ULTRASOUND-GUIDED TRANSVAGINAL FOLLICULAR ASPIRATION TO PROVIDE A SOURCE OF BOVINE OOCYTES FOR GENE MICROINJECTION

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ABSTRACT

Three experiments were conducted to determine an efficient method of obtaining oocytes from cows via ultrasound-guided transvaginal follicular aspiration. Transvaginal oocyte recovery combined with in vitro maturation, fertilization, and culture (IVM/IVF/IVC) can produce pre-implantation stage bovine embryos and also supply a source of ova for gene microinjection. In Experiment 1, once- (1X) vs. twice-weekly (2X) oocyte recovery frequencies were compared. No differences in recovery per session were observed (1X = 6.8 vs. 2X = 6.3 oocytes/session; $p > 0.10$). However, the 2X group generated more oocytes on a weekly basis (1X = 6.8 vs. 2X = 12.6 oocytes/week; $p < 0.05$). In Experiment 2, in vitro embryo production was compared among the groups aspirated once-weekly (1X), twice-weekly (2X), and twice-weekly after receiving 15 mg FSH (2XF). No differences existed among the aspiration groups in the proportion of blastocysts produced following IVM/IVF/IVC (1X = 23.1%, 2X = 26.1%, 2XF = 18.0%, % viable). However, the 2X group generated more oocytes and embryos throughout the experiment (2X = 83/318, 2XF = 38/211, 1X = 58/251 viable blastocyst/total oocytes; $p < 0.05$) than the other groups. A higher proportion of 2X or 2XF generated embryos were of excellent quality (2X = 60.2%, 2XF = 60.5%) compared to the 1X group (37.9%)

In Experiment 3, in vitro embryo development rates were compared among oocytes from follicular aspiration (TVFA) and oocytes derived from slaughterhouse ovaries (SHD). Oocytes (65%) from both sources were subjected to pronuclear-microinjection of foreign DNA while the remainder served as non-injected controls. Only control oocytes differed, with TVFA-derived oocytes developing to blastocyst more successfully than SHD oocytes (40.8% vs. 30.0%; $p < 0.05$). Microinjected embryo development was similar between the groups (TVFA = 15.9%, vs. SHD = 12.8% viable blastocyst/total;) with the TVFA oocytes holding a slight but non-significant numerical advantage ($p > 0.10$) In these experiments, twice-weekly follicular aspiration without exogenous FSH, was the best scheme of oocyte recovery. This program provided a source of consistent, high quality oocytes that responded favorable to the IVM/IVF/IVC system and microinjection.

Last, but certainly not least, I wish to recognize Dr. Rebecca Krisher for her undying friendship. She deserves most of the credit, or blame, for educating me in the ways of scientific research. I look forward to many years with Rebecca as my wife and colleague. I really consider myself fortunate to be associated with someone with whom I share so many interests. Between the two of us there is very little we cannot accomplish.

I feel I must also offer a thank you to my fellow students in Dairy Science. I was accepted immediately by them, even though I have only a basic concept of the dairy industry. I appreciate the patience and togetherness of all the students in this department, and I can only hope that I will find this "bonding" in future institutions that I am associated with. I think that the adhesiveness that the students here have for each other is probably the strongest, and certainly the most memorable, aspect of my education at Virginia Tech.

Before I get off my soapbox, I must also acknowledge my parents for their patience and advice throughout my graduate work. Their continuing support and friendship are certainly necessary ingredients in a successful pursuit of life. I know they don't always understand my reasoning for continuing in this path, but I appreciate the latitude and help that they have administered to aid in the search for my goals.

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Chapter I

INTRODUCTION

The most popular method of producing animals that carry foreign genes is to insert the DNA during the early life of the embryo. Producing transgenics that are capable of producing a protein or hormone that they would not normally is very inefficient, primarily due to the microinjection of DNA. Typically, the gene construct is microinjected into the pronucleus shortly after the embryo is fertilized. If the genomic DNA of the embryo is disrupted and the foreign DNA can become incorporated, this early insertion may help to eliminate mosaics as each subsequent cell replication should propagate this "new" DNA. If each cell of the developing embryo contains the proper compliment of foreign DNA then the resulting animal should posses the transgene and have the capabilities to produce the protein encoded for by the microinjected gene construct.

The gene constructs used in the following experiments are directed by a mammary-specific promoter. If the DNA is incorporated and the promoter is successful in directing activity to the developing mammary gland, then the animal should "express", or produce the protein encoded for by the construct, in the milk. The mammary gland is competent to produce many types of proteins and if the genome of the animal has been properly altered then the production of this foreign protein will not be distinguishable from normal milk proteins. Because of reasons not entirely understood, some transgenic animals produce different amounts of the new protein than do others. There is probably

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a certain genetic component involved with the different expression levels among transgenics. The amount of milk that an animal can produce is certainly controlled to a degree by the genetics of the animal. The level of expression of complex proteins and the amount of milk that can be harvested are two key factors in producing appreciable amounts of desirable, engineered, mammary-specific proteins and both can be predicted providing the identity of the genetic donor are known.

Microinjection at an early stage (15 h post fertilization) of development requires that the time of fertilization be known. This is most easily controlled by producing the embryos in vitro. Embryos can be surgically recovered from donors (of known genetics) and microinjected at the proper time relative to the putative time of fertilization. However, the in vivo maturation, ovulation and fertilization events can be greatly variable among donors. Also, the number of surgical recoveries from a single donor are limited due trauma. In vitro maturation, fertilization, and embryo culture seem to be the method of choice for microinjection of DNA and transgenic embryo production, providing a source of oocytes is readily available.

Recovering immature oocytes from cows at slaughter can provide many oocytes for entry into the in vitro fertilization system. Due to the inefficiencies of the multi-step process of producing transgenic embryos, many oocytes are necessary. However, with slaughterhouse material the identity of the donor is normally lost. Even if the donor's identity could be maintained, the slaughter process is very rigorous (not to mention

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terminal) and physiological changes at the time of slaughter may effect the quality and competency of the oocytes. The genetic component would be lost for any future oocyte collections. Although microinjection of slaughterhouse-derived ova is the most common method of producing transgenic cattle, it may not be the best source. The inefficiencies of the entire process prevents much research in the area of genetic or health status effects (from oocyte donors) on microinjected embryos. It seems obvious that a system that allows repeated removal of known genetic material, in the form of an immature oocyte, from a healthy donor may be advantageous for decreasing the inefficiencies associated with producing transgenic calves.

The overall goal for the following experiments was to develop a system that allows for the repeated removal of oocytes from donor cows. This involves the use of an ultrasound-guided transvaginal follicular aspiration procedure. This procedure has gained much popularity in the commercial field as a method to access genetics from "problem" cows, or cows that are genetically superior, but have some acquired reproductive inabilities. Follicular aspiration first gained acceptance in the human field and the technology has gradually been adopted as an alternate method of bovine reproduction. The procedure is conceptually simple but does require a great deal of practice to become an accomplished technician. The follicular aspiration and in vitro embryo production system is very labor intensive and this factor had to be evaluated when formulating the experimental design.

Harvesting oocytes from a donor cow while visualizing the ovaries and follicles with ultrasound provides a source of oocytes that are potentially at a similar stage of development. Comparing oocyte recovery rates between once- versus twice-weekly recovery frequencies was the goal of the initial experiment. The second experiment was designed to improve on the recovery rates from the first experiment by the administration of FSH to the cows aspirated twice-weekly. This experiment contained two twice-weekly aspiration groups (FSH and control) and a once-weekly group to link the two experiments. Developmental rates to the blastocyst stage were explored in the second experiment to determine if the more synchronous population of follicles and oocytes (twice-weekly) would respond differently to in vitro fertilization and culture than the once-weekly group. In the third experiment development rates from oocytes harvested through follicular aspiration were directly compared to the development rates of oocytes recovered from slaughtered cattle. The addition of the DNA microinjected groups to both sources of oocytes allowed for the investigation of the effects that microinjection had on oocytes recovered from cows of known genetics and health status as well as slaughterhouse-derived ova.

Chapter II

LITERATURE REVIEW

Animal biotechnology has radically changed in the past decade (34). The advances being made in DNA manipulation since the beginning of the 1990's have dramatically increased the potential for application of mammalian biotechnology. The genetic engineering capabilities that scientist have only fantasized about in the past are now becoming commonplace in the effort to aid humankind (34) as well as impact the animal livestock industry (106). Although challenged by animal rights activists, the advances in animal biotechnology may be the key to unlocking some of the mysteries of mammalian biology and certainly the salvation to many endangered species (27). The most intensively investigated area of genetic manipulation in mammals is the study of transgenics (24).

Overview of Transgenics

The microinjection of foreign DNA into the pronuclei of zygotes is the most common (but not the only) method of producing transgenic offspring (72,79,89). This technology first appeared in the literature with the production of transgenic mice in the early 1980's (32). Other methods of initiating foreign DNA integration such as embryonic stem cell, and retroviral transfection have been explored but are not as efficient as microinjection, especially in livestock species (73,92). The mechanisms involved for

successful production of animals that express the protein or hormone encoded for by the injected gene construct are not widely understood. It is known that the gene sequence must be integrated into the genomic DNA of the zygote in order for the expression event to be noticed (10). The site of integration is apparently random (10,79,89) and appears to be at a single rather than at multiple chromosomal sights (10). With every cell division the modified DNA replicates and the individual cannot distinguish the engineered gene sequence form it's own (89). Therefore, if the sequence is integrated into the proper site the animal will produce the encoded protein as if it were under inherent control of the original genome. Promoter regions can be attached to the construct to attempt to direct expression to a certain target region such as the mammary gland or muscle tissue (76,99).

Producing animals that express a foreign protein (especially a human protein) in their milk have enormous financial potential (42). These "bioproducers" have the capability to produce large volumes of therapeutic proteins that can be harvested from the milk and administered to humans (42). Because of the complexities of the human proteins being produced, there are very few sources (aside from other humans) that can duplicate these proteins. Post-translational glycosylations and tertiary folding and binding modifications are very complicated and prevent microbacteria from making these proteins. The mammary directed gene constructs are used for two reasons. The mammary gland has the inherent capabilities to produce very complex proteins (42,103) and the exploitation of the massive volumes of milk produced by livestock allows even low

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expression levels to yield an appreciable amount of protein (103). If the foreign integration site is appropriate, the animal may have germ-line transmission of the transgene to the subsequent offspring (73). The bovine model is considered to be the best for these reasons but, unfortunately is the least understood in terms of genetic modification (80).

Transgenics in the Bovine

Because of the complexity of the livestock species, the expression levels and integration events experienced with the mouse as a model cannot be readily duplicated (76). Transgenic mouse pups can be produced at an efficiency of 10 to 30% of the total live-born (15,105) with an integration frequency of 18 to 25% (13). The efficiencies in the larger animals (cows, pigs, and sheep) are considerably lower (4 to 10%; 79,89). In fact, considering the overall efficiency of the multi-step process of in vitro maturation, fertilization, microinjection, embryo culture, and transfer to create transgenics, the efficiencies range from .02% to .2% transgenic individuals from the total number of oocytes at the onset (11,12,38). Several putative transgenic pregnancies (67,91), some fetuses (9,11), and only a few actual transgenic calves (38,44) have been produced, suggesting that there is much about the integration events and subsequent embryo and fetal development to term that is unknown.

In Vitro Maturation and Fertilization

Recent advances in technology have increased the potential for producing

transgenic livestock entirely in vitro. Although not as efficiently as with the in vivo instance, bovine oocytes maintain the potential to mature when removed from the follicles (6,22). The medium that the oocytes are matured in is paramount for the oocyte to have the competence to undergo fertilization (33). Supplementation of the medium with exogenous hormones typically found in the follicular fluid (FSH, LH, and E_2; Estrogen) seems to be the most advantageous method of creating an environment suitable for maturation. There also is a relationship between the oocyte and the cumulus cells that surround the oocyte. This relationship can be intimate when the cumulus oocyte complex is intact (16,94) as in the natural case, but there also is evidence that the oocyte and cells can be separated from each other without hindering oocyte maturation, provided the cells are cultured in the same vessel as the maturing oocyte (36). This suggests that a communication event from the cumulus cells to the oocyte (or vice-versa) can be mediated through a synthetic environment (36). In either instance it is obvious that the presence of cumulus cells is important for the oocyte to maintain the meiotic competence for fertilization (87).

The coincubation of mature oocytes and capacitated sperm cells can produce fertilized ova. There are many factors that must be controlled in order to achieve maximum normal sperm penetration without multiple sperm penetration or polyspermy. Heparin added to the medium was shown to be very effective to facilitate capacitation (66). The presence of a protein substrate in the fertilization medium is important to decrease the incidence of polyspermy. The addition of fetal calf serum has been shown

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to be especially advantageous for normal sperm penetration (90%) while decreasing the high incidence of polyspermy (72%) observed with BSA as the protein substrate (94). Other agents (such as gonadotropins) have been added to the fertilization medium to promote normal fertilization, but have not been as effective as calf serum (29). Considering the theory that ovulation is an inflammatory response in vivo (23), the addition of anti-inflammatory drugs has been examined. Phenylbutazone (PB) and Flunixin-Meglumin (FM) have been tested in in vitro maturation and fertilization (47). Oocytes matured at a normal rate (90 to 95%) with low (<100μg/ml) doses of PB but a negative threshold was reached at higher levels. The FM had deleterious effects on maturation and fertilization at all doses (47). A penicillamine, hypotaurine, and epinephrine (PHE) mixture seems to yield the best normal fertilization rates (>70%) without high incidence of polyspermy, and has become the method of choice for fertilization additives (60,66).

Microinjection

Microinjection of DNA into the ova has been examined at several stages of development. To insure that the DNA is present during the first genomic replication, constructs have been injected into the germinal vesicle of the oocyte (45). Although some embryos have developed from this procedure, DNA injection after fertilization was shown to be more advantageous (38). Microinjection into the pronuclei of mouse embryos has readily produced transgenics (15). However, the bovine zygote does not act the same during the injection process (30). In order to assume multiple copies of the construct are

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deposited during pronuclear swelling, the pronuclear membrane must be visible (57). This can be accomplished by centrifugation of the zygote to displace the opaque lipid material (57). This process alone does not hinder further development to the morula or blastocyst stage (30). Microinjection of DNA is the single-most deleterious event in the production of transgenics (105). Immediate ova survival following microinjection can be as high as 80% (57), but subsequent in vivo (57) and in vitro (46) development are effected. In vitro development of 5 to 15% of microinjected embryos to the blastocyst stage is common (30,45,46,91).

Embryo Culture

In vitro embryo development (noninjected) is perhaps the most intensively investigated area in bovine embryo technology. Many different culture systems have been used to mimic the conditions found during the first 7 days of pregnancy in the bovine uterus. Simple defined medium with only salts plus fatty-acid free BSA supplementation was reported to support development to the blastocyst stage. In these experiments (81) the addition of energy substrates such as pyruvate or lactate actually decreased development in a dose dependent manner form 34% (control) to as little as 5.5% (lactate; 5mM) and 3.0% (pyruvate; 2.5mM). To more closely mimic early culture conditions in the oviduct, bovine oviductal epithelial cells (BOEC) have been used to produce a monolayer to culture embryos on (2). Development was enhanced using this culture system (39% blastocyst) compared to cumulus cell coculture (19.5%;2). The cumulus cell coculture system does have advantages in terms of labor intensity by using the cumulus

cells removed from the embryos following IVF (108). Regardless of media supplementation, the cumulus-oocyte interaction prior to and during culture is important to achieve normal development rates. Denuded oocytes not exposed to cumulus cells during culture were shown to produce blastocysts at a low rate (denuded = 3.1% vs. intact 37.0%; 96). Conditioning of culture medium by exposure to BOEC was effective in promoting enhanced development to the 8-cell stage (37) in both CZB and TCM-199 medium. Development to this stage, however, may not be an accurate measure of an embryo culture medium. Trophoblastic vesicle cells also have been examined as a coculture cell type, and while enhancing development over medium alone, these cell types may not be as beneficial as buffalo rat liver (BRL) cells in providing an appropriate coculture environment (95). The BRL cell line has been intensively investigated and as a coculture component can support development of transferable-quality embryos with repeatable and consistent results (24% to 30%; 35). Additionally, embryos produced on a BRL monolayer were shown to be less susceptible to freeze/thaw damage (100).

Growth factors such as transforming growth factor (TGF; 107), epidermal growth factor (EGF; 65), platelet derived growth factor and insulin-like growth factor-one (PDGF and IGF-1, respectively; 26) have enhanced embryo development rates. However, these results are often unreproducible. Certain growth factors may enhance development over the base media alone, but the specific mechanisms and biochemical events involved are not entirely understood. These factors may activate amino acids already existing in the culture medium and present them in a useable form to the developing embryo. This

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seems feasible as the addition of glycine (10mM) and alanine (1mM) to culture medium has increased embryo development rates from 24% to 42% in the absence of coculture cells (62). The majority of this literature provides data obtained using oocytes from slaughterhouse cattle. It is possible that oocytes recovered from live cows may be of higher quality and may respond even more favorably to growth factor supplementation.

Ultrasound-guided Transvaginal Follicular Aspiration

Many different schemes have been used in an attempt to remove oocytes from live cows. The concept of harvesting follicles of known genetic origin and performing in vitro maturation and fertilization to a desirable bull could potentially change the way genetically superior embryo donors are selected. Follicular aspiration combined with in vitro embryo production could be a major breakthrough in bovine embryology as well as biotechnology (34).

Prior to ultasound-assisted aspiration technology, oocytes were harvested from cows by means of laparoscopy. This procedure involved an incision in the paralumbar fossa region large enough (>2cm) for insertion of an endoscope (90). The operator observed the ovaries through the objective located in the endoscope with light provided by fiberoptics. The ovaries were manipulated with either a pair of long forceps, a blunt-end stylet, or both (51). When the ovaries were manipulated into the proper position, the follicles were punctured and the follicular fluid removed. This procedure was very efficient in recovering oocytes from Holstein heifers with recovery rates around 7.3

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oocytes per cow per session (90). The animals in these experiments were administered FSH to promote follicle growth prior to laparoscopy. They seemed to respond better to the FSH than PMSG, as the recovery rates decreased dramatically when PMSG was used (3.6 oocytes per cow per session; 90). Other research has shown that the morphological appearance of oocytes recovered with this method was very good. Recovering in vivo matured oocytes approximately 25 h after administration of hCG has yielded about 7 oocytes per cow that had expanded cumulus (67% of total oocytes) while 24% or 2.5 oocytes per cow had only the corona radiata or compact cumulus cells. The remaining 9% of the oocytes were without cumulus cells (51). Since the time of ovulation cannot be easily predicted, aspiration of in vivo matured oocytes may not be a reliable source of ova for microinjection. Although, this procedure was shown to produce fertilizable oocytes, the problems associated with an invasive, surgical approach to aspiration may be inhibitory on the reproducability of these results with the same donors. Adhesions and inter-experiment variability, combined with large laparoscope technician variability seemed to be the largest negative effects from this research (51,90). Also, because of the nature of the procedure, repeated recoveries were usually limited to 3 to 5 per donor and suggested recovery times between surgeries seemed to lead to inefficiency (90).

A procedure similar to the laparoscopy method has been used to generate oocytes from young (3 wk) calves (3). These oocytes were competent to undergo fertilization and subsequent embryo culture, but the development rates were lower than oocytes derived from mature cows (calves = 16% vs cows = 22% culture to the blastocyst stage; 64).

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These development percentages have reached as high as 30% to the blastocyst in some laboratories with proper pre-aspiration hormone priming to create a synthetic estrus cycle (40,41). If the goal is to decrease the generation interval these development percentages may be acceptable. The long-term effects on the calves is not entirely understood. However, limited research has provided the unique ability of "mothers" and calves to have concomitant first lactations (3).

Increasing popularity of in vitro technology has provided financial support for the use of ultrasound as a tool for human oocyte recovery. This procedure first appeared in the literature in the early 1970's (93) and has gained approval in many in vitro fertilization clinics. Oocyte aspiration in humans was first performed with endoscope technology, and the ultrasound method was shown to be only slightly more advantageous for oocyte recovery (25). However, the actual procedure is much less traumatic when the ultrasound is substituted for endoscopy (52). An ultrasound-guided transvaginal aspiration route is effective in women, However, if the ovaries cannot be accessed, a laparoscopy also may be performed (18). Other problems associated with oocyte aspiration in women include mishaps in the puncturing plane because of ultrasound malfunction or misalignment of the transducer and needle guide (18). Despite these problems, oocyte recovery rates are usually quiet high on hormone-treated women donors. Lewin and coworkers (53) have reported recovery rates of 7 oocytes per patient. The fate of these oocytes is not favorable as pregnancy rates range from 20 to 25%. The patients serve as both donors and recipients in most cases, and the women have had histories of infertility which could lead

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to the reduced pregnancy rates (53).

In an attempt to eliminate the trauma associated with the surgical procedure involved in bovine laparoscopy, alterations have been made in the placement of the aspiration device (74,75). With the aid of an external monitor, and by inserting the trochar through the vagina, ovaries and follicles can be visualized. This procedure was efficient in a once- or twice-weekly aspiration schedule without long-term damage to the tissue (74,75). Since the oocyte recovery rates per session were not significantly different (5.2 vs. 6.1 oocytes/session), the weekly rates were higher in the twice-weekly group (once-weekly = 5.2 vs twice-weekly = 12.2; 74,75). The twice-weekly method allows more frequent aspiration without the added expense associated with ultrasound. The images seen on the monitor are apparently high quality since the ovary is visualized directly. The results using this method are readily repeatable and the recovery rates (expressed as oocytes/visible follicles) are high (60-70%; 74,75). The ability of these donors to maintain normal reproductive function has yet to be explored.

Another method of recovering oocytes that has been examined involves a finger tip ultrasound transducer that is packaged in a vaginal probe. The ovaries are visualized with ultrasound and the follicles punctured and contents removed into a permanently rinsed tubing device (88). This needle and tubing system was designed to eliminate the "dead space" in the long needles. However, the short needle and finger tip probe are difficult to manipulate possible affecting recovery (88). A shorter interval between

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aspiration sessions (48h vs. 96h) was shown to generate more oocytes but the morphological quality of the oocytes was jeopardized (88).

Ultrasound technology was introduce into the animal industry as a pregnancy diagnostic tool, primarily in horses (43). Since that time (late 1970's), the uses of ultrasound have increased dramatically. Ultrasound is readily used today to monitor reproductive physiology of cattle (7), horses (43), and sheep (86). As the field of ultrasound grows, so does the application, and real-time ultrasound has been used very efficiently in dolphins (104), monkeys (84), deer (77,78),and even turtles (82). As a tool to determine the sex of the developing fetus in the bovine, ultrasound was found to be very accurate (~97%; 20,21). A technique also has been developed to determine if the site of semen deposition during artificial insemination is appropriate (7). Perhaps the most advanced use of ultrasound is to monitor bovine ovaries during follicular aspiration.

Bovine ultrasound-guided transvaginal follicular aspiration was first reported in the late 1980's (69). Since that time, largely with the financial support of industry (35,55), use of this technique has become widespread. Initial oocyte recovery results from small follicles (3 -5mm) were poor (13%). However, oocyte recovery rates were higher (66%; 69) from larger follicles (15mm) possibly, because they were easier to transect. The cumulus oocyte complexes (COC) recovered were competent to be matured, fertilized, and cultured in vitro to produce pre-implantation stage bovine embryos (48,49,50,97,98). The cumulus-oocyte interaction was important for continued development, since the COCs

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with little or no cumulus cells developed at a lower rate (15.3 vs 24% blastocyst) than the COCs with a healthy cumulus cell investment (48,49). Donor variation was also important, as the cows that consistently produced more oocytes typically had a higher rate of development to blastocyst (98). van der Schans et. al. (98), consistently harvested as many as 26 oocytes per session from certain donors and had development rates of 43% to the blastocyst. This research has been difficult to repeat with or without exogenous follicle stimulation, in recent literature.

Many exogenous agents have been used to promote follicle development for aspiration. Pregnant mare's serum gonadotropin (5), FSH (55,56), vaccination against inhibin (28), and ECG (68,70,71), had no major effects on the recovery rates in a once-weekly aspiration schedule. A more frequent (twice-weekly) scheme was suggested (102) to maintain a synchrony among the cohort of developing follicles. Although recovery rates per session were not shown to be enhanced by the more aggressive system, an accelerated endogenous follicle stimulation was noticed throughout the experiment (102). Whether this superstimulation is inherent to the frequent aspirations or an increased sensitivity to exogenous gonadotropins has not been elucidated (31,70,102). Harvesting oocytes at times relative to the LH surge has been examined in ECG-treated donors, and recovery rates were higher (23,4% vs. 47%) 22 h after the LH peak compared to 12 h after the LH surge (101). This research involved an initial recovery session approximately 30 h prior to the LH surge in both groups. These results appear to indicate that the LH surge alone does not promote sufficient follicle growth. It was hypothesized that the post

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ovulatory surge of FSH may be implicated in promoting subsequent follicle growth throughout the estrus cycle (101). With aspiration occurring only 22 h after the LH surge, the post-ovulatory FSH rise was not experienced prior to aspiration. Initiating aspiration sessions without regard to the stage of the estrus cycle was examined and shown to have no effect on the cyclicity of the donors (68,71). This observation was only evident in the once-weekly aspiration cows.

More recently, embryo development from aspirated oocytes has been consistent (18%; 50) and the concept of replacing conventional embryo transfer with follicular aspiration has been suggested (14,48,50). In the commercial aspects of oocyte aspiration, recovery and development rates are highly variable primarily because "problem" cows are used as donors (55,56). These cows are those which have valuable genetics, but have some acquired reproductive problems affecting their ability to produce embryos naturally or to maintain a pregnancy (35,55,56).

The combination of aspiration technology and conventional embryo transfer has been intensively investigated recently. Two major ideas are at work to reach a common goal. The concept of the dominant follicle having inhibitory actions on subordinant follicles (4,39,85) has prompted the use of aspiration to eliminate the dominant follicle prior to a typical FSH superstimulation (54). By removing the largest follicle (or all follicles) the FSH response may be more uniform and increase the number of transferable embryos at the time of collection (54). This theory is supported by data showing that

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previously-aspirated animals have a higher degree of ovulation synchrony following prostaglandin administration than do non-aspirated individuals (8). Another approach involves intensive aspiration (twice-weekly) in an attempt to cause the donor to have multiple ovulations and multiple embryos (1). Without exogenous FSH, this system does not seem to produce as many ovulations or embryos as a conventional embryo transfer procedure. Only 2 to 3 ovulations per animal have been observed after long-term, twice-weekly ablations (1). If transferable quality embryos are recovered as a result of these ovulations, this method has an economical advantage over the exogenous FSH administration system. Also, since no exogenous FSH is administered, the possibilities of overstimulation or reproductive damage as a result of the ovarian trauma of multiple ovulation, are reduced. Both of these systems have potential to make more efficient use of these donor cows.

Other efforts have concentrated on increasing the efficiency of gestating animals. Hormone (FSH) treatment during pregnancy can increase follicle number and potentially the availability of oocytes for IVM/IVF (83). Pregnant cows generated more oocytes per ovary than nonpregnant cows (63). Specifically, the ovary ipsilateral to the gravid uterine horn (with a CL), had significantly more oocytes than the ovary with a CL in non-pregnant cows (pregnant = 11.7 vs. open = 7.7). The oocytes from pregnant cows were shown to have higher morphological quality than oocytes from open cows (26 and 33%, respectively; 63). The increased response to FSH and high quality oocytes of pregnant cows compared to non-pregnant cows, has piloted efforts to increase the embryo

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productivity of pregnant cows (59).

In addition to the ovarian dynamics and embryo production research in the bovine, ultrasound-guided follicular aspiration has been used to recover oocytes from horses. Ultrasound technology has been slowly adopted by the equine industry, possibly due to lack of commercial support. In vitro maturation and fertilization in the equine recently has gained acceptance and the oocyte recovery methods are rapidly being pursued (17). Recent experiments have suggested that oocyte recovery from mares is more efficient during estrus compared to diestrus (19). Those mares aspirated during estrus or during estrus and diestrus had a decrease in the number of large follicles (10 to 20 mm) compared to control mares that were not aspirated (19). This would seem to indicate that a more aggressive approach to aspiration in the mare may be inhibitory to large follicle development, possibly due to an increase in small and medium follicle synchrony as in the bovine (19). Follicular aspiration during the first 40 days of gestation in the mare has generated a higher number of oocytes than normally recovered from cyclic mares (58). The prolific nature of the ovary to generate follicles and oocytes during pregnancy seems to be similar among cows and horses.

Chapter III

EFFECTS OF ONCE VERSUS TWICE WEEKLY TRANSVAGINAL FOLLICULAR ASPIRATION ON BOVINE OOCYTE RECOVERY AND EMBRYO DEVELOPMENT

ABSTRACT

Ultrasound-guided transvaginal follicular aspiration (TVFA) combined with in vitro maturation/in vitro fertilization (IVM/IVF) and culture was used to obtain bovine preimplantation stage embryos. These experiments evaluated the effects of aspiration frequency on oocyte recovery and embryo development following IVM/IVF. In the first experiment TVFA was performed once (1X; n=5) or twice (2X; n=5) weekly in multiparous Angus cows with the aid of a transvaginal sector transducer (5MHz). In the second experiment Angus cows had TVFA performed once weekly (1X; n=6), twice weekly (2X; n=4), or twice weekly after treatment with FSH (15 mg; 2XF; n=4). Follicles (>2mm) were punctured using a 55-cm needle (17 ga) and oocytes were aspirated through the needle and silastic tubing (2m) by vacuum suction (75mmHg). Oocytes were examined for morphology and placed into the IVM/IVF procedure. Following IVF, all ova were co-cultured in vitro (7 d) on Buffalo Rat liver cells. Oocyte recovery rates per aspiration session for Experiment 1 were not different between 1X and 2X groups (6.8±2.0 vs. 6.3±1.1 oocytes/session; x ± SEM) or between the 1X, 2X, or 2XF (7.7±1.8 vs. 9.5±1.1 vs. 6.2±1.1) in Experiment 2 (p>0.10). In vitro development

to the blastocyst stage was not different between the 1X, 2X, 2XF, or control oocytes obtained from cows at slaughter (23.1 vs. 26.1 vs 18.0 vs 27.9%; p>0.10). Oocytes from the 2X and 2XF aspiration groups generated a higher percentage of grade 1 quality embryos than the 1X group (p<0.05). In commercial bovine oocyte aspiration, more transferable embryos can be generated from 2X aspirations than from once-weekly aspiration.

INTRODUCTION

Laparoscopic follicular oocyte aspiration first gained acceptance as a method of recovering human oocytes in the 1970s (28). Bovine oocyte aspiration using this technique followed in the early 1980s (27). Since the late 1980s, with the aid of commercial interests (21), much research has been conducted to develop a non-surgical method of bovine oocyte recovery from live animals (19). Ultrasound-guided transvaginal follicular aspiration (TVFA) combined with in vitro maturation/in vitro fertilization and in vitro culture (IVM/IVF/IVC) is emerging as a method for producing preimplantation stage bovine embryos that are suitable to transfer and establish pregnancies (8,16). This system may have the potential of replacing conventional superstimulation and embryo transfer as a method to propagate desirable genetics (13).

Oocyte recovery rates associated with TVFA are variable among laboratories and donor cows (24,25). The most popular aspiration schedule is once weekly (19) starting on day 3 to 4 (day 0=estrus) of the estrous cycle, which corresponds with the emergence of the first follicular wave (3). Reported recovery rates range from 2 to 3 oocytes per cow per aspiration session (21) up to 25 oocytes per session (25). Exogenous agents, such as pregnant mare's serum gonadotropin (PMSG; 4,18,24) or FSH (16,29), have been used in an attempt to increase oocyte recovery. Transvaginal follicular aspiration has been performed at all stages of the estrous cycle (24), during the 1st trimester of gestation (17),

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and the possibility of oocyte aspiration from pre-pubertal calves has been explored (2).

In vitro development of oocytes to the blastocyst stage is important for TVFA to be commercially viable. Initial reports indicated that oocytes resulting from TVFA did not develop to the blastocyst stage at a higher rate than oocytes recovered from bovine ovaries at slaughter (24). However, more recent results indicate that the rate of development of oocytes generated from TVFA may be superior to those collected at slaughter (13).

The purpose of the first experiment was to determine a schedule for obtaining oocytes that was efficient, continuous, and not detrimental to the animal. Once a week (1X) versus twice a week (2X) TVFA frequencies were compared to determine if there were differences in oocyte recovery per session and per week. The purpose of Experiment 2 was to examine the effects of exogenous FSH on ova recovery and to compare the developmental competence of aspirated oocytes with that of oocytes collected at slaughter.

MATERIALS AND METHODS

Estrus Synchronization

In Experiment 1, estrus was synchronized in 12 non-lactating, multiparous Angus cows (4 to 10 yr). A PRID remained in place for 7 d. Cows were injected (im) with

PGF$_{2\alpha}$ (Lutalyse®, Upjohn Co., Kalamazoo, MI) at 36 (25 mg) and 24 h (12.5 mg) prior to PRID removal. All cows were examined twice daily for visible signs of estrus. All cows responded and exhibited estrus within 60 h following PRID removal.

In Experiment 2, estrus was synchronized in 22 non-lactating, multiparous Angus cows (2 to 11 yr) using norgestomet ear implants (Sanofi Animal Health, Overland Park, KS). Ear implants were in place for 7 d and PGF$_{2\alpha}$ (25 mg; Lutalyse®) was administered (im) 24 h prior to implant removal. Cows were examined twice daily for signs of estrus for 4 d following implant removal. Eighteen cows exhibited estrus. For both experiments, TVFA was performed on all cows initially between day 3 and 5 of their estrous cycle (day 0=estrus).

Treatment Groups

In Experiment 1, 10 cows were randomly allocated to either the once (1X; n=5) or twice weekly (2X; n=5) TVFA. the TVFA was performed on Mondays for the 1X group and on Sundays and Wednesdays for the 2X group throughout the 5 wk experiment (1X=6 and 2X=10 TVFA sessions). This schedule provided alternating 3 and 4 d intervals between aspiration sessions for the 2X cows. Cows were aspirated in random order during each TVFA session.

Experiment 2 consisted of three TVFA schedules: once weekly (1X; x=6), twice weekly (2X; n=4), and twice weekly following administration of FSH (2XF; n=4). Both

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twice weekly TVFA groups (2X, 2XF) were aspirated in random order on Mondays and Thursdays allowing for a 3 to 4 d interval between sessions as in Experiment 1. The 1X group had TVFA performed on Tuesdays. Experiment 2 was conducted over a 5.5 wk period (1X=6 and 2X or 2XF=11 TVFA sessions).

The 2XF group was administered 2.5 mg FSH-P (Schering-Plough Animal Health; Omaha, NE) twice daily (im) for 3 d prior to aspiration. This schedule resulted in administration of 15 mg FSH-P per cow prior to each TVFA session for the duration of the experiment.

Blood Sampling and Ultrasound Ovarian Examination

In Experiment 1 the ovaries of all cows were examined daily (5) with a linear-array real-time ultrasound unit (5MHz; Corimetrics Medical Supply, Inc., Wallingford, CT). Blood was collected daily (10ml) from the jugular vein, allowed to stand at room temperature for 3 to 5 h and centrifuged for 30 min to separate plasma. Plasma samples were frozen and used for determination of progesterone concentrations (P4) by RIA. RIA was performed by solid-phase RIA kits (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA) as described by Holt et al., (9). The intra- and inter-assay coefficients of variation were 8.7 and 15.2%, respectively.

Ultrasound-Guided Transvaginal Follicular Aspiration

Prior to TVFA all animals were sedated with acepromazine maleate (44mg/100 kg

body weight, iv; Aveco Co., Inc.; Fort Dodge, IA). The vagina was rinsed with sterile saline (.9%; 120ml). The vulva and perianal area were thoroughly cleaned and disinfected. An epidural anesthesia was administered (6ml, 2% Lidocaine Hydrochloride; Phoenix Scientific, Inc.; St. Joseph, MO) to restrict peristaltic movement.

Ovaries were visualized with an Aloka 500V ultrasound machine (Corimetrics Medical Systems, Inc., Wallingford, CT) equipped with a 5-mHz sector transducer packaged in a vaginal probe with a dorsal mounted needle guide (16ga). The ovaries were manipulated per rectum and positioned to align follicles on the ovary into the path of the needle. A 17-ga, 55-cm needle with echogenic tip (RAM Consulting, Madison, WI) was inserted via the needle guide, through the wall of the vagina, and into the ovarian follicles. Silastic tubing (2m) connected the needle to an embryos filter (Professional Embryo Transfer Supply, Canton, TX). A vacuum pump, attached to a rubber stopper tightly sealed on top of the embryo filter, provided 75 to 85 mmHg suction at a flow rate of 27 to 29 ml/min. The vacuum was applied as the tip of the needle pierced the wall of the vagina and remained on until the needle was removed from the peritoneal cavity. Follicular contents were aspirated through the needle, silastic tubing, and into the embryo filter. This procedure was repeated until all visible follicles (>2mm) had been aspirated from the ovary. The needle was withdrawn from the animal periodically and the needle and tubing rinsed with PBS (Gibco, Grand Island, NY) supplemented with 10% vol/vol newborn calf serum (Gibco), 1% vol/vol penicillin-streptomycin (Gibco), and 25 µg/ml heparin (Sigma Chemical, St. Louis, MO). After

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follicles on both ovaries had been punctured and aspirated, the embryo filter was rinsed and the oocytes located using 10X stereo microscopy.

Oocyte Evaluation

All oocytes were scored for morphology after recovery and were classified as either good or poor. Good oocytes were those exhibiting an organized cytoplasm and at least three layers of cumulus cells. The poor oocytes had less than three layers of cumulus cells or a disorganized cytoplasm. Oocytes that had over-expanded cumulus were categorized as good.

In Vitro Maturation, Fertilization, and Culture

After oocytes were collected, they were rinsed three times in a TL HEPES buffered wash media supplemented with BSA (3mg/L; Sigma Chemical). Oocytes from cows in the same treatment group were combined and placed in maturation media of TCM199 (#400-1100 EB, Gibco) supplemented with 10% vol/vol fetal calf serum (FCS; HyClone, Logan, UT) bFSH and bLH (0.01 U/ml each; Nobl Labs, Sioux Center, IA), and 1% vol/vol penicillin-streptomycin (Gibco). Oocytes were maintained for 22 to 24 h at 39°C in 5% CO_2 and air(12).

A Percoll-separation procedure was used to prepare frozen/thawed semen for IVF (12). Frozen 0.5-ml straws of semen from a previously-characterized ejaculate of a single Holstein bull were thawed at 35°C for 1 min. Sperm were layered on top of a Percoll

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(Sigma Chemicals, St. Louis, MO) density gradient (90%:45%) in a 15-ml centrifuge tube and centrifuged for 30 min at 700 x g. After centrifugation live sperm concentration was determined using a hemocytometer. Sperm (20μl) were added to fertilization wells with the washed oocytes to give a final concentration of 1.0×10^6 sperm/ml. Heparin (5 mg/ml) and PHE (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine, 1 mM) were included (12).

After an 18 to 20 h incubation with enriched sperm, all ova were collected, vortexed to remove cumulus cells, and co-cultured on Buffalo Rat Liver (BRL) cells. Medium used for co-culture consisted of TCM199, 10% FCS, 1% BSA, 0.1 mM non-essential amino acids (Gibco), 2 mM glycine, 1 mM alanine (Sigma Chemical), and 1% vol/vol penicillin-streptomycin (Gibco). On day 4 of culture all embryos were moved to fresh culture wells.

Seven d after co-incubation with enriched sperm, all embryos were removed from culture and scored for development. The grade and stage of each embryo was recorded. All embryos were evaluated as follows: grade 1 = very little (<5%) or no extruded material, consistent, organized cell mass and trophoblast with no discolored regions, grade 2 = less than 10% of total cell mass extruded, some disorganization or discoloration, grade 3 = less than 25% of cell mass extruded, considerable disorganization or discoloration. All other embryos were considered non-viable.

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Oocytes Collected at Slaughter

Oocytes collected from cows at slaughter (11) were purchased (Em Tran, Inc. Elizabethtown, PA) and matured as described above, in transit in a temperature-controlled portable incubator (Minitub, Madison, WI). These oocytes were fertilized and cultured in vitro on the same days as the oocytes collected by TVFA. The development rate of slaughterhouse-derived embryos was compared to that of embryos produced from TVFA-derived oocytes.

Ovariectomy

Two cows from each group (1X, 2X) in experiment 1 were ovariectomized via vaginal incision. In each group, 1 cow was ovariectomized 1 d and the other cow 1 wk after the last TVFA session. The vulva and perianal area were cleaned thoroughly and the cows were given an epidural anesthesia (6ml, 2% lidocaine). An incision was made through the dorsal surface of the vagina and the ovaries extended through that incision. A curved blade ecraseur was inserted into the vagina and the ovaries removed. After gross examination the ovaries were fixed in neutral buffered formalin, embedded in paraffin (EM 400 paraffin embedding media, Surgipath Medical Industries, Richmond, IL) and sectioned (5 μm). To aid microscopic evaluation, the sections were stained with Masson's Trichrome as described by Carson (6).

Rebreeding

Twenty cows from both experiments, including representatives from all groups (1X, 2X, 2XF) and 18 control cows that had not been aspirated were injected with $PGF_{2\alpha}$ (25 mg; im) to synchronize estrus. The cows in the aspirated groups were given 1 to 3 months without TVFA prior to estrus synchronization. Cows that exhibited behavioral signs of estrus within 48 to 72 h post $PGF_{2\alpha}$ injection were inseminated at 12 and 24 h after observed estrus using Holstein semen from the same ejaculate as described for IVF. Pregnancy was diagnosed via ultrasound at 30 d post insemination.

Statistical Analysis

Oocyte recovery data were analyzed using a general linear model (GLM) with the model statement including cow, treatment group (1X, 2X, or 2XF), experiment, and error. No effects or interactions involving aspiration technician were found in the preliminary analysis so technician was not included in subsequent analyses. Embryo development and quality data were also analyzed using GLM, with the model including treatment, experiment, and error. Progesterone data were compared by analyzing the area under the curve with a similar GLM model. All analyses were performed with the Statistical Analysis System (SAS, 26). Comparison of oocyte quality and rebreeding data were analyzed using Chi-Square.

RESULTS

Oocyte Recovery

Oocyte recovery rates per session were not different among groups (p>0.10, Table 1). In the second experiment ova recovery increased slightly in both groups, but this increase was not significant (p>0.10). Both twice weekly groups (2X, 2XF) yielded more oocytes ((p<0.01) per week than the 1X groups. Treatment with FSH did not increase (p>0.10) oocyte recovery in Experiment 2.

Table 1. Transvaginal oocyte recovery rates for Angus cows aspirated once weekly (1X), twice weekly (2X) and twice weekly after receiving 15mg FSH (2XF).

ASPIRATION GROUP	EXPERIMENT	MEAN OVA RECOVERY ± SEM	
		OVA/SESSION	OVA/WEEK
1X	1	6.8 ± 2.0^a	6.8 ± 2.0^a
1X	2	7.7 ± 1.8^a	7.7 ± 1.8^a
2X	1	6.3 ± 1.1^a	12.5 ± 2.2^b
2X	2	9.5 ± 1.1^a	19.0 ± 2.2^b
2XF	2	6.2 ± 1.1^a	12.5 ± 2.2^b

[a,b] Values with different superscripts within column differ (p<0.01).

Oocyte Quality

There were no interactions and no differences among treatment groups or between experiments in the proportion of oocytes classified as good quality (p>0.10). The oocyte quality data from both experiments were combined and are in Table 2.

Table 2. Quality of oocytes recovered from Angus cows aspirated once weekly (1X), twice weekly (2X) and twice weekly after receiving FSH (15 mg;2XF).

GROUP	No. GOOD[a]/TOTAL	PERCENT[b]
1X	211/481	43.9
2X	354/770	46.0
2XF	137/299	45.8
TOTALS	702/1550	45.3

[a] Good oocytes are those with at least three layers of cumulus cells and an organized cytoplasm.
[b] There were no differences among treatment groups in the proportion of oocytes classified as good (p>0.10).

Embryo Development

Embryos developing to the compact morula or blastocyst stage on day 7 of in vitro culture were regarded as viable. These embryos were graded and embryo quality was compared among treatment groups. Oocytes recovered in the first experiment were used to establish in vitro maturation and fertilization protocols, and are not reflected in this analysis. Development data from Experiment 2 are in Table 3. Development rates were similar regardless of whether oocytes were generated by TVFA or recovered from the ovaries of cows at slaughter (p>0.10).

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Table 3. Development rates of embryos derived from oocytes recovered from Angus cows aspirated once weekly (1X), twice weekly (2X), twice weekly after receiving 15mg FSH (2XF), or from oocytes recovered from cows at slaughter (SLA).

ASPIRATION GROUP	MORULAE and BLASTOCYSTS	
	VIABLE/TOTAL	PERCENT[a]
1X	58/251	23.1
2X	83/318	26.1
2XF	38/211	18.0
SLA	158/565	27.9

[a] There were no differences in developmental rates among treatments (p>.010).

Embryo Quality

A higher proportion of the embryos derived from twice weekly oocyte aspiration (2X,2XF) was of Grade 1 quality at the time of evaluation (p<0.01; Table 4). Consequently, a lower percentage of the embryos from the 2X cows were of Grade 3, or poor quality (p<0.05). The percentage of Grade 3 embryos from 1X and 2XF cows was not different (p>0.10) and there were no differences among groups in the proportion of Grade 2 embryos (p>0.10).

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Table 4. Comparison of embryo quality from oocytes derived from aspiration of Angus cows once weekly (1X), twice weekly (2X), or twice weekly after receiving 15 mg FSH (2XF).

ASPIRATION GROUP	No. GRADE 1[a]/No. TOTAL EMBRYOS (%)	No. GRADE 2[b]/No. TOTAL EMBRYOS (%)	No. GRADE 3[c]/No. TOTAL EMBRYOS (%)
1X	22/58 (37.9)[d]	22/58 (37.9)[d]	14/58 (24.1)[d]
2X	50/83 (60.2)[e]	26/83 (31.3)[d]	7/83 (8.4)[d,e]
2XF	23/38 (60.5)[e]	9/38 (23.7)[d]	6/38 (15.8)[d,f]

[a] Grade 1 embryos are those with very little (<5%) or no extruded material, exhibiting a consistent, organized cell mass and trophoblast with no discolored regions.

[b] Grade 2 embryos are those with some (>5% but <10%) extruded material, and slight discoloration or disorganization.

[c] Grade 3 embryos are those with considerable (>10% but <25%) extruded material, and a higher degree of discoloration or disorganization.

[d,e,f] Values in the same column with different superscripts are different at $p < 0.05$.

Ovarian Examination

Gross examination of the ovaries excised from cows that had been aspirated either 1 d or 1 wk prior to ovariectomy revealed that there was visible trauma associated with prolonged follicular aspiration. A fibrous net was present around 6 of the 8 ovaries examined. Sectioning, confirmed the presence of blood filled follicles that were identified as hemorrhagic follicles by ultrasound examination. Histological examination revealed several (3 to 4) fluid-filled structures (<20mm) on each ovary had developed a thin layer of luteal tissue surrounding the cavity. Microscopic examination confirmed the diagnosis of luteal cyst formation (>25mm) from the cow in the 1X group. No luteal cysts were observed in the 2X group, but luteal tissue was observed in the ovaries of these cows.

There was no obvious differences in ovaries which had been punctured 1 d or 1 wk prior to inspection. Although the effects were not quantified, it appeared based on this superficial evaluation that there was some short-term trauma associated with follicular aspiration.
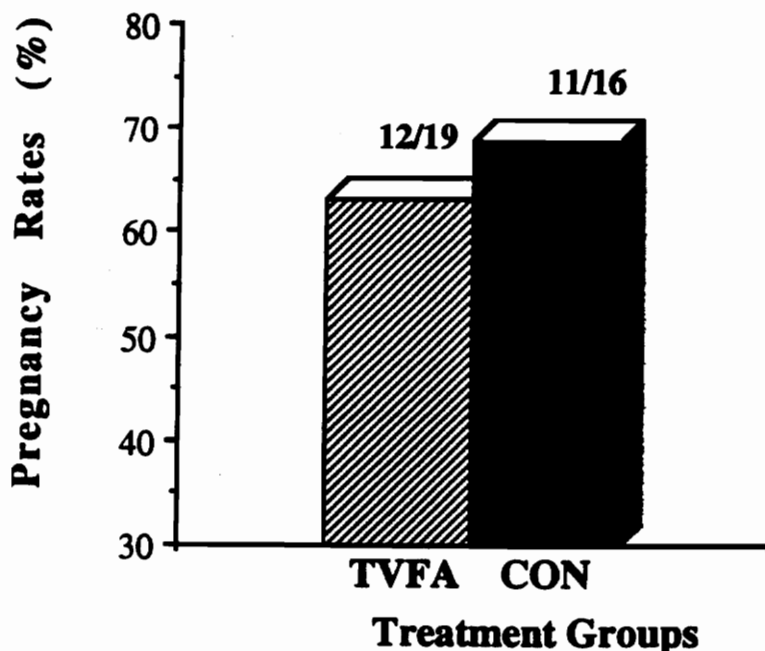
Figure 1.   Pregnancy rates of cows following transvaginal follicular aspiration (TVFA) and non-aspirated control cows (CON).

Rebreeding

Nineteen of 20 cows in the aspirated groups responded to the $PGF_{2\alpha}$ treatment and were inseminated. Ultrasound examination at d 30 of gestation revealed 12 pregnancies (Figure 1). Sixteen cows that had not been previously aspirated displayed estrus and were inseminated. Eleven were confirmed pregnant by ultrasound.

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Progesterone and Estrous Cycle Analyses

Estrous cycles for the 1X group in Experiment 1 were normal (19 to 22d) and all members of the group formed a new CL within 3 to 5 d after estrus. Corpora lutea for the subsequent estrus cycle appeared normal via ultrasound but P4 production may have been comprimised (Figure 2). No cows in the 2X group returned to estrus and cows did not form a subsequent CL after regression of the initial CL (Figure 3) until after TVFA had ended.
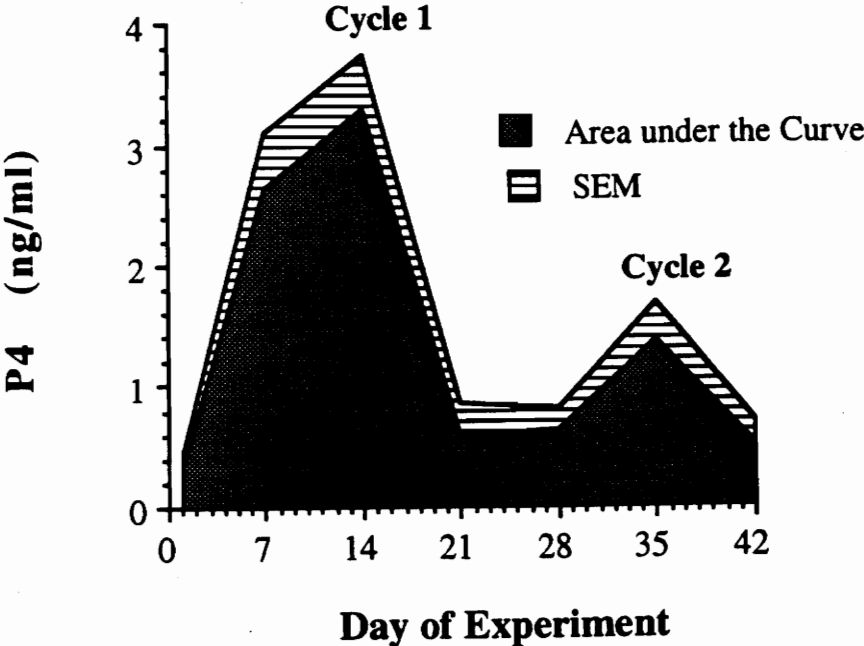
Figure 2.   Progesterone concentrations (P4) for cows aspirated once weekly (1X).

Area under the curve (AUC) P4 concentrations were not different between 1X and 2X cows in Experiment 1 (Figures 2 and 3). The 1X group had 2 estrous cycles and the AUC values for Cycle 1 were higher than Cycle 2 (p<0.08). Only P4 data from Experiment 1 are displayed in these figures.
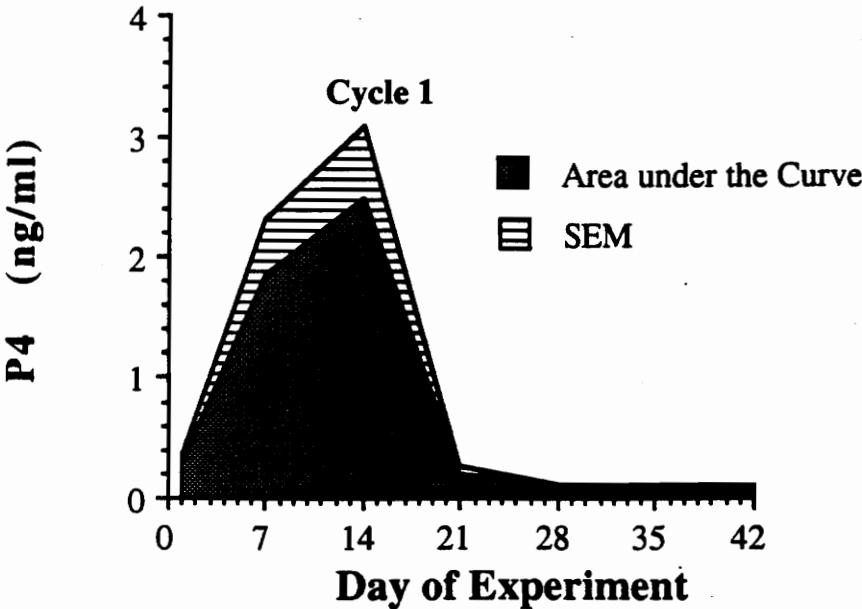
Figure 3.   Progesterone concentrations (P4) for cows aspirated twice weekly (2X).

DISCUSSION

The twice-weekly schedule for TVFA employed in these experiments was more efficient in producing transferable bovine embryos than once weekly aspirations. These

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results are consistent with the research by van der Schans (25) and Walton (29). The once-weekly systems currently in use, combined with IVM/IVF/IVC, are adequate for generating embryos but the more intensive twice-weekly aspiration schedule would generate additional oocytes and embryos.

Although there was no difference in the rate of development to morula or blastocyst between the 1X and 2X TVFA treatments, over a given time more oocytes were recovered and more embryos produced by 2X TVFA. Oocytes from cows aspirated twice weekly (2X, and 2XF) were not adversely affected by any trauma associated with the more frequent procedure. The oocytes recovered from these cows did not appear morphologically inferior to those recovered from the 1X as shown by the proportion of good oocytes. Oocytes harvested from the 2X or 2XF resulted in a higher percentage of embryos that were of Grade 1 and fewer of Grade 3 quality than oocytes from 1X TVFA. Grade 1 embryos have been shown to result in a higher pregnancy rate after transfer to synchronous recipients (8,16).

The rate of development to morula or blastocyst was not different among any of the treatment groups. The slaughterhouse-derived oocytes were screened prior to selection and only good quality oocytes were carried into the IVM/IVF/IVC system. Oocytes derived from TVFA were not screened but were placed into the system regardless of quality. Since embryos development was not compromised due to lack of selection in the TVFA groups, it is possible that TVFA oocytes are inherently of higher quality. The

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TVFA procedure also may be associated with oocyte quality since oocytes are recovered from live animals instead of a highly variable scource, such as a slaughterhouse.

A more frequent aspiration schedule may be important for TVFA oocytes to maintain developmental competence. With only a shortened interval between aspiration sessions (2X) the follicular population does not normally establish a dominant follicle (10,23). In the absence of a dominant follicle the levels of inhibin (7,10) would be expected to be low, decreasing the incidence of atresia in developing follicles. Also, with only 3 to 4 d between aspiration sessions, the follicle population may be synchronized when oocytes are harvested yielding a group of oocytes that are similar in developmental stage and may respond more consistently to IVM/IVF/IVC (14).

The 2XF TVFA group did not show an increase in the number of oocytes recovered per session, and the in vitro development of embryos with previous exposure to exogenous FSH was not increased. These findings are similar to studies performed by Hasler (8) and only slightly different to results in other laboratories (16). Cows treated with exogenous FSH may not respond as expected because they may have elevated endogenous FSH levels due to the frequency of aspiration. Follicles were being formed more frequently in the 2X than in the 1X TVFA and without a dominant follicle and high levels of inhibin to direct FSH suppression an increase in FSH may be noticed. An increase of endogenous FSH due to TVFA frequency probably overshadowed any effects from exogenous FSH. With more frequent TVFA (2X) more excellent quality (grade 1)

embryos were generated. This may be related to oocytes harvested from a growing, synchronous, follicular population and not from an atretic environment that may exist in the 1X TVFA.

Although gross examination of excised ovaries indicated some visible damage associated with TVFA, a 63% pregnancy rate on TVFA cows that were given at least a 1 month recovery period, shows that this trauma is short term. These data suggest that there is no erreparable damage to the reproductive tract from TVFA. This finding is consistent with that of Hasler (8) and Looney et al. (16).

Progesterone for Cycle 1 in Experiment 1 was normal (22) for both groups. The 1X group had two separate estrous cycles, but P4 production was decreased in the latter indicating a decrease in normal CL function. The 2X group did not ovulate or form a subsequent CL and therefore had no second estrus or ovarian cycle. It is unclear if this is linked to the manipulation of the ovaries or CL, frequent loss (2X) of follicular fluid during TVFA, or a consequence of TVFA on the hormonal level. Additionally, cows in the 2X group formed luteal structures but no increase in P4 was observed, which seems to suggest incomplete or abnormal luteinization which is hormonally regulated (10,20).

Investigation is warranted into the combination of conventional superstimulation and embryo collection and TVFA. Aspirating follicles from the ovary prior to FSH stimulation may be a method of removing the dominant follicle so that a new

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synchronous, follicular population emerges from the ovary without inhibitory dominant follicle effects (15). The aspiration or ablation of follicles increases the number of follicles recruited and may increase the number of ovulations and embryos collected (1).

In order to produce large numbers of grade 1 quality bovine embryos through TVFA a twice weekly system should be adopted. The current systems of once weekly or monthly aspiration may be more accommodating commercially, but could be counterproductive for generating large numbers of high quality embryos through TVFA and IVM/IVF/IVC.

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Chapter IV

IN VITRO EMBRYO PRODUCTION AND OVARIAN DYNAMICS FOLLOWING TRANSVAGINAL FOLLICULAR ASPIRATION

ABSTRACT

Ultrasound-guided transvaginal follicular aspiration (TVFA) of oocytes from live cows combined with in vitro maturation, fertilization, and culture (IVM/IVF/IVC) has become an accepted method of producing pre-implantation stage bovine embryos. This method provides a source of ova of known genetics and donor health status. Pronuclear microinjection of DNA into slaughterhouse-derived zygotes has been used for producing transgenic cattle. However, a less variable source of high quality oocytes may be required to decrease the inefficiency of progeny produced. This experiment was designed to compare in vitro embryo development rates between oocytes derived from TVFA and those generated from cows at slaughter. Nine cows (8 Holstein, 1 Jersey) were subject to a twice-weekly TVFA. Oocytes were aspirated with the aid of a 5 MHz ultrasound transducer packaged in a vaginal probe equipped with a dorsal-mounted needle guide (16-ga). Ovaries were manipulated through the rectum and aligned with the path of a 17-ga 55-cm needle with an echogenic tip. All visible follicles (>2 mm) were punctured at each TVFA session and the contents removed under vacuum suction (75 to 85 mmHg) at a flow rate of 27 to 29 ml/sec. Oocytes were examined to determine morphology and entered into the IVM/IVF/IVC system. Microinjection of DNA (1 to 3 pl) was performed during the pronuclear stage of development (14 to 16 h post IVF) and the zygotes

cocultured on BRL Buffalo Rat Liver (BRL)cells in modified M199 at 39°C in 5% CO_2 and air. After 7 d in culture, embryos were removed and scored for development. A 2 X 2 factorial design was used to analyze embryo development, comparing TVFA-derived oocytes (microinjected and not) and slaughterhouse-derived oocytes (SHD; microinjected and not) by Chi Square analysis. Non-microinjected embryos resulting from IVF of TVFA-derived oocytes developed to blastocysts at a higher rate than SHD oocytes (40.0% vs. 30.8%; $p<0.05$). There was no difference in development rates between the microinjected groups (TVFA = 15.9% vs. SHD = 12.8%). A higher proportion of the embryos generated from the TVFA-derived oocytes were of excellent or good quality following the 7 d culture period ($p<0.05$). The effects of microinjection may overshadow some effects of ova source, but TVFA may provide a more consistent, synchronous population of oocytes for entry into the in vitro system.

KEYWORDS: Ultrasound, microinjection, in vitro fertilization, embryo culture.

INTRODUCTION

Recovering oocytes from cows at slaughter is a reliable method of providing ova for in vitro fertilization (IVF) and embryo culture (IVC). Cumulus oocyte complexes (COC) may be obtained from the ovaries in several ways. The follicular fluid and other contents can be aspirated from visible follicles using a needle and syringe or a vacuum apparatus, or follicles may be dissected out of the ovary and then ruptured in a petri dish to expel the oocytes (14). A slicing pattern across the ovary also has been described as a method of recovering oocytes from slaughtered cow ovaries, and although more labor intensive, it does provide more COC's than aspiration (10). After COC's are recovered they are usually classified by morphological appearance according to the amount of cumulus cell investments. The COC's with many dense cumulus cell layers were shown to have higher in vitro maturation (IVM) rates (97%) while those that were partially or totally denude of cumulus cells matured at a lower rate (90% and 53%, respectively; 25). Typically only high quality oocytes with dense cumulus cell investments are used in the IVM/IVF/IVC systems. This selection process may aid in eliminating some lower quality oocytes. However, the inherent variability of oocytes from slaughtered cows may be eliminated if oocyte donor identification was possible. Transvaginal-follicular aspiration (TVFA) is an efficient method of obtaining oocytes from live cows to be used with IVM/IVF/IVC (19). Oocytes recovered by TVFA have resulted in embryos that are of sufficient quality to establish and maintain pregnancies when transferred to recipients (11). Many exogenous stimulation regimes (21,28,29) and aspiration frequencies (30) have

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been examined in an attempt to increase oocyte recovery rates. Recent research involved oocyte aspiration relative to a certain phase of the estrus cycle (29). However, there is a relative paucity of information on the effects of TVFA oocyte recovery programs on the potential in vitro development of oocytes.

Microinjection of foreign DNA, coding for a specific protein, prior to fertilization (before germinal vesicle breakdown; GVBD) has been examined (16) but was less efficient than microinjection of DNA at the pronuclear stage of embryo development, which is the most common method of producing transgenic animals (26). Oocytes matured in vivo (27) and in vitro (17) have been proposed as a source of oocytes for microinjection. However, only the in vitro method provides oocytes in which the pronuclear stage of development can accurately be predicted. Microinjection involves inserting a glass needle into one of the pronuclei and depositing sufficient amounts of DNA (1 to 2 mg/ml in the bovine; 6,8) to allow foreign DNA integration. The injection of this volume of the DNA solution (1 to 2 pl) normally causes pronuclear membrane swelling and results in deposition of tens to hundreds of copies of the specific DNA construct into the pronucleus (8,26). The time of microinjection of DNA was shown to be important, with 14 to 16 h post IVF resulting in the highest embryo development rates (16). Microinjection can be an efficient method of transgenic mouse production, with 50 to 85% of the zygotes surviving the process and 10 to 30% of the live born mice carrying the inserted gene (31). However, in the livestock species (cows, sheep, and pigs) efficiencies are much lower with only 4 to 10% of the offspring integrating the transgene

(22). Production of transgenic animals, regardless of the inefficiencies, has great potential for the generation of human pharmaceutical proteins from the mammary gland that cannot be produced by other methods (13). The production of transgenic dairy cattle specifically, would allow exploitation of increased milk production and a higher yield of mammary-expressed human proteins. The efficiency of meat production also may be enhanced by production of transgenic animals containing muscle expressed transgenes (7). The financial rewards from producing these "bioreactors" (23) are offset because only .02 to .2% of microinjected bovine zygotes continue to develop and become live-born calves (7,12,15). A source of oocytes with an inherently greater developmental potential may be one method of decreasing the inefficiencies associated with gene microinjection.

The following experiment was designed to explore the feasibility of using oocytes recovered during ultrasound-guided TVFA for microinjection of DNA and to describe ovarian dynamics following long-term TVFA in donor animals.

MATERIALS AND METHODS

Transvaginal Follicular Aspiration

Ultrasound-guided TVFA was conducted on 9 dairy cows (8 Holstein and 1 Jersey) twice-weekly (2X) as described by Gibbons et al. (9). This recovery frequency was shown to produce a higher proportion of grade 1 or excellent quality embryos after 7 d in culture than once-weekly aspiration (9). Briefly, 2X TVFA was performed by manipulating the ovaries per rectum while puncturing the follicles (>2 mm) with a 17-ga

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55-cm needle with an echogenic tip (RAM Consulting, Madison, WI). The ovaries are visualized with a sector ultrasound transducer (5 MHz) packaged in a vaginal probe equipped with a dorsal-mounted needle guide (16 ga). As the follicles were observed on the ultrasound monitor (Aloka 500V; Corimetrics, Wallingford, CT), they were pierced with the needle after it passed through the vaginal wall. The contents of the follicle were removed under vacuum suction (85 mmHg), aspirated through silastic tubing (2.0 mm ID X 3.2 mm OD; 2 m), and collected into an embryo filter (Professional Embryo Transfer Supply, Canton, TX). The oocytes were rinsed from the filter with phosphate buffered saline (PBS; Gibco, Long Island, NY) enriched with 10% vol/vol newborn calf serum (NCS; Gibco), 1% vol/vol penicillin-streptomycin (P/S, Gibco) and 25 U/ml heparin (Sigma Chemical, St. Louis, MO). After recovery the oocytes were washed (3X) in TL HEPES medium and microscopically-examined for morphology. Oocytes exhibiting at least 3 layers of cumulus cells and an organized cytoplasm were categorized as good and the remaining oocytes were classified as poor. All oocytes harvested through TVFA (regardless of morphological quality) were carried through the IVM/IVF/IVC procedure. Oocytes were then placed into 0.5 ml maturation medium under equilibrated mineral oil (1.0 ml; Sigma) for transport to the laboratory in a temperature-controlled (39°C) portable incubator (Minitube of America, Madison, WI). Oocytes were maintained in maturation medium which consisted of TCM 199 (Gibco) supplemented with 10% vol/vol fetal calf serum (FCS; HyClone, Logan, UT), bFSH and bLH (0.01 U/ml each; Nobl Labs, Sioux Center, IA) and 1% vol/vol P/S (Gibco) for 24 h prior to IVF (3).

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In Vitro Fertilization

A Percoll-separation procedure was used to prepare frozen/thawed semen from a single ejaculate of a well-characterized Holstein bull for IVF (15). After thawing (35°C for 1 min), semen from a 0.5 ml straw was layered on top of a Percoll (Sigma) density gradient (90%:45%) in a 15 ml centrifuge tube and centrifuged at 700 x g for 30 min. After centrifugation, live sperm cell concentration was determined and sperm were added to fertilization wells containing the washed oocytes (3X in TL HEPES) at a final concentration of 1.0×10^6 sperm/ml. Heparin (5 mg/ml) and PHE (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine, 1 mM) were also added at this time to aid sperm capacitation and motility (4).

Microinjection of DNA

After 14 to 16 h of co-incubation with enriched sperm, ova were removed from fertilization wells and vortexed (2 min, 15 sec) to remove cumulus cells. Approximately 20% of the zygotes were placed directly into the coculture system to serve as noninjected controls. The remaining zygotes were centrifuged (12,00 x g for 6 min) to enable visualization of the pronuclei. Microinjection of DNA was performed in TL HEPES on a heated stage (39°C). The gene construct used for microinjection was a 12 kb fragment made up of genomic human protein C (HPC) under the direction of the murine whey acidic protein (WAP) promoter (16,17). One to three pl of DNA solution (1.5 mg/ml DNA; 100 copies/pl) was injected into the pronucleus of each zygote. Oocytes in which 2 pronuclei were not visible were considered unfertilized and discarded.

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In Vitro Culture

After microinjection, embryos were cocultured in wells (~25 embryos/well) containing Buffalo Rat Liver (BRL; 200,000 cells/well) cells and modified culture medium (500 μl). This medium consisted of TCM 199, 10% FCS, 1% BSA (Sigma), 1% P/S, and was fortified with 0.1 mM non-essential amino acids (Gibco), 2 mM glycine, and 1 mM alanine (Sigma). Embryos were moved to a fresh culture well containing newly prepared BRL cells on day 4 of culture. On day 7 of IVC embryos from the injected and control groups were removed from culture and scored for development.

Slaughterhouse Oocytes

Oocytes recovered from cows at slaughter (14) served as controls for the IVM/IVF/IVC system. These oocytes were purchased (Em Tran, Inc; Elizabethtown, PA) and matured in transit in a temperature-controlled (39°C) portable incubator (Minitube of America, Madison, WI). Oocytes were received twice-weekly and recovered from slaughtered cows on the same days as the TVFA-derived oocytes were harvested. In vitro fertilization and microinjection of the slaughterhouse-derived (SHD) oocytes was concomitant with the IVF and microinjection of TVFA-derived oocytes. A similar number of oocytes (~65%) from each source was microinjected as described above. Non-microinjected oocytes were placed into coculture wells at 16 h after initial co-incubation with enriched sperm. Embryos produced from microinjected and control groups from each respective oocyte source (TVFA vs. SHD) were scored for development on day 7 of culture.

Follicular aspiration was performed twice-weekly for 12 weeks on 9 cows. Only development and embryo quality data from the first 5 TVFA sessions are presented in these results, as oocytes recovered subsequent to these sessions were used in other experimentation. Oocyte recovery data reflects the entire 12 week experiment.

Post TVFA Ovarian Examination

Following the cessation of TVFA, cows were observed for visible signs of estrus. Ovaries were monitored with ultrasound (5 MHz real-time linear array) every 12 h following the onset of estrus for the detection of ovulation (5). Cows that maintained large follicles (>12 mm) for 72 h were administered 100μg (im) GnRH (Cystorelin; Sanofi Animal Health, Overland Park,KS) to induce ovulation. Ovaries were scanned twice daily for 5 d beginning 10 d after GnRH therapy to determine the effectiveness of treatment.

Statistical Analysis

A 2 X 2 completely randomized block arrangement was used to examine in vitro embryo production efficiencies from two oocyte sources (TVFA and SHD) with two post-fertilization treatments (microinjection and non-microinjection). Comparison of developmental and embryo quality data was performed using Chi-Square analysis. Correlation values were calculated between mean number of good oocytes (dependent variable), and mean number of total oocytes (independent variable).

RESULTS

Oocyte Recovery

Mean oocyte recovery rates (x = 9.7 +/- .8 oocytes/cow/session) varied greatly among donors (range = 6.7 to 16.1 oocytes/cow/session). Recovery rates also varied from week to week, although no statistical difference in weekly ova recovery was found throughout the 12 week experiment ($p > 0.05$). The number of good quality oocytes recovered also varied among donors (Figure 1) and was positively correlated (r = .96; $p < 0.01$) with the mean number of oocytes recovered from individual donors. The mean number of good quality oocytes recovered per donor was 5.1 +/- .6, or 52.5% of the total number of oocytes recovered.
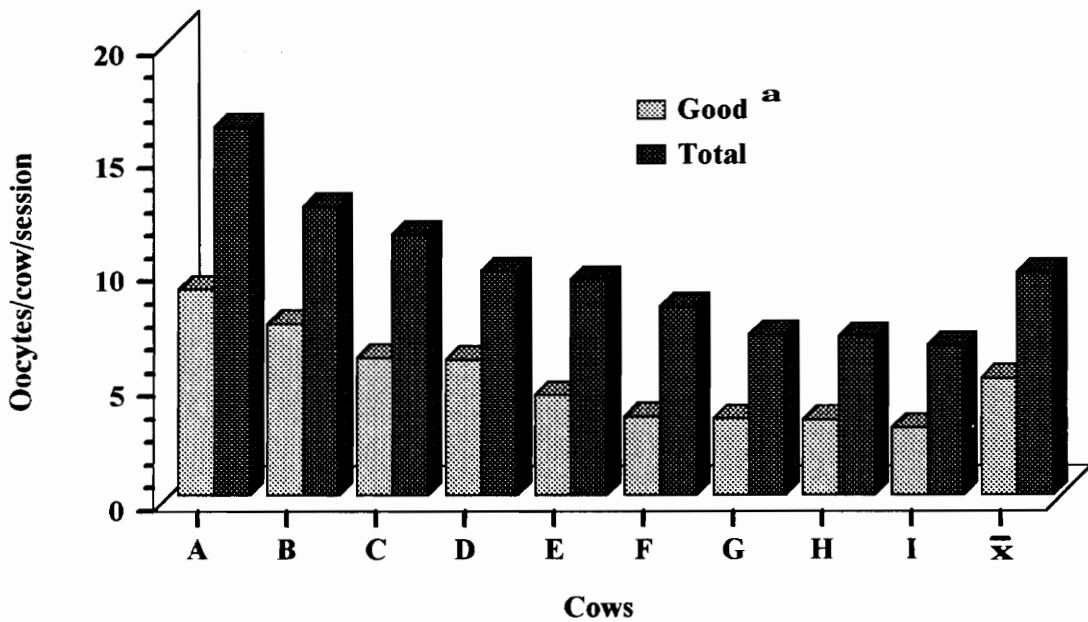
Figure 1. Average number of good quality and total oocytes recovered per cow via twice-weekly TVFA.

[a] Good quality oocytes are those with at least 3 layers of cumulus cells and an orgainized cytoplasm.

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In Vitro Embryo Development

In the noninjected control groups, a higher percentage of morula and blastocyst-stage embryos were produced in vitro from the TVFA source of oocytes ($p<0.05$) compared to SHD oocytes. The developmental rates for embryos in the microinjected groups were not different ($p>0.05$), although the trend was in the same direction with the oocytes derived from TVFA having a higher numerical value (Table 1) than SHD oocytes. A significant interaction between oocyte source and treatment (microinjected and control) was discovered ($p<0.05$). This interaction was due primarily to the similar ($p>0.10$) combined (microinjected and control) embryo development between oocyte source. Control oocytes, regardless of source, developed to the morula and blastocyst-stage at a higher rate than microinjected embryos (32.8 vs. 14.3%, respectively; $p<0.01$).

Table 1. In vitro embryo development rates from Ultrasound-Guided Transvaginal Follicular Aspiration-derived (TVFA) or Slaughterhouse-derived (SHD) oocytes microinjected with DNA.

Treatment Group	Morula and Blastocyst [a]/Total	
	Viable / total	Percent
TVFA microinjected	44 / 276	15.9 [d]
TVFA non-microinjected	49 / 120	40.8 [b]
SHD microinjected	40 / 313	12.8 [d]
SHD non-microinjected	101 / 337	30.0 [c]

[a] Morula and blastocyst were considered viable only after 7 d in culture.
[b,c,d] Values within column with a different superscript are different ($p<0.05$).

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Embryo Quality

In addition to a higher in vitro embryo development rate, the nonmicroinjected TVFA oocytes produced a higher proportion (p<0.05) of good and excellent quality embryos than the nonmicroinjected SHD oocytes (Table 2). There was no difference in embryo quality between the microinjected groups regardless of the oocyte source (p>0.05).

Table 2. Embryo quality comparison following IVM/IVF/IVC from Ultrasound-Guided Transvaginal Follicular Aspiration-derived (TVFA) or Slaughterhouse-derived (SHD) oocytes microinjected with DNA.

Treatment Group	Excellent and Good Quality Embryos [a]/Total	Percent
TVFA microinjected	27 / 44	61.4 [b]
TVFA non-microinjected	45 / 49	91.8 [c]
SHD microinjected	29 / 40	72.5 [b]
SHD non-microinjected	80 / 101	79. 2 [bd]

[a] Excellent or Good quality embryos are those with very little extruded cellular debris (<25%) and no substantial discolorization or disorganization at day 7 of in vitro culture.
[b,c,d] Values within column with a different superscript are different (p<0.05).

Ovarian Dynamics Post-TVFA

Following cessation of TVFA, 8 cows returned to estrus within 7 d. Two cows ovulated within 36 h of estrus while the remaining 6 received GnRH at 72 h following estrus. Five of these cows ovulated within 13 d of GnRH treatment. Mean number and sizes of follicles for the 72 h period following estrus are in Figure 2. The mean number

of small follicles (< 6 mm) decreased (65%) between 36 and 48 h post estrus (p<0.01).

Multiple ovulations (unilaterally or bilaterally) were found in 5 of the 7 cows that

ovulated (x = 2.0 ovulations).
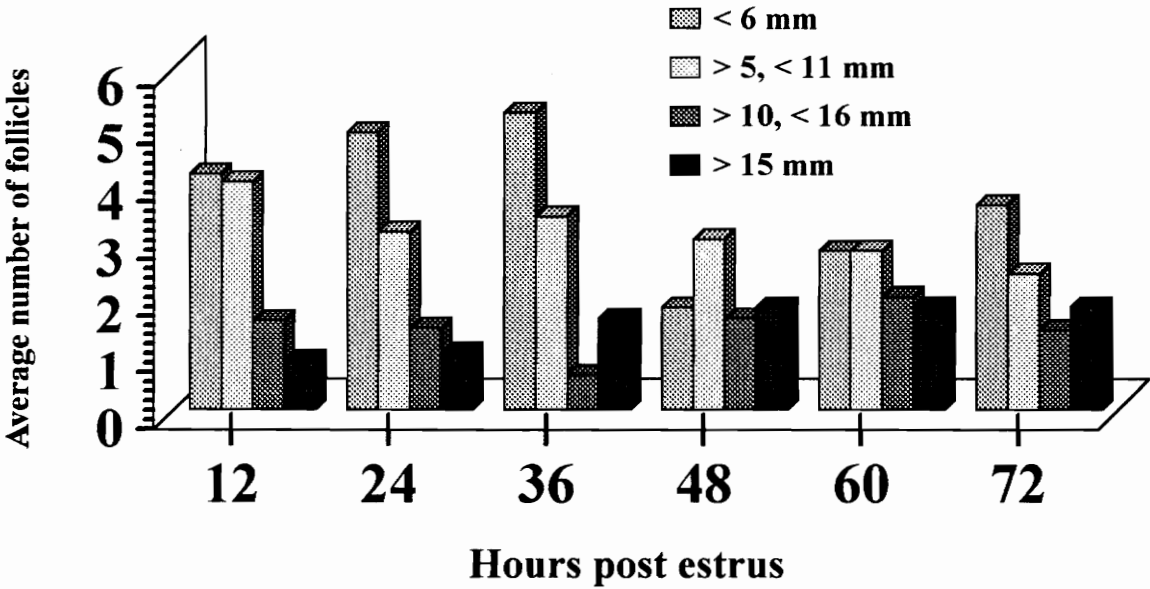
Figure 2. Average size and number of follicles following first estrus after cessation of long-term TVFA.

DISCUSSION

Recovering oocytes from ovaries of slaughtered cows, although it is the most common source for ova, has many inherent difficulties that are not entirely understood. Many biochemical and physiological pathways may be immediately interrupted at the time of slaughter which could lead to rapid decline in the viability of the oocytes within the cow's ovaries (Hasler, personal communication). The greatly variable health and genetic status of the oocyte "donors" in a slaughterhouse also can be an unknown factor in the subsequent IVM/IVF/IVC of bovine oocytes. A more consistent method for recovery of bovine oocytes may increase the overall efficiencies of in vitro embryo production.

Through TVFA, oocytes from cows of known health status and genetics can be routinely harvested and used in in vitro systems. The time that the oocytes are out of a favorable environment can be dramatically reduced. This potentially could allow for a more developmentally competent oocyte. A more developmentally sound oocyte may respond more effectively to the challenge of DNA microinjection. Although recovery of oocytes via TVFA is more labor intensive than recovery of SHD oocytes, an oocyte with greater potential for subsequent development is obtainable.

As implied in this report, by previous experimentation in this laboratory (9), and through the commercial industry (Hasler, personal communication), oocytes from TVFA appear of inherently higher quality and are potentially capable of development to the pre-

implantation stage more readily than oocytes recovered from cows at slaughter. It is unclear whether the actual procedure of follicular aspiration of oocytes from a live cow is different from aspiration of the follicles from an ovary removed post mortem (2). It is reasonable to expect the changes that occur at the time of slaughter are detrimental to the oocyte and these changes potentially reduce the possibility that the oocyte will continue to mature and develop.

Oocyte recovery rates via TVFA (9.7 oocytes /cow/session) were consistent with previous experimentation in this laboratory (9). Twice-weekly oocyte recovery may have been beneficial in increasing the number of oocytes harvested throughout the duration of this experiment. Results have shown that once- or twice-weekly oocyte recovery rates are similar in number of oocytes per session (9). However, it is unclear if exogenous follicle-promoting agents (FSH or ECG) combined with a once-weekly recovery scheme can be more advantageous than the twice-weekly system (9,19,28,29). The oocyte recovery data presented in this experiment were similar to recovery rates in the literature (11,19,30) and was obtained without any exogenous follicle stimulation treatment.

In vitro embryo development (non-injected groups) was higher for oocytes harvested through TVFA compared to oocytes from SHD (TVFA = 40.8% vs SHD = 30.0%), suggesting that the TVFA oocytes may be inherently of higher quality. These development data are consistent with previous development data from TVFA oocytes in our laboratory (9), and may be slightly higher than development data from commercial

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entities (11,19). The SHD development was similar to other in vitro embryo development data (9,11,17). Additionally, only 52.5% of the TVFA oocytes were of good quality morphologically but they developed at a higher rate than the SHD oocytes, from which 100% were considered good quality. The lack of selection among the TVFA oocytes did not hinder in vitro development. However, it is not clear how the oocytes classified as poor quality contributed to the developmental efficiencies.

Microinjection of DNA into the pronucleus of a newly fertilized oocyte can be very deleterious. The procedure itself causes lysis of the cytoplasm or the pronucleus in a large percentage of oocytes (~30%; 17) and subsequent embryo development declines as well (16). In our data, the effects of microinjection may have overshadowed the effects due to the source of the oocytes. The microinjected oocytes harvested through TVFA had a slight numerical advantage in embryo development (TVFA = 15.9% vs. SHD = 12.8%), although not statistically significant (p>0.05). These development data for microinjected embryos are similar to previous results (6,7,13,17). Further experimentation is necessary to determine if the effects of microinjection can be altered to be less traumatic to the oocyte.

A higher proportion of embryos produced in vitro from oocytes harvested via TVFA were of excellent or good quality when compared with embryos generated from SHD oocytes (p<0.05). Embryos that are of this quality are more desirable for transfer to recipients in order to establish a pregnancy (11,19). Poor quality embryos are still

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considered viable and may still establish a pregnancy but the efficiencies of producing pregnancies from poor quality embryos are reduced. Higher quality embryos were produced primarily because a more consistent population of oocytes was recovered in a twice-weekly TVFA collection frequency (9). Oocytes that are in a high degree of synchrony with each other may respond more favorable and consistently for embryo production. In vivo studies involving the removal of the dominant follicle indicate that the remaining synchronous follicles may yield a higher number of transferable quality (grade 1 and 2) embryos at the time of conventional embryo recovery (1,18).

The decrease (65%) in follicle numbers (< 5 mm) observed between 36 and 48 hr following estrus after long-term TVFA was not accounted for by the increase in large follicles (35%; > 10 mm). This decrease combined with a slight decrease (12%) in medium-sized follicles (> 6 mm to < 11 mm) may indicate that a short-term artificial follicular wave has been induced. It is not known if these follicles are undergoing atresia, or if elevated inhibin levels exists to selectively inhibit these smaller follicles. A multiple-dominant follicle hypothesis (20,24) is supported by the multiple ovulation observations. However, more research is needed to thoroughly examine the possible anovulatory nature of cows subjected to long-term twice-weekly TVFA.

Microinjection of DNA into oocytes, although inefficient, is still the most widely used method of producing transgenic offspring (22). Through TVFA, a more consistent source of oocytes may be helpful in offsetting a small portion of this inefficiency. Donor

identity, as indicated by the recovery rates and oocyte quality from individual cows, may also be a method of reducing variability and increasing efficiency of producing transgenic individuals. The effects of microinjection on oocyte development may be impossible to overcome. However, TVFA oocytes with a less variable genetic background and health status may be more suited to challenge by DNA microinjection and in vitro culture.

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Chapter V

SUMMARY

These three experiments demonstrate that ultrasound-guided transvaginal follicular aspiration can provide a source of bovine oocytes for in vitro maturation, fertilization, microinjection and embryo culture. The approach to producing transgenic calves of known genetics capable of producing human proteins in their milk is greatly advanced by the foundation laid in these three experiments. Oocyte donors can be chosen on a health and genetic basis and their identity maintained through the use of follicular aspiration and in vitro embryo production. This system also provides for further experimentation into the fields of early embryo development in microinjected (and control) embryos, ovarian dynamics in previously aspirated donors, and follicular ablation combined with superstimulation and the effects on the number of transferable quality embryos in conventional embryo transfer.

The first experiment demonstrated that oocytes could be routinely recovered from donors on a twice-weekly schedule. There was no significant difference in the number of oocytes recovered per session between the once- and twice-weekly groups. However, the twice-weekly group did yield a higher number of oocytes throughout the duration of the experiment and the weekly oocyte recovery was higher in the twice-weekly group. From this experiment, twice-weekly was determined to be the recovery frequency of choice.

In an attempt to further enhance oocyte recovery, FSH was administered in the second experiment. There was not a statistical difference observed between the FSH and control groups (both aspirated twice-weekly) in oocyte recovery rates. In fact, the "stimulated" group actually had a numerical disadvantage in weekly and per session oocyte recovery rates, although this difference was not statistically significant. The biological significance of this finding has yet to be elucidated. The developmental efficiencies of oocytes recovered from each of the aspiration groups (1X, 2X, 2XF) did not differ statistically from each other or from oocytes recovered from slaughterhouse cattle. Also, a higher proportion of the embryos produced in vitro from the 2X group were of excellent quality compared to the embryos generated from 1X oocytes. This experiment demonstrated that the lack of selection among aspirated oocytes did not hinder future embryo development to the blastocyst stage. This experiment also clearly established twice-weekly oocyte recovery without exogenous follicle promoting agents as the recovery method most efficient to produce high quality embryos following in vitro culture.

In the third experiment aspirated oocytes and slaughterhouse-derived oocytes were more closely compared. Oocytes from both of these sources were subjected to microinjection to determine the effects of ova source on control and microinjected oocytes. The nonmicroinjected oocytes derived from follicular aspiration demonstrated a higher developmental efficiency than those oocytes recovered from slaughtered cows. The development from the slaughterhouse-derived ova was consistent between the second

and third experiments. The higher developmental efficiencies in the aspirated group of oocytes was not extended to the microinjected groups. The aspirated oocytes held a slight numerical advantage over the slaughterhouse ova, but this was not statistically significant. In this experiment it seems the effects of microinjection outweighed the effects of ova source.

These experiments show that ultrasound-guided oocyte aspiration can be used to recover ova for in vitro maturation, fertilization, DNA microinjection, and embryo culture. The data demonstrate that weekly recovery rates and quality of the resulting embryos are enhanced if oocytes are recovered on a twice-weekly schedule. Using this system a developmental efficiency higher than oocytes from slaughtered cows can be obtained. Potentially the inefficiencies of producing transgenics by microinjection can be reduced without having deleterious effects on the genetically-superior donors.

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APPENDIX A

RETROSPECTIVE EVALUATION OF NON-SURGICAL EMBRYO TRANSFER USING MICROINJECTED BOVINE EMBRYOS

ABSTRACT

Producing transgenic calves from in vitro generated embryos microinjected with foreign DNA at the pronuclear stage of development is very inefficient compared to embryo transfer of in vivo derived embryos. The insult of microinjection process can be quite deleterious to the developing embryo and the effects may not be fully realized until several days into gestation. The purpose of these observations was to establish embryo transfer procedures and efficiencies for microinjected embryos either directly from In Vitro Culture, or after subjected to a freeze/thaw procedure. Embryos were created using oocytes from slaughtered cows, or from live cows via Ultrasound-Guided Transvaginal Follicular Aspiration. All oocytes were matured and fertilized in vitro. Some of the embryos were cultured in vivo in the ligated oviducts of mature sheep, while the bulk of the embryos were cocultured on a monolayer of Buffalo Rat Liver (BRL) cells. Embryos were transferred to synchronous recipients to the uterine horn ipsilateral to the ovary with a palpable Corpus Luteum. Pregnancy was diagnosed using ultrasound at 30 d of gestation, and the pregnant animals were checked again at 60 d. There was no difference in initial (30 d.) pregnancy rates between the fresh or frozen embryos ($14/45 = 31\%$ vs.$8/32 = 25\%$; $p>0.05$). The frozen embryo group experienced a significant decline in the number of pregnancies between 30 and 60 d ($8/32 = 25\%$, to $3/32 = 9.4\%$; $p<0.05$)

and was significantly lower than the fresh group at 60 d ($12/45 = 26.6\%$ vs.$3/32 = 9.4\%$; $p<0.025$). It is not clear why these viable pregnancies are lost or why frozen embryos seem to be more susceptible to early embryonic mortality, but the death lose percentage may be higher than in a typical embryo transfer situation. Both transfer groups experienced a further decrease in number of pregnancies between 60 d. and parturition. If possible, microinjected embryos should be transferred fresh in order to establish and maintain a higher number of pregnancies.

INTRODUCTION

Microinjection of foreign DNA coding for a specific protein into the pronucleus of a bovine zygote, although inefficient, is the most popular method of producing transgenic cattle (21). As in any multi-step process, inefficiencies are magnified at each level and the overall feasibility of producing transgenics suffers dramatically. In the bovine, the efficiency of producing a transgenic calf is between .02% and .2% of the total number of zygotes microinjected (2,11). Higher efficiencies have been reported for the sheep (12.2%; 6) possibly due to the multiple transfer of embryos to a litter bearing species. The mouse model represents the highest zygote to transgenic animal efficiency of approximately 25% (21), in part due to multiple embryo transfer (5) and a high DNA integration rate (5,21). In the bovine, multiple microinjected embryo transfer was effective in producing transgenic calves (2). However, the possibility of freemartinism may be a deterrent when injecting a mammary specific gene construct. Fresh, non-microinjected, in vitro produced bovine embryos were dependent on each other for survival when transferred two per recipient (18). This all-or-none twin-transfer theory (18) may magnify the already high abortion rate common to microinjected bovine embryos (11). In vivo produced embryos may have a higher success rate than in vitro produced embryos, in terms of pregnancies (23). However, when multiple transfer of in vivo embryos is performed the abortion rate may still be higher than single in vivo embryo transfer (12).

Ultrasound-guided transvaginal follicular aspiration (TVFA) of bovine oocytes provides a source of oocytes competent to continue to develop and establish pregnancies (10,15,16). Fresh transfer of in vitro produced embryos from TVFA-derived oocytes has resulted in pregnancy rates comparable to in vivo produced embryos (10). Frozen/thawed in vitro produced embryos (from TVFA and slaughterhouse oocytes) appear more susceptible to freezing trauma than in vivo collected embryos, and result in a slightly lower success rate when transferred to recipients either singly (10) or in tandem (20). The grade and stage of development of in vitro produced embryos (especially on d 7) may be very important as grade 1 or 2 morula stage embryos result in pregnancies at a lower rate than do grade 1 expanded blastocysts (10,19).

Microinjected embryos exhibit a higher rate of abortion or absorption during the first trimester of pregnancy (11). It is possible that the disruption of the pronuclei during the microinjection procedure may result in immediate (14) as well as long-term trauma (2,11). The embryo may be competent to establish a pregnancy, but subsequent need for certain on/off genetic mechanisms may have been disrupted at microinjection and the fetus is terminated when these mechanisms are needed (7). Multiple embryo transfer of microinjected embryos, although more efficient in terms of recipients, may not be the best method for producing germ line transgenics because of the possibility of freemartinism and one unstable embryo may cause the ultimate demise of the viable embryo (18). It seems obvious that microinjected embryos are very different from noninjected embryos in terms of the recipient embryo relationship. A recent report has suggested that the non-

pregnant recipient following embryo transfer maintains the ability to become pregnant by artificial insemination (4). Thus, it seems that the stage, quality, and manipulation status (microinjected or not) of the in vitro produced embryo may have more impact on the resulting potential for pregnancy than the recipient, providing the animals are reproductively sound (4). There is little difference in pregnancy rates between maiden heifers as recipients compared to once-calved animals if both are given an adequate plane of nutrition (3).

The following data are a retrospective summary of pregnancy rates from fresh and frozen microinjected embryos transferred to cows and heifers.

MATERIALS AND METHODS

In Vitro Maturation and Fertilization (Slaughterhouse-derived oocytes)

After oocytes were collected, they were rinsed three times in a TL HEPES buffered wash media supplemented with BSA (3mg/L; Sigma Chemical). Oocytes were combined and placed in maturation media of TCM199 (#400-1100 EB, Gibco) supplemented with 10% vol/vol fetal calf serum (FCS; HyClone, Logan, UT) bFSH and bLH (0.01 U/ml each; Nobl Labs, Sioux Center, IA), and 1% vol/vol penicillin-streptomycin (Gibco). Oocytes were maintained for 22 to 24 h at 39°C in 5% CO_2 and air (13).

A Percoll-separation procedure was used to prepare frozen/thawed semen for IVF (13,14). Frozen 0.5-ml straws of semen from a previously-characterized ejaculate of a single Holstein bull were thawed at 35°C for 1 min. Sperm were layered on top of a Percoll (Sigma Chemicals, St. Louis, MO) density gradient (90%:45%) in a 15-ml centrifuge tube and centrifuged for 30 min at 700 x g. After centrifugation live sperm concentration was determined using a hemocytometer. Sperm (20µl) were added to fertilization wells with the washed oocytes to give a final concentration of 1.0×10^6 sperm/ml. Heparin (5 mg/ml) and PHE (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine, 1 mM) were included (13,14).

TVFA-derived oocytes

Oocytes harvested through TVFA were treated as described except were matured in 500 µl of maturation medium in 1.8 ml cryotubes (Corning, McGaw Park, IL) overlaid with mineral oil (1.0 ml). Oocytes were transported to the laboratory in a temperature-controlled (39°C) portable incubator (Minitube of America, Madison, WI). In vitro fertilization was conducted as described for slaughterhouse-derived oocytes. The TVFA method of recovery was twice-weekly as described by Gibbons et al. (19).

Pronuclear Microinjection

After 14 to 16 h of co-incubation with enriched sperm, ova were removed from fertilization wells and vortexed (2 min, 15 sec) to remove cumulus cells. Approximately 20% of the zygotes were placed directly into the coculture system at this time to serve

as noninjected controls. The remaining zygotes were centrifuged (12,00 x g for 6 min) to enable visualization of the pronuclei. Microinjection of DNA was performed in TL HEPES on a heated stage (39°C). Two gene construct were used for microinjection. The first was a 12 kb fragment made up of genomic human protein C (HPC) under the direction of the murine whey acidic protein (WAP) promoter (14,24). The second gene construct used for microinjection was a combined construct consisting of three different fibrinogen (FIB) fragments. All of the FIB constructs were under the direction of the WAP promoter and contained alpha (α; 5.6 kb), beta (β; 5.5 kb), and gamma (γ; 5.4 kb) chains (Velander, unpublished data). One to three pl of DNA solution (1.5 mg/ml DNA; 100 copies/pl) was injected into the pronucleus of each zygote. Oocytes in which 2 pronuclei were not visible were considered nonfertilized and discarded.

Germinal Vesicle Microinjection

To create the Germinal Vesicle (GV) microinjection-derived embryos, oocytes were removed from meiotic arrest medium (6-diethylaminopurine; DMAP, 8) approximately 18 hr after aspiration from the ovary. Oocytes to be injected were washed 4 times in TL HEPES and vortexed to remove cumulus cells. After centrifugation (12,000 x g; 6 min) the GV was visualized and the DNA solution deposited. Maturation of these oocytes was assisted by combining the cumulus-free oocytes with cumulus-intact oocytes at a ratio of 1:1. In vitro fertilization was performed in the same manner as in the pronuclear microinjected zygotes.

In Vivo culture

Following microinjection, some (~20%) zygotes were cultured in the ligated oviducts of mature ewes as described by Wolfe and Kraemer (26). Briefly, The microinjected zygotes were embedded in agar chips (~25 zygotes/chip) and placed in the ligated sheep oviducts by means of a positive pressure plunger device. Agar chips were recovered via retro-grade flushing of the oviducts after 5 d of culture and the viable embryos removed from the chips. Embryos considered suitable for transfer were transferred to recipients at this time.

In Vitro Culture

After microinjection, embryos were cocultured in wells (~25 embryos/well) containing Buffalo Rat Liver (BRL; 200,00 cells/well) cells and modified culture medium (500 μl). This medium consisted of TCM 199, 10% FCS, 1% BSA (Sigma), 1% P/S, and was fortified with 0.1 mM non-essential amino acids (Gibco), 2 mM glycine, and 1 mM alanine (Sigma). Embryos were moved to a fresh culture well containing newly prepared BRL cells on day 4 of culture. On day 7 of IVC embryos from the injected and control groups were removed from culture and scored for development.

The grade and stage of each embryo was recorded. All embryos were evaluated as follows: grade 1 = very little (<5%) or no extruded material, consistent, organized cell mass and trophoblast with no discolored regions, grade 2 = less than 10% of total cell mass extruded, some disorganization or discoloration, grade 3 = less than 25% of cell

mass extruded, considerable disorganization or discoloration. All other embryos were considered non-viable.

Embryo Freezing and Thawing

When no synchronous recipients were readily available for transfer, viable embryos were frozen for later transfer. Embryos were allowed to equilibrate in a 1.4 M glycerol (Sigma) solution as described by Voelkel (25). Embryos were loaded into a .25 ml straw (1 per straw) in PBS plus 10% vol/vol glycerol and placed in an alcohol-bath embryo freezer. Straws were equilibrated at -7°C for 3 min and then manually seeded. Straws were cooled at a rate of -.5°C/min to -35°C and plunged in liquid nitrogen. Straws were thawed in 30°C water and the embryos discharged from the straw. Embryos were moved through 3 sucrose rehydration steps (25) and maintained in 39°C PBS until transfer to recipients (< 1 hr).

Embryo Transfer and Pregnancy Evaluation

Embryos were loaded into .25 ml straws in PBS supplemented with .4% BSA and 1% vol/vol Pen Strep, and transferred to the uterine horn ipsilateral to the ovary with a palpable corpus luteum. Embryo transfers were conducted with the aid of a side-port transfer gun (IMV, "Mini Syringe"; Professional Embryo Transfer Supply, Canton, TX). Deposition site of the embryos was cranial to the external bifurcation of the uterus. Only dairy-type (Holsteins and Jerseys) recipients exhibiting estrus within 24 hr (+/-) to the time of IVF were used. Pregnancy was evaluated at 30 d of gestation using a 5 MHz

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linear transducer and a real-time ultrasound monitor (Aloka 500-V; Corimetrics, Wallingford, CN). Only fetuses having a detectable heartbeat were considered viable (1). Pregnant animals were re-examined at 60 d of gestation to determine maintenance of the pregnancy. No further examination was performed on pregnant animals unless parturition was overdue (10 d). One recipient (Jersey) received assistance during parturition.

Statistical Analysis

Variables such as type of construct, embryo age at transfer, and type of pre-transfer culture method were altered to determine the environment best suited for establishment of a pregnancy from a microinjected embryo. Fresh vs. frozen embryo and stages of gestation data were analyzed using Chi Square analysis.

RESULTS

Fresh vs. Frozen/Thawed Embryos

The embryos resulting from GV microinjected oocytes produced no pregnancies (0/14; pregnancies/transfers) and that data was excluded from Tables 1 and 2. Initial pregnancy rates (30 d) were not different between fresh and frozen/thawed embryos (14/45 vs. 8/32; Figure 1). However, the pregnancy rates for the frozen/thawed embryos declined significantly ($p < 0.05$) between day 30 and 60 of gestation (8/32 to 3/32; Figure 1). Because of this decrease, the embryos transferred fresh held a significant advantage ($p < 0.05$) over the frozen/thawed embryos at 60 d of gestation.

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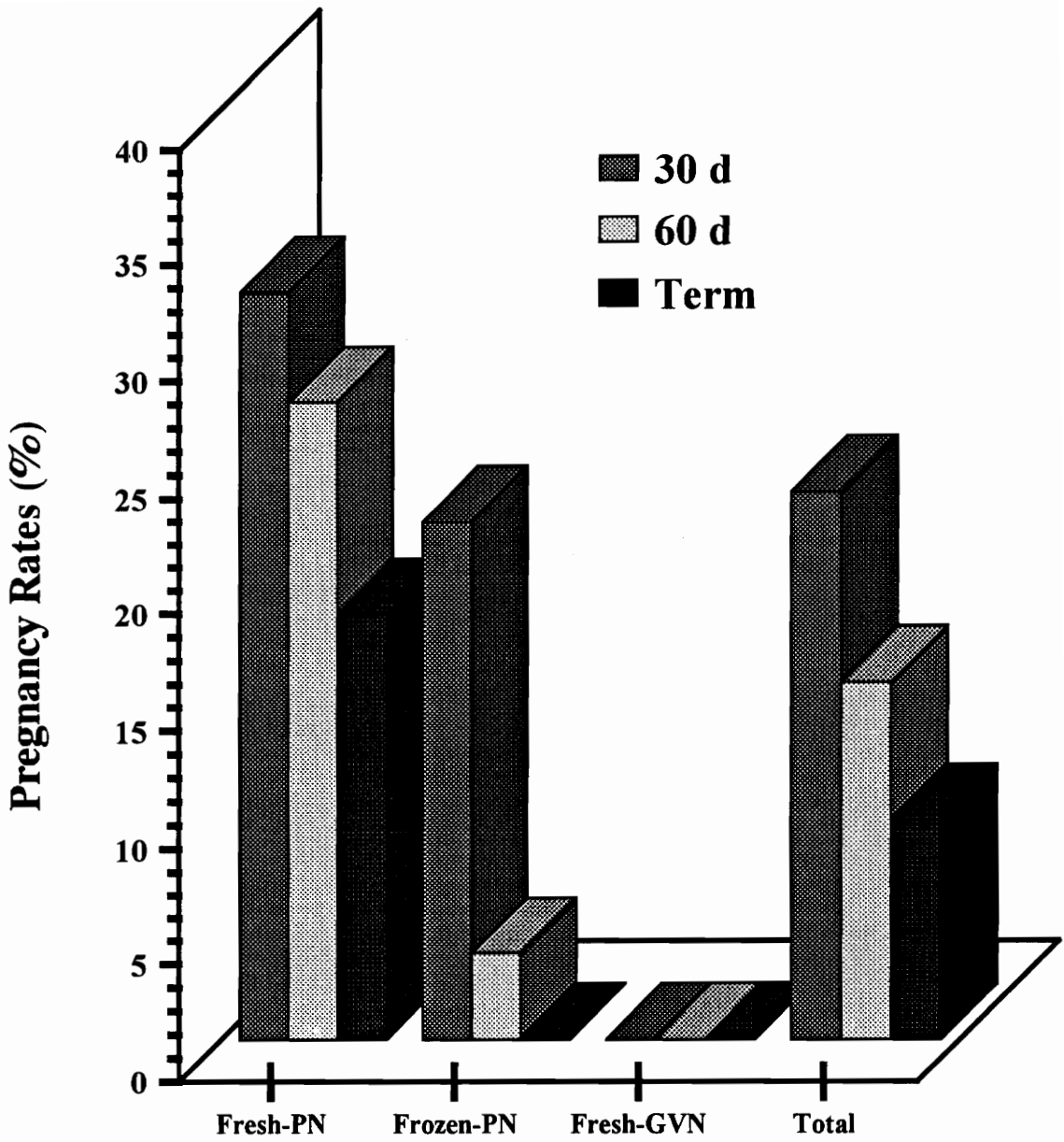
Figure 1. Type of Embryo Transferred

PN=Pronuclear microinjected
GVN=Germinal vesicle microinected

Four of the 8 term pregnancies are ongoing and are about 200 d of gestation. The fresh embryo pregnancy rates were not different between cows (19/60) and maiden heifers (6/20) used as recipients (p>0.10), although, heifers were only transferred to once while cows may have been transferred to many times throughout these observations.

Gene Construct Effects

There was no noticeable differences in pregnancy rates (30 d) between those embryos which had been microinjected with FIB and those microinjected with HPC constructs (p>0.10). Fresh and frozen embryo data was combined for the comparison of the two constructs and the data are displayed in Table 1.

Table 1. Pregnancy rates using embryos microinjected with human protein C (HPC) construct and fibrinogen (FIB) construct.

Gene Construct	# Pregnant / Total transferred	Percent Pregnant[a]
HPC	13 / 51	25.5
FIB	9 / 26	35.6

[a] Pregnancy rates (30 d) were not different between constructs used for microinjection (p>0.10)

Effects of Oocyte Source

The source of the oocytes had no significant effects on the pregnancy rates (30 d; p>0.10). The data comparing oocytes recovered from cows at slaughter and TVFA-

derived oocytes are displayed in Table 2. There was no significant interaction between source of oocytes and construct, or between construct and type of embryo transferred (fresh or frozen/thawed;p>0.10).

Table 2. Pregnancy rates resulting form oocytes recovered from slaughtered cows (SHD) and oocytes recovered during TVFA.

Oocyte Source	# Pregnant / Total transferred	Percent Pregnant[a]
SHD	7 / 26	26.9
TVFA	15 / 51	29.4

[a] Pregnancy rates (30 d) were not different between sources of oocytes used for microinjection (p>0.10)

Effects of Culture Method

There was no difference in pregnancy rate between embryos cultured in vivo and embryos produced in vitro (p>0.10). There seemed to be no recipient synchrony (+24, +12, 0, -12, -24) effect on pregnancy rate and a synchrony by construct, embryo type (fresh or frozen), or source of oocyte (SHD or TVFA) interaction was not evident (p>0.10). Pronuclear microinjected zygotes produced more early pregnancies (25/80 vs. 0/14; 30 d gestation) than the GV microinjected zygotes (p<0.05).

Abortion Observations

A fetus from a SHD microinjected (HPC) tested positive for the transgene in all of the tissues tested. The recipient carrying this fetus was found dead of unknown causes

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at 6.5 mo gestation. There were several other incidences of "late" abortions (>100 d) and at least one recipient aborted approximately 200 d into gestation. Another recipient was diagnosed with cystitis and pyonephritis and treated but subsequently lost the fetus. This fetus tested negative for the presence of the transgene. The recipient aborted on day 96 of gestation and it is unclear if the infection or the treatment contributed to the abortion.

DISCUSSION

The data from these observations suggest that transgenic individuals can be produced through pronuclear microinjection of bovine oocytes. With only a limited number of recipients available for transfer and subsequently only a small number of pregnancies, it is not known if the percentage of transgenics produced as a function of the number of zygotes microinjected in this data is comparable to the current literature (2). The pregnancy rates from pronuclear microinjected embryos seem to parallel current (2) and previous experimentation (11).

The decrease in viable pregnancies from 30 d to 60 d of gestation in the frozen/thawed embryos is confusing. The pregnancies from embryos transferred fresh experienced a slight decline from 30 to 60 d. However, this decline was less than with the previously frozen embryos. It is not clear if the significant decrease in the frozen embryo group is a function of the freezing process or an artifact created by few

observations. The overall abortion loss (fresh and frozen) appears higher for in vitro produced microinjected embryos than for non-microinjected in vivo generated embryos (12). However, this loss (~30%) is consistent with other studies involving in vitro generated microinjected embryos (2,11). The effects of gene construct used in microinjection on overall pregnancy rate in these observations cannot be readily compared with previous findings due to the complexity of individual constructs (2,11,17,24). It is possible that some gene constructs because of there size and structure may be less harmful to the zygote and the developing embryos may be more able to maintain a pregnancy when transferred to a recipient.

The overall initial pregnancy rates from in vitro produced microinjected embryos in these observations are consistent with previous findings in this and other laboratories (2,11,22). The data seems to suggest that embryos should be transferred fresh if possible. This requires a great number of recipients which may be financially limiting. In an attempt to increase the efficiency of these recipients an embryo biopsy can be taken and only the embryos testing positive for the presence of the transgene actually be transferred (2). However, the method of transgene detection must be further refined in order to establish a confidence that the embryos testing positive for the transgene are actually transgenic and not "false positives" or mosaics.

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APPENDIX B

CONSUMER COST ANALYSIS
CONVENTIONAL EMBRYO TRANSFER VS. TRANSVAGINAL FOLLICULAR
ASPIRATION AND IVM/IVF/IVC

EMBRYO TRANSFER (FRESH TRANSFER)

COLLECTION FEE (DRUGS, SUPPLIES, LABOR)	$350.00/DONOR
TRANSFER FEE (SUPPLIES, LABOR)	$75.00/EMBRYO
TRANSFERABLE EMBRYOS PER COLLECTION	5
TOTAL FEES FOR 5 TRANSFERS FROM 1 DONOR	$725.00
PREGNANCY RATE (FRESH)	55%
CALVES PER COLLECTION	2.5
COLLECTIONS PER DONOR PER YEAR	
(1 COLLECTION EVERY OTHER MONTH)	6
NUMBER OF CALVES PER DONOR PER YEAR	15
TOTAL COST OF PRODUCING CALVES	$4,350.00

COST PER CALF PER YEAR $290.00

TRANSVAGINAL FOLLICULAR ASPIRATION

COLLECTION FEE (ASP, U/S USE, SUPPLIES, LABOR)	$75.00/COLL
NUMBER OF OVA RECOVERED PER DONOR	8.5
PROCESSING FEE PER COLLECTION	
(IVM/IVF/IVC SYSTEM, SEMEN, LABOR)	$50.00/COLL
DEVELOPMENT OF OOCYTES TO BLASTOCYST	20%
TRANSFERABLE EMBRYOS PER COLLECTION	1.7
TRANSFER FEE	$75.00/EMBRYO
PREGNANCY RATE (FRESH)	50%
TOTAL COST FOR 1 ASP AND TRANSFER OF	
RESULTING 1.7 EMBRYOS	$252.50
NUMBER OF CALVES PRODUCE FROM SINGLE ASP	.85
ASPIRATIONS PERIODS PER YEAR	
(2X WEEK FOR 4 WEEKS WITH 4 WEEK REST)	6
NUMBER OF SESSIONS PER YEAR	48
NUMBER OF CALVES PER DONOR PER YEAR	40.8
COST OF PRODUCING CALVES ($252.50 X 48)	$12,120.00

COST PER CALF PER YEAR $297.05

OTHER COSTS

SPERM CHARACTERIZATION

RECIPIENT AND DONOR MANAGEMENT

RECIPIENT COST

EMBRYO FREEZING FEES

ON THE FARM COLLECTION AND/OR TRANSFER

TAXES AND INTEREST ON CAPITAL EXPENDITURE

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CAREER OBJECTIVES:

I am interested in research and development of new products and services as they relate to technological advances in the area of artificial or assisted reproduction in domestic livestock, specifically the Bovine species.

EDUCATION

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY, BLACKSBURG, VIRGINIA

Master's of Science, Dairy Science Department. Thesis research involves ultrasound- guided transvaginal oocyte collection and in vitro embryo production in the Bovine. Explored several aspiration schedules to obtain the maximum number of favorable quality oocytes for In Vitro Maturation, Fertilization and Culture. Degree expected in May, 1994.

TEXAS A&M UNIVERSITY, COLLEGE STATION, TEXAS

Bachelor of Science, Animal Science Department. Industry option with influence on artificial insemination and non-surgical bovine embryo collection and transfer. Performed research projects involving reproduction and maintenance of miniature horses. Received undergraduate credits while learning experimental design and logistics. B.S., December, 1988.

WORK EXPERIENCE

TRANSPHARM, INC., Blacksburg, VA. - March 1993-present,

Duties include ultrasound examination of Angus heifers to determine reproductive soundness, presence of corpus luteum, and pregnancy status. Perform in vitro maturation, fertilization, and culture (IVM/IVF/IVC) of bovine oocytes to produce transferable quality pre-implantation stage embryos. Aid in microinjection of DNA into 1-cell embryos to produce transgenic embryos. Involved in embryo biopsy, Polymerase Chain Reaction (PCR), and cell culture technologies.

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AMERICAN BREEDERS SERVICE, DeForest, WI, January, 1992 - August, 1992

Lab Technician IV, Specialty Genetics Division
Accomplished in all aspects of nuclear transfer. Exposed to experimental design schemes related to different methods of performing nuclear transfer. Involved in several research projects to increase the efficiency of cloning through new methods of electrofusion and various chromatin recognition regimes. This involved both chemical and fluorescent microscopy methods. Trained on Nikon and Zeiss inverted microscopes equipped with Narishegee micromanipulators. Performed ultrasound-guided transvaginal oocyte recovery and In Vitro maturation and fertilization to provide a source of 32 cell-stage embryos to be used in the cloning process.

GRANADA BIOSCIENCES, INC., Marquez, TX, March, 1989 - September, 1991.

Lab Technician, Nuclear Transfer Laboratory, Jan. 1991 - September 1991.
Trained in all aspects of bovine nuclear transfer micromanipulations. Experienced in oocyte recovery form abattoir ovaries, In Vitro maturation, aging, and selection of oocytes to be used as recipient cytoplasm. Efficient in preparation of oocytes for the nuclear transfer procedure, as well as donor embryo selection, separation, recombination, electrofusion, and in vivo or in vitro culture. Accomplished in collection and transfer surgeries for in vivo ovine culture.

Lab Technician, Embryo Transfer Production Unit, March 1989 - Jan. 1991.
Two years experience in commercial bovine embryo transfer. Skilled in all modern aseptic laboratory techniques associated with bovine embryo transfer. Experienced in embryo collection, evaluation, processing, freezing, and transfer. Versed in embryo biopsy for sex determination and splitting micromanipulations. Travelled extensively assisting veterinarian in on the farm bovine embryo transfer. Also associated with several research projects including:
                    - IVM/IVF/IVC embryo freezing, thawing, and transfer
                    - Apical trophoblast recognition in 14 d in vivo derived embryos
                    - Ethylene Glycol freezing and direct transfer procedures
                    - Bovine embryo cryoprotectant analysis
                    - Polar body enucleation procedure

TEXAS AGRICULTURE EXTENSION SERVICE, Overton, Texas,
Beef Cattle Research Technician. Summer, 1988; Spring. 1989
Responsible for 600 acres and from 300 to 450 Braford cows and calves. Fed, monitored and weighed test animals in several forage and grain studies, including stocking rates, nutrient recycling, nitrogen and potassium absorption tests, and seed and clover analysis. Aided in calving and managing 125 Brahman cows and heifers.

PUBLICATIONS AND PRESENTATIONS

Betthauser, J.M., Scott, B.M., **GIBBONS, J.R.,** Stice, S.L., The use of Hoechst 3342 to stain donor nuclei used in the Bovine Nuclear Transfer procedure. Theriogenology 39:187 (1993, abstr.)

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Attended International Embryo Transfer Society (IETS) meeting, Baton Rouge, LA. January, 1993

Presented research at IETS meeting, Melbourne, Australia, January, 1994

Seminar; Dairy Reproduction Cow College, VPI&SU, Blacksburg, VA. Feb, 1994

AWARDS AND HONORS

Artificial Insemination Certificate - May, 1988
Distinguished Student Award - Spring, 1988
Quantico Community Scholarships - June, 1984
Eagle Scout - 1984

ACTIVITIES AND PERSONAL DATA

Intramural Sports, TAMU, VPI
Saddle and Sirloin Club, TAMU
Horsemanship Club, TAMU
Klein Hometown Club, Charter member, TAMU
    - membership co-chairman
Born September 29, 1966, Dallas Texas
Enjoy sports of all kinds

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