IN-VITRO DEVELOPMENTAL POTENTIAL OF BOVINE OOCYTES OBTAINED BY TRANSVAGINAL FOLLICULAR ASPIRATION AS RELATED TO THEIR MORPHOLOGICAL QUALITY AND AFTER MICROINJECTION OF DNA

by

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(ABSTRACT)

The development of oocytes of differing quality retrieved using transvaginal follicular aspiration (TVFA) and following DNA injection was examined. Eight cows were subjected to twice weekly TVFA for 16 wk. Oocytes retrieved were graded and placed in an in-vitro maturation, fertilization and co-culture (IVM/IVF/IVC) program. Two thirds of oocytes were injected with DNA. Good quality oocytes from slaughtered cows (SH) were obtained once monthly and processed the same way. Good quality TVFA oocytes had a higher mean development score than poor quality oocytes, but not different from that of good quality SH oocytes. Good quality TVFA oocytes produced more viable embryos (31.7% blastocysts) than poor quality oocytes or SH oocytes (12.8% and 20.4% blastocysts, respectively). Embryo development following injection of DNA was the same for oocytes for each source-quality group (TVFA-good, 8.4; TVFA-poor, 5.5; SH-good, 6.3 % blastocysts). Development of good quality TVFA oocytes increased

during the last 9 wk of the 16 wk collection period. Poor oocyte development increased slightly to 9 wk and then decreased. Development of TVFA oocytes injected with DNA did not vary during the experiment. However, development of controls increased from a mean score of 2.50 at wk 1 to 4.17 at wk 16. Oocytes from TVFA produced more PCR positive blastocysts (95.0%) than SH oocytes (61.5%). More calves were born from the transfer of embryos injected with DNA from TVFA oocytes (3/5) than from SH oocytes (1/6), although not statistically significant. One calf was PCR positive in bone-marrow, but was negative in other tissues. The use of oocytes obtained by TVFA may improve the efficiency of producing transgenic cattle.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The establishment of in-vitro maturation (IVM), fertilization (IVF) and culture (IVC) systems for bovine oocytes has allowed for the production of transferable bovine embryos, and has provided a reliable source of embryos for gene transfer experiments. Large numbers of embryos at the same stage of development can be produced, manipulated and cultured to the blastocyst stage entirely in-vitro. Development of in-vitro produced embryos following microinjection of DNA remains low, as does the efficiency of producing calves from the transfer of such embryos. Thus, thousands of embryos must be injected with DNA and a large herd of recipient animals must be maintained to produce a single transgenic calf. Improving the development of in-vitro produced DNA microinjected embryos would increase the efficiency of producing transgenic cattle.

Previously, most oocytes for in-vitro embryo production have been obtained from the ovaries of slaughtered cows. Thus the reproductive history, genetics and general status of donor animals is unknown and may affect the developmental potential of oocytes obtained. The development of ultrasound-guided transvaginal follicular aspiration (TVFA) techniques has made it possible to obtain oocytes repeatedly from the same donor over extended periods of time. However, quality of such oocytes varies when they are classified by their layers of cumulus cells and cytoplasmic appearance. Previous studies using TVFA oocytes have utilized all oocytes obtained, whereas experiments using slaughter-house oocytes (SH) have generally used only those oocytes classified as good quality. Thus, it is unknown how TVFA oocytes compare to SH oocytes when they are

matured, fertilized and cultured in-vitro. In addition, it is unclear whether different quality grades of TVFA oocytes have higher rates of development following microinjection of DNA than those obtained from ovaries of slaughtered cows.

The objectives of this study were 1) to determine the developmental potential of TVFA derived oocytes as related to their morphological quality when they are matured, fertilized, and cultured in-vitro, and following DNA microinjection; and 2) to compare developmental potential of good quality TVFA derived oocytes to that of SH derived oocytes in the same IVM/IVF/IVC, DNA-microinjection program. In addition, viable DNA-microinjected embryos were transferred when recipient cows were available to produce calves transgenic for a microinjected whey acidic protein-human fibrinogen DNA construct.

REVIEW OF LITERATURE

Bovine In-Vitro Embryo Production

Bovine oocytes can now be routinely fertilized and matured in-vitro (Trounsen, 1992; Trounsen et al., 1994). However, the bovine embryo exhibits an irreversible developmental block at the 8 to 16 cell stage when cultured in-vitro (Thibault, 1966; Eyestone and First, 1986). To overcome this block, various co-culture systems have been developed. Most of these have utilized cells of the reproductive tract. Kuzan and Wright (1982) used uterine fibroblast monolayers and found that a higher percentage of morula developed to hatched blastocysts than with medium alone. Also, an endometrial fibroblast monolayer system supported viability of demi-embryos (Voelkel et al., 1985). Kuzan and Wright (1982) suggested that fibroblasts were either releasing a development promoting factor or removing a toxic substance from the medium. However, medium conditioned with fibroblasts had no effect on development in their study.

In the mid nineteen-eighties, Camous et al. (1984) developed a co-culture system utilizing trophoblastic vesicles. When harvested at the proper time from bovine conceptuses, these trophoblastic vesicles in co-culture produced more blastocysts than medium alone (Camous et al., 1984; Heyman et al., 1987). Also, it was proposed that direct contact between the developing embryo and the trophoblastic cells was not necessary (Heyman et al., 1987). Although this technique has successfully produced viable embryos and a live calf, obtaining trophoblastic vesicles is difficult and time consuming.

Cumulus cells have been used successfully in co-culture and were used to produce the first calves from bovine oocytes matured, fertilized and cultured in-vitro (Fukuda et al., 1990). However, the proportion of blastocysts developed was only 9% (Fukuda et al., 1990) and 15.1% (Goto et al., 1988) with cumulus cell co-culture. Therefore, even though this system produced viable embryos, efficiency of production was low, and would not be acceptable in a gene transfer program where there is additional embryonic death due to embryo manipulation.

Co-culture systems using bovine oviductal epithelial (BOE) cells, introduced by Eyestone et al. (1987), have been used widely. Eyestone et al. (1987) found that 46% of 5-8 cell embryos cultured with BOE cells reached late morula or blastocyst stages, compared to none in medium alone. In addition, development in medium conditioned with BOE cells was equal to that in the co-culture system (Eyestone and First, 1989). Subsequent studies have shown oviductal epithelial cells to be superior to other tested cell types for co-culture (Aoyagi et al., 1990; Rexroad, 1989). Also, embryos co-cultured with BOE cells developed faster than those cultured on other cell type monolayers (Goto et al., 1992). Thibodeaux and Godke (1992) concluded that oviductal cell monolayers were the most effective system for early stage farm animal embryos, while fibroblasts were adequate only for development of later stage embryos.

Inconsistent results using co-culture systems have made duplication of experiments difficult. While some researchers have shown reproductive cells to be superior to non-reproductive cells in co-culture (Rexroad, 1989), others have found no difference in development to blastocyst when using cells from different embryological origin and

species (Goto et al., 1992). Bavister et al. (1992) reported different results using the same conditions as others. He saw no block to development with medium alone (TCM-199 + serum) and no improvement in development with oviductal cell co-culture. Therefore, he proposed that the block was due to some factor introduced into the medium (perhaps by heating of serum) which was overcome by co-culture removing the factor. Bavister et al. (1992) and others (Thibodeaux and Godke, 1992; Brackett and Zuelke, 1993) have called for the development of a more defined culture system for bovine embryos and characterization of cell types and factors used, thus allowing for less variability from laboratory to laboratory. Synthetic oviductal fluid (SOF) plus amino acids was shown to yield development rates to morula and blastocyst stages not different from co-culture methods (Trounsen et al., 1994). However, embryos produced in cell-free culture were of lower quality than those from co-culture (Goto et al., 1994).

Reducing the variability of bovine embryo co-culture may be possible using a permanent, established cell line. Buffalo Rat Liver (BRL) cells have been successfully used to culture one-cell bovine embryos to the late morula and blastocyst stages (Voelkel and Hu, 1992; van Inzen et al., 1992) and produce calves (Krisher et al., 1994). These cells are easily maintained in culture and cell numbers are highly reproducible between replicates and experiments. Hasler et al. (1994) reported that different populations of BRL cells provide comparable support for bovine in-vitro culture. The need for primary cells from oviductal or uterine tissues is eliminated, and the same cell lines can be purchased and used in many laboratories. BRL cells secrete multiplication-stimulating activity, now termed rat IGF-II (Marquardt al., 1981), as well as transforming growth

factor β (TGF-β; Massague et al., 1985). They also produce differentiation-inhibiting activity, which exhibits a negative control over embryonic stem cell differentiation (Smith and Hooper, 1987). The production of these growth factors may account for the ability of BRL cells to enhance embryo development in-vitro, whereas little is known about other cell types used in co-culture.

Van Inzen et al. (1992) directly compared BRL cell co-culture to BOE cell co-culture and found no difference in the production of blastocysts or in the number of cells in the blastocysts. There was also no difference between the number of blastocysts formed in BRL cell co-culture or in culture in ligated sheep oviducts. Additionally, Voelkel et al. (1992) showed that embryos produced by BRL cell co-culture have enhanced capacity to survive freezing. More recently, several laboratories have reported equivalent (Reed et al., 1996; van Inzen et al., 1995; Collins and Wright, 1994; Hawk and Wall, 1994b) or increased (Rehman et al., 1994) development of embryos to the blastocyst stage when comparing culture with BRL cells to BOE cells. BRL cell co-culture has been used successfully in our laboratory, producing 40.8% viable morulae and blastocysts (Gibbons et al., 1995). Calves produced from oocytes matured and fertilized in-vitro and co-cultured with BRL cells have been born as well. (Krisher et al., 1994). Alanine and glycine were shown to enhance embryonic development in a co-culture system (Moore and Bondioli, 1993) and non-essential amino acids were shown also to be beneficial (Rosenkrans and First, 1994; Brackett and Keskintepe, 1994). These compounds have been added to embryo culture medium during BRL cell co-culture (Krisher et al., 1994).

Using BRL cells to co-culture bovine embryos ensures good development rates

to the blastocyst stage. In addition, there is consistency in results throughout the duration of experiments and across experiments and laboratories. This would not be possible using media alone or primary cell co-culture methods such as BOE cells. Embryos produced by IVM, IVF and BRL cell co-culture are therefore good candidates for entry into a gene transfer program.

Ultrasound-Guided Transvaginal Follicular Aspiration

The development of ultrasound-guided transvaginal follicular aspiration techniques has provided a source of bovine oocytes from live animals (Pieterse et al., 1988; Pieterse et al., 1991). In addition, oocytes can be collected repeatedly from the same donor animal over extended periods of time. Briefly, ovaries and follicles are visualized by ultrasound, and a 17g needle is passed through a needle guide in a vaginal probe to collect oocytes and follicular fluid under a vacuum pressure. Ovaries are rectally manipulated to allow the needle access to each visible follicle. Oocytes obtained in this manner can be placed in an IVM/IVF/IVC program to produce embryos of transferable quality (van der Schans et al., 1992; Pieterse et al., 1991). Embryos produced using this technique also have been microinjected with DNA, frozen, and subsequently transferred, producing 7 pregnancies and two calves born (J.R. Gibbons, unpublished data).

Laparoscopic follicular aspiration has been performed on calves (Armstrong et al., 1992), and transvaginal follicular aspiration has been performed on pregnant cows (Mientjes et. al, 1995), heifers (Bergfelt et al., 1994), and cows at all stages of the estrous cycle (van der Schans et al., 1991). TVFA was used to collect follicular fluid from

individual follicles (Vos et al., 1994), and induce subsequent follicular development and synchrony of ovulation in heifers following $PGF_{2\alpha}$ administration. The procedure has been used commercially to produce embryos and pregnancies from infertile and problem cows that fail to respond to traditional superovulation and embryo transfer protocols (Hasler, 1993; Looney et al., 1994). Commercial breeders have modified the equipment (Matthews et al., 1994) and procedures for manipulating ovaries (Hill, 1995) for easier use in the field. It is clear that this procedure holds promise for providing a source of oocytes for in-vitro embryo production.

A large amount of research has focused on establishing aspiration schedules that are most efficient at recovering oocytes without being detrimental to the donor animal. Van der Schans et al. (1991) found that cows treated once a week with 500 IU of PMSG produced more cumulus oocyte complexes (COCs) per week with twice weekly aspiration than with once weekly aspiration. He also found no damage to genital tracts of these cows upon slaughter. Simon et al. (1993) aspirated cycling cows at either 48 or 96 h intervals and found higher numbers of follicles and oocytes available with the shorter interval. It was proposed that shorter intervals prevented the formation of a dominant follicle, thus allowing for other follicular development. Gibbons et al. (1994) aspirated follicles of beef cattle once or twice weekly, or twice weekly after exogenous follicle stimulating hormone (FSH) stimulation. The twice weekly aspiration schedule yielded more ova per week than the once weekly group and in addition produced more transferable embryos of higher quality following IVM/IVF/IVC. There was no effect of FSH treatment on number of ova recovered per week.

Other researchers have shown that exogenous hormone stimulation does not necessarily improve the efficiency of TVFA. Pieterse et al. (1992) found that cows stimulated with PMSG had more follicles available for collection, but the recovery rate of ova was low, thus negating any advantage of PMSG stimulation. Stubbings and Walton (1995) compared cows stimulated with FSH subjected to once weekly TVFA with unstimulated cows subjected to twice weekly TVFA. They found no difference in the average number of follicles available per week for aspiration between the two groups. Over the eight week experimental period, only the unstimulated twice weekly aspirated cows showed a significant increase in follicles available for aspiration. It was concluded that bi-weekly aspiration resulted in an increased number of follicles available which was comparable to the average weekly number of follicles in stimulated cows. In addition, by the end of the 8 wk period, FSH stimulated cows had enlarged ovaries with luteal masses which made manipulations during TVFA more difficult. Similarly, Bungartz et al. (1995) found that for twice weekly aspiration, FSH treatment increased the number of follicles available, but did not increase oocyte yields. Meintjes et al. (1995) suggested that the low recovery of oocytes from FSH stimulated cows may be due to them having larger follicles which may require needle size or vacuum pressure adjustment.

To be used as a source for embryo production for transfer, TVFA oocytes must be able to undergo IVM/IVF/IVC and develop to the blastocyst stage as well as those from other sources. Some reports have shown that development was equal to that of embryos produced from oocytes collected from slaughtered cows (27% vs 25-40% and 26.1% vs 27.9% blastocysts; van der Schans et al., 1992; Gibbons et al., 1994;

respectively). In a study by Kruip et al. (1990), SH oocytes appeared developmentally superior to TVFA oocytes (24.3% vs. 15.3% blastocysts, respectively). However, when numbers were adjusted because SH oocytes were selected and aspirated ones were not, there were significantly more transferable embryos produced by TVFA. Armstrong et al. (1992), using oocytes aspirated from calves, had higher rates of development to morula and blastocysts (as a proportion of cleaved ova) than with SH oocytes processed concomitantly (91% morulae, 27% blastocysts vs. 59% morulae, 18% blastocysts, respectively). In a later study no difference in development to the blastocyst stage was found between aspirated calf oocytes and slaughter cow oocytes (30% vs 25% blastocysts, respectively; Armstrong et al., 1994). The developmental potential of aspirated bovine oocytes appears good although more research is needed.

In another study, Gibbons et al. (1995) graded TVFA oocytes into two categories: good: having 3 or more layers of cumulus cells and an organized cytoplasm; and poor: having less than 3 layers of cumulus cells or a disorganized cytoplasm. These categories were used to compare oocytes aspirated from different cows over a three month period. Overall, 52.5% of total oocytes collected from 9 cows were of good quality, and the remainder were graded poor quality. Some oocytes were designated for DNA microinjection and others served as non-injected controls. Non-injected oocytes of both quality grades were cultured together and yielded 40.8% development to morula and blastocyst. It is not known how many of these embryos developed from poor quality oocytes, if any, since they were cultured together. Development of the poor quality TVFA oocytes may have been enhanced by placing them in culture with oocytes having

good cumulus investments (Mermillod et al., 1992). Slaughter cow oocytes processed at the same time developed at significantly lower rates (30.0% to morula and blastocyst) even though they were all of good quality. In addition, a higher proportion of embryos generated from TVFA oocytes was of excellent or good quality following culture. To date, there have been no studies directly comparing the developmental potential of good quality TVFA oocytes to selected good quality SH oocytes.

Oocyte Quality and Developmental Potential

In-vitro maturation, fertilization and culture of bovine oocytes of varying quality have been studied using oocytes from ovaries of slaughter cows. Liebfried and First (1979) showed than only 44% of oocytes lacking cumulus investments matured in vitro, whereas 71% of cumulus intact oocytes matured. Shioya et al. (1988) observed significantly lowered maturation and fertilization rates for naked oocytes. However, they pointed out that naked oocytes already were degenerate before maturation and therefore lack of cumulus cells could not be blamed for low maturation rates. Behalova and Greve (1993) reported polyspermy increased in denuded eggs. This could account for subsequent developmental arrest. A study by Sirard et al. (1988) showed that the number of cumulus cells per unit volume in culture did not significantly effect later development. Removal of cumulus cells from oocytes before maturation did have adverse effects on maturation and development in-vitro. However, 40% of denuded oocytes cultured with cumulus cells cleaved and 11% of these developed to morula or blastocyst in ligated sheep oviducts. Yang and Lu (1990) found 48% cleavage of denuded oocytes after

IVM/IVF, but none developed to blastocysts when co-cultured with granulosa cells. The authors concluded that cumulus cells are necessary for in-vitro oocyte maturation, to enhance IVF and subsequent in-vitro development. Similarly, Younis and Brackett (1991) found a lower cleavage rate of denuded ova following IVF and reported they rarely developed into morula or blastocysts, even with co-culture on cumulus monolayers. Liu et al. (1995) showed that addition of cumulus cells during IVM does not improve development of denuded oocytes although it did improve cleavage rates following IVF. Staining procedures have shown that maturation and penetration of denuded oocytes can take place at rates similar to those of oocytes with intact cumulus cell layers (Chian et al, 1994). However, cumulus cell layers associated with the oocyte have proven to be important for proper maturation, male pronuclear formation, and subsequent development in-vitro (Cox et al.,1993; Chian and Niwa, 1994; Chian et al., 1994). Brackett and Zuelke (1993) proposed that cumulus cells affect the metabolic activity of the oocyte, providing a beneficial metabolic influence during IVM. It was suggested that in the absence of cumulus cells, sperm penetrate ova less effectively (Younis and Brackett, 1991). Cox et al. (1993) further postulated that the cumulus provides a capacitation-inducing mechanism and facilitates interaction between the capacitated sperm and the zona pellucida surface.

Mermillod et al. (1992) showed that the cumulus effect was independent of attachment to the embryo when embryos were cultured in BOE cell conditioned medium. However, the cumulus cells were intact during maturation and fertilization in this study and were removed prior to co-culture. Other studies also have shown cumulus cells to be less important during later stages of embryo development (Seidel et al.,1991; Thomas

and Seidel,1993).

The importance of cumulus cells during IVM, IVF and early in-vitro culture is well established. So, classification and selection of bovine oocytes for in-vitro embryo production usually focuses on the cumulus investments of the oocyte. Perhaps the most widely known classification system was devised by de Loos et al. (1989). It classifies oocytes into 4 categories based on cumulus investment and ooplasm appearance. Category 1 has a compact, multilayered cumulus investment, a homogeneous ooplasm and a total light and transparent appearance. Category 2 is similar however the ooplasm is slightly darker. In the third category oocytes are less compact and have a darker color than categories 1 and 2. Finally, category 4 have expanded or scattered cumulus cells and irregular ooplasm with dark clusters. In the study of de Loos et al. (1989), only the category 4 oocytes had a decreased developmental capacity. Other studies have shown marginal quality oocytes develop as well as good quality oocytes (Hawk and Wall, 1993;1994a; Wurth and Kruip, 1992).

Oocytes derived from TVFA range in quality, with generally half being considered of good quality (Gibbons et al., 1995; van der Schans et al., 1991) No previous studies have been conducted on oocyte quality and subsequent development using aspirated oocytes. Aspiration procedures may strip away some cumulus cells. However, in many oocytes partial layers remain, and may be enough to allow for successful IVM/IVF/IVC.

Production of Transgenic Embryos and Cattle

Production of transgenic livestock is expensive and inefficient (Wall and Seidel, 1992). Only 1% of injected ova become transgenic young in farm animal species (Wilmut et al., 1990). In cattle, the efficiency of producing transgenic offspring was only .2% (Bondioli et al., 1988, Krimpenfort et al., 1991). Low embryo survival rate after transfer and low integration frequencies hinder production of transgenic livestock most (Wall and Seidel, 1992). Until these problems are overcome, a large number of embryos and recipients will be needed. In-vitro embryo production can provide a relatively less expensive source and more embryos than superovulation (Thomas and Seidel, 1993; Sparks et al., 1994). In addition, embryos produced in this manner are in predictable stages of the cell cycle and therefore can be microinjected more efficiently (Thomas et al., 1993). Development rates are predictable and allow for better synchronization of embryo transfer as well. Several laboratories have already produced transgenic cattle using in-vitro embryo production (Krimpenfort et al., 1991; Hill et al., 1992; Bowen et al., 1994).

One problem with using in-vitro produced bovine embryos for gene transfer experiments is that they have been shown to have reduced developmental potential following microinjection (Gagne et al., 1990; Krisher et al., 1994). However, centrifugation of embryos, to visualize pronuclei prior to microinjection did not reduce embryo development (Wall and Hawk, 1988; Gagne et al., 1990), suggesting that the lack of development may be due to the microinjection procedure (Peura et al., 1993; Krisher et al., 1994) and increased mortality of embryos carrying mutations after integration of

foreign DNA (Roschlau et al., 1989). Additional methods to improve development of microinjected bovine embryos would greatly increase the efficiency of producing transgenic cattle.

Since development of TVFA oocytes may be better than oocytes from slaughter cows (Kruip et.al, 1990; van der Schans et al., 1992; Gibbons et al., 1994), it is possible that these oocytes may have an increased ability to develop following DNA microinjection. In addition, selected good quality TVFA oocytes may provide superior development rates when used in in vitro embryo production and gene transfer programs.

In-vitro maturation and fertilization of bovine oocytes, and culture of the resulting zygotes to the blastocyst stage is possible and can produce pregnancies and live births. In addition, transgenic calves can be produced by pronuclear microinjection of in-vitro produced embryos. Production of transgenic cattle remains inefficient, however, mostly due to low embryo survival rate following microinjection and low DNA integration efficiency. Improving the developmental capacity of microinjected bovine embryos would reduce the number of embryos that must be manipulated to make a transgenic animal, thus reducing the cost and labor involved in this pursuit. In-vitro produced embryos derived from TVFA oocytes may have superior developmental potential and may better tolerate embryo manipulation and microinjection procedures. The objectives of this study were to determine the developmental potential of TVFA oocytes of good and poor morphological quality when they are matured, fertilized and cultured in-vitro and subjected to DNA microinjection and to compare these findings to those using selected good quality SH oocytes processed in the same way.

MATERIALS AND METHODS

Synchronization of Cattle and Ultrasound-Guided Transvaginal Follicular Aspiration

Estrus was synchronized in six previously aspirated non-lactating Holstein cows (Gibbons et al., 1995) and 16 non-lactating Holstein cows using Synchromate B (Sanofi Animal Health, Overland Park, KS) ear implants. Ear implants were kept in place for 7 d. $PGF_{2\alpha}$ (25 mg; Lutalyse®; The Upjohn Company, Kalamazoo, MI) was administered i.m. 24 h prior to implant removal. Cows were watched for signs of estrus twice daily for 4 d following implant removal. Initial TVFA was performed between d 3 and 5 of their estrous cycle.

TVFA was performed on eight cows (four previously aspirated and four not previously aspirated, as part of an additional study) twice weekly (Sundays and Wednesdays) for 16 wk. The order of the cows was random for each aspiration day. Animals were sedated and cleaned, and all follicles > 2mm were aspirated as described in detail by Gibbons et al. (1994) using an Aloka 500V ultrasound machine (Corimetrics Medical Systems, Inc., Wallingford, CT) and a 5 mHz sector transducer packaged in a vaginal probe with dorsal mounted needle guide. Follicular fluid and oocytes were collected by vacuum (75-85mm Hg) through a 17g needle (RAM Consulting, Madison, WI) into an embryo filter (Professional Embryo Transfer Supply, Canton, TX). The embryo filter was 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) and oocytes were located with a 10X stereo microscope.

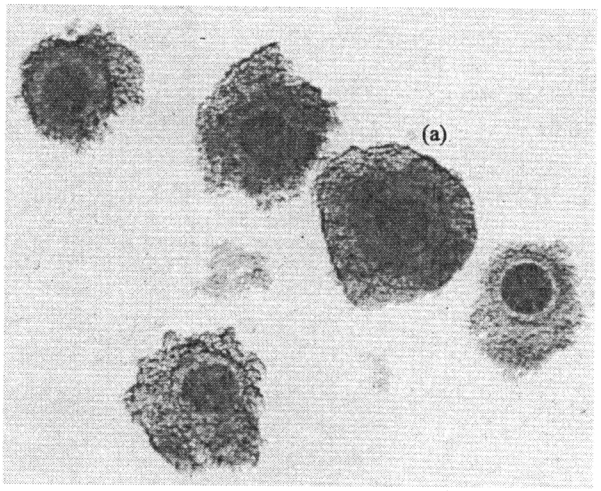
Oocyte Evaluation

Oocytes were classified as described by Gibbons et al. (1995), with good oocytes having an organized cytoplasm and 3 or more layers of cumulus cells. Poor oocytes were classified as having less than 3 layers of cumulus cells or a disorganized cytoplasm. Oocytes with over expanded cumulus cells were categorized as poor also (Figure 1).

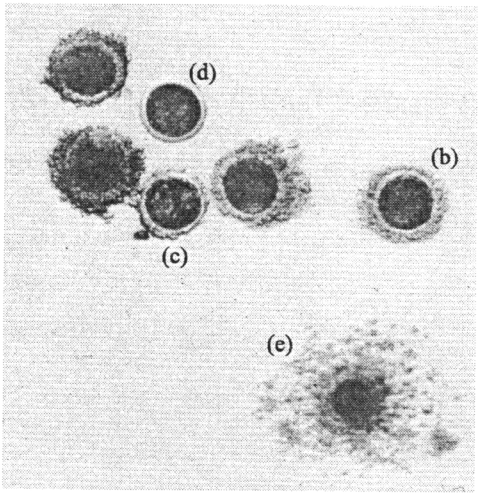
Oocyte Maturation, Fertilization, Culture and Microinjection

Oocytes were immediately rinsed three times in a TL Hepes buffered medium supplemented with BSA (3 mg/ml; Sigma) and 1% vol/vol penicillin-streptomycin (Gibco). Oocytes in the same classification group were placed in maturation medium of TCM-199 (Gibco) supplemented with 10% vol/vol fetal calf serum (FCS; Hyclone, Logan UT), bFSH and bLH (.01 IU each; NOBL Labs, Sioux Center, IA), and 1% vol/vol penicillin-streptomycin. Two to three clusters of free cumulus cells recovered from the aspiration fluid were added to each poor groups' maturation media. Oocytes in groups of 50 or less were maintained in 1 ml of maturation media with a silicone oil overlay for 22 to 24 h at 39^{o}C and 5% CO_2 and air (Krisher et al., 1994).

Frozen/thawed Holstein sperm was prepared using a Percoll separation procedure (Krisher et al., 1994). Frozen 0.5 ml straws of semen from a previously characterized ejaculate of a single bull were thawed in a 35^{o}C water bath for 1 min. Sperm were 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. Live sperm concentration was determined using a hemocytometer and sperm were added to fertilization wells (Nunclon 4-well



Good qualtiy oocytes



Poor quality oocytes

(a) Oocyte with organized cytoplasm and 3 or more layers of cumulus cells.

(b) Oocyte with less than 3 layers of cumulus cells.

(c) Oocyte with less than 3 layers of cumulus cells and a disorganized cytoplasm.

(d) Nude oocyte (no cumulus cells).

(e) Oocyte with over expanded cumulus cells.

Figure 1. Oocytes recovered from ultrasound-guided transvaginal follicular aspiration.

plates, Roskilde, Denmark) containing up to 50 washed (3X in TL Hepes) oocytes in 500 µl of fertilization medium (Bavister and Yanagimachi, 1977) giving a final concentration of 1.0 x 10^6 sperm per ml. Heparin (5 mg/ml) and PHE (penicillamine, .20 mM; hypotaurine, 10 mM; epinephrine, 1 mM) were included (Krisher et al., 1994). Throughout the procedure, oocytes in each quality group were kept separate.

Fourteen and one half hours following incubation with sperm, all groups of ova were removed and vortexed in 1 ml of TL Hepes media for 2 min 15 sec to remove any cumulus cells. Two-thirds of each quality group were randomly selected and centrifuged at 12,000 x g for 6 min to allow for visualization of pronuclei and microinjection of DNA. Remaining zygotes were placed in BRL cell co-culture. Microinjections were performed (15-18 h following fertilization) in TL Hepes medium on a heated stage at 37°C. Three constructs, designated WAP5FibAα1, WAP5FibBβ1 and WAP5Fibγ1, were injected. The constructs were hybrids containing the cDNA for each human fibrinogen chain with 3' UTR and 5' WAP promoter elements. All three constructs were mixed equal molar in injection buffer (10mM Tris-HCl, 0.25 mM EDTA, at pH 7.4) to a final concentration of 5.0 µg/ml corresponding to 800 total copies/pl. One to three pl of DNA solution was injected into the most visible pronucleus of each zygote. Oocytes without visible pronuclei were considered unfertilized and discarded. Following injection, zygotes were placed in the BRL cell co-culture system.

Embryos were co-cultured on BRL cells with TCM-199 (Gibco) supplemented with 10% FCS, 1% BSA, 1 mM alanine, 10 mM glycine (Sigma), .1 mM non-essential amino acids and 1% vol/vol penicillin-streptomycin (Gibco). The culture environment

was 39°C in a 5% CO_2 in air atmosphere. Wells were plated with 200,000 BRL cells in 500 µl medium 24 h prior to use (Voelkel and Hu, 1992). In addition, embryos were moved to fresh co-culture wells on d 4 of culture. These procedures have been described in detail by Krisher et al. (1994).

Slaughter Cow Oocytes

Once monthly during the experimental period (4X), 200 oocytes aspirated from the ovaries of slaughtered cows were purchased (Ken White, Logan, UT), matured in maturation medium (TCM-199 supplemented with 10% vol/vol fetal calf serum (FCS; Hyclone), bFSH and bLH (.01 IU each; NOBL Labs), and 1% vol/vol penicillin-streptomycin) while in transit in a portable incubator (Minitube of America, Madison, WI). They were put through the same IVF/IVC microinjection procedures as TVFA oocytes. All slaughterhouse oocytes were previously selected as good quality.

Embryo Evaluation and PCR Analysis

Seven days post-fertilization embryos were assessed for stage of development. Embryos were assigned a numerical value corresponding to developmental stage as follows: 1=1-cell, 2=2-cell, 3=4-cell, 4=8-cell, 5=12-16-cell, 6=morula, 7=blastocyst. All embryos (except those transferred to recipients) were placed individually in 0.5 ml microtubes containing 1 µl of TL Hepes medium and 4 µl of embryo lysing solution (400 µg Protease K/ml, 20mM Tris, 0.9% Nonidet P-40 and 0.9% Tween 20, pH 9.0) overlayed with 1 drop (~25 µl) of mineral oil. They were stored frozen (-80°C) for later

PCR analysis. Ten or fewer (depending on amount available) embryos within each source (TVFA vs SH), quality (good vs poor), treatment (injected vs control) and development stage 1-5, were analyzed by PCR. All morulae and blastocysts produced and a matching number of controls in each category were analyzed as well.

PCR analysis was performed using the general method of Saiki et al. (1989). Initial digestion of the embryos was performed at 55°C for 30 min, followed by a 15 min denaturation period at 98°C, and holding at 85°C until 20 µl of the reaction mixture was added (water, 1X *Taq* buffer, 0.2 mM of each dNTP, 0.5 µM oligonucleotide primers, 1.5 mM $MgCl_2$, 0.625 units *Taq* polymerase) for a final volume of 25 µl. Only primers for the WAPFibAα1 construct were used, as primers for the other two constructs require different reaction conditions. The primers consisted of a WAP specific sense primer (WAP S2, 5' CTGTGTGGCCAAGAAGGAAGTGTTG) plus FibAα-A1 (5' GATGTCTTTCCACAACCCTTGGGC) which amplify a 278 bp sequence. In addition, primers for the bovine β-casein gene were included as internal controls to verify that amplification of bovine DNA took place. These primers were BCS2 (5' TGCCCTTCCCTAAATATCCAGTTG) and BCA2 (5' ACATCAGAATCTCCACGGGTAAG) which amplify a 524 bp sequence. Following addition of the reaction mixture, samples were subjected to 40 cycles of denaturation (15 sec at 96.5°C), annealing (1 min at 55°C) and elongation (30 sec at 75°C). Positive controls consisted of serial dilutions of 30 ng of bovine DNA mixed with 10,000 copies of construct. Control bovine DNA as well as the non-injected bovine embryos served as negative controls. Positive and negative controls were included in each reaction.

Amplification products were evaluated concurrently with those from positive and negative controls on 1% agarose gels stained with 0.5% ethidium bromide.

Embryo Bisection

Several DNA injected embryos reaching the blastocyst stage were bisected and each part subjected to separate PCR analysis. Each embryo was placed in serum free PBS in a 30 mm petri dish which had been scored with a razor blade, creating a crevice for the embryo to rest on. Embryos were rotated such that the inner cell mass (ICM) was located on one side and the trophectoderm on the other side. A microsurgical blade (Special Blade S; Storz, Heidelberg, Germany) was passed vertically through the zona pellucida, dividing the ICM and the trophectoderm. BSA (Sigma) was added to the dish to free the demi-embryos and each portion was placed in a separate microtube and processed for PCR analysis as described previously.

Embryo Transfer to Recipients

Four times during the duration of the experiment, recipient cattle were available and were treated such that estrus was synchronized with the day of fertilization of oocytes. Open cows and heifers were either treated with 40 mg of $PGF_{2\alpha}$ i.m. on d 6-16 of the estrous cycle or synchronized using Synchromate B ear implants and $PGF_{2\alpha}$ injection as described previously. Cattle were observed for signs of estrus twice daily and cattle showing estrus within 24 h of the time of oocyte fertilization were used as recipients.

After culture for 7 d, 11 DNA microinjected embryos at the blastocyst stage of development were non-surgically transferred, one embryo per recipient, to the uterine horn ipsilateral to a palpable corpus luteum of the recipient. Cattle were examined for signs of pregnancy using ultrasound at 30 and 60 d post fertilization of oocytes.

DNA Isolation from Calf Tissue Samples and PCR Analysis

Following the birth of calves from embryo transfer, samples of placental and ear tissues, and blood were taken from each calf. In addition tissue samples of kidney, spleen, liver, muscle, bone marrow and testes were taken from one bull calf that was euthanized at 5 mo age due to illness (bovine viral diarrhea). All tissue samples were immediately frozen and stored for later analysis. DNA was isolated from tissue samples using an adaptation of the procedure developed by Marmur (1961). Briefly, tissue samples were incubated overnight at 55^{o}C in 840 µl of lysing solution (50mM Tris-HCl, pH 8.0; 0.15 M NaCl, 1M Na_2ClO_4, 10 mM EDTA, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 100 µg/ml proteinase K). Samples were then extracted with 250 µl of phenol:chloroform:isoamyl alcohol (25:24:1) by mixing for 15 sec on a "Mini Bead-Beater" (Biospec products, Bartlesville, OK) and centrifuging for 10 min at 11,000 x g. DNA was precipitated from the supernatant by adding 500 µl of isopropyl alcohol which was then decanted. The DNA was then washed in 80% ethanol, which was decanted, and followed by a second wash with ethanol and subsequent centrifugation at 11,000 x g. The ethanol was aspirated and the DNA pellet was allowed to dry. It was then resuspended in 150 µl of TE buffer (10mM Tris-HCl, pH 8.0; 1 mM EDTA) and stored at -20^{o}C.

Concentration of DNA was measured spectrophotometrically at 260 nm absorbance.

Semen samples were obtained by electro-ejaculation of bull calves when they were approximately 18 mo of age. Semen was centrifuged at 11,000 x g to separate sperm from seminal fluid. DNA was isolated from the sperm pellet using the above procedures except that samples were incubated with lysing solution for only 2 h. Blood samples (20 ml blood + 100 µl 0.5 M EDTA) obtained from the jugular vein of calves were spun at 1,100 x g for 10 min and the plasma was removed. Next the white blood cell layer was removed and placed in a 1.5 ml microcentrifuge tube. This was then spun at 13,000 x g for 2 min and any remaining plasma was removed. DNA was isolated from the white cell pellet using the same procedures as for tissue isolation.

Thirty nanograms of DNA in 1 µl solution served as the template for PCR analysis This volume was placed in 5 µl of pretreatment solution (20 mM Tris, 0.9% Nonidet P-40 and 0.9% Tween 20, pH 9.0) and subjected to 96^{o}C for 5 min. Reaction volume was brought up to 25 µl by the addition of components (water, 1X *Taq* buffer, 0.2 mM of each dNTP, 0.5 µM oligonucleotide primers, 1.5 mM $MgCl_2$, 0.625 units *Taq* polymerase). Three different primer sets were used to screen for the fibrinogen constructs, all sharing a common WAP specific sense primer (WAP S2, 5' CTGTGTGGCCAAGAAGGAAGTGTTG). WAPS2 + FibAα-A1 (5' GATGTCTTTCCACAACCCTTGGGC) amplifies a 278 bp sequence, WAPS2 + FibBβ-A1 (5' CCCGATAGCCACCTCCACTGATG) a 369 bp sequence, and WAPS2 + FibG-A1 (5' CCTGGACTTCAAAGTAGCAGCGTC) a 488 bp sequence. The WAPS2 + FibAα-A1 and WAPS2 + FibG-A1 PCR reactions were subjected to 40 cycles of denaturation

(96.5°C for 20 sec), annealing (57°C for 1 min) and elongation (75°C for 30 sec). The WAS2 + FibBβ-A1 conditions were the same except the annealing temperature was raised to 63°C. Positive controls consisting of 30 ng of bovine DNA mixed with appropriate copy number of construct and negative controls were included in each reaction. Products were evaluated concurrently with those of positive and negative controls on 1% agarose gels stained with 0.5% ethidium bromide. Molecular weight standards (123 bp ladder, Sigma) were also included on each gel.

Statistical Analysis

Statistical analysis was performed using GLM procedures in the Statistical Analysis System (SAS, 1985). Two different models were used to analyze embryo development data. The first model examined only TVFA oocytes. The model statement included TVFA session (1-32), oocyte quality (good vs poor), treatment (injected vs control) and their interactions. Linear and quadratic regressions on session number replaced the discrete session effect. The second model included all oocytes treated in the experiment and contained treatment, source-quality (SH-good, TVFA-good, TVFA-poor), session within source-quality, and the interactions treatment by source-quality, and treatment by session within source-quality. Differences in percent viable embryos (percent embryos reaching the blastocyst stage) for each source, quality and treatment, as well as number of calves born per transfer of SH and TVFA derived embryos were analyzed by Chi-square analysis.

Embryo PCR results were coded as follows: 0=negative 1=positive for both amplification of the transgene and amplification of bovine β-casein. PCR results were analyzed using GLM with the model containing treatment, source-quality, stage of development and their interactions. Bovine β-casein results were analyzed using GLM with the model containing treatment, source-quality, stage of development and the interaction term treatment by source-quality. There were insufficient data for statistical analysis of the embryo bisection results.

RESULTS

The number of oocytes collected and manipulated during the experimental period and the mean number of oocytes per session for each source-quality group are shown in Table 1. The number of oocytes processed in the IVM/IVF/IVC and DNA microinjection system was slightly lower than the number recovered due to handling losses and degeneration of oocytes.

Evaluation of the development of DNA injected and control oocytes obtained by TVFA resulted in a treatment by quality interaction ($P<.01$) for development score. Embryos injected with DNA and derived from TVFA oocytes developed similarly, regardless of oocyte quality, with good quality oocytes reaching a mean (least-squares means; LSM) score of 1.94 and poor quality oocytes attaining a mean score of 1.84 (Table 2). However, development of good quality control embryos derived from TVFA oocytes exceeded development of poor quality controls (3.64 vs 2.66; Table 2). Control embryos had a higher score (3.17) than DNA injected embryos (1.89). However, only 12.8 percent of control embryos from poor quality oocytes reached the blastocyst stage. This was not different ($P>.05$) from the percent blastocysts produced after injection of DNA in embryos from good or poor quality TVFA oocytes (8.4% and 5.5%, respectively; Table 2). More than twice as many blastocysts were produced from good quality controls than from poor quality control oocytes.

Aspiration session significantly ($P<.01$) affected development of embryos derived from good and poor quality oocytes (Figure 2). Regression analysis showed that good

Table 1. Total oocytes and mean number of oocytes per session collected and processed during the 16 wk experimental period for each source-quality group.

Total Oocytes	Collected	IVM/IVF	Injected	Control	UFO[a]
Source-Quality:					
TVFA-Good	346	311	202	104	5
TVFA-Poor	960	781	451	266	64
SH-Good	800	764	449	252	63
TOTAL	2106	1856	1102	622	132
Mean No. Oocytes/Session					
Source-Quality:					
TVFA-Good	10.8	10.0	6.5	3.3	.2
TVFA-Poor	30.0	25.2	14.5	8.6	2.1
SH-Good	200.0	191.0	112.2	63.0	15.8

[a] UFO = unfertilized oocytes

Table 2. Least-squares means (± SE) for development score of oocytes, and percent viable embryos produced from ultrasound-guided transvaginal follicular aspiration (TVFA).

Treatment Group	Development Score[d]	% Viable Embryos[e]
Injected with DNA[f]:		
Good quality	1.94 ± .13[a]	8.4[a] (17/202)
Poor quality	1.84 ± .09[a]	5.5[a] (25/451)
Control:		
Good quality	3.64 ± .19[c]	31.7[b] (33/104)
Poor quality	2.66 ± .12[b]	12.8[a] (34/266)

[a.b.c] Values in the same column with different superscripts are different ($P < .05$).
[d] Development score is defined as stage of development reached after 7 days in culture. 1=1-cell embryo, 2=2-cell embryo, 3=4-cell embryo, 4=8-cell embryo, 5=12-16-cell embryo, 6=morula, 7=blastocyst.
[e] Viable embryos are those reaching the blastocyst stage after 7 days in culture.
[f] Fibrinogen construct consisting of equal parts of WAP5FibAα1, WAP5FibBβ1 and WAP5Fibγ.

quality oocytes had higher development scores than poor quality oocytes throughout most of the experimental period, with their development increasing during the last 18 sessions. The highest mean development score occurred at session 27 for good quality oocytes (5.33 ± 1.07). Poor quality oocytes had a slight increase in development score for the first half of the experimental period, peaking on sessions 3, 13, 17 and 25 with mean development scores of 3.17 ± .65, 3.12 ± .43, 3.11 ± .38 and 3.16 ± .40, respectively. This was followed by a slight decrease in development score in the latter half of the experimental period, with mean development scores ranging from 1.54 ± .46 to 2.67 ± .38 from sessions 26 to 32.

There was a session by treatment interaction ($P<.01$) for development scores of TVFA oocytes. Oocytes injected with DNA did not vary significantly ($P>.05$) over sessions. However, control oocytes had increasing development scores over the 32 aspiration sessions with a maximum of 5.22 at session 30 (Figure 3).

Development of good and poor TVFA oocytes injected with DNA was not different ($P>.05$) from development of SH oocytes that were injected with DNA (Table 3). Development score of SH control oocytes was not different ($P>.05$) from good quality TVFA control oocytes (3.46 vs 3.81; Table 3). Development score of poor quality TVFA control oocytes (2.66 ± .13) was lower ($P<.05$) than development of the other control groups. The percent of viable embryos (blastocysts) produced following microinjection of DNA was the same for SH oocytes, TVFA-good oocytes and TVFA-poor oocytes and did not differ from development of TVFA-poor control oocytes ($P>.05$). However, good quality TVFA control oocytes produced over 50% more ($P<.05$)

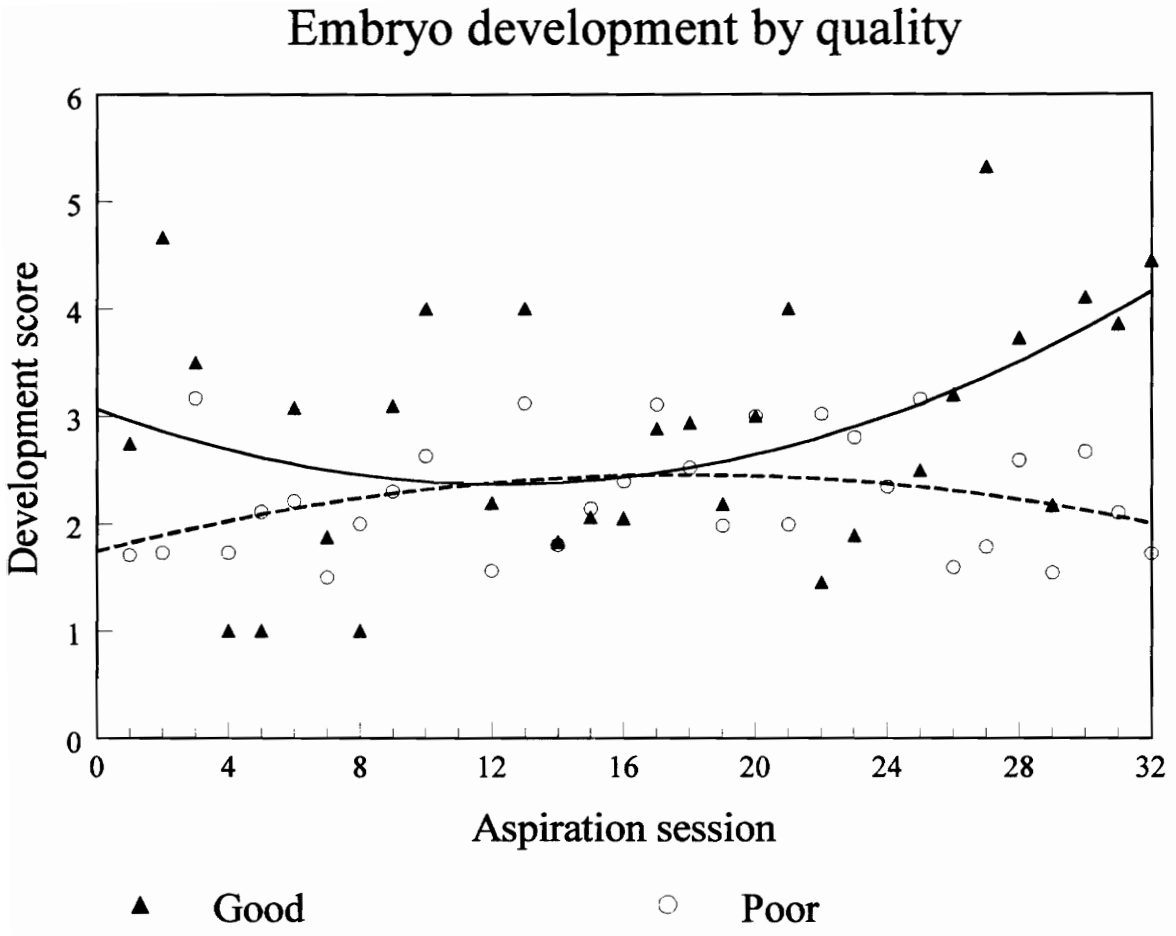


Figure 2. Curvilinear relationship of good and poor quality oocytes obtained by ultrasound-guided transvaginal follicular aspiration for each twice weekly aspiration session (symbols represent LSM).

Equation for good quality oocytes (solid line): $y = 3.072-.114x+.005x^2$.

Equation for poor quality oocytes (dotted line): $y = 1.745+.080x-.002x^2$.

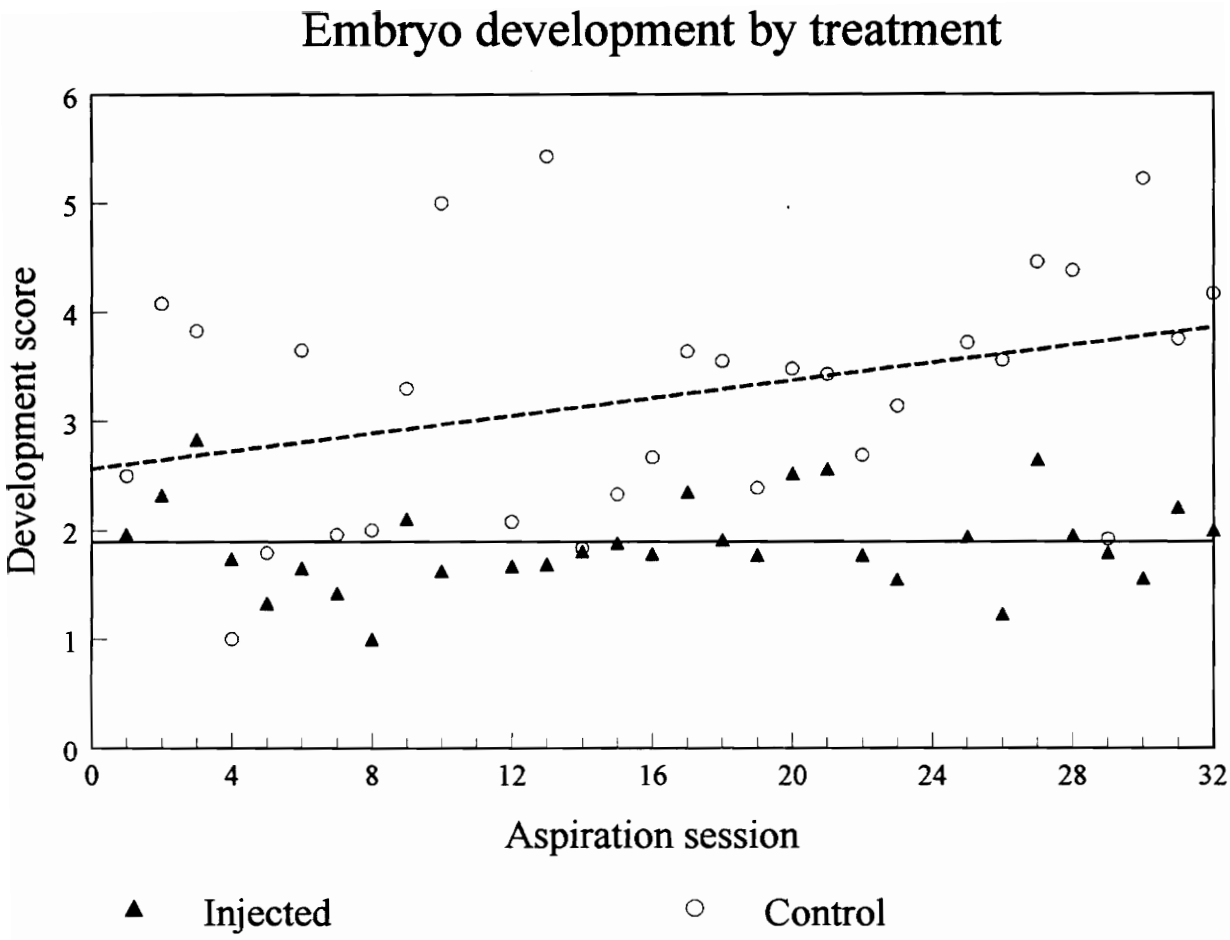


Figure 3. Linear relationships over time for development of oocytes microinjected with DNA obtained from ultrasound-guided transvaginal follicular aspiration for each twice weekly aspiration session (symbols represent LSM).

Equation for injected oocytes (solid line): y = 1.89.

Equation for control oocytes (dotted line): y = 2.566+.040x.

Table 3. Least-squares means (± SE) for development of oocytes and percent viable embryos produced after microinjection of DNA for oocytes of good (G) or poor (P) quality from ultrasound-guided transvaginal aspiration (TVFA), and for good (G) quality oocytes obtained from slaughtered cows (SH).

Treatment Group	Development Score[d]	% Viable Embryos[e]
Injected with DNA[f]:		
TVFA-G	1.91 ± .17[a]	8.4[a] (17/202)
TVFA-P	1.86 ± .10[a]	5.5[a] (25/451)
SH-G	2.09 ± .09[a]	6.3[a] (28/443)
Control:		
TVFA-G	3.81 ± .22[c]	31.7[c] (33/104)
TVFA-P	2.66 ± .13[b]	12.8[ab] (34/266)
SH-G	3.46 ± .12[c]	20.4[b] (51/250)

[a.b.c] Values in the same column with different superscripts are different ($P < .05$).
[d] Development score is defined as stage of development reached after 7 days in culture. 1=1-cell embryo, 2=2-cell embryo, 3=4-cell embryo, 4=8-cell embryo, 5=12-16-cell embryo, 6=morula, 7=blastocyst.
[e] Viable embryos are those reaching the blastocyst stage after 7 days in culture.
[f] Fibrinogen construct consisting of equal parts of WAP5FibAα1, WAP5FibB1 and WAP5Fibγ.

blastocysts than good quality SH control oocytes. There was no difference in development to blastocyst of poor quality TVFA control oocytes and SH control oocytes of good quality.

There were significant (P<.01) session effects on the development scores of SH oocytes, with development increasing over the first three sessions from 2.64 to 3.11. Mean development score (Table 4) for session four was lower (2.42) than for sessions two (2.95) and three (3.11).

Results from the PCR analysis of embryos are shown in tables 5 and 6. There was a treatment by stage of development by source-quality interaction (P<.05) for amplification of the transgene (WAP5FibAα1). All control embryos analyzed were PCR negative, therefore the results presented are for embryos injected with DNA only. For embryos derived from TVFA oocytes (Table 5), a higher percentage of embryos at later stages of development (8-cell through blastocyst) were PCR positive, except for poor quality oocytes in which all two-cells were PCR positive as well, and morulae in which only half were positive for the transgene. For SH derived oocytes, embryos at the morula stage produced the highest percentage of PCR positives, while putative one-cell embryos produced the least percentage of positives. SH oocytes produced more PCR positive morulae than TVFA oocytes. Oocytes derived from TVFA produced a higher percentage of PCR positive blastocysts (95%) than SH oocytes (62%). Good quality TVFA oocytes produced a greater percentage of PCR positive 1-cell embryos (67%) than SH (30%) and poor quality TVFA oocytes (none).

Stage of embryo development was the only variable affecting (P<.01) the

Table 4. Least-squares means (± SE) for development of good quality oocytes from slaughtered cows (SH-G) for each session[e].

Session	Development Score[f]
1	$2.64 \pm .15^{ab}$
2	$2.95 \pm .15^{bc}$
3	$3.11 \pm .16^{cd}$
4	$2.42 \pm .15^{a}$

[a,b,c,d] Values in the same column with different superscripts are different ($P < .05$).
[e] SH-G oocytes were processed once a month during the experimental period for a total of four sessions over the 16 wk period.
[f] Development score is defined as stage of development reached after 7 days in culture. 1=1-cell embryo, 2=2-cell embryo, 3=4-cell embryo, 4=8-cell embryo, 5=12-16-cell embryo, 6=morula, 7=blastocyst.

Table 5. Percentage PCR[f] positive ($\bar{X}$ ± SE) DNA microinjected embryos from TVFA oocytes of good (G) or poor (P) quality or SH oocytes of good (G) quality for each stage of embryo development after 7 days in culture.

Stage of development	TVFA-G	(n)	TVFA-P	(n)	SH-G	(n)
1-cell	67 ± 11^{aC}	(6)	0 ± 12^{aA}	(5)	30 ± 9^{aB}	(10)
2-cell	78 ± 9^{abA}	(9)	100 ± 10^{deA}	(7)	80 ± 9^{bcA}	(10)
4-cell	56 ± 9^{aA}	(9)	78 ± 9^{bcdA}	(9)	57 ± 10^{bA}	(7)
8-cell	100 ± 28^{abA}	(1)	100 ± 9^{dA}	(10)	86 ± 10^{bcA}	(7)
12-16-cell	100 ± 12^{bA}	(5)	71 ± 10^{bceA}	(7)	78 ± 9^{bcA}	(9)
morula	n.d[g]		50 ± 14^{bA}	(4)	92 ± 8^{cB}	(13)
blastocyst	100 ± 11^{bB}	(6)	93 ± 7^{deB}	(14)	62 ± 8^{bA}	(13)

[a,b,c,d,e] Values in the same column with different superscripts are different ($P < .05$).
[A,B,C] Values in the same row with different superscripts are different ($P < .05$).
[f] PCR results were coded as follows: negative=0, positive=1.
[g] No data available.

percentage of embryos with positive PCR results for amplification of bovine β-casein. Amplification of β-casein was higher in embryos at the 8-cell stage or beyond, and was greatest in blastocysts. (Table 6).

Five blastocysts (three from TVFA poor quality oocytes, one from a TVFA good quality oocyte and one from a SH oocyte) were successfully bisected and their ICM and trophectoderm analyzed for the DNA construct by PCR. There was no instance of the transgene being found in the trophectoderm of an embryo and not in the embryo's ICM. In two cases (both TVFA poor quality oocytes) the construct was found in ICM only. The blastocysts derived from the other two TVFA oocytes were negative for the transgene in both ICM and trophectoderm and the SH derived blastocyst was positive in both ICM and trophectoderm.

Five embryos produced from TVFA oocytes were transferred to suitable recipients, resulting in 4 pregnancies, for a pregnancy rate of 80%. Three of these pregnancies went to term, producing 3 bull calves. The other recipient was open after d 60 of gestation. No fetus was recovered. Six embryos produced from SH oocytes were transferred and produced two pregnancies (33.3% pregnancy rate). One bull calf was born by cesarian section. The other pregnant cow aborted her calf at ~ d 70 of gestation. No fetus was recovered. The remaining 4 recipients were open at d 30 of gestation.

Placental and ear tissue samples, as well as blood samples from all four live calves tested negative for the presence of the transgene by PCR . One of the calves obtained from TVFA oocytes, calf 2541 (Figure 4), became ill at 5 mo age and was diagnosed with bovine viral diarrhea (BVD). After euthanasia and upon necropsy he had gross lesions

Table 6. Percentage PCR[c] positive ($\bar{X}$ ± SE) for presence of endogenous bovine β-casein for embyos at each stage of development after 7 days in culture.

Stage of development	% PCR Positive	(n)
1-cell	23 ± 6[a]	(51)
2-cell	39 ± 6[a]	(46)
4-cell	36 ± 6[a]	(53)
8-cell	77 ± 7[b]	(39)
12-16-cell	81 ± 6[b]	(42)
morula	80 ± 8[b]	(28)
blastocyst	93 ± 5[b]	(66)

[a,b] Values in the same column with different superscipts are different ($P < .05$).
[c] PCR results were coded as follows: negative=0, positive=1 for amplification of bovine β-casein.

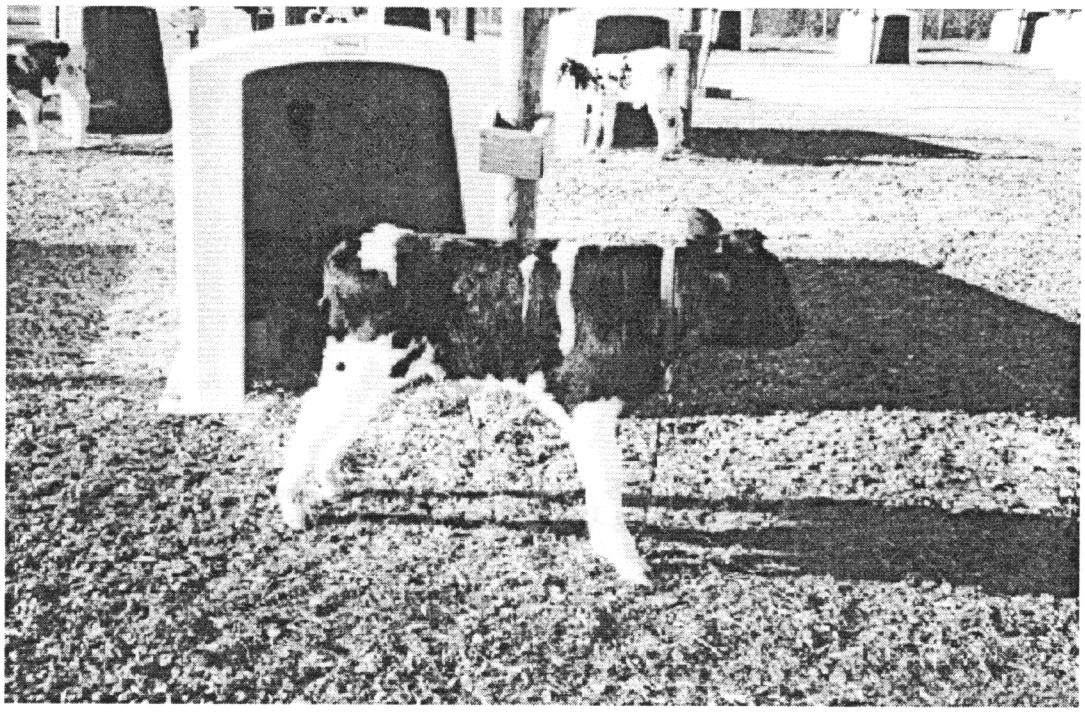
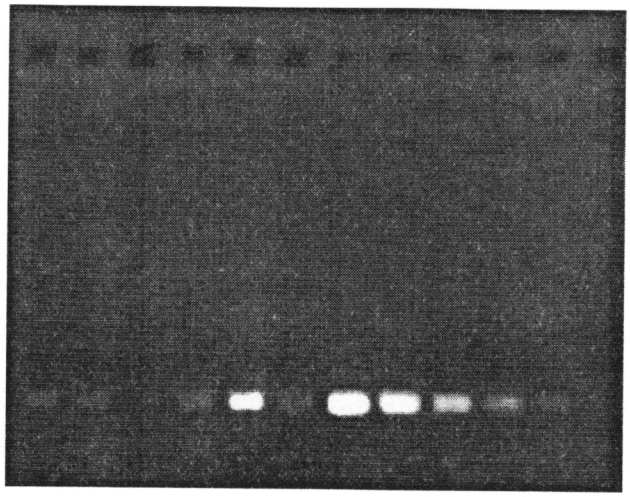


Figure 4. Bull calf 2541 born 1/18/95 and subsequently found mosaic for the transgene

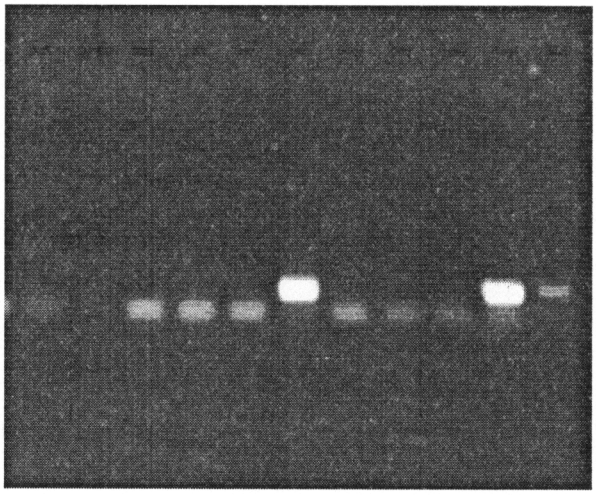
of ulcers in the esophagus, rumen and intestines and lymphoid depletion in the intestines. Immunohistochemistry revealed that all tissues analyzed were positive for BVD (personal communication, Geoffrey Saunders, DVM). At necropsy six different tissues were obtained and analyzed for presence of the transgene. Liver, kidney, spleen, muscle, and testes tested negative by PCR for all three constructs. Bone-marrow tested positive for all three constructs (Figures 5,6,7), with the strongest positive result for the WAP5FibBβ construct (Figure 6). Semen samples obtained from the remaining three calves were negative for all three constructs.



Lane:		
	1	kidney
	2	spleen
	3	liver
	4	muscle
	5	bone marrow
	6	testes
	7-10	fibrinogen construct[a] (10K, 1K, 100, 10 copies)
	11	bovine control DNA (10K copies)
	12	no DNA

[a] Fibrinogen construct is equal parts of WAP5FibAα1, WAP5FibBβ1 and WAP5Fibλ.
[b] Markers not shown

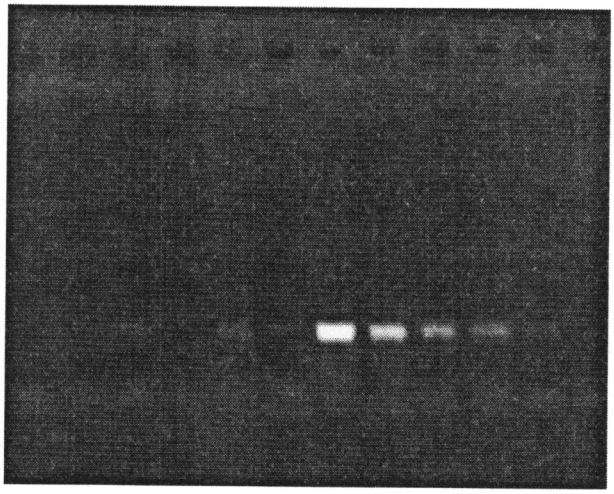
Figure 5. PCR for WAP5FibAα1 in tissue samples of calf 2541.



Lane:		
	1	kidney
	2	kidney
	3	liver
	4	spleen
	5	muscle
	6	bone marrow
	7	testes
	8	bovine control DNA (10K copies)
	9	no DNA
	10	fibrinogen construct[a] (10K copies)
	11	fibrinogen construct[a] (1K copies)

[a] Fibrinogen construct is equal parts of WAP5FibAα1, WAP5FibBβ1 and WAP5Fibλ.
[b] Markers not shown.

Figure 6. PCR for WAP5FibBβ1 in tissue samples of calf 2541.



Lane:		
	1	kidney
	2	spleen
	3	liver
	4	muscle
	5	bone marrow
	6	testes
	7-10	fibrinogen construct[a] (10K, 1K, 100, 10 copies)
	11	bovine control DNA (10K copies)
	12	no DNA

[a] Fibrinogen construct is equal parts of WAP5FibAα1, WAP5FibBβ1 and WAP5Fibλ.
[b] Markers not shown.

Figure 7. PCR for WAP5Fibλ in tissue samples of calf 2541.

DISCUSSION

Morphological characteristics of oocytes, such as layers of cumulus cells and cytoplasmic appearance were shown to be important for subsequent development in-vitro (de Loos et al., 1989; Liebfried and First, 1979). Therefore, it is not surprising that development of poor quality TVFA oocytes was lower than development of good quality oocytes. However, mean development score and percent viable embryos produced were similar between good and poor TVFA oocytes following DNA injection. The trauma of the microinjection procedure may be so great that it overshadows the effect of oocyte quality on embryo development. Microinjection into the pronucleus causes membrane disruption with lysis of the cell or pronucleus occurring in a large number of oocytes. As a result a large percentage of oocytes microinjected with DNA fail to cleave, and few go on to develop to morulae and blastocysts (Krisher et al.,1994). Development data from this experiment are similar to that of previous work conducted in this laboratory (Gibbons et al, 1995; Krisher et al., 1994).

When examining the number of transferable embryos produced following DNA injection, good quality TVFA oocytes have a numerical advantage over those of poor quality, although not statistically different (8.4% vs 5.5% blastocysts, respectively). Less than half as many good oocytes (26.5%) as poor oocytes (73.5%) were obtained by TVFA in this experiment, differing from the results of others who have retrieved approximately 50% good quality oocytes through TVFA (Gibbons et al., 1995; Looney et al., 1994; van der Schans et al., 1991). Therefore, during most of the aspiration sessions there were

fewer good quality oocytes per treatment group, and fewer good quality oocytes were fertilized and cultured together. It has been shown that embryos cultured in groups develop at higher rates to the blastocyst stage than those cultured singly, probably due to the secretion of growth factors (Canseco et al., 1992; Keefer et al., 1994). Therefore, development of the DNA injected good quality TVFA oocytes may have been hindered by a lower group size (average size 6.5 embryos/group; range 1-23 per group) in culture than the poor quality oocytes (average size 14.5 embryos/group; range 3-28 per group). With larger numbers of good quality TVFA oocytes differences may have been statistically significant.

The development score of good quality TVFA oocytes increased during the latter half of the experimental period. During long term twice-weekly TVFA, development of a dominant follicle and ovulation is prevented (Simon et al., 1993). Therefore, there is no subsequent luteal tissue formation, and after regression of the CL which exists at the start of TVFA, circulating progesterone levels remain low (Gibbons et al., 1994). Lack of a dominant follicle allows for development of more follicles (Stubbings and Walton, 1995), and more oocytes are retrieved with twice-weekly than once weekly aspiration (Gibbons et al., 1994). In this experiment, the number of oocytes recovered per session varied during the 16 wk period (Carlin, 1995), and therefore development data may have been affected by varying numbers of oocytes cultured together over the 32 sessions. However, with twice weekly TVFA a consistent population of oocytes was recovered, and once the effects of a dominant follicle were removed, subsequent follicular development may have been more synchronous, yielding oocytes which develop more consistently in

an IVM/IVF/IVC program (Gibbons et al., 1995). Poor quality oocytes on the other hand, were probably aspirated from atretic follicles (Wurth and Kruip, 1992), therefore compromising their development such that it did not increase to the degree that good oocyte development did with twice-weekly aspiration.

The increase in development of poor quality TVFA oocytes occurred in the early part of the experiment, corresponding to the time when the initial CL was regressing in these cows. Machatkova et al. (1996) showed that SH oocytes collected at the end of the luteal phase of the estrous cycle had greater development rates. Previous research reported no difference in oocyte development for oocytes collected from ovaries early or late in the luteal phase of the estrous cycle (Tan and Lu, 1990). Another study found that SH oocytes collected from cyclic cattle with a CL had higher development rates (Thonon et al., 1993) than those from cattle without a CL. Development of poor quality oocytes may have been compromised by lack of a CL during the latter half of the experiment, whereas good quality oocytes were able to overcome this effect.

Development of TVFA oocytes injected with DNA did not vary over the 16 wk of this study. Procedures were performed consistently and without modification. Development data for embryos injected with DNA did not differ from that of other studies (Krisher et al., 1994; Gibbons et al., 1995; de Loos et al, 1996). Therefore, it was not expected to vary over the course of the experiment. Development of control embryos did increase over time. The effects of twice weekly TVFA on ovarian dynamics and oocyte development in-vitro discussed previously could be responsible for this increase in development. Another possibility is that the increasing rate of embryo development over

the course of the experiment was due to increasing proficiency in the IVM/IVF/IVC procedures and general embryo handling techniques. This is also reflected in the increasing development scores of the SH oocytes over the first three sessions. The exception is session 4 which had the lowest development score. Since there were no substantial changes in procedure or detrimental occurrences in this laboratory, lower development of this shipment of oocytes may be due to something that occurred during the collection or maturation of the oocytes prior to shipment. Another possibility is that higher environmental temperature affected the development of session 4 oocytes, which were obtained in June. In-vitro development of SH oocytes collected in the summer months was reduced (W.H. Eyestone, personal communication) as was development of oocytes collected by TVFA during hot periods of the year (Broussard et al., 1996) compared to development of oocytes collected during cool temperature periods.

Embryos produced from SH oocytes had equal development scores to those produced from good quality TVFA oocytes. Since oocytes from both sources were graded as good quality, this is not an unreasonable result. Development of poor oocytes was inferior to that of good quality oocytes from both sources. In a previous study, good and poor TVFA oocytes cultured together had development exceeding that of SH oocytes (Gibbons et al, 1995). It was suggested that when cultured together, some of the poor oocytes may be "rescued" and go on to develop. The results of this study show that poor TVFA oocytes do not have much ability to develop on their own, even when cumulus cells are added to the maturation medium. Moreover, their development to the blastocyst stage was not different from DNA injected oocytes.

Good quality TVFA oocytes were able to produce more blastocysts than good quality SH oocytes. Even though SH oocytes had a similar mean development score (i.e. the same number developed to cleavage stage embryos as TVFA oocytes), less of them survived in-vitro to the blastocyst stage; a stage which is desirable to attain so they can be successfully transferred. Therefore, for commercial in-vitro embryo production, there is an advantage to using TVFA derived oocytes. These source differences in oocyte development may be due to the size and status of follicles from which the oocytes are retrieved. It has been reported that oocyte development is greatest for oocytes aspirated (post mortem) from follicles 2.7-5 mm and ≥ 5 mm in diameter or > 6 mm in diameter (Blondin and Sirard, 1994; Lonergan et al., 1994, respectively). With ultrasound-guided TVFA, it is more likely that smaller follicles were not detected in visualization and puncture by the aspiration needle (personal observation) than when aspirating from an ovary on a laboratory bench. Therefore, in general, TVFA oocytes may be from larger follicles and therefore have increased developmental potential. The use of ultrasound with TVFA allows for visualization and puncture of follicles within the ovarian stroma as well as on the surface. Twice weekly TVFA prevents the formation of a dominant follicle which inhibits other follicular development (Simon et al., 1993). Therefore, small follicles within the ovary punctured during TVFA are more likely growing than regressing. Oocytes from these follicles may have increased developmental potential (Wurth and Kruip, 1992). In addition, with TVFA, status of the donor animal is known, and when performed twice-weekly on the same animals, a more synchronous population of follicles may be aspirated yielding oocytes at similar developmental stages which may better

respond to IVM/IVF/IVC (Gibbons et al., 1995).

Oocytes from both sources (TVFA and SH) and qualities did not differ in development following injection of DNA. The microinjection procedure itself may have greater effects on embryo development than oocyte source or quality. Good quality TVFA oocytes do have a slight numerical advantage, followed by SH oocytes for producing viable microinjected embryos for transfer. Recently it was reported that TVFA oocytes produced more viable d 7 embryos after injection of DNA than SH oocytes (de Loos et al., 1996). It is notable that these researchers injected a similar number of TVFA oocytes as in this study (726 vs 653), however, they injected nearly 16 times as many SH oocytes (7032 vs 449). Therefore, their results of 3.4% SH oocyte development to day 7, may better represent the developmental potential of SH oocytes following injection of DNA .

PCR analysis of embryos injected with DNA at later developmental stages yielded a high percent of PCR positives. This is consistent with previous results reporting 50% PCR positive morulae (Sparks et al., 1994), and 89% PCR positive morulae and blastocysts (Krisher et al., 1994). Various researchers have discussed the limitation of PCR analysis of embryos and its inability to differentiate between integrated and non-integrated DNA (Krisher et al., 1994; 1995; Horvat et al., 1993). However, when used with embryo biopsy techniques, its advantage is in eliminating embryos which do not contain the transgene at all, thus reducing the number of recipients needed by as much as 79% (Bowen et al., 1994). Recently, a positive selection system for transgenic embryos was described in which the construct contained a neomycin resistance gene

(Bondioli and Wall, 1996). This selection system enriched the number of blastocysts expressing the transgene in the majority of their cells following culture in the presence of G418, a neomycin derivative. Preliminary use of a reporter construct to screen for transgenic bovine embryos was reported as well (Menck et al., 1996). Optimization of such techniques will likely make screening of transgenic bovine embryos more efficient in the near future.

Although SH derived oocytes produced the highest percentage PCR positive morulae, there were only 4 morulae analyzed by PCR from TVFA oocytes (2 were positive) compared to 13 from SH oocytes. The superior ability of TVFA oocytes to develop to the blastocyst stage meant that fewer TVFA derived embryos were at the morulae stage on day 7 when they were prepared for PCR analysis.

Oocytes from TVFA produced more PCR positive blastocysts than SH oocytes. If PCR positive results do represent integrated transgene, then this result is of importance. It was reported that embryos produced from TVFA are of higher quality than SH derived embryos (Gibbons et al., 1994; 1995). Perhaps, the higher quality TVFA derived embryo is better able to integrate foreign DNA and continue its development.

Primers for bovine β-casein were used as internal controls to co-amplify bovine genomic DNA during PCR analysis of embryos, as suggested by Bowen et al. (1994). It was discovered that embryos reaching the 8-cell stage and later stages of development had a higher rate of amplification of β-casein than earlier development stage embryos. The primers were designed to reduce the incidence of false negative results from PCR; i.e., to show that bovine DNA is present. These results show that this technique is only

worthwhile when eight or more cells are present. Fewer cell numbers did not provide for adequate amplification using these primers. However, the low incidence of amplification of genomic DNA from early cleavage stage embryos could have been due to their degeneration after 7 days in culture.

Although statistically speaking, an insufficient number of DNA injected blastocysts were bisected and analyzed by PCR, it is notable that the transgene was never detected in trophectoderm cells without being detected in the ICM of the same embryo. In mice it was shown that transgenes can be detected more frequently in placental tissue than in the fetus (Canseco et al., 1994). This lead to speculation that a PCR positive result from embryo biopsy could lead to production of a non-transgenic offspring. Results here indicate that biopsy of trophoblast cells for PCR analysis may accurately reflect the PCR status of the ICM. However, the problem of differentiating whether the construct DNA is integrated in the genome or persisting in cells without integrating still remains.

More pregnancies were produced and more calves were born from the transfer of TVFA oocytes injected with DNA than SH oocytes, although not statistically significant. This is in agreement with recent results which reported a higher (although not significant) pregnancy rate with TVFA oocytes injected with DNA (de Loos et al, 1996). When combined with other embryo transfer data from this laboratory (F.C. Gwazdauskas, unpublished data), the live birth rate of calves from embryos injected with DNA (number calves born/number embryo transfers) is 21% from SH derived oocytes (4/19) and 35% from TVFA oocytes (9/26). A higher pregnancy rate is most likely due to the higher quality of TVFA derived embryos upon transfer (Gibbons et al., 1995), as high quality

embryos optimize establishment of a pregnancy (Looney et al., 1994). However, the greater frequency of live births from the transfer of embryos injected with DNA derived from TVFA oocytes suggests that these embryos may have an enhanced ability to develop past the blastocyst stage in-vivo compared to SH derived embryos injected with DNA. If this is true, this benefit may help outweigh the labor intensive nature of TVFA when used in gene transfer programs.

One of four calves born was PCR positive for all constructs in it's bone-marrow, but was negative for all three in other tissues sampled (placenta, skin, blood, liver, kidney, spleen, muscle, testes). This highly mosaic animal likely would not have been discovered had it not become ill requiring euthanasia. Since the constructs were only found in bone-marrow and not in the other tissues of mesodermal origin such as muscle and testes, integration must have occurred late in development. Burdon and Wall (1992) showed that injected DNA forms head to tail concatamers prior to integration which segregate independently until they are integrated into the genome or degraded. Bovine embryos specifically, have been reported to produce large ligation products from injected DNA in pronuclear stage embryos (Powell et al., 1992). In mice it was shown that 62% of integrations following DNA microinjection result in mosaic embryos (Whitelaw et al., 1993). Kubisch et al. (1995) found that nearly all bovine embryos injected with a LacZ construct had a mosaic pattern of expression. Production of transgenic cattle continues to be complicated by mosaicism, most probably caused by late integration of DNA. To be commercially useful, transgenic founder cattle must be able to transmit transgenes to their offspring. Therefore it is essential that transgenes integrate into germ line cells.

Several new methodologies make this more probable. Recently, transgenic bovine fetuses were generated by retroviral infection of early embryos (Haskell and Bowen, 1995). Although each of four fetuses analyzed was mosaic, a large fraction of cells from each of six tissues was transgenic in three of the four fetuses, and mosaicism appeared uniform among tissues representing all three germ layers. Therefore, the authors proposed that genetic transmission rates from animals such as these would be high. Another study conducted by Brinster and Avarbock (1994) showed that transplanted spermatogonial cells generated sperm capable of fertilizing oocytes and producing offspring. If methods are developed allowing spermatogonia to be successfully cultured, transfected with transgenes and selected, this system could be used to successfully produce transgenic farm animals. Problems of mosaicism would be eliminated because transgenes would be introduced at the time of fertilization. Finally, researchers are currently seeking to isolate pluripotent embryonic stem cells in cattle. Stem cell technology, combined with nuclear transfer techniques would allow for establishment of a production herd of transgenic cattle in less time than with current methods (Eyestone, 1994).

In conclusion, the use of oocytes retrieved using transvaginal follicular aspiration techniques shows promise for increasing the efficiency of transgenic cattle production. More embryos develop to the blastocyst stage from good quality TVFA oocytes than from good quality SH oocytes. Effects of microinjection of DNA may undermine this benefit; however, injected TVFA derived embryos may also develop better following transfer to recipients leading to a larger number of putative transgenic calves born. In addition, oocytes from TVFA produce more PCR positive blastocysts than SH oocytes. With the advent of better embryo screening procedures, this may prove important.

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APPENDIX

Table 7. Analysis of variance for developmental score of oocytes recovered from transvaginal follicular aspiration.

Source	df	Mean Squares	P Value
Session	30	8.47	**
Quality	1	52.03	**
Session×Quality	29	7.84	**
Treatment	1	260.84	**
Session×Treatment	30	5.97	**
Quality×Treatment	1	42.73	**
Session×Quality×Treatment	29	4.44	
Error	902	3.41	

* $P < .05$
** $P < .01$

Table 8. Analysis of variance for developmental score of oocytes recovered from transvaginal follicular aspiration and slaughterhouse ovaries.

Source	df	Mean Squares	P Value
Source-quality	2	48.61	**
Session (Source-quality)	62	8.01	**
Treatment	1	455.75	**
Source-quality×Treatment	2	24.94	**
Session×Treatment (Source-quality)	62	4.31	
Error	1587	3.69	

* $P < .05$
** $P < .01$

Table 9. Analysis of variance for quadratic regression of mean development score on TVFA session for oocytes of good and poor quality.

Good quality

Source	df	Mean Squares	P Value
Session	1	2.04	
Session^2	1	3.89	*
Error	27	1.12	
Parameter	**Estimate**		
Intercept	3.072		**
Session	-.114		
Session^2	.005		*

Poor quality

Source	df	Mean Squares	P Value
Session	1	1.01	*
Session^2	1	.91	*
Error	28	.28	
Parameter	**Estimate**		
Intercept	1.745		**
Session	.080		*
Session^2	-.002		*

* $P < .1$
** $P < .05$

Table 10. Analysis of variance for linear regression of mean development score on TVFA session for oocytes from each treatment group (injected with DNA, control).

Injected

Source	df	Mean Squares	P Value
Session	1	.02	
Error	28	.18	
Parameter	**Estimate**		
Intercept	1.828		**
Session	.003		

Control

Source	df	Mean Squares	P Value
Session	1	4.33	*
Error	28	1.22	
Parameter	**Estimate**		
Intercept	2.566		**
Session	.040		*

* $P < .1$
** $P < .05$

Table 11. Analysis of variance for PCR of embryos of different source-quality (SH-G; TVFA-P; TVFA-G) stages of development, and treatments (injected with DNA, non-injected).

Source	df	Mean Squares	P Value
Treatment	1	31.19	**
Stage	6	.46	**
Treatment×Stage	6	.46	**
Source-quality	2	.09	
Treatment×Source-quality	2	.09	
Stage×Source-quality	11	.14	*
Treatment×Stage×Source-quality	11	.14	*
Error	285	.08	

* $P < .05$
** $P < .01$

Table 12. Analysis of variance for PCR amplification of bovine β-casein in embryos of different source-quality (SH-G; TVFA-P; TVFA-G) stages of development, and treatments (injected with DNA, non-injected).

Source	df	Mean Squares	P Value
Treatment	1	.18	
Stage	6	3.74	**
Source-quality	2	.21	
Treatment×Source-quality	2	.38	
Error	313	.17	

* P < .05
** P < .01

VITA

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