The viability and fertility of stallion semen after extension and storage

by

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INTRODUCTION

Artificial insemination has a great deal of potential as a management tool in the equine breeding industry, but some aspects of equine artificial insemination need further research and development. One such area is the handling, extension and storage of semen after it has been collected. There is often a lag period of several hours between the time semen is collected and inseminated. It is thought by most authors involved with artificial insemination in the equine that an extender or diluent is needed during this lag period to protect and maintain the viability of the spermatozoa. A problem arises, however, in that there is little agreement as to what type of extender best serves this function. A great variety of extender formulas have been reported in the literature as being satisfactory.

This study attempts to evaluate certain individual extender ingredients on the basis of their ability to maintain the viability of equine spermatozoa, and to compare the effectiveness of certain extender formulas by means of microscopic observations and fertility trials.

REVIEW OF THE LITERATURE

Chemical Composition of Stallion Semen

Generally, the biochemical constituents of stallion seminal plasma are similar to those found in the semen of other domestic animals, but there are some unique features. Season of the year appears to significantly affect the concentrations of some of the seminal constituents of the stallion[37].

The large ampullary glands, unique to the horse, are responsible for the production of ergothionine, which accounts for a large portion of the non-protein sulfhydryl in stallion semen[17,28]. Ergothionine is highly reducing in nature and is responsible for protecting spermatozoa against oxidizing or peroxidizing agents. One report suggests that ergothionine levels are significantly affected by season with levels being higher in the early spring and fall[28], while another report indicates that the levels tend to remain constant throughout the year[17]. Levels of ergothionine in stallion semen range from 0.2 to 0.6 mg/ml[28].

The seminal vesicles are responsible for the citric acid content of the semen. The function of the citric acid is not completely known, but it may complex with calcium ions and act as a buffer[28]. Citric acid appears to be subject to seasonal fluctuations and is usually present in levels of below 1 mg/ml[28].

The seminal vesicles are also responsible for the production of the gel fraction of stallion semen. The quantity of gel produced varies considerably among stallions, with the season of the year, and with the frequency of ejaculation[42,51].

A highly characteristic feature of stallion semen is its very low level of fructose compared to the bull, boar and ram. It is difficult to understand this difference as both glucose and sorbitol (an intermediary product in the conversion of glucose to fructose) are present, and lactic acid (a by-product of glycolysis) appears in high levels[28]. The enzymes necessary for fructolysis are all present in adequate quantities, but in anaerobic conditions, the rate of fructolysis is low[28]. This low carbohydrate level in the stallion, compared to the bull, may exist because both fructose and gel are secreted by the seminal vesicles, and in the stallion, the gel is usually separated from the rest of the ejaculate before analysis[17]. In the bull there is no gel. Stallion spermatozoa may use lipid as an energy source, as cholesterol levels in stallion semen are much higher than in other domestic animals[28].

Another unique characteristic of stallion semen is the presence of glycerylphosphorylcholine (GPC) which is produced by the epididymus. The function of GPC is not completely understood. It may play a role in sperm maturation, or it may be involved in biosynthesis or metabolism of lipids in epididymal semen[28].

The protein content of stallion semen ranges from 20.1 mg/ml to 9.8 mg/ml[17]. Bull semen appears to have a much higher protein level than does stallion semen, and it is suggested that the species with higher levels of seminal plasma protein (bull, ram, goat and man) respond better to freezing than species with low seminal plasma protein levels (stallion, boar and turkey)[20].

The ejaculate of the stallion is divided into three fractions. The clear pre-sperm fraction has a high sodium chloride content. The sperm-rich fraction contains

a high concentration of spermatozoa, ergothionine, GPC, and trace amounts of sodium chloride and citric acid. The post sperm-rich fraction contains a high level of citric acid and very little spermatozoa, ergothionine and GPC[28].

Sperm Morphology

Stallion spermatozoa have the same general form as spermatozoa from other species. They consist of a single head containing the nuclear material, an acrosomal cap and a single flagellum or tail.

The head of an equine spermatozoa is somewhat elliptical in shape with the posterior end slightly thicker than the anterior end[13,48]. When stained it measures 6.62 $\pm$ 0.22 um in length, 3.26 $\pm$ 0.19 um in width at the widest part, and has an area of 16.28 $\pm$ 0.8 um^{2}[13]. Measurements of wet spermatozoa with phase contrast microscopy are significantly less than that of stained spermatozoa[13]. Slightly different measurements, length 7.0 $\pm$ 0.936 um, width 3.91 $\pm$ .225 um and area 16.54 $\pm$ 1.994 um^{2} have also been suggested[34]. Many abnormalities of the sperm head have been described in the equine, including micro and macro heads, and abnormal shapes. An incidence of 1.8% head abnormalities in sixty normally fertile stallions has been reported[5].

There is a nuclear membrane which surrounds the nuclear material contained in the head of the sperm cell. The anterior two-thirds of the head is also covered by the acrosome, which is a double membrane structure containing the acrosomal enzymes[13]. The posterior one-third of the head is covered by a distinct post-acrosomal cap[13]. The posterior border of the acrosome and the anterior edge of

the post-acrosomal cap lie very close together but are not continuous[13]. In the ram, bull and boar, the inner and outer acrosomal membranes near the posterior edge of the acrosome are very close together and form the equatorial segment visible with the light microscope as a half moon-shaped area in the middle of the head. This equatorial segment is not normally visible with the light microscope in the stallion[13]. Acrosomal defects do occur in the stallion, but the incidence is not reported.

The tail of equine spermatozoa is similar in structure to that of other species. It's attachment is often abaxial, and this is considered normal[5]. The mid-piece consists of the typical 9 + 9 + 2 fibrillar arrangement surrounded by a helical mitochondrial sheath, and is 9.83 ± 0.33 um long[34]. The main piece is approximately 44 um long, and the end piece is 2 to 3 um long[13]. Most abnormalities of the tail are secondary in nature and usually involve bending or coiling.

Like other species, immature equine spermatozoa contain a cytoplasmic droplet which moves down the tail and is lost as the sperm matures. Retention of this droplet occurs with spermatozoa immaturity. Proximal droplets occur in 4.2% of spermatozoa from normal stallions, and distal droplets in 7.2%[5].

The entire sperm cell, including the tail, is surrounded by a cell membrane or glycocalyx containing mucoplysaccharides[23]. This glycocalyx contains sialic acid, and it has been suggested that capacitation of sperm may include the elimination of sialic acid by specific enzymes in follicular fluid[23].

Semen Characteristics

The volume and concentration of stallion semen varies considerably between individual animals, much more so than in other species. The volume of raw semen ejaculated ranges from 30 to 250 ml[25]. In the normal animal, sperm concentration ranges from 100 x 10^6 to 300 x 10^6 cells/ml with progressive motility in 60-80% of the cells[46]. These parameters are all dependent upon season of the year, with most stallions showing lower semen volume, gel content, and concentration during the winter months[37,42,55].

The pH of stallion semen also seems to be quite variable. It ranges from 6.8 to 8.4[25,46]. Unlike other species, stallion semen tends to become alkaline with storage because of the low glucose levels, resulting in very little lactic acid accumulation[25].

Clumping or agglutination of sperm cells appears to be a normal phenomenon in the stallion, as well as the rabbit, guinea pig and boar[41,43,50]. The clumping usually involves the sperm heads, although tail to tail attachments are seen[43]. The concentration of electrolytes in the media may influence the degree of clumping, and the degree of clumping appears to be highly seasonal, probably due to changes in the chemical composition of the seminal plasma[43]. The effects of antibiotics on equine spermatozoa have been studied and head to head and tail to tail agglutination was noted when solutions of gentamicin sulfate and neomycin sulfate plus polymixin B sulfate were used[49]. The cause of agglutination in stallion semen is not really known, but it has been suggested that it is due to differences in surface properties of the plasma membrane over the acrosomal and post-acrosomal regions[43,56].

Although it is obvious agglutination of sperm cells interferes with their progressive motility, it is not known how it affects fertility. One study indicated that fertility rates are not affected[41].

The role of seminal plasma in stallion semen is confusing. There appears to be a factor present in stallion seminal plasma that has an adverse effect on spermatozoa. The factor responsible for this adverse effect is extremely unstable, and has been identified as being related to sulfhydryl compounds in the seminal fluid, which can be measured by the total non-protein sulfhydryl concentration[29]. Stallions vary according to the non-protein sulfhydryl content of their seminal plasma, and this can be correlated to fertility. A decline in fertility (conception rates of less than 55-60%) is reported if seminal plasma contains greater than 10 ug sulfhydryl/ml, and almost complete infertility if the levels exceed 20 ug/ml[22].

Some workers feel it is necessary to remove some of the seminal plasma in stallion semen if the concentration of spermatozoa is low, and that dilution of semen or removal of some seminal plasma is necessary for prolonged storage[36]. It was concluded that seminal plasma concentration, not extender concentration, was the dominant factor in obtaining maximum motility during storage. Plasma concentrations of 10-20% or dilution rates of 1:4 to 1:8, semen to extender, appear to provide maximum motility and fertility of stored stallion semen[39].

Centrifugation of semen is necessary for removal of seminal plasma, but this may cause extensive damage to sperm cells[15]. It has been reported, however, that the

fertility of stallion semen is unaffected by centrifugation of undiluted semen at 300-310 g (1500 r.p.m.) for 3-3.5 minutes[11,39].

Washing spermatozoa of any species can cause excessive sperm damage, but this can apparently be significantly decreased in the bull, ram and rabbit if the washing solution contains egg yolk[39]. Centrifugation in an egg yolk medium, however, seems to be extremely detrimental to the motility of stallion spermatozoa and the fertility of bull spermatozoa[39].

Semen Extenders

Stallion semen in the raw state has very little ability to withstand stresses such as temperature changes or storage[7]. This lack of resistance necessitates the use of extenders or diluents, which not only protect the semen, but also allow treatment of the semen with antibiotics and extension of the seminal volume[3,4,7,24,25,26].

The following is a list of requirements that an extender should meet in order to be successful in preserving the viability and fertility of stored semen[20,30]:

1. It must be near the isotonicity of blood and be capable of maintaining that approximate osmotic pressure during storage. Osmolarity of stallion semen is 302 mOs[41].

2. The extender must provide the minerals essential to the life of the spermatozoa. Calcium and magnesium ions added to the tissue culture media of spirochetes have

been shown to increase viability, and since the metabolism of these cells is similar to that of sperm cells, the addition of these ions to semen extenders may be beneficial[32]. However, some ions have a deleterious effect on sperm survival. Potassium and sodium have been shown to decrease the motility of bull and stallion spermatozoa, and excess or insufficient calcium ions also adversely affect motility[7,20].

3. It must have a pH of between 6 and 8, and have the buffering capacity to prevent deleterious shifts in pH. Stallion semen can tolerate a rise in pH fairly well, but is severely affected by a drop in pH[7,11]. Sodium citrate appears to be a better buffer in semen extenders than does sodium phosphate, sodium carbonate or potassium phosphate[7]. This may be because sodium citrate has been shown to be less highly ionizable than the others and consequently less harmful ions are released in solution.

4. Extenders must provide nutrients for both aerobic and anaerobic metabolism of spermatozoa. In most cases, glucose is added in concentrations that are close to isotonic levels. It has been suggested that, because stallion semen seems to have a low level of glycolytic activity, glucose may not be acting as an energy source, but may be acting as a buffer with a low degree of ionization[7]. Pyruvate, as an intermediate in glycolysis, has been added to spirochete tissue culture media for an alternate

energy source and may also be of benefit in semen extenders[32].

5. It must provide lipoproteins and/or lecithin to protect against cold shock. Egg yolk or milk products are most often used as sources of these materials[30].

Egg yolk appears not only to protect spermatozoa from drastic temperature changes, but also protects them from many types of adverse conditions, such as pH changes, osmotic pressure changes, and the accumulation of harmful substances[7]. Based on motility studies, egg yolk plus an isotonic solution of glucose has been suggested as the ideal extender[7]. A lipoprotein from egg yolk has been isolated that was superior to the egg yolk itself in maintaining the viability of spermatozoa[16].

Milk products contain a substance called lactinin that is harmful to spermatozoa, and that must be destroyed by heating to 95°C for 4-5 minutes[15,30]. Phospholipids appear to be the protective factors in milk[30].

6. The extender must be free of substances, bacterial products, or infectious organisms that are harmful to spermatozoa, the female reproductive tract, or the fertilization process. Most semen extenders contain some type of antibiotic to retard bacterial growth. The growth of bacteria in semen stored in sterile saline and in an extender containing antibiotics has been compared. There was a slight increase

in the number of bacteria in the saline after two hours and a 91% and 99% reduction of bacterial growth in the extended semen after fifteen minutes and two hours respectively[10,49]. Based on motility studies, it has been determined that levels of up to 2,500 ug/ml of gentamicin sulfate, lincomycin, nalidixic acid, polymyxin B sulfate and sodium penicillin, and 1 mg/ml of streptomycin were effective for control of bacterial growth without harmful effects on spermatozoa[3].

Attempts to freeze equine semen have led to the discovery that stallion semen, like boar semen, is very sensitive to the effects of glycerol[35]. Semen diluted in an extender containing 7% glycerol had a significant decrease in fertility compared to raw semen, and fresh semen extended without glycerol[35,41,43].

One group of workers has done fairly extensive studies on the comparison of different extender formulas[12,41,43,56]. The fertility of semen in two extender formulas; 2.4% Tris or .349% Tris in 45% glucose, 1.25% citric acid, 22.8% egg yolk, and 5.25% glycerol was compared to raw, unextended semen. The semen was diluted in 10 ml of extender and incubated at 38°C for one to two hours before insemination. Mares were inseminated with 500 x 10^6 motile sperm. It was found that these two Tris extenders depressed fertility significantly compared to raw semen, even though good motility was maintained, especially in the .349% Tris extender. Raw semen was then compared to semen extended in a cream-gel extender containing 88.7% half and half cream, and 1.3% gelatin, using the same protocol. The fertility of the

semen extended in cream-gel was not significantly different from that of raw semen. In further studies, four seminal treatment groups were compared: fresh extended semen in cream-gel extender, extended semen cooled for 2 hours at 5°C then warmed to 38°C, extended semen cooled for 2 hours then 7% glycerol added, and extended semen stored at 5°C for 24 hours. There was no significant difference in conception rates between fresh, extended semen and that stored for 2 hours at 5°C. There was, however, a significant decrease in the fertility of mares bred with semen containing 7% glycerol or semen stored for 24 hours. A study was then undertaken to compare the cream-gel extender to one containing heated skim milk, and one containing heated skim milk plus 1.3% gelatin. Only fresh extended semen was studied. Although there was statistically no significant difference in fertility, conception rates with extenders containing skim milk tended to be better.

Other breeding trials using skim milk extended semen stored at 5°C for up to 24 hours, and cream-gel extended semen stored at 5°C for up to 96 hours have been reported[24]. Ten of 14 mares conceived with 24 hour old semen, 12 of 14 with 48 hour old semen, 3 of 6 with 72 hour semen, and 2 of 3 with 96 hour old semen. However, the number of spermatozoa inseminated and the number of times the mares were bred was not mentioned. On another breeding farm, the use of fresh extended semen in a skim milk-gelatin extender resulted in good fertility, but again, insemination dose and frequency was not elaborated upon[11].

One group of researchers reported that semen stored at 5°C in cream-gel extender can retain its fertilizing capacity up to 3 days with conception rates equal

to those obtained with raw semen and natural service[1]. It was their opinion that milk product extenders are superior to egg yolk-sugar extenders.

An extender composed of 5% buttermilk and 5% glucose in distilled water has been evaluated[4]. Viability of stallion semen lasted up to four days at 4°C in a 1:4 dilution of this extender. Viability estimates were based solely on motility.

Egg yolk has been used extensively in stallion semen extenders. An 85% conception rate has been claimed with semen stored in an extender composed of 5% glucose and .8% egg yolk for 12 to 42 hours, and a 72% conception rate with semen stored in it for 48 hours[10]. Another extender containing 1.4% glucose and 25% egg yolk in sterilized cow's milk maintained 30% motility for 48 hours. Five mares bred with this semen conceived[24]. A more complex extender containing .1 gm gelatin, .05 gm sodium phosphate, .025 gm potassium chloride, .25 gm potassium sodium tartrate, .7 gm glycine, 4.5 gm glucose, 0.5 gm casein and 7% egg yolk in 100 ml distilled water has been reported to maintain excellent motility up to 48 hours[33]. Fertility was good after 2 to 8 hours of storage in this extender, but after 24 hours of storage, conception rates were very poor. Good conception rates were obtained using semen extended in an extender containing 5.7 gm glucose, 0.67 gm potassium sodium tartrate, 25 ml egg yolk and 25 ml distilled water[54]. This extended semen was stored at 17°C for 5 to 10 hours before usage.

The fertility of semen stored in what was termed hydrogen ion extenders containing multiple combinations of BIS-TRIS, BES, TRIS, glycyl-glycine, citric acid, sodium citrate and dextrose was evaluated[35]. One hundred million

sperm per insemination with fresh, extended semen was used and fertility results were suboptimal compared to un-extended semen.

Insemination Parameters

It appears that the volume of the insemination dose is not as important in the mare as it is in the sow. Volumes as low as 1.5 ml do not adversely affect fertility rates, although care must be taken during the insemination procedure to insure that losses are minimal[43,56]. Volumes as small as 0.6 ml have also been shown to provide normal conception rates[1,42]. Contrary results, however, suggest that at least 10 ml must be inseminated, as anything less appears to decrease conception rates[45].

The desired number of live sperm per insemination has not been completely determined, but it has been recommended that 100×10^6 is sufficient for semen used fresh, but semen subjected to any stress such as extension or storage should be inseminated at a dose of 500×10^6 live cells[56]. It has also been determined that 50×10^6 live sperm per insemination significantly depresses conception rates[56]. Another study indicates that conception rates with 80×10^6 live cells per insemination were better than with 40×10^6, but increasing the dose to 160×10^6 live cells did not further improve fertility[35].

Timing of insemination is important in the mare since her estral periods are relatively long and ovulation occurs close to the end of heat. Studies on the timing of insemination revealed that most fertilizations occurred in mares inseminated within 24 hours of ovulation[35]. Stallion semen appears to survive at least 48 hours in the mare's reproductive tract, and it is generally recommended that

a mare be bred at least every other day while she is in heat to insure maximum fertility rates with the most efficient use of the stallion[41,56].

METHODS AND MATERIALS

Experiment 1 - Evaluation of Extender Ingredients

This portion of the study was undertaken to determine if certain substances enhance the viability of equine semen under short term storage conditions, and to use these substances to formulate an extender.

Semen from four mature stallions (two Thoroughbreds, one Quarterhorse and one Arabian) was used for this experiment. The stallions were collected with a Missouri model artificial vagina using mares in heat as mount animals. After collection, any gel was removed from the sample using aspiration, and the semen was stored at 37°C until extension. All samples were evaluated on the basis of concentration, live:dead ratio and morphology, and were used in the study only if they appeared to be of good or excellent quality.

Protocol I - washing

Equine spermatozoa were treated in saline-tris buffer and verinol buffer (VB). In both cases, the spermatozoa were non-motile after one wash and centrifugation at 1500 r.p.m. for fifteen minutes. Semen was also washed in three different semen extenders: CGH-27, cream-gelatin (see Appendix I), and equine seminal plasma. They were centrifuged only once using a speed of 1500 r.p.m. for fifteen minutes. The samples were then diluted 1:1, 1:3 and 1:9 with the corresponding extender and stored at 37°C. Samples were examined every two hours and motility estimates made using a light microscope.

Protocol II - gelatin

Semen washed once in Ontario extender (see Appendix I) was diluted 1:17 in verinol buffer containing

.5%, 1%, or 2% gelatin. Verinol buffer alone was used as a control. These samples were stored at 35°C and motility estimates using a light microscope made at 0 time and 35 minutes.

Protocol III - glucose

An attempt was made to study the effects of glucose concentration on equine spermatozoa. One milliliter of raw semen was incubated with 450 mg of powdered glucose in 10 ml of verinol buffer at 5°C and 22°C to determine if glucose was beneficial to the cells. An attempt was made to determine the best concentration of glucose by incubating semen extended in 2% gel in verinol buffer with different dilutions of 5% dextrose in water. The ratios of 5% dextrose:2% gel in verinol buffer were 1:1, 2:1, 3:1, 1:2, 1:3. Semen was diluted in each of these formulas by a ratio of 1:10, and all samples incubated at both 22°C and 5°C. Motility estimates were made at 0 hours, 6 hours and 24 hours.

Pyruvate was examined as an alternate energy source. Pyruvate acid sodium was used as a source of pyruvate at a .01 M, .001 M and .0001 M concentration in 10 ml of 1% gelatin in verinol buffer. One ml of semen was added to each 10 ml aliquot and they were incubated at 35°C for 35 minutes before an estimation of motility was made.

Protocol IV - Tris

Tris buffer was evaluated as to its effect upon equine spermatozoa. Five different solutions were made: 2% gel in VB plus .01 M Tris, 2% gel in VB plus .01 M Tris and 500 mg glucose, 2% gel in VB plus .01 M Tris and 250 mg glucose, 2% gel in VB plus .01 M Tris and 500 mg glucose with 7% egg yolk, and 2% gel in VB plus .01 M Tris and 500 mg glucose with 7% cream. Semen was diluted with

these solutions at a rate of 1:10 and they were incubated at 5°C and 22°C. Motility estimates were taken at 0, 6, 24 and 48 hours.

Protocol V - formulation

Six different formulas were fabricated using information obtained in previous protocols and ingredients from other extenders reviewed in the literature:

1. 5.0 gm glucose
   2.2 gm sodium citrate
   2.0 gm gelatin
   1.3 gm sodium bicarbonate
   0.4 gm potassium chloride
   .12 gm Tris
   .02 gm gentamicin sulfate
   .001 M magnesium chloride
   .003 M calcium chloride
   q.s. 100 ml with distilled water

2. Formula #1 - with .24 gm Tris instead of .12 gm.

3. Formula #1 - with 3.5 gm glucose instead of 5.0 gm.

4. Formula #1 - with 3.5 gm glucose plus 5 ml of 0.1 M pyruvate acid sodium.

5. Formula #4 - with 10 ml of 0.1 M pyruvate acid sodium.

6. Formula #4 - with 13 ml of 0.1 M pyruvate acid sodium.

Semen was added to these extenders at a ratio of 1:10.

Samples in formulas 1. and 2. were incubated at 5°C and 22°C while samples in the other formulas were incubated at 22°C only. Motility estimates were made every 24 hours. The following formula gave the best results at room temperature (22°C):

3.5	gm glucose
2.2	gm sodium citrate
1.3	gm sodium bicarbonate
0.4	gm potassium chloride
.12	gm Tris
2.0	gm gelatin
.02	gm gentamicin sulfate
0.02	M pyruvate acid sodium
0.001	M magnesium chloride
0.003	M calcium chloride
q.s. to 100 ml with distilled water	

It was called AE extender. The original pH of this formula was 8.3. The pH was adjusted with the addition of 1M HCL in an attempt to determine at what pH the formula gave the best protection to the spermatozoa. Seven aliquots of the AE extender were prepared, each with a different pH reading, including pH 8, 7.7, 7.3., 7.0, 6.7, 6.4 and 6.1. Semen was extended 1:10 in each of these aliquots and stored at 22°C. Motility was estimated at 24 hours and the pH of the solution determined.

Semen was then extended in the AE formula at different rates to determine if the effect of the extender was better at a certain concentration. Dilution ratios of 1:1, 1:3, 1:5, 1:7 and 1:9 were used. Extended semen was stored at 22°C and motility estimated at 12, 24 and 48 hours.

Experiment 2 - Comparison of Extender Formulas

Experiment two was devised to compare different extender formulas reported in the literature in an attempt to find one with superior activity. Two formulas containing egg yolk, Ontario and CGH-27 (see Appendix I), and two formulas using a milk base, skim milk and cream-gelatin (see Appendix I), were used. These were compared to equine seminal plasma.

Semen was collected from the same stallions used in Experiment 1, using the same collection techniques. The criteria for evaluation and storage of the unextended samples were also the same as used for Experiment 1. Semen was mixed with extender only when the temperatures of each were the same. Dilution ratios (semen:extender) of 1:1, 1:4 and 1:9 were used. Three aliquots of each dilution were made and one was stored at 5°C, one at 30°C, and the other at 37°C. Motility estimates were made at 12 hours, 24 hours, and every 24 hours until less than 10% live sperm cells were present in the sample.

Experiment 3 - Fertility Trials

A fertility trial was undertaken to test the results of *in vitro* studies done in Experiments 1 and 2. Forty Shetland pony mares, two years of age and older, and of unknown fertility, were used for these trials. Both dry and foaling mares were included. The mares were housed in a dry lot and fed hay free choice. They were teased daily by leading an aggressive pony stallion through the lot. Mares were inseminated artificially on day 4 and day 6 of their heat but were eliminated from the trial if they stayed in heat longer than 9 days. Some mares were

bred during their foal heats. Mares which did not conceive were randomly reassigned to a new experimental group. The mares were examined for pregnancy 40-60 days post breeding if they did not return to estrus.

A mature Shetland pony stallion of known fertility was trained to mount a dummy and serve a miniature Missouri model artificial vagina (Fig. I). Semen was collected from him daily. Semen concentration was evaluated using a spectophotometer and the percent of live cells and motility estimated under a light microscope. The semen from this stallion was generally of good quality with an average concentration of between 200 x 10^6 and 500 x 10^6 cells per ml with 65% to 75% of the cells alive.

The two extenders used for fertility evaluations were CGH-27, stored at 5°C and AE formula, stored at 22°C. Raw, unstored semen was used as a control. Between 400 x 10^6 and 600 x 10^6 live sperm cells were inseminated at each breeding with a dilution rate of 1:6 to 1:10. Volume per insemination ranged from 2 to 15 mls.

Initially mares were randomly assigned to five treatment groups as they came into heat; CGH-27 and AE at 0-4 hours post extension and 48 hours post extension with raw semen as a control (Table I).

Table I: Number of mares in the five semen treatment groups

	0-4 hrs. storage	48 hrs. storage
CGH-27	10	10
AE	10	10
Control	5	

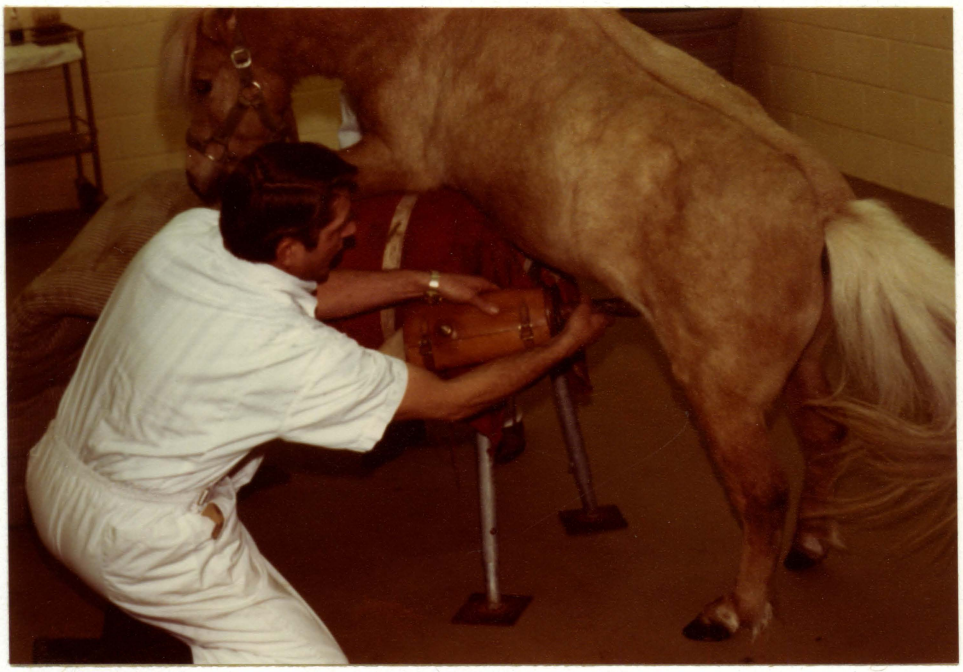

Fig. I: Collection of semen from the Shetland pony stallion mounted on a dummy

A twenty-four hour storage group was omitted because of the shortage of experimantal animals and because it was thought that if conception occurred at 48 hours it was likely to occur at 24 hours.

Results of the initial trial indicated that semen stored for 24 hours in each extender should be evaluated. Mares were randomly assigned to two more groups as they came into heat (Table II).

Table II: Number of mares in 24 hour semen treated groups

	24 hour storage
CGH-27	5
AE	6

This second trial was equally as unsuccessful as the first, so an attempt was made to see if any pregnancies could be caused by stored, extended equine semen. Cream-gelatin extender was chosen for this trial as it has been used extensively in the clinic at Iowa State University and it is known to have caused a substantial number of pregnancies when used at 0-4 hours and after storage at 5^{o}C for up to 24 hours. Six mares were inseminated with semen extended in cream-gelatin extender, according to the same criteria used for the other two extenders, and stored for 48 hours.

Experiment 4 - Morphological Evaluation of Extended Semen

Since the motility and fertility of semen extended in the CGH-27 extender containing egg yolk did not coincide, an attempt was made to determine if there were

any morphological changes associated with the sperm cells which interfered with their ability to fertilize the ovum. Semen was collected from the same pony stallion as was used for experiment 3 and was extended 1:9 with the CGH-27 extender. Two ml of the extended semen were added to 2 ml of 2% buffered formalin immediately. The remaining extended semen was stored at 4°C. Two ml aliquots of the stored, extended semen were added to 2 ml of 2% buffered formalin after 24 hours of storage and after 48 hours of storage. Raw semen mixed with 2% buffered formalin (1:1) was used as a control.

After all samples were collected, smears of each were made and stained with eosin analine dye. The identity of the samples was concealed and the morphology of the sperm cells on each slide examined using the oil immersion lens of a light microscope. One hundred cells were counted on each slide and were categorized into normal cells, cells with acrosomal defects, or cells with non-acrosomal defects, including cytoplasmic droplets, bent and coiled tails, and misshapen heads.

Semen was then collected from the pony stallion daily for three days. Semen was extended at a ratio of 1:9 in the CGH-27 extender each of these days and was stored at 4°C. Samples of 48 hour old semen, 24 hour old semen, fresh extended semen (0-4 hours), and raw semen were prepared for scanning election microscopy using the procedure outlined in Appendix II. The prepared specimens were examined with a JEOL 35 scanning electron microscope operating at 15 KV.

RESULTS

Experiment 1

Results of this experiment indicate that stallion semen is more sensitive to many of the procedures used in handling semen of the other domestic species. Washing of the spermatozoa in straight verinol buffer or a saline-Tris buffer resulted in death of all of the spermatozoa after the first spin. When semen was washed in CGH-27, cream-gelatin or equine seminal plasma, approximately 40% to 60% of the viability was maintained. When these washed samples were diluted with the corresponding extender and stored at 37°C, 3% to 5% viability was maintained for two hours in the equine seminal plasma and for four hours in the CGH-27 and cream-gelatin.

When gelatin was added to semen washed with Ontario extender (see Appendix I) in concentrations of .5%, 1% and 2% in VB, and stored at 37°C for 35 minutes, viability was maintained considerably better than in Verinol buffer alone (Table III).

Table III: Viability and motility of semen stored in different concentrations of gelatin

	0 Minutes	35 Minutes
2% gelatin	60% live good motility	40% live good motility
1% gelatin	60% live slow	40% live good motility
.5% gelatin	40% live slow	10% live slow
Verinol buffer alone	10% live slow	0

From these results it appears that 1% and 2% gelatin are superior to .5% gelatin in maintaining the viability of stored stallion semen.

Investigation into the effect of glucose on stored stallion semen indicated that it too had a beneficial effect. Four hundred and fifty mgs of glucose added to 10 ml of verinol buffer maintained the viability of stallion semen at 35% after 8 hours compared to 0 for the control. These results were identical at 5°C and 22°C. The effect of the different concentrations of glucose and the addition of 2% gelatin are summarized in Table IV. It appears that glucose in concentrations of 250 mg/10 ml to 750 mg/10 ml is beneficial to stallion semen stored at 5°C and 22°C, but a concentration of less than 125 mg/10 ml is not.

The work done with pyruvate seemed to indicate that in a .001 M concentration it had a beneficial effect on semen stored in verinol buffer plus 2% gelatin at 35°C. Motility was estimated at 60% in the .001 M concentration after 35 minutes storage compared to 35% to 40% with the control sample and samples containing .01 M and .0001 M pyruvate. The activity of sperm cells in solutions containing pyruvate was more vigorous than in those that did not contain pyruvate.

The addition of .01 M Tris to the gel/glucose did not have any detrimental effects on the motility of equine semen. The sample containing 2% gel, 500 mg glucose and .01 M Tris in verinol buffer maintained at least 30% motility at 5°C and 15% at 22°C for 24 hours.

The six formulas that were devised were evaluated according to their ability to maintain motility of stallion semen under storage conditions (Table V). Since Formulas #1 and #2 were superior at 22°C, the rest of the formulas

Table IV: Viability estimate of semen stored in different concentrations of dextrose

Ratio of 5% Dextrose to VB/2% gel	Viability at 5°C			Viability at 22°C		
	0 hrs.	8 hrs.	24 hrs.	0 hrs.	8 hrs.	24 hrs.
1:1	40%	20%	10%	40%	50%	5%
2:1	40%	30%	0	40%	50%	0
3:1	40%	40%	0	40%	45%	0
1:2	5%	5%	0	5%	10%	0
1:3	5%	5%	0	5%	5%	0

Table V: Evaluation of experimental extender formulas at 5°C and 22°C

Formula	5°C			22°C		
	0 hrs.	24 hrs.	48 hrs.	0 hrs.	24 hrs.	48 hrs.
#1	75%	0		75%	40%	10%
#2	75%	0		75%	30%	0
#3				70%	0	
#4				70%	60%	5%
#5				70%	60%	10%
#6				70%	50%	5%

were only evaluated at that temperature. There is little difference in the motility estimates after storage in these formulas, but #5 appears slightly better, so it was used as a basis for the final formulation called AE.

The AE extender contains:

3.5	gm glucose
2.2	gm sodium citrate
1.3	gm sodium bicarbonate
0.4	gm potassium chloride
0.12	gm Tris
2	gm gelatin
.02	gm gentamicin sulfate
0.02	M pyruvate acid sodium
0.001	M magnesium chloride
0.003	M calcium chloride
q.s.	100 ml with distilled water

Altering the pH of this solution showed that stallion semen survived well in a wide pH range (6.7-8.3) with the best results seen in samples with a pH of close to 7 or slightly alkaline. Acidic pH readings tended to result in poor sperm survival (Table VI).

The pH of each solution after 24 hours of storage did not change appreciably from its original pH. The pH of the AE extender was subsequently altered to approximately 7.3 with 1 M HCl when used in any further investigations.

The final investigation with the AE extender was to determine the ratio of semen to extender that maintained the best viability. This appeared to be 1:9, as 35% of sperm cells were still alive at 48 hours of storage at 22°C.

Table VI: Motility estimates in AE extender at multiple pH readings

pH	6.5 hrs.	24 hrs.	Final pH
8.3	30%	20%	7.7
7.7	30%	20%	7.45
7.3	50%	20%	7.3
7.0	40%	15%	7.2
6.7	40%	15%	6.9
6.4	30%	5%	6.9
6.1	20%	5%	6.6

Experiment 2

The results of the comparison studies involving extender formulas reported in the literature are summarized in Table VII. Equine seminal plasma was used as a control. It appears that all extenders evaluated were most successful at an incubation temperature of 5°C. All extenders tested seemed to function best at dilution ratios of between 1:4 and 1:9. The cream-gelatin extender and the CGH-27 were far superior to either the Ontario or skim milk extender. There seemed to be little difference between the cream-gelatin extender and CGH-27, although slightly better motility was evident at 48 hours storage at 5°C in the CGH-27 than in the cream-gelatin. The CGH-27 did maintain at least 10% of the motility of the sperm cells for up to 108 hours, whereas motility in the cream-gelatin extender had ceased at 72 hours.

Experiment 3

The CGH-27 was chosen to be compared to the AE extender as it appeared to be the most capable of maintaining the motility of stored equine semen of all the extenders examined, including the AE extender. It was stored at 5°C, whereas the AE extender was stored at 22°C.

The results of the fertility trials are summarized in Table VIII:

Table VII: Evaluation of motility of semen stored in five different extenders at different concentrations and temperatures

		5°C					30°C					37°C				
		0	12	24	48	72	0	12	24	48	72	0	12	24	48	72
Ontario	1:1	60%	25%	15%	0		60%	25%	5%			60%	0			
	1:4	60%	35%	15%	0		60%	35%	5%			60%	0			
	1:9	60%	25%	10%	0		60%	25%	0			60%	0			
CGH-27	1:1	70%	30%	20%	10%	10%	70%	10%	0			70%	0			
	1:4	70%	60%	35%	30%	30%	70%	15%	0			70%	0			
	1:9	70%	65%	40%	35%	30%	70%	10%	0			70%	0			
Cream gel[a]	1:1	exc	gd	fr	pr	0	exc	fr	0			exc	pr	0		
	1:4	exc	gd	gd	gd	pr	exc	fr	0			exc	pr	0		
	1:9	exc	gd	gd	gd	pr	exc	fr	0			exc	pr	0		
Skim Milk	1:1	60%	10%	5%	0		60%	15%	5%	0		60%	0			
	1:4	60%	25%	10%	5%		60%	15%	5%	0		60%	0			
	1:9	60%	25%	10%	5%		60%	15%	5%	0		60%	0			
Equine seminal pl[b]	1:1	60%	0				60%	0								
	1:4	60%	0				60%	0								
	1:9	60%	0				60%	0								

[a]excellent (exc), fair (fr), good (gd) and poor (pr) as accurate motility estimates could not be made due to interference by fat globules in the extender.
[b]seminal plasma was only evaluated at 5°C and 30°C.

Table VIII: Conception rates of seminal treatment groups

	0-4 hrs.	48 hrs.	24 hrs.
CGH-27	40%	0	0
AE	60%	0	17%
Control	60%		
Cream-gelatin		20%	

Statistical analysis using Chi Square reveals no statistical difference between semen treatment groups at 0-4 hours with either extender or control ($Px^2=6.7=.010$). The conception rates in these groups approach the expected value for any group of mares of unknown fertility. There is also no statistical differences between the 24 and 48 hour seminal treatment groups of all extenders. All five of these groups had either one pregnancy or none and were obviously much below the expected conception rate. There was an obvious statistical difference in results of the 0-4 hour group compared to the 24 and 48 hour groups. ($Px^2=217.7<<.005$).

Experiment 4

Table IX summarizes the morphological evaluation of the four semen treatment groups using a light microscope. Statistical analysis of these results using Chi Square revealed that there is no significant difference between the percent of normal cells, the percent of acrosomal defects, and the percent of non-acrosomal defects in each semen treatment group.

Table IX: Morphological evaluation of stained semen from four treatment groups

Sample	Normal Cells	Acrosomal Defects	Non-acrosomal Defects
Raw semen	83%	9%	8%
0 hrs.	92%	1%	7%
24 hrs.	87%	5%	8%
48 hrs.	91%	1%	8%

Figures II through V are electron micrographs of stallion semen taken with a scanning electron microscope. Only raw, unextended semen and semen stored for 48 hours in the CGH-27 extender were examined. There does not appear to be any morphological differences in these two groups. Figures II and III are photos of raw, unextended semen and Figures IV and V are photos of semen after storage for 48 hours in the CGH-27 extender. Figure V is a side view, and very adequately reveals no swelling or defects of the acrosomal cap.

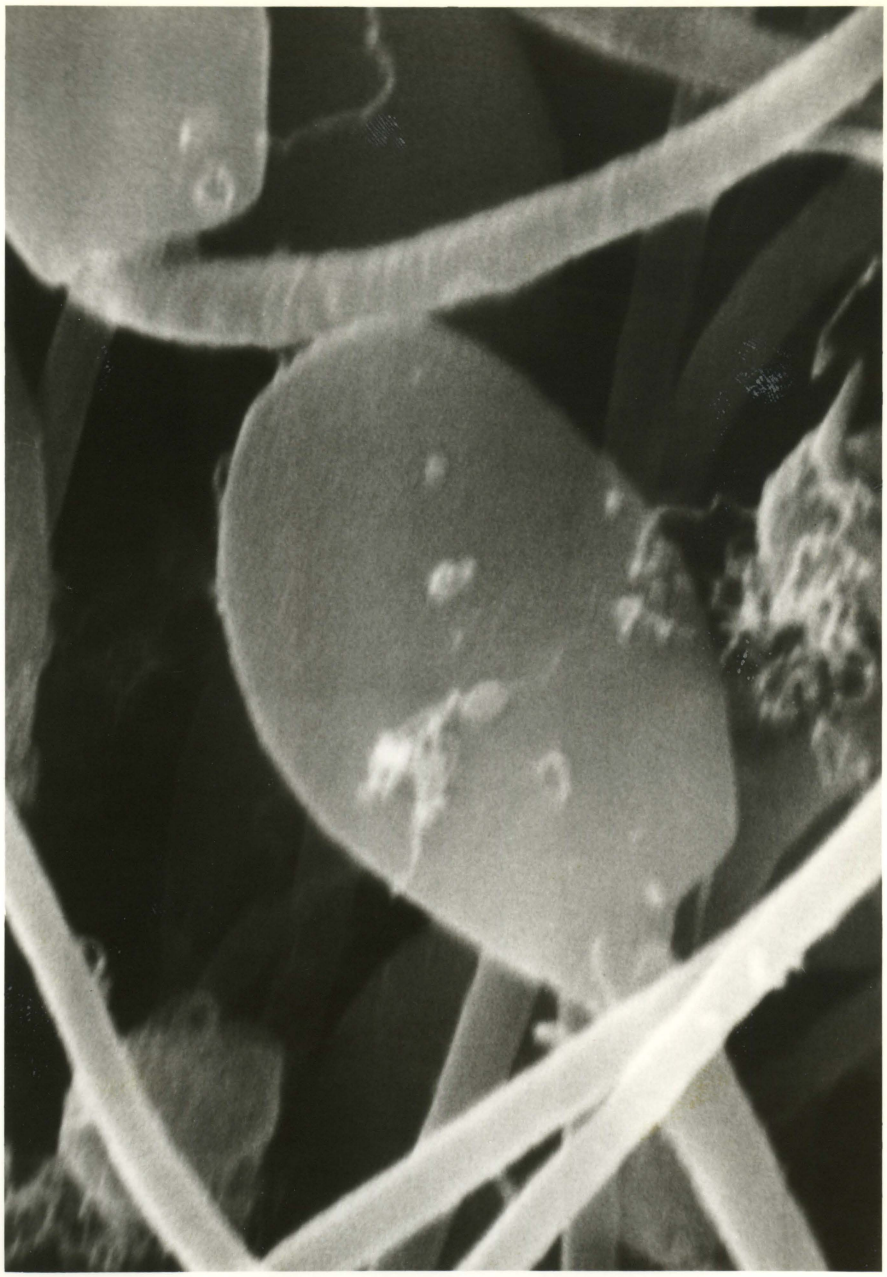

Figure II: Raw unextended stallion semen magnified 10,000X

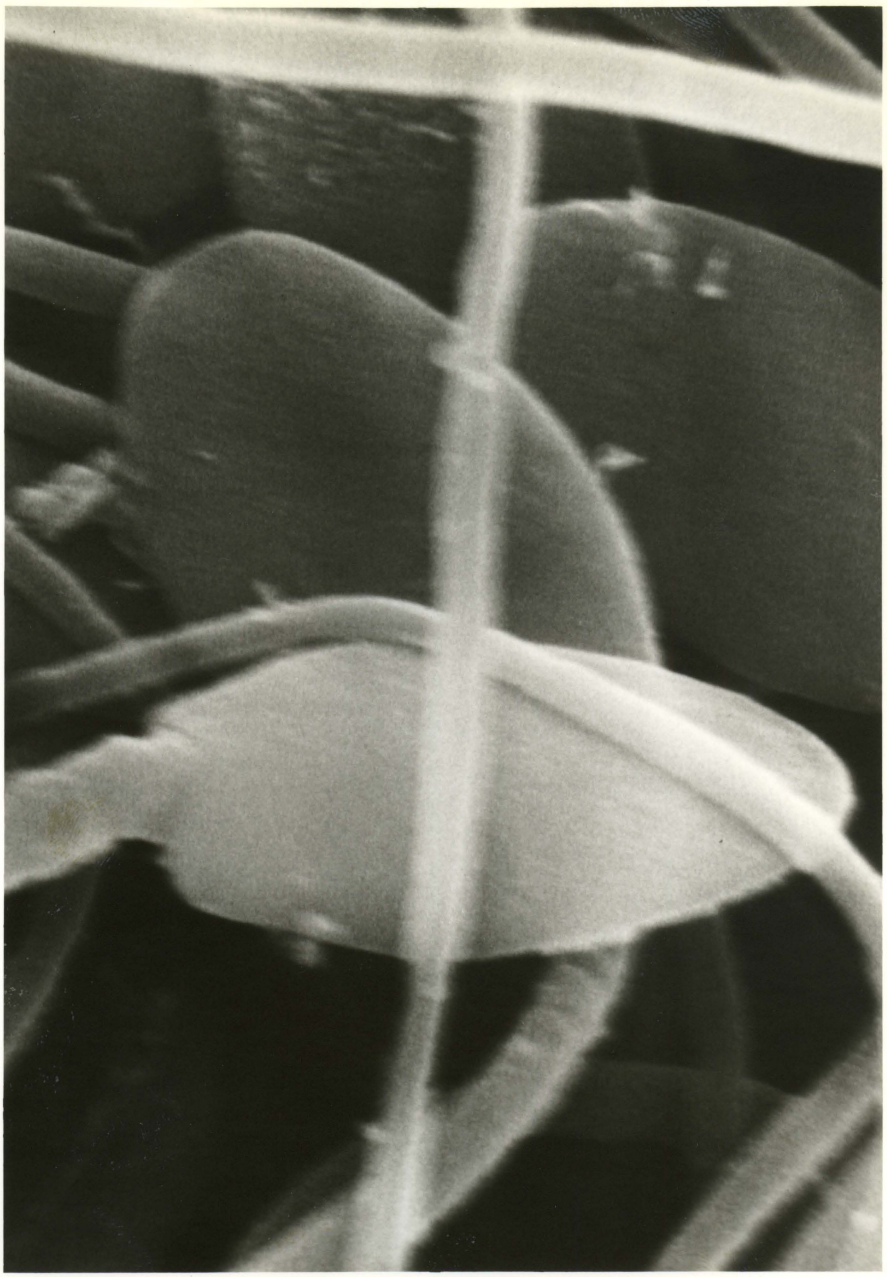

Figure III: Raw unextended stallion semen magnified 10,000X

Figure IV: Stallion semen stored 48 hours in CGH-27 extender magnified 6,600X

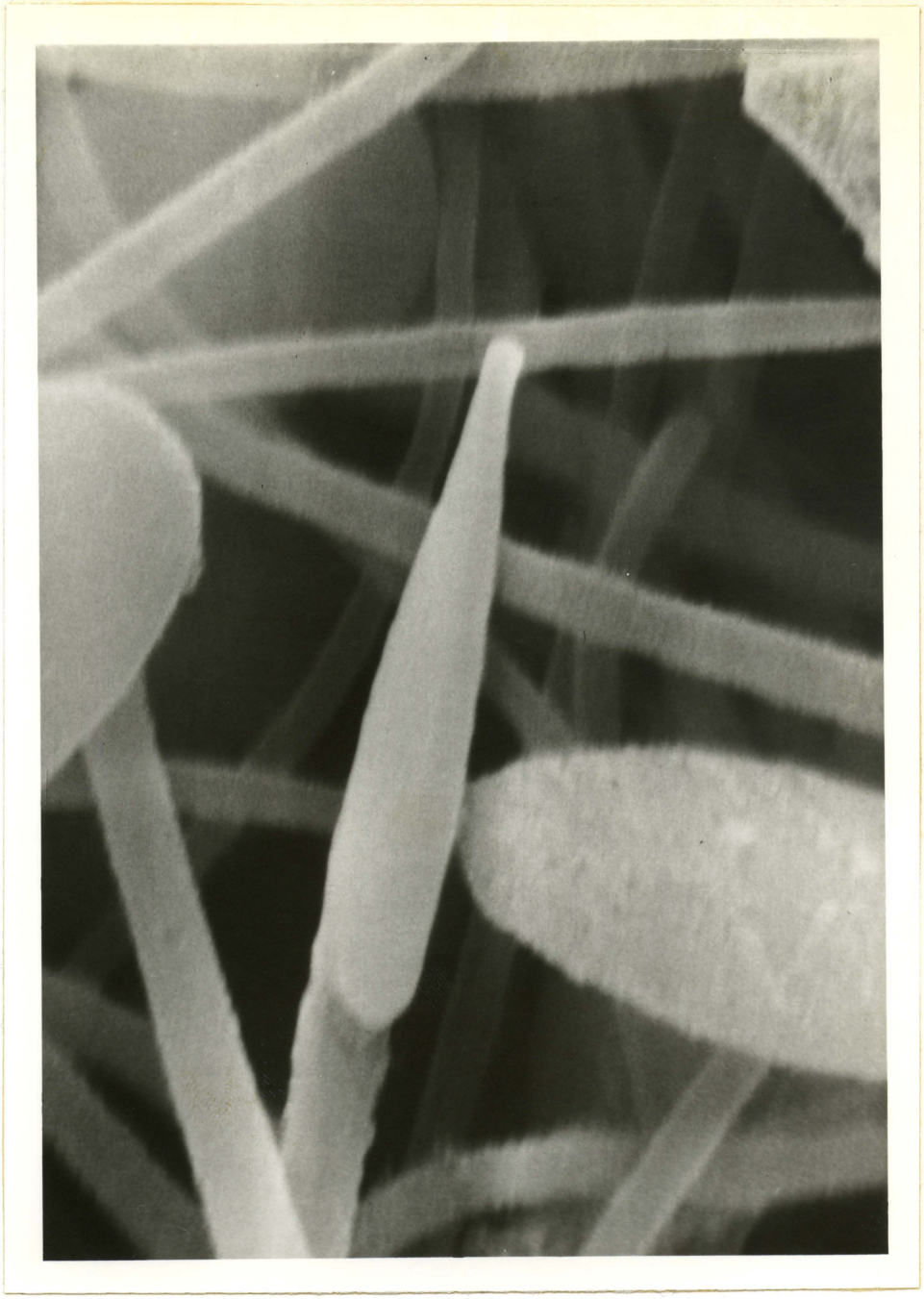

Figure V: Side view of stallion spermatozoa stored for 48 hours in CGH-27 extender magnified 10,000X

DISCUSSION

The results of this study indicate that stallion spermatozoa are fragile and are easily destroyed by adverse handling, temperature changes and storage. The effects of these conditions can be offset by the use of extenders or diluents containing ingredients that protect the spermatozoa against these detrimental effects and allow them to survive longer.

Stallion semen seems especially sensitive to the effects of centrifugation and washing. When washed in a saline-Tris or verinol buffer, in which they are relatively unprotected, the sperm cells did not survive centrifugation at 1500 r.p.m. for 15 minutes. Washing the spermatozoa in various extenders seemed to afford them a certain amount of protection. Equine seminal plasma as a washing solution preserved sperm viability better than the buffer solutions. It was determined that the gelatin portion of the extenders was responsible for protecting the spermatozoa during the washing procedure. Ontario extender, containing dextrose, sodium citrate, EDTA and egg yolk (see Appendix I), used as a washing solution did not maintain the viability of the spermatozoa during the centrifugation process. However, the addition of .5%, 1% and 2% gelatin to the Ontario extender did significantly improve the viability of the spermatozoa post centrifugation. Gelatin concentrations of 1% and 2% were superior to the .5% concentration in protecting the spermatozoa against the effects of centrifugation.

Glucose appears to have a beneficial effect on the survival of stallion semen under storage conditions. Semen extended in a solution of 4.5% glucose in verinol buffer maintained 35% of its motility for 8 hours compared

to almost immediate loss of motility after extension in verinol buffer alone. The addition of 2% gelatin further improved the maintenance of motility, especially at 22°C. After 8 hours of storage at 22°C in the 4.5% glucose and 2% gelatin in verinol buffer solution, there were still 50% of the spermatozoa motile.

There appears to be a wide range of efficacy for the effects of glucose on stallion spermatozoa, and the strict maintenance of isotonic levels does not seem to be necessary. Identical results were obtained with semen extended in 2.5% glucose, 5% glucose and 7.5% glucose in verinol buffer, with no toxic effects evident at the higher concentration. There was, however, a significant loss of the ability to maintain the motility of stored spermatozoa when glucose concentrations were 1.25% and lower.

The addition of pyruvate acid sodium to a 1% gelatin in verinol buffer solution seemed to improve the motility of stallion semen, especially in a .001 M concentration. The pyruvate was more effective in improving the activity of the sperm cells than actually increasing the longevity of them. Sperm cells stored in solutions containing pyruvate acid sodium moved much more vigorously than those stored in solutions that did not contain pyruvate.

Tris buffer has been used effectively in extenders for other species, particularly porcine. It appeared to have no detrimental effects on stallion spermatozoa and in combination with glucose and gelatin was effective in maintaining the motility of stored spermatozoa. The addition of .01 M Tris to a solution containing 5% glucose and 2% gelatin in verinol buffer maintained at

least 30% motility of spermatozoa at 5°C and 15% at 22°C after 24 hours of storage.

Formulation of the AE extender involved the manipulation of the concentrations of Tris, glucose and gelatin until the most satisfactory combination was obtained. The basic formula consisted of Tris, glucose and gelatin with sodium citrate, sodium bicarbonate and potassium chloride as additional buffers, and magnesium chloride and calcium chloride to provide calcium and magnesium ions. Gentamicin sulfate was included to prevent bacterial overgrowth. No egg yolk or milk products were added to this formula in an attempt to determine if they were necessary for a successful extender for equine semen.

The final AE formula consisted of:

3.5 gm glucose
2.2 gm sodium citrate
1.3 gm sodium bicarbonate
0.4 gm potassium chloride
.12 gm Tris
2.0 gm gelatin
0.02 gm gentamicin sulfate
0.02 M pyruvate acid sodium
0.001 M magnesium chloride
0.003 M calcium chloride
q.s. to 100 ml with distilled water

The motility of semen stored in this extender was maintained for approximately 48 hours. Five percent of the spermatozoa were still motile at this time.

The original pH of the formula was 8.3. Altering the pH of multiple samples indicated that stallion semen

can survive a wide range in pH (6.7-8.3). This range is almost entirely on the alkaline side of neutral. This coincides with the fact that stallion semen has a wide range of pH depending upon individual animals and the season of the year, but it always tends to be slightly alkaline. The buffering capacity of the AE extender appears to be adequate as the pH readings of the samples after 24 hours of storage were not significantly different from the original readings.

Comparisons of the effect of several different extender formulas at different concentrations indicated that dilution ratios of between 1:4 and 1:9 semen to extender gave the best results for all extenders evaluated. This appears to be consistent with the findings of other investigators who believe that the seminal plasma must be diluted with extender at the ratio of 1:4 to 1:8 before the viability of spermatozoa can be maintained with storage[39]. It is also substantiated by the fact that semen extended with equine seminal plasma alone had a complete loss of motility after only six hours of storage.

Most of the extenders examined functioned best at 5°C or refrigerator temperature. At this temperature the metabolic rate of the sperm cells has slowed considerably. This results in a decreased uptake of metabolites from the media and less metabolic by-products being released into it. Subsequently, the osmotic pressure and pH of the extender remains relatively constant for a longer period of time. The AE extender did not function well at 5°C but was effective at 22°C. The lack of lipoproteins or phospholipids such as are found in milk or egg yolk products probably left the spermatozoa relatively unprotected against the temperature changes associated with cooling to 5°C. None of the extenders evaluated were effective at

30°C or 37°C. The rapid uptake of metabolites and accumulation of metabolic by-products due to the increase in metabolic rate of the sperm cells, and the increase in bacterial and fungal growth that would occur at this temperature accounts for the early death of these cells.

The cream-gelatin extender and the CGH-27 extender (see Appendix I) were superior to the other three extenders in maintaining the viability of stored equine spermatozoa. There was 30% motility of semen stored for 48 hours in the CGH-27 extender and good motility of semen stored for 48 hours in the cream-gelatin extender. There were still approximately 5% of the cells motile in CGH-27 extender after 108 hours of storage, whereas motility had ceased in the cream-gelatin extender at 72 hours. Motility was 5% or less in the skim milk, Ontario (see Appendix I), and AE extenders after 48 hours of storage. Neither the skim milk extender or the Ontario extender contain gelatin, and this may account for their ineffectiveness in maintaining the viability of stored semen. Gelatin has been shown in this thesis to be very effective in protecting spermatozoa against certain adverse conditions such as centrifugation and storage. The AE extender does contain gelatin, but since it cannot be stored at 5°C, the metabolic rate of the spermatozoa remains high and there is probably a relatively rapid accumulation of metabolic by-products that change the osmotic concentration of the extender resulting in cell death. The buffers included in the AE extender appear to be quite effective in preventing a significant change in the pH due to a buildup of these metabolic by-products.

In spite of the good motility of semen stored in the CGH-27 extender, conception rates were very poor. The conception rate for semen stored for 0-4 hours in the

CGH-27 extender was not statistically different from that obtained in the control group inseminated with raw, unextended semen, although the conception rate for the control group was slightly better. There were, however, no pregnancies obtained with semen stored 48 hours or 24 hours in the CGH-27 extender despite insemination of adequate numbers of live spermatozoa. Thirty-five to forty percent of the 500 x 10^6 live cells extended were still alive after 48 hours of storage, which indicates that 175 x 10^6 to 200 x 10^6 cells were alive at insemination. After 24 hours of storage in the CGH-27 extender, 45% to 55% of the sperm cells were motile, indicating that 250 x 10^6 to 275 x 10^6 live cells were inseminated. It has been shown that 80 x 10^6 to 100 x 10^6 live cells per insemination are sufficient to cause a 55% to 60% conception rate in the mare[35,56].

Conception rates were equally as poor for the mares bred with semen stored in the AE extender. In this case, however, the motility of the samples inseminated were such that adequate numbers of live spermatozoa were probably not inseminated. After storage for 24 hours in the AE extender, the average motility of the semen was 20%. After 48 hours of storage, this had dropped to 5-10%. The conception rate obtained with semen stored for 0-4 hours in the AE extender was identical to that obtained for raw, unextended semen.

The significance of the small trial done with semen stored for 48 hours in the cream-gelatin extender is somewhat questionable. This trial was undertaken at the end of the summer when the fertility of the ponies was beginning to decline. As well, many of the pony mares included in this group were repeat breeders which probably had somewhat lowered fertility. The breeding records for

the theriogenology horse herd at Iowa State University will substantiate that good conception rates can be obtained with semen extended for 24 to 36 hours in cream-gelatin extender.

The discrepancy between the motility of semen extended in the CGH-27 extender and its fertility points out that motility is not necessarily a good estimation of fertility. Conception does not occur with semen stored over 24 hours in this extender even though adequate live spermatozoa are inseminated. Extenders containing egg yolk are therefore contraindicated in the stallion, despite their ability to maintain the motility of spermatozoa. Most authors reporting on the use of egg yolk with equine semen agree with this finding[33,41,43], although a few claim good conception rates using equine semen stored in egg yolk extenders[10,24]. The effect of egg yolk on the fertility of semen seems to be somewhat species specific, as bovine semen is routinely extended and frozen in an egg yolk-citrate extender, with very good results[20].

The ability of the equine spermatozoa to fertilize ova is somehow interfered with by the presence of egg yolk in the CGH-27 extender. Morphological evaluation of raw, unextended semen and that stored in the CGH-27 extender revealed no changes in the extended cells. Emphasis was placed on the acrosome, as acrosomal abnormalities have been shown to cause lowered fertility in bulls[47]. With a light microscope it was determined that there was no significant difference in the number of acrosomal defects and non-acrosomal defects in raw semen and semen stored for 0 hours, 24 hours, and 48 hours in the CGH-27 extender. Detailed examination of the surface of spermatozoa using a scanning electron microscope verified the lack of morphological changes associated with the semen stored in

the CGH-27 extender. The surface membranes were smooth in both raw and extended samples and there was no evidence of acrosomal swelling or vacuolization associated with semen storage.

The measurement of certain acrosomal enzymes such as glutamic oxaloacetic transaminase (GOT) and lactic dehydrogenase (LDH), that are released with cell damage, has been used as a means of estimating fertility[20,21]. In most cases, success was limited.

It is not within the realm of this thesis to explore other effects that egg yolk may have on equine spermatozoa. Further investigation is obviously needed into the biochemistry of the acrosomal enzymes and the effect that egg yolk proteins may have on the activation or release of these enzymes. Egg yolk is known to contain many non-specific, innate antibodies. The effect of these antibodies on sperm cells is also an area where more research is needed.

The success of artificial insemination in the equine depends not only upon the proper handling and insemination of semen, but also upon the proper timing of insemination. Mares generally ovulate 24 to 36 hours before the end of heat and insemination should coincide with that time. It was determined in this study that pony mares have a slightly different estrus cycle than do horse mares. They have a cycle length of approximately 23 days compared to 21 days in the horse. Pony mares are in heat for 8-9 days with the diestral period lasting 14-15 days. Horse mares are generally in heat for 5 to 6 days and out of heat for 15 to 16 days. Other investigators have documented this difference between the horse and pony mare[18].

SUMMARY AND CONCLUSIONS

Individual extender ingredients were evaluated on the basis of their ability to maintain the viability of equine spermatozoa under adverse conditions such as centrifugation and storage. One or two percent gelatin is required in the washing solution to protect stallion semen from the deleterious effects of centrifugation. The inclusion of gelatin, glucose and the buffer Tris in a storage medium significantly improved the length of time motility was maintained in stored equine spermatozoa.

Semen is best stored at 5°C to reduce metabolic activity of the cells and the build-up of metabolic by-products in the media. Milk or egg yolk products are required in the extender to protect the spermatozoa against the effect of cooling to this temperature. The cream-gelatin extender and the CGH-27 extender containing egg yolk and gelatin were the best extenders for maintaining the motility of stallion spermatozoa at 5°C. Extenders containing egg yolk and milk products, but no gelatin, were much less effective. The AE extender containing gelatin, but no egg yolk or milk products, functioned only at 22°C and was also much less effective than cream-gelatin or CGH-27 at 5°C.

The fertility of semen stored in the CGH-27 extender was considerably poorer than was expected considering how well motility was maintained. There appear to be several factors in egg yolk that influence equine spermatozoa. Some factors protect the sperm cells against certain adverse conditions such as temperature changes and storage, while others interfere with their ability to fertilize ova. Changes in surface morphology of the spermatozoa were eliminated as a cause of this

lack of fertility by detailed studies using light microscopy and scanning electron microscopy. Further studies are needed into the effect of egg yolk on acrosomal membranes and enzymes.

The fertility of the AE extender and the cream-gelatin extender was concurrent with their ability to maintain the motility of spermatozoa.

The results of this thesis indicate that an extender used for the equine should contain gelatin, glucose, some type of buffer system such as Tris, and milk or cream products. Egg yolk should be avoided as it appears to have deleterious effects on the fertility of equine semen. Extended semen should be stored at 5^{o}C and a significant reduction in conception rates should be expected with semen stored for longer than twenty-four hours.

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APPENDIX I: EXTENDER FORMULAS

Cream-gel

- 1 oz. Knox unflavored gelatin dissolved in 90 cc. sterile distilled water heated to 145°F.
- 900 cc. commercially prepared half cream/half milk heated to 105-110°F in a water bath.
- these ingredients are added together slowly while stirring.
- the mixture is allowed to cool then 2 million units of crystalline penicillin and 2 grams crystalline streptomycin is added.

Skim milk

- 1000 ml. of nonfortified skimmilk is heated to 95°C for 4 minutes.
- it is allowed to cool, then 2 million units of crystalline penicillin and 2 grams crystalline streptomycin is added.

CGH-27

- 1 gram of unflavored gelatin is dissolved in 500 cc. of distilled water (in a water bath if desired).
- then the following ingredients are added:

.5	gm Na phosphate (disbasic)
.25	gm potassium chloride
2.5	gm potassium sodium tartrate
7	gm glycine
45	gm glucose
5	gm casein
70	ml egg yolk
2	million units crystalline penicillin
2	gm crystalline streptomycin

- distilled water is added to a volume of 1000 ml.
- the pH is adjusted to 6.7-6.9 with 2% citric acid.

Ontario

- the following ingredients are added to 1000 ml. of sterile distilled water:

  50 gm anhydrous dextrose
  3 gm EDTA
  50 ml egg yolk
  1 ml gentamicin (50 mg/ml)

APPENDIX II: PROCEDURE FOR PREPARING SEMEN FOR SCANNING ELECTRON MICROSCOPY

1. Semen was centrifuged at 1500 r.p.m. for 10 minutes.
2. A couple of drops from each sample were put on pre-cut millipore filters.
3. The filter paper with sperm was put into a vial containing 5% glutaraldehyde in 0.1 M PO_4 buffer (pH 7.2).
4. This was fixed for 2½ hours at 4°C.
5. The samples were rinsed three times for 10 minutes each with PO_4 buffer.
6. They were then dehydrated using the following steps:

   30% EtOH - ½ hour (2 x 15 minutes)

   50% EtOH - ½ hour

   75% EtOH - ½ hour

   95% EtOH - ½ hour

   100% EtOH - 3 x ½ hour washes

   1:3 Freon TF:100% EtOH - ½ hour

   1:1 Freon TF:100% EtOH - ½ hour

   3:1 Freon TF:100% EtOH - ½ hour

   Pure Freon - overnight
7. These dehydrated samples were critical point dried and;
8. Coated with carbon and gold in vacuum evaporator.