TEMPERATURES ON THE TOLERANCE OF GLYCEROL AND TRIS LEVELS FOR A GIVEN PERCENT INTACT ACROSOME (MEAN OF 0, 3, 6 AND 9 HR OF INCUBATION)

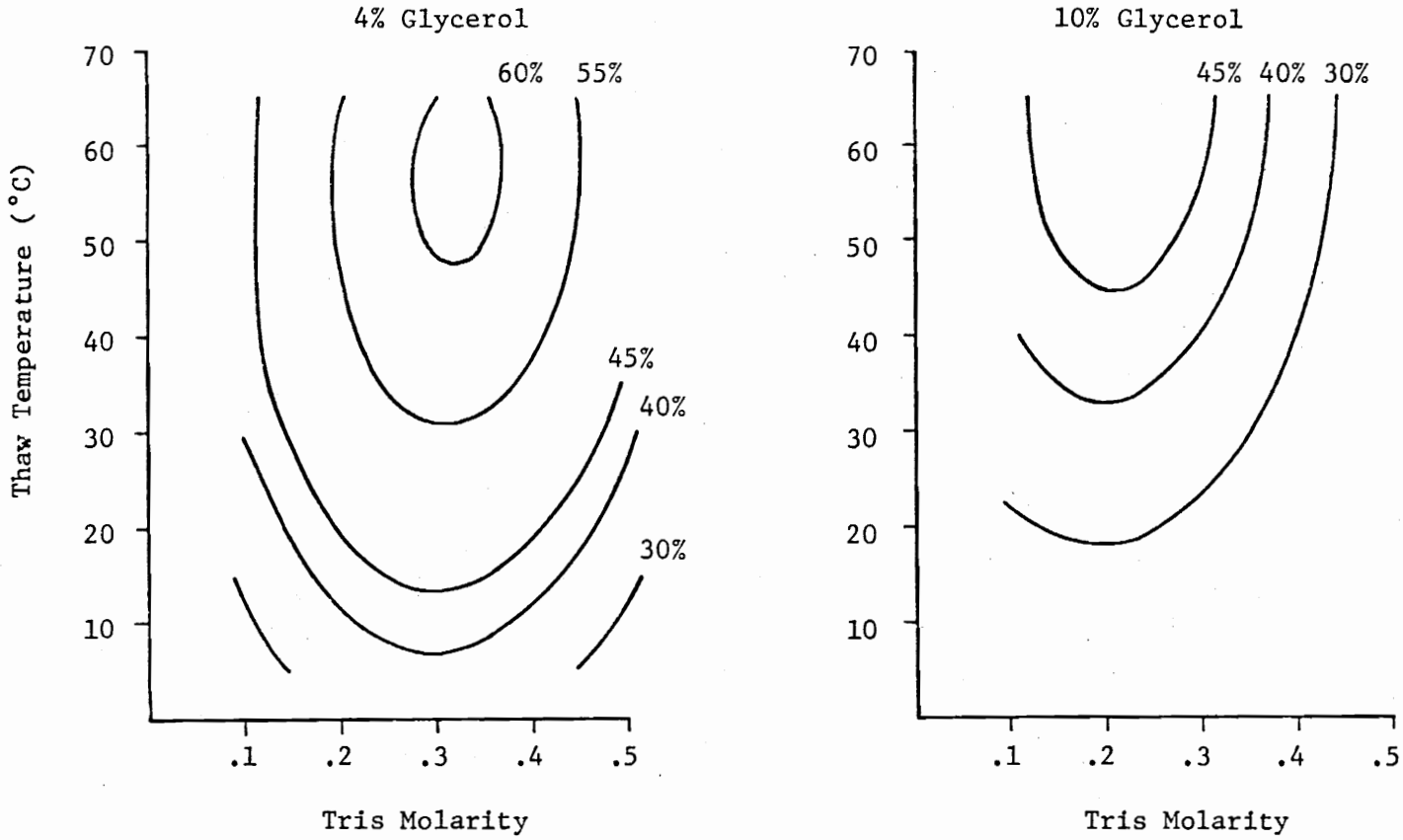

FIGURE 8. THE INFLUENCE OF 4 AND 10% GLYCEROL ON THE TOLERANCE OF THAW TEMPERATURE AND TRIS MOLARITY FOR A GIVEN PERCENT INTACT ACROSOMES (MEAN OF 0, 3, 6 AND 9 HR OF INCUBATION)

motility when all incubation periods were analyzed as a whole. The thaw water bath temperature had a similar effect on the percent motility as it did on intact acrosomes. Faster thaws produced higher percentages of motile cells and did not interact with Tris or glycerol levels. Figure 9 illustrates the effect of thaw temperature on the glycerol levels and Tris molarities needed to produce a mean of 20% motile sperm over 0, 3, and 6 hr of incubation. The 9-hr incubation was excluded since zero motility was observed in almost all treatments. With faster thaw rates, less restrictions were placed on both glycerol and Tris levels to obtain a mean response of 20% motile sperm. The 5 C thaw temperature produced by far the most limited tolerance to glycerol and Tris levels as indicated by the area within the center circle. However, the center point of all the circles representing different thaw temperatures lie close together.

The effect of glycerol and Tris levels on sperm motility over 0, 3, and 6 hr of incubation is shown in Figure 10. The thaw temperature was set at 50 C for this illustration. Glycerol levels of 4 and 7% were superior in maintenance of motility to the other glycerol levels at all Tris molarities. Motile life of spermatozoa were sustained better at 10% than 1%; however, 13% (which does not appear in this figure) was lethal at all Tris molarities. Also, extreme Tris molarities were detrimental to motility. The relationship between glycerol and Tris may also be observed in Figure 11. In this illustration, thaw temperature was fixed at 65 C. The optimum combination lies near 6% glycerol and .31 M Tris which are near the center of the experimental treatments.

An optimum combination of glycerol, Tris and thaw temperature for

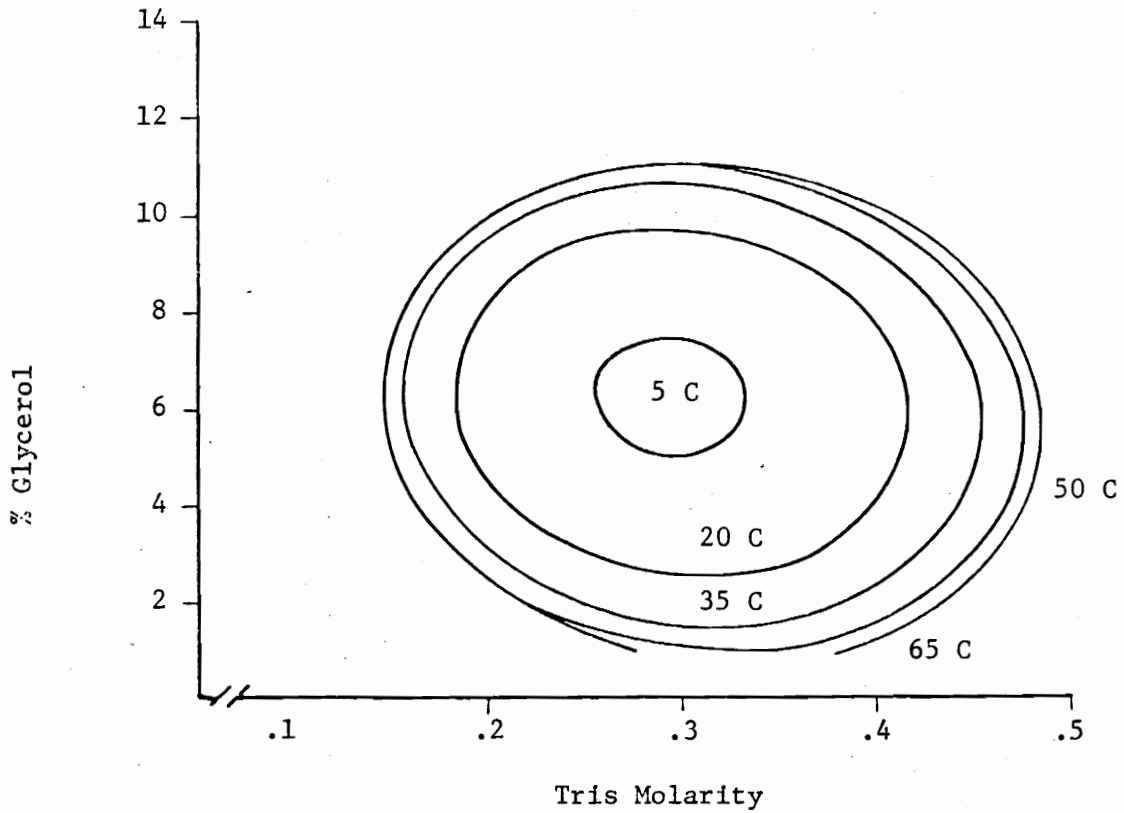

FIGURE 9. THAW TEMPERATURE, GLYCEROL AND TRIS LEVELS REQUIRED FOR 20% MOTILE SPERM OR BETTER (MEAN OF 0, 3, AND 6 HR OF INCUBATION)

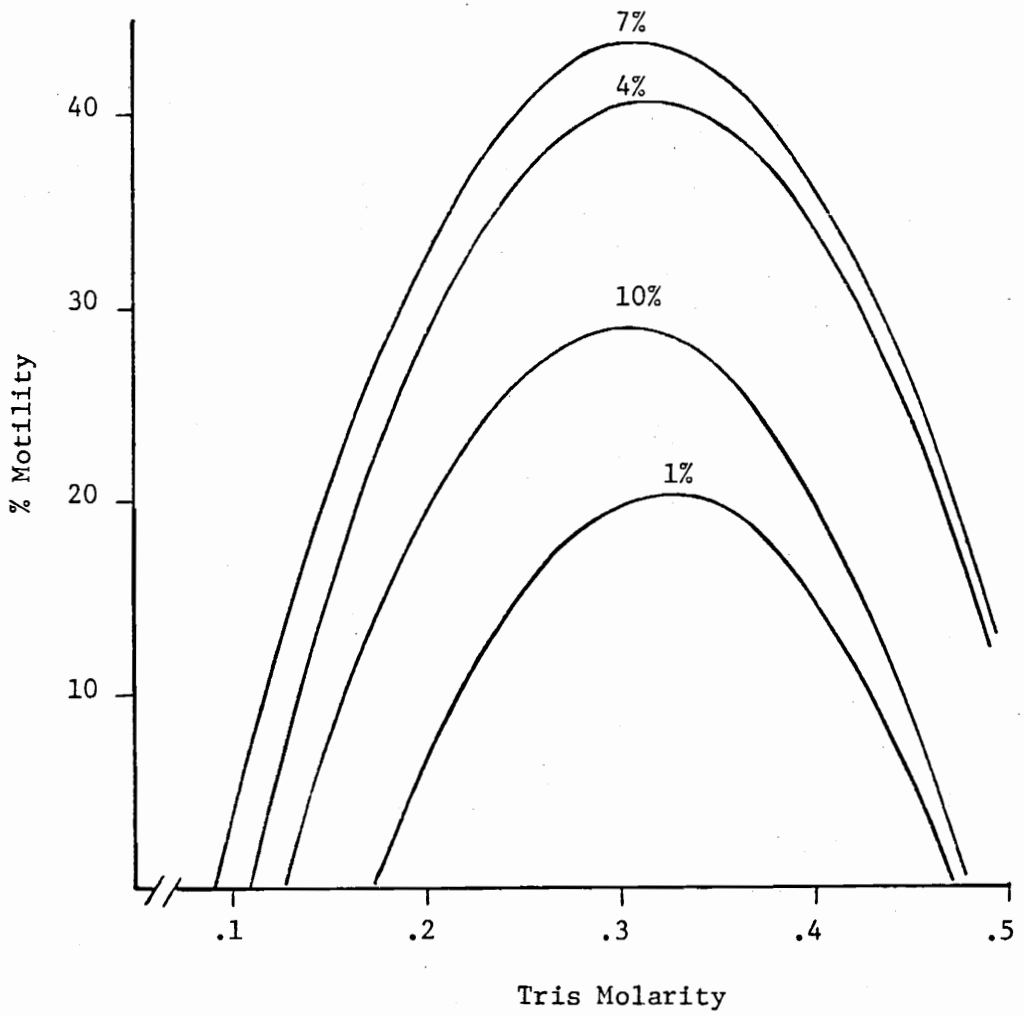

FIGURE 10. THE EFFECT OF GLYCEROL LEVEL AND TRIS MOLARITY AT 50 C THAW ON SPERM MOTILITY (MEAN OF 0, 3 AND 6 HR OF INCUBATION)

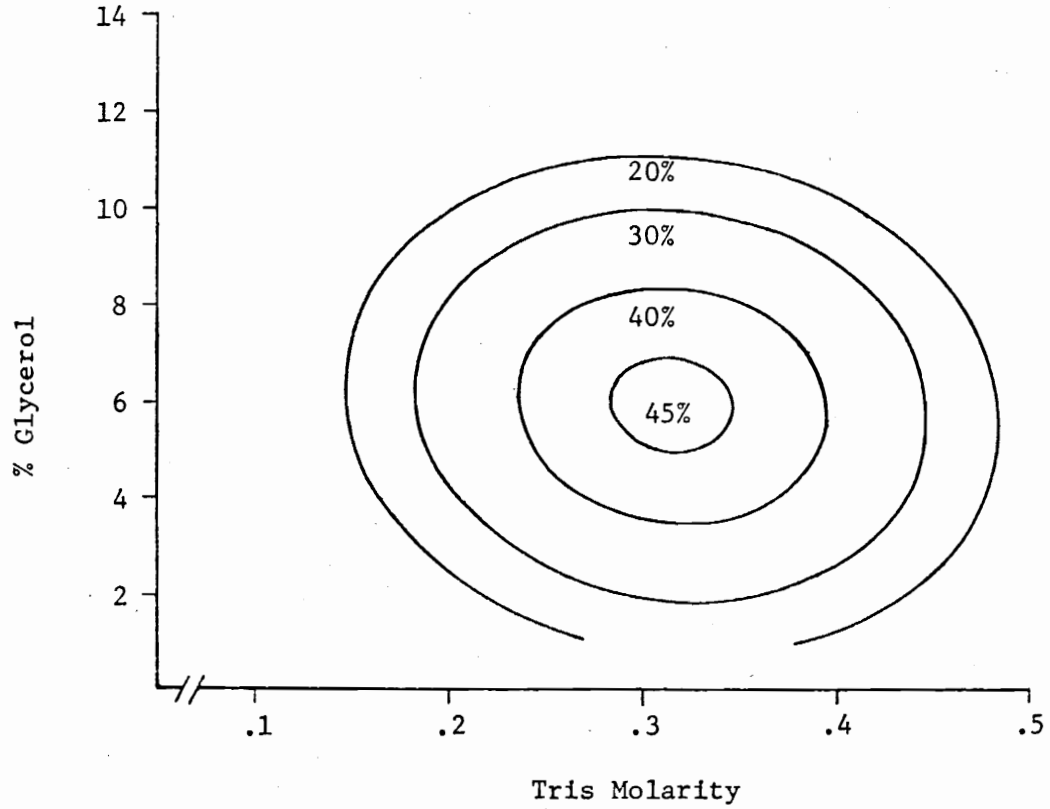

FIGURE 11. THE EFFECT OF GLYCEROL LEVEL AND TRIS MOLARITY AT 65 C THAW ON POST-THAW SPERM MOTILITY (MEAN OF 0, 3, AND 6 HR OF INCUBATION)

percent intact acrosomes was calculated from three equations drawn from the multiple regression equation as discussed in the Experimental Design. The optimum conditions for intact acrosomes at each hour of incubation and for the mean of all hours are shown in Table 13. Throughout all incubations, thaw temperature was relatively constant except at the 3-hr incubation at which point it was outside the experimental limits. As incubation progressed, lower glycerol levels and higher Tris molarities were needed to produce maximum intact acrosomes. Based on this table, the best combination of glycerol and Tris was dependent upon the hour of incubation chosen.

The optimum set of conditions of the 3 independent variables for maximal maintenance of motility is shown in Table 13. The optimum conditions based on the total of all incubations is relatively close to the combinations at each incubation. The exception was the 9-hr incubation which was dropped since most treatments exhibited zero motility. There was little change in glycerol and thaw temperature with incubation, though they did fluctuate. There was essentially no change in Tris molarity with incubation. Table 13 also compares the effect of incubation on percent intact acrosomes with motility. Though incubation influenced optimums for motility very little, there was a great change in optimums for percent intact acrosomes. The importance of these differences to the fertility of ram semen can only be realized by fertility studies.

Discussion

A comparison of the results of Experiments 1 and 2 vividly demonstrates the vulnerability of ram spermatozoa to the stress of freeze-

Table 13. CALCULATED OPTIMUM COMBINATION OF GLYCEROL, TRIS AND THAW TEMPERATURE FOR THE % INTACT ACROSOMES AND % MOTILITY MEAN AND EACH INCUBATION PERIOD

	Hours	Glycerol[a]	Tris[b]	Thaw[c]
Intact Acrosomes (%)				
	0	3.8	.30	45.6
	3	4.4	.31	72.7
	6	1.5	.42	51.7
	9	1.0	.49	65.0
	$\bar{X}$[e]	3.1	.34	55.8
Motility (%)				
	0	6.3	.31	74.6
	3	5.8	.32	65.6
	6	5.5	.33	57.1
	9[d]	---	---	----
	$\bar{X}$[e]	5.7	.32	69.5

[a]Percent glycerol by volume of final extender.

[b]Tris molarity of buffer portion.

[c]Degrees C of thaw water bath.

[d]Optimum was not calculated since essentially all treatments exhibited zero motility.

[e]Mean based on 0, 3, 6 and 9 hr of incubation.

thawing. When ram spermatozoa were subjected to freeze-thaw stress, there was more variation among treatments reflected in maintenance of both intact acrosomes and motile life (Experiment 2, Table 9) than when unfrozen sperm were studied (Experiment 1, Table 5). The influence of glycerol and Tris are particularly noteworthy. Though rate of thaw is not part of the surrounding media, it does control the amount of time the cells are subjected to an even more hostile environment (high salt concentrations) encountered near the melting point of ice. Therefore, the benefit of higher thaw temperatures and resulting faster thaw rates was undoubtedly due to minimizing exposure time of spermatozoa to this adverse condition.

Lower pH's produced minimal injury to ram spermatozoa diluted in yolk-Tris-glycerol extender and stored at 6 C. The extender pH exerted significant linear and quadratic effects on sperm motility. The percent motile sperm was maximum at a pH of 6.25 (lowest tested pH) for all glycerol and Tris levels. The pH had no effect on percent intact acrosomes per se, but there was a highly significant interaction between glycerol and pH. With a pH of 6.25, 1% glycerol was most beneficial in acrosomal maintenance. However, as the extender pH was increased to 7.25, more glycerol was required to minimize acrosomal damage. Working with bull semen, Steinback and Foote (1967) reported a protective effect of higher than conventional glycerol levels (7%) against the adverse effect of high pH and osmotic pressure.

The finding that lower pH levels produced minimal spermatozoan injury was not in complete agreement with studies of liquid stored bovine and porcine semen (Davis et al., 1963a; Foote, 1970; and Igboeli,

1970). Davis et al. (1963a) and Foote (1970) found the pH of 6.75 superior to 6.50 and 7.00 in the maintenance of bull sperm motility. Foote (1970) also found no significant difference in the fertility of bull semen diluted in ris-yolk-glycerol at pH of 6.50 or 6.75. However, the lower pH was associated with an insignificantly higher level of fertility. Also, Steinback and Foote (1967) found that pH 6.50 maintained motility of extended bull spermatozoa in Tris-yolk-glycerol better than pH's of 6.0, 7.0 or 7.5. Igboeli (1970) observed that pH 6.5 maintained Tris extended boar sperm motility significantly less than pH's of 6.75 or 7.0. Freeze-thawed ram spermatozoa exhibited best survival at pH of 6.8 rather than 6.2, 7.1 or 7.4 pH's (Blackshaw, 1960).

Tris molarity significantly affected the survival of ram sperm stored at 6 C and -196 C. The greater tolerance to varied Tris molarity for maximum response was observed in Experiment 1 than in Experiment 2 for both intact acrosomes (Figures 2 and 7) and motile spermatozoa (Figures 3 and 9). Tris molarities did not interact with either glycerol or pH based on intact acrosomes at 6 C storage. But for motility, Tris did interact with glycerol at 6 C storage. There was a direct relationship between glycerol and Tris levels. An increase in Tris molarity must be accompanied by an increase in glycerol for maximum motility. At a Tris molarity range from .25 to .33 M, all glycerol levels gave maximum motility (Figure 3). However, at a Tris range from .24 to .31 M, only lower levels of glycerol (<4%) produced maximum acrosomal maintenance.

In contrast to Experiment 1, the optimum Tris level for frozen storage of ram spermatozoa was dependent upon the glycerol level and hour of incubation, based on intact acrosomes. Figure 4 illustrates the highly

significant interaction between glycerol and Tris as well as the dependence of these constituents on duration of incubation. There was an inverse relationship between glycerol and Tris for maximum acrosome maintenance. Cragle et al. (1955) reported that optimum recovery of bull sperm was achieved when levels of glycerol and sodium citrate were increased together rather than independently (direct relationship). On the other hand, Blackshaw (1960) found no interaction between glycerol and salt content (sodium citrate) of an extender on the post-thaw motility of either ram or bull spermatozoa. Based on acrosomal evaluation immediately post thaw (0 hr) maximum results were achieved with 3.8% glycerol, .30 M Tris and a 45.6 C thaw temperature. After 9 hours of incubation, maximum results were achieved with 1% glycerol, .49 M Tris and a 65 C thaw temperature (Table 13).

Thus, higher glycerol levels (4%) were needed to protect the acrosomes through the freeze-thaw process; however, this advantage (over 1% glycerol) was overcome by high glycerol toxicity during the liquid storage of incubation. This theory was supported by the finding that maximum maintenance of intact acrosomes was achieved with lowest levels of glycerol in Experiment 1. It seems reasonable that freeze-thawing in one environment might be supplemented by reconcentration in another, since the best freezing media was different from the best storage media. Pellet freezing offers each possibility through the use of a thawing solution, due to the separation of the freezing media and thawing media. The use of two different media is apart from the conventional thinking based on the work on bovine by Robbins (1973).

Thaw temperature had a similar effect on intact acrosome and motility.

Faster thaw rates seem to act only in increasing the $\hat{Y}$ response of intact acrosomes (Figure 6) and motility (Figure 9). However, there was a significant ($P<.01$) interaction between glycerol and thaw at the 0-hr incubation, based on intact acrosomes. This interaction was significant ($P<.05$) over all incubations (Figure 7). At 20 C thaw temperature, the point of maximum response (center point of the partial circle) lies just below 2% glycerol. For the 50 C thaw temperature, the point of maximum response lies just below 3% glycerol. This difference in glycerol level due to thaw temperature was small, but is consistent with the findings of Robbins (1973). He found an interaction between glycerol and thaw temperature. Maximum maintenance of intact acrosomes was achieved with higher glycerol levels, but only when accompanied by faster thaw rates.

Increased acrosome retention was realized with increasing thaw temperature from 5 to 50 C with 4% glycerol (Figure 5). No advantage was seen with thaw temperatures above 50 C. Robbins et al. (1972) reported no improved recovery of intact acrosomes with thaw temperatures above 35 C when 7% glycerol was used in the extender for bovine sperm. However, when glycerol levels were increased to 10%, a thaw temperature of 65 C was advantageous (Robbins, 1973).

Faster thaws increase sperm tolerance to varying levels of glycerol and Tris (Figures 7, 8, and 9). In Figure 7, 50% intact acrosomes or better were maintained with 20 C thaw in conjunction with restricted glycerol and Tris levels. However, with 50 C thaw temperatures, 50% intact acrosomes or better were met with a relatively wide tolerance of Tris and glycerol levels. A more dramatic effect of thaw was seen in Figure 9 where 5 C was compared to 65 C to produce 20% motility or

better over 0, 3, and 6 hr of incubation. When semen was thawed at 5 C, there was a very small tolerance to Tris and glycerol levels. However, when semen was thawed at 65 C, over half of the experimental range of glycerol and Tris levels were tolerated to produce 20% motility. This wider tolerance to varied glycerol and Tris levels, by faster thaw rates, was reflective of reduced exposure time of spermatozoa to adverse environments created by these levels. These environments were created by high concentration of salts prior to the melting of ice. Glycerol also aids in preventing adversely high salt concentrations since it does not crystallize and, therefore, reduces the exposure of cells to adverse conditions (Rapatz, 1966).

In view of these findings on the thaw temperatures and levels of glycerol and Tris tested, it appears that minimal post-thaw ram spermatozoan injury could be realized when thawed at 65 C after freezing in an extender containing 4% glycerol and .30 M Tris. However, it appears that prolonged spermatozoan preservation post thaw could best be accomplished when semen is extended in 1% glycerol and .49 M Tris. A breeding trial is needed to determine which combination of glycerol and Tris will produce better fertility.

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Appendix

Appendix Table 1. THE ACTUAL MEAN (7 EJACULATES) RESPONSES IN PERCENT INTACT ACROSOMES TO EACH TREATMENT FOR THE MEAN AND EACH INCUBATION PERIOD IN EXPERIMENT 1

Treatment no.	Treatment combination			Hours of incubation 0	4	8	$\bar{X}$
1	4[a]	6.50[b]	.20[c]	91.4	87.6	83.7	87.5
2	10	6.50	.20	89.5	84.8	83.8	86.0
3	4	7.00	.20	84.1	84.4	81.2	83.2
4	10	7.00	.20	87.8	84.6	82.4	84.9
5	4	6.50	.30	92.0	91.6	88.3	90.6
6	10	6.50	.30	92.7	90.9	87.1	90.2
7	4	7.00	.30	89.2	87.5	84.3	87.0
8	10	7.00	.30	89.4	90.3	87.7	89.1
9	7	6.75	.25	89.6	87.0	85.9	87.5
10	1	6.75	.25	89.8	90.0	87.6	89.1
11	13	6.75	.25	90.4	87.9	86.4	88.2
12	7	6.25	.25	92.5	89.1	86.8	89.5
13	7	7.25	.25	87.4	85.5	82.5	85.1
14	7	6.75	.15	82.1	77.3	74.0	77.8
15	7	6.75	.35	88.6	85.6	85.1	86.4

[a]Percent glycerol by volume of buffer portion of final extender.

[b]pH of final extender.

[c]Tris molarity of buffer portion of final extender.

Appendix Table 2. THE ACTUAL MEAN (7 EJACULATES) RESPONSES IN PERCENT MOTILITY FOR THE MEAN AND EACH INCUBATION PERIOD IN EXPERIMENT 1

Treatment no.	Treatment combination			Hours of incubation 0	4	8	$\bar{X}$
1	4[a]	6.50[b]	.20[c]	63.6	59.3	45.7	56.2
2	10	6.50	.20	60.0	58.6	35.7	51.4
3	4	7.00	.20	59.3	45.7	25.7	43.6
4	10	7.00	.20	57.1	44.3	16.4	39.3
5	4	6.50	.30	69.3	68.6	53.6	63.8
6	10	6.50	.30	71.4	70.0	58.6	66.7
7	4	7.00	.30	70.0	57.1	29.3	52.1
8	10	7.00	.30	67.9	57.1	32.9	52.6
9	7	6.75	.25	66.4	62.9	42.9	57.4
10	1	6.75	.25	68.6	66.4	47.9	61.0
11	13	6.75	.25	65.0	62.1	35.7	54.3
12	7	6.25	.25	70.7	69.3	57.1	65.7
13	7	7.25	.25	62.1	44.2	12.1	39.5
14	7	6.75	.15	44.6	18.6	3.6	22.3
15	7	6.75	.35	68.6	57.9	41.4	56.0

[a]Percent glycerol by volume of buffer portion of final extender.

[b]pH of final extender.

[c]Tris molarity of buffer portion of final extender.

Appendix Table 3. PARTIAL REGRESSION COEFFICIENTS BASED ON INTACT ACROSOMES AND MOTILITY FOR LINEAR, QUADRATIC, AND LINEAR X LINEAR EFFECTS AT THE 3-HR INCUBATION

Component	Partial regression coefficients ± s.e.	
	Intact acrosomes	Motility
Intercept		
b_0	-0.111567±.1643	-1.273162±.2355**
Linear		
b_1 (Glycerol)	0.058795±.0181**	0.156966±.0259**
b_2 (Tris)	2.455506±.5900**	6.035972±.8456**
b_3 (Thaw)	0.006468±.0036	0.011215±.0052*
Quadratic		
b_{11} (Glycerol)	-0.004264±.0009**	-0.010988±0.0013**
b_{22} (Tris)	-3.285139±.8394**	-9.763889±1.2030**
b_{33} (Thaw)	-0.000055±.0000	-0.000095±0.0001
Interaction		
b_{12} (Gly x Tris)	-0.124000±.0329**	-0.054167±.0472
b_{13} (Gly x Thaw)	0.000234±.0002	-0.000194±.0003
b_{23} (Tris x Thaw)	0.001700±.0066	0.007500±.0094

*P<.05

**P<.01

Appendix Table 4. PARTIAL REGRESSION COEFFICIENTS BASED ON INTACT ACROSOMES AND MOTILITY FOR LINEAR, QUADRATIC, AND LINEAR X LINEAR EFFECTS AT THE 6-HR INCUBATION

Component	Partial regression coefficients ± s.e.	
	Intact acrosomes	Motility
Intercept		
b_0	-0.207812±.1774	-0.804794±.1989**
Linear		
b_1 (Glycerol)	0.060647±.0195**	0.102333±.0219**
b_2 (Tris)	2.324778±.6369**	3.432083±.7142**
b_3 (Thaw)	0.009341±.0039*	0.007578±.0044
Quadratic		
b_{11} (Glycerol)	-0.003831±.0010**	-0.005972±0.0011**
b_{22} (Tris)	-2.709028±.9061**	-5.375000±1.0161**
b_{33} (Thaw)	-0.000106±.0000**	-0.000083±0.0000
Interactions		
b_{12} (Gly x Tris)	-0.152000±.0355**	-0.079167±.0399*
b_{13} (Gly x Thaw)	0.000267±.0002	-0.000194±.0033
b_{23} (Tris x Thaw)	0.002967±.0071	0.009167±.0080

* $P<.05$

** $P<.01$

Appendix Table 5. THE ACTUAL MEAN (10 EJACULATES) RESPONSE IN PERCENT INTACT ACROSOMES FOR EACH TREATMENT FOR THE TOTAL AND EACH INCUBATION PERIOD IN EXPERIMENT 2

Treatment no.	Treatment combination			Hours of incubation				
				0	3	6	9	$\bar{X}$
1	4[a]	.19[b]	20[c]	65.1	49.2	43.0	39.1	49.1
2	10	.19	20	46.5	37.4	35.2	28.7	39.6
3	4	.39	20	63.6	50.4	46.6	40.0	50.2
4	10	.39	20	24.8	21.5	14.5	15.3	19.0
5	4	.19	50	70.8	58.5	52.7	48.2	57.6
6	10	.19	50	59.2	48.7	43.6	39.0	47.6
7	4	.39	50	70.4	58.5	52.0	50.8	57.9
8	10	.39	50	44.8	36.1	30.8	28.2	35.0
9	7	.29	35	64.0	45.7	41.2	34.1	46.2
10	1	.29	35	71.0	49.1	45.5	43.1	52.2
11	13	.29	35	20.2	16.8	14.1	12.8	16.0
12	7	.09	35	43.8	38.7	33.4	32.8	37.2
13	7	.49	35	32.3	31.6	32.1	30.8	31.7
14	7	.29	5	46.0	25.8	16.1	12.2	25.0
15	7	.29	65	68.6	60.9	52.0	46.4	57.0

[a]Percent glycerol by volume of final extender.

[b]Tris molarity of buffer portion of final extender.

[c]Degrees C of thaw water bath.

Appendix Table 6. THE ACTUAL MEAN (10 EJACULATES) RESPONSE IN PERCENT MOTILITY FOR EACH TREATMENT FOR THE TOTAL AND EACH INCUBATION PERIOD IN EXPERIMENT 2

Treatment no.	Treatment combination			Hours of incubation				
				0	3	6	9	$\bar{X}$
1	4[a]	.19[b]	20[c]	31.0	18.0	11.0	3.0	15.8
2	10	.19	20	13.0	5.0	0.0	0.0	4.5
3	4	.39	20	45.0	33.0	12.0	1.0	22.8
4	10	.39	20	19.0	4.0	0.0	0.0	5.8
5	4	.19	50	44.0	33.0	10.0	3.0	22.5
6	10	.19	50	19.0	7.0	4.0	0.0	7.5
7	4	.39	50	51.0	43.0	25.0	10.0	32.3
8	10	.39	50	35.0	20.0	1.0	0.0	14.0
9	7	.29	35	53.0	39.0	22.0	2.0	29.0
10	1	.29	35	14.0	1.0	0.0	0.0	3.8
11	13	.29	35	5.0	0.0	0.0	0.0	1.3
12	7	.09	35	1.0	0.0	0.0	0.0	0.3
13	7	.49	35	8.0	2.0	0.0	0.0	2.5
14	7	.29	5	41.0	10.0	0.0	0.0	12.8
15	7	.29	65	60.0	53.0	28.0	7.0	37.0

[a]Percent glycerol by volume of final extender.

[b]Tris molarity of buffer portion of final extender.

[c]Degrees C of thaw water bath.

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FACTORS AFFECTING PRESERVATION OF LIQUID AND FROZEN RAM SPERMATOZOA

by

Larry Johnson

(ABSTRACT)

A three-dimensional central composite design was employed in Experiment 1 to determine the effect of pH, glycerol and Tris on acrosomal alteration and motile life of ram spermatozoa stored a 6 C. By altering the amount of citric acid monohydrate 5 pH's (6.25, 6.50, 6.75, 7.00 and 7.25) were achieved. Glycerol levels tested were 1, 4, 7, 10 and 13% by volume. Five Tris molarities tested were: .15, .20, .25, .30 and .35 M. Stored semen was evaluated on days 1 and 3 for percent intact acrosomes (presence of the apical ridge) and percent sperm motility at 0, 4, and 8 hours of 37 C incubation. Using the same design, Experiment 2 was conducted to determine the effect glycerol, Tris and thaw temperature had on sperm motility and intact acrosomes. Glycerol levels were 1, 4, 7, 10 and 13%. Tris molarities tested were .09, .19, .29, .39 and .49 M. Five thaw rates were achieved by plunging the frozen straws into a water bath at 5 C/2 min, 20 C/ 1 min, 35 C/30 sec, 50 C/15 sec, and 65 C/7.5 sec. Three to four hours post glycerolation, the semen was frozen in .5-ml French straws 2.5 cm above liquid nitrogen in a 250-liter refrigerator for 8.5 min. Then the straws

were plunged into liquid N_2 and stored until evaluation 3 to 4 weeks later. Semen was evaluated immediately post thaw as well as after 3, 6, and 9 hours of incubation at 37 C.

In Experiment 1, the percent intact acrosomes was affected by glycerol and Tris as linear components of the regression model in addition to a glycerol x pH interaction. With increased pH's, higher levels of glycerol were needed for maximum acrosomal maintenance. Sperm motility was affected by pH and Tris as linear and quadratic components of the model. Also, a glycerol x Tris interaction affected motility. With increased Tris molarity, higher levels of glycerol were needed for minimal loss of motility. For 6 C stored semen, it appeared that lower pH's (6.25 to 6.50) and a relatively wide range of glycerol and Tris molarity would produce optimum spermatozoan preservation. In Experiment 2, glycerol, Tris and thaw significantly affected intact acrosomes as linear and quadratic components of the model. There was a glycerol x thaw and a glycerol x Tris interaction effect on intact acrosomes. Higher glycerol levels were better at higher thaw temperatures based on the evaluation immediately post thaw. There was an inverse relationship between glycerol and Tris for maximum acrosomal maintenance. This interaction was seen throughout all hours of incubation. However, the optimum level of glycerol and Tris changed with incubation. At 0 hr, 3.8% glycerol with .30 M Tris maintained intact acrosomes best, but at 9 hr, 1% glycerol with .49 M Tris produced maximum retention. The mean optimum was: 5.7% glycerol, .32 M Tris and 69.5 C thaw temperature. This optimum was representative of each hour of incubation (excluding 9 hr). Higher thaw temperatures (50 to 65 C) were more

beneficial for both intact acrosomes and motility than lower temperatures.