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To the Graduate Council:

I am submitting herewith a thesis written by Thomas William Lamb entitled "The Effects of Glycerol Levels, Equilibration Times, and Their Interrelationship with Bulls on the Ability of Bovine Semen to Survive the Freezing Process." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairying.

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THE EFFECTS OF GLYCEROL LEVELS, EQUILIBRATION TIMES, AND THEIR INTERRELATIONSHIP WITH BULLS ON THE ABILITY OF BOVINE SEMEN TO SURVIVE THE FREEZING PROCESS

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Thomas William Lamb

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CHAPTER I

INTRODUCTION

In the past few years, artificial insemination has become one of the most important tools that the cattle breeder has at his disposal to improve the quality of his animals.

The artificial insemination organizations invest a considerable amount of time and money in locating sires. A portion of these bulls are rejected after semen test because of low fertility or a low rate of survival of spermatozoa during the semen freezing process.

The loss of many valuable bulls and much time and money might be avoided if the causes of freezing failures in some bulls could be determined. With this in mind, an experiment was conducted to determine optimum glycerol levels, optimum equilibration times, the importance of their interactions, and to estimate the sources of variability in the ability of spermatozoa from different bulls to survive the freezing process.

CHAPTER II

REVIEW OF LITERATURE

Glycerol effects on semen stored at 5°C. Fertility of semen from some bulls declines rapidly during storage at 5°C. In an attempt to increase fertility, glycerol was added to the semen from several bulls (18). The semen from bulls with low fertility was significantly improved upon the addition of glycerol, whereas, the semen from bulls with normal fertility was unchanged when stored at 5°C.

Flipse and Almquist (14) found that the addition of glycerol or fructose to a diluent of reconstituted nonfat dry milk solids--0.5 molar (M) glycine was superior to the same diluent without either glycerol or fructose.

Albright, et al. (1) observed that when glycerol was added stepwise to semen partially diluted in yolk-citrate, yolk-glycine, whole milk, or skim milk the motility did not decline as rapidly as when no glycerol was added, but that when the glycerol was added all at one step, the motility was reduced below that of the semen with no glycerol added.

Almquist (2) and Almquist and Wickersham (3) tested levels of glycerol from 5 to 25 per cent using either homogenized milk or skim milk diluent. They observed that motility on the third, fourth, and fifth days after collection was improved over

the control semen to which no glycerol was added. They also found that optimum results in terms of livability and motility were obtained when the glycerol was added stepwise to the partially diluted semen at 5°C. Dilution at room temperature into completely formulated skim milk-glycerol diluents containing more than 5 per cent glycerol also resulted in abnormal sperm motility (circular or backward movement). Glycerol levels of 5 to 15 per cent gave approximately equal results in a skim milk diluter but levels above 15 per cent gave lower motility and livability than was observed for the control semen to which no glycerol was added.

Shannon (46) obtained higher conception rates with semen diluted in four experimental diluents containing glycine than with 2.9 per cent sodium citrate with semen used on the day of collection and the day after collection. The four experimental diluents were:

I. Cornell University Extender;

II. Cornell University Extender plus 1.25 per cent glycerol;

III. Shannon's diluent number 14;

IV. Shannon's diluent number 14 plus 1.25 per cent glycerol.

Shannon (46) also obtained a slightly better conception rate with the diluents containing glycerol than with those containing no glycerol. It was concluded that glycine and glucose added to a buffer solution significantly increase conception rates and that glycerol added to some diluents may improve conception rates.

Effects of levels of glycerol on post-freeze motility. By chance observation, Polge, et al. (37) observed that glycerol had a beneficial effect on protecting spermatozoa against the effects of low temperature. Since that observation many different levels of glycerol have been tested and a fairly wide range has given satisfactory results.

Steinbach and Foote (49) studied several levels of glycerol in three different diluents, citrate-yolk-glycerol (CYG), Tris-yolk-glycerol (TYG), and skim milk-glycerol (SMG). The TYG extender consistently resulted in a higher percentage of sperm surviving the freezing process than did the CYG or SMG extenders. The optimum level of glycerol in TYG was about 8.8 per cent; in CYG, about 7.2 per cent; and in SMG it was less than 9 per cent.

Four basic diluents and four glycerol levels for each diluter were studied by Saroff and Mixner (44). The diluter that gave the highest mean motility had the following final concentration: 18.5 per cent egg yolk, 2.22 per cent sodium citrate dihydrate, and 7.5 per cent glycerol.

Amann and Almquist (4) found a highly significant interaction between the per cent of total solids in milk and the optimum level of glycerol. In fresh skim milk containing 9 per cent solids, 11 per cent glycerol was significantly better than either 9 or 13 per cent. However, when the skim milk had been fortified with condensed skim milk to 11 per cent solids, the use of 13 or 15 per cent glycerol gave slightly better results.

Erickson, et al. (12) used glycerol levels of 7 and 10 per cent for both yolk-citrate (2.9 per cent) and milk. The most satisfactory glycerol levels were 7 per cent for yolk citrate and 10 per cent for milk.

Addition of glycerol in 5 equal portions at intervals of either 6 or 12 minutes gave revival and survival rates superior to 3 equal additions at 10 minute intervals or addition of the glycerol all at once (30). Glycerol levels of 10 and 13 per cent were slightly better than 4, 7, and 16 per cent when used with yolk-citrate diluents but levels of 10-13 per cent were required with skim milk (30, 31). Several other investigators (13, 19) found that glycerol levels of 6-8.5 per cent gave the best post-freeze motility when levels of 0 to 15 per cent were tested with heated homogenized milk.

Many investigators (6, 8, 9, 22, 23, 26, 27, 36, 39, 45, 54) observed that optimum glycerol levels were within the range of 3.5 to 8 per cent with best results between 6 and 8 per cent when yolk-citrate diluters were used.

Stallcup, et al. (46) tested levels of glycerol from 15 to 30 per cent with yolk-citrate diluent at a storage temperature of -15°C. Motility of semen treated with the 20 and 25 per cent levels of glycerol, was superior to that treated with 15 and 30 per cent, and there was no survival of the control, to which no glycerol was added.

Swanson (52) found that slightly higher post-thawing motility rates were obtained when 1-2 per cent glycerol was added to the semen at the time of the initial dilution.

Factors contributing to cell damage during freezing. Saacke and Almquist (42) studied the structure of normal bovine spermatozoa with the aid of an electron microscope. The sperm head consists of a homogeneous, flattened nucleus covered anteriorly by a three-layered head cap and posteriorly by a loose, thin, dense post-nuclear cap. The cell membrane constitutes the outermost covering which extends around the entire cell. The sperm tail consists of the classical nine + nine + two fiber pattern, bound anteriorly by the mitochondrial helix and posteriorly by the fibrous helix. The outer ring of nine coarse fibers originates as laminated columns from a common matrix located in a recess at the base of the head. The fibers taper posteriorly, ending at different levels in the principal piece. Sufficient evidence exists to conclude that the alternate contraction and expansion of the laminated segments of the coarse fibers is the probable mechanism underlying initiation of motility.

Several investigators (38, 41, 43) have studied the effects of freezing and damage caused by freezing on sperm cells with the aid of an electron microscope. Some of these researchers (41, 43) found that there were two principal types of damage to

the cell during the freezing process, either loss or damage of the cell membrane and complete disruption of the cristae within the mitochondrial helix. When the semen was frozen with 10 per cent glycerol, the degree of damage during freezing ranged from damage to the cell membrane and disruption of the cristae to no damage of cell membrane or cristae. Rapatz (38) believes that cell damage during rapid freezing is due to intracellular ice formation, particularly in the neck and tail.

Saacke and Almquist (43) in an attempt to determine whether the observed injury to the cell membranes and mitochondrial cristae was unique to freeze-thawing or similar to other sources of injury, compared the ultrastructure of sperm exposed to a lethal dosage of heat, hypertonicity, hypotonicity, or mercury (mercuric chloride), to that of untreated control sperm. Cells exposed to excess heat showed general disorganization of the entire cell. Cells subjected to a hypertonic or hypotonic medium showed damaged or missing cell membranes. Mitochondrial cristae were damaged in cells subjected to hypertonic medium and mercury, but not in those exposed to a hypotonic medium. In general, mercury-treated cells possessed intact cell membranes.

Several researchers (25, 32, 38, 40) believe that the cell damage during slow freezing is due either to a hypertonic electrolyte balance inside the cell caused by the freezing out of the water or to increased concentrations of cryoprotective

agents. These processes in turn disrupt the nuclear material, namely the mitochondrial cristae. If glycerol is added to the diluent containing the spermatozoa, the slow freezing technique is a satisfactory freezing method. It is believed that the glycerol enters the cells and prevents the freezing out of water by binding it and by this binding process protects the cells from the hypertonic situation which develops without glycerol.

Pickett and Komarek (34) have found evidence that there is a loss of lipid from the sperm cell during freezing. They extracted 1.26 milligrams (mg.) per milliliter (ml.) of lipid from seminal plasma separated from spermatozoa prior to freezing and 1.92 mg. per ml. of lipid from seminal plasma separated from spermatozoa after freezing and thawing. There was also a significant difference between the 2.34 and 1.63 mg. per ml. extracted for the pre- and post-freeze spermatozoa, respectively. Freezing of whole raw semen before separation apparently results in an alteration in the quality and possibly the class of lipid found in the sperm cells and seminal plasma.

Effects of lengths of equilibration time on post-freeze motility. Many studies involving lengths of equilibration time with glycerol have been conducted.

Many investigators (8, 9, 17, 24, 27, 28, 29, 31, 36, 39, 44, 45, 50, 54) have found optimum lengths of glycerol equilibration to range from 0 to 18 hours.

O'Dell and Hurst (28, 29) compared 0, 0.5, and 18 hours of equilibration with glycerol and found that 0 and 0.5 hours gave significantly better post-freeze motility than 18 hours.

O'Dell and Almquist (31) tested lengths of equilibration of 0.5, 4, and 18 hours using a skim milk diluter and observed that 0.5 and 4 hours of equilibration were equal to or better than 18 hours.

White (54) obtained best results with 2 to 4 hours of equilibration when 2, 4, 8, and 20 hours were compared.

Several investigators (8, 24, 27, 36, 50) obtained optimum results with equilibration times of six to eight hours. Cragle and Myers (8) found 6.5 to 16 hours of equilibration to be satisfactory and below 4 and above 28 hours to be detrimental. Sullivan and Mixner (50) found 6, 12, and 18 hours to be superior to a 2-hour equilibration period.

A glycerol equilibration period of 12 hours was found to be superior to 4, 6, 8, and 18 hours by two investigators (17, 39).

Cragle, et al. (9) found the optimum glycerol equilibration time to be 14.9 hours and that as equilibration time decreased, per cent survival decreased slowly.

Saroff and Mixner (44, 45) compared glycerol equilibration times of 2, 6, 12, and 18 hours and found a progressive increase in sperm survival as equilibration time increased from 2 to 18 hours.

Two investigators (30, 48) found no significant differences between glycerol equilibration times of 0.5, 4, 6, and 18 hours, but 5 equal additions of glycerol at intervals of either 6 or 12 minutes gave revival and survival rates superior to 3 equal additions at 10 minute intervals or a single glycerol addition.

Several investigators (9, 30, 31, 39, 45, 50) studied the interaction between length of glycerol equilibration time and other factors. O'Dell and Almquist (30) observed a significant difference in optimum equilibration time for different bulls and Roussell, et al. (39) found a highly significant interaction between length of glycerol equilibration time and per cent glycerol. Other studies (9, 31, 45, 50) indicated that no significant interaction between glycerol equilibration time and other variables exists.

Since such a wide range of optimum glycerol equilibration times have been reported, one could conclude that there is either a bull-equilibration interaction or a diluent-equilibration interaction. Enough evidence has been found to indicate that equilibration time may be varied to adjust total freezing time to the labor conditions existing at each individual bull stud without very much variation in semen quality.

Effects of irradiation on semen production. Irradiation effects on semen production were studied by several investigators (10, 11, 15, 16, 53). Freund and Murphree (15) studied the

effects of whole-body gamma irradiation on semen production characteristics of mature Hereford bulls. A single dose of 400 roentgens (r) from a cobalt^{60} source did not result in any apparent change in semen characteristics (sperm concentration and initial fructose level) or in sperm activity (initial motility and fructolytic activity) during a three-week post-irradiation period. It was concluded that apparently, those cells which were past the radiosensitative spermatogonial stage at the time of irradiation were metabolically unaffected.

Erickson (10) and Erickson, et al. (11) found that the greatest male germ-cell sensitivity in swine and cattle extends from approximately days 50 and 80 prenatally, respectively, to days 70 to 80 postnatally in each species. It was also observed that the bull and boar differ from the rat in that sublethal doses of irradiation usually only diminish their sperm producing capacities and do not cause sterility.

Effects of fractionated doses of whole-body gamma irradiation at semi-weekly or weekly intervals on sperm output, testis histology and blood constituents in mature Hereford bulls were studied by Welch and Murphree (53). Treatment groups were: Group I, control; Group II, 50 r semi-weekly (1,100 r total dose); Group III, 100 r weekly (1,100 r total dose), and Group IV, 100 r weekly (600 r total dose). Sperm production began to decline after nine weeks following the initial exposure and all bulls

were aspermic by the sixteenth week. After ten weeks of aspermia, a gradual recovery began. Groups II and III were unilaterally castrated at 18 and 24 months and Group IV at 12 and 18 months following the initial exposures. During the five-week period preceeding the final castration, sperm production of Groups II, III, and IV was 28, 57, and 68 per cent, respectively, of the control level.

Testes of bulls in Group III had a higher per cent of seminiferous tubules producing sperm than did the testes from Groups II and IV. Based on sperm production and histological data, two doses of 50 r per week were more deleterious than a single dose of 100 r per week. An accumulated dose of 1,100 r was no more deleterious than 600 r when both were delivered at the same rate. Although histological studies showed evidence of severe damage to the seminiferous epithelium, the irradiated bulls were producing sufficient numbers of motile and morphologically normal sperm that it could be assumed that they would have been fertile.

Gillette and Corwin (16) estimated the time periods for various segments of spermatogenesis by observing the changes in bull semen after X-irradiation of the testicles. The most severe alterations were observed in ejaculated semen 14 weeks after irradiation. It was estimated that, on the average, nine weeks were required for development of spermatozoa from spermatogonia, three weeks for passage of spermatozoa through the

testes, and two weeks for passage of spermatozoa through the epididymis. The rate and degree of recovery after this period is related to the type and extent of original damage to spermatogonia. Recovery from damage was practically complete by 30 weeks after irradiation except for those treatments of 800 r or more.

Other factors affecting semen quality after freezing. Several other factors not discussed above have been evaluated to determine their effects on semen quality after freezing.

Lanz, et al. (21) tested various levels of cholesterol, diolein, triolein, milk fat triglycerides, and lecithin to determine if any of them exhibited a protective effect during the freezing process. Only lecithin protected the cells during freezing and thawing and cholesterol reduced motility.

Kampschmidt, et al. (20) extracted the lipoprotein portion from egg yolk and tested it for cold shock protection. The principle lipoproteins in egg yolk are cephalin and lecithin. These lipoproteins obtained from egg yolk or a variety of other sources were effective in protecting bull spermatozoa from cold shock. The protection from temperature shock in the presence of phospholipids, was more effective in citrate or phosphate solutions than in solutions of non-electrolytes such as glucose.

Bialy, et al. (5) found that adding lipoproteins at the 5 per cent level significantly increased motility and livability when compared to the control to which no lipoprotein was added.

Pickett, et al. (33) compared 20 and 30 million progressively motile spermatozoa per milliliter (ml.). A slightly higher but non-significant difference in conception rate in favor of 30 million per ml. was found when a 60-90 day nonreturn rate was used as a basis for evaluation.

Buch, et al. (7) conducted a study of the differences in freezability of semen among bulls and among different inbred lines. A marked difference was observed among bulls in the ability of their spermatozoa to withstand freezing, although there were no significant differences prior to freezing. No line differences were observed. Although the rate of decline in motility of spermatozoa during storage at the temperature used in this study differed among bulls, the per cent of motile sperm after 12 weeks of storage generally could be predicted immediately after freezing. A highly significant decline in the per cent survival of spermatozoa was observed from period to period with the highest rate of decline occurring during the first two weeks after freezing.

Sullivan and Mixner (51) studied the effects of length of storage time upon rate of decline of sperm motility. With increasing time of storage, the mean motility of the semen

declined linearly. For every three months of storage in dry ice-alcohol, the motility of the semen decreased 2.02 percentage units.

Summary. Whole-body irradiation in sub-lethal amounts causes a period of aspermia in all bulls with some extent of recovery occurring eventually in most bulls. Local irradiation to the testes causes greater damage to the testes and a lower percentage of recovery or no recovery usually follows a period of aspermia.

It is believed that most of the damage caused during freezing is due to the freezing out of water from the diluent and leaving the cells exposed to hypertonic salt solutions too long at high sub-zero temperatures. Glycerol and certain phospholipids, namely lecithin and cephalin, help protect spermatozoa against hypertonic salt solution during freezing.

Optimum glycerol levels in the diluent vary with the diluent used. Glycerol levels of approximately 7 per cent have been found to be satisfactory in a yolk-citrate diluent and levels of approximately 10 per cent are best in milk diluents.

Glycerol equilibration times between 4 and 18 hours have been found to be satisfactory for freezing bull semen. There is some indication that the optimum equilibration time may vary between bulls (29). There is also evidence that there is a difference in the ability of semen from different bulls to survive freezing (7).

CHAPTER III

EXPERIMENTAL PROCEDURE

An experiment was conducted in the Dairy Department of the University of Tennessee to determine the optimum glycerol levels and equilibration times and the importance of interactions of bulls with various glycerol levels, equilibration times, and to estimate the sources of variability in the survival of spermatozoa from different bulls during the freezing process.

The UT-AEC Laboratory made available 26 bulls ranging in age from about 14 to 18 months for semen studies. These bulls were being used concurrently in a study to determine the effects of gamma irradiation at varying levels at various ages on sperm production and testis histology. Since the fact that irradiation will result in diminished sperm output has been well established, these bulls were collected weekly for a two week period to determine which bulls were producing sufficient sperm to be used in this experiment. Ten bulls produced in excess of 1.2 billion sperm which was arbitrarily established as the minimum level sufficient for the study. Four of the bulls were non-irradiated controls. The other 6 bulls were irradiated at varying ages up to 244 days with irradiation levels ranging from 300 r to 800 r.

The semen from these ten bulls was used in a split ejaculate design to determine the effects of various glycerol levels, various equilibration times, and their interaction on the ability of spermatozoa to survive the freezing process. The treatments were as follows:

I. Six per cent glycerol and four hours equilibration time;

II. Seven per cent glycerol and four hours equilibration time;

III. Eight per cent glycerol and four hours equilibration time;

IV. Six per cent glycerol and sixteen hours equilibration time;

V. Seven per cent glycerol and sixteen hours equilibration time;

VI. Eight per cent glycerol and sixteen hours equilibration time.

Preparation of diluents. The same basic diluent, egg yolk-citrate, was used for the entire experiment.

The egg yolk-citrate was prepared by adding 2.9 grams of sodium citrate dihydrate to 100 ml. of glass double distilled water. The sodium citrate was thoroughly dissolved and this buffer solution was stored in a glass stoppered bottle in a refrigerator until used. The diluent was made on the day of

collection by adding one part of egg yolk to four parts of the buffer solution.

The egg yolk was separated from the white with an egg separator and rolled on filter paper to remove any remaining white that was left by the separator. The yolk was punctured and the internal portion was used in making the diluent.

The experiment involved three levels of glycerol and two glycerol equilibration periods. The diluents used for freezing were made by dividing the diluent for each level of glycerol into two equal portions. One portion was used for the initial dilution of the semen and either 12, 14, or 16 per cent by volume of the other portion was removed and replaced with glycerol so that after the final dilution with the glycerol portion, the semen samples contained either 6, 7, or 8 per cent glycerol.

Collection, preparation, and cooling of semen. The semen used in this experiment was obtained from a group of Hereford and Hereford-Angus crossbred bulls at the UT-AEC Laboratory in Oak Ridge. The semen was collected using the electro-ejaculation technique. Two ejaculates were collected from each bull weekly for three weeks. A colorimeter (Klett-Summerson) reading was taken on each ejaculate immediately after collection for the purpose of estimating the number of sperm present. The colorimeter reading was made on a one ml. sample of the semen diluted

at the rate of 1:40 or 1:10 in 2.8 per cent sodium citrate. The per cent progressive motility was also estimated immediately after collection under a microscope. At this point, if the best ejaculate contained sufficient progressively motile sperm cells to make at least 40 ml. of diluted semen containing approximately 30 million progressively motile spermatozoa per ml., the semen was diluted at the rate of one part semen to two parts diluent and placed in a water bath at 35°C. The semen and water bath were placed in a refrigerator at 5°C. After all semen had been collected, it was transferred from the refrigerator to a styrene box maintained at approximately 5°C. by crushed ice and transported from the Oak Ridge laboratory to the main laboratory at the Dairy Department in Knoxville and again placed in a refrigerator at 5°C.

The final dilution rate was calculated for each sample and the semen was diluted to one-half the final volume at approximately 25°C. The cooling process was continued until the semen reached 5°C. which required a total of about 4.5 hours.

Glycerol addition and equilibration. The glycerol portion of the diluent was added in 4 equal portions at 15-minute intervals. The semen was allowed to equilibrate for four hours during which that portion of semen used for Treatments I, II, and III was placed in one milliliter glass ampules and sealed. At the

end of the four-hour equilibration period the semen used for Treatments I, II, and III was frozen to -79°C. in a dry ice-alcohol bath. The frozen semen was transferred to a dry ice-alcohol storage freezer immediately after freezing.

The portion of semen used for Treatments IV, V, and VI was allowed to equilibrate for an additional 12 hours for a total equilibration time of 16 hours during which the remainder of the semen was placed in one milliliter glass ampules and sealed. The semen was frozen at the end of the 16-hour equilibration period and transferred to the storage freezer described above. All freezing was done according to the freezing schedule outlined in Table I.

Semen evaluation. In addition to initial motility rating, progressive motility was estimated immediately prior to freezing, immediately after freezing, and at one, two, three, and four weeks after freezing. A minimum of ten ampules of each treatment was frozen to provide two ampules for evaluation at each of the post-freezing periods. The motility of two separate samples from each of two ampules was estimated for each treatment at each observation period, and a single average motility value was recorded for each treatment. Motility ratings were estimated to the nearest 5 per cent. All semen was thawed in ice water at approximately 5°C.

TABLE I

TIME SCHEDULE FOLLOWED FOR FREEZING BULL SEMEN WITH DRY ICE-ALCOHOL

Time Since Beginning	Temperature	Rate of Deline of Temperature
	(degrees centrigrade)	(degrees per minute)
0	+ 5	1/2
20	- 5	
20	- 5	1
30	-15	
30	-15	2
38	-31	
38	-31	4
39	-35	
39	-35	5
47	-75	

Since 10 ampules of semen were needed for each treatment and 6 treatments were used, at least 60 ampules of semen were needed from each bull. However, if as many as 40 ampules per bull could be obtained from a collection, the bull was included in the experiment. For those bulls that produced only 40 ampules of semen on the first week, Treatments III and VI were omitted. All bulls produced 60 ampules on the second week and for those bulls that produced only 40 ampules on the third week, Treatments I and IV were omitted. The pattern of omitting different treatments on different weeks was to permit at least two replicates of all treatments.

CHAPTER IV

RESULTS AND DISCUSSION

Semen production during the preliminary collection period is summarized in Table II. Semen volume was essentially equal for the control and irradiated bulls. Mean sperm production for ejaculate one, 4,059 X 10^6 for the controls was significantly higher ($P<0.05$) than 1,322 X 10^6 for the irradiated bulls. Sperm production for the controls was not significantly higher than for the irradiated bulls for Ejaculate II. Sperm motility for Ejaculate I was higher for the controls, 71.3 per cent, than for the irradiated bulls, 38.2 per cent. Sperm motility for Ejaculate II was approximately the same as for Ejaculate I. The suggestion from the above data that irradiation reduced the number of spermatozoa produced agrees with earlier findings (10,11). Sperm motility ratings were considerably lower for the irradiated than non-irradiated bulls because several bulls were completely sterile and motility ratings of zero were assigned to those bulls.

After the preliminary collection period, all bulls that did not produce a minimum of 1.2 billion spermatozoa in the best ejaculate were eliminated from the study. Only ten bulls produced at least the minimum number of spermatozoa required for this experiment.

TABLE II

MEANS AND STANDARD DEVIATIONS OF EJACULATE CHARACTERISTICS FROM PRELIMINARY COLLECTIONS

	Mean	Standard Deviation
Ejaculate 1		
Volume in ml.	9.9	1.5
Control	9.7	2.5
Irradiated	10.0	1.4
Total Sperm X 10^6	1,744	2,165
Control	4,059	2,514
Irradiated	1,322	1,863
Progressive Motility Ratings	43.3%	34.3
Control	71.3%	11.8
Irradiated	38.2%	34.7
Ejaculate 2		
Volume in ml.	10.1	1.3
Control	10.1	0.9
Irradiated	10.1	1.5
Total Sperm X 10^6	1,486	1,667
Control	2,468	2,806
Irradiated	1,303	1,633
Progressive Motility Ratings	40.6%	24.7
Control	73.8%	12.5
Irradiated	39.5%	30.9

Semen was collected and frozen from the remaining ten bulls once weekly for three weeks. Only the best ejaculate was used each week. Mean initial volume, concentration, and progressive motility ratings were 10.4 ml., 398×10^6, and 67.9 per cent, respectively, as shown in Table III. A slight progressive decrease in motility before freezing was observed as glycerol levels increased from 6 to 8 per cent. A decrease of approximately 7 per cent in progressive motility ratings before freezing was noted when equilibration time was increased from 4 to 16 hours.

Treatment and bull differences were highly significant ($P<0.01$) as shown in Table IV. The collection periods were not significantly different, but the interactions between collection and treatments and collection and bulls were highly significant ($P<0.01$). The interaction between treatments and bulls was also significant ($P<0.05$). A further breakdown of the treatment effects indicated that glycerol equilibration time was highly significant ($P<0.01$) and that glycerol levels were not significant.

A comparison of the progressive motility ratings of the various treatments immediately post-freezing (Table III) indicates that the 7 per cent glycerol level had the highest progressive motility ratings when equilibrated for 16 hours and that the 8 per cent level is better than the 6 per cent glycerol level

TABLE III

MEANS AND STANDARD DEVIATIONS OF THE EJACULATE CHARACTERISTICS AND THE PROGRESSIVE MOTILITY RATINGS BEFORE FREEZING, AFTER FREEZING, AND AFTER FOUR WEEKS OF STORAGE

	Mean	Standard Deviation
Initial Volume Per Ejaculate	10.4 ml.	1.3
Initial Sperm Concentration	398 X 10^6	149.1
Initial Per Cent Motile Sperm	67.9	9.3
Treatment I		
Pre-Freezing	64.4%	7.7
Post-Freezing	30.3%	7.9
Four Weeks Post-Freezing	23.1%	7.9
Treatment II		
Pre-Freezing	63.8%	9.2
Post-Freezing	33.8%	9.7
Four Weeks Post-Freezing	23.6%	7.8
Treatment III		
Pre-Freezing	63.7%	9.1
Post-Freezing	33.3%	7.8
Four Weeks Post-Freezing	23.8%	6.8
Treatment IV		
Pre-Freezing	56.9%	6.9
Post-Freezing	26.4%	10.5
Four Weeks Post-Freezing	18.6%	11.3
Treatment V		
Pre-Freezing	56.7%	10.4
Post-Freezing	37.0%	10.2
Four Weeks Post-Freezing	23.0%	10.4
Treatment VI		
Pre-Freezing	56.4%	10.9
Post-Freezing	32.2%	10.9
Four Weeks Post-Freezing	27.3%	11.1

TABLE IV

ANALYSIS OF VARIANCE OF SPERM MOTILITY BEFORE FREEZING

Source	DF	Variance	F Value
Total	142		
Among Collection Periods	2	684.00	2.51
Among Treatments	5	423.60	18.14[c]
Among Glycerol Levels	2	15.00	0.64
Among Equilibration Times	1	2,072.00	88.74[c]
Glycerol X Equilibration	2	8.00	0.61
Among Bulls	9	300.33	22.93[c]
Collection X Treatment	10	74.00	5.65[c]
Collection X Bulls	18	272.89	20.84[c]
Treatment X Bulls	45	23.36	1.78[b]
Error	53	13.09	

[b]Probability < 0.05.

[c]Probability < 0.01.

for either equilibration time. Higher motility ratings were obtained with the 7 per cent glycerol level when the semen was equilibrated for 16 hours rather than 4 hours. However, both the 6 and 8 per cent glycerol levels gave higher motility ratings when equilibrated only four hours.

Treatment and bull differences were highly significant at the post-freezing examination ($P < 0.01$) but collection period differences were not significant (Table V). The interaction between collection and bulls was also highly significant ($P < 0.01$), but the other interactions were not significantly different. A further breakdown of the treatment effects indicated that differences due to both glycerol levels and equilibration times were highly significant ($P < 0.01$).

The observation that the 7 per cent glycerol level is better than either the 6 or 8 per cent level agrees with data obtained by several workers who used an egg yolk-citrate diluter (12, 44, 49). Research reports indicate that a rather wide range of equilibration times, 0 to 18 hours, are all satisfactory. However, a large number of the reported studies favor the shorter equilibration times, 8 hours or less, (8, 24, 27, 28, 29, 31, 36, 50, 54) than for the longer equilibration times, 12 hours or more, (9, 44, 45). The results obtained in this experiment agree with the latter studies. No absolute reason can be established for the fact that 16 hours of equilibration

TABLE V

ANALYSIS OF VARIANCE OF SPERM MOTILITY
IMMEDIATELY POST-FREEZING

Source	DF	Variance	F Value
Total	142		
Among Collection Periods	2	87.00	0.52
Among Treatments	5	206.40	5.21[c]
Among Glycerol Levels	2	230.00	5.80[c]
Among Equilibration Times	1	466.00	11.75[c]
Glycerol X Equilibration	2	34.00	1.01
Among Bulls	9	626.44	18.75[c]
Collection X Treatment	10	25.20	0.75
Collection X Bulls	18	165.83	4.96[c]
Treatment X Bulls	45	39.64	1.18
Error	53	33.37	

[c]Probability < 0.01.

gave better results than 4 hours. However, one difference between this study and others that have been published is worthy of mentioning. Most of the bulls used in the studies that have been published were in regular use at bull studs and thus had been selected for their ability to produce semen capable of withstanding freezing, whereas, the bulls used in this experiment were not selected for semen survival or fertility.

Progressive motility ratings after four weeks of storage indicated that the 8 per cent glycerol level maintained higher motility after a period of storage in dry ice-alcohol than either the 6 or 7 per cent levels. Data presented in Table III (page 25) also indicates that the 16-hour equilibration period with 8 per cent glycerol was better than a 4-hour equilibration time. Survival ability through the storage period was approximately equal for 4 and 16 hours of equilibration with the 7 per cent glycerol level. However, when 6 per cent glycerol was used, best survival was obtained with a 4-hour equilibration time.

These results were with semen stored in dry ice-alcohol. Pickett, et al. (35) reported a drop in mean motility ratings from 44.0 per cent to 33.1 per cent after three weeks of storage in dry ice-alcohol (-79°C.). Pickett, et al. (35) also reported that semen stored in liquid nitrogen (-196°C.) declined only slightly in motility, from 44.0 to 42.5 per cent. Thus the relationship of glycerol levels used in this study might be different in semen stored at -196°C.

Differences in motility between treatments and collection periods for the four-weeks post-freezing evaluation were significant ($P<0.10$) as indicated in Table VI. Differences between bulls were highly significant ($P<0.01$). All of the interactions were also highly significant ($P<0.01$). A further breakdown of treatments indicated that the differences in glycerol levels were significant ($P<0.05$) and that differences in equilibration times were not significant. Progressive motility ratings after four weeks of storage were about equal for all levels of glycerol when a 4-hour equilibration interval was used; but when a 16-hour equilibration interval was used, motility after four weeks of storage increased as glycerol levels increased. The interaction observed between glycerol levels and equilibration time was in accordance with that observed by Roussell, et al. (39).

A conflict exists between data collected immediately after freezing and after four weeks of storage as to the best level of glycerol. Post-freezing results indicated that the 7 per cent glycerol level was superior, but the results after four weeks of storage indicated that the 8 per cent glycerol level yielded higher progressive motility ratings. Sixteen hours of equilibration were better at both times. Rapatz (38) believes that much of the damage to sperm cells during storage may be due to the fact that frozen semen is not stable and that the ice crystals may change in size and shape during storage. An explanation as

TABLE VI

ANALYSIS OF VARIANCE OF SPERM MOTILITY AFTER FOUR WEEKS OF STORAGE

Source	DF	Variance	F Value
Total	140		
Among Collection Periods	2	613.50	5.27[a]
Among Treatments	5	174.00	2.45[a]
Among Glycerol Levels	2	244.50	3.44[b]
Among Equilibration Times	1	12.00	0.17
Glycerol X Equilibration	2	184.50	8.59[c]
Among Bulls	9	529.56	24.61[c]
Collection X Treatment	10	78.30	3.54[c]
Collection X Bulls	18	116.39	5.42[c]
Treatment X Bulls	45	71.07	3.30[c]
Error	51	21.47	

[a]Probability $<$ 0.10.

[b]Probability $<$ 0.05.

[c]Probability $<$ 0.01.

to the cause of the above stated conflict as to the best glycerol levels is that although the 7 per cent glycerol level may be sufficient to prevent damage to the sperm cells during the freezing process, it may not be sufficient to prevent the recrystalization described by Rapatz (38).

The components of variance attributable to various factors are summarized in Table VII. The major source of variation among the progressive motility ratings before freezing was the interaction between collection periods and bulls, 61.5 per cent, with a somewhat random distribution of the remainder of the variation among all other factors. However, when pre-freezing motility was analyzed within irradiation levels, the distribution of the variation was somewhat different between the two groups. Most of the variation in the controls was contributed by the difference in treatments, collection periods, and bulls, and the interaction between collection periods and bulls. The variation in the irradiated bulls was primarily due to the interaction between collection periods and bulls, with differences in treatments and the interaction between treatments and collection periods contributing an approximately equal but small amount of the total variation.

Post-freezing variations were due primarily to bull differences and the interaction between time periods and bulls. Treatment differences also contributed a small amount of the

TABLE VII

COMPONENTS OF VARIANCE ATTRIBUTABLE TO DIFFERENT FACTORS AT BOTH PRE-FREEZING AND POST-FREEZING

	$\sigma^2_{Tr^a}$	$\sigma^2_{C^b}$	$\sigma^2_{B^c}$	$\sigma^2_{Tr^a \times C^c}$	$\sigma^2_{Tr^a \times B^c}$	$\sigma^2_{C^b \times B^c}$
	(per cent)	(per cent)	(per cent)	(per cent)	(per cent)	(per cent)
Pre-Freezing	16.06	7.57	1.35	8.64	4.85	61.49
Controls	21.80	20.27	13.47	5.90	0.43	38.10
Irradiated	11.56	0.00	0.00	11.01	3.44	73.97
Post-Freezing	10.11	0.00	45.92	0.00	3.80	40.16
Controls	17.61	0.00	0.87	0.00	5.19	76.31
Irradiated	4.65	1.65	63.38	0.00	4.47	25.83

[a]Treatments

[b]Collections

[c]Bulls

variation. When post-freezing variations were broken down into the contributions of the control and irradiated bulls, distribution of variations between the two groups was different. Most of the variations in the controls could be attributed to treatment differences and the interaction between collection periods and bulls, whereas, for the irradiated bulls, bull differences and the interaction between collection periods and bulls contributed most of the variation.

Evidence stated above indicates that irradiation may affect the ability of spermatozoa to survive the freezing process since the variation attributable to bulls was zero for the irradiated bulls before freezing and 63.4 per cent post-freezing.

Decline in motility during freezing and storage. The mean decline for all treatments during freezing and storage are presented in Table VIII. The mean decline in sperm motility during the freezing process for Treatments I, II, III, IV, V, and VI was 34.7, 30.0, 30.3, 30.6, 28.3, and 24.1 per cent, respectively, and mean decline during storage was 7.2, 10.6, 9.6, 7.8, 6.0, and 4.9 for Treatments I, II, III, IV, V, and VI, respectively.

Variations in decline in motility during freezing were analyzed statistically and the results are shown in Table IX. Differences in treatments, collection periods, and bulls were highly significant ($P < 0.01$). None of the interaction terms

TABLE VIII

DECLINE IN PROGRESSIVE MOTILITY RATING OF BOVINE SEMEN DURING FREEZING AND STORAGE

	Mean	Standard Deviation
Treatment I		
Decline During Freezing	34.7%	9.0
Decline During Storage	7.2%	8.5
Treatment II		
Decline During Freezing	30.0%	11.0
Decline During Storage	10.6%	9.7
Treatment III		
Decline During Freezing	30.3%	10.6
Decline During Storage	9.6%	7.3
Treatment IV		
Decline During Freezing	30.6%	9.5
Decline During Storage	7.8%	11.1
Treatment V		
Decline During Freezing	28.3%	11.7
Decline During Storage	6.0%	8.7
Treatment VI		
Decline During Freezing	24.1%	10.8
Decline During Storage	4.9%	8.0

TABLE IX

ANALYSIS OF VARIANCE OF DECLINE IN PROGRESSIVE MOTILITY OF BOVINE SEMEN DURING FREEZING

Source	DF	Variance	F Value
Total	142		
Among Collection Periods	2	425.78	5.69[c]
Among Treatments	5	266.20	5.03[c]
Among Glycerol Levels	2	333.00	6.45[c]
Among Equilibration Times	1	530.00	10.27[c]
Glycerol X Equilibration	2	67.50	0.89
Among Bulls	9	1,066.50	8.53[c]
Collection X Treatments	10	52.93	0.69
Collection X Bulls	18	125.00	1.60
Treatments X Bulls	45	51.60	0.70
Error	53	75.11	

[c]Probability < 0.01.

was significant. When treatment differences were separated into glycerol levels and equilibration times, both were highly significant ($P < 0.01$).

When the decline was examined by irradiation levels, a slightly different pattern of variation was noted between the two groups as indicated in Tables X and XI. For the irradiated bulls, differences in collection periods and bulls, and the interaction between collection and treatment and collection and bulls were highly significant ($P < 0.01$). Treatment differences and the interaction between treatments and bulls were also significant ($P < 0.10$). For the control bulls, differences in collection periods and treatments were highly significant ($P < 0.01$). Bull differences were also significant ($P < 0.05$), but none of the interaction terms was significant. Evidence stated above indicates that irradiation may magnify the importance of the interactions during the freezing process.

The decline during a four-week storage period was analyzed statistically and the results are presented in Table XII. Bull differences were highly significant ($P < 0.01$). Differences in collection periods were also significant ($P < 0.05$), but treatment differences were not significant. None of the interaction terms was significant. When treatments were broken down into glycerol levels and equilibration times, differences due to glycerol levels were not significant but equilibration times were significantly different ($P < 0.05$).

TABLE X

ANALYSIS OF VARIANCE OF DECLINE IN PROGRESSIVE MOTILITY OF BOVINE SEMEN DURING FREEZING--IRRADIATED BULLS

Source	DF	Variance	F Value
Total	85		
Among Collection Periods	2	1,019.00	8.08[c]
Among Treatments	5	141.00	2.29[a]
Among Bulls	5	551.80	16.98[c]
Collection X Treatment	10	171.20	5.27[c]
Collection X Bulls	10	126.10	3.88[c]
Treatment X Bulls	25	61.52	1.89[a]
Error	28	32.50	

[a]Probability < 0.10.

[c]Probability < 0.01.

TABLE XI

ANALYSIS OF VARIANCE OF DECLINE IN PROGRESSIVE MOTILITY OF BOVINE SEMEN DURING STORAGE--CONTROL BULLS

Source	DF	Variance	F Value
Total	56		
Among Collection Periods	2	349.50	5.45[c]
Among Treatments	5	165.00	6.73[c]
Among Bulls	3	332.33	3.41[b]
Collection X Treatments	10	68.80	0.70
Collection X Bulls	6	64.17	0.66
Treatment X Bulls	15	24.53	0.25
Error	15	97.60	

[b]Probability < 0.05.

[c]Probability < 0.01.

TABLE XII

ANALYSIS OF VARIANCE OF DECLINE IN PROGRESSIVE MOTILITY OF BOVINE SEMEN DURING STORAGE

Source	DF	Variance	F Value
Total	140		
Among Collection Periods	2	216.11	2.39[b]
Among Treatments	5	106.60	2.57
Among Glycerol Levels	2	12.50	0.30
Among Equilibration	1	296.00	7.15[b]
Glycerol X Equilibration	2	106.00	1.17
Among Bulls	9	221.00	4.30[c]
Collection X Treatments	10	53.00	0.58
Collection X Bulls	18	51.39	0.57
Treatments X Bulls	45	41.42	0.46
Error	51	90.54	

[b]Probability < 0.05.

[c]Probability < 0.01.

The decline in progressive motility during storage was found to vary among bulls by Buch, et al. (7). The greatest decline occurred during the first two weeks after freezing (7). Sullivan and Mixner (51) observed that for every three months of storage in dry ice-alcohol, the motility of semen decreased 2.02 percentage units.

The rate of decline in progressive motility ratings during storage for semen used in this experiment was above that observed by Sullivan and Mixner (51) but the fact that the greatest decline was found to be during the first two weeks cannot be overlooked (7). If this experiment had involved a longer storage time, a rate of decline more nearly like that of Sullivan and Mixner (51) may have been achieved.

Sufficient evidence is presented above to indicate that 8 per cent glycerol and 16 hours of equilibration are the optimum values of those tested for use with an egg yolk-citrate diluent when motility after storage or per cent decline during freezing and storage is used as a basis for drawing conclusions.

All semen evaluations in this experiment were based on progressive motility ratings. However, the ultimate test of semen quality is the fertility of the semen and in this study it was not possible to test this criteria.

CHAPTER V

SUMMARY AND CONCLUSIONS

An experiment was conducted in the Dairy Department of the University of Tennessee to determine the optimum glycerol levels and equilibration times and the importance of interactions of bulls with various glycerol levels, equilibration times, and to estimate the sources of variability in the survival of spermatozoa from different bulls during the freezing process.

Split ejaculates from ten bulls, six irradiated and four controls, at the UT-AEC Laboratory, Oak Ridge, were frozen. The prefreezing procedure was the same for all treatments with the exception of glycerol levels and equilibration time which varied according to the treatment schedule as follows:

I. Six per cent glycerol with four hours equilibration time;

II. Seven per cent glycerol with four hours equilibration time;

III. Eight per cent glycerol with four hours equilibration time;

IV. Six per cent glycerol with sixteen hours equilibration time;

V. Seven per cent glycerol with sixteen hours equilibration time;

VI. Eight per cent glycerol with sixteen hours equilibration time.

The basic diluent used consisted of 2.9 per cent sodium citrate, 20 per cent egg yolk, and either 6, 7, or 8 per cent glycerol. All semen was collected by the electro-ejaculation technique.

Semen evaluated for progressive motility after equilibration but before freezing indicated that a significantly smaller decline in motility ratings occurred with an equilibration time of 4 hours as compared to 16 hours. Treatment and bull differences were highly significant for progressive motility ratings before freezing. The interactions between bull and treatment, bull and collection periods, and treatment and collection periods were also significant.

A comparison of the progressive motility ratings of the various treatments immediately after freezing indicated that the 7 per cent glycerol level had the highest progressive motility rating when equilibrated for 4 or 16 hours and that 8 per cent glycerol was better than 6 per cent for either equilibration time. Higher motility ratings were obtained with 7 per cent glycerol when the semen was equilibrated for 16 hours rather than 4 hours. Treatment and bull differences and the interaction between collection and bulls were highly significant. All other factors were not significant. A further breakdown of the treatment effects indicated that differences due to both glycerol levels and equilibration times were highly significant.

Motility ratings after 4 weeks of storage indicated that the 8 per cent glycerol level maintained a higher progressive motility rating after a period of storage than either 6 or 7 per cent, and that 8 per cent glycerol was more effective with 16 hours of equilibration than with 4 hours of equilibration. Differences in bulls and all interactions were highly significant. Treatment effects, when considered in terms of glycerol levels and equilibration times, indicated that differences due to glycerol levels were significant, but that differences in equilibration times were not significant.

An analysis of the components of variance indicated that the interaction between collection periods and bull contributed a large portion of the variance at all times. In most cases, the remainder of the variance was primarily due to collection periods, treatments, and bulls. However, when comparing the sources of variation before and after freezing for the irradiated bulls, a large difference existed in the portion of the variation attributed to bulls, 0 and 63.4 per cent, respectively. This suggests that irradiation may affect the ability of spermatozoa to survive the freezing process.

The decline in motility ratings during freezing and storage followed approximately the same pattern as the motility ratings at the specified intervals except that the amount of decline was inversely related to the motility rating. The

treatment with the highest motility rating after four weeks of storage was accompanied by the smallest amount of total decline. When total amount of decline in progressive motility ratings from pre-freezing to four weeks after freezing were considered, observations showed that the amount of decline decreased progressively from Treatment I to Treatment VI, which pointed out that 8 per cent glycerol with an equilibration time of 16 hours was more satisfactory than any other treatment studied.

The reason for the conflict between post-freezing and after four weeks of storage as to the best level of glycerol may be explained by an observation of Rapatz (38) that semen frozen with glycerol is not stable and that ice crystals may change in size and shape, and thus damage the sperm cells. Seven per cent glycerol may be sufficient to protect the cells during the freezing process, but may not be sufficient to prevent damage during storage.

Since the bull interactions were statistically significant in most cases, indications are that the best method of freezing for semen of one bull may be unsatisfactory for semen from other bulls.

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APPENDIX

TABLE XIII

MEAN SPERM MOTILITY BY BULL BY SUBCLASS

Bull Identification Number	Observation Period	Treatments I	II	III	IV	V	VI
16	Prefreeze	62	62	60	55	57	55
	Post-freeze	28	34	37	32	30	34
	Four Weeks Post-freeze	32	25	19	18	28	29
34	Prefreeze	70	73	70	65	70	65
	Post-freeze	35	44	37	30	37	43
	Four Weeks Post-freeze	34	26	32	35	33	45
47	Prefreeze	65	67	67	50	50	53
	Post-freeze	34	35	31	19	21	29
	Four Weeks Post-freeze	21	25	24	15	18	28
110	Prefreeze	65	63	63	53	50	50
	Post-freeze	20	20	22	19	17	19
	Four Weeks Post-freeze	15	17	18	8	9	10
73	Prefreeze	60	65	60	60	60	60
	Post-freeze	33	38	30	8	18	33
	Four Weeks Post-freeze	20	20	13	2	5	15
79	Prefreeze	70	70	75	60	65	60
	Post-freeze	23	27	35	28	27	38
	Four Weeks Post-freeze	19	27	33	21	24	30
222	Prefreeze	70	62	65	65	60	58
	Post-freeze	30	37	35	29	32	33
	Four Weeks Post-freeze	19	20	23	13	23	24

TABLE XIII (continued)

Bull Identification Number	Observation Period	Treatments I	II	III	IV	V	VI
96	Prefreeze	67	70	68	62	62	62
	Post-freeze	35	41	42	40	43	45
	Four Weeks Post-freeze	24	22	26	28	29	38
188	Prefreeze	65	57	53	53	48	53
	Post-freeze	32	27	25	25	22	22
	Four Weeks Post-freeze	22	26	25	18	21	28
152	Prefreeze	60	57	57	53	55	57
	Post-freeze	37	39	39	23	33	35
	Four Weeks Post-freeze	24	28	29	25	31	35