# THE INFLUENCE OF SEMEN QUALITY, AS DETERMINED BY PERCENT INTACT ACROSOMES, ON FERTILIZATION RATES IN SUPEROVULATED COWS

by

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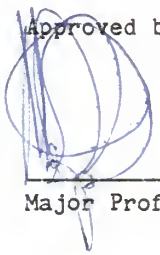

Major Professor

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## LITERATURE REVIEW

Semen quality, historically indicating primarily percent motile spermatozoa and recently incorporating cellular morphology, is a direct indication of potential fertilization rates. The influence of semen quality on fertilization rates in normal, single-egg ovulating cows has been extensively studied.[1,2,3,4,5] Semen quality evaluation has allowed the prediction of fertilization rates in single-egg ovulating cows and has shown that superior semen quality correlates to higher fertilization rates. In contrast, the influence of semen quality and semen quality evaluation in relation to fertilization rates in multiple-egg ovulating cows has not been correlated.[6]

Fertilization of ova in multiple-ovulating cows is achieved almost exclusively through artificial insemination. Insemination with single or multiple doses of semen does not significantly vary the fertilization rate of ova from multiple-ovulating cows;[6,7] however, insemination of semen of high quality has shown a trend toward higher fertilization rates. The exclusive use of processed semen in the embryo transfer industry should demand and would allow the correlation of specific semen quality evaluation standards to fertilization rates in super-ovulated cows.

The acrosome has been implicated and subsequently identified as a spermatozoal cellular characteristic that relates directly to fertility.[1,2,3] This relationship is made most evident with review of the reduced fertility associated with spermatozoa with abnormal acrosomal caps. In one study, semen from subfertile bulls (subfertility identified with 40-59%, 60-90 day non-return rates, as compared to

control bulls with greater than 70%, 60-90 day non-return rates) was examined for incidence of abnormal acrosomes, hereditary continuation of the defect, and site of acrosomal cap malformation.[8] The identified acrosomal defect consisted of knobbed, ruffled, and incomplete acrosomes which varied between 30.7 and 56.0% of ejaculates collected from subfertile bulls. The knobbed acrosome characteristic observed in this study was traced to sons of one particular bull, indicating the heritable nature of the defect. This developmental acrosomal defect had previously been reported and found to be heritable.[9,10] Histological examination of the testes of subfertile bulls showed the acrosomal defects were detectable during spermiogenesis. Examination of ova for spermatozoal attachment after insemination with knobbed spermatozoa and normal spermatozoa revealed 0-1 spermatozoa on ova after exposure to knobbed spermatozoa, as compared to spermatozoa in numbers too many to count on ova exposed to normal spermatozoa. Lack of evidence of ova activation was similar between ova exposed to knobbed spermatozoa and ova not exposed to any spermatozoa. (Activation was determined by cellular cleavage within the zona and was consistent with fertilization.) This potential reduction in fertility may have occurred due to the inability of the sperm to attach to or to penetrate the intact zona pellucida, as evidenced by this study of complete sterility in boars.[11]

An acrosomal developmental defect associated with sterility in mice has been induced.[13] X-irradiation of mature male mice induces a spermatozoal acrosomal mutation. This developmental defect is displayed in male offspring carrying the homozygous mutant gene, which produces high proportions of spermatozoa with the acrosomal defect.

The influence of the acrosomal cap on fertility is best explained by reviewing the anatomy and the cellular physiology of the acrosome. The bovine acrosomal cap is a sac-like structure into which the spermatocyte nucleus invaginates. Developing from the confluence of small vacuoles and granules in the Golgi apparatus, the acrosome spreads out over half the nucleus early in spermiogenesis.[12] The bovine acrosomal cap covers approximately 60% of the anterior portion of the nucleus and displays a typical apical body and apical fold which are a continuation of the acrosomal cap beyond the apex of the nucleus.[14] The posterior portion of the acrosomal cap is identical to the equatorial segment which can be seen with prepared slides under light microcopy (Appendix Figure 1).

The physiological components of the acrosomal cap continue to be identified.[1,15,16] Extraction of the acrosomal contents produces a mixture of protein, phospholipid, cholesterol, glucosamine, and glucuronic acid.[15] Disruption of the acrosome was shown to be accompanied by a release of hyaluronidase, neuraminidase, and trypsin-like enzymes. These acrosomal proteins from bovine spermatozoa have been shown to cause a dispersion of the cumulus oopherus and the corona radiata cells on rabbit ova.[15] Proteolytic activity is necessary for penetration of the zona pellucida, and evidence of proteolytic enzyme present in the acrosomal cap has directed speculation of this function.

In contrast to the developmental structural defects identified with the acrosome, induced acrosomal structural deterioration occurs in a number of situations: 1) spontaneously in cases of prolonged sexual rest; 2) artificially, due to invitro aging or injury; and 3) during the semen freezing-thawing process.[14] The rate of these acrosomal changes

varies between bulls, between ejaculates from individual bulls, and between different semen processing techniques.[1,2,17] The degenerative changes that occur with aging or injury have been described.[1,2,18]

These acrosomal changes vary but are distinctive from a normal motile spermatozoa which is characterized by an intact acrosomal cap with an evident apical ridge. In degenerative spermatozoa, a progressive deterioration of the anterior portion of the acrosome occurs with a loss of membrane integrity and a gradual loss of acrosomal contents. On morphological examination, degenerating spermatozoa are especially evident due to the loss of the apical ridge and due to the retention of the equatorial segment. Acrosomal cap changes observed with Giemsa-stained semen examined under direct light microscopy identified initially an increase in stainability of the acrosomal cap, followed by a loss of staining in the anterior acrosomal cap with retention of stain in the posterior region of the acrosome.[19] Electron microscopic examination in this study by Saacke and Marshall revealed the following sequential deterioration of the acrosomal cap: 1) loss of the apical ridge, breakdown of the membrane, slight to severe swelling of the anterior acrosomal cap; 2) ruffling of, followed by a breakdown of the outer membrane of the anterior acrosomal cap, retention of the posterior region of the acrosome; and 3) loss of moderately electron-dense ground substance in the anterior acrosomal cap. Differential interference contrast microscopy on unfixed semen reveals changes similar to those identified by electron microscopy (Appendix Figure 2).

As the use of artificial insemination plays a major role in cattle reproduction and in the embryo transfer industry, the potential for the semen freezing-thawing process to create semen quality changes must be

understood. Spermatozoa can be stored in liquid nitrogen at temperatures as low as -196°C and survive with relatively high fertility after thawing. However, a significant portion of the spermatozoa from a particular semen extension may be killed or damaged during freezing and thawing.[18]

The damage to the spermatozoa in the freezing-thawing process occurs: 1) by intracellular ice crystal formation; 2) by solute concentration gradiants across cell membranes; and 3) by the interactions of these two factors.[18] Freezing of semen creates an environment where intracellular and extracellular water is removed from solution in the form of ice crystals, resulting in an increasing solute concentration that can be damaging to the spermatozoa. These solvent-solute changes, and the associated changes in semen quality, are influenced by cryoprotective agents, osmolality, extender pH, freezing rate, and thawing rate.[20]

The cryoprotective agent most often used in the processing of bovine semen for freezing is glycerol. Glycerol has been shown to be one critical variable in the maintenance of semen quality through freezing and thawing.[18] The protective influence of glycerol is attributed to its water-binding properties and the reduction in solute concentrations achieved at any given temperature during freezing.

The exposure of extended semen to glycerol prior to freezing is necessary to achieve cryoprotection. Glycerol equilibration time, the interval from spermatozoal exposure to glycerol to freezing, has been shown to interact with thawing rate.[21] Shorter glycerol equilibration time (0.5 hr. vs 2.0 hr.) yields higher quality semen (quality defined as percent intact acrosomes and percent motility) when thawing at higher temperatures (35°C vs 75°C) and when incubating post-thaw at 37°C.

Optimal glycerol level has been shown to also be related to total solid content in the extender. Optimal glycerol level in the extender increases (6.4% to 8.8%) as extender solid content is increased (Tris concentration from 150 mosm. to 350 mosm).[23]

Glycerol volume in the total semen extension has been shown to influence thaw rates. As glycerol levels are increased above 4% by volume of the total semen extension, it has been shown that thaw rates should be increased (achieved in higher temperature water baths, 35°-75°C vs 5°-20°C) to maintain a minimal spermatozoal injury, as measured by percent intact acrosomes and percent motility.[22] Semen stored in plastic straws, protected with 6-8.5% glycerol, fast frozen and thawed at 35°C for 20 seconds or 65°C for 7.5 seconds, provide post-thaw spermatozoa with minimal cellular damage.[22,24]

An interaction between glycerol level and freezing rate has also been shown. Optimal glycerol levels decreased (9-11% vs 5-7%) as freezing rate increased for semen frozen in plastic straws.[25] This glycerol level-freezing rate interaction has also been shown in semen frozen in pellets and in ampules.[26] In the latter study, the optimal glycerol level for semen frozen rapidly in pellets was lower than semen frozen slowly in ampules.

Commonly used extender contains variable amounts of egg yolk as a major contribution to final extender volume. Components of egg yolk have been shown to be important in the cryoprotection of spermatozoa.[27] In the absence of egg yolk, extender buffer nor extender buffer plus glycerol protected spermatozoal motility through freezing. However, egg yolk plus extender buffer without glycerol yielded semen with progressive motility post-thaw. Egg yolk, extender buffer, plus

glycerol resulted in a significantly higher ($p < 0.05$) percent motility post-thaw than egg yolk and extender buffer without glycerol.

The osmolality and the pH of semen extender and the changes in these properties that occur during freezing and thawing influence semen quality.[28] Hypertonic extenders damage spermatozoal cell membranes significantly at freezing temperatures of -10° to -15°C, compared to similar damage seen initially at -30°C with hypotonic extenders. This variation in critical temperature appears to be related to the concentration of residual solution as pure water freezes out of solution. Osmolality of extended semen is preferred to be isosmotic or, if necessary, slightly hyposmotic.

Optimal pH of semen extender differs at various stages in the processing of extended semen. Prior to freezing, extender with a pH value of 6.5 proved to be least damaging to spermatozoa.[23] However, the post-thaw motility was improved in semen extended in an extender with a pH value of 7.5. An interaction between pH and osmotic pressure is evident as higher pH values tended to more adversely influence semen quality at lower solute concentrations, and lower pH values tended to more adversely influence semen quality at higher solute concentrations.

Freezing rate influences the maintenance of the acrosomal cap; however, variable rates of extreme temperature change due to the variation in the dissipation of the heat of fusion allow a range of freezing conditions to be acceptable.[22] The influence of freezing rate on post-thaw semen quality was determined by comparing the quality of semen extended in egg yolk-citrate-glycerol, packaged in .25ml plastic straws, and then frozen from 5°C to -130°C in 3.5 minutes, 20 minutes, and 37.8 minutes.[25] Based on post-thaw motility, the fast freezing rate

resulted in the best post-thaw semen quality. However, a separate study using similar freezing rates with semen extended in egg yolk-Tris-glycerol did not show any significant advantage to any of the three freezing rates.[29] Only after 3 hours incubation post-thaw at 37°C did the slow freezing rate result in a lowered spermatozoal motility.

The thawing of frozen bovine semen converts the stored semen into an immediately usable product. The technique of thawing frozen semen has been successful in a range from "in vivo" thawing to thawing in a 75°C water bath for less than 7 seconds. Thawing procedures have been shown to influence the fertility of bovine spermatozoa, and techniques that maintain optimal semen quality have been identified.[30]

Thawing procedures comparing the thawing of semen in .25ml plastic straws in a 75°C water bath for 7 seconds versus "in the cow" or "in the pocket" produced significantly higher pregnancy rates in favor of the 75°C/7 second thaw (38.6% vs 23.0% and 82.8% vs 61.6%, respectively).[30]

Thawing semen at 38°C for 25 seconds has been shown to produce significantly ($p < 0.08$) higher non-return rates than a 38°C for 8 seconds thaw.[31] This 25-second equilibration time increased the resistence of the semen to post-thaw acrosomal changes when incubated at 37°C or when incubated at a reduced temperature of 5°C.

Semen stored in .25ml plastic straws and thawed at 35°C for 60 seconds maintained a higher percent intact acrosomes ($p < 0.01$) after exposure to 20°C or 37°C post-thaw incubations than semen thawed at 5°C for 3 minutes. Acrosomal retention was significantly lowered ($p < 0.01$) in semen thawed at 5°C and then exposed to sudden temperature increases to 20°C or 37°C, as compared to post-thaw exposure to 1°C.[32]

Semen packaged in 1.0ml glass ampules and thawed at various temperature/time scales showed a significantly higher ($p < 0.01$) retention of acrosomes and percent motility after a 50°C/75 second thaw versus a 5°C/10 minute thaw or 20°C/4 minute thaw.[33]

Acrosomal damage has been attributed to a sudden reversal in the post-thaw incubation temperature of semen. This damage, cold shock injury, is more evident if changes in percent intact acrosomes are examined rather than percent motility.[34] Semen thawed at 35°C and then exposed to 5°C experienced a significant ($p < 0.01$) reduction in percent intact acrosomes, 67.7% to 57.9%. The corresponding reduction in percent motility was not significant. Rapidly thawed straws of semen (35°C/10 sec., 20 sec., 80 sec.) maintained significantly ($p < 0.01$) higher quality after exposure to 5°C than identical semen thawed at 5°C for 50 seconds.

The accurate estimation of fertility of a bull is the basis for performing semen quality evaluations and for establishing evaluation standards that can direct the industry. Semen evaluation standards vary in accuracy and repeatability and in the case of making the evaluation. A very accurate fertility estimate has been non-return rate data on particular bulls. This data indicates the percentage of cows that do not return to estrus after a specific period of time following insemination with semen from a specific bull. A group of bulls selected for fertility based on non-return rates proved there was a significant ($p < 0.01$) advantage in fertilization rates for bulls of estimated high fertility.[35]

The accuracy of semen evaluation standards in predicting fertility was tested in a group of Holstein bulls with a range of apparant

fertility as indicated by non-return rates.[36] Comparisons were made between non-return rate, percent intact acrosomes, and percent motility. Acrosomal retention was significantly related to fertility immediately post-thaw, as well as through 10 hours of incubation at 37°C. Percent motility was also significantly related to fertility immediately post-thaw, but became less related to fertility through incubation. The percent intact acrosomes evaluation proved to persist as a significant indicator of fertility after individual bull variation was removed from the data. This study indicated that 65% of the variation in fertility between bulls could be accounted for by measuring percent intact acrosomes. In addition, a similar study comparing semen evaluation standards to non-return rate data on a group of Holstein bulls showed that 60% of the non-return rate variance could be accounted for by combining percent motility and percent dead spermatozoa with percent morphologically abnormal spermtozoa parameters.[3]

Semen evaluation utilizing acrosomal retention and percent motility shows a small coefficient of variation, .06, for percent intact acrosomes as opposed to .25 for percent motility on estimates of the same semen.[2] This difference, due to the subjectivity of motility estimations when compared to acrosomal retention estimates, indicate evaluating percent intact acrosomes should provide a repeatable post-thaw estimate of bull fertility and if progressive motility is present, should provide an accurate estimate of bull fertility.

Other semen evaluation tests are currently being employed in laboratory settings to identify bull fertility and may eventually increase the accuracy of semen quality evaluations. Sperm penetration assays, the ability of a capacitated bovine spermatozoa to penetrate a

zona-free hampster ovum, have proven to correlate well with percent intact acrosomes and percent motility when estimating semen quality.[37] The ability of bovine spermatozoa to disperse cumulus oopherus cells and to penetrate the zona pellucida of mice ova has been positively correlated to competitive-mating indices, the ability of one bull's semen to fertilize ova when inseminated with semen of another bull.[38] This test may also prove an accurate estimate of bull fertility.

In limited studies involving the insemination of multiple-egg ovulating cows, the use of non-return rates or non-specific quality estimates has been shown to correlate well with fertilization rates. In a trial evaluating the role of insemination timing, sperm numbers, and semen quality in fertilization rates in superovulated cows, higher quality semen showed a tendency towards higher fertilization rates.[7] This trial utilized 40 cross-bred beef cows and 8 beef bulls. Semen quality was classified as either high or low quality, with no specific quality characteristics noted. A similar trial utilizing semen from a bull of above average fertility and a bull of below average fertility showed a significant advantage ($p < 0.01$) in fertilization rates achieved with semen from the high fertility bull when inseminated in superovulated cows.[39]

The incorporation of specific semen evaluation standards to semen processed for use in normal, single-egg ovulating cows has been a positive factor directing the improvement of the beef and dairy breeds. The same concentration of effort to establish specific quality standards appropriate to the multiple-egg ovulating cow should be encouraged and supported by the bovine industry. The accurate estimation of fertility of a bull is of vital importance to the embryo transfer industry, as

technical procedures are timely and expensive. The use of specific standards, such as percent intact acrosomes, or future standards, such as the sperm penetration assay, can only allow the embryo transfer industry to be more accurate and more efficient in reproducing quality cattle.

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## INTRODUCTION

Genetic advancement through the use of embryo transfer in cattle has developed into an enormous industry. Any genetic loss due to the collection of unfertilized ova increases the expense of the procedure. Embryo transfer demands the use of only superior sires in a breed, and artificial insemination allows the widespread distribution of semen from these bulls. The influence that the quality of this semen has on genetic loss through unfertilized ova should be identified.

A direct correlation has been shown between quality of semen and increased fertilization rates in single-ovulating cows.[1,2,3,4,5] This correlation has not been accurately identified in multiple-ovulating cows.[6] The insemination of multiple-ovulating cows with semen of variable quality has indicated that semen quality may directly relate to increased fertilization rates.[7,39]

Artificial insemination is used almost exclusively in embryo transfer to achieve fertilization. Processed, packaged semen is known to suffer spermatozoal damage due to the freezing and thawing procedures required for its storage and use.[18,20] The identification of spermatozoal structural changes that occur due to freezing and thawing, as described by Saacke and Marshall,[19] has allowed a standardization of specific quality estimates on processed semen. Semen quality deterioration, particularly percent intact acrosomes, can be predicted by the handling techniques employed, and techniques that maintain optimal semen quality have been identified.[22,24,30]

The use of percent intact acrosomes and percent motility as estimates of semen quality have been shown to correlate well with

fertility of bulls when compared to non-return rate data.[36] The estimation of percent intact acrosomes shows a small coefficient of variation, .06 for estimates between observers, on the same semen.[2] Percent intact acrosomes with progressively motile spermatozoa should, therefore, provide a repeatable, accurate estimate of bull fertility.

The genetic and economic loss suffered by the embryo transfer industry, through the failure to fertilize ova, should be minimized. The identification of specific semen quality estimates that accurately predict fertility in multiple-ovulating cows should be an immediate goal. The purpose of this research is to determine the influence of a specific semen quality estimate, percent intact acrosomes, on the fertilization rate in multiple-ovulating beef females.

## MATERIALS AND METHODS

Semen Pool - Semen used was that collected from one ejaculate from one bull. Collection, processing, and freezing of this semen was done at the Kansas Artificial Breeding Service Unit in Manhattan, Kansas. Collection of this semen was accomplished using a mounting stimulus and a warm, air-filled artificial vagina. The semen was held in a 27°C water bath during the raw semen evaluation phase. Raw semen extension, using egg yolk-citrate-glycerol with antibiotics, was calculated to yield 27 million live spermatozoa per breeding unit prior to freeze. Initial processing consisted of 50% extension, without glycerol, of the raw semen followed by a four hour incubation at 4°C. Following this incubation interval, final extension with the addition of 6%, by volume, glycerol was completed. Extended semen was then packaged in 0.5ml plastic straws. Freezing was accomplished in liquid nitrogen vapor, under pressure (8psi), for 10 minutes to create a temperature drop from +4°C - -196°C.

Semen Handling - Three different temperature/time scale thaws were utilized to produce three levels of percent intact acrosomes. High quality semen was that semen thawed in a 65°C water bath under observed thaw to maintain 75.75 ± 3.95 percent intact acrosomes. The observed thaw technique required a light source that would disperse through the water bath and allow direct visualization of thawing. Semen was removed from this water bath as the ice-ribbon disappeared. Medium quality semen was that semen thawed in a 35°C water bath for 18 seconds, to maintain 64.50 ± 3.87 percent intact acrosomes. Poor quality semen was

that semen thawed in a 4°C water bath for 90-100 seconds to maintain 46.00 ± 10.52 percent intact acrosomes.

Donor Pool - Experiment I. Twelve cross-bred beef cows were utilized in the initial experiment. These cows were mature, multiparous, and non-lactating.

Experiment II. Eleven cross-bred beef heifers were utilized in this experiment. These heifers were puberal and nullparous.

Procedure - Experiment I. Each of the twelve cows was subjected to three consecutive superovulatory stimulations and was randomly assigned to be inseminated with semen with high percent intact acrosomes, medium percent intact acrosomes, and low percent intact acrosomes. Multiple ovulatory stimulation was achieved in each of the twelve cows using 5mg follicle stimulating hormone,[a] administered intramuscularly, BID, for a total of 10 doses; starting on day 9 following estrus. In conjunction with the seventh and eighth doses of follicle stimulating hormone, 35mg and 10mg prostaglandin $F_2\alpha$[b] was administered intramuscularly, respectively. All cows received 100mg gonadotropin releasing hormone,[c] administered intramuscularly, at first observed standing estrus (Appendix Table I).

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[a]FSH-P. Burns-Biotec Laboratories, Inc., Omaha, Nebraska 68103

[b]Lutalyse.® The Upjohn Company, Kalamazoo, Michigan 49001

[c]Cystorelin.® CEVA Laboratories, Inc., Overland Park, Kansas 66212

Experiment II. Each of the eleven heifers was subjected to two consecutive superovulation stimulations and was randomly assigned to be inseminated with semen of high percent intact acrosomes and low percent intact acrosomes. Multiple ovulatory stimulation was achieved in each of the eleven heifers using a decreasing dose schedule of follicle stimulating hormone, administered intramuscularly over four days, for a total of 36mg, starting on day nine following estrus. In conjunction with the fifth and sixth doses of follicle stimulating hormone, 750mg and 250mg cloprostenol[d] was administered intramuscularly, respectively. All heifers received 100mg gonadotropin releasing hormone, administered intramuscularly, at first observed standing estrus (Appendix Table I).

Breeding Schedule - Experiment I. Following each multiple ovulatory stimulation, each cow was inseminated with two 0.5ml straws of specific quality semen at 0 hour post-standing estrus, and with two 0.5ml straws of specific quality semen at 12 hours post-standing estrus.

Experiment II. Following each multiple ovulatory stimulation, each heifer was inseminated with one 0.5ml straw of specific quality semen at 12 hours post-standing estrus, and with one 0.5ml straw of specific quality semen at 24 hours post-standing estrus.

Ova Collection - Ova from each cow and heifer were collected on day seven post-standing estrus using a non-surgical uterine flushing technique. Cows and heifers were prepared for collection with epidural

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[d]Estrumate.® Bayvet Division, Cutter Laboratories, Inc., Shawnee, Kansas 66201

regional anesthesia utilizing 0.5 - 0.75ml of 2% xylocaine[e] per 45.4 kilograms of body weight, and with a thorough cleansing of the perineum with an iodine-base disinfectant scrub.[f] A three-way, 18 French Foley catheter,[g] made rigid with a stainless steel stylet, was inserted vaginally through the cervix. The inflatable cuff on the Foley catheter was filled with 4-12ml of flushing media to allow the catheter to rest anterior to the internal cervical os.

Using a 60cc plastic syringe equipped with a three-way fluid transfer valve, flushing media was forced retrograde through the Foley catheter to the utero-oviductal junction and allowed to return antegrade through the Foley catheter into collection bowls. The flushing media consisted of phosphate-buffered saline[h] into which was added heat-inactivated fetal calf serum[i] at 2% by volume, and penicillin-dihydrostreptomycin-amphotericin-B[j] at 1% by volume. Forty to 60ml increments of flushing media was used to dilate the horns of the uterus, with a total of 240-320ml of flushing media used per cow or heifer. Physical manipulation of the uterine horns, via rectal massage, and gravity flow aided antegrade flow of the flushing media out the Foley catheter to be collected in flat-bottomed plastic bowls.[k]

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[e]2% Lidocaine. Elkins-Sinn, Inc., Cherry Hill, New Jersey 08034

[f]Betadine Surgical Scrub. The Purdue-Frederick Company, Norwalk, Connecticut 06856

[g]Travenol Laboratories, Inc., Deerfield, Ilinois 60015

[h,i,j]Gibco Laboratories, Grand Island, New York 14072.

[k]Ova-Trans, Veterinary Concepts, Spring Valley, Wisconsin 54767

Ova Evaluation - Each bowl of flushing media was examined in a grid fashion using an inverted-light microscope at 40x power. Identified ova were removed from the flushing media and placed into a holding media containing phosphate-buffered saline, heat-inactivated fetal calf serum (22% by volume), and penicillin-dihydrostreptomycin-amphotericin B (1% by volume). Final evaluation of collected ova was completed by one hour after collection using 40x, 100x, and 200x power. Each ova was evaluated for evidence of cellular division, indicating fertilization had occurred.

Data Analysis - Data accumulated, identifying fertilized or unfertilized ova, was analyzed by ANOVA. Incomplete data, that being zero total ova collected, was removed from analysis. Zero data, that being zero fertilized or zero unfertilized ova collected, was included in analysis. Cow-heifer/semen interactions were assumed to be negligible.

## RESULTS

Experiment I - Thirty-six multiple ovulatory stimulations were carried out, of which twenty-four responses yielded collectible ova. One hundred five ova were collected in 24 uterine flushes, with the mean number of ova collected per uterine flush being 4.38 ± 2.84. Twenty-seven, 42, and 36 total ova were collected after exposure to semen with high, medium, and low percent intact acrosomes, respectively, Percent fertilized ova collected was 63.0%, 78.6%, and 77.8% for ova exposed to semen with high, medium, and low percent intact acrosomes, respectively (Appendix Table II). Analysis of variance showed no significant difference ($p > 0.10$) between the proportion of fertilized ova collected in each treatment group.

Experiment II - Twenty-two multiple ovulatory stimulations were carried out, of which twelve responses yielded collectible ova. One hundred three ova were collected in 12 uterine flushes, with the mean number of ova collected per uterine flush being 8.58 ± 4.93. Forty-nine and 54 total ova were collected after exposure to semen with high and low percent intact acrosomes, respectively. Percent fertilized ova collected was 77.6% and 77.8% for ova exposed to semen with high and low percent intact acrosomes, respectively (Appendix Table III). Analysis of variance showed no significant difference ($p > 0.10$) between the proportion of fertilized ova collected in each treatment group.

## DISCUSSION

The influence of thawing procedures on post-thaw semen quality was made evident in this study. The decrease in percent intact acrosomes, from 75.75% at a 65°C thaw to 64.50% at a 35°C thaw and then to 46.00% at a 4°C thaw, is a characteristic indication of the acrosomal deterioration that occurs at slower thawing rates. This acrosomal deterioration rate (deterioration as described by Saacke and Marshall[19]) agrees with trials comparing 35°C, 60-second thaws to 5°C, 3-minute thaws where percent intact acrosomes was significantly higher in the 35°C thawed semen, 69.2% vs 55.7%.[32] The percent intact acrosomes maintained through each thawing procedure was relatively constant in this study, as indicated by standard errors of 3.95%, 3.87%, and 10.52% for thaws at 65°C, 35°C, and 4°C, respectively. The use of semen from one ejaculate from one bull increased the accuracy of prediction of the spermatozoal changes occurring as a result of freezing and thawing. Saacke has shown a significant ($p < 0.01$) difference in percent intact acrosomes between ejaculates from the same bull when the semen was maintained at 37°C post-thaw.[2]

The definition of semen quality as percent intact acrosomes in this study allowed the use of a semen evaluation parameter that has been shown to be accurately estimated and which plays an apparent direct role in fertilization. The acrosome is suggested to have a role in fertilization due to the proteolytic nature of its intracellular contents. This particular role of the acrosome is currently being evaluated in studies determining the ability of bovine spermatozoa to disperse cumulus oopherus cells and to penetrate the zona pellucida of

mice ova; a quality test that correlates well with other indices of fertility.[38] The identification of heritable acrosomal structural defects associated with subfertility in bulls and boars and the evidence that these defective spermatozoa cannot attach to or penetrate an intact zona pellucida indicate the role of the acrosome in fertilization.[8,11]

The coefficient of variation between estimates of percent intact acrosomes on identical semen samples has been reported to be .06, compared to .25 for motility estimates on the same semen.[2] This small estimate variation indicates that percent intact acrosomes is a repeatable and accurate estimate of semen quality.

Ovulation rates in the donor females used in this study were increased using follicle stimulating hormone. The use of follicle stimulating hormone to increase ovulations has been shown to produce a progressive increase in the number of ovulations with increasing total dosage levels.[40] However, in one study[41] a shortened follicle stimulating hormone treatment, administering 28mg FSH over 4 days as compared to 32mg FSH over 5 days, gave a higher response in total ova collected. Total ova collected per donor cow in the study by Garcia[41] was 7.44 in the 4-day FSH treatment group compared to 3.23 in the 5-day FSH treatment group. The variability in donor female response is also shown in reported summaries of total ova collected per donor. Massey[42] reports 7.8 ± 0.3 total ova collected per donor for 904 donors stimulated with variable dosage treatments of follicle stimulating hormone. A similar summary reported by Critser[43] showed 4.7 ± 0.3 total ova collected per donor from 68 donor females stimulated with follicle stimulating hormone. Variability in donor female response was expressed in collections from both experiments. In experiment I, 4.38 ± 2.84

total ova were collected from donors yielding at least one ovum. This was considered a below average response. In experiment II, 8.58 ± 4.93 total ova were collected from donors yielding at least one ovum. This was considered an above average response. The follicle stimulating hormone treatments were different between groups, as was the age of the donor females. Experiments I and II were not designed to be compared, but the increased ovulation response in experiment II may substantiate that reported by Garcia[41] where a 4-day FSH treatment yielded more total ova collected than a 5-day FSH treatment.

The percent fertilized ova collected in both experiments showed no significant effects on fertilization rate by varying the percent intact acrosomes. These fertilization rates, 63.0 - 78.6% in experiment I and 77.6 - 77.8% in experiment II, agree well with the percent fertilized ova collected by Callaghan and King,[44] 76.8 - 89.5%, in superovulated beef heifers. The percent fertilized ova collected in both experiments tended to be higher than the percent fertilized ova from superovulated donors, 70.7 ± 2.5%, as reported by Massey.[42]

The maintenance of the acrosomal cap in bovine semen has been shown to account for as much as 65% of the variability in fertility between bulls.[1] In comparisons between non-return rate data and percent intact acrosomes, the percent intact acrosomes has been shown to be significantly related to fertility.[36] The evidence reported indicates the influence of the acrosome on fertility does not explain the similar fertilization rates in both experiments when using semen of such varied percent intact acrosomes. It is of consideration here that a more precise estimate of potential fertilization rate would be the total number of normal spermatozoa with which the donor females were

inseminated, rather than just the percent normal spermatozoa inseminated.[45]

The use of multiple insemination doses of semen at variable times post-standing estrus is a common clinical practice when inseminating multiple-ovulating cows. The sperm numbers required for optimal fertilization in multiple-ovulating cows has not been studied; however, multiple insemination doses have been shown to be of no significant advantage in achieving increased fertilization rates.[7,46] The sperm numbers required for maximum breeding efficiency in single-ovulating cows has been reviewed.[5] Sullivan[5] has demonstrated that 10-15 million motile spermatozoa per insemination dose may in fact produce significantly higher 60-90 day non-return rates than insemination doses of 5 million motile spermatozoa.

The semen utilized in both experiments was of known concentration (27 million live spermatozoa prior to freeze, minimum) and of known response to thawing. In experiment I the total spermatozoa with intact acrosomes inseminated in these multiple-ovulating cows ranged from 38 million to 77 million, with no significant differences in fertilization rates. In experiment II the total spermatozoa with intact acrosomes inseminated in these multiple-ovulating heifers ranged from 19 million to 38 million, with no significant differences in fertilization rates. The regime of inseminating one dose of semen at 12 and at 24 hours post-standing estrus was shown by West[46] to produce significantly higher fertilization rates than two insemination doses at 12 hours post-standing estrus. Average fertilization rates of 73% after one dose insemination at 12 and 24 hours agrees well with the 77.6 - 77.8% fertilization rates achieved in experiment II. This

particular study by West indicates that inseminations should be carried out over a period of time for optimal fertilization, but does not indicate appropriate sperm numbers required for optimal fertilization.

Although experiments I and II were not designed to be compared because of different insemination timing schedules, insemination doses, and donor female ages, similar results in percent fertilized ova collected were found. These findings would indicate that a concentration of at least 19 million spermatozoa with intact acrosomes post-thaw, inseminated in split doses at 12 and 24 hours post-standing estrus, will produce acceptable fertilization rates in multiple-ovulating beef females.

The spermatozoa numbers required for optimal fertilization in multiple-ovulating cows is another factor in semen quality that needs to be identified. The interactions of spermatozoal morphology and adequate numbers of spermatozoa required for optimal fertilization and the interactions of specific bull and adequate numbers of spermatozoa required for fertilization, as reviewed by Sullivan,[5] remain to be identified. The interactions are of importance as to their influence on fertilization rates in multiple-ovulating cows.

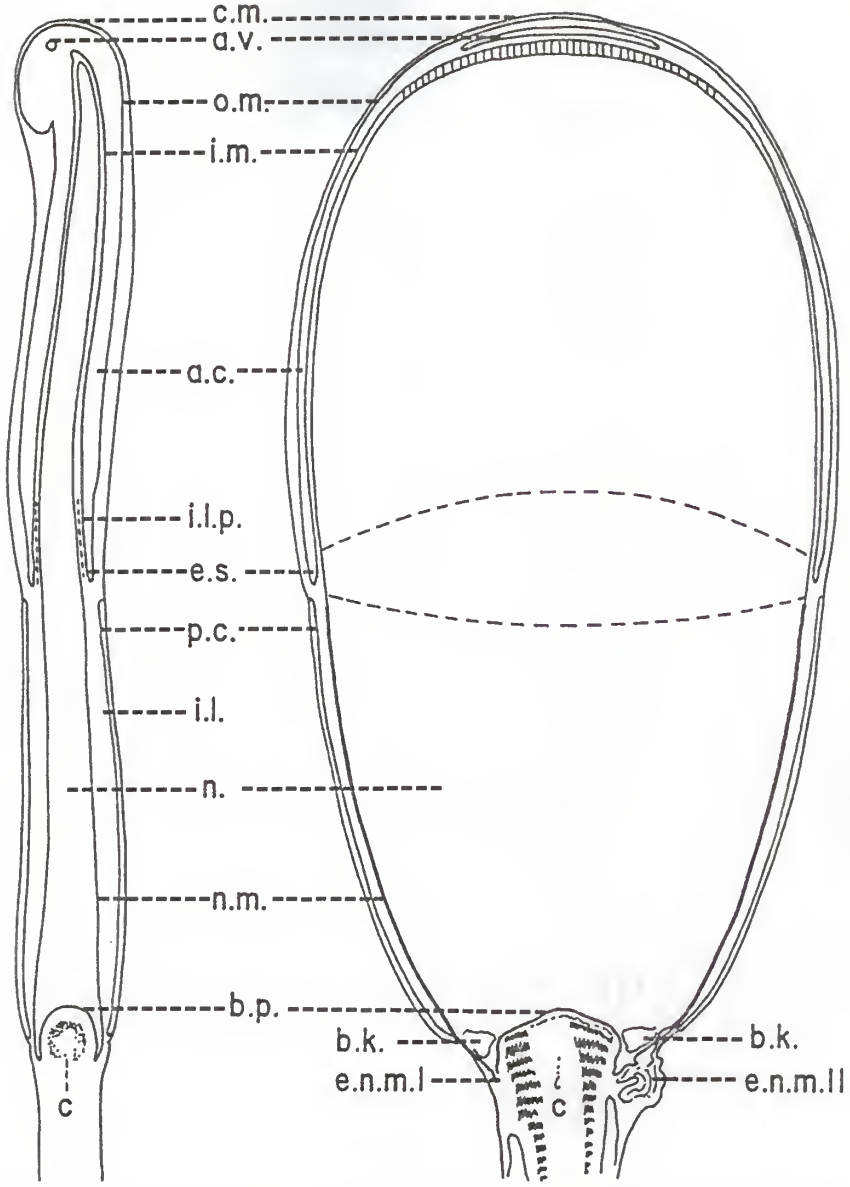


Figure 1

Abbreviations: c.m. - cell membrane, a.v. - apical vacuole within the apical body, o.m. - outer membrane of the acrosome cap (compare galea capitis), i.m. - inner membrane of the acrosome cap, a.c. - acrosome cap (contents), i.l.p. - intermediate layer (dense material found between e.s. and n.m. -- perforatorium substance?), e.s. - equatorial segment or pars intermedia, p.c. - postnuclear cap, i.l. - intermediate layer between p.c. and n.m., n. - nucleus, n.m. - nuclear membrane, b.p. - basal plate or basal lamella lining the implantation groove, b.k. - basal knob, e.n.m.I - evaginated nuclear membranes (more simple type), e.n.m.II - evaginated nuclear membranes (more convoluted type), and c. - centriole.

From: Nord. Vet.-Med. 1965, 17, 193-212.

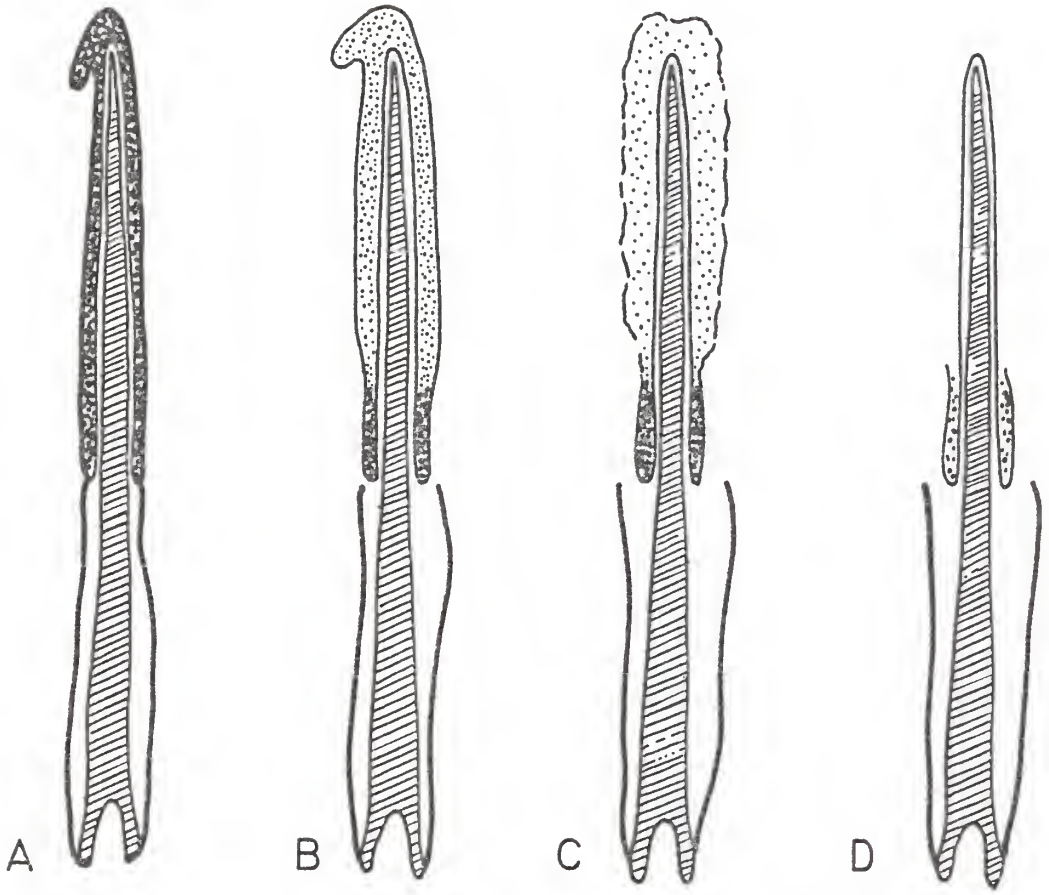


Figure 2. Schematic representations of sagittal sections through the bovine sperm head as seen using the electron microscope. A through D show the sequential alterations of sperm aging and injury which result in formation of the equatorial segment and loss of the anterior acrosome.

From: Proceedings of the Third Tech. Conf. on A.I. & Repro., 1970

Appendix Table I. Superovulatory Stimulation Schedules: Donor Pools

| Days Post-Estrus | Donor Pool | |
|---|---|---|
| | Cow Group | Heifer Group |
| 0 | Estrus | Estrua |
| 9 | AM 5.0 mg FSH[a]<br>PM 5.0 mg FSH | AM 6.0 mg FSH<br>PM 6.0 mg FSH |
| 10 | AM 5.0 mg FSH<br>PM 5.0 mg FSH | AM 5.0 mg FSH<br>PM 5.0 mg FSH |
| 11 | AM 5.0 mg FSH<br>PM 5.0 mg FSH | AM 4.0 mg FSH<br>750 mcg cloprostenol[b]<br>PM 4.0 mg FSH<br>250 mcg cloprostenol |
| 12 | AM 5.0 mg FSH<br>35 mg $PGF_2\alpha$[c]<br>PM 5.0 mg FSH<br>10 mg $PGF_2\alpha$ | AM 3.0 mg FSH<br>PM 3.0 mg FSH<br>Detect estrus* |
| 13 | AM 5.0 mg FSH<br>PM 5.0 mg FSH<br>Detect estrus* | AM Detect estrus<br>PM Detect estrus<br>Breed** |
| 14 | AM Detect estrus<br>PM Detect estrus<br>Breed*** | AM Breed<br>PM Breed |
| 15 | AM Breed<br>PM Breed | |

[a]FSH-P, Burns-Biotec Laboratories, Inc., Omaha, Nebraska 68103.

[b]Estrumate.® Bayvet Division, Cutter Laboratories, Inc., Shawnee, Kansas 66201.

[c]Lutalyse.® The Upjohn Company, Kalamazoo, Michigan 49001.

*Each cow and heifer received 100 mcg GnRH (Cystorelin.® CEVA Laboratories, Inc., Overland Park, Kansas 66212) at first detected standing estrus.

**Each heifer was inseminated with 1-1/2 cc straw of semen of 12 hours and at 24 hours post-standing estrus.

***Each cow was inseminated with 2-1/2 cc straws of semen at 0 hours and at 12 hours post-standing estrus.

Appendix Table II. Fertilized and Unfertilized Ova Collected: Experiment I - Cows

| | Semen Quality (% intact acrosomes) | | | | | |
|---|---|---|---|---|---|---|
| | High | | Medium | | Low | |
| Cow | Fert | UnFert | Fert | Unfert | Fert | Unfert |
| 1 | 1 | 0 | 6 | 1 | 3 | 0 |
| 2 | 1 | 0 | -* | - | -* | - |
| 3 | 2 | 0 | 3 | 0 | 3 | 1 |
| 4 | 2 | 0 | - | - | - | - |
| 5 | 3 | 1 | 6 | 1 | - | - |
| 6 | -* | - | 1 | 0 | - | - |
| 7 | 3 | 0 | - | - | 5 | 0 |
| 8 | 1 | 0 | 8 | 1 | 4 | 5 |
| 9 | 3 | 7 | 1 | 4 | 8 | 1 |
| 10 | - | - | 0 | 2 | 5 | 1 |
| 11 | 1 | 2 | 5 | 0 | - | - |
| 12 | - | - | 3 | 0 | - | - |
| Total | 17 | 10 | 33 | 9 | 28 | 8 |
| % Fertilized** | 63 | | 78.6 | | 77.8 | |

*Incomplete data due to:
1) Lack of ovarian response, no uterine flush (1 cow).
2) Zero ova collected by uterine flush (10 cows).
3) Inability to catheterize cervix, no uterine flush (1 cow).

**No significant differences in proportion of fertilized ova between treatment groups ($p > 0.10$).

Appendix Table III. Fertilized and Unfertilized Ova Collected: Experiment II - Heifers

| | Semen Quality (% intact acrosomes) | | | |
|---|---|---|---|---|
| | High | | Low | |
| Heifer | Fert | Unfert | Fert | Unfert |
| 1 | 10 | 0 | -* | - |
| 2 | 2 | 5 | 6 | 0 |
| 3 | 5 | 4 | 1 | 2 |
| 4 | 5 | 2 | 8 | 3 |
| 5 | 12 | 0 | - | - |
| 6 | 4 | 0 | - | - |
| 7 | -* | - | 17 | 0 |
| 8 | - | - | 10 | 6 |
| 9 | - | - | 0 | 1 |
| 10 | - | - | - | - |
| 11 | - | - | - | - |
| Totals | 38 | 11 | 42 | 12 |
| % Fertilized** | 77.6 | | 77.8 | |

*Incomplete data due to:
1) Lack of ovarian response, no uterine flush (4 heifers).
2) Zero ova collected by uterine flush (6 heifers).

**No significant differences in proportion of fertilized ova between treatment groups ($p > 0.10$).

# THE INFLUENCE OF SEMEN QUALITY, AS DETERMINED BY PERCENT INTACT ACROSOMES, ON FERTILIZATION RATES IN SUPEROVULATED COWS

by

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B.S., Kansas State University, 1980
D.V.M., Kansas State University, 1982

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The influence of semen quality on fertilization rates in the single-egg ovulating cow has been extensively studied. However, this influence in relation to the multiple-egg ovulating cow has not been defined. This research is designed to identify the influence of semen quality, specifically related to varying percent intact acrosomes, on the fertilization rates in multiple-ovulating cows.

In both experiments, a randomized complete block design was utilized to analyze the influence of different semen qualities on the fertilization rate of ova from 12 different cows superovulated three consecutive times and from 11 different heifers superovulated two consecutive times. In the initial experiment, 12 non-lactating crossbred beef cows were superovulated and inseminated with semen of three qualities, as determined by percent intact acrosomes. The semen used was from one bull, was extended to 27 million live spermatozoa per breeding unit in the fresh stage, was frozen, and was thawed at three specific temperature/time scales (65°C/observed thaw, 35°C/18 seconds, 4°C/90-100 seconds) to obtain percent intact acrosomes of 76%, 64%, and 46%, respectively. Eleven crossbred beef heifers were then superovulated and inseminated with semen of two qualities, as determined by percent intact acrosomes. The semen used was from the same bull with percent intact acrosomes of 76% and 46%.

Superovulation of the initial 12 cows was achieved using a constant 5mg FSH BID IM for a total of 10 doses, starting at day 9 following estrus. Thirty-five mg and 10mg $PGF_2\alpha$ were given in conjunction with the seventh and eighth doses of FSH, respectively. All cows received 100mcg GnRH at standing estrus. Two 1/2-cc straws of specific quality semen were inseminated at 0 hour and 12 hours post-standing estrus.

Superovulation of the 11 heifers was achieved with a decreasing dose of FSH, over four days, for a total of 36mg FSH, starting at day 9 following estrus. Cloprostenol was given in conjunction with the fifth and sixth doses of FSH. All heifers received 100mcg GnRH at standing estrus. One 1/2-cc straw of specific quality semen was inseminated at 12 hours and 24 hours post-standing estrus. Embryos and ova from each cow and heifer were collected non-surgically at day 7 post-insemination. Embryos and ova collected were examined under an inverted light microscope for the presence of cellular division, indicating fertilization.

Data from both experiments was analyzed by ANOVA. Cow/heifer treatments with incomplete data were removed from analysis. Cow/heifer treatments with zero fertilized or unfertilized ova were included in the analysis. Cow/heifer-semen interactions were assumed to be negligible.

Results of the initial experiment were 63.0%, 78.6%, and 77.8% fertilized ova for specific percent intact acrosomes of 76%, 64%, and 46%, respectively. Results of the subsequent experiment were 77.6% and 77.8% fertilized ova for specific percent intact acrosomes of 76% and 46%, respectively. No significant differences between treatments were noted in either experiment ($p > .10$). In conclusion, the insemination of this many spermatozoa is sufficient to override the influence of decreased percent intact acrosomes induced by various thaw rates.