FACTORS IMPORTANT TO THE EFFICIENCY OF ARTIFICIAL INSEMINATION IN SINGLE-OVULATING AND SUPEROVULATED CATTLE

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ABSTRACT

To identify factors important to the efficiency of artificial insemination in cattle, four studies were conducted. In the first study, the addition of cream to the inseminate was used in an attempt to increase accessory sperm number. On d 6 after insemination, 60 embryos were evaluated. The addition of cream to the inseminate had no effect on accessory sperm number. In the second study, cryopreserved semen of a marked bull (spermatozoa exhibiting a semi-flattened anterior head) was matched with semen from an unmarked bull (conventional sperm head shape) to determine competitively the effect of a deep uterine insemination on accessory sperm number. Forty embryos were recovered 6 d after insemination and the ratio of accessory sperm observed was different: 62:38 for unmarked semen in the uterine body and marked semen in the uterine horn, and 72:28 for unmarked semen in the uterine horn and marked semen in the uterine body ($P < .05$). In the third study, superovulated cows were utilized to determine the effect of artificial insemination time on fertilization status and accessory sperm number. Cows were inseminated once at 0 h (n=10), 12 h (n=10), or 24 h (n=10) after the first standing event. On d 6 after insemination, 529 embryos(ova) were recovered. Fertilization

rates were 29% (0 h); 60% (12 h); and 81% (24 h)($P < .01$). Percentages of embryos with accessory sperm were: 5 (0 h); 8 (12 h); and 41(24 h) ($P < .01$). In the fourth study, three experiments utilizing superovulated cows were conducted to provide a basis for distinguishing unfertilized ova from very early embryonic death. In Exp. 1, recovered d 6 unfertilized ova were classified morphologically as either: 1) typical, 2) satellite, or 3) fragmented. In Exp. 2, recovered d 6 unfertilized ova from the third study were classified morphologically, and typical ova were fixed. In Exp. 3, ultrastructural features of preovulatory, tubal-stage, and typical d 6 unfertilized ova were investigated. Preovulatory ova revealed normal ultrastructure; tubal-stage ova exhibited evidence of degeneration; typical d 6 ova were degenerated and contained no discernable organelles. The first three studies support the use of accessory sperm evaluation as an alternative measure of fertility. The final study provides a basis from which future embryologists may distinguish fertilization failure from very early embryonic death.

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CHAPTER 1

INTRODUCTION

Worldwide, the most heavily used reproductive technologies in cattle are artificial insemination (AI) and embryo transfer (ET). Semen selection and AI can result in milk production increases of approximately 100 kg/yr (Peel and Bauman, 1987), while ET can cause additional increases of 40 kg/yr (Van Vleck, 1981).

In 1991, 44 million cows received first service AI worldwide (Chupin and Thibier, 1994). Greater than 12 million units of dairy semen were sold domestically in 1997, with an additional 8.7 million units sold for export (NAAB, unpublished). In 1997, 82,307 uterine flushes of cattle resulted in the recovery of 456,258 transferable embryos (Thibier, 1998). The total number of embryos transferred was 360,656 (168,373 fresh and 192,283 frozen-thawed)(Thibier, 1998). Europe and North America accounted for 70% of all bovine embryo collection and transfer activity in 1997.

Milk production and reproductive performance are the most important factors determining profitability of dairy cattle. Reproductive inefficiency is second to low milk production as the leading cause of culling in dairy herds. For optimal milk production and reproductive efficiency, dairy cattle should calve at 12 to 13.5 month intervals. To achieve this goal, dairy cattle must conceive within approximately 100 days of calving. This can be a difficult task for many producers because first service

conception rates (all cows pregnant to a first service in a period divided by the total number of first services in the same period) rarely exceed 50% in lactating cows. Although many personnel factors contribute to low conception rates, including heat detection accuracy, semen handling, site of semen deposition, and time of insemination, many producers have lost enthusiasm for AI (Chupin and Thibier, 1995). Furthermore, as herd sizes continue to increase without new employees being hired, many producers have erroneously chosen to save time and money in labor costs and use herd bulls. Hunter and Greve (1997) argue that enthusiasm for AI might be rekindled by research findings fostering increased success to AI, thus allowing the continuation of genetic progress and safeguarding of animal health.

Statement of the Problem

Two common methods used to monitor fertility are nonreturn rate and pregnancy rate. Rycroft (1992) defined nonreturn rate as the percentage of cows that are not subsequently rebred within a specified period of time after an insemination, typically 60 to 90 d. Pregnancy rate is defined as the number of cows diagnosed pregnant (by rectal palpation) divided by the total number of cows inseminated. Typical nonreturn rates vary between 60 and 75%, while pregnancy rates are usually below 50%. Nonreturn is an indirect measure of fertility, and can be influenced by heat detection efficiency, herd management, and accuracy of record keeping. Thus, nonreturn rates tend to overestimate fertility. Although

pregnancy rates provide a more accurate measure of fertility than nonreturn data, pregnancy rates encompass fertilization success or failure, and early embryonic death. Pregnancy rates do not describe why inseminations are successful or unsuccessful. Furthermore, pregnancy rates do not give insight into how physiologists and producers might optimize fertilization rates, decrease the incidence of early embryonic death, and ultimately increase pregnancy rates.

Accessory sperm quantification is a research tool which may provide a more sensitive measure of fertility than either nonreturn or pregnancy rate. In this procedure, embryos(ova) are recovered by uterine flush 6 d after insemination. The fertilization rate is calculated, the morphological embryo quality grade is judged, and the number of sperm trapped in the zona pellucida of each embryo(ova) is quantified. The number of accessory sperm in the zona pellucida has been positively associated with fertility in cattle (Hunter and Wilmut, 1984; Hawk and Tanabe, 1986; DeJarnette et al., 1992; Nadir et al., 1993). Fertilization rates on d 6 after insemination are typically greater than 75% for single-ovulating cattle. In contrast, pregnancy rates as determined by rectal palpation between 35 to 70 d after insemination rarely exceed 50%. By recovering embryos(ova) 6 d after insemination, physiologists may gain insight into whether the disparity between fertilization rates and pregnancy rates occurs because of fertilization failure or early embryonic death. By virtue of the aforementioned positive relationship between

accessory sperm and fertility, accessory sperm studies which seek to increase accessory sperm numbers may lead to increased fertilization rates, embryo quality, and pregnancy rates.

In efforts to improve the efficiency of AI in single-ovulating and superovulated cattle and further refine accessory sperm number as an alternative, more descriptive indicator of fertility than nonreturn and pregnancy rates, four studies have been performed. The first three studies focus upon an applied approach to reproduction: 1) supplementation of the inseminate with cream in an effort to aid sperm transport and retention; 2) a competitive insemination trial investigating the site of semen deposition; and 3) the time of insemination of superovulated cows relative to the true onset of estrus. The final study focuses on the morphology of unfertilized ova recovered from superovulated cattle. This investigation into the structural characteristics of unfertilized ova was initiated in an attempt to provide a basis from which future embryologists will be able to investigate and distinguish between fertilization failure and very early embryonic death. Recognizing that fertilization failure and very early embryonic death are two components of pregnancy rate, distinguishing between these two problems is of utmost importance to the evaluation of reproductive efficiency of the male and female.

Literature Cited

Chupin, D., and M. Thibier. 1994. Résultats d'une enquête sur l'état de l'insémination Artificielle dans les pays développés. Elevage and Insémination. 263:1.

Chupin, D., and M. Thibier. 1995. Survey of the present status of the use of artificial insemination in developed countries. World Animal Review. 82:58.

DeJarnette, J.M., R.G. Saacke, J. Bame, and C.J. Vogler. 1992. Accessory sperm: Their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J. Anim. Sci. 70:484.

Hawk, H.W., and T.Y. Tanabe. 1986. Effect of unilateral cornual insemination upon fertilization rate in superovulating and single-ovulating cattle. J. Anim. Sci. 63:551.

Hunter, R.H.F., and T. Greve. 1997. Could artificial insemination of cattle be more fruitful? Penalties associated with ageing eggs. Reprod. Dom. Anim. 32:137.

Hunter, R.H.F., and I. Wilmut. 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reprod. Nutr. Dev. 24:597.

Nadir, S., R. G. Saacke, J. Bame, J. Mullins, and S. Degelos. 1993. Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle. J. Anim. Sci. 71:199.

Peel, C.J., and D.E. Bauman. 1987. Somatotropin and lactation. J.Dairy Sci. 70:474.

Rycroft, H. 1992. Factors influencing non-return data. In: Proc. XIV Tech. Conf. Artif. Insem. and Reprod., pp.43-46. NAAB, Columbia, MO.

Thibier, M. 1998. The 1997 embryo transfer statistics from around the world: A data retrieval committee report. In: Embryo Transfer Newsletter, Ed. M.B. Wheeler, pp. 17-20. International Embryo Transfer Society, Savoy, Illinois.

Van Vleck, L.D. 1981. Potential genetic impact of artificial insemination, sex selection, embryo transfer, cloning, and selfing in dairy cattle. In: New technologies in animal breeding, Eds. B.G. Brackett, G.E. Seidel, Jr., and S.M. Seidel, pp 221-241. Academic Press, Inc., New York.

CHAPTER 2

LITERATURE REVIEW

Historical Perspective of AI

In 1677 Anton van Leeuwenhoek discovered sperm with the aid of a crude microscope. As described by Gilbert (1991), although Leeuwenhoek initially assumed sperm to be parasitic animals having nothing to do with reproduction, he later came to believe that each sperm contained a preformed human. Nicolas Hartsoeker concurred, and in 1694 drew what he envisioned was contained within human sperm, a preformed human which he labelled "homunculus"(as cited in Gilbert, 1991). This belief never gained much acceptance, most likely because it implied an enormous waste of potential life.

The first scientific research in AI of domestic animals was performed by L. Spallanzani in 1780. As recounted by Perry (1968), Spallanzani deposited fresh dog semen in the uterus of a bitch utilizing a pointed syringe. Sixty-two days later the bitch whelped, and all three pups resembled the bitch and the sire. Spallanzani was also the first to filter semen and recognize that the fertilizing capability resided in the sperm fraction which remained on the filter, not in the seminal fluid which passed through (Perry, 1968; Herman, 1981). Furthermore, in 1803 Spallanzani observed that cooling stallion semen in snow did not kill sperm, but rather held them motionless until exposed to heat (Perry, 1968; Herman, 1981).

As described by Perry (1968), Hoffman of Stuttgart, Germany, following the advice of the French veterinarian Repiquet, practiced "supplementary AI" after natural mating in horses during the 1890's. This was accomplished by: 1) collecting semen naturally deposited in the vagina using a speculum and spoon; 2) diluting the semen with cow's milk; 3) loading the extended semen in a special syringe; and 4) depositing the semen into the uterus (Perry, 1968).

In 1897, W. Heape of England reported on the work of dog breeder E. Millais, who successfully artificially inseminated 19 bitches between 1884 and 1896. According to Perry (1968), Heape (1897) argued that since 15 bitches whelped, it appeared that AI was not difficult and that a single ejaculate could serve several bitches.

As noted by Perry (1968), Sand and Stribolt of Denmark artificially inseminated eight mares near the turn of the century, which resulted in four conceptions. Although Sand is the first on record to consider AI for the improvement of farm animals, it was E. I. Ivanov who, in 1899 was requested by the chief of the Royal Russian Stud to investigate the feasibility of AI in horse breeding (Perry, 1968). In 1912, Ivanov successfully inseminated 39 mares, 31 of which conceived (Perry, 1968). Ivanov also headed the first AI school during the years prior to World War I, in which approximately 350 veterinarians were trained as AI technicians (Perry, 1968). Approximately 40,000 mares, 1.2 million cows and 15 million sheep in Russia received AI in 1938 (Herman, 1981).

G. Amantea, a physiologist at the University of Rome, worked with sperm from the dog, cock, and pigeon, and is credited with the development of the first artificial vagina (Perry, 1968; Herman, 1981). Circa 1914, this artificial vagina was used to collect semen from the dog. In the 1920's, Milovanov and coworkers developed artificial vaginas to collect semen from the bull, stallion, and ram (Perry, 1968). In 1948, Laplaud and Cassou, and Thibault et al. reported on the use of electroejaculation to collect semen from those bulls unwilling or unable to mount a teaser animal.

As discussed in historical reviews by Perry (1968) and Herman (1981), the first cooperative AI organization was formed in Denmark by E. Sorenson and J. Gylling-Holm in 1936. Over 1,000 cows were inseminated during the first year. The first service conception rate was 59% (Perry, 1968). Also during this time period, Danish veterinarians developed the rectovaginal method of AI for cows, with semen deposition deep in the cervix or into the uterus (Perry, 1968; Herman 1981). Previous to the development of this technique, semen was deposited in the vagina, or intracervically with the aid of a vaginal speculum.

Domestically, the first calf born as a result of AI was owned by R. L. Hughey of Alva, Oklahoma. Immediately following the natural service of one cow, semen was collected from the vagina, placed into a gelatin capsule, and inserted into another cow in heat (Herman, 1981). The year was 1907.

By the 1930's the use of AI in the United States had progressed as 130

calves were born at Pabst Farms, Wisconsin, between 1934 and 1938, and 98 cows conceived to AI during 1937-38 at the North Central School of Agriculture and Experiment Station in Grand Rapids, Minnesota (Perry, 1968). In 1938, a critical event in domestic AI occurred in New Jersey when E. J. Perry, a Rutgers University extension specialist, helped open and manage the first AI organization in the United States, the Cooperative Artificial Breeding Association # 1 (Perry and Bartlett, 1955; Herman, 1981). During this same time period, many agricultural schools such as Missouri-Columbia, Nebraska, Minnesota, Wisconsin, Tennessee, and Cornell began using AI in their herds (Herman, 1981). As a result, a flurry of research regarding semen extension, storage, shipping, and AI techniques followed over the ensuing years.

Extension and Freezing of Semen

Phillips and Lardy (1940) reported on the use of egg yolk as a semen extender to protect sperm cells against damage during cooling, consequently allowing fresh semen to be preserved for a few days. Salisbury et al. (1941) added a buffer, sodium citrate, to the egg yolk extender, allowing high extension rates of semen and the possibility of inseminating larger numbers of cows. There was a multitude of research regarding semen extenders at end of the 1940's and the beginning of the 1950's. Almquist et al. (1946;1949) reported on the addition of antibiotics to semen to control pathogenic microorganisms; Mijachilov (1950) and Thacker and Almquist (1953) developed heated cow's milk extender. The addition

of antibiotics to semen resulted in a 10% increase in breeding efficiency of cattle during this time period (Perry, 1968), and represents the most important contribution to the enhancement of fertility to AI. Currently, there are many variations of either egg yolk-citrate or milk-based extenders for bull semen in use worldwide.

Polge et al. (1949) reported on the long-term preservation of semen by freezing to -79°C with dry ice. In 1952, Polge and Rowson achieved acceptable fertility following extension of bull semen with egg yolk citrate and equilibration with glycerol prior to freezing. Currently the common refrigerant used in freezing and storage of bull semen is liquid nitrogen (-196°C). In the United States, the biggest proponent of frozen semen was J. Rockefeller Prentice, the founder of American Breeders Service, currently known as ABS Global. According to Herman (1981b), Prentice and the staff at the American Foundation for the Study of Genetics, in Madison, Wisconsin, worked during the early 1950's to perfect frozen semen processing techniques. Consequently, the first calf resulting from frozen semen use in the United States, "Frosty, " was born 29 May 1953 to "Janey, " a grade Holstein owned by J. and M. Hill of Janesville, Wisconsin. The sire was the ABS-owned bull "Pabst Burke Tritomia Fryslan"(Herman, 1981b).

Site of Semen Deposition and Numbers of Sperm Deposited

Prior to the development of the rectovaginal method of AI for cows, with

semen deposition deep in the cervix or into the uterus, semen was deposited in the vagina, or intracervically with the aid of a speculum. Early experiments (Salisbury and VanDemark, 1951; Olds et al., 1953) used unfrozen semen with relatively high spermatozoal concentrations (15 $\times 10^6$ and 4 $\times 10^6$ to 48 $\times 10^6$ total sperm cells, respectively) and showed little difference in fertility when semen was deposited in the uterine horns, uterine body or cervically. It is possible the large numbers of spermatozoa may have masked treatment differences. Salisbury et al. (1978) proposed that fertility increases with increasing numbers of viable sperm inseminated up to a threshold level. After this threshold level has been attained, the female population becomes the limiting factor and further increases in fertility do not result from increases in numbers of sperm. Therefore, large numbers of spermatozoa could mask treatment differences because this threshold value has already been surpassed. In dosage comparison studies of unfrozen extended semen (5 $\times 10^6$ with 10 $\times 10^6$ motile sperm; Salisbury, 1957; and 4 $\times 10^6$ with 8 $\times 10^6$ motile sperm; Foote, 1970) nearly equal 60 to 90 d nonreturn rates (~ 75%) were achieved following thousands of first services. In hindsight, the earlier experiments of Salisbury and VanDemark (1951) and Olds et al. (1953) may not have been sensitive to exposing treatment differences because of the high numbers of sperm utilized. The number of spermatozoa per insemination recommended for semen frozen in straws is between 10 $\times 10^6$ to 30 $\times 10^6$ total sperm cells, as evidenced by the nearly equal nonreturn rates (~78%) reported by Stewart and Bennett (1968).

There appears to be a relationship between the site of semen deposition and the number of viable sperm inseminated necessary to attain optimum fertility, e.g., higher numbers of sperm are necessary for inseminates deposited in the vagina than for those deposited into the uterine body or uterine horns. Comparing AI and natural service, Senger (1993) argued that deposition of semen into the uterus allowed much lower sperm numbers in each dose of semen, by virtue of passing the most formidable barrier to sperm transport, the cervix. Nevertheless, the lowest numbers of sperm per AI needed to attain threshold differs between bulls. Utilizing 20 bulls and over 85,000 first and second services, den Daas et al. (1998) determined that sperm numbers needed to obtain 95% of the maximal conception rate varied among bulls, ranging from 1×10^6 to 11×10^6 viable sperm. In addition, den Daas et al. (1998) reported that the bulls differed in their maximal nonreturn rate at high sperm numbers, and in the rate at which they approached this maximum as sperm numbers per dose were increased.

Macpherson (1968) investigated the depth of insemination with frozen semen (50×10^6 total sperm cells) by utilizing plastic washers cemented to insemination rods, resulting in semen deposition 4 cm (cervically), 8 cm (uterine body), and 12 cm (uterine horn) anterior to the external cervical os. Based on 60 to 90 d nonreturn rates, the greatest fertility was achieved in those animals inseminated in the uterine body (Macpherson, 1968).

To further increase the efficiency of artificial insemination, many studies

have compared semen deposition near the greater curvature of the uterine horns with conventional deposition into the uterine body. In a study by Senger et al. (1988), nine herdsman-inseminators in four commercial dairy herds were trained to deposit semen in the body of the uterus. After using this method for six months, the herdsman- inseminators were retrained and instructed to deposit one-half of the semen into the right uterine horn and one-half into the left uterine horn (both at the greater curvature) for the following six months. Following over 4,000 services, an increase in the conception rate was reported when semen was deposited in the uterine horns rather than the uterine body (65% vs. 45%). In other studies, semen was deposited 2.5 cm (Williams et al., 1988), and 5 cm (McKenna et al., 1990) anterior to the internal cervical os, in the greater curvature following straightening of the horns (Hawk and Tanabe, 1986), and in the cranial half after straightening the horns (Lopez-Gatius, 1996). In the aforementioned studies, Lopez-Gatius (1996) reported an increase in pregnancy rates, while Williams et al. (1988), McKenna et al. (1990), and Hawk and Tanabe (1986) found no difference in the conception rate, nonreturn rate or fertilization rate, respectively, when comparing uterine body and uterine horn inseminations.

In superovulated cows, Hawk et al. (1988) used a modified insemination device requiring 2 technicians to deposit semen near the uterotubal junction. This device consisted of a stainless steel sheath for passage through the cervix, a flexible tube with a rounded epoxy bead attached to the end to facilitate passage of

the tube through the uterine horn, and a nylon rod to expel semen from the tube. When compared with traditional uterine body deposition, they found no effect upon the fertilization rate.

Retrograde Loss of Sperm

Following natural service, the majority of sperm are lost from the reproductive tract shortly after deposition. Those sperm which remain in the tract enter the uterus against the uterine mucosa after following privileged paths within grooves originating in the fornix vagina (Mullins and Saacke, 1989). Intrauterine AI bypasses the cervix, the major barrier to sperm transport, and allows for the use of low numbers of sperm relative to those required in natural service. Nevertheless, within 12 h after insemination approximately 60% of sperm artificially inseminated are lost by retrograde flow (Mitchell et al., 1985).

Gallagher and Senger (1989) investigated whether greater numbers of sperm might be retained in animals inseminated in the uterine horn approximately 7 cm anterior to the internal cervical os. Using a vaginal sampling technique, Gallagher and Senger (1989) found that sperm retention did not differ between uterine horn and uterine body insemination. Furthermore, when semen was deposited mid-cervically, sperm loss was nearly twice that reported for uterine horn deposition.

Sperm Transport

Sperm transport occurs in two distinct phases. The first, or rapid transport

phase, has been shown to occur within a few minutes of insemination in cattle (Hawk, 1987) and rabbits (Overstreet and Cooper, 1978). During this phase, muscle contractions of the female reproductive tract transfer sperm from the site of deposition to the oviducts. In rabbits, many of these sperm are dead, and subsequently cleared to the peritoneal cavity (Overstreet and Cooper, 1978). Thus, sperm in the rapid transport phase are not thought to be involved in fertilization.

The second, or sustained phase of sperm transport, brings sperm capable of fertilizing ova to the isthmus of the oviduct between 6 to 12 hours after insemination (Hunter and Wilmut, 1983; Wilmut and Hunter, 1984; Hawk, 1987). Sperm numbers in the isthmus progressively increase between 8 to 18 h after insemination (Hawk, 1987;Wilmut and Hunter, 1984). In rabbits, sperm colonizing the isthmus during this phase are predominantly live, and are thought to compete for fertilization based upon their motility (Overstreet et al., 1978).

Accessory Sperm

The number of accessory sperm in the zona pellucida has been positively correlated with fertility in cattle (Hunter and Wilmut, 1984; Hawk and Tanabe, 1986; DeJarnette et al., 1992; Nadir et al., 1993). Although accessory sperm are not directly involved in fertilization, they represent sperm able to access the oviduct, undergo capacitation, recognition, binding and the true acrosome reaction, and partially penetrate the zona pellucida. Accessory sperm are trapped in the zona pellucida by the "zona reaction", a functional block to polyspermy which occurs

immediately following fertilization by the fertilizing sperm. Thus, accessory sperm are thought to be an indirect measure of sperm transport, and a quantitative measure of sperm available and competing for fertilization (DeJarnette et al., 1992).

Utilizing a device consisting of a standard .5-mL French insemination gun encased in a 24-gauge Foley catheter, DeJarnette et al. (1992) attempted to increase the number of accessory sperm by mechanical blockage of retrograde sperm loss at artificial insemination. Following passage of the catheter tip into the uterine body, the cuff of the Foley catheter was inflated and the insemination device was pulled posteriorly to ensure blockage occurred at the internal cervical os. Semen was deposited in the uterine body and the block was left in place for up to three hours. This technique failed to increase the number of accessory sperm per embryo(ovum); however, the number of accessory sperm per embryo was positively related to fertilization and embryo quality (DeJarnette et al., 1992).

Nadir et al. (1993) investigated the use of fresh or frozen semen at 20×10^6 or 100×10^6 sperm per dose on the number of accessory sperm per embryo(ovum). Freezing semen had no effect on fertility or any accessory sperm measurements at either dosage. In addition, there was no interaction between freezing semen and dosage. Therefore, Nadir et al. (1993) combined the embryos(ova) recovered from inseminations with fresh and frozen semen and reexamined the data for a possible effect of dosage on accessory sperm. An increase in the median number of accessory sperm per embryo(ovum) resulted

from insemination with the high dose (Nadir et al., 1993). Furthermore, insemination with the high dose improved the percentage of embryos(ova) with accessory sperm, fertilization rate, and embryo quality.

Oviductal Proteins, Ova, and Accessory Sperm

It is also possible that accessory sperm number is related to the oviductal microenvironment at the time of fertilization. Oviductal fluid proteins derived from oviductal epithelium are thought to play a role in sperm capacitation and the acrosome reaction, two mandatory events in the fertilization process (Way et al., 1997;Staros and Killian, 1998). Although the specific function of these proteins has not been elucidated, they have been shown to associate with the bovine sperm plasma membrane (McNutt et al., 1992) and the bovine zona pellucida (Wegner and Killian, 1991;Staros and Killian, 1998). Deficiencies in oviductal fluid protein composition and(or) volume within an animal may lead to: 1) a reduction in capacitated sperm, and(or) 2) a decreased ability of ova to bind sperm, resulting in diminished sperm-ova interaction and a concomitant decrease in the number of accessory sperm.

Accessory sperm number may also be regulated by the relative composition of the zona pellucida. Protease digestion of the zona pellucida of superovulated embryos(ova) requires half the time necessary to digest the zona pellucida of single-ovulated embryos(ova) (Saacke et al., 1994). "Hardness" (relative resistance to protease digestion) of the zona pellucida may be dependent on the source of the

ovum, and may play a role in the time required for sperm transit through the zona pellucida. In this scenario, single-ovulated ova exhibit more accessory sperm than superovulated ova due to increased transit times prior to fertilization and the zona reaction. The putative compositional differences of the zona pellucida between superovulated and single-ovulated ova are unknown. Nevertheless, these apparent differences may be important to the selection of sperm by the zona pellucida, as previously described by Howard et al. (1993).

Compensable and Uncompensable Seminal Traits

Compensable traits of semen quality relate to the ability of inseminated sperm to not only reach the ovum, but also initiate and complete fertilization. Uncompensable traits of semen quality relate to the competence of fertilizing sperm to complete fertilization and(or) sustain early embryonic development. Nuclear vacuoles (craters or diadem) on otherwise normally shaped sperm heads and sperm with subtle head distortions appear as accessory sperm in vivo at the same frequencies as they occur in the inseminate (Saacke et al., 1998). Furthermore, the use of semen with these traits has been shown to depress embryo quality and fertilization rates compared with control semen where these traits were not evident or were minimized (Miller et al., 1982; DeJarnette et al., 1992; Saacke et al., 1992). Therefore, nuclear vacuoles (craters or diadem) and subtle sperm head distortions are considered uncompensable seminal traits. Severely misshapened sperm do not appear as accessory sperm (Saacke et al., 1998), and thus are not thought to be

able to traverse the barriers of the female reproductive tract. Consequently, severely misshapened sperm are considered a compensable seminal trait. Impaired progressive sperm motility may be one of the reasons for the exclusion of these sperm as Dresdner and Katz (1981) reported that even small geometrical differences in sperm head morphology can cause large differences in sperm motility.

Compensable seminal traits cannot be explained completely by morphology and present-day in vitro viability measurements. Bulls whose sperm are able to access the ovum in vivo at low insemination dose based on fertility data (den Daas et al., 1998) or accessory sperm numbers per embryo(ova) (Nadir et al., 1993) may differ from sperm of other bulls in motility patterns or sperm surface modifications. Hyperactivated motility, instead of progressive motility, is thought to be more important for penetration of the zona pellucida in mice (Suarez and Dai, 1992). Killian et al. (1993) argued that sperm surface modifications may involve seminal plasma proteins, while Bellin et al. (1994) reported that heparin-binding proteins in sperm membranes and seminal fluid were positively related to fertility in bulls.

Historical Perspective of the Discovery of the Ovum

Regnier de Graaf described and illustrated rabbit blastocysts in 1672 (translated by Jocelyn and Setchell, 1972). Although de Graaf also described follicles and corpora lutea in cattle, it was not until 1827 that the mammalian ovum was found. According to Betteridge (1981), in 1827 Karl Ernst von Baer described

"opening his pet dog" and finding "a small yellow fleck" within each follicle. von Baer opened a follicle, removed "the fleck" with a knife, transferred "the fleck" to a watchglass filled with water, and upon examination with a microscope, discovered the ovum. In 1840 Hausmann observed the tubal ova of sheep (as cited in Betteridge, 1981); however, ninety-one years passed before the tubal ova of cattle were described (Hartman et al., 1931).

Historical Perspective of Superovulation, Embryo Recovery and Transfer

In the late 1920's Smith and Engle (1927) and Zondek and Aschheim (1927) separately demonstrated the pituitary-gonadal relationship. In one of a series of studies, Engle and Smith (1927) injected rabbit and rat pituitaries into the legs of mice over a period of several days. Consequently, the mice were induced to superovulate, as up to 58 ova were observed in the uterine tubes during necropsy. As cited by Casida et al. (1943), Rumjancev (1938) utilized "fresh pituitary substance" to induce superovulation in cattle. Research in Wisconsin utilizing pituitary extracts (Casida et al., 1943) and studies in Britain utilizing pregnant mare serum gonadotropin (PMSG) and pituitary extracts (Parkes and Hammond, 1940; Folley and Malpress, 1944) established the superovulatory effects of both PMSG and pituitary extracts in cattle and sheep.

Although the first successful embryo recovery and transfer in rabbits was championed by W. Heape in 1891 (as cited by Betteridge, 1981), the first embryo

transfer calf was not born until 1951, the result of a joint study between the University of Wisconsin and the USDA (Willett et al., 1951). A d 5 embryo was collected at slaughter and surgically transferred to the uterine horn contralateral to the corpus luteum. In hindsight this was nearly a fatal experimental error, given that later studies would provide evidence that endogenous prostaglandin, originating from the ipsilateral uterine horn and acting locally, contributes to the regression of the corpus luteum unless muted or negated by the conceptus. Even though the recipient had not been inseminated, coat-color descriptions and blood-typing was used to give evidence of parentage.

Previous to the work by Willett et al. (1951), early work in bovine embryo recovery and transfer was initiated by G. Pincus and R. Umbaugh with the Foundation of Applied Research in San Antonio, Texas during the 1940's (Chang, 1971; Betteridge, 1981). Umbaugh achieved the first 4 pregnancies with transferred embryos in cattle, although they all aborted prior to eight months (as reviewed by Betteridge, 1981).

Attempts to conserve embryos began when M.C. Chang (1947) reported on the low temperature (10°C) preservation of rabbit embryos for several days. According to Betteridge (1981) the experimental progression from cooling embryos to freezing embryos occurred slowly during the 1950's and 1960's. Indeed, twenty-six years elapsed before Wilmut and Rowson (1973) reported the birth of the first live calf derived from a frozen-thawed embryo. The calf was named "Frosty II," an

obvious reference to the first calf produced as a result of the use of frozen semen.

Insemination Regimens of Superovulated Cattle

Previous insemination studies with superovulated cattle vary widely with respect to a suggested insemination regimen. Early work by Elsden et al. (1976) utilized: 1) fresh, extended semen containing 50×10^6 motile sperm inseminated at 12 and 24 hours after observed estrus; or 2) two, three, and one frozen-thawed doses inseminated at 12, 24, and 36 hours after observed estrus. Elsden et al. (1976) achieved an overall 70% fertilization rate but failed to report a fertilization rate resulting from each insemination schedule.

Critser et al. (1980) superovulated 76 heifers and reported a 60% fertilization rate following insemination with either one dose of semen 12 hours after observed estrus, three doses at 12 hours, or one dose at 0, 12, and 24 hours. Each dose contained approximately 20×10^6 total sperm cells (Critser et al., 1980).

A few insemination studies with superovulated cattle have associated the time of observed estrus synonymously with the onset of estrus. In a study utilizing nearly 400 cows and six insemination schemes, Donaldson (1985) reported a 74% fertilization rate in superovulated animals inseminated with one dose of semen 12 hours after onset (observed estrus). In contrast, based on an 89% fertilization rate resulting from the use of frozen-thawed semen from six bulls bred to six cows, Schiewe et al. (1987) recommended insemination with two doses (approximately 40 $\times 10^6$ total sperm cells) of semen 24 hours after onset (observed estrus).

The recommended insemination regimens cited here must be viewed cautiously because: 1) Elsden et al. (1976) and Critser et al. (1980) did not mention the frequency of estrus observations; and 2) the timing of the onset of estrus has an 8 to 12 h error based on the frequency of visual observation by Donaldson (1985) (three times daily) and Schiewe et al. (1987)(twice daily).

The Morphological Evaluation of Embryos(Ova)

Morphological evaluation of embryos with a stereomicroscope is used to identify embryos of transferable and freezable quality. Current evaluation systems score embryos either on a scale of 1 to 4 (International Embryo Transfer Society), or excellent, good, fair, or poor (Lindner and Wright, 1983). Grade 1(excellent) embryos are routinely frozen for later use, while grade 2(good) embryos are primarily transferred fresh. Grade 3(fair) embryos may also be transferred fresh, but are not suitable for freezing (International Embryo Transfer Society). High quality embryos consistently yield the highest pregnancy rates, reported to be greater than 45% (Elsden et al., 1978; Shea, 1981; Lindner and Wright, 1983), while low quality embryos yield pregnancy rates of only 20 % (Lindner and Wright, 1983).

Regardless of the evaluation system used, embryos are classified relative to their anticipated stage of development at the time of recovery. On d 6 after insemination, the anticipated stage of embryonic development is the compact morula exhibiting a homogeneous cell mass. On d 7 after insemination, the

anticipated stage of embryonic development is the blastocyst. Those ova with no sign of cleavage are designated as unfertilized, while those exhibiting underdeveloped blastomeres with nuclei visible under phase contrast or differential interference contrast optics are classified as degenerate embryos (DeJarnette et al., 1992; Nadir et al., 1993). Although bovine embryo morphology has been described for the compact morula (Shea et al., 1976; Elsden et al., 1978; Lindner and Wright, 1983) and the blastocyst in superovulated cattle (Linares and King, 1980), there is little information on the structural characteristics of unfertilized ova (Church and Shea, 1976; Shea, 1981; Hytell et al., 1988). Thus, the ability to distinguish very early embryonic death from spontaneous, degenerative embryo fragmentation is not possible.

Structural Characteristics of Preovulatory and Tubal-stage Bovine Ova

In 1972, Fleming and Saacke reported on the fine structure of oocytes from mature Graafian follicles of unstimulated cattle in natural estrus. These authors described the oocytes based on organelle morphology and distribution, formation of the perivitelline space, and structural configuration of nuclear components. Fleming and Saacke (1972) reported that as the oocytes matured, the mitochondria and lipid inclusions relocated from a peripheral to a general distribution, the perivitelline space became apparent, the chromosomes condensed, and the nuclear envelope broke down. The work of Fleming and Saacke (1972) preceded the discovery of the

importance of the LH surge upon the maturation of the ovum and ovulatory follicle.

Kruip et al. (1983) observed the presence of the perivitelline space from .5 h after the LH peak, while germinal vesicle breakdown (GVBD) occurred approximately 4 to 8 h after the peak LH level. These authors also reported the relocation of mitochondria and lipid inclusions as the oocytes matured, between 19 and 25 h after the LH peak. During the same time period, Kruip et al. (1983) noted that the cortical granules were present immediately below the plasma membrane.

In general, the ultrastructural observations of the preovulatory maturation of oocytes from superovulated cattle follow the same patterns as described for single-ovulating cattle. Hytell et al. (1986) reported GVBD occurred about 9 to 12 h after the LH peak, while the mitochondria assumed an even spatial distribution approximately 15 h after the LH peak. About 22 h after the LH peak the cortical granules were present immediately below the plasma membrane (Hytell et al., 1986).

There are no studies which have specifically investigated the structural characteristics of bovine tubal-stage ova. Brackett et al. (1980), in a study of fertilization and early embryo development utilizing superovulated cattle, reported on the ultrastructural characteristics of one tubal-stage ovum recovered between approximately 56 to 64 h after observed estrus. Brackett et al. (1980), described this ovum as exhibiting "an indefinite perivitelline space and severely degenerated ooplasm." Brackett et al. (1980) postulated that these ultrastructural features

resulted from the ovulation of an abnormal ovum, due to "unusual hormonal influences."

Structural Characteristics of In Vivo Fertilization

Hytell et al. (1988), using superovulated heifers, described the ultrastructural aspects of in vivo fertilized, possibly fertilized, and unfertilized ova recovered from the oviducts within 24 h from the estimated time of ovulation. Fertilized ova exhibited activated female chromatin, and at least one sperm head or its succeeding stages were found in the ooplasm (Hytell et al., 1988). These authors described possibly fertilized ova as having activated female chromatin; however, no sperm head or its succeeding stages were found in the ooplasm. Although the sperm heads could not be traced, the possibly fertilized ova were thought to be fertilized because the cortical granule reaction occurred in all fertilized and possibly fertilized ova (Hytell et al., 1988). Unfertilized ova were typically in metaphase II, exhibited retained cortical granules, and usually were without accessory sperm. These authors also reported that the first polar body was degenerating in most unfertilized ova, and was not observed as time increased (> 7 h) from the estimated time of ovulation. In one-third (4 of 12) of the unfertilized ova recovered, deviations of nuclear and(or) cytoplasmic oocyte maturation were noticed (Hytell et al., 1988). Three unfertilized ova recovered between 4 and 15 h after the estimated time of ovulation displayed a peripheral distribution of mitochondria. This was interpreted

as evidence of cytoplasmic immaturity because mature bovine oocytes exhibit a general distribution of mitochondria (Hytell et al., 1986). One unfertilized ovum recovered approximately 15 h after the estimated time of ovulation displayed deviations in both nuclear and cytoplasmic maturation. The oocyte nucleus was at the GVBD stage, the mitochondria were located peripherally, and the cortical granules were in big clusters. These occurrences were interpreted as signs of nuclear and cytoplasmic immaturity as a metaphase II plate, a general distribution of mitochondria, and the presence of cortical granules immediately below the plasma membrane were evident in all other mature bovine ova recovered (Hytell et al., 1988).

Restatement of the Problem

Artificial insemination has a rich history and has played a significant role in the improvement of domestic animals. Early investigations into increasing the efficiency of AI included inseminations with above threshold numbers of sperm, and utilized nonreturn rates and pregnancy rates as measures of fertility. Nonreturn rates may be influenced by management factors and tend to overestimate fertility. Although pregnancy rates provide a more accurate measure of fertility than nonreturn data, pregnancy rates encompass fertilization success or failure, and early embryonic death. Consequently, pregnancy rates do not give insight into how physiologists and producers might optimize fertilization rate, and decrease the incidence of early embryonic death. By virtue of the aforementioned positive

relationship between accessory sperm and fertility, studies which seek to increase accessory sperm numbers are necessary and may lead to increased fertilization rates, embryo quality, and pregnancy rates. Therefore, by recovering embryos(ova) 6 d after insemination, physiologists may gain insight into whether the disparity between fertilization rates and pregnancy rates occurs because of fertilization failure or early embryonic death. Lastly, the ability to distinguish between fertilization failure and very early embryonic death (two components of pregnancy rate), is of utmost importance to future efforts to increase the efficiency of AI in single-ovulating and superovulated cattle.

Literature Cited

Almquist, J.O., W.T.S. Thorp, and C.B. Knodt. 1946. The effect of penicillin upon the livability, fertility and bacterial content of bull semen. J. Anim. Sci. 5:400.

Almquist, J.O., P.J. Glantz, and H.E. Shaffer. 1949. The effect of a combination of penicillin and streptomycin upon the livability and bacterial content of bovine semen. J. Dairy Sci. 32:183.

Bellin, M.E., H.E. Hawkins, and R.L. Ax. 1994. Fertility of range beef bulls grouped according to presence or absence of heparin-binding proteins in sperm membranes and seminal fluid. J. Anim. Sci. 72:2441.

Betteridge, K.J. 1981. An historical look at embryo transfer. J. Reprod. Fertil. 62:1.

Brackett, B.G., Y.K. Oh, J.F. Evans, and W.J. Donawick. 1980. Fertilization and early development of cow ova. Biol. Reprod. 23:189.

Casida, L.E., R.K. Meyer, W.H. McShan, and W. Wisnicky. 1943. Effects of pituitary gonadotropins on the ovaries and the induction of superfecundity in cattle. Am. J. Vet. Res. 4:76.

Chang, M.C. 1947. Normal development of fertilized rabbit ova stored at low temperature for several days. Nature London 159:602.

Chang, M.C. 1971. Experimental studies of mammalian spermatozoa and eggs. Biol. Reprod. 4:3.

Church, R.B., and B.F. Shea. 1976. Some aspects of bovine embryo transfer. In: Egg Transfer in Cattle, Ed. L.E.A. Rowson, pp 73-86. Commission of the European Communities, Luxembourg.

Critser, J.K., R.F. Rowe, M.R. Del Campo and O.J. Ginther. 1980. Embryo transfer in cattle:Factors affecting superovulatory response, number of transferable embryos, and length of post-treatment estrous cycles. Theriogenology 13:397.

den Daas, J.H.G., G. DeJong, L.M.T.E. Lansbergen, and A.M. van Wagtendonk-de Leeuw. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J. Dairy Sci. 81:1714.

DeJarnette, J.M., R.G. Saacke, J. Bame, and C.J. Vogler. 1992. Accessory sperm: Their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J. Anim. Sci. 70:484.

Donaldson, L. 1985. Effect of insemination regimen on embryo production in superovulated cows. Vet. Rec. 117:35.

Dresdner, R.D., and D.F. Katz. 1981. Relationship of mammalian sperm motility and morphology to hydrodynamic aspects of cell function. Biol. Reprod. 25:920.

Elsden, R.P., J.F. Hasler, and G.E. Seidel, Jr. 1976. Non-surgical recovery of bovine eggs. Theriogenology 6:523.

Elsden, R.P., L.D. Nelson, and G.E. Seidel. 1978. Superovulating cows with follicle

stimulating hormone and pregnant mare's serum gonadotrophin. Theriogenology 9:17.
Fleming, W.N., and R.G. Saacke. 1972. Fine structure of the bovine oocyte from the mature Graafian follicle. J. Reprod. Fertil. 29:203.
Foote, R.H. 1970. Fertility of bull semen at high extensive rates in tris-buffered extenders. J. Dairy Sci. 53:1475.
Folley, S.J., and F.H. Malpress. 1944. The response of the bovine ovary to pregnant mare's serum and horse pituitary extract. Proc. R. Soc. B. 132:164.
Gallagher, G.R., and P.L. Senger. 1989. Concentrations of spermatozoa in the vagina of heifers after deposition of semen in the uterine horns, uterine body or cervix. J. Reprod. Fertil. 86:19.
Gilbert, S.F. 1991. Fertilization: Beginning a new organism. In: Developmental Biology, Third Edition, p.34. Sinauer Associates, Sunderland, Massachusetts.
Hartman, C.G., W.H. Lewis, F.W. Miller, and W.W. Swett. 1931. First findings of tubal ova in the cow, together with notes on oestrus. Anat. Rec. 48:267.
Hawk, H.W. 1987. Transport and fate of spermatozoa after insemination of cattle. J. Dairy Sci. 70:1487.
Hawk, H.W., and T.Y. Tanabe. 1986. Effect of unilateral cornual insemination upon fertilization rate in superovulating and single-ovulating cattle. J. Anim. Sci. 63:551.
Hawk, H.W., H.H. Conley, R.J. Wall, and R.O. Whitaker. 1988. Fertilization rates in superovulating cows after deposition of semen on the infundibulum, near the uterotubal junction or after insemination with high numbers of sperm. Theriogenology 29:1131.
Heape, W. 1897. The artificial insemination of mammals and subsequent possible fertilization or impregnation of their ova. Proc. Royal Soc. London. 61:52.
Herman, H.A. 1981. History and development of artificial insemination. In: Improving cattle by the millions: NAAB and the development and worldwide application of artificial insemination, pp 1-13. University of Missouri Press, Columbia, Missouri.
Herman, H.A. 1981b. Technical developments:past and future. In: Improving cattle by the millions: NAAB and the development and worldwide application of artificial insemination, pp 83-103. University of Missouri Press, Columbia, Missouri.
Howard, J.G., A.M. Donoghue, L.A. Johnston, and D.E. Wildt. 1993. Zona pellucida filtration of structurally abnormal spermatozoa and reduced fertilization in teratospermic cats. Biol. Reprod. 49:131.
Hunter, R.H.F., and I. Wilmut. 1983. The rate of functional sperm transport into the oviducts of mated cows. Anim. Reprod. Sci. 5:167.
Hunter, R.H.F., and I. Wilmut. 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reprod. Nutr. Dev. 24:597.

Hytell, P., H. Callesen, and T. Greve. 1986. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. J. Reprod. Fertil. 76:645.

Hytell, P., T. Greve, and H. Callesen. 1988. Ultrastructure of in-vivo fertilization in superovulated cattle. J. Reprod. Fertil. 82:1.

Jocelyn, H.D., and B.P. Setchell. 1972. Regnier de Graaf on the human reproductive organs. J. Reprod. Fertil. Suppl. 17.

Killian, G.J., D.A. Chapman, and L.A. Rogowski. 1993. Fertility-associated proteins in Holstein bulls. Biol. Reprod. 49:1202.

Kruip, T.A.M., D.G. Cran, T.H. van Beneden, and S.J. Dieleman. 1983. Structural changes in bovine oocytes during final maturation in vivo. Gamete Res. 8:29.

Laplaud, M., and R. Cassou. 1948. Recherches sur l'électro-éjaculation chez le taureau et le verrat. Compt. Rend. Soc. Biol. (Paris). 142:726.

Linares, T., and W.A. King. 1980. Morphological study of the bovine blastocyst with phase contrast microscopy. Theriogenology 14:123.

Lindner, G.M., and R.W. Wright. 1983. Bovine embryo morphology and evaluation. Theriogenology 20:407.

Lopez-Gatius, F. 1996. Side of gestation in dairy heifers affects subsequent sperm transport and pregnancy rates after deep insemination into one uterine horn. Theriogenology 45:417.

Macpherson, J.W. 1968. Semen placement effects on fertility in bovines. J. Dairy Sci. 51:807.

McKenna, T., R.W. Lenz, S.E. Fenton, and R.L. Ax. 1990. Nonreturn rates of dairy cattle following uterine body or cornual insemination. J. Dairy Sci. 73:1779.

McNutt, T.L., L. Rogowski, R. Vasilatos-Younken, and G. Killian. 1992. Adsorption of oviductal fluid proteins by the bovine sperm membrane during in vitro capacitation. Mol. Reprod. Dev. 53:313.

Michajilov, N.N. 1950. Sperm dilution in the milk. The Czecho-Slovak Vet. Mag., (Abstr.) J. Am. Vet. Med. Assoc. 117:337.

Miller, D.M., F. Hrudka, W.F. Cates, and R. J. Mapletoft. 1982. Infertility in a bull with a nuclear sperm defect:A case report. Theriogenology 17:611.

Mitchell, J.R., P.L. Senger, and J.L. Rosenberger. 1985. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract. J. Anim. Sci. 61:956.

Mullins, J., and R.G. Saacke. 1989. Study of the functional anatomy of the bovine cervical mucosa with special reference to mucus secretion and sperm transport. Anat. Rec. 225:106.

Nadir, S., R. G. Saacke, J. Bame, J. Mullins, and S. Degelos. 1993. Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle. J. Anim. Sci. 71:199.

Olds, D., D.M. Seath, M.C. Carpenter, and H.L. Lucas. 1953. Interrelationships between site of deposition, dosage and number of spermatozoa in diluted semen and fertility of dairy cows inseminated artificially. J. Dairy Sci. 36:1031.

Overstreet, J.W., and G.W. Cooper. 1978. Sperm transport in the reproductive tract of the female rabbit. I. The rapid transit phase of transport. Biol. Reprod. 19:101.

Overstreet, J.W., G.W. Cooper, and D.F. Katz. 1978. Sperm transport in the reproductive tract of the female rabbit. II. The sustained phase of transport. Biol. Reprod. 19:115.

Parkes, A.S., and J. Hammond. 1940. Induction of fertility by the injections of gonadotrophic preparations. Vet. Rec. 52:540.

Perry, E.J. 1968. Historical Background. In: The Artificial Insemination of Farm Animals, Fourth Edition, pp 3-12. Rutgers University Press, New Brunswick, New Jersey.

Perry, E.J., and J.W. Bartlett. 1955. The artificial insemination of dairy cows. N.J. Agr. Col. Ext. Bul. 284.

Phillips, P.H., and H. A. Lardy. 1940. A yolk-buffer pabulum for the preservation of bull semen. J. Dairy Sci. 23:399.

Polge, C., A.U. Smith, and A.S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 164:666.

Polge, C., and L.E.A. Rowson. 1952. Fertilizing capacity of bull spermatozoa after freezing at -79 degrees C. Nature. 169:626.

Rumjancev, N.V. 1938. The influence of total pre-pituitary preparation on the genitalia of the cow (Translated from Russian). Anim. Breed. Abstr. 8:130.

Saacke R.G., J. Bame, C.J. Vogler, S. Nadir, and J. Mullins. 1992. Association of sperm nuclear vacuoles (craters) with failure of sperm to sustain embryonic development after fertilization in cattle. J. Anim. Sci. 70:256(abstr.).

Saacke, R.G., S. Nadir, J. Dalton, J. Bame, J.M. DeJarnette, S. Degelos, and R.L. Nebel. 1994. Accessory sperm evaluation and bull fertility (An Update). In: Proc. XV Tech. Conf. Artif. Insem. and Reprod., pp. 57-67. NAAB, Columbia, MO.

Saacke R.G., J.M. DeJarnette, J.H. Bame, D.S. Karabinus, and S.S. Whitman. 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super- and single-ovulating cattle? Theriogenology 50:117.

Salisbury, G.W., H.K. Fuller, and E.L. Willett. 1941. Preservation of bovine spermatozoa in yolk-citrate diluent and field results from its use. J. Dairy Sci. 24:905.

Salisbury, G.W., and N.L. VanDemark. 1951. The effect of cervical, uterine and cornual insemination on fertility of the dairy cow. J. Dairy Sci. 34:68.

Salisbury, G.W. 1957. Recent developments with bull semen diluents. Animal

Breeding Abstr. 25:111.
Salisbury, G.W., N.L. VanDemark, and J.R. Lodge. 1978. Significance of semen quality. In: Physiology of Reproduction and Artificial Insemination of Cattle, Second edition, pp 428-430. W.H. Freeman and Company, San Francisco.
Schiewe, M.C., C.R. Looney, C.A. Johnson, K.G. Hill, and R.A. Godke. 1987. Transferable embryo recovery rates following different insemination schedules in superovulated beef cattle. Theriogenology 28:395.
Senger, P.L., W.C. Becker, S.T. Davidge, J.K. Hillers, and J.J. Reeves. 1988. Influence of cornual insemination on conception in dairy cattle. J. Anim. Sci. 66:3010.
Senger, P.L. 1993. Site of semen deposition and its effect on fertility and sperm retention: A review. Reprod. Fertil. Dev. 5:659
Shea, B.F., D.J. Hines, D.E. Lightfoot, G.W. Ollis, and S.M. Olson. 1976. The transfer of bovine embryos. In: Egg Transfer in Cattle, Ed. L.E.A. Rowson, pp 145-152. Commission of the European Communities, Luxembourg.
Shea, B.F. 1981. Evaluating the bovine embryo. Theriogenology. 15:31.
Smith, P.E., and E.T. Engle. 1927. Experimental evidence reporting the role of the anterior pituitary in the development and regulation of the genital system. Am. J. Anat. 40:159.
Staros, A.L., and G.J. Killian. 1998. In vitro association of six oviductal fluid proteins with the bovine zona pellucida. J. Reprod. Fertil. 112:131.
Stewart, D.L., and G.H. Bennett. 1968. The minimum number of spermatozoa per frozen semen insemination compatible with normal fertility in cattle. Sixth Int'l. Cong. Anim. Reprod. and Artif. Insem. Paris. II:1167.
Suarez, S.S., and X. Dai. 1992. Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. Biol. Reprod. 46:686.
Thacker, D.L., and J.O. Almquist. 1953. Diluters for bovine semen. Fertility and motility of bovine spermatozoa in boiled milk. J. Dairy Sci. 36:173.
Thibault, C., M. Laplaud, and R. Ortevant. 1948. L'Electro-éjaculation chez le taureau: Technique et résultats. Compt. Rend. Acad. Sci. (Paris) 226:2006.
Way, A.L., A.M. Schuler, and G.J. Killian. 1997. Influence of bovine ampullary and isthmic oviductal fluid on sperm-egg binding and fertilization in vitro. J. Reprod. Fertil. 109:95.
Wegner, C.C., and G.J. Killian. 1991. In vitro and in vivo association of an oviduct estrus-associated protein with bovine zona pellucida. Mol. Reprod. Dev. 29:77.
Willet, E.L., W.G. Black, L.E. Casida, W.H. Stone, and P.J. Buckner. 1951. Successful transplantation of a fertilized bovine ovum. Science, N.Y. 113:247.
Williams, B.L., F.C. Gwazdauskas, W.D. Whittier, R.E. Pearson, and R.L. Nebel. 1988. Impact of site of inseminate deposition and environmental factors that influence reproduction of dairy cattle. J. Dairy Sci. 71:2278.

Wilmut, I., and R.H.F. Hunter. 1984. Sperm transport into the oviducts of heifers mated early in oestrus. Reprod. Nutr. Dev. 24:461.

Wilmut, I., and L.E.A. Rowson. 1973. Experiments on the low-temperature preservation of cow embryos. Vet. Rec. 92:686.

Zondek, B., and S. Aschheim. 1927. Das hormone des hypophysenvorderlappens. Klin. Wschr. 6:248.

CHAPTER 3

EFFECT OF THE ADDITION OF CREAM TO THE INSEMINATE ON ACCESSORY SPERM NUMBER IN ARTIFICIALLY INSEMINATED CATTLE

Abstract

Ejaculates of four bulls were cryopreserved in .5-mL French straws (50 x 10^6 sperm) using clarified egg yolk-citrate-glycerol extender. Upon thawing and immediately prior to insemination, contents of the straw were mixed 1:1 with egg yolk-citrate (control), homogenized cream-egg yolk-citrate, or homogenized cream, each of which contained glycerol equivalent to the thawed semen. The experimental inseminates were carefully drawn into .5-mL French straws and contained 25 x 10^6 sperm and 0%, 3.5%, or 8.3% milk fat. From 116 inseminations, 66 embryos(ova) were recovered on d 6 after estrus. To avoid the potential influence of any one bull it was necessary to randomly balance embryos(ova) recovered across treatments by bull (n=60). The addition of 3.5% or 8.3% fat to the inseminate post-thaw had no effect on accessory sperm number, fertilization status and embryo quality. Mean ± SD and median values for accessory sperm per embryo(ovum) were: 26.9 ± 36.1 and 11 for control, 0% fat; 18.8 ± 29.9 and 5 for 3.5% fat; 18.6 ± 24.1 and 10.5 for 8.3% fat. The percentage of embryos(ova) with accessory sperm was higher for the control than for the 3.5% fat treatment (95% vs. 75%; $P = .07$), however, there were no differences between either the control or the 3.5% fat treatments and the 8.3% fat treatment (95% vs. 85%; and 75% vs.

85% respectively). The addition of cream to the inseminate post-thaw did not aid the retention or increase the availability of spermatozoa for fertilization as measured by accessory sperm number per embryo(ovum).

Introduction

The number of accessory sperm in the zona pellucida has been positively associated with fertility (Hunter and Wilmut, 1984; Hawk and Tanabe, 1986) and embryo quality in cattle (DeJarnette et al., 1992;Nadir et al., 1993). Although not directly involved in fertilization, accessory sperm are able to access the oviduct, undergo capacitation, ovum recognition and binding, the true acrosome reaction, and partially penetrate the zona pellucida. Thus, accessory sperm are thought to be a quantitative measure of sperm available for fertilization (DeJarnette et al., 1992).

The use of milk to extend bovine semen has been thoroughly researched (Thacker and Almquist, 1953; Almquist et al., 1954; for a review see Salisbury et al., 1978). Nonreturn rates differ between skim milk (69%, McFee and Swanson, 1960) and homogenized milk (~76%, VanDemark, 1950;~ 71%, Foote and Bratton, 1949) as extenders for fresh semen. In contrast, Adler and Rasbech (1956) reported no difference in nonreturn rates when skim milk and sterilized homogenized cream (9% fat) were compared as fresh semen extenders. The reason for the conflicting results between studies regarding skim milk, homogenized milk and cream is not known.

Pickett et al. (1975) reported that mares inseminated with fresh stallion semen extended in an 11% cream-gel extender had increased first cycle and three cycle pregnancy rates compared to those inseminated with semen extended in TRIS (75% vs. 37.5% and 96% vs. 75%, respectively). Pickett et al. (1975) defined first cycle pregnancy rate as the number of mares diagnosed pregnant divided by the total number of mares inseminated in one estrous cycle. Three cycle pregnancy rates were defined as the total number of mares diagnosed pregnant divided by the total number of mares inseminated in three estrous cycles (Pickett et al., 1975). Although the use of fresh bovine semen has become rare in the United States, an increase of similar magnitude utilizing cream as an extender or supplement to the frozen-thawed inseminate would be of great importance to the artificial insemination and cattle industries. Taken together with the realization that previous studies investigating skim milk, homogenized milk, and cream as extenders for fresh bovine semen utilized nonreturn data which may be influenced by management factors (Rycroft, 1992), this study was undertaken to revisit the effect of the addition cream to the inseminate on fertility in cattle. The objective of this experiment was to determine if an emulsion such as cream, could aid sperm transport and increase the availability of sperm for fertilization following AI as measured by the number of accessory sperm per embryo(ovum).

Materials and Methods

Semen

Four Holstein bulls, ranging from 2 to 9 years of age, were selected for their ability to produce neat semen with characteristics equal to or greater than 70% morphologically normal sperm and 60% estimated progressive motility. Ejaculates were collected by artificial vagina, and having met the minimum criteria, were extended to 100×10^6 sperm per mL with clarified egg yolk-citrate-glycerol. The extended semen was packaged and cryopreserved in .5-mL French straws (Instruments de Médecine Vétérinaire, l'Aigle, France) according to the optimum procedures for this extender system established by Robbins et al. (1976).

Treatments

Homogenized cream (236 mL;18% fat) (Pace Dairy Foods Co., Cincinnati, OH) was heated to 95°C for 10 minutes in a double boiler (Thacker and Almquist, 1953; Appendix A). After cooling to 25°C, two cream fractions were prepared. The first was prepared by mixing clarified egg yolk-citrate (58 mL) with homogenized cream (18% fat; 42 mL), yielding a 100-mL mixture of egg yolk-citrate and homogenized cream containing 7.5% fat. A 7-mL aliquot was removed, and a 7-mL aliquot of glycerol was added, yielding a 100-mL mixture containing 7% fat and 7% glycerol. The second cream fraction was prepared by removing a 7-mL aliquot from 100 mL of homogenized cream (18% fat). Subsequently, a 7-mL aliquot of glycerol was added, yielding a 100- mL mixture of homogenized cream and glycerol

containing 16.7% fat and 7% glycerol. These cream fractions were prepared to yield 3.5% and 8.3% fat upon mixing (1:1,v/v) with the frozen-thawed semen. Both cream fractions contained glycerol equivalent to the thawed semen (7%,v/v) and were packaged in .5-mL French straws and stored in liquid nitrogen until use. Clarified egg yolk-citrate-glycerol (EYC) extender was used as the control and was stored in 1-mL vials at -20°C.

Frozen straws and vials were thawed by plunging into 35°C water for 45 s. Immediately prior to insemination, contents of the semen straw were mixed (1:1,v/v) with either: a) egg yolk-citrate-glycerol (control); b) homogenized cream-egg yolk-citrate-glycerol; or c) homogenized cream-glycerol. Experimental inseminates were carefully drawn into .5-mL French straws and contained 25×10^6 sperm and either 0%, 3.5%, or 8.3% milk fat.

Artificial Insemination, Embryo Recovery and Evaluation, and Acquisition of Accessory Sperm

Estrus was induced by a single injection (25 mg i.m.) of $PGF_{2\alpha}$ (Lutalyse®, Pharmacia and Upjohn Co., Kalamazoo, MI) in nonlactating Holstein cows ranging from 2 to 10 years of age. Cows were observed for signs of estrus twice daily (early morning and evening) and were considered to be in estrus if they stood to allow mounting by a herdmate. At first observation of estrus, cows were randomly assigned to be inseminated approximately 12 hr later at the next estrus observation period.

Embryos(ova) were recovered using standard nonsurgical uterine flushing techniques 6 d after insemination. The initial search and evaluation of embryos(ova) was carried out by a single observer with a stereomicroscope. The anticipated stage of embryonic development was a compact morula. Recovered viable embryos were evaluated as described by Lindner and Wright (1983), resulting in designations of excellent, good, fair, and poor based on compactness and homogeneity of the cell mass. Degenerate embryos and unfertilized ova were designated according to DeJarnette et al. (1992). Briefly, embryos with blastomeres that contained nuclei but were too underdeveloped to be considered viable according to Lindner and Wright (1983) were designated as degenerate. An unfertilized ovum was designated when there was no indication of cleavage.

Accessory sperm per embryo(ovum) were visualized using the procedure of DeJarnette et al. (1992). Following partial digestion of the zona pellucida with .5% protease (Pronase, Behring Diagnostics, La Jolla, CA) in a hanging drop preparation, the embryo(ovum) was compressed with a coverslip and the smear examined with differential interference contrast optics at $\times$500 magnification. This procedure renders the heads of accessory sperm flat to the viewer, and their numbers per embryo(ovum) were obtained by direct count.

Statistical Analyses

DeJarnette et al. (1992) first reported the skewed distribution associated with accessory sperm data and recommended that median accessory sperm values

were more important than mean values. Therefore, median accessory sperm numbers per embryo(ovum) were analyzed for treatment differences using the Wilcoxon Two-Sample Test (SAS®) in which the population distribution is not assumed to be normal. Chi-square analyses were used to detect differences due to treatments in fertilization rate (2 df), embryo quality (6 df), and the percentage of embryos with accessory sperm (2 df).

Results

From 116 inseminations, 66 embryos(ova) were recovered 6 d after insemination. To avoid the potential influence of any one of the four bulls, embryos(ova) were equally balanced, i.e., excess embryos (ova) from a specific bull were randomly removed such that the number of embryos(ova) recovered following inseminations to any one of the four bulls was the same for each treatment. Therefore, 60 embryos(ova) were utilized for evaluation and accessory sperm count. The frequency distribution of accessory sperm per embryo(ovum) was highly skewed (Figure 1). Across all treatments, the range was 0 to 147 accessory sperm per embryo(ovum) with a mean ± SD, median, and mode of 21.4 ± 30.1, 8, and 0, respectively. The addition of cream to the inseminate did not improve the median accessory sperm number per embryo(ovum) (Table 1). Although the fertilization rate was unaffected by treatment, the percentage of embryos(ova) with accessory sperm was higher for the control (EYC, 0% fat) than for the 3.5% fat treatment (P = .07) (Table 2). The median accessory sperm

number per embryo(ovum) was not different between embryo quality designations.

Discussion

This is the first attempt to supplement the frozen-thawed bovine inseminate with cream. As measured by accessory sperm number per embryo(ovum), the addition of cream to the inseminate post-thaw did not aid the retention or increase the availability of spermatozoa for fertilization. Milk extender (3.5% fat) is commonly used by AI organizations and 8.3% fat approaches the level used previously by Adler and Rasbech (1956), in a study comparing skim milk, cream, and egg yolk-citrate as fresh semen extenders. It is possible the desired effect of aiding sperm transport and increasing the availability of sperm for fertilization following AI was not achieved due to placement and volume of the dose. At natural service the bull deposits semen in a spiral motion against the mucosa of the fornix vagina and in a pool on the ventral aspect of the vagina (Seidel and Foote, 1967; Seidel, 1968). This placement, coupled with a large volume and high numbers of sperm, likely allows many sperm access to privileged paths within grooves originating in the fornix vagina, thus enabling these sperm to traverse the cervix and enter the uterus along the mucosa, effectively escaping much of the retrograde flow of mucus (Mullins and Saacke, 1989). In contrast, the artificial inseminate in this study was deposited conventionally into the uterine body, presumably in a pool on the ventral aspect of the uterus.

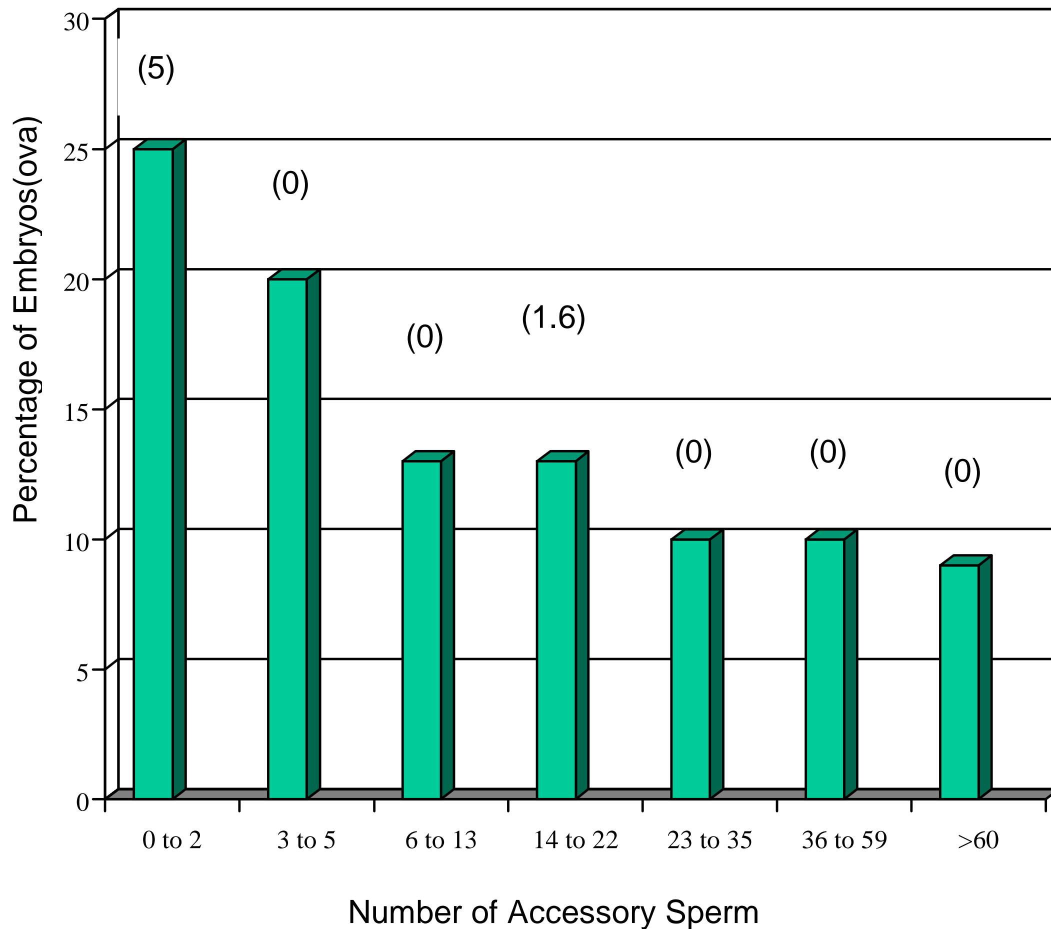

Figure 1. Frequency distribution of accessory sperm per embryo(ovum). The mean, median, and mode for 60 embryos(ova) were 21.4, 8, and 0 respectively. Numbers above each bar represent the percentage of the bar composed of unfertilized ova.

Table 1. Effect of cream added to the inseminate on accessory sperm per embryo(ovum)

Treatment	n[a]	Accessory sperm per embryo(ovum)		
		Median	Mean ± SD	Range
EYC	20	11	26.9 ± 36.1	0-147
3.5% fat	20	5	18.8 ± 29.9	0-121
8.3% fat	20	10.5	18.6 ± 24.1	0-84

[a]Number of embryos(ova).

Table 2. Effect of cream added to the inseminate on the fertilization rate and proportion of embryos with accessory sperm

Treatment	n[a]	Embryos with accessory sperm, %	Fertilization rate, %
EYC	20	95[b]	100
3.5% fat	20	75[c]	90
8.3% fat	20	85[b, c]	90

[a]Number of embryos(ova).
[b,c]Values within a column with different superscripts differ (*P* = .07).

This placement, coupled with the small, but normal volume and concentration for AI, may not have allowed sperm to benefit from the possible effect of an emulsion such as cream to mimic sperm transport along the uterine mucosa as occurs following natural service. Furthermore, the increased pregnancy rate reported by Pickett et al. (1975) in mares inseminated with stallion semen extended in an 11% cream-gel extender compared to those inseminated with semen extended in TRIS may have been due to the large volume (10 mL) deposited. It is possible that any benefits to utilizing cream as an extender or supplement to the inseminate may be related to volume of the dose.

In summary, the addition of cream (3.5% or 8.3% fat) to the inseminate post-thaw did not improve the availability of spermatozoa for fertilization as measured by accessory sperm number per embryo(ovum). The results reported here do not indicate the need for continued research into the supplementation of bull semen with cream.

Literature Cited

Adler, H.C., and N. O. Rasbech. 1956. Undersøgelser over skummetmaelk og flødesom saedfortyndingsvaedsker. (Investigations on skim milk and cream as a semen diluent). Nord. Vetmed. 8:497.

Almquist, J.O., R.J. Flipse, and D.L. Thacker. 1954. Diluters for bovine semen. IV. Fertility of bovine spermatozoa in heated homogenized milk and skim milk. J. Dairy Sci. 37:1303.

DeJarnette, J.M., R.G. Saacke, J. Bame, and C.J. Vogler. 1992. Accessory sperm: Their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J. Anim. Sci. 70:484.

Foote, R.H., and R.W. Bratton. 1949. The fertility of bovine semen cooled with and without the addition of citrate-sulfanilamide-yolk extender. J. Dairy Sci. 32:856.

Hawk, H.W., and T.Y. Tanabe. 1986. Effect of unilateral cornual insemination upon fertilization rate in superovulating and single-ovulating cattle. J. Anim. Sci. 63:551.

Hunter, R.H.F., and I. Wilmut. 1984. Sperm transport in the cow: Peri-ovulatory redistribution of viable cells within the oviduct. Reprod. Nutr. Dev. 24:597.

Lindner, G.M., and R.W. Wright. 1983. Bovine embryo morphology and evaluation. Theriogenology 20:407.

McFee, A.F., and E.W. Swanson. 1960. The influence of cooling rates and storage temperatures on sperm activity. A.I. Digest 8(10):12.

Mullins, J., and R.G. Saacke. 1989. Study of the functional anatomy of the bovine cervical mucosa with special reference to mucus secretion and sperm transport. Anat. Rec. 225:106.

Nadir, S., R.G. Saacke, J. Bame, J. Mullins, and S. Degelos. 1993. Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle. J. Anim. Sci. 71:199.

Pickett, B.W., L.D. Burwash, J.L. Voss, and D.G. Back. 1975. Effect of seminal extenders on equine fertility. J. Anim. Sci. 40:1136.

Robbins, R.K., R.G. Saacke, and P.T. Chandler. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in french straws. J. Anim. Sci. 42:145.

Salisbury, G.W., N.L. VanDemark, and J.R. Lodge. 1978. Extenders and extension of unfrozen semen. In: Physiology of Reproduction and Artificial Insemination of Cattle Second Edition, p 442. W.H. Freeman and Company, San Francisco, CA.

SAS®. 1985. User's Guide: Statistics.SAS® Inst. Inc., Cary, NC.

Seidel, G.E. 1968. Effect of collection interval and artificial vagina length and temperature on the bovine ejaculate, compartmental analysis using

biochemical markers, and motion picture analysis of ejaculation. M.S. Thesis, pp. 97-104. Cornell University, Ithaca, NY.
Seidel, G.E., and R. H. Foote. 1967. Motion picture analysis of bovine ejaculation. J. Dairy Sci. 50:970(Abstr.).
Thacker, D.L., and J.O. Almquist. 1953. Diluters for bovine semen. I. Fertility and motility of bovine spermatozoa in milk. J. Dairy Sci. 36:173.
VanDemark, N.L. 1950. Extension of semen before and after cooling and its effect on fertility. Illinois Agr. Exp. Sta. Ann. Proj. Rep.

CHAPTER 4

EFFECT OF A DEEP UTERINE INSEMINATION ON SPERMATOZOAL ACCESSIBILITY TO THE OVUM IN CATTLE: A COMPETITIVE INSEMINATION STUDY

Abstract

A competitive insemination study was conducted to determine the effect of a deep uterine insemination on accessory sperm number per embryo in cattle. Cryopreserved semen of a fertile bull characterized by spermatozoa with a semi-flattened region of the anterior sperm head (marked bull) was matched with cryopreserved semen from an unmarked bull having spermatozoa with a conventional head shape. Using .25-mL French straws and a side delivery embryo transfer device, deep uterine insemination (.125 mL deposited in each horn) was performed 2 cm from the uterotubal junction. Immediately after, the uterine body was artificially inseminated using semen (.25 mL) from an alternate bull and a conventional insemination device. The complete dose (both inseminations) was 50×10^6 total sperm cells consisting of an equal number of spermatozoa from each bull. Single-ovulating cows (n = 95) were inseminated with either the unmarked semen in the uterine body and marked semen in the uterine horn, or the unmarked semen in the uterine horn and marked semen in the uterine body. Sixty-one embryos(ova) were recovered nonsurgically 6 d after insemination, of which 40 were fertilized and contained accessory spermatozoa. The ratio and total number of accessory spermatozoa recovered was different among treatments: 62:38 (326) for

the unmarked semen in the uterine body and marked semen in the uterine horn, and 72:28 (454) for the unmarked semen in the uterine horn and marked semen in the uterine body ($P < .05$). Deep uterine insemination using this semen in a split dose and a side delivery device favors accessibility of spermatozoa to the ovum compared with conventional uterine body insemination.

(Theriogenology 51:5:883-890)

Introduction

The site of semen deposition is an important factor in the success of artificial insemination in cattle. Macpherson (1968) reported that deposition of semen in the uterine body resulted in a 10% higher nonreturn rate than did cervical deposition. To further increase the efficiency of artificial insemination, many studies have compared semen deposition near the greater curvature of the uterine horns with conventional deposition into the uterine body. Senger et al. (1988) reported an increase in the conception rate when semen was deposited in the uterine horns rather than the uterine body. In contrast, Hawk and Tanabe (1986), Williams et al. (1988), and McKenna et al. (1990) found no difference in the fertilization rate, conception rate or nonreturn rate, respectively, between uterine body and uterine horn inseminations. In superovulated cows, Hawk et al. (1988) used a modified insemination device requiring two technicians to deposit semen near the uterotubal junction. When compared with traditional uterine body deposition, they found no effect upon the fertilization rate.

In 1992, Munkittrick et al. reported that a uniform morphological difference between spermatozoa of different bulls could be used to describe the competitiveness of spermatozoa in accessing the ovum based on accessory spermatozoa attributed to the competing bulls. The number of accessory spermatozoa in the zona pellucida has been positively correlated with fertility in cattle (Hawk and Tanabe, 1986; Hunter and Wilmut, 1984) as well as embryo quality at 6 to 7 d after insemination (DeJarnette et al., 1992). Although not directly involved in fertilization, accessory spermatozoa are able to traverse the barriers of the female reproductive tract, undergo capacitation, ovum recognition and binding, the true acrosome reaction, and partially penetrate the zona pellucida during the time the ovum is receptive (DeJarnette et al., 1992).

This study was conducted to determine the effect of deep uterine insemination on accessibility of spermatozoa to the ovum. Although studies investigating the site of semen deposition that yields optimum fertility have been performed, this study is the first in which two morphologically distinct spermatozoal populations were used competitively to evaluate the site of semen deposition by measuring accessory sperm number per embryo in cattle.

Materials and Methods

Semen

Ejaculates from two mature bulls (1 Holstein and 1 Brown Swiss) with semen characteristics equal to or greater than 70% morphologically normal

spermatozoa and 60% estimated progressive motility were collected by artificial vagina and extended to 100×10^6 total cells/mL with clarified egg yolk-citrate-glycerol. The extended semen was packaged and cryopreserved in .5-mL French straws (Instruments de Médecine Vétérinaire, l'Aigle, France) according to the optimum procedures for this extender system established by Robbins et al. (1976).

Cryopreserved spermatozoa of the marked bull exhibited a semi-flattened region of the anterior spermatozoal head, a subtle abnormality which does not affect sperm transport or fertility (Saacke et al., 1998). Cryopreserved spermatozoa of the unmarked bull displayed a uniform, conventional spermatozoal head shape.

Animals, Treatments and Artificial Insemination

Estrus was induced with $PGF_{2\alpha}$ (25 mg, i.m. Lutalyse®, Pharmacia and Upjohn Co., Kalamazoo, MI) in cyclic nonlactating Holstein cows ranging from 2 to 10 yr of age. The cows were observed for signs of estrus twice daily (early morning and evening) and were considered to be in estrus if they stood to be mounted by a herdmate. At first observation of estrus, the cows were randomly assigned to one of two insemination treatments to be performed 10 to 12 hr later at the next estrus observation period.

The two treatments consisted of the deposition of : 1) unmarked semen in the uterine body and marked semen in the uterine horns, and 2) unmarked semen in the uterine horns and marked semen in the uterine body. Frozen straws were

thawed by plunging into 35°C water for 45 s. Immediately prior to insemination, the contents of a .5-mL French straw were deposited into a vial. Experimental inseminates were carefully drawn into .25-mL French straws and contained 25×10^{6} total sperm cells. Using a side delivery embryo transfer device, deep uterine insemination (.125 mL deposited in each horn) was performed 2 cm from the uterotubal junction. Immediately following this, uterine body insemination was completed using semen (.25 mL) from the alternate bull and a conventional insemination device. The complete dose (both inseminations) was 50×10^{6} total sperm cells consisting of an equal number of spermatozoa from each bull. A side delivery embryo transfer device was used for the deep uterine inseminations to minimize potential trauma to the endometrium during manipulation of the uterine horns. As is traditionally performed in the field, a conventional insemination device was used for the uterine body inseminations. This study was not designed to examine the two insemination devices at each insemination site.

Embryo Recovery and Evaluation

Embryos(ova) were recovered using standard nonsurgical uterine flushing techniques 6 d after insemination. The initial search and evaluation of embryos(ova) was carried out by a single observer with a stereomicroscope. The anticipated stage of embryonic development was a compact morula. Recovered viable embryos were evaluated as described by Lindner and Wright (1983),

resulting in designations of excellent, good, fair, and poor based on compactness and homogeneity of the cell mass. Degenerate embryos and unfertilized ova were designated according to DeJarnette et al. (1992). Briefly, embryos with blastomeres that contained nuclei but were too underdeveloped to be considered viable according to Lindner and Wright (1983) were designated as degenerate. An unfertilized ovum was designated when there was no indication of cleavage.

Acquisition of Accessory Spermatozoa and Statistical Analysis

Accessory spermatozoa per embryo were visualized using the procedure of DeJarnette et al. (1992). Following partial digestion of the zona pellucida with .5% protease (Pronase, Behring Diagnostics, La Jolla, CA) in a hanging drop preparation for 2 to 10 min, the embryo was compressed with a coverslip and the smear examined with differential interference contrast optics at $\times$500 magnification. This procedure caused the heads of accessory spermatozoa to appear flat to the viewer, and their numbers per embryo were obtained by direct count. Immediately following the first direct count, the smear was further examined using $\times$1250 magnification and oil immersion. This facilitated distinguishing between the two morphologically distinct spermatozoal populations, and accessory sperm numbers attributed to each bull were obtained by differential count. A Chi-square test of independence was used to detect differences due to treatments in the competitive ratio and total number of accessory spermatozoa recovered (1 df), and embryo quality distribution (2 df).

Results

From 95 inseminations to single-ovulating cows, 61 embryos(ova) were recovered, of which 40 were fertilized and contained accessory spermatozoa (Table 1). Fourteen unfertilized ova were recovered, of which one exhibited a single accessory sperm. The quality of embryos which contained accessory spermatozoa was not different due to treatments (Table 2).

The ratio (%) of unmarked to marked accessory spermatozoa was different between treatments ($P < .05$, Table 3). It is evident from Table 3 that the competitive ratio (%) of accessory spermatozoa shifted in favor of those spermatozoa deposited deep in the uterine horns at the expense of spermatozoa deposited in the uterine body.

Table 1. Number of embryos and ova with or without accessory sperm resulting from uterine body and deep uterine horn inseminations using equal numbers of unmarked and marked sperm

Treatment	n[a]	Embryos with accessory sperm	Embryos without accessory sperm	Unfertilized ova
Unmarked body Marked horn[b]	33	20	5	8
Unmarked horn Marked body[c]	28	20	2	6

[a]Number of embryos(ova).
[b]Unmarked body Marked horn: unmarked sperm deposited in the uterine body, marked sperm in the uterine horns.
[c]Unmarked horn Marked body: unmarked sperm deposited in the uterine horns, marked sperm in the uterine body.

Table 2. Quality and number of embryos recovered 6 days after uterine body and deep uterine horn inseminations with equal numbers of unmarked and marked sperm

Treatment	Embryos		
	Excellent/good	Fair/poor	Degenerate
Unmarked body Marked horn[a]	12	5	3
Unmarked horn Marked body[b]	13	6	1

[a]Unmarked body Marked horn: unmarked sperm deposited in the uterine body, marked sperm in the uterine horns.

[b]Unmarked horn Marked body: unmarked sperm deposited in the uterine horns, marked sperm in the uterine body.

Table 3. Effect of uterine body and deep uterine horn inseminations with equal numbers of unmarked and marked sperm on accessibility of sperm to the ovum

Treatment	No. of unmarked sperm	No. of marked sperm	Total no. of sperm	Ratio unmarked:marked sperm (%)
Unmarked body Marked horn[a]	202	124	326	62:38[c]
Unmarked horn Marked body[b]	328	126	454	72:28[d]

[a]Unmarked body Marked horn: unmarked sperm deposited in the uterine body, marked sperm in the uterine horns.

[b]Unmarked horn Marked body: unmarked sperm deposited in the uterine horns, marked sperm in the uterine body.

[c,d]Values within a column with different superscripts differ ($P < .05$).

Discussion

Deep uterine insemination using this semen in a split dose and a side delivery device favors accessibility of spermatozoa to the ovum when compared with conventional uterine body artificial insemination, as measured by the competitive ratio of unmarked to marked accessory spermatozoa. The biologic reason for this is not clear. A possible explanation is that the deep uterine insemination allows for a shift in the ratio of spermatozoa in the reservoir of the oviductal isthmus. The number of accessory spermatozoa is thought to parallel the number of potential fertilizing spermatozoa available to the ovum (Saacke et al., 1994). It is unlikely, however, that the total number of spermatozoa available for fertilization was increased due to deposition of semen adjacent to the uterotubal junction. Gallagher and Senger (1989), using a vaginal sampling technique, found that spermatozoal retention did not differ when uterine horn insemination was compared with uterine body insemination.

Although numerous studies have investigated the site of semen deposition that yields optimum fertility, the present study is the first in which semen deposition was made 2 cm from the uterotubal junction in multiparous, single-ovulating cattle. In previous studies, semen was deposited 2.5 cm (Williams et al., 1988) and 5 cm (McKenna et al., 1990) anterior to the internal cervical os, at the greater curvature of the uterine horns (Senger et al., 1988), in the greater curvature following straightening of the horns (Hawk and Tanabe, 1986), and in the cranial half after

straightening the horns (Lopez-Gatius, 1996). In the aforementioned studies, Senger et al. (1988) and Lopez-Gatius (1996) reported an increase in conception and pregnancy rates, respectively. Williams et al. (1988), McKenna et al. (1990), and Hawk and Tanabe (1986) found no difference in the conception rate, nonreturn rate or fertilization rate, respectively, when comparing uterine body and uterine horn inseminations.

It is not clear why a few studies have shown a fertility advantage following uterine horn insemination while others have not. A possible explanation for the positive effect of uterine horn inseminations may be related to a bull effect on spermatozoal transport. Saacke et al. (1998), in a study which assessed characteristics of spermatozoa capable of traversing barriers in the female tract, reported the accessory sperm population was enriched with spermatozoa of normal head shape relative to the inseminate. Furthermore, Saacke et al. (1998) concluded that morphologically abnormal spermatozoa are excluded from the accessory sperm population based upon severity of head shape distortion. In the present study, however, cryopreserved spermatozoa of the marked bull exhibited a semi-flattened region of the anterior spermatozoal head, a subtle abnormality which does not affect sperm transport or fertility (Saacke et al., 1998). Another possible explanation for the positive effect of uterine horn inseminations may be related to the minimization or elimination of cervical semen deposition. Cervical insemination errors account for approximately 20% of attempted uterine body depositions

(Peters et al., 1984). Furthermore, Senger et al. (1988) reported no decline in successful uterine horn depositions 6 mo following inseminator retraining. In contrast, a decline of 20% in successful uterine body depositions was reported 6 mo after retraining (Senger et al., 1988). The lack of a decline in successful uterine horn inseminations was attributed to a higher repeatability of uterine horn insemination due to the ease of identifying the anatomical location (Senger et al., 1988).

The number of spermatozoa per inseminate may also affect measures of fertility. Median accessory sperm values per embryo, fertilization rate and embryo quality were increased when the total number of spermatozoa was increased from 20×10^6 to 100×10^6 in single-ovulating cows (Nadir et al., 1993). In 1988, Hawk et al. reported an increase in fertilization rate and the proportion of embryos(ova) with accessory spermatozoa in superovulated cows inseminated with fresh semen (4.4×10^9 total sperm cells per cow) compared with cows inseminated with frozen semen (70×10^6 total sperm cells per cow), apparently overcoming problems with spermatozoal transport. In contrast, median accessory spermatozoal values, fertilization rates and embryo quality were not changed when the total number of spermatozoa inseminated increased from 20×10^6 to 40×10^6 in single-ovulating cows (DeJarnette et al., 1992). In the current study, each inseminate consisted of : 1) 25×10^6 total sperm cells to ensure that each bull competed at an acceptable

concentration according to current AI practices, and 2) that the complete dose inseminated (50×10^6 total sperm cells) did not confound the data by overcoming problems related to spermatozoal transport in normal cyclic cattle.

Deep uterine insemination may be the most advantageous in cases of impaired spermatozoal transport or where low numbers of spermatozoa are necessary. It has been demonstrated that the side of previous gestation affects subsequent spermatozoal transport after deep insemination into one uterine horn (Lopez-Gatius, 1996). Lopez-Gatius (1996) reported an increase in the pregnancy rate when deep ipsilateral insemination (relative to ovulation) was performed in the previously nongravid horn. Seidel et al. (1996) reported similar pregnancy rates resulting from a split semen dose with low numbers of spermatozoa (5×10^5 or 10×10^6) deposited at the greater curvature of the uterine horns in nulliparous heifers. The efficacy of insemination with low numbers of spermatozoa due to semen sexing or post-mortem harvest of epididymal spermatozoa might be increased by deep uterine insemination. Further research is needed to assess these possibilities.

In summary, deep uterine insemination using this semen in a split dose and a side delivery device favors accessibility of spermatozoa to the ovum compared with conventional uterine body insemination, as measured by the number of accessory spermatozoa per embryo. Nevertheless, it is not known whether the

differences reported here would be translated into increased fertility or pregnancy rate following homospermic insemination with the semen from these or other bulls.

Literature Cited

DeJarnette, J.M., R.G. Saacke, J. Bame, and C.J. Vogler. 1992. Accessory sperm: Their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J. Anim. Sci. 70:484.

Gallagher, G.R., and P.L. Senger. 1989. Concentrations of spermatozoa in the vagina of heifers after deposition of semen in the uterine horns, uterine body or cervix. J. Reprod. Fertil. 86:19.

Hawk, H.W., and T.Y. Tanabe. 1986. Effect of unilateral cornual insemination upon fertilization rate in superovulating and single-ovulating cattle. J. Anim. Sci. 63:551.

Hawk, H.W., H.H. Conley, R.J. Wall, and R. O. Whitaker. 1988. Fertilization rates in superovulating cows after deposition of semen on the infundibulum, near the uterotubal junction or after insemination with high numbers of sperm. Theriogenology. 29:1131.

Hunter, R.H.F., and I. Wilmut. 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reprod. Nutr. Dev. 24:597.

Lindner, G.M., and R.W. Wright. 1983. Bovine embryo morphology and evaluation. Theriogenology. 20:407

Lopez-Gatius, F. 1996. Side of gestation in dairy heifers affects subsequent sperm transport and pregnancy rates after deep insemination into one uterine horn. Theriogenology. 45:417.

Macpherson, J.W. 1968. Semen placement effects on fertility in bovines. J. Dairy Sci. 51:807-808.

McKenna, T., R.W. Lenz, S.E. Fenton, and R.L. Ax. 1990. Nonreturn rates of dairy cattle following uterine body or cornual insemination. J. Dairy Sci. 73:1779.

Munkittrick, T.W., R.L. Nebel, and R.G. Saacke. 1992. Accessory sperm numbers for cattle inseminated with protamine sulfate microcapsules. J. Dairy Sci. 75:725.

Nadir, S., R. G. Saacke, J. Bame, J. Mullins, and S. Degelos. 1993. Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle. J. Anim. Sci. 71:199.

Peters, J. L., P. L. Senger, J. L. Rosenberger, and M. L. O'Connor. 1984. Radiographic evaluation of bovine artificial inseminating technique among professional and herdsman-inseminators using .5- and .25-mL French straws. J. Anim. Sci. 59:1671.

Robbins, R.K., R.G. Saacke, and P.T. Chandler. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in french straws. J. Anim. Sci. 42:145.

Saacke, R. G., S. Nadir, J. Dalton, J. Bame, J. M. DeJarnette, S. Degelos, and R. L. Nebel. 1994. Accessory sperm evaluation and bull fertility (An update). In:

Proc. 15th Tech. Conf. on A.I. and Reprod., pp 57-67. NAAB, Columbia, Missouri.

Saacke, R.G., J.M. DeJarnette, J.H. Bame, D.S. Karabinus, and S.S. Whitman. 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super- and single-ovulating cattle? Theriogenology 50:117.

Seidel, Jr., G.E., C.H. Allen, Z. Brink, M.D. Holland, and M.B. Cattell. 1996. Insemination of heifers with very low numbers of frozen spermatozoa. J. Anim. Sci. 74(Suppl. 1):235 abstr.

Senger, P.L., W.C. Becker, S.T. Davidge, J.K. Hillers, and J.J. Reeves. 1988. Influence of cornual insemination on conception in dairy cattle. J. Anim. Sci. 66:3010.

Williams, B.L., F.C. Gwazdauskas, W.D. Whittier, R.E. Pearson, and R.L. Nebel. 1988. Impact of site of inseminate deposition and environmental factors that influence reproduction of dairy cattle. J. Dairy Sci. 71:2278.

CHAPTER 5

ESTRUS CHARACTERISTICS OF SUPEROVULATED CATTLE AND THE EFFECT OF ARTIFICIAL INSEMINATION AT 0, 12, OR 24 H AFTER ONSET OF ESTRUS ON FERTILIZATION STATUS, EMBRYO QUALITY AND PERCENTAGE OF EMBRYOS WITH ACCESSORY SPERM

Abstract

Thirty nonlactating Holstein cows were superovulated to determine the effect of time of artificial insemination on fertilization status, accessory sperm number per embryo, and embryo quality. Beginning on day 8, 9, 10 or 11 of the estrous cycle, cows were administered 38 mg Armour units FSH-P™ in a four-day descending dose regimen. Luteolysis was induced with two injections of prostaglandin on the last day of FSH-P™ treatment. All cows were continuously monitored for behavioral estrus by HeatWatch®. For 30 cows, the elapsed time (mean ± SD) from the first prostaglandin dose to the first standing event was 39 h 23 min ± 7 h 42 min. The (mean ± SD) duration of behavioral estrus was 13 h 11 min ± 4 h 05 min. The (mean ± SD) number of standing events was 27.03 ± 16.97. All cows were inseminated once with one .5-mL straw (50×10^6 sperm from a single bull) at either 0 h (n=10), 12 h (n=10), or 24 h (n=10) after the first standing event. Five hundred twenty-nine embryos(ova) were recovered nonsurgically 6 d after insemination. Fertilization rates were 29% (0 h); 60% (12 h); and 81% (24 h)($P < .01$). Percentages of embryos with accessory sperm were: 5 (0 h); 8 (12 h); and 41(24 h)

and differed between the 0 and 24 h, and 12 and 24 h inseminations (*P* < .01). Number (and percentages) of excellent and good, fair and poor, and degenerate embryos were not different: 25(44%), 20(35%), 12(21%) (0 h); 48(39%), 53(43%), 23(18%) (12 h); and 43(42%), 34(33%), 26(25%) (24 h) (*P* > .05). Artificial insemination of superovulated nonlactating Holstein cattle 24 h after onset of estrus as determined by HeatWatch® increased fertilization rate and percentage of embryos with accessory sperm when compared to AI at 0 or 12 h after onset of estrus. Embryo quality was not affected by time of insemination using this semen.

Introduction

Hawk et al. (1988) utilized high numbers of sperm from pooled, fresh ejaculates to demonstrate that nearly all superovulated oocytes are capable of being fertilized. Nevertheless, it is still not clear at which time after onset of estrus that artificial insemination with conventional frozen-thawed semen provides optimal fertilization status and embryo quality. Nearly 35% of ova recovered from superovulated cows are unfertilized (Seidel et al., 1978; Donaldson, 1985; Hasler, 1992), while an additional 15% of embryos are of questionable viability, and therefore are nontransferable (Hasler, 1992).

Previous insemination studies with superovulated cattle have associated the time of observed estrus synonymously with the onset of estrus. Donaldson (1985) recommends insemination with one dose of semen 12 h after onset (observed

estrus). In contrast, Schiewe et al. (1987) recommend insemination with two doses of semen 24 h after onset (observed estrus). Earlier work (Critser et al., 1980) found that one dose of semen 12 h after observed estrus, three doses at 12 h, or one dose at 0, 12, and 24 h gave similar results. These recommendations are difficult to interpret and implement because the timing of the onset of estrus has an 8 to 12 h error based on the frequency of visual observation.

Accessory sperm trapped in the zona pellucida by the zona reaction following penetration of the fertilizing sperm cell are believed to reflect the number of spermatozoa competing for fertilization in cattle (Saacke et al., 1998). The accessory sperm number is an indirect measure of sperm transport, and has been positively associated with fertility (Wilmut and Hunter, 1984) and embryo quality (DeJarnette et al., 1992; Nadir et al., 1993) in single-ovulating cattle. Nevertheless, previous efforts (Hawk et al., 1988; Saacke et al., 1998) to assess the importance of accessory sperm in superovulated cattle have been unfruitful.

There are no studies which provide a basis for insemination time of superovulated cattle relative to the true onset of estrus, as defined by the first standing event of a heat period. Taken together with the realization that heat detection is the primary problem limiting reproduction of dairy cattle, the present study utilized the HeatWatch® estrus detection system (DDx Inc., Denver, CO). The objective was to determine the optimal time of artificial insemination of

superovulated cows as measured by fertilization status, embryo quality, and percentage of embryos with accessory sperm relative to the true onset of estrus.

Materials and Methods

Animals

Thirty-five Holstein cows, ranging in age from 2 to 9 years, were selected at random from a research herd of normally cycling, nonlactating animals. These animals were free of gynaecological abnormalities, and previous to this experiment exhibited estrous cycles between 18 and 24 d in length.

Ultrasound Examination

Immediately prior to the initial superovulatory treatment, ovaries were examined with a real-time B-mode ultrasound scanner equipped with a 5-MHz transrectal probe (Aloka 500V; Corometrics Medical Systems, Inc., Wallingford, CT) to determine the presence or absence of a dominant follicle as described by Bungartz and Niemann (1994). Briefly, animals with ten or more follicles 3-8 mm in diameter (on both ovaries) were designated as being without a dominant follicle. Animals with less than ten follicles 3-8 mm in diameter were designated as having a dominant follicle.

Semen

A single ejaculate was collected by artificial vagina from one fertile Holstein bull. The ejaculate used met the minimum criteria of greater than 70%

morphologically normal sperm and 60% estimated progressive motility. The ejaculate was extended to 100×10^6 sperm per mL with clarified egg yolk-citrate-glycerol. The extended semen was packaged and cryopreserved in .5-mL French straws (Instruments de Médecine Vétérinaire, l'Aigle, France) according to the optimum procedures for this extender system established by Robbins et al. (1976).

Superovulatory Treatment,
System for Detecting Estrus, and Artificial Insemination

Eight to eleven days following estrus, the cows were superovulated with 38 mg Armour units of FSH-P™ (Lot Number 548F93;Schering-Plough Animal Health Corp., Kenilworth, NJ). The cows received twice daily injections (2×6 mg, 2×6 mg, 2×4 mg and 2×3 mg Armour units FSH-P™ i.m.) for 4 d. Luteolysis was induced with an injection of $PGF_{2\alpha}$ (Lutalyse®, Pharmacia and Upjohn Co., Kalamazoo, MI) at 72 h (40 mg i.m.) and 84 h (25 mg i.m.) after the initial FSH-P™ injection. All injections i.m. were performed in the gluteal muscle.

All cows were continuously monitored for behavioral estrus by HeatWatch®, which utilizes radio frequency data communications. This system included battery-powered, reusable pressure-sensing transmitters with a .4-km range, a repeater with a .8-km range, a signal receiver, a buffer that received and stored mounting activity data, and software that sorted information by cow, date, and time using a

personal computer with an 80386 microprocessor. Transmitters were contained in a nylon pouch on a 35- x 20-cm nylon mesh patch, which was glued with contact-type adhesive to the hair of the sacral region just anterior to the tailhead. At all times, the cows were housed in a pasture within working range of the transmitters and repeater. Transmitters were activated by continuous pressure (minimum of 2 s) from a mounting herdmate. Transmitted data included transmitter number, date, time and duration of standing events. Beginning 32 h after the first $PGF_{2\alpha}$ injection, the HeatWatch® software was reviewed every 3 h to determine the onset of estrus as defined by the time of the first standing event.

Frozen straws were thawed by plunging into 35°C water for 45 s. All cows were artificially inseminated by the same experienced technician with one dose of semen (50×10^6 sperm) at either 0 h (n=10), 12 h (n=10), or 24 h (n=10) after onset of estrus as determined by HeatWatch®. The body of the uterus was the site of semen deposition. The order of insemination times (treatments) to be performed were randomized each week.

Embryo Recovery and Evaluation

Embryos(ova) were recovered by an experienced technician using standard nonsurgical uterine flushing techniques 6 d after insemination. The initial search and evaluation of embryos(ova) was carried out by a single observer with a stereomicroscope at magnifications of $\times$10 and $\times$70. The anticipated stage of

embryonic development was a compact morula. Recovered viable embryos were evaluated at ×70 magnification based on compactness and homogeneity of the cell mass as described by Lindner and Wright (1983). This results in designations of excellent, good, fair, and poor. Degenerate embryos and unfertilized ova were designated according to DeJarnette et al. (1992). Briefly, embryos with blastomeres that contained nuclei but were too underdeveloped to be considered viable according to Lindner and Wright (1983) were designated as degenerate. An unfertilized ovum was designated when there was no indication of cleavage, or when all cytoplasmic fragments were without nuclei.

Accessory Sperm Evaluation

Accessory sperm per embryo were visualized using the procedure of DeJarnette et al. (1992). Following partial digestion of the zona pellucida with .5% protease (Pronase, Behring Diagnostics, La Jolla, CA) in a hanging drop preparation, the embryo was compressed with a coverslip and the smear examined with differential interference contrast optics at a magnification of ×500. This procedure renders the heads of accessory sperm flat to the viewer, and their numbers per embryo were obtained by direct count. This technique also allowed for the simultaneous quantification of accessory sperm tracks in the zona pellucida as previously mentioned by Saacke et al. (1998).

Statistical Analyses

A Chi-square test of independence was used to detect differences due to

treatments in fertilization rate (2 df), embryo quality (6 df), percentage of embryos with accessory sperm (2 df), and percentage of embryos with accessory sperm tracks (2 df). In data from single-ovulating cattle, the Wilcoxon Two-Sample Test (SAS®) has been used previously (Nadir et al., 1993) to detect differences between median accessory sperm values. In our data from superovulated cattle, the predominance of zero accessory sperm values resulted in an excessive number of ties in the Wilcoxon Two-Sample Test (SAS®), rendering the analysis invalid. Consequently, with the central limit theorem as a guideline, a Student's *t* test was performed to detect differences between mean accessory sperm values per embryo and considered appropriate with a skewed distribution provided that sample sizes were > 40 (Ott, 1988; Moore and McCabe, 1989). A Student's *t* test was also performed to determine if the presence of a dominant follicle on the day of initiation of superovulation affected the number of embryos(ova) recovered. Lastly, the correlation coefficient between the pooled number of small follicles (≤ 8 mm) in both ovaries and the number of embryos(ova) recovered was calculated according to Pearson (SAS®).

Results

The interval from the first prostaglandin injection to the onset of estrus was 39 h 23 min ± 7 h 42 min (mean ± SD). The characteristics of behavioral estrus are shown in Table 1. The mean ± SD and range in time from onset of estrus to AI for

Table 1. Characteristics of behavioral estrus according to HeatWatch® in cows treated for superovulation (n=30)

Characteristic	Mean	SD	Range
Time to onset from first $PGF_{2\alpha}$	39 h 23 min	7 h 42 min	23 h 21 min to 58 h 20 min
Duration of behavioral estrus	13 h 11 min	4 h 05 min	5 h 25 min to 20 h 56 min
No. of mounts	27.03	16.97	5 to 67

Table 2. Time to AI following the onset of estrus as defined by the first standing event recorded by HeatWatch® in cows treated for superovulation

		Time to AI		
Treatment	n[a]	Mean	SD	Range
0 h AI	10	2 h 03 min	57.6 min	40 min to 3 h 15 min
12 h AI	10	12 h 03 min	14.9 min	11 h 44 min to 12 h 31 min
24 h AI	10	23 h 53 min	13.4 min	23 h 16 min to 24 h 04 min

[a]Number of animals

each of the treatments is shown in Table 2.

From 35 cows superstimulated with FSH-P™, 30 cows yielded ≥ 3 embryos(ova). The total number, mean ± SD and range of embryos(ova) recovered from 30 animals was 529, 17.6 ± 10.9 and 3 to 47. A summary of the total number of embryos(ova) and total embryos recovered, by treatment, is shown in Table 3. Fertilization rates were different among the insemination times ($P < .01$). The greatest fertilization rate was achieved with the 24 h insemination (Figure 1). Embryo quality, however, was not different among treatments (Figure 2). The number of excellent/good, fair/poor, and degenerate embryos that comprise the percentages shown in figure 2 were: 25, 20, and 12 (0 h); 48,53, and 23 (12 h); and 43, 34, and 26 (24 h).

The percentage of embryos with accessory sperm increased parallel to the increase in fertilization rate, with the highest percentage of embryos with accessory sperm recovered from those animals inseminated 24 h after the onset of estrus ($P < .01$, Table 4). Furthermore, the percentages of embryos exhibiting sperm tracks in zonae pellucidae (indicative of accessory sperm loss between fertilization and evaluation) also increased parallel to the increase in fertilization rate: 21% (0 h), 31% (12 h), 52% (24 h) ($P <.01$, Table 4).

A single ultrasound evaluation at the time of superovulatory induction allowed the number of small follicles (3-8 mm in diameter) in both ovaries to be

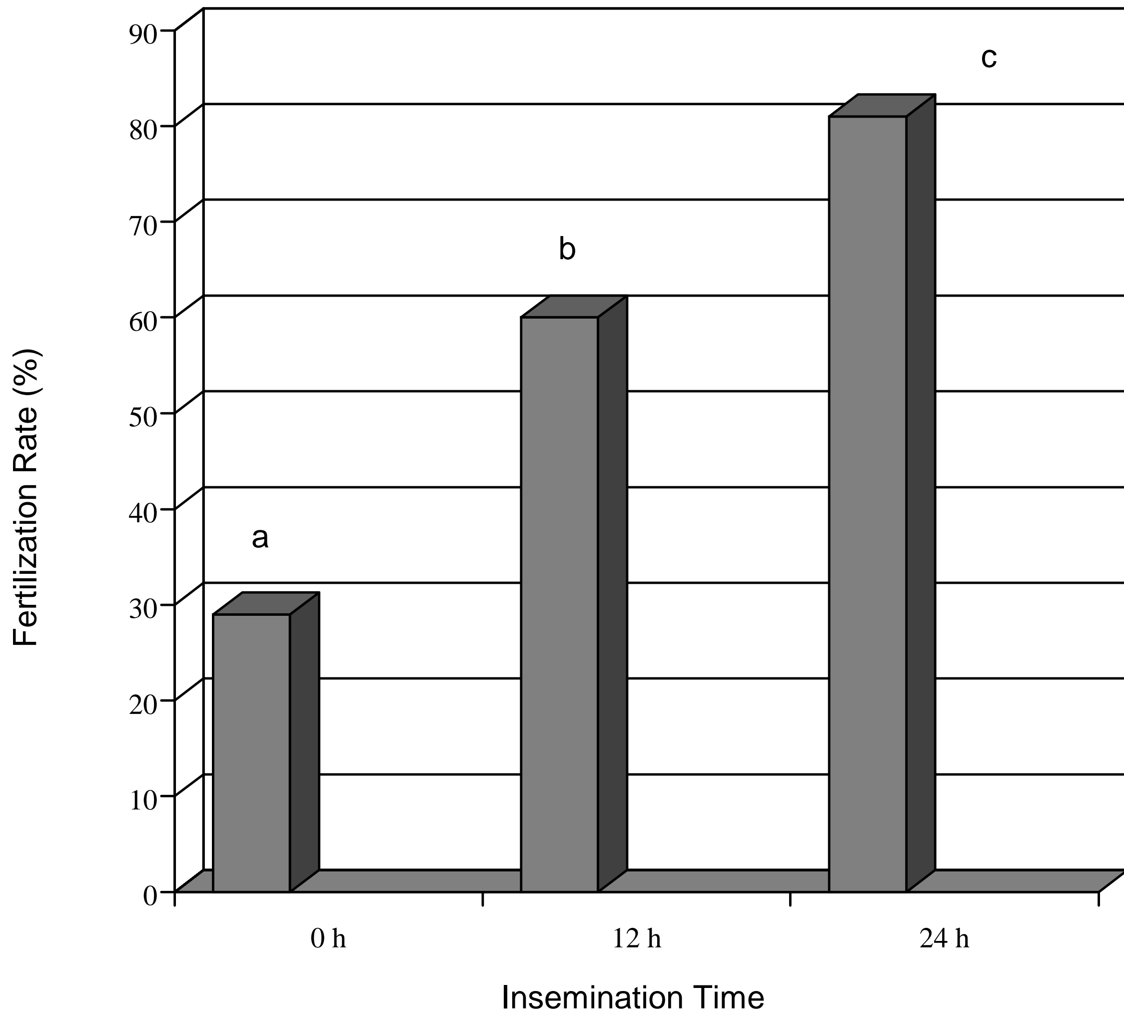

Figure 1. Effect of AI at 0, 12, or 24 h after onset of estrus on fertilization rate. Bars with different letters (a, b, c) differ (*P*<.01).

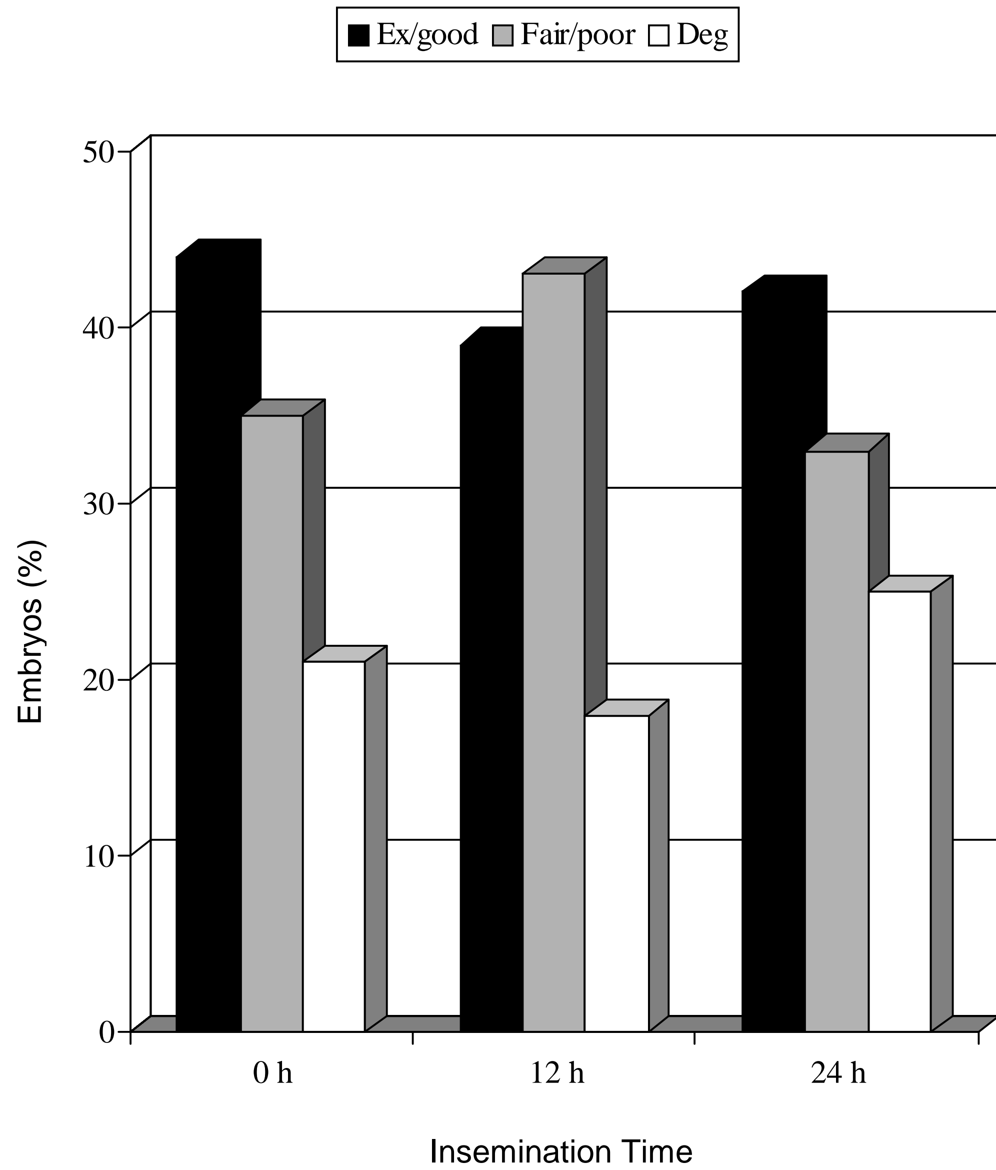

Figure 2. Effect of AI at 0, 12 or 24 h after onset of estrus on embryo quality.

Table 3. Total number of embryos(ova) and total embryos recovered on d 6 after artificial insemination of superovulated cattle at 0, 12, or 24 h after onset of estrus

Time of AI (h)	n[a]	Total embryos(ova)	Total embryos
0	10	195	57
12	10	207	124
24	10	127	103

[a]Number of animals

Table 4. Effect of artificial insemination of superovulated cows at 0, 12, or 24 h after onset of estrus on accessory sperm values

			Accessory sperm			
Time of AI (h)[a]	n[b]	Total embryos	Mean no. per embryo	SD	Embryos with accessory sperm,%	Embryos with accessory sperm tracks,%
0	195	57	.07[c]	.31	5[c]	21[c]
12	207	124	.24[c]	1.3	8[c]	31[c]
24	127	103	2.9[d]	8.3	41[d]	52[d]

[a] 10 animals per treatment.
[b] Total number of embryos and ova.
[c,d]Values within a column with different letters differ ($P < .01$).

counted. Twenty animals were categorized as having a dominant follicle, as indicated by < 10 follicles 3-8 mm in diameter, while 10 animals were without a dominant follicle. The presence of a dominant follicle tended to have a negative effect on the number of embryos(ova) recovered (P = .08, Table 5). The pooled number of follicles (≤ 8 mm) in both ovaries at the time of superovulatory induction was correlated with the number of embryos(ova) recovered (r = 0.51, P = .004).

Table 5. Embryo yields in cows following FSH injections given twice a day over 4 days and a single ultrasound examination to determine the presence or absence of a dominant follicle at the time of superovulatory induction

	Dominant Follicle[a]	
Characteristic	Present	Absent
Number of animals	20	10
Number of embryos(ova) (mean ± SEM)	15.2 ± 2.1[b]	22.5 ± 3.9[c]

[a] < 10 follicles 3-8 mm in diameter (dominant follicle present); ≥ 10 follicles 3-8 mm in diameter (dominant follicle absent).

[b,c]Values within a row with different superscripts tend to differ (P = .08).

Discussion

This is the first study to describe estrus characteristics of superovulated cattle and investigate the time of insemination of superovulated cattle based on the onset of estrus, as defined by the first standing event of a heat period. Estrus

characteristics monitored by HeatWatch® (Table 1) differ markedly from those reported by Walker et al. (1996) for single-ovulating cows. The time from administration of $PGF_{2\alpha}$ to the first standing event was shorter in our superovulated cattle (39 h 23 min) compared to the previously reported time of 73 h 6 min for lactating, single-ovulating cattle (Walker et al., 1996). The mean duration of behavioral estrus was longer for our superovulated cows (13 h 11 min vs. 9 h 30 min) and the mean number of standing events was greater (27.03 vs. 10.1) compared with single-ovulating cows (Walker et al., 1996). These discrepancies are likely due, at least in part, to the increased numbers of large follicles resulting from superstimulation with gonadotropins. The differences in mean duration of behavioral estrus and number of standing events could also be due to lactational status, numbers of herdmates simultaneously in estrus, and footing surface.

The biologic reason the fertilization rate to AI at 0 and 12 h was lower than 24 h AI may be related to sperm selection pressure and resulting depletion of potential fertilizing sperm, prior to the completion of ovulations. The estimated time required for sustained sperm transport is between 6 to 12 hours (Hunter and Wilmut, 1983; Wilmut and Hunter, 1984; Hawk, 1987). In the cases of 0 and 12 h AI, it is possible that many sperm were removed from the tract, not only through initial retrograde loss (Mitchell et al., 1985), but also through the loss of functional sperm reservoirs over the time span required for ovulations to occur. The estimated

start of ovulations in superovulated cattle is 24 h after visual observation of estrus (Yadav et al., 1986). Taking into account that these authors observed for estrus every 6 h, the actual start of ovulations in superovulated cattle is most likely between 24 to 30 h after the true onset of estrus. By quantifying populations of ovulated and nonovulated follicles immediately after slaughter, Yadav et al. (1986) accounted for nearly ninety percent of the ovulations which occurred over a 22 h period. In the current study, the 24 h insemination apparently allowed enough time for sustained sperm transport, and perhaps less time for loss of sperm from the functional sperm reservoirs, as evidenced by the increase in fertilization rate and the percentage of embryos with accessory sperm. In single-ovulating cattle artificially inseminated at 0, 12, or 24 h after the onset of estrus, the trend in fertilization rates was similar to those reported in the current study (Dalton et al., 1998).

In the present study, 41% of embryos recovered from the 24 h treatment contained accessory sperm. In contrast, Hawk et al. (1988) and Saacke et al. (1998) reported that 12% and 10% of embryos recovered from superovulated cows contained accessory sperm, respectively. The current results demonstrate an increase in the percentage of embryos with accessory sperm parallel to the increase in fertilization rate. This agrees with previous work by Hawk et al. (1988) in which the fertilization rate and the percentage of embryos with accessory sperm were increased when animals were inseminated with high numbers of sperm from

pooled, fresh ejaculates. In the 0 and 12 h AI treatments, many sperm were most likely removed from the tract through initial retrograde loss (Mitchell et al., 1985) and also loss of functional sperm reservoirs over the time span required for ovulations to occur. Therefore, in the 0 and 12 h AI treatments lower sperm numbers may have been at the site of fertilization, resulting in a lower percentage of embryos with accessory sperm and lower numbers of accessory sperm per embryo. Depleted sperm numbers may also explain the decreased fertilization rates for the 0 and 12 h AI treatments.

Differences remain, however, between species in distribution of accessory sperm across embryos within a recovery. Soede et al. (1995) reported nearly uniform distribution of accessory sperm (57 ± 77, mean ± SD) across porcine embryos classified as normal within a recovery, while relatively few embryos within a recovery from superovulated cattle exhibit accessory sperm. The biologic reason for the differences between the species in accessory sperm distribution is unknown. Cattle are naturally a monotocous species, which when induced to superovulate, appear to perform as a polytocous species. Nevertheless, the inherent reproductive strategy of cattle is undoubtedly refined for the optimization of fertilization of one ovum. In contrast, the inherent reproductive strategy of swine is refined for the optimization of fertilization of many ova. Although cattle can be easily induced to superovulate, it is possible that the mechanism which governs movement of sperm from the oviductal isthmus to the site of fertilization may not be changed. In this

scenario, the movement of sperm to the site of fertilization would be in a manner optimal for a single ovulation. Therefore, in the superovulated cow, the first few ova ovulated would be fertilized and exhibit accessory sperm; any ova ovulated later may be fertilized but would exhibit no accessory sperm due to depletion of sperm numbers in the oviductal isthmus and at the site of fertilization. In the sow, it is apparent that the mechanism(s) which govern movement of sperm to the site of fertilization are optimized to fertilize a large number of ova over a span of time. Therefore, the inherent fertilization strategy of the sow may be related to the retention and release of discrete numbers of sperm over the time span required for ovulation, without the depletion of sperm numbers prior to the last ovulation. It must be noted that the mechanism(s) which control sperm movement from the isthmus to the site of fertilization are currently unknown. Hunter (1998) proposed a model in which the release of sperm from the oviductal isthmus may be under ovarian endocrine control and occur just before ovulation, ensuring that sperm are at the site of fertilization when the ovum arrives. Further research will be required to establish the mechanism(s) involved in movement of sperm from the oviductal isthmus to the site of fertilization in the sow and the superovulated cow, which undoubtedly contributes to the differences between species in distribution of accessory sperm across embryos within a recovery.

Another possible reason for the disparity in distribution of accessory sperm across embryos within a recovery may be related to the oviductal microenvironment

at the time of fertilization. Oviductal fluid proteins derived from oviductal epithelium are thought to play a role in sperm capacitation and the acrosome reaction, two mandatory events in the fertilization process (Way et al., 1997;Staros and Killian, 1998). Although the specific function of these proteins has not been elucidated, they have been shown to associate with the bovine sperm plasma membrane (McNutt et al., 1992) and the bovine zona pellucida (Wegner and Killian, 1991;Staros and Killian, 1998). Deficiencies in oviductal fluid protein composition and(or) volume within an animal over the timespan required for ovulations may lead to: 1) a reduction in capacitated sperm, and(or) 2) a decreased ability of ova to bind sperm, resulting in diminished sperm-ova interaction and a concomitant decrease in the number of accessory sperm.

Accessory sperm number may also be regulated by the relative composition of the zona pellucida. Protease digestion of the zona pellucida of superovulated embryos(ova) requires half the time necessary to digest the zona pellucida of single-ovulated embryos(ova) (Saacke et al., 1994). Thus, relative resistance to protease digestion may be an indirect measure of the time required for sperm transit through the zona pellucida. In this scenario, embryos(ova) from superovulated cattle would exhibit less accessory sperm than an embryo(ovum) from a single-ovulated cow due to a decrease in transit time through the zona pellucida prior to fertilization and the zona reaction.

Regardless of the mechanism(s) involved in the regulation of accessory

sperm number, the results reported here provide evidence that accurate timing of insemination may help overcome potential problems with sperm transport and depletion of potential fertilizing sperm, prior to the completion of ovulations in superovulated cows. Consequently, accurate timing of AI could reduce the number of inseminations performed, doses used, and handling time of superovulated cattle.

Providing further evidence the 24 h AI may have overcome impaired sperm transport and(or) depletion of numbers of potential fertilizing sperm, sperm tracks in zonae pellucidae were most evident in embryos recovered from the 24 h AI group. Sperm tracks in the zonae pellucidae of embryos recovered from superovulated cattle have been reported previously (Hawk et al., 1988;Saacke et al., 1998), and are thought to represent accessory sperm loss. Apparently, sperm become attached to the zona pellucida at the site of each track but become dislodged prior to embryo recovery (Hawk et al., 1988).

It is still not clear at which time artificial insemination with conventional frozen-thawed semen provides optimal embryo quality. Although embryo quality was not affected by time of insemination using this semen, there appears to be a trend toward an increase in degenerate embryos in the 24 h AI group. This may be related to ova that were ovulated early, and underwent ageing prior to the completion of sustained sperm transport and fertilization. Delayed fertilization has been shown to lead to an increase in the degeneration of embryos recovered from superovulated cattle (Schiewe et al., 1987). In single-ovulating cows artificially

inseminated at 0, 12, or 24 h after the onset of estrus, a significant increase in degenerate embryos was noted in the 24 h AI group (Dalton et al., 1998).

This study was designed to focus on the time of insemination, while controlling the variability associated with semen. The bull chosen to provide semen for this study has been used previously to measure accessory sperm number per embryo following insemination with high and low sperm numbers (Nadir et al., 1993) and different times of insemination in single-ovulating cows (Dalton et al., 1998). The work by Nadir et al. (1993) gave insight into the ability of this bull to achieve consistently high accessory sperm values in embryos recovered from single-ovulating cows. Furthermore, numerous observations with the semen from this bull have shown a similarity with other bulls in the directionality of all traits tested, (e.g. fertilization rate, accessory sperm number per embryo, embryo quality) in response to different treatments (R.G. Saacke, unpublished). Taken together with previous reports of low percentages of embryos with accessory sperm recovered from superovulated cows (Saacke et al., 1998), this bull was chosen in order to provide the best possibility of determining differences between the insemination times, should they exist. Further research will be necessary to assess the effect of cryopreserved semen from different bulls, given that semen quality varies among bulls. Nevertheless, the current study provides an appropriate model in which semen from other bulls may be compared.

Guilbault et al. (1991) and Bungartz and Niemann (1994) reported that

superovulatory responses were reduced in animals treated in the presence of a dominant follicle. The single ultrasound examination performed in this experiment was an effort to describe the follicular status of the animals at the onset of treatment, as previously described by Bungartz and Niemann (1994). In the current study, based on the number of embryos(ova) recovered, the superovulatory response tended to be reduced in animals treated in the presence of a dominant follicle ($P = .08$, Table 4).

Previous recommendations on the optimal insemination time of superovulated cattle were made based on visual observation of estrus twice or three times daily (Donaldson, 1985; Schiewe et al., 1987). Comparisons with the present study must be made carefully because the timing of the onset of estrus in the previously mentioned studies has up to a 12 h error. Nevertheless, it appears that our best results fall in between 1) Donaldson (1985) in which AI with one dose of semen most likely occurred prior to 20 h after onset of estrus; and 2) Schiewe et al. (1987) in which AI with two doses of semen most likely occurred between 24 to 36 h after the onset of estrus. In the current study, artificial insemination of superovulated nonlactating Holstein cattle with one unit of semen 24 h after onset of estrus (as defined by the first standing event of a heat period) yielded an acceptable fertilization rate, embryo quality, and percentage of embryos with accessory sperm. The results reported here, coupled with further research utilizing

semen from other bulls, will help to determine a recommended insemination schedule for superovulated cattle.

Literature Cited

Bungartz, L., and H. Niemann. 1994. Assessment of the presence of a dominant follicle and selection of dairy cows suitable for superovulation by a single ultrasound examination. J. Reprod. Fertil. 101:583.

Critser, J.K., R.F. Rowe, M.R. Del Campo, and O.J. Ginther. 1980. Embryo transfer in cattle:Factors affecting superovulatory response, number of transferable embryos, and length of post-treatment estrous cycles. Theriogenology 13:397.

Dalton, J.C., S. Nadir, J.H. Bame, M. Noftsinger, R.L. Nebel, and R.G. Saacke. 1998. Effect of artificial insemination time and natural service on accessory sperm number, fertilization status and embryo quality in cattle. J. Anim. Sci. 76(Suppl 1):239(Abstr.).

DeJarnette, J.M., R.G. Saacke, J. Bame, and C.J. Vogler. 1992. Accessory sperm: Their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J. Anim. Sci. 70:484.

Donaldson, L. 1985. Effect of insemination regimen on embryo production in superovulated cows. Vet. Rec. 117:35.

Guilbault, L.A., F. Grasso, J.G. Lussier, P. Roullier, and P. Matton. 1991. Decreased superovulatory responses in heifers superovulated in the presence of a dominant follicle. J. Reprod. Fertil. 91:81.

Hasler, J.F. 1992. Current status and potential of embryo transfer and reproductive technology in dairy cattle. J. Dairy Sci. 75:2857.

Hawk, H.W. 1987. Transport and fate of spermatozoa after insemination of cattle. J. Dairy Sci. 70:1487.

Hawk, H.W., H.H. Conley, R.J. Wall, and R.O. Whitaker. 1988. Fertilization rates in superovulating cows after deposition of semen on the infundibulum, near the uterotubal junction or after insemination with high numbers of sperm. Theriogenology 29:1131.

Hunter, R.H.F. 1998. Sperm-epithelial interactions in the isthmus and ampulla of the fallopian tubes and their ovarian control. In: Gametes:Development and Function, A. Lauria, F. Gandolfi, G. Enne, and L. Gianaroli (Eds.), pp. 355-367. Proc. 50th Int'l. Cong. of Anim. Reprod. and Artif. Insem., Milano, Italy.

Hunter, R.H.F., and I. Wilmut. 1983. The rate of functional sperm transport into the oviducts of mated cows. Anim. Reprod. Sci. 5:167.

Lindner, G.M., and R.W. Wright. 1983. Bovine embryo morphology and evaluation. Theriogenology 20:407.

McNutt, T.L., L. Rogowski, R. Vasilatos-Younken, and G. Killian. 1992. Adsorption of oviductal fluid proteins by the bovine sperm membrane during in vitro capacitation. Mol. Reprod. Dev. 53:313.

Mitchell, J.R., P.L. Senger, and J.L. Rosenberger. 1985. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital

tract. J. Anim. Sci. 61:956.
Moore, D.S., and G.P. McCabe. 1989. Inference for distributions. In: Introduction to the Practice of Statistics, pp 510-579. W.H. Freeman and Company, New York.
Nadir, S., R.G. Saacke, J. Bame, J. Mullins, and S. Degelos. 1993. Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle. J. Anim. Sci. 71:199.
Ott, L. 1988. Inferences about μ_1-μ_2. In: An Introduction to Statistical Methods and Data Analysis, Third Edition, pp 170-184. PWS Kent, Boston.
Robbins, R.K., R.G. Saacke, and P.T. Chandler. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. J. Anim. Sci. 42:145.
Saacke, R.G., S. Nadir, J. Dalton, J. Bame, J.M. DeJarnette, S. Degelos, and R.L. Nebel. 1994. Accessory sperm evaluation and bull fertility (An Update). In: Proc. XV Tech. Conf. Artif. Insem. and Reprod., pp. 57-67. NAAB, Columbia, MO.
Saacke, R.G., J.M. DeJarnette, J.H. Bame, D.S. Karabinus, and S.S. Whitman. 1998. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super- and single-ovulating cattle? Theriogenology 50:117.
SAS®. 1985. User's Guide: Statistics.SAS® Inst. Inc., Cary, NC.
Schiewe, M.C., C.R. Looney, C.A. Johnson, K.G. Hill, and R.A. Godke. 1987. Transferable embryo recovery rates following different insemination schedules in superovulated beef cattle. Theriogenology 28:395.
Seidel, G.E., R.P. Elsden, L.D. Nelson, and J.F. Hasler. 1978. Methods of ovum recovery and factors affecting fertilization of superovulated bovine ova. In: J.R. Sreenan (Ed.), Control of Reproduction in the Cow, pp 268-280. M. Nijhoff, The Hague.
Soede, N.M., C.C.H. Wetzels, W. Zondag, M.A.I. de Konig, and B. Kemp. 1995. Effects of time of insemination relative to ovulation, as determined by ultrasonography, on fertilization rate and accessory sperm count in sows. J. Reprod. Fertil. 104:99.
Staros, A.L., and G.J. Killian. 1998. In vitro association of six oviductal fluid proteins with the bovine zona pellucida. J. Reprod. Fertil. 112:131.
Walker, W.L., R.L. Nebel, and M.L. McGilliard. 1996. Time of ovulation relative to mounting activity in dairy cattle. J. Dairy Sci. 79:1555.
Way, A.L., A.M. Schuler, and G.J. Killian. 1997. Influence of bovine ampullary and isthmic oviductal fluid on sperm-egg binding and fertilization in vitro. J. Reprod. Fertil. 109:95.
Wegner, C.C., and G.J. Killian. 1991. In vitro and in vivo association of an oviduct estrus-associated protein with bovine zona pellucida. Mol. Reprod. Dev.

29:77.
Wilmut, I., and R.H.F. Hunter. 1984. Sperm transport into the oviducts of heifers mated early in estrus. Reprod. Nutr. Dev. 24:461.
Yadav, M.C., J.S. Walton, and K.E. Leslie. 1986. Timing of the onset and duration of ovulation in superovulated beef heifers. Theriogenology 26:509.

CHAPTER 6

THE MORPHOLOGICAL EVALUATION OF UNFERTILIZED OVA RECOVERED FROM SUPEROVULATED CATTLE

Abstract

Three experiments were conducted to provide a foundation to distinguish between unfertilized ova and very early embryonic death. In Exp. 1, cows (n=16) were induced to superovulate and inseminated with killed spermatozoa. Two hundred twenty-three d 6 unfertilized ova were recovered and a morphological classification system was devised to separate all ova into one of three categories: 1) typical, 2) satellite, or 3) fragmented. Percentages of ova classified into each category were: 85% typical, 7% satellite, and 8% fragmented. In Exp. 2, 30 superovulated cows were artificially inseminated with live spermatozoa to determine the morphological classification of d 6 unfertilized ova recovered following insemination with live spermatozoa. Two hundred forty-two ova were recovered; Percentages of ova classified into each category were: 95% typical, 2% satellite, and 3% fragmented. In Exp. 3, cows were induced to superovulate, monitored for behavioral estrus by HeatWatch®, and were subjected to ovariectomy (n = 2), or recovery of tubal-stage ova (n = 4). Ultrastructural features of preovulatory, tubal-stage, and d 6 ova (typical, from Exp. 2) were investigated to provide a reference point for future investigations into very early embryonic death. Preovulatory ova exhibited normal ultrastructure. Evidence of degeneration was apparent in tubal-stage ova as the ooplasm exhibited a flocculent appearance and membranes of microvilli and

mitochondria were not distinct. Evidence of degeneration was apparent in all d 6 ova evaluated, with a heavily flocculent, degenerated ooplasm and no discernable organelles the only recognizable features common to all sections. Combining the first two experiments, 90% of all d 6 ova were readily identified as unfertilized and classified as typical. Cytological changes associated with degeneration were seen in tubal-stage ova 22 to 28 h after the beginning of ovulations, and in d 6 unfertilized ova recovered by uterine flush. This work provides: 1) a classification system which will help embryologists distinguish unfertilized ova from very early embryonic death; 2) a cytological reference point for future investigations into very early embryonic death; and 3) evidence the lifespan of superovulated ova is less than 24 h.

Introduction

The primary goal of superovulation, whether for research or commercial embryo transfer, is to recover a large number of high quality embryos. Superovulation and embryo transfer offer the food animal producer the potential for rapid genetic gain, and a means to market select offspring. For the physiologist, superovulation and embryo transfer provide a means to explore early embryonic development and mortality. Nevertheless, superovulation remains an inefficient process that has failed to reach its maximum potential for the industry and researcher alike. Nearly 35% of ova recovered from superovulated cows are unfertilized (Seidel et al., 1978; Donaldson, 1985; Hasler, 1992), while an additional

15% of embryos are of questionable viability, and therefore are nontransferable (Hasler, 1992).

Rapid, accurate morphological (ova)embryo evaluation is important to the success of embryo transfer. When evaluating ova(embryos) following recovery, considerable difficulty can be experienced by embryologists equipped with only a stereomicroscope. Unfertilized ova are routinely group-classified with fertilized degenerate embryos as "nontransferable" and discarded by embryologists. Consequently, very little information is available on the characteristics of ovulated, unfertilized ova (Church and Shea, 1976; Shea, 1981; Hytell et al., 1988). Providing further difficulty for embryologists, ova(embryos) with underdeveloped blastomere-like cytoplasmic fragments that do not exhibit nuclei (as seen only under phase contrast or differential interference contrast optics) have been classified as unfertilized/degenerate (UFO/DEG) (Saacke et al., 1992). According to Saacke et al. (1992), the fertilization status of ova(embryos) classified as UFO/DEG is unknown.

Saacke et al. (1994) reported a 10 to 27% incidence of ova(embryos) classified as UFO/DEG recovered from superovulated cows inseminated with semen having spermatozoa with a high proportion of protoplasmic droplets and tailless heads. The use of semen with sperm exhibiting the aforementioned traits or with nuclear vacuoles (craters or diadem) has been shown to depress embryo quality and fertilization rates (Saacke et al., 1992;1994). The decreased fertility reported by Saacke et al. (1992;1994) has been attributed to uncompensable

seminal traits, i.e., abnormal sperm able to initiate fertilization and(or) early embryonic development but not sustain either process.

In an attempt to build a foundation to distinguish unfertilized ova from very early embryonic death, an investigation into the gross morphology of d 6 unfertilized ova as viewed with the light microscope was undertaken (Exp. 1). The objectives of this experiment were: 1) to devise a classification system that would describe the gross morphology of d 6 unfertilized ova recovered from superovulated cattle; and 2) to quantify the incidence of the morphological types observed in superovulated cattle inseminated with killed spermatozoa.

Utilizing the classification system devised in Exp. 1, Exp. 2 was conducted to determine the incidence and morphological classification of d 6 unfertilized ova recovered from superovulated cattle artificially inseminated with live spermatozoa at 0, 12, or 24 h after onset of estrus.

Although light microscopic morphological evaluation of embryos(ova) has traditionally been used to identify embryos of transferable and freezable quality, much can be learned about early embryonic death, the inefficiency of superovulation regimens, and the potential impact of the male by the further investigation of unfertilized ova. An understanding of the morphology and ultrastructure of unfertilized ova may allow future veterinary practitioners and physiologists to decrease the incidence of unfertilized ova, thereby improving the efficiency of superovulation and embryo transfer. In Exp. 3, the preliminary objective was to establish the structural characteristics of the most common

morphological type of d 6 unfertilized ovum using transmission electron microscopy, and compare and contrast those characteristics with ova recovered surgically from the oviducts. After an extensive comparison of electron micrographs of the most common morphological type of d 6 unfertilized ova and tubal-stage ova, this experiment evolved into an ultrastructural survey of preovulatory ova obtained by ovariectomy, ova recovered surgically from the oviducts, and the most common morphological type of d 6 unfertilized ova. The evolution of this investigation was necessary to ensure the observations made were accurate and did not result from artifacts due to procedural errors.

Materials and Methods

Superovulatory Treatment and Detection of Estrus (Exp. 1, 2 and 3)

Eight to eleven days following estrus, the cows (Exp. 1: n=16; Exp. 2: n=30; Exp. 3: n=2 for bilateral ovariectomy; n=4 for tubal-stage ova recovery; and n=30 from Exp. 2) were superovulated with 38 mg of FSH-P™ (Schering-Plough Animal Health Corp., Kenilworth, NJ). The cows received twice daily injections of FSH-P™ (2×6 mg, 2×6 mg, 2×4 mg and 2×3 mg i.m.) for 4 d. Luteolysis was induced with an injection of $PGF_{2\alpha}$ (Lutalyse®, Pharmacia and Upjohn Co., Kalamazoo, MI) at 72 h (40 mg i.m.) and 84 h (25 mg i.m.) after the initial FSH-P™ injection. All injections i.m. were performed in the gluteal muscle. All cows used for the first experiment were observed for evidence of behavioral estrus twice daily, in the early morning

and late evening. Cows that stood to allow mounting by a herdmate were considered to be in estrus. All cows utilized for Exp. 2 and 3 were continuously monitored for behavioral estrus by HeatWatch® (DDx Inc., Denver, CO), which utilizes radio frequency data communications. This system included battery-powered, reusable pressure-sensing transmitters with a .4-km range, a repeater with a .8-km range, a signal receiver, a buffer that received and stored mounting activity data, and software that sorted information by cow, date, and time using a personal computer with an 80386 microprocessor. Transmitters were contained in a nylon pouch on a 35- x 20-cm nylon mesh patch, which was glued with contact-type adhesive to the hair of the sacral region just anterior to the tailhead. At all times, the cows were housed in a pasture within working range of the transmitters and repeater. Transmitters were activated by continuous pressure (minimum of 2 s) from a mounting herdmate. Transmitted data included transmitter number, date, time and duration of standing events.

Freeze-thaw Procedure and Artificial Insemination (Exp. 1)

All cows were artificially inseminated with frozen-thawed killed spermatozoa at first observation of standing estrus and 12 h later. Cryopreserved semen (egg yolk-citrate-glycerol extender) in .5-mL French straws were removed from storage in liquid nitrogen and allowed to air-thaw for 15 min at room temperature. The semen straws were then plunged into liquid nitrogen for 3-7 min. Lastly, the semen was air-thawed for 15 min at room temperature and multiple smears were

examined under phase contrast microscopy at $\times$250 magnification. Death of the spermatozoa was confirmed by separation of the head and tail at the implantation socket and 0 % motility.

Artificial Insemination Schedule (Exp. 2)

Beginning 32 h after the first $PGF_{2\alpha}$ injection, the HeatWatch® software was reviewed every 3 h to determine the onset of estrus as defined by the time of the first standing event. Characteristics of the semen used in this experiment have been described previously (Chapter 5). Frozen straws were thawed by plunging into 35°C water for 45 s. All cows were artificially inseminated by the same experienced technician with one dose of semen (50×10^6 sperm) at either 0 h (n=10), 12 h (n=10), or 24 h (n=10) after onset of estrus as determined by HeatWatch®. The body of the uterus was the site of semen deposition.

Recovery of d 6 Unfertilized Ova (Exp. 1 and 2)

Ova were recovered by an experienced technician using standard nonsurgical uterine flushing techniques 6 d after insemination. The initial search and evaluation of ova was carried out by a single observer with a stereomicroscope at magnifications of $\times$10 and $\times$70.

Preoperative and Postoperative Regimens (Exp. 3)

Feed and water were withheld from each animal for 24 and 12 h, respectively, prior to surgery. Postoperatively, the cows were allowed free choice grass hay for 1 h and then were given both hay and water. All cows received

Procaine Penicillin G (20,000 units/kg, i.m.) once daily for the first five postoperative days. Sutures were removed on the tenth postoperative day. The cows were housed in stalls for 14 d postoperatively before returning to pasture.

Ovariectomy (Exp. 3)

Within 20 hours of the onset of estrus as determined by HeatWatch®, the left paralumbar fossa and caudal flank regions of the cows selected for bilateral ovariectomy were clipped, washed and prepared for surgery. Analgesia for laparotomy was accomplished with local infiltration of lidocaine HCL 2% (100 mL). Approximately 5 cm cranial and on a line parallel to the quadriceps femoris muscle, an incision was begun 20 cm ventral to the transverse processes of the lumbar vertebrae and extended for approximately 20 cm toward the flank. After blunt separation of the external and internal abdominal oblique muscles, and the transverse abdominal muscle, the peritoneum was perforated to expose the abdominal cavity. The left and right ovaries were located and removed by chain ecrasure. Closure was performed as previously described by Wolfe et al. (1990). The ovaries were placed into a vacuum flask containing phosphate-buffered saline with 1% fetal calf serum (PBS-FCS). The contents of follicles greater than 8 mm in diameter were aspirated with a 23 gauge needle attached to a 1 mL syringe and transferred to a plastic dish containing PBS-FCS. Cumulus-enclosed oocytes were isolated with a stereomicroscope at magnifications of ×10 and ×70.

Recovery of Tubal-stage Ova (Exp. 3)

Within 72 hours of the onset of estrus as determined by HeatWatch®, the right and left paralumbar fossa and caudal flank regions of the cows selected for bilateral caudal flank surgery were clipped and washed. Xylazine (.05 mg/kg i.v.) was administered as a preanesthetic. Anesthesia was induced with 10% guaifenesin and ketamine (2.2 mg/kg) i.v., and maintained with 3% isoflurane. The cows were positioned in right lateral recumbency, the left rear leg was pulled caudally, and the left caudal flank was prepared and draped for aseptic surgery. The incision and exteriorization of the reproductive tract were performed as previously described by Wolfe et al. (1990). Following the location of the infundibulum, the uterine tube was cannulated with a sterile pipette attached to i.v. extension tubing. A 60-mL syringe containing PBS-FCS was then attached to the i.v. extension tubing. Following the blunt insertion of a scalpel handle into the uterine lumen approximately 6 cm distal to the uterotubal junction, a canine endotracheal tube (6 Fr) was introduced into the uterus. The endotracheal tube was positioned against the uterotubal junction to facilitate fluid collection. Fifty milliliters of PBS-FCS media were gently flushed into the i.v. extension tubing set, through the oviductal pipette and the oviduct, out the endotracheal tube, and into a plastic dish. Closure was performed as previously described by Wolfe et al. (1990). The cow was rolled into left lateral recumbency, and the right rear leg was pulled caudally. The procedure was repeated to flush the right oviduct. Immediately

following each oviductal flush, recovered ova were isolated with a stereomicroscope at magnifications of ×10 and ×70.

Specimen Processing for
Transmission Electron Microscopy (Exp. 2 and 3)

The manipulation of ova in preparation for transmission electron microscopy was as described by Hytell and Madsen (1987)(Appendix B). Beginning immediately after recovery and isolation, the ova were fixed in 3% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in 0.1 M phosphate buffer (Appendix C) for 1 hr at 0°C. The ova were then washed three times in 0.1 M phosphate buffer, and held overnight at 4°C in fresh phosphate buffer. Following pre-embedding in 2% agar (BBL Inc., Cockeysville, MD)(Appendix D) as described by Hytell and Madsen (1987) and Janisch (1974), the ova were washed twice in 0.1 M phosphate buffer, and post-fixed in 2% osmium tetroxide (Polysciences, Inc., Warrington, PA) for 1 hr at 0°C. After three washes in fresh buffer and two washes in deionized water, the ova were stained en bloc with 2% uranyl acetate (Fisher Scientific Co., Fair Lawn, NJ). Following two washes in deionized water, the ova were dehydrated in 50, 70, 95, 100 and 100% ethanol. The ova were then transferred to a 1:1 mixture of propylene oxide (Polysciences, Inc., Warrington, PA) and Spurr resin (Polysciences, Inc., Warrington, PA) for ten minutes. The ova were held overnight in 100% Spurr resin at room temperature to allow for infiltration. Next the ova were placed in BEEM® capsules (Polysciences, Inc., Warrington, PA) with fresh 100% Spurr resin. After polymerization for 16 hours at 70°C, ultrathin sections were

obtained with a diamond knife and a Porter-Blum MT2-B ultramicrotome. The sections were picked up on uncoated copper grids, stained with 2% uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Jeol transmission electron microscope.

Results and Discussion

Experiment 1

Two hundred twenty-three unfertilized ova were recovered from 16 superovulated animals using standard nonsurgical uterine flushing techniques. Ova were evaluated at ×10 and ×70 magnification with a stereomicroscope, and a classification system was devised which separated all recovered ova into one of three categories: 1) typical, 2) satellite, or 3) fragmented (Figure 1). Ova with a readily defined vitellus exhibiting no sign of fragmentation were designated as typical. Ova exhibiting two cytoplasmic masses (one larger than the other) were classified into the satellite category. Ova with three or more large cytoplasmic fragments were designated as fragmented. The majority of ova recovered (85%) were classified as typical (Table 1). Ova classified into the satellite category (7%) were considered to have a residual polar body and appeared to be cow related (Table 1). Fragmented ova accounted for 8% of all ova recovered (Table 1) and also appeared to be cow related.

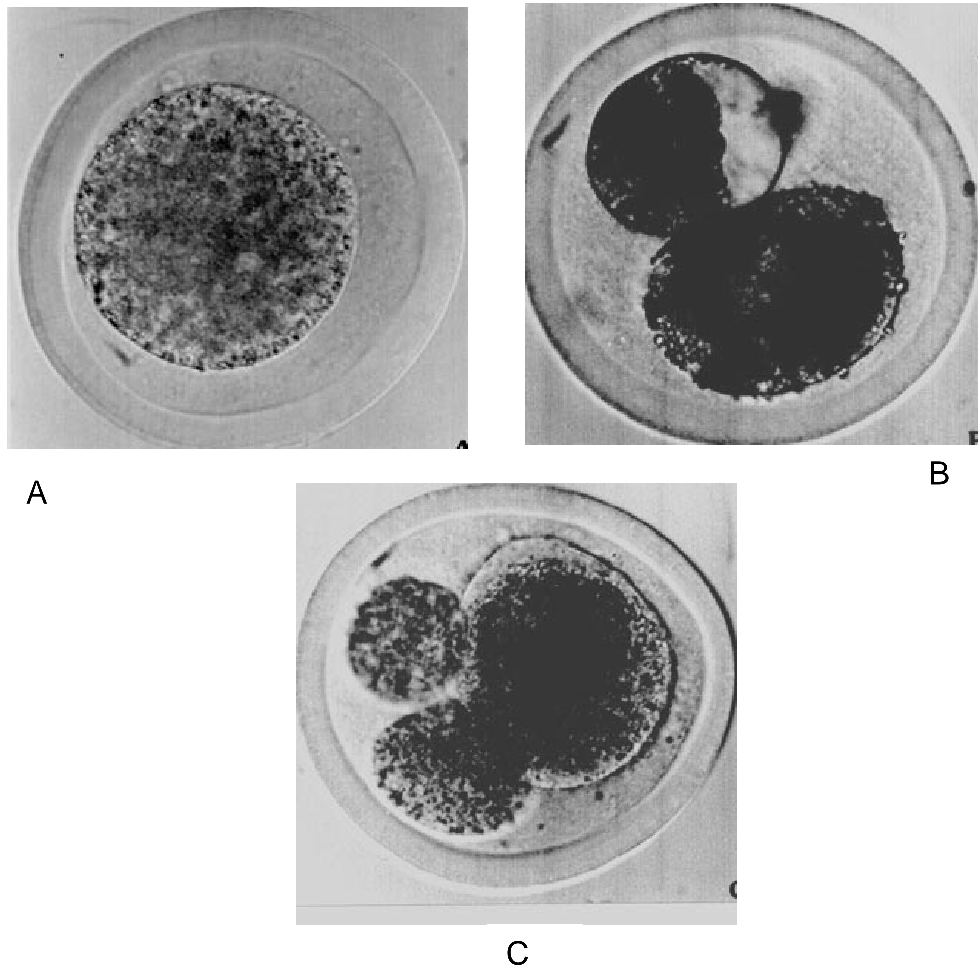

Figure 1. Light micrographs of the three morphological classifications of d 6 unfertilized ova recovered from superovulated cattle. (A) typical, (B) satellite, (C) fragmented. ×1289.

Experiment 2

The classification system established during Exp. 1 was used to morphologically classify 242 d 6 unfertilized ova recovered from superovulated cows artificially inseminated with live spermatozoa at 0, 12, or 24 h after onset of estrus as determined by HeatWatch® (Tables 2, 3 and 4). The incidence of the most common morphological type of unfertilized ovum, classified as typical, ranged from 90 to 100% of the ova recovered. As noted in Exp. 1, the incidence of ova classified as satellite or fragmented was low, and appeared to be cow related. When data from the three different insemination times was combined, 95% of all recovered d 6 ova were readily identified as unfertilized and classified as typical (Table 5). Therefore, the most likely causes of ova(embryos) of questionable fertilization status observed in other studies are cow related and(or) male related, e.g., due to uncompensable seminal traits which might lead to very early embryonic death. Lastly, combining Exp. 1 and 2, 90% of all unfertilized ova recovered were classified as typical (Table 5). These ova recovered from superovulated cattle should be readily identified by embryologists as unfertilized.

Table 1. Morphological classification of d 6 unfertilized ova recovered from cows treated for superovulation and receiving two inseminations with killed spermatozoa (Exp. 1)

Cow	No. of ova recovered	Morphological Classification[a]		
		Typical	Satellite	Fragmented
A	16	8	7	1
B	25	24	1	0
C	17[b]	13	2	2
D	19	18	0	1
E	2	2	0	0
F	7	7	0	0
G	4	3	1	0
H	8	7	0	1
I	13	12	0	1
J	24	24	0	0
K	10	10	0	0
L	24	21	1	2
M	2	2	0	0
N	25	16	1	8
O	18[b]	16	0	2
P	9	7	2	0
Total (%)	223 (100)	190 (85)	15 (7)	18 (8)

[a]Morphological Classification: **Typical:** Ova with a readily defined vitellus exhibiting no sign of fragmentation. **Satellite:** Ova exhibiting a large and small cytoplasmic mass. **Fragmented:** Ova with three or more distinct cytoplasmic fragments.

[b]One empty zona pellucida was also recovered.

Table 2. Morphological classification of d 6 unfertilized ova recovered from cows treated for superovulation and receiving a single AI with frozen-thawed live spermatozoa at 0 h after onset of estrus as determined by HeatWatch®(Exp. 2)

				Morphological Classification[a]		
Cow No.	n[b]	No. embryos	No. ova	Typical	Satellite	Fragmented
592	21	1	20	17	1	2
832	6	6	0	0	0	0
119	12	3	9	9	0	0
965	10	2	8	8	0	0
986	28	1	27	27	0	0
871	6	5	1	0	1	0
968	22	3	19	16	0	3
90	39	18	21	21	0	0
841	17	1	16	16	0	0
908	34	17	17	17	0	0
Total (%)	195	57	138 (100)	131 (95)	2 (1)	5 (4)

[a]Morphological Classification: **Typical:** Ova with a readily defined vitellus exhibiting no sign of fragmentation. **Satellite:** Ova exhibiting a large and small cytoplasmic mass. **Fragmented:** Ova with three or more distinct cytoplasmic fragments.

[b]Total number of embryos(ova) recovered.

Table 3. Morphological classification of d 6 unfertilized ova recovered from cows treated for superovulation and receiving a single AI with frozen-thawed live spermatozoa at 12 h after onset of estrus as determined by HeatWatch®(Exp. 2)

				Morphological Classification[a]		
Cow No.	n[b]	No. embryos	No. ova	Typical	Satellite	Fragmented
968	24	15	9	8	1	0
834	29	21	8	5	2	1
841	9	8	1	1	0	0
90	29	29	0	0	0	0
959	12	10	2	2	0	0
642	6	4	2	2	0	0
870	23	20	3	*	*	*
694	47	1	46	46	0	0
832	7	4	3	3	0	0
870	21	12	9	8	0	1
Total (%)	207	124	83 (100)	75 (90)	3 (4)	2 (1)

[a]Morphological Classification: **Typical:** Ova with a readily defined vitellus exhibiting no sign of fragmentation. **Satellite:** Ova exhibiting a large and small cytoplasmic mass. **Fragmented:** Ova with three or more distinct cytoplasmic fragments.

*Missing data.

[b]Total number of embryos(ova) recovered.

Table 4. Morphological classification of d 6 unfertilized ova recovered from cows treated for superovulation and receiving a single AI with frozen-thawed live spermatozoa at 24 h after onset of estrus as determined by HeatWatch®(Exp. 2)

				Morphological Classification[a]		
Cow No.	n[b]	No. embryos	No. ova	Typical	Satellite	Fragmented
908	12	12	0	0	0	0
581	23	18	5	5	0	0
868	5	5	0	0	0	0
969	12	3	9	9	0	0
805	13	13	0	0	0	0
51	4	4	0	0	0	0
222	21	20	1	1	0	0
920	18	11	7	7	0	0
855	3	1	2	2	0	0
119	16	16	0	0	0	0
Total (%)	127	103	24 (100)	24 (100)	0 (0)	0 (0)

[a]Morphological Classification: **Typical:** Ova with a readily defined vitellus exhibiting no sign of fragmentation. **Satellite:** Ova exhibiting a large and small cytoplasmic mass. **Fragmented:** Ova with three or more distinct cytoplasmic fragments.

[b]Total number of embryos(ova) recovered.

Table 5. Summary of morphological classifications of d 6 unfertilized ova recovered from cows treated for superovulation

Treatment	No. cows	No. ova	Morphological Classification[d]		
			Typical	Satellite	Fragmented
Exp. 1[a]	16	223	190(85)	15(7)	18(8)
Exp. 2[b]	29[c]	242	230(95)	5(2)	7(3)
Total (%)	45	465	420(90)	20(4)	25(6)

[a]All animals inseminated with frozen-thawed killed spermatozoa.
[b]All animals received a single AI with frozen-thawed live spermatozoa from the same bull.
[c]Due to missing data (cow 870 12 h), the total for this study is 29.
[d]Morphological Classification: **Typical:** Ova with a readily defined vitellus exhibiting no sign of fragmentation. **Satellite:** Ova exhibiting a large and small cytoplasmic mass. **Fragmented:** Ova with three or more distinct cytoplasmic fragments.

Experiment 3

From two superstimulated cows subjected to bilateral ovariectomy, 14 oocytes were aspirated from follicles greater than 8 mm in diameter. All oocytes recovered were enclosed by cumulus cells. The ultrastructure of a cumulus cell is shown in Figure 2. The nucleus is readily apparent as are golgi complexes, lipid droplets, and a multivesicular body. Smooth endoplasmic reticulum can also be seen surrounding one of the lipid droplets. Lipid metabolism is one of a variety of functions performed by smooth endoplasmic reticulum (Bozzola and Russell, 1992).

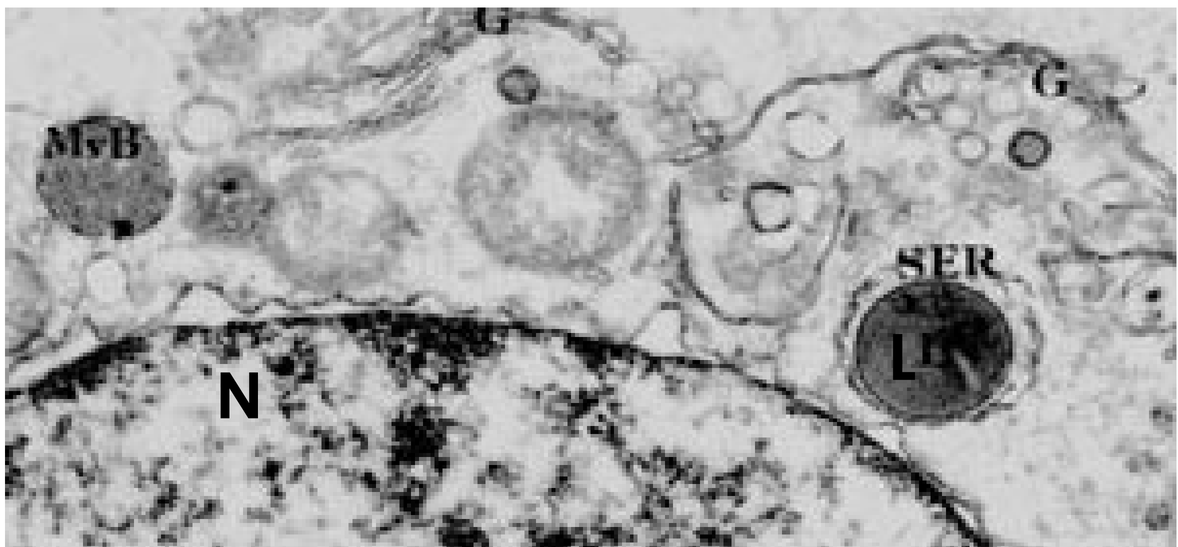

Figure 2. Ultrastructural features of a cumulus cell showing the nucleus (N), a multivesicular body (MvB), golgi complexes (G), and a lipid droplet (L). Note the smooth endoplasmic reticulum (SER) surrounding the lipid droplet. ×23,125.

The age of the oocyte shown in Figure 3 at the time of fixation relative to the onset of estrus as determined by HeatWatch® was approximately 15 h. Mitochondrial clusters in cortical positions were evident in all sections examined, in agreement with the morphology of oocytes recovered from unstimulated (Kruip et al., 1983) and superstimulated cattle (Hytell et al., 1986) estimated to be approximately 15 h after the LH peak (Figures 3 and 4). A large proportion of the mitochondrial population exhibited a "hood-like" process, as described by Senger and Saacke (1970) and Fleming and Saacke (1972)(Figures 3 and 4). Smooth endoplasmic reticulum was visualized within the extramitochondrial cavity formed by the hood, in agreement with Fleming and Saacke (1972), Kruip et al. (1983), and Hytell et al. (1986) (Figure 4). The function of the hooded mitochondria is not known. Fleming and Saacke (1972) postulated that the close apposition of endoplasmic reticulum with the surface of the hood may facilitate the exchange of metabolic intermediates between mitochondria and endoplasmic reticulum.

Microvilli arising from the preovulatory oocyte were also observed, as was the perivitelline space, a flocculent layer easily differentiated from the zona pellucida. The cumulus cell projection end appears to have begun retraction, as previously estimated to occur within 9 to 12 h after the LH peak (Hytell et al., 1986)(Figure 3). Surface contact between the cumulus cell projection end and the oocyte appeared to be enhanced by microvilli extended

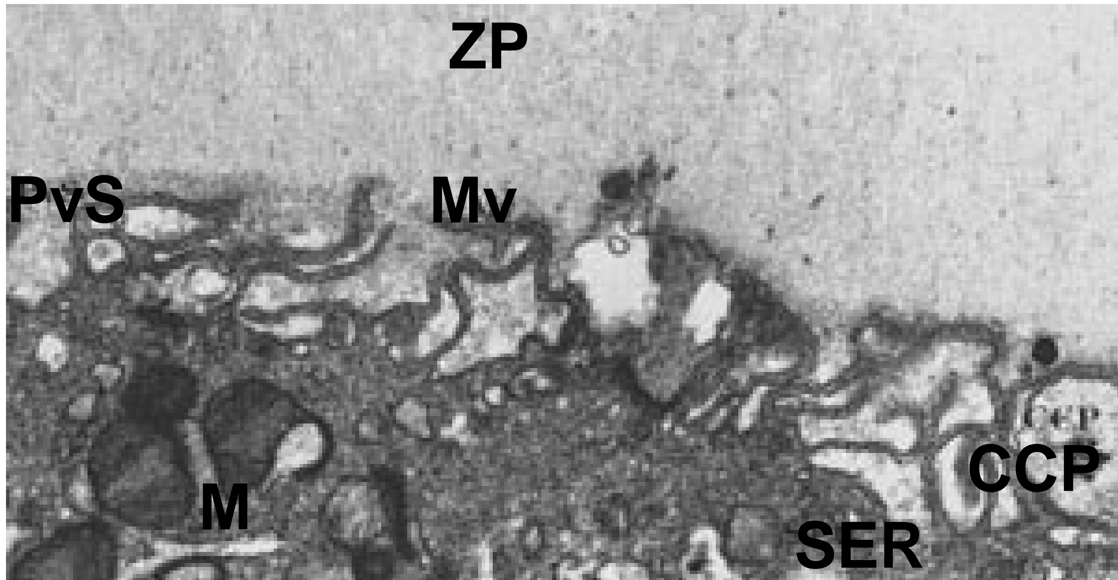

Figure 3. Electron micrograph of an oocyte approximately 12 h after the LH peak (15 h after onset of estrus) recovered following ovariectomy and follicular aspiration. The zona pellucida (ZP) is readily apparent as are numerous microvilli (Mv) extending from the surface of the oocyte into the perivitelline space (PvS). A cumulus cell process (CCP) is observed within the perivitelline space. Numerous mitochondria (M) with "hood-like" appendages are apparent. Smooth endoplasmic reticulum (SER) is also present. $\times$21,600.

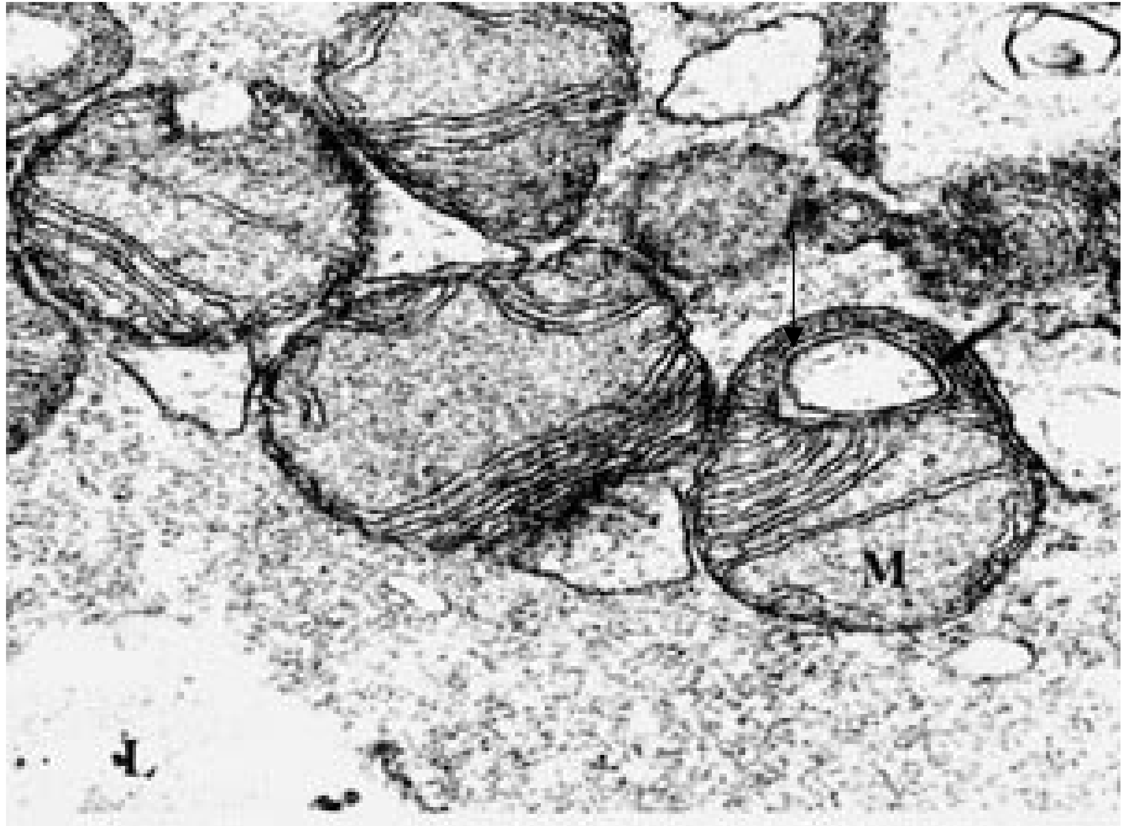

Figure 4. Detail of numerous mitochondria (M) with "hood-like" appendages. The cross section of the hooded mitochondria reveals a cavity formed by the hood and a portion of smooth endoplasmic reticulum closely apposed to the outer mitochondrial membrane within the cavity (arrow). Remnants of a lipid droplet can also be observed. $\times$61,625.

over the projection end. This intimate association between cumulus cell projection ends and the oocyte has been described previously (Fleming and Saacke, 1972; Assey et al., 1994).

Using the work of Stevenson et al. (1998) as a template, in which the LH surge in unstimulated heifers occurred 3.5 ± 0.6 h following the onset of estrus as determined by HeatWatch®, the estimated age of the oocyte shown in Figure 3 at the time of fixation relative to the presumptive LH peak was approximately 12 h. This age estimate is further supported by the observations of cortical granules only in clusters, and not in close proximity to the plasma membrane, in agreement with Kruip et al. (1983) for unstimulated cattle and Hytell et al. (1986) for superstimulated cattle. Furthermore, the presence of smooth endoplasmic reticulum, the lack of rough endoplasmic reticulum, and infrequently observed, poorly developed golgi complexes (not shown) agrees with previous descriptions of oocytes of similar estimated ages (Kruip et al., 1983; Hytell et al., 1986). Lastly, although the nucleus was not observed in the sections examined, it would be expected to be at the germinal vesicle breakdown stage as previously described by Kruip et al. (1983) and Hytell et al. (1986) for oocytes of the same approximate age. A total of 32 ova were recovered, fixed, and processed for electron microscopy from two superovulated cows subjected to surgical recovery of tubal-stage ova. unfortunately, glutaric acid contamination of the fixative resulted in sections of

extremely poor contrast, with little evidence of membrane integrity in all ova examined. Therefore, two additional cows were superovulated and subjected to surgical recovery of tubal-stage ova. A total of thirty-five ova were recovered. At the level of gross morphology as determined with a stereomicroscope, all ova recovered were classified as typical. Ova were randomly chosen from both cows and numerous sections were examined. Readily visible were randomly-distributed mitochondria, many of which exhibited "hood-like" appendages; lipid droplets, and multivesicular bodies. Nevertheless, evidence of degeneration was apparent. The mitochondrial membranes, and membranes of the microvilli protruding into the perivitelline space were not distinct (Figure 5). Giving further evidence of degeneration was the lack of smooth endoplasmic reticulum and the flocculent appearance of the ooplasm. The observations discussed here agree in part with Brackett et al. (1980) in which a tubal-stage ovum recovered between approximately 64 to 72 h after observed estrus exhibited degenerated ooplasm. No mention was made by Brackett et al. (1980) as to which organelles, if any, were observed. In the current study, to guard against procedural problems, all new solutions were used for these ova and resulting sections. Therefore, the loss of membrane integrity and flocculent appearance of the ooplasm are

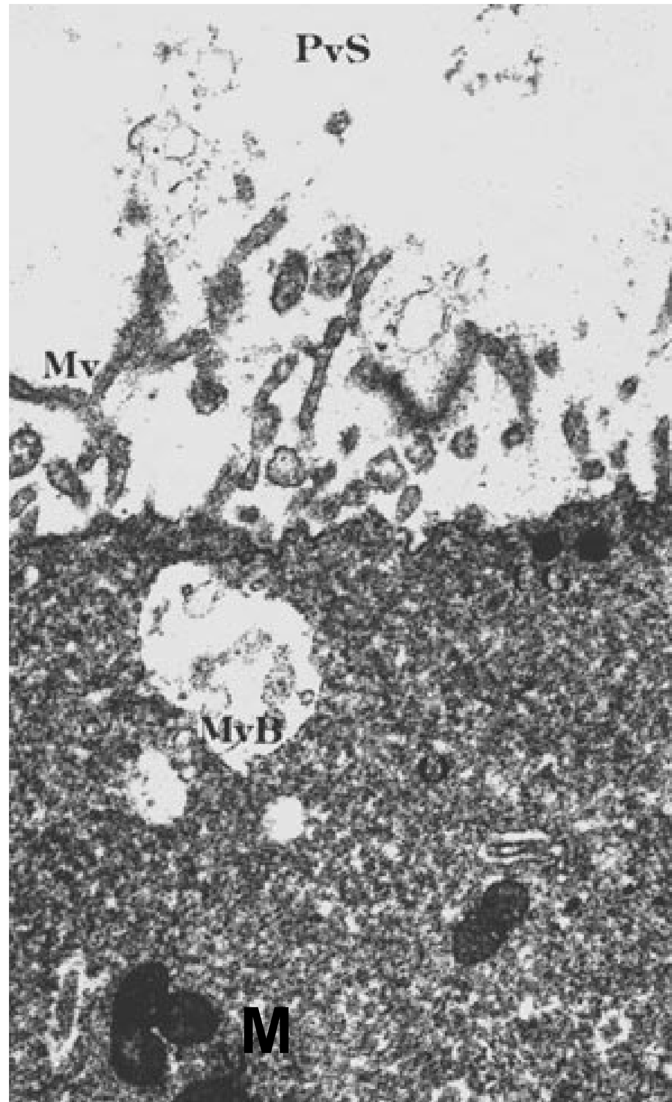

Figure 5. Ultrastructure of an oocyte recovered surgically from the oviducts. The perivitelline space (PvS) is readily apparent as are numerous microvilli (Mv). Cortical granules (CG) are observed immediately below the surface of the oocyte. Mitochondria (M), lipid (L), and remnants of a multivesicular body (MvB) are evident in the ooplasm (O). Membranes of mitochondria, microvilli, and multivesicular bodies are not distinct, giving evidence of degeneration. Note the heavily flocculent appearance of the ooplasm. $\times$18,900.

considered to be real and attributed to degeneration, rather than artifacts esulting from a processing error.

Although it is not possible to know when each ovum was ovulated and the time span that elapsed prior to recovery and fixation, the elapsed time from heat onset to surgery was approximately 52 h for the first animal and 67 h for the second animal. Using the work of Yadav et al. (1986) as a template, in which ovulations in superovulated cattle began between 24 and 30 after onset of estrus, the approximate range in elapsed time from the beginning of ovulations to surgery was 22 to 28 h for the first cow and 37 to 43 h for the second cow. As previously stated, ova were randomly chosen from both cows and numerous sections were examined. The oocyte shown in Figure 5 is from the first cow, placing the estimated age between 22 and 28 h after ovulation. The fertilizable lifespan of the bovine egg has been estimated at up to 24 h after ovulation (Thibault, 1967), while the functional lifespan, during which normal fertilization results in a viable embryo, has been suggested to be less than 8 to 12 h (Casida, 1950; Hunter, 1988). The distinction between the functional and fertilizable or "penetrable" lifespan of ova, as mentioned by Hunter (1988), is important as delayed fertilization has been shown to lead to an increase in the degeneration of embryos recovered from superovulated cattle (Schiewe et al., 1987). It is clear from the observations made here that evidence of degeneration is apparent in tubal ova within 22 to 28 h after ovulation (Figure 5). This supports the importance of insemination time in

superovulated and single-ovulating cattle. In the previous study (Chapter 5) the optimal time of insemination of superovulated cattle was 24 h after the onset of estrus. In single-ovulating cattle, the optimal time of AI has been determined to be between 4 and 12 h after the onset of estrus (Dransfield et al.,1998). These insemination times allow for sustained sperm transport with resulting colonization of the oviductal isthmus, and for fertilization to occur during the functional lifespan of the ovum.

Throughout Exp. 2 recovered d 6 unfertilized ova classified as typical were fixed and processed for electron microscopy. At the ultrastructural level, the only recognizable features common to all sections examined were the presence of the zona pellucida, perivitelline space, and the heavily flocculent, degenerated ooplasm (Figure 6). There were no recognizable organelles or structures other than lipid droplets in any of the sections examined.

The evolution of this investigation from the establishment of structural characteristics of the typical d 6 unfertilized ovum to a survey of preovulatory ova obtained by ovariectomy, ova recovered surgically from the oviducts, and d 6 unfertilized ova was necessary to provide a meaningful morphologic foundation of the most common unfertilized ova recovered. Although further investigation into the ultrastructure of unfertilized d 6 ova is not necessary, this work not only provides points from which comparisons may be made in the future, but also gives insight into the lifespan of the bovine ovum.

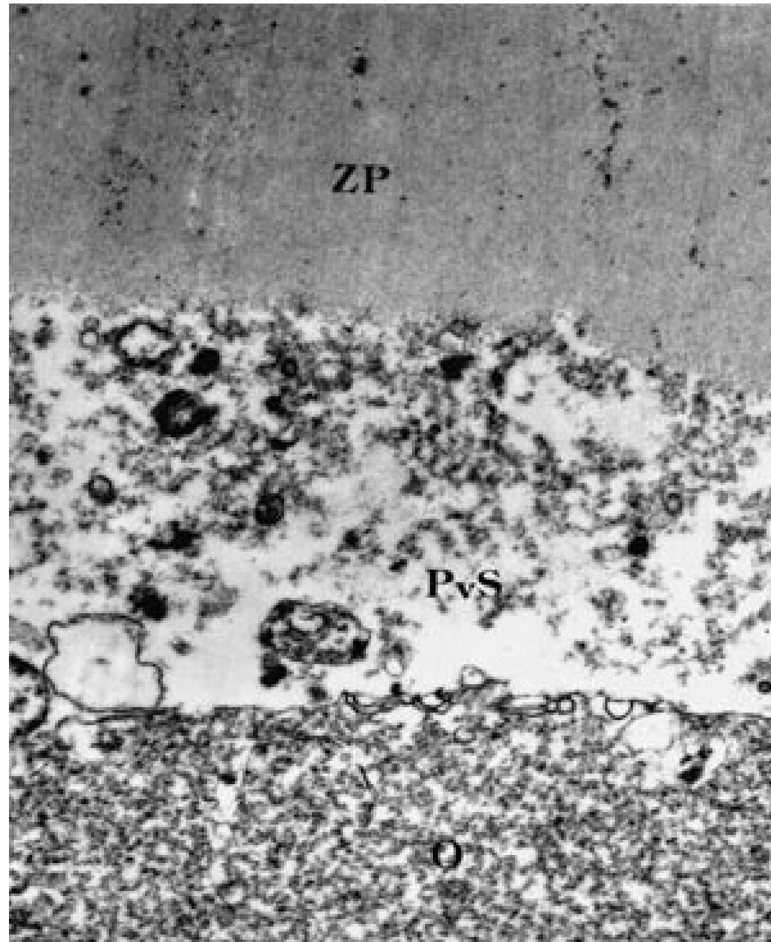

Figure 6. Electron micrograph of a d 6 unfertilized ovum. The zona pellucida (ZP), perivitelline space (PvS), and ooplasm (O) are apparent. Note the advanced degenerative state as evidenced by debris within the perivitelline space, the heavily flocculent appearance of the ooplasm, and the absence of recognizable organelles within the ooplasm. $\times$14,400.

In summary, d 6 unfertilized ova were classified morphologically into one of three categories: 1) typical, 2) satellite, or 3) fragmented. Four hundred sixty-five ova were recovered in the first two experiments, 90% of which were classified as typical (Table 5). These superovulated ova should be readily identified by embryologists as unfertilized. The most likely causes of ova(embryos) of questionable fertilization status and(or) viability are cow related and(or) male related, e.g., due to uncompensable seminal traits which might lead to very early embryonic death. This work provides a foundation to distinguish between two components of pregnancy rate, unfertilized ova and very early embryonic death. On an ultrastructural level, cytological changes associated with degeneration were seen in tubal-stage ova and in d 6 unfertilized ova recovered by uterine flush. Evidence of degeneration was observed in tubal-stage ova as early as 22 to 28 h after the beginning of ovulations. Thus, the fertilizable lifespan of bovine ova appears to be less than 22 h.

Literature Cited

Assey, R.J., P. Hytell, T. Greve, and B. Purwantara. 1994. Oocyte morphology in dominant and subordinate follicles. Mol. Reprod. Dev. 37:335.

Bozzola, J.J., and L.D. Russell. Survey of biological ultrastructure. In: Electron Microscopy, p 450. Jones and Bartlett Publishers, Boston, MA.

Brackett, B.G., Y.K. Oh, J.F. Evans, and W.J. Donawick. 1980. Fertilization and early development of cow ova. Biol. Reprod. 23:189.

Casida, L.E. 1950. The repeat breeder cow. Vlaams Diergeneeskundig Tijdschrift 19:273.

Church, R.B., and B.F. Shea. 1976. Some aspects of bovine embryo transfer. In: Egg Transfer in Cattle, Ed. L.E.A. Rowson, pp. 73-86. Commission of the European Communities, Luxembourg.

Donaldson, L.E. 1985. Effect of insemination regimen on embryo production in superovulated cows. Vet. Rec. 117:35.

Dransfield, M.B.G., R.L. Nebel, R.E. Pearson, and L.D. Warnick. 1998. Timing of insemination for dairy cows identified in estrus by a radiotelemetric estrus detection system. J. Dairy Sci. 81:1874.

Fleming, W.N., and R.G. Saacke. 1972. Fine structure of the bovine oocyte from the mature Graafian follicle. J. Reprod. Fertil. 29:203.

Hasler, J.F. 1992. Current status and potential of embryo transfer and reproductive technology in dairy cattle. J. Dairy Sci. 75:2857.

Hunter, R.H.F. 1988. Transport of Gametes, Selection of Spermatozoa and Gamete Lifespans in the Female Tract. In: The Fallopian Tubes: Their Role in Fertility and Infertility, pp 53-80. Springer-Verlag, Berlin.

Hytell, P., H. Callesen, and T. Greve. 1986. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. J. Reprod. Fertil. 76:645.

Hytell, P., and I. Madsen. 1987. Rapid method to prepare mammalian oocytes and embryos for transmission electron microscopy. Acta Anat. 129:12.

Hytell, P., T. Greve, and H. Callesen. 1988. Ultrastructure of in-vivo fertilization in superovulated cattle. J. Reprod. Fertil. 82:1.

Janisch, R. 1974. Oriented embedding of single-cell organisms. Stain Technol. 49:157.

Kruip, T.A.M., D.G. Cran, T.H. van Beneden, and S.J. Dieleman. 1983. Structural changes in bovine oocytes during final maturation in vivo. Gamete Res. 8:29.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain for electron microscopy. J. Cell Biol. 17:208.

Saacke R.G., J. Bame, C.J. Vogler, S. Nadir, and J. Mullins. 1992. Association of sperm nuclear vacuoles (craters) with failure of sperm to sustain embryonic development after fertilization in cattle. J. Anim. Sci. 70:256(abstr.).

Saacke, R.G., S. Nadir, J. Dalton, J. Bame, J. M. DeJarnette, S. Degelos, and R.L. Nebel. 1994. Accessory sperm evaluation and bull fertility: An update. In: Proc. XV Tech. Conf. Artif. Insem. and Reprod. pp. 57 - 67. NAAB, Columbia, MO.

Schiewe, M.C., C.R. Looney, C.A. Johnson, K.G. Hill and R.A. Godke. 1987. Transferable embryo recovery rates following different insemination schedules in superovulated beef cattle. Theriogenology 28:395.

Seidel, G.E., R.P. Elsden, L.D. Nelson, and J.F. Hasler. 1978. Methods of ovum recovery and factors affecting fertilization of superovulated bovine ova. In: Control of Reproduction in the Cow, Ed. J.R. Sreenan, pp. 268-280. M. Nijhoff, The Hague.

Senger, P.L., and R.G. Saacke. 1970. Unique mitochondria in the bovine oocyte. J. Cell Biol. 46:405.

Shea, B.F. 1981. Evaluating the bovine embryo. Theriogenology. 15:31.

Stevenson, J.S., G.C. Lamb, Y. Kobayashi, and D.P. Hoffman. 1998. Luteolysis during two stages of the estrous cycle: Subsequent endocrine profiles associated with radiotelemetrically detected estrus in heifers. J. Dairy Sci. 81:2897.

Thibault, C. 1967. Analyse comparee de la recondation et de ses anomalies chez la brebis, la vache et la lapine. Ann. Biol. Anim., Biochim., Biophys. 7:5.

Wolfe, D.F., M.G. Riddell, P.W. Mysinger, D.A. Stringfellow, R.L. Carson, and P.D. Garrett. 1990. A caudal flank approach for the collection of oviductal-stage bovine embryos. Theriogenology 34:167.

Yadav M.C., J.S. Walton, and K.E. Leslie. 1986. Timing of the onset and duration of ovulation in superovulated beef heifers. Theriogenology 26:509.

Appendix A

Double-boiler protocol for heating cream beyond pasteurization

1. Fill a large beaker approximately 1/3 full with water.
2. Pour cream into a small beaker.
3. Place small beaker into large beaker.
4. Place beakers "double boiler" on a hot plate.
5. Monitor the temperature of the cream with a thermometer.
6. Maintain the temperature at 95°C for 10 minutes.
7. Remove the small beaker from the large beaker.
8. Turn the hot plate off.
9. Place the small beaker in cold water to bring temperature down to 25°C.
10. Remove the top layer with a glass rod or spoon.
11. Obtain pH and osmolarity readings from a sample of the cream.
12. Cream is now ready for use as an extender component or as a supplement to extended semen.

Appendix B

Specimen processing protocol for electron microscopy

Fix the ova in 3% glutaraldehyde in 0.1 M phosphate buffer at 0°C ....1 h

Wash the ova three times in 0.1 M phosphate buffer .... 10 min each

Wash the ova in fresh 0.1 M phosphate buffer at 4°C .... overnight

Pre-embed the ova in 2% agar.

Wash the ova twice in 0.1 M phosphate buffer .... 10 min each

Post-fix in 2% osmium tetroxide at 0°C ....1 h

Wash the ova three times in fresh 0.1 M phosphate buffer .... 10 min each

Wash the ova twice in deionized water .... 10 min each

Stain the ova en bloc with 2% uranyl acetate ....1 h

Wash the ova twice in deionized water .... 10 min each

Dehydrate the ova in 50, 70, 95, 100 and 100% ethanol .... 10 min each

Transfer the ova to a 1:1 mixture of propylene oxide and Spurr resin ....10 min

Transfer the ova to fresh 100% Spurr resin and hold at room temperature to allow for infiltration .... overnight

Transfer the ova to BEEM® capsules with fresh 100% Spurr resin and polymerize at 70°C .... min.16 h

Obtain ultrathin sections with a diamond knife and an ultramicrotome.

Pick sections up on uncoated copper grids and stain with 2% uranyl acetate and lead citrate.

Examine sections in a transmission electron microscope.

Appendix C

Solutions

A. Phosphate buffer

1. Solution A: (0.2 M) (pH ~4.4; ~340 mOsm)
    a. 2.78 g monobasic sodium phosphate
    b. Deionized water to make 100 mL
2. Solution B: (0.2 M) (pH~8.9; ~380 mOsm)
    a. 2.84 g dibasic sodium phosphate
    b. Deionized water to make 100 mL
3. 0.1 M phosphate buffer:(pH 7.2 to 7.4; ~200 mOsm)
    a. 23 ml stock solution A (0.2 M)
    b. 77 ml stock solution B (0.2 M)
    c. 1 ml 1% $CaCl_2$ solution
    d. Deionized water to make 200 ml

B. 3% glutaraldehyde in 0.1 M phosphate buffer (pH ~7.3; ~560 mOsm)

1. 10 ml glutaraldehyde (25% in water)
2. 73 ml 0.1 M phosphate buffer
3. NOTE: If necessary, adjust the osmolarity of the 0.1 M sodium phosphate buffer to equal the osmolarity of the fixative, according to the following formula:

$$\frac{\text{mOsm Fixative - mOsm Buffer}}{30} = \text{\% sucrose needed}$$

C. 1% calcium chloride ($CaCl_2$) (pH ~8.0; ~200 mOsm)

1. 1g $CaCl_2$ (anhydrous)
2. Deionized water to make 100 mL

D. 2% agar

1. 2g agar
2. Deionized water to make 100 mL

Appendix D

Pre-embedding ova in 2% agar

With the aid of a stereomicroscope, and following primary fixation in 3% glutaraldehyde and washes in fresh buffer:

1. Add one drop of agar (40 - 50°C) on a clean glass slide.
2. Let the agar solidify.
3. Place the ovum on the agar drop.
4. Place one drop of agar (40 - 50°C) on top of the ovum.
5. Let the agar solidify.
6. Cut out a cube of agar approximately 2 $\times$ 2 $\times$ 2 mm or smaller containing the ovum.
7. Place a small piece of paper on the slide, pointing towards the ovum.
8. Add one drop of agar (40 - 50°C) on top of the paper and agar cube containing the ovum.
9. Let the agar solidify.
10. Trim away excess agar.
11. Pick agar cube up by the attached piece of paper and continue with processing regimen.

VITA

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The author was born 24 April 1963 in Inglewood, California to Frederick John Dalton and Irma Dalton de Balderrama. The author grew up in Inglewood and Downey, California where he attended St. Jerome's Catholic Grade School and Our Lady of Perpetual Help Catholic Grade School. He completed college preparatory work at St. John Bosco High School in 1981. The author has a bachelor's degree from Cal Poly, San Luis Obispo in Dairy Science (1986) and a master's degree from Utah State University in Dairy Science (1990). This work represents partial fulfillment of the requirements for the Ph. D. degree in Animal Science from Virginia Tech to be awarded May 1999. The author married the former Nell Johnson in June 1989 at Mission San Luis Obispo de Tolosa. The author has a brother, Frederick John Dalton, who lives with his wife and family in San Jose, California, and a sister Rita Dalton Baumann who lives with her husband and family in Visalia, California.