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CAPRINE SPERM CELLS AS VECTORS FOR GENE TRANSFER

A Dissertation

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in

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by

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ABSTRACT

Goat sperm cells were used as a model to evaluate sperm-mediated gene transfer. Fresh and frozen-thawed goat sperm cells were co-incubated with radio-labelled foreign DNA. After 10 extensive washings, the compacted sperm retained foreign DNA. Confocal microscopy confirmed that the position on the sperm cell responsible for binding of foreign DNA was located in both the anterior and the posterior heads of caprine sperm cells. Furthermore, confocal longitudinal sections of the sperm cells showed that foreign DNA had penetrated into goat sperm nuclei. In this study, 37% of the fresh goat sperm and 33% of the frozen-thawed goat sperm cells were able to bind foreign DNA. In vitro fertilization experiments were conducted with goat sperm cells co-incubated with foreign DNA and the results showed that 3% of the fertilized and cleaved embryos expressed foreign DNA. Electroporation was applied to sperm cells co-incubated with foreign DNA and at various voltages (range of 0 to 1,200 volts/cm). Even though the sperm cells were immobilized by electroporation, all of them were able to bind foreign DNA. A further study showed that the sperm membranes play a very important role in sperm cell binding of foreign DNA. Southern blotting results showed that enzymes in the sperm cells were able to degrade foreign DNA.

To further evaluate the potential use of sperm-mediated gene transfer, intracytoplasmic injection of goat oocytes with immotile sperm was evaluated. Results indicated that goat oocytes need exogenous stimuli, such as calcium ionophore A23187 for activation. Overall, 21% of cleaved sperm-injected embryos developed to the blastocyst stage, while sham-injected oocytes only developed to 16- to 32-cell stages. With this goat intracytoplasmic sperm injection methodology, sperm-mediated gene transfer was further studied. Fluorescent in situ hybridization results showed that without electroporation of sperm cells, only 14% of the cleaved embryos had foreign
DNA (BC31), while with electroporation, 25% cleaved embryos had foreign DNA (BC31). Furthermore, foreign DNA (BC31) had integrated into goat genomic DNA.
CHAPTER 1

INTRODUCTION

Gordon et al. (1980) first introduced new DNA sequences into mouse embryos. The offsprings that integrated these foreign DNA sequences were termed as "transgenic animals" (Gordon et al., 1980; Gordon and Ruddle, 1981). The mice that had a transgene for growth hormone grew to nearly twice normal size of the control animals (Palmiter et al., 1982, 1983). This physiological response greatly excited animal scientists.

Genetic engineering of farm animals is expected to have a major effect on animal agriculture in the future. The annual gross receipts for the sale of livestock in the United States is ~$60 billion. Improving farm animal economic characteristics such as growth performance, wool production and milk production with transgenic technology will likely increase the efficiency of animal agriculture. It has been predicted that genetically engineered embryos with superior economic traits would appear on the market by the year 2000 (Hansel, 1986). With pronuclear microinjection gene transfer method (Gordon et al., 1980), much effort has been put toward producing transgenic farm animals over the last 15 years. The first transgenic farm animals (sheep and swine) were reported in 1985 (Hammer at al., 1985). Since then, there has been much effort to improve productivity traits of farm animals with transgenic techniques over the last 10 years (Reviewed by Pursel and Rexroad, 1993; Wall, 1996).

The vast majority of research on transgenic farm animals has focused on growth enhancement. Most genes being used in this effort are genes coding for growth hormone (GH) and growth hormone releasing factors with a metallothionein (MT) promoter. These genes were expressed at high levels and foreign growth hormone
gene can be expressed in successive generations of transgenic pigs (Pursel et al., 1989). Pigs and lambs with the foreign GH gene did not grow larger than their siblings like the giant mouse model. However, some transgenic pigs gained weight faster and were 17% more efficient in converting feed into muscle, while containing only one-fifth as much carcass fat as litter mates at market weight (Pursel et al., 1990).

Transgenic techniques can also be used to modify wool growth and fiber quality. Cysteine is a rate-limiting amino acid for wool growth (Reis and Schinkel, 1963). However, direct dietary supplementation is not effective because cysteine is degraded in the rumen of sheep by the microflora. The genes that encode the cysteine biosynthetic pathway have been isolated and cloned from bacteria and from Salmonella typhimurium (Rogers, 1990). These genes have been introduced into sheep, and recently, it has been established that these transgenic sheep can enhance wool production characteristics (Bullock et al., 1995).

Application of transgenic technology to improve animal health holds considerable promise in the future. Genes encoding mouse α heavy and κ light chains of antibodies against phosphorylcholine have been introduced into pigs and lambs and high levels of mouse IgA were expressed in pig plasma (Lo et al., 1991). The IgA secreted was assumed to be a chimeric antibody including endogenous light chains with mouse heavy chains, since it showed little binding specificity for phosphorylcholine. In transgenic sheep, IgA was detectable in peripheral lymphocytes but not in the plasma.

The possibility of using the mammary gland as a "bioreactor" to produce rare pharmaceutical products is another very attractive feature of transgenic animals. A transgenic mammary gland production system would have a multitude of advantages over other common tissue culture systems. A mammary gland production system would not require the vast capital expenditure necessary for the establishment of manufacturing facilities required in a tissue culture production system. Furthermore,
high gene expression levels and low expendable production costs involved in making these specialized transgenic animals make it a cost efficient production system. Several pharmaceutical protein genes have been expressed in transgenic goats (Ebert et al., 1991), sheep (Clark et al., 1989) and pigs (Shamay et al., 1991).

Dairy goats are ideal candidates for pharmaceutical protein production. They produce, on an average, of 4 L of milk per day, and are more economical to purchase and maintain than cows. More importantly, goats have gestation and postnatal development intervals of moderate length (5 to 8 months). Thus, the use of goats would enable transgenic product production to quickly adapt to the market demand. Like that of cow’s milk, goat milk, has been characterized for its biochemical components (Simos et al., 1991).

Over the last 15 years the production of transgenic animals has enabled scientists to better understand the regulation of gene expression (Palmiter and Brinster, 1986), the process of tumorigenesis (Hanahan, 1988), the functioning of the immune system (Storb, 1987) and the molecular basis of embryo development (Wagner, 1990).

The usefulness of transgenic animals is only limited by human imagination. A crucial step involved in the production of transgenic farm animals is the introduction of the foreign gene into the genomic DNA. The pronuclear microinjection method originally used to produce transgenic mice (Gordon et al., 1980), is now the predominant method used to produce transgenic farm animals. The procedure requires special equipment for DNA microinjection and considerable experience. The problems using this method for farm animals include: 1) unlike with mice, fewer oocytes are available from farm animals, 2) greater variability in the development of embryos make even fewer ova available for microinjection, and 3) more cytoplasmic vesicles are present in farm animal ova obscuring the view of the pronuclei for microinjection. These factors together make the efficiency of producing of transgenic farm animals production very low (0.5-1%).
Based on information presently available, only a few transgenic pigs, sheep, goat and cattle have been produced by this procedure. This low success rate also reflects that many microinjected embryos are damaged by the act of injection and that gene integration is a random but infrequent event. Inefficiency makes production costs range from $25,000 for a single founder pig to over $500,000 for a single functional founder calf (Wall et al., 1992). The efficiency of this procedure must be improved or alternative methods must be sought.

Embryo stem (ES) cell technology may offer alternative for the production of transgenic farm animals. The most exciting possibility with ES cell technology involves homologues recombination to insert transgenes into targeted loci or to inactivate endogenous genes (Doetschman et al., 1987; Mansour et al., 1988), so that a specific gene can be modified. Chimeric mice have been produced by introducing transformed ES cells into blastocysts (Capecchi, 1989). Once farm animal ES cells have been established, clones of transgenic farm animals could be produced by nuclear transfer of ES cells with known desired genotypes.

Since the major function of the sperm cell during the fertilization process is to deliver its own DNA into an oocyte, it seems logical that there may be a way to induce sperm cell to assimilate foreign DNA and further to deliver this foreign DNA, along with its own DNA, into oocytes during the fertilization process.

Sperm-mediated gene transfer was first conducted in mammals by Lavitrano et al. (1989). In this study, 30% of the mouse progeny obtained foreign DNA during in vitro fertilization (IVF) of oocytes with sperm cells co-incubated with foreign DNA. When compared with the pronuclear microinjection procedure, the sperm-mediated gene transfer approach offers a much simpler and more efficient gene transfer method. Experiments conducted by Brinster et al. (1989) and Baringa (1989) to duplicate the experiments of Lavitrano et al. (1989) are the source of some skepticism regarding this method. However, in most recent years progress on sperm-mediated gene transfer has
been made. It has been shown that sperm cells transfer foreign DNA into oocytes during fertilization in various species (sea urchin, Arezzo, 1989; cattle, Perez et al., 1991, Gagné et al., 1991; mouse, Perez et al., 1991, de la Fuente et al., 1991; Hochi et al., 1992; Chicken, Nakanishi and Iritani, 1993). Transgenic farm animal offsprings have been produced (lambs, Sun et al., 1994; calves, Schellander et al., 1995). These preliminary findings indicate that sperm-mediated gene transfer is a method that shows promise for further study.

The objective of this study was to use caprine sperm cells as a model for evaluating sperm-mediated gene transfer in the goat. The ability of goat sperm cells to bind foreign DNA will be evaluated. A series of experiments will be conducted using confocal microscopy to ascertain the following: 1) the effect of seminal plasma on goat sperm cell binding of foreign DNA, 2) the binding patterns and the position of foreign DNA on sperm cells, 3) the percentage of sperm cell binding of foreign DNA and the differences between fresh goat sperm cells and frozen-thawed sperm cells on binding of foreign DNA. An experiment involving in vitro fertilization of goat oocytes with sperm cells co-incubated with foreign DNA will be conducted to evaluate whether goat sperm cells can introduce foreign DNA into oocytes during the fertilization process.

Based on information from in vitro fertilization experiments, the mechanism of goat sperm cell binding of foreign DNA will be evaluated. The fate of foreign DNA after binding to sperm cells will be evaluated with Southern blotting procedures. The possibility of increasing the efficiency of sperm cell binding of foreign DNA with electroporation will also be evaluated. Based on this information from these objectives, a high efficiency of sperm cell binding of foreign DNA could be achieved.

Intracytoplasmic sperm injection (ICSI) for goat oocytes will be developed to further evaluate sperm-mediated gene transfer. Sperm-mediated gene transfer via intracytoplasmic sperm injection will be evaluated. The integration of foreign DNA
into goat genomic DNA will be evaluated by the biopsy of early stage goat embryos and the detection of foreign DNA in individual embryonic cells with a fluorescent in situ hybridization (FISH) procedure.
Transgenic animals are produced by introducing foreign DNA into the genomic DNA of the animal. Identification of the peptide or protein and cloning of the gene must be accomplished before considering the use of gene transfer. The strategy for constructing a transgene (fusion gene) involves selecting a genetic regulatory element (promoter and enhancer) that will determine the tissue in which the gene is to be expressed. In addition to regulatory elements, a transgene consists of a DNA sequence encoding the desired protein or peptide (such as the frequently used growth hormone gene). Transgenes are usually inserted into plasmid vectors for further amplification and purification. The amplification process produces many copies of the transgene for gene transfer. The presence of a eukaryotic promoter and polyadenylation signal in the vector will help ensure expression of the gene in eukaryotic cells.

Majority of the structural genes being tested in farm animals are those with growth enhancement characteristics, such as the growth hormone (GH) gene. Others being evaluated for growth performance include growth hormone releasing factor (GnRH), insulin like growth factor I (IGF-I), cellular SKI (cSKI) and estrogen receptor genes. Also under consideration are genes that enhance disease resistance, such as the influenza virus resistant gene Mx1 and genes encoding antibodies against phosphorylcholine (reviewed by Pursel and Rexroad, 1993). The regulatory elements for these structural genes commonly used are derived from metallothionein (MT) genes from various species. Metallothionein is an inducible liver enzyme, and its gene is usually quiescent until a threshold level of circulating zinc or cadmium triggers
transcription of MT genes. Thus the expression of an MT-GH fusion gene in transgenic animals can be controlled by feeding zinc to the animals. Long terminal repeats (LTR) from two retroviruses (MLV and RSV) and sequences from CMV (a DNA virus) also have served as regulatory components of transgenes. Promoters from albumin, prolactin, skeletal actin and phosphoenol pyruvate carboxykinase (PEPCK) genes have also been used as promoter/enhancer elements. These promoters were much less responsive to dietary manipulation in transgenic pigs and sheep than in transgenic mice (Murray et al., 1989; Wieghart et al., 1990).

Other genes such as whey acid protein, human protein C, tissue plasminogen activator and human alpha-1-antitrypsin have also been introduced into transgenic farm animals (reviewed by Pursel and Rexroad, 1993). Since these pharmaceutical proteins are designed to be secreted from the mammary glands, specific regulatory sequences have been used. Sheep β-lactoglobulin, cow α-S1-casein, β-casein gene from rabbit, rat, cow and goat, and mouse whey acidic protein (WAP) promoter/regulatory sequences have been investigated. Since all these genes are expressed in the mammary glands, the transgenes have been designed to be directed by regulatory sequences of these tissue specific expression genes. Thus, transgene expression is restricted to the mammary glands. Several pharmaceutical protein transgenes have been designed in this manner and have been expressed in the milk of transgenic goats (Ebert et al., 1991), sheep (Clark et al., 1989) and pigs (Shamay et al., 1991).

Gene Transfer

Pronuclear Microinjection

Transgenic animals are now recognized as powerful tools both for analyzing the basic biological function of genes and gene regulation in vivo (reviewed by Palmiter and Brinster, 1986) and for improving the efficiency of animal agriculture (reviewed by Rexroad, 1992). A popular method used in production of transgenic animals is the microinjection of several hundred copies of the gene into a pronucleus of sperm-
activated ova. This method was first described in mice (Gordon et al., 1980). Briefly, donor animals are superovulated and sperm-activated ova at the pronuclear stage are recovered from the donor females. Individually, these ova are secured via a holding pipette and examined for the presence of pronuclei with the use of an inverted phase-contrast microscope equipped with differential interference contrast (DIC) optics. Sperm-activated ova from farm animals such as pigs and cows contain cytoplasmic vesicles that obscure the view of the pronuclei. The ova from these animals must be centrifuged before the pronuclei can be visualized. Once a pronucleus is located, a pipette filled with a buffer containing the transgene is introduced into one pronucleus of the ovum for direct injection of the transgene. The pipettes used for gene injection with farm animals are less than 0.5 μm diameter and are slightly tapered (less than 5 degrees) to easily pass through the membranes and minimize damage to the ova. After injection a volume of 2 femto liter DNA, containing several hundred or more copies of DNA, the pronucleus becomes distended. The injected ova are then transferred into the oviducts of the recipient animal. A few transgenic pigs, sheep, goats, cattle have been produced by this procedure. However, the production of transgenic animals by pronuclear microinjection is still problematic in large domestic animals and the efficiency of production of transgenic farm animals is very low (0.5-1%) (Brinster et al., 1985; Rexroad et al., 1989; Roschlau et al., 1989).

Recent progress has been made to reduce the cost of transgenic animal production. Embryos can be produced by in vitro fertilization (IVF) methodologies. Today, the production of embryos from oocytes matured in vitro has been successfully developed for cattle (Lu et al., 1987; Goto et al., 1988; Zhang et al., 1992). Offsprings have been produced from IVF in sheep (Cheng et al., 1986; Crozet et al., 1987), pigs (Cheng et al., 1986; Nagai et al., 1988) and goats (Hanada, 1985). Even though current success in goat IVF is behind that of bovine IVF, recent progress has been made in goat IVF methodologies. Oocytes have been successfully retrieved by the transvaginal

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method and goat offspring has been produced from in vitro matured oocytes, in vitro fertilized, in vitro cultured and frozen-thawed goat embryos (Han et al., 1996). Progress on additional technologies now would offer a less expensive source of sperm-activated ova from large animals for DNA microinjection procedures.

Injected embryos could also be cultured in vitro to a later stage to help reduce the embryos damaged by microinjection, thus reducing the total recipient animals needed for transfer. This would reduce the cost of maintaining the number of animals needed per transgenic embryo produced (Bowen et al., 1994). In recent years, the most notable development in embryo culture systems has been the use of "helper" cells to co-culture early stage embryos in vitro. The helper cells used in co-culture systems can be derived from several origins including cells derived from the trophoblast, oviduct, uterus, follicle and liver and cells from chicken embryos (see review by Thibodeaux and Godke, 1992).

Zhang et al. (1995) showed the benefits of cumulus cells to support bovine oocyte maturation and early stage embryo development. Recent progress has also been made on the in vitro co-culture of goat embryos. A recent study has shown that helper cells, such as bovine oviduct epithelial cells (BOEC), goat oviduct epithelial cells, buffalo rat liver cells (BRLC) and goat cumulus cells (GCC) were effective in improving the in vitro development of IVF-derived goat embryos (Han et al., 1996). Co-culture of embryos for a short period has become a routine method to in vitro develop IVF-derived embryos of the farm animals (Godke et al., 1993).

The mechanism on the helper cells benefit embryos is not very clear. It has been suggested that helper cells release embryotropic factors into the co-culture system (Gandofi et al., 1992). Helper cells are thought to produce and release metabolites or growth factors that benefit embryonic development. Earlier, Bavister (1988) had reported that helper cells may detoxify the medium by consumption of oxygen around the embryos in the culture.
A number of growth factors have been shown to benefit embryo development in vitro. Exogenous IGF-I was added to culture mouse embryos in vitro and resulted in an increase in the inner cell mass of the mouse embryo (Harvey and Kaye, 1992). Epidermal growth factor (EGF) significantly improved bovine embryo development to the blastocyst stage when supplemented in culture medium (Flood and Bunch, 1992). Leukemia inhibitory factor (LIF) improved the viability of cultured sheep blastocysts when added to the culture medium (Fry et al., 1991). These results and those of others suggest that the benefit of the helper cells may be related to growth factors secreted from these cells. With further understanding of how helper cells improve embryo viability and development, efficiency of in vitro embryo culture will be improved and the cost of gene transfer will likely be reduced.

In vitro culture could also offer another opportunity to further increase the efficiency of production of transgenic animals. New techniques have been developed that make it possible to identify transgenic embryos at an early embryo developmental stage. After isolating cells from the potential transgenic embryo, the cells are analyzed for the transgene. After the transgenic embryos are identified and cultured, only transgenic embryos are transferred to the recipient, greatly reducing the number of recipient animals needed during the procedure.

The polymerase chain reaction is an extremely sensitive method that can with a few cells detect a single copy of a gene (Saiki et al., 1988). This approach has been used successfully to detect foreign DNA in cells from biopsied embryos with mice and cattle (Ninomiya et al., 1989; Burdon and Wall, 1992; Bowen et al., 1994). It has been found that PCR technique used for analyzing biopsies was incapable of differentiating integrated DNA from residual unintegrated foreign DNA, which can persist in early embryos for a relatively long period of time (Ninomiya et al., 1989). Consequently, embryos shown to be PCR-positive at biopsy developed into fetus that were negative (Ninomiya et al., 1989; Burdon and Wall, 1992; Bowen et al., 1994).
Recently another technique, fluorescence in situ hybridization (FISH), has been shown to be capable of distinguishing integrated transgenes from unintegrated residual DNA in biopsy of mouse embryos (Lewis-Williams et al., 1996). This technique may improve the efficiency of detection of embryos with truly integrated transgenes and reduce the false positive results encountered with the PCR technique. With these molecular technologies, the production efficiency of transgenic animals should be improved in the near future.

Stem Cell Technology

Pluripotent embryonic stem (ES) cells may offer another method for the production of transgenic farm animals. Pluripotent embryonic cells are cells isolated from the inner cell mass of the blastocyst. Embryonic stem cells from mice can be maintained in an undifferentiated state in culture, allowing the introduction of transgenes (Robertson et al., 1986). One advantage of using stem cell technology is that the cells can be screened or selected for the integration of the transgene (Doetschman et al., 1987; Mansour et al., 1988) prior to insertion into the embryo. Genetically modified embryonic stem cells can then be microinjected into the blastocyst of a recipient embryo. Chimeric mice were produced using this procedure (Capecchi, 1989). These chimeric mice have been used to produce pure transgenic strains through natural mating.

The most exciting use of ES cells involves homologous recombination to insert a transgene into a targeted locus or to inactivate an endogenous gene (Doetschman et al., 1987; Mansour et al., 1988). This is because homologous recombination is a very rare event in mammalian cells, and stem cells lines can be selected with a desired gene type. Embryonic stem cells have not yet been established and verified in farm animals. Recent derivation of embryonic stem cell lines from pig and sheep promises that ES technique may soon be applied to farm animals (Notarianni et al., 1991). Recently, lambs were produced by nuclear transfer of embryonic disc cells, but the efficiency
was still very low (Campbell et al., 1996). For farm animals, nuclear transfer of ES cells with a known desired genotype could be used as a strategy to avoid chimeric offspring and thus, producing clones of identical animals (Wilmut and Smith, 1988).

Retroviral Vector

Retroviral vectors represent another approach to gene transfer technology. Retroviruses infect cells and then incorporate their genome into the chromosomes of the infected host cells (Varmus, 1988). Some fragments of retroviruses DNA insert themselves into chromosomes of infected host cells while other fragments are responsible for replication of retrovirus and dispersal of retroviral DNA from the host chromosome. Recombinant viruses are being designed that only have the ability to insert themselves into the chromosomes (Varmus, 1988). In principle, retroviral vector-mediated gene transfer should have a high frequency of integration.

The retrovirus vector has been an attractive approach for producing transgenic chickens. Chicken embryos after oviposition consist of 10,000 to 60,000 cells, which makes microinjection unfeasible. Transgenic chickens have been produced with replication-competent and replication-defective retroviral vectors (Salter et al., 1986, 1987; Salter and Crittenden, 1989; for reviews see Bosselman et al., 1990; Crittenden, 1991).

Retrovirus-mediated gene transfer has been used to introduce genes into mouse embryos at various stage by infecting preimplantation embryos with a retroviral vector (Eglitis et al., 1985; Soriano et al., 1986; Jaenisch, 1988). In the farm animals, viral sequences have been artificially introduced into both sheep embryos (Hettle et al., 1989) and bovine embryos (Haskell and Bowen 1995). Mosaic founder animals are the major problem with this approach. Furthermore, the size of the transgene is a limiting factor for most retroviruses. With the possibility of a defective vector reverting to an infective state, safety is still a concern with the retrovirus technology.
This gene transfer method will likely remain unattractive for the production of transgenic farm animals.

Sperm-Mediated Gene Transfer

The fertilization process involves that sperm cell delivers its DNA into an oocyte, it seems possible that there may be a way to induce sperm cells to associate foreign DNA, and to deliver the foreign DNA into oocytes during fertilization. Recently, transgenic sheep and calves have been produced with sperm-mediated gene transfer (Sun et al., 1994; Schellander et al., 1995). This method and recent progress will be reviewed later in this chapter.

Transgene Integration

Integration of the microinjected transgenes occur at random sites in the chromosome of the recipient nucleus. Therefore, unless the transgene is constructed to target a specific chromosomal locus (Thomas et al., 1986), the foreign gene will act as a third allele or a new allele in the transgenic animals. The transgene is usually integrated into the genome at a single location, but multiple integration can occur (Wilkie et al., 1986; Shamay et al., 1991). Multiple copies of the transgene are most often arranged in a head-to-tail or head-to-head arrangement at a single location.

The mechanism that governs gene integration is not fully understood. Palmiter and Brinster (1986) suggested that DNA integration was the result of damage to the chromosomes during injection and that incorporation of the gene into one of these sites occurred during DNA repair. It has also been suggested that transgene integration occurs during DNA replication (Bishop and Smith, 1989). In mice, the frequency of integration of transgene is affected by factors such as transgene buffer composition, conformation of the transgene and concentration of transgene used for microinjection (Brinster et al., 1985).

Pronuclear injection can generate a hemizygous or mosaic embryo with respect to the transgene, depending on when the injected transgene integrates into the genomic
component (Wilkie et al., 1986). Hemizygous transgenic animals can be produced if the integration event occurs before the first round of DNA replication after pronuclear microinjection; while mosaic embryos can be produced if integration events occur after this period. There are two possible fates for mosaic embryos: 1) if transgene-containing cells segregate to the inner cell mass at the blastocyst stage, the offspring (derived from a proportion of the ICM cells) will likely become transgenic; 2) if transgene-containing cells segregate to trophectoderm cells at blastocyst stage, offsprings will not become transgenic. Approximately 62% of transgenic mouse embryos produced by microinjection have been found to be mosaic (Bruce et al., 1993). Other studies have shown that 70% of transgenic mice produced by microinjection carry the transgene in all cells, and the remaining 30% of the embryos were mosaic (Wilkie et al., 1986; Palmiter and Brinster, 1986). In another study, none of the 8-cell mouse embryos had transgenes in more than four blastomeres (Burdon and Wall, 1992), while other results showed that as embryos developed, the incidence of mosaicism significantly increased from 35% post-microinjection at 21 hours to 61% at 67 to 69 hours post-microinjection (Lewis-Williams et al., 1996).

Mosaicism is also a problem in transgenic farm animals. Pursel et al. (1990) reported that 20% of founder transgenic pigs and sheep fail to transmit the gene to progeny and another 20 to 30% transmit the transgene to less than 50%, due to mosaicism in the germ cells. Transgene integration efficiency is less than 1% in farm animals (reviewed by Pursel and Rexroad, 1993). This low efficiency may be attributed to microinjection apparently being performed during late S-phase or later in the cell cycle of farm animals (reviewed by Wall, 1995).

Alteration of integrated foreign DNA has also been reported in mice (Gordon et al., 1980; Wagner et al., 1981; Ross and Solter, 1985; Strojek, 1986). It has been proposed that before foreign DNA can be integrated into the genome, deletions and

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recombinatons of foreign DNA by DNA-active enzyme and the linearization of circular DNA at different sites could occur (Roschlau et al., 1989).

Transgenes introduced by microinjection are randomly integrated into the genome. In contrast, the stem cell method could offer the potential for production of transgenic animals with site-specific gene insertion (Doetschman et al., 1987; Mansour et al., 1988). Until ES cells are established or an alternative method is found, DNA integration will remain as a major problem in the production of transgenic farm animals.

Transgene Expression

To have a successful gene transfer, the transgene must perform its function and the foreign peptide/protein must be expressed in transgenic animals. If the gene functions normally, expression will occur in a tissue-specific fashion according to the promoter/enhancer. Transgenes are sometimes activated in unintended tissues (ectopic expression) and the timing of expression can be shifted in relation to morphological development.

The aberrant expression patterns (no expression or atypical expression) have been attributed to the so-called "position effect" in which transgene expression is influenced by neighbor endogenous genes (Jahner and Jaenisch, 1985; Soriano et al., 1986; Shani, 1986). A transgene with boundary DNA or isolator sequences has been used to block the influence of the surrounding genes (McKnight et al., 1992; Huber et al., 1994).

In one study, 66% of founder MThGH and MTbGH transgenic pigs produced detectable levels of hGH or bGH in plasma at birth (Pursel et al., 1989). It has been suggested that the variability of expression among founder animals was due to the influence of chromosomal position on tissue specificity. Variation resulted from random insertion of transgenes into different loci of the genome and from activity of the MT promoter (Palmiter et al., 1982). In one study, it was found that transgenic
pigs with foreign growth hormone gene did not grow larger than their siblings (Pursel et al., 1990). However, in the same study, some transgenic pigs grew faster and were 17% more efficient in converting feed into muscle, and at market weight contained only one-fifth as much carcass fat as littermates (Pursel et al., 1990). The second generation of the transgenic pigs was also 18% more efficient in food conversion than the control pig line (Pursel et al., 1989). Reduction in lipid in intramuscular tissue was also detected in transgenic pigs (Pursel et al., 1990). The presence of either human growth hormone or bovine growth hormone in the plasma of pigs was accompanied by a decrease in the levels of pig growth hormone (Miller et al., 1989). However, an increase of endogenous IGF-I was detected in these transgenic pigs (Miller et al., 1989). An increase in endogenous IGF-I was probably the result of stimulation of transgenic GH on the GH hepatocyte receptor (Hughes and Friesen, 1985; Mathews et al., 1986) while the decrease in endogenous GH was due to feedback inhibition by transgenic GH and endogenous IGF-I on the hypothalamus and pituitary (Mathews et al., 1986).

Several studies showed that transgenic lambs did not grow faster or utilize feed more efficiently than control lambs, however they were much leaner (Rexroad et al., 1989, 1991; Nancarrow et al., 1991). Long term exposure to elevated GH also stimulated organ growth. Pursel et al. (1989) showed that some transgenic pigs had enlarged livers, hearts, kidneys, adrenal glands and thyroids. Long bone weight and circumference were also increased in this study. Expression of continuously elevated hGH and bGH in transgenic pigs was associated with several notable pathologic changes including joint pathology, gastric ulcers and infertility (Pursel et al., 1987, 1989; Ebert et al., 1988, 1990; Wieghart et al., 1990). Transgenic lambs with elevated GH also had a number of pathological problems including joint problems and degenerative kidney disease (Nancarrow et al., 1991).
Targeted expression of transgene into specific tissues such as mammary gland has also been achieved with pharmaceutical proteins expressed in the milk of transgenic goats (Ebert, et al., 1991), sheep (Clark et al., 1989) and pigs (Shamay et al., 1991).

A key factor responsible for high levels of transgene expression is the inclusion of the introns in the structural gene (Wright et al., 1991). The apparent requirement for introns poses major problems for the exploitation of gene transfer. Many gene sequences are too large to be transferred by microinjection or retroviruses. Different structural genes have different expression levels and promoters also play significant roles in controlling gene expression. Ebert et al. (1991) showed that plasminogen activator yields increased 1000-fold in transgenic goat milk when the transgene promoter was switched from the promoter of the gene encoding mouse whey acid protein to the promoter of the goat β-casein gene.

Transgenic sheep and goats producing pharmaceutical proteins in their milk have been generated and to date exhibit no physiological or reproductive problems (Clark et al., 1989; Ebert et al., 1991). However, when mouse whey acidic protein gene was transferred into farm animals, some transgenic pigs and goats exhibit agalactic (Shamay et al., 1991; Pursel and Rexroad, 1993). Successful targeted transgene expression has also been achieved in skeletal muscle with promoter sequences associated with the skeletal muscle actin gene (Shani, 1986) or myosin light chain kinase (Shani, 1985). Recombinant human hemoglobins have also been successfully produced in the blood of transgenic pig (Sharma et al., 1993). Although some success has occurred, the precise control of transgene expression needs to be further studied to improve the efficiency of transgenic animals.

**Transgene Transmission**

Since founder animals are usually hemizygous for the transgene, it would be expected that 50% of their offspring would inherit a copy of the transgene locus.
However, non Mendelian inheritance can be caused by transgene mosaicism in the germ cells. It has been suggested that the mosaicism is caused by late integration of transgenes during embryonic development (Wall and Seidel, 1992). In contrast to the variability in transgene expression in founder animals, the transgene expression in porcine successive generations of progeny from the founder animals was maintained at a relatively consistent level (Pursel et al., 1989).

Part II. Sperm Cell Structure and Functions

Sperm Cell Formation

Prespermatogenesis

In mammalian animals, prespermatogenesis is initiated during development of the male fetus, primordial germ cells divide mitotically as they migrate to the gonadal ridge, where they then proliferate for a while and start differentiating. The differentiation of fetal male germ cells is arrested at the G1 phase of mitosis before the male fetus reaches full development.

Spermatogenesis

After spermatogonia is formed, spermatogenesis is initiated. Spermatogenesis is initiated at puberty in most mammalian species and continues throughout the reproductive life of the male. At the early stage of spermatogenesis, type A spermatogonia involves several mitotic divisions to form type B spermatogonia. While type A spermatogonia keeps mitotic divisions to provide type B spermatogonia, type B spermatogonia divide to form primary spermatocytes. After primary spermatocytes are formed, they will undergo meiosis twice instead of mitotic divisions and ultimately form haploid spermatids. The first meiotic division is different from the second meiotic division. In the first meiotic division, the prophase is very long and this process can be further divided into preleptotene, leptotene, zygotene and pachytene stages. At the preleptotene stage, DNA synthesis is very active. At the
leptotene stage, primary spermatocytes become tetraploid. In animals, DNA synthesis becomes inactive after the leptotene stage. After the first meiotic division, one tetraploid primary spermatocyte forms two diploid secondary spermatocytes. Diploid secondary spermatocytes undergo another reduction division cycle without DNA synthesis and form haploid spermatids. The entire divisional process of spermatocytogenesis, from spermatogonia to spermatid, takes ~45 days in the bull (reviewed by Curtis and Amann, 1981).

Spermiogenesis

Once spermatids are formed, they do not divide further, but differentiate to form spermatozoa. Spermiogenesis starts from the formation of the spermatid at the second meiotic division and ends with the release of the spermatozoon from the seminiferous epithelium. During this process, round spermatid develops into a mature spermatozoon with tadpole shapes. These morphogenic events are accompanied with development of an acrosome, development of a flagellum, nuclear shaping, nuclear condensation, mitochondrial reorganization and elimination of the residual cytoplasm. In the rat it takes ~21 days to complete this process, while other species show similar events at analogous stages (Russell et al., 1990). A thorough description of the events is given by Russell et al. (1990).

Sperm Cell Structure

Fully formed sperm cells are elongated cells consisting of a flattened head and a tail. The flattened head contains the nucleus and a tail has the apparatus (mitochondria) necessary for sperm motility.

Acrosome

The acrosome of mammalian sperm originates from the Golgi apparatus during spermiogenesis. The acrosome contains various hydrolytic enzymes. The acrosome together with the plasma membrane make membrane of the sperm head unique and complicated. The front head of sperm is covered by both acrosomal membranes and
the plasma membrane. During normal fertilization process, this region is responsible for initial interaction of the sperm with the cytoplasmic membrane of the oocyte. The postnuclear cap and the posterior portion of plasma membrane of the sperm head cover postacrosomal region of the sperm head. The plasma membrane of the posterior region of the sperm head will be involved in gamete fusion between the sperm and the oocyte in the final steps of the fertilization process (Bedford et al., 1979; Shalgi and Phillips, 1980; Yanagimachi, 1981).

Sperm Nucleus

The sperm head is filled with tightly packed chromatin material consisting almost entirely of deoxyribonucleoproteins (DNPs), which are composed of deoxyribonucleic acid (DNA) conjugated to certain highly basic nuclear proteins (Gledhill, 1975). The DNA organization in mammalian sperm is not quite clear compared with somatic cell DNA organization. Compared to the double helix in somatic cells, the double helix in sperm DNA is similar except that telomeres in sperm DNA is longer (De Lange et al., 1990) and DNA hypermethylation is much more extensive with sperm DNA (Groudine and Conkin, 1985). The most significant difference between sperm DNA and somatic DNA is reflected in DNA binding proteins. The DNA binding proteins of mammalian sperm DNA are protamines while DNA binding proteins of somatic DNA are histones. Protamines are highly basic proteins with positively charged arginine residues (Balhorn, 1982). Protamines bind to the DNA lengthwise with each positively charged arginine residue neutralizing one negatively charged residue of the phosphodiester backbone of the DNA. This interaction makes the polyanionic DNA into a neutral polymer, and at the molecular level, the DNA-protamine complexes can then bind together by van der Waals forces in a linear fashion (Balhorn, 1982).

How DNA-protamine complexes form the tertiary structure in sperm nuclei is still not clear even though the sperm DNA loop doughnut model has been proposed.
The DNA of sperm is so tightly bound to the nuclear proteins that the activity of transcription is not expected. Contrast to somatic nucleus, typical properties of the mammalian sperm nucleus are high stability and inertness. Furthermore, during maturation of the sperm in the epididymis, the number of disulfide bonds between the sperm nuclear proteins are increased, which in turn increases the thermal stability of the DNP complex of the sperm cell.

The nuclei of mammalian sperm are genetically inactivated and structurally stabilized by the association of sperm DNA with protamines, highly basic proteins that replace somatic histones during spermiogenesis (reviewed by Bellve, 1979; Poccia, 1986). As sperm pass through the epididymis, protamine sulfhydryls are oxidized to disulfides, which further stabilize the sperm nuclei (Calvin and Bedford, 1971; Marushige and Marushige, 1975; Meistrich et al., 1976). Disruption of these disulfide bridges is a prerequisite for decondensation of the fertilizing sperm nucleus (Perreault et al., 1984). The initial process of sperm decondensation during fertilization is protamine replacement by histones and subsequent reactivation of the sperm genome in the oocyte (reviewed by Poccia, 1986).

**Sperm Cell Function**

Sperm Capacitation and Acrosome Reaction

Austin (1951) and Chang (1951) first reported that the sperm cells released from the male reproductive tract of most mammals are not immediately fertile, and that sperm cells have to undergo a physiological change called capacitation that normally occurs in the female reproductive tract.

Capacitation and acrosome reaction are required in vivo for the development of the fusogenic ability of sperm (Rogers and Bentwood, 1982). The membrane alterations of capacitation normally precipitate the acrosome reaction, which involves the exocytotic release of hydrolases and exposure of the fusogenic region. Induction
of the acrosome reaction has been found to be universally dependent upon the presence of calcium ions.

Several regimens have been devised to achieve capacitation and acrosome reaction in vitro. These include preincubation of sperm in ligated rodent or rabbit uteri (Austin, 1951) in simple or complex culture medium supplemented with albumin or serum (Miyamoto and Chang, 1973) in the presence of glycosaminoglycans such as heparin (First and Parrish, 1988). Capacitation also results in response to exposure to high ionic medium to increase permeability or to phosphatidylcholine liposomes (Brackett et al., 1978; Graham et al., 1986), long time exposure sperm cells to chemically defined isotonic medium (Iritani et al., 1984) or treatment of the sperm with calcium ionophore A23187 for a short period (Atiken et al., 1984). In general, any agent that causes calcium influx into the sperm acrosome and causes a pH increase within the sperm cell induces capacitation (First and Parrish, 1988).

Activation of Oocytes at Fertilization

Fertilization normally occurs when the oocyte is meiotically mature, and is initiated after the sperm cell membrane fuses with the membrane of the ovum. The sperm receptor on the oocyte surface interacts with oocyte-binding protein located on the sperm surface to form a complex and results in the species-specific adhesions between the sperm cell and the oocyte (Kinsey et al., 1980). Resultant changes of the mature oocyte include cortical granule exocytosis, which usually hardens the zona to block polyspermy and extrusion of the second polar body. In mammalian species studied to date, the sperm cell triggers repetitive rises in the Ca\(^{2+}\) at regular intervals for several hours during fertilization in the mice (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992), in the hamster (Igusa and Miyazaki, 1986, Miyazaki, 1990), in the cattle (Fissore et al., 1992; Sun et al., 1994), in the pig (Sun et al., 1992) and in the human (Taylor et al., 1992). It is suggested that these oscillations of intracellular free
calcium $\text{Ca}^{2+}$ leads to oocyte activation, DNA synthesis and further embryonic development.

The mechanism by which the sperm activates the oocyte is still in controversial. Various factors from the sperm cell have been suggested as the trigger of oocyte activation. Currently there are two major hypotheses to explain how sperm cells trigger the free calcium $\text{Ca}^{2+}$ elevation and oocyte activation. The first is that the leakage of extracellular calcium through calcium channels originating from sperm plasma membranes occurs during gamete fusion (Miyazaki et al., 1986; Speksnijder et al., 1989; Jaffe, 1990), which then introduces a soluble factor directly into the ooplasm (Dale et al., 1985; Swann and Whitaker, 1990). The second is that there are complementary receptors on sperm cell membrane and oocyte plasma membrane to activate the G-protein-phospholipase C pathway (Miyazaki et al., 1990, Williams et al., 1992).

Recently results from direct sperm injections into cytoplasm of an oocyte indicated that the sperm soluble factor hypothesis was more reasonable. It was suggested that this factor is formed during sperm cell maturation after secondary spermatocyte formation (Kimura and Yanagimachi, 1995).

The amplitude and duration of the $\text{Ca}^{2+}$ elevations inside oocytes evoked by sperm cells were similar among several mammalian species studied. Amplitude of the $\text{Ca}^{2+}$ elevation ranged 240 to 4,500 nM in mouse, hamster and bovine oocytes (Cuthbertson and Cobbold, 1985; Igusa and Miyazaki, 1986; Fissore et al., 1992) while the average duration was approximately 3 minutes (Kline and Kline, 1992; Fissore et al., 1992). Mammalian oocytes also can be activated with exogenous stimuli. Injecting $\text{Ca}^{2+}$ directly into oocytes, electropereabilization in the presence of $\text{Ca}^{2+}$ or addition of ionophore A23187 could all trigger $\text{Ca}^{2+}$ exocytosis of cortical granules and completion of meiosis and early development in mouse, hamster and pig oocytes (Steinhardt et al., 1974; Fulton and Whittingham, 1978; Sun et al., 1992).
Other parthenogenetic stimuli such as ethanol may also activate oocytes. However, parthenogenetic stimulated oocytes have no calcium oscillation pattern or were totally different from that of the oocytes stimulated by sperm cells (Cuthbertson et al., 1981). This may explain that parthenogenetic activated mammalian oocytes cannot develop to full term even though they go through early development like sperm fertilized oocytes. Factors inducing parthenogenetic activation but mimicking the pattern of Ca\(^{2+}\) oscillation similar to that occurring during fertilization could prolong considerably the activated oocyte development (Ozil, 1990). In summary, sperm cells are required to activate oocytes and to complete the early fertilization process and full development in mammalian species.

Pronuclear Formation

Once the sperm penetrates into the oocyte cytoplasm, the earliest chromosomal event is decondensation of protamine and nucleosome-bound supercondensed sperm chromatin (Benford and Calvin, 1974; Longo and Kunkle, 1978; Balhorn, 1982). In addition to sperm chromatin dispersion, removal of sperm specific basic proteins, such as protamine, remodeling of chromatin with histones and recondensation of chromatin are necessary steps toward the formation of a male pronucleus. The control mechanisms of male pronuclear formation are presently not clear. Thibault and Gerard (1973) indicated that there are some factors (known as the male pronuclear growth factor) in the activated oocyte cytoplasm controlling male pronucleus formation. Other groups call these factors sperm pronucleus development factors (Yanagimachi, 1981). There are three morphological events that take place for sperm nuclei to form a male pronucleus. These include breakdown of sperm nuclear envelope, decondensation of the sperm nucleus and the formation of a male pronuclear envelope (Longo, 1987).

The stability of mammalian sperm nuclei differ markedly among species. When various sperm nuclei were injected into hamster oocytes, human sperm and...
mouse sperm decondensed within 15 to 30 minutes of injection, while sperm of the bull and rat decondensed more slowly (Perreault et al., 1988). It was found that this difference was related to the extent and/or efficiency of disulfide bonding in the sperm nuclei (Perreault et al., 1988). Once the sperm nuclear envelope has broken down, protamine in sperm chromatin begins being replaced by histone (Ecklund and Levine, 1975). The formation of a male pronucleus from the sperm is accompanied by the formation of a female pronucleus from the ovum. At the same time, two pronuclei migrate toward each other and their bilaminar membranes interdigitate. Inside the pronuclei, chromatin from maternal and paternal origin begins to condense and intermix to form the metaphase arrangement of the first mitotic division (see review by Gwatkin, 1977).

Following fertilization, the sperm chromatin becomes reorganized in a manner such that the appropriate genes are transcribed in an orderly fashion as the embryo develops and differentiates. It is not clear if the transfer of sperm nuclear DNA is all that is required to accomplish mammalian fertilization and development. The mitochondrial genome is maternally inherited (Hutchison et al., 1974; Hecht et al., 1984) and mitochondria of rat, hamster and rabbit sperm were found degenerated at the early stage embryo after fertilization (before 4-cell stage) (Hiraoka and Hirao, 1988; Szollosi, 1965; Bedford, 1972). Full term development of mice have been obtained after secondary spermatocytes were injected into mature oocytes (Kimura and Yanagimachi, 1995). This finding indicated that male genomic imprinting is complete, at least by the secondary spermatocyte stage, and that all the post-modification and maturational change, such as formation of sperm tail, into mature sperm cells play only a role in the transportation and deliverance of male genome into ova during fertilization.
Sperm Function during Micromanipulation Assisted Fertilization

Sperm injection, subsequent oocyte activation and in vitro fertilization procedures have been used for years as a tool in echinoderm (Hiramoto, 1962) and amphibian research (Brun, 1974). Further understanding of sperm function during fertilization has been enhanced by sperm injection technology. The first sperm injection experiments with mammals were conducted in rodents. The microinjected sperm head was observed to decondense into pronuclei and followed with subsequent early cleavage of the injected ova in the hamster (Uehara and Yanagimachi, 1976).

It was reported that motile mouse sperm was required to fertilize the ovum, while immotile sperm failed to fertilize even when the sperm was injected under the zona (Kobayashi et al., 1992). Capacitation of microinjected motile sperm is thought not to be required, however, using a Ca²⁺ ionophore did increase the fertilization rate after subzonal sperm injection of mouse ova (Kobayashi et al., 1992). The latter study suggested that "motile" function was required for sperm penetration of zona and fusion with ovum cytoplasmic membrane. Intracytoplasmic sperm injection (ICSI) procedures further revealed the role of sperm organelles during fertilization. Induction of the acrosome reaction by different treatments, such as electroporation or incubation of sperm with pentoxifylline did not enhance the fertilizing ability of human sperm after injection into the cytoplasm (Liu et al., 1994). This finding suggested that under the conditions of ICSI, the acrosome reaction is unnecessary for fertilization. However, the fate of acrosomal components and their impact on the development of fertilized ovum are unknown. It is not clear what elements of sperm are essential during fertilization with ICSI.

Ogura et al. (1994) have shown that the birth of normal mice can be achieved from oocytes injected with immature spermatids. Furthermore, the same research group showed that development of normal mice could be produced from oocytes injected with secondary spermatocyte nuclei (Kimura and Yanagimachi, 1995). These
experiments verified that all of the post-meiotic modifications that result in the differentiation of a mature sperm cell serve only to deliver a competent haploid male genome to the oocyte. This may indicate the genomic imprinting of the male is completed by at least the secondary spermatocyte stage. However spermatid and the secondary spermatocytes apparently require artificial stimulation to activate oocytes after ICSI (Ogura et al., 1994; Kimura and Yanagimachi, 1995).

These observations indicate that one very important function during post-meiotic modification is the formation of an oocyte activation factor. It has been shown that immobilized (killed) bovine sperm can fertilize oocytes, and after artificial activation of the oocyte result in the birth of live calves (Goto et al., 1990). This may indicate that the oocyte activation factor has been damaged in immobilized sperm and that it can be replaced with an artificial activation stimulus.

**Goat Semen Characteristics**

The prepubertal development period of the goat is very short. For example, in Boer goats it was reported that 157 ± 9.6 days of age sperm cells began to appear in the ejaculate with a volume of 0.17 ml and a sperm concentration of 0.09 x 10⁹/ml. After another 12 weeks, sperm production reached levels characteristic of maturity. At 8 months of age, male goats reach maturity and produce good quality semen. The goat is a seasonal breeder. Seasonal variation in semen characters such as sperm motility and concentration have been reported by Sahni and Roy (1969) for Barbari and Jamnapari bucks. Other semen characteristics such as volume, sperm concentration and proportion of live sperm cells were also significantly affected by season (Vinha, 1975; Patil and Raja, 1978). In general, reasonable expectations for normal semen ejaculate from goats appear to be ejaculate volume of 0.5 to 1.0 ml, sperm motility at collection of 50 to 90% and sperm number of 18 x 10⁸ to 40 x 10⁸ /ml.

For storage of goat semen at temperatures above freezing, cow milk, goat milk and coconut milk extender can be used for up to 48 hours with good motility (Tewari
et al., 1968; Sahni and Roy, 1972b; John and Raja, 1973; Pillai et al., 1978). Deep freezing goat semen can be accomplished using a glycerol and egg yolk extender. However, when egg yolk was used in the extender, an enzyme produced by the bulbo-urethral glands could catalyze the hydrolysis of lecithins in egg yolk to fatty acids (Roy, 1957), and lysolecithins are toxic to goat sperm cells (Aamdal et al., 1965). Sahni and Roy (1972 a,c) reported 6% glycerol was superior as a cryoprotectant to either 3% or 9% glycerol. Rossouw (1974) reported that the best post-thawing motility was achieved when semen was equilibrated for 2.5 hours in Tris diluent and thawed at 40°C or 90°C for 10 seconds.

Part III. Current Statues of Sperm-Mediated Gene Transfer

When eukaryotic cells were exposed to purified foreign DNA in culture, some of these cells occasionally took up foreign DNA (Pellicer et al., 1980). Foreign DNA can be integrated into the genomic of the cells, expressed and transmitted to their progeny with a very low transfecting efficiency (in the order of 1:10^8 exposed cells) (Pellicer et al., 1980).

Sperm cells were first used to introduce foreign DNA (SV40) into oocytes during the fertilization process in the rabbit (Brackett et al., 1971). Some years later, Lavitrano et al. (1989) showed that in vitro fertilization of mouse oocytes with sperm co-incubated with foreign DNA resulted in transgenic mice. In this study, 30% progeny were transgenic. Since pronuclear microinjection technology requires a great deal of experience and produces an extremely low integration rate (0.5 to 1% in farm animals), using sperm cells may be an attractive alternative method to introduce foreign DNA during fertilization, especially in farm animals. During microinjection, foreign DNA is introduced prior to syngamy, while sperm-mediated gene transfer first occurs during meiosis.
Sperm Cell Binding of Foreign DNA

The ability of sperm cells to bind foreign DNA has been shown in several animal species. Castro et al. (1990) showed that bull, pig, buffalo, ram, goat and rooster sperm cell pellets had radioactivity after they were incubated with radiolabelled foreign DNA. Results from different research groups confirmed this general feature of sperm cells in mammalian animals, such as cattle (Atkinson et al., 1991; Lavitrano et al., 1991; Gagné et al., 1991; Bird et al., 1992; Camaioni et al., 1992), swine (Horan et al., 1991; Camaioni et al., 1992; Bird et al., 1992), human (Camaioni et al., 1992; Chan et al., 1995) and mice (Lavitrano et al., 1989). Sperm cells from chickens (Nakanashi and Iritani, 1993), insects (such as Australian sheep blowfly, honeybee) (Atkinson et al., 1991) and fish (Khoo et al., 1992; Sin et al., 1993; Symonds et al., 1994) also had the ability to bind foreign DNA. However, from these studies it was not clear whether the foreign DNA molecules were on sperm cell surfaces or inside sperm nuclei.

Foreign DNA Binding Patterns and Binding Rate

Binding Patterns

Light microscopic autoradiography first indicated that foreign DNA binding was mainly at the postacrosomal region of the sperm heads of rabbits (Brackett et al., 1971), mice (Wu et al., 1990; Lavitrano et al., 1992b; Camaioni et al., 1992), bulls (Atkinson et al., 1991) and pigs (Horan et al., 1991) and not on the sperm tails. Other studies indicated that the sites of foreign DNA uptake are the postacrosomal and equatorial regions of the mouse sperm head (Francolini et al., 1993). Nakanishi and Iritani (1993) reported that chicken sperm cells had three binding patterns: either the anterior head, the posterior head or the whole head. These results indicated that different binding patterns may result when different animals are evaluated.

It was not clear which binding pattern is more important for successful sperm-mediated gene transfer. Electron microscopic autoradiography showed that $^{3}$H-end-
labelled plasmids were in the nuclear region of mouse sperm cells (Lavitrano et al., 1992b; Camaioni et al., 1992; Francolini et al., 1993) suggesting that plasmids were able to penetrate into the sperm head. However, these results also suggested that the majority of foreign DNA molecules appeared to be localized externally on the membrane surface. A portion of foreign DNA molecules was protected from digestion of sperm cells with DNase I (Castro et al., 1990; Atkinson et al., 1991; Nakanishi and Iritani, 1993). These results again suggest that a portion of foreign DNA did penetrate the sperm cells.

Controversial results indicated the DNase could abolish binding of foreign DNA to sperm cells. In this case, Bird et al. (1992) indicated that foreign DNA was only bound to sperm membranes. It remains unknown whether foreign DNA molecules must penetrate into sperm nuclei to perform sperm-mediated gene transfer. Transfer of sperm surface molecules into oocyte cytoplasm has been demonstrated in fertilized sea urchin eggs (Gundersen et al., 1986). While the rest of the sperm membrane has been reported to incorporate into the oocyte plasma membrane, the apical sperm membrane and the equatorial segment of the acrosome were internalized into the oocyte in rabbits (Bedford, 1972), hamsters (Yanagimachi and Noda, 1970; Clark and Koehler, 1990) and humans (Sathananthan et al., 1986). Whether foreign DNA associated with these parts of the membrane can be internalized into the oocyte during fertilization remains to be verified.

**Binding Rate**

It was demonstrated that foreign DNA association did not occur in all sperm cells and that DNA association with sperm cells occurs at a very low rate (Atkinson et al., 1991). Sperm cells from different species showed different capacities for binding of foreign DNA. Castro et al. (1990) indicated that among species such as pig, buffalo, ram, goat, rooster and mouse, rooster sperm cells had the greatest ability to retain foreign DNA, while buffalo sperm cells had the weakest ability. In a
preliminary study, 30 to 35% of rabbit sperm cells were found to bind foreign DNA (Brackett et al., 1971). In situ hybridization studies showed that 30 to 70% of motile pig sperm carried foreign DNA on the postacrosomal region of the sperm (Horan et al., 1991) and this percentage could be increased 10% with electroporation (Horan et al., 1992a). In mice, 85 to 90% of sperm cells bound foreign DNA (Francolini et al., 1993; Wu et al., 1990), while 39 to 47% of bull sperm cells showed foreign DNA binding ability (Atkinson et al., 1991; Camaioni et al., 1992). In chickens, the proportion of the sperm cell binding of foreign DNA was very low (6.3%). However, after sperm cells were treated with electroporation or lipofectin, the percentage of chicken sperm cell binding of foreign DNA increased to approximately 60% (Nakanishi and Iritani, 1993).

Factors Affecting Sperm Cell Binding of Foreign DNA

There are a lot of factors affecting sperm cell binding of foreign DNA. Longer DNA fragments (7 kb) were taken up by mouse sperm cells more effectively than shorter ones (150 to 750 bp) in one study (Lavitrano et al., 1992a). Certain DNA fragments were easier to interact with human sperm cells than other DNA fragments (Chan et al., 1995). When foreign DNA of different sizes was co-incubated with mouse sperm cells, results indicated that larger fragments (several to 23 kb) were retained in the sperm pellet and smaller sizes fragments (0.5 to 0.125 kb) were not (Bird et al., 1992).

Castro et al. (1990) indicated that sperm cells from different animal species had different abilities to bind foreign DNA. Uptake averaged between 2,100 and 2,700 molecules of foreign DNA per rabbit sperm cell was reported in one study (Clausen et al., 1991). Some studies reported that DNA was primarily taken up by motile sperm cells (Lavitrano et al., 1989; Clausen et al., 1991; Nakanishi and Iritani, 1993). There is a controversy over whether only live sperm cells can bind foreign DNA. After co-incubation with foreign DNA for 30 minutes, the separation of live and dead bovine
sperm fractions on percoll gradients revealed that more foreign DNA molecules were associated with the dead sperm fraction rather than the live sperm fraction (Bird et al., 1992). Binding to dead sperm was further confirmed by a supravital stain and light autoradiographs (Bird et al., 1992). Lavitrano et al. (1989) showed that only live mouse sperm cells can bind foreign DNA, but once sperm cells are treated with 4% buffered formaldehyde, they lose the ability to bind foreign DNA. Similar conclusions indicating that only live sperm cells can bind foreign DNA have been made by several other groups (Wu et al., 1990; Castro et al., 1990).

The incubation time and temperature do not seem to play crucial roles in sperm cell binding of foreign DNA. A very short co-incubation period seems enough for mouse sperm cell binding of foreign DNA (Lavitrano et al., 1989; Castro et al., 1990; Lavitrano et al., 1992b; Camaioni et al., 1992; Francolini et al., 1993). Wu et al. (1990) found that optimal mouse sperm binding of foreign DNA was obtained after 60 minutes incubation at 37°C while at 4°C binding was reduced, but other researchers reported that there was no difference whether co-incubation temperature was at 37°C or at 0°C for mouse sperm binding of foreign DNA (Lavitrano et al., 1989).

By means of radioimmunoassay, it was found that mouse major histocompatibility complex (MHC) class II molecules on sperm cells were responsible for the posterior region of sperm head binding of foreign DNA (Wu et al., 1990). In another study, biochemical analysis of proteins extracted from sperm heads showed the presence of a 30 to 35 kDa protein conserved in various mammals could specifically bind foreign DNA (Lavitrano et al., 1992d). This 30 to 35 kDa protein was proposed to be the substrate for the interaction between sperm cells and foreign DNA (Lavitrano et al., 1992d). Whether MHC class II in mouse sperm are the same molecules as the 30 to 35 kDa protein remains to be verified.

Seminal plasma also affects sperm cell binding of foreign DNA (Camaioni et al., 1992). Only after extensive washing, ejaculated mouse sperm, boar and human

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sperm could bind foreign DNA. Positively charged polyamine and highly sulfated polymers glycosaminoglycan in seminal plasma were considered to be the potential substrates interfering foreign DNA binding to sperm cells (Camaioni et al., 1992). Sperm cells were prevented from binding of foreign DNA when 10 µg/ml heparin was added to the sperm mixture (Camaioni et al., 1992).

**Sperm Cells as Vectors to Introduce Foreign DNA into Oocytes during Fertilization**

Lavitrano et al. (1989) reported that 30% transgenic mice can be produced with sperm-mediated gene transfer. It was concluded that transgenic mice could be produced using sperm cells as vectors of foreign DNA. However, other research groups failed to produce similar results and concluded that there was no simple solution for making transgenic mice by this method (Brinster et al., 1989; Baringa, 1989).

Recent results showed that sperm cells from different species can be used as vectors to introduce foreign DNA into ova during fertilization. Sea urchin sperm have been reported to introduce pSV-CAT and pRSV-CAT plasmids into eggs during fertilization. Chloramphenicol acetyltransferase (CAT) enzyme activity was detected at the swimming blastula stage in the sea urchin (Arezzo, 1989). Perez et al. (1991), de la Fuente et al. (1991) and Hochi et al. (1990) have also reported that most of early stage mouse embryos retained foreign DNA after sperm-mediated gene transfer. Human sperm cells were used to deliver foreign DNA into hatched blastocysts and results showed that sperm cells deliver only one type foreign DNA [HPV type 18 (length 80 bp)] (Chan et al., 1995). Among the four types of foreign DNA evaluated in the experiment were HPV type 16 (98 bp), type 18 (80 bp), type 31 (162 bp) and type 33 (103 bp) (Chan et al., 1995).

There is also evidence showing that bovine sperm cells can introduce foreign DNA into oocytes during fertilization. Perez et al. (1991) showed that bovine sperm cells were capable of transferring foreign DNA into blastocysts. Gagné et al. (1991)
electroporated frozen-thawed bovine sperm cells in the presence of foreign DNA plasmids at 1 mg/ml and the electroporated sperm cells were used to fertilize in vitro-matured bovine oocytes. At the zygote stage, autoradiography showed that foreign DNA was inside the cytoplasm of the oocytes.

Results further showed that without electroporation of bovine sperm cells, most of foreign DNA was retained by the zona pellucida after fertilization (Gagné et al., 1991). This may indicate that foreign DNA had a better chance of penetrating into sperm cells during electroporation, as a result, these foreign DNA molecules were more stable in binding to sperm cells when they pass through zona pellucida and went into cytoplasm of oocytes. The same experiments also showed that at morula and blastocyst stages after fertilization, PCR detected 17 to 22% of the embryos containing foreign DNA. However, it was not known whether the foreign DNA was integrated into the genomic DNA. Electroporation also significantly reduced the sperm fertilizing ability and early embryonic development in this study.

Schellander and Peli (1994) incubated fresh bovine sperm cells with plasmid pSV2CAT at 1 μg/ml concentration for 1 hour at 37°C, and then artificially inseminated 210 synchronized cows with DNA-treated sperm cells. Southern blotting detected 2 of the 45 calves positive to pSV2CAT. In this experiment, the unexpected size of the restriction fragments in offspring was detected indicating that before foreign DNA was integrated into the genomic components, deletions and recombinatons of foreign DNA by DNA-active enzyme could have occurred. Furthermore, circular DNA may have been linearized at different sites before integration (Roschlau et al., 1989).

Schellander et al. (1995) showed that after in vitro fertilization of bovine oocytes with foreign DNA treated sperm cells, 3% early blastocysts had retained the whole plasmid and 10% retained up to a 1 kb fragment. This result suggests that foreign DNA was deleted or truncated before integration. Schellander et al. (1995)
also artificially inseminated 210 synchronized heifers with foreign DNA pSV2-cat-treated sperm cells. In this study, 2% (1/41) of the calves had retained foreign DNA after a Southern blot analysis was performed on DNA samples from these calves. However, when another plasmid, RSV-lac Z, was used, none of the calves had a positive response in spite of the use of RSV-lac Z-treated sperm cells. The reason for this was not clear.

In one field study, 203 sheep were artificially inseminated with sperm cells co-incubated with foreign DNA plasmid oMTbGH, and 3% (3/98) of the lambs showed positive results after Southern blot and PCR analysis (Sun et al., 1994).

Gandolfi et al. (1989) reported that transgenic pigs could be obtained by artificial insemination with foreign DNA treated sperm cells and the transgene could be transmitted into the F1 progeny. The same research group reported that 12% (12/102) of the fetuses and the piglets resulting from the 22 sows were surgically inseminated with pSV2CAT plasmid treated sperm cells had foreign DNA, but integration was not proved (Lauria and Gandolfi, 1993).

One study reported that foreign DNA can be transferred into newly laid eggs when hens were inseminated with sperm cells co-incubated with foreign DNA (Nakanishi and Iritani, 1993). Lipofection and electroporation were used to enhance chicken sperm cell binding of foreign DNA. In this study, 67% (34/45) of the eggs had foreign DNA after sperm cells were treated by lipofection, while electroporation gave a poor result, only 23% (7/31) with foreign DNA. It was suggested that electroporation might damage the acrosome, preventing electroporated sperm cells from participating in fertilization in vivo (Nakanishi and Iritani, 1993).

Sperm cells from a variety of species other than farm animals also can be used as vectors for foreign DNA. Khoo et al. (1992) successfully introduced foreign DNA into zebrafish (Brachydanio rerio) using sperm cells as vectors. In this study, 20 to 30% of the fish showed the presence of foreign DNA. Müller et al. (1992) showed
that sperm cells from the common carp (*Cyprinus carpio* L.), the African catfish (*Clarias gariepinus*) and the tilapia (*Oreochromis niloticus*) could introduce foreign DNA into fish eggs after they were incubated with foreign DNA and were electroporated. These electroporation results indicated that foreign DNA must penetrate into sperm nuclei; then and only then can sperm cells introduce foreign DNA molecules into ova during fertilization. Sin et al. (1993) reported that salmon sperm electroporated in the presence of pRSV-lacZ DNA could introduce foreign DNA into eggs after fertilization. However, only 7% (3/40) of the fry had foreign DNA. Unfortunately, integration was not analyzed.

Our understanding of sperm-mediated gene transfer is far from complete. A better understanding the mechanism of sperm cell binding of foreign DNA and the mechanism of integration from further studies should improve the efficiency of production of transgenic farm animals.
CHAPTER 3

ASSOCIATION OF FOREIGN DNA WITH FRESH AND FROZEN-THAWED GOAT SPERM CELLS

Introduction

It has been proposed that transgenic animals will someday increase the efficiency of livestock production (Hansel, 1986). The current methodology used to introduce foreign DNA into the sperm-activated oocytes is pronuclear microinjection. Although much effort has been executed to develop gene transfer techniques in farm animals, few transgenic pigs, sheep, goats, cattle and chickens have been produced. The efficiency of production of transgenic farm animals remains very low (0.5 to 1%) (reviewed by Pursel and Rexroad, 1993). Researchers agree that microinjection gene transfer needs to be improved or alternative methods for inserting foreign genes need to be found (Wilmut. et al., 1990).

Lavitrano et al. (1989) first reported that the production of transgenic mice using a simple technique in which foreign DNA was co-incubated with sperm cells prior to the in vitro fertilization procedure. With this approach, 30% embryo transfer offsprings were transgenic and the transgene was subsequently inherited by their progeny. In contrast, other research laboratories have failed to reproduce these results in mice (Brinster et al., 1989; Baringa, 1989).

Recent results show with sperm-mediated gene transfer, foreign DNA can be detected at the swimming blastulae in the sea urchin (Arezzo et al., 1989), in the mouse embryos (Hochi et al., 1990) and in the bovine embryos (Gagné et al., 1991). Most recent results show that foreign DNA can be detected in the offsprings of sheep (Sun et al., 1994) and of cattle (Schellander et al., 1995) when sperm cells co-incubated with foreign DNA were used for artificial insemination. A preliminary study indicated that sperm cells from different species have different capacities in...
binding of foreign DNA (Castro et al., 1990). The characterization of this interaction is very important before considering the use of sperm cells as vectors to transfer foreign DNA into ova.

The objectives of this study were: 1) to evaluate whether goat sperm cells could bind foreign DNA, 2) to evaluate the effect of removal of seminal plasma from goat sperm cells on binding of foreign DNA, 3) to evaluate whether foreign DNA is retained on sperm cells after extensive washing and 4) to identify the difference between fresh goat sperm cells and frozen-thawed goat sperm cells on binding of foreign DNA.

Materials and Methods

Experimental Design

Experiment 3.1. Association of Foreign DNA with Fresh Goat Sperm Cells

This experiment was designed to evaluate whether fresh goat sperm cells could bind foreign DNA, and whether this foreign DNA could still be retained on sperm cells after 10 times of post-washing after co-incubation with foreign DNA (post-washing: washing sperm cells after co-incubation with foreign DNA). The effect of seminal plasma on sperm cell binding of foreign DNA was evaluated using the following treatments: semen was either directly co-incubated with foreign DNA without removing seminal plasma or semen samples were first pre-washed with Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975) then co-incubated with foreign DNA (pre-washing: washing sperm cells before co-incubation with foreign DNA). The experimental design is outlined in Table 1.

Experiment 3.2. Association of Foreign DNA with Frozen-Thawed Goat Sperm Cells

This experiment was designed to evaluate the interaction between frozen-thawed goat sperm cells and foreign DNA. The effect of freezing extender on the sperm cell’s ability to bind foreign DNA was evaluated by the following treatments.
Table 1. Experimental design for Experiment 3.1

<table>
<thead>
<tr>
<th>Pre-washing treatment(^a)</th>
<th>Incubation with foreign DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh semen without pre-washing (F)</td>
<td>Yes</td>
</tr>
<tr>
<td>Fresh semen pre-washed once (F1W)</td>
<td>Yes</td>
</tr>
<tr>
<td>Fresh semen pre-washed twice (F2W)</td>
<td>Yes</td>
</tr>
<tr>
<td>Fresh semen (Control)</td>
<td>No</td>
</tr>
</tbody>
</table>

\(^a\)Three replicates per treatment.
Semen was either directly co-incubated with foreign DNA without removing the extender or frozen-thawed semen samples were first pre-washed with B-O medium, then co-incubated with foreign DNA. This experimental design is presented in Table 2.

Foreign DNA

In Experiments 3.1 and 3.2, 7.2 kb pCMVβ plasmids (Clontech, Palo Alto, CA) were used as the foreign DNA. This plasmid is a mammalian reporter vector designed for the expression of β-galactosidase in mammalian cells as transcribed by the cytomegalovirus promoter. The pCMVβ contains a RNA splice site, a polyadenylation signal, and full length *E. coli* β-gal gene.

Preparation of Radio-Labelled DNA

Plasmid pCMVβ was first linearized with Hind III restriction enzyme, then pCMVβ was end-labelled with 32P-dATP. The labelling procedure followed was similar to the method previously described (Maniatis et al., 1982). Briefly, 1 μg Hind III linearized pCMVβ was incubated with 5 μl of 10X Klenow buffer, 1 μl of each unlabelled dNTPs (10 mM), 5 μl 32P-dATP (10 mCi/ml) and 5 units of Klenow enzyme in a volume of 50 μl for 1 hour at 37°C. The end-labelled pCMVβ plasmids were purified with Bio-Spin Chromatography Columns (Bio-Red Laboratories, Richmond, CA).

Sperm Cell Preparation

Goat semen for sperm sample preparation was collected from healthy Spanish bucks with an artificial vagina maintained at 37°C. Frozen semen used in the Experiment 3.2 was similarly collected and the sperm samples were frozen at approximately 3 x 10^8 cells/ml in an egg yolk-citrate glycerol diluent and stored under liquid nitrogen.

Prior to the experiment, French straws (0.25 ml each) of sperm cells were thawed for 1 minute at 37°C. The sperm samples were then processed as described.
Table 2. Experimental design for Experiment 3.2

<table>
<thead>
<tr>
<th>Pre-washing treatment</th>
<th>Incubation with foreign DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen-thawed semen without pre-washing (T)</td>
<td>Yes</td>
</tr>
<tr>
<td>Frozen-thawed semen pre-washed once (T1W)</td>
<td>Yes</td>
</tr>
<tr>
<td>Frozen-thawed semen pre-washed twice (T2W)</td>
<td>Yes</td>
</tr>
<tr>
<td>Frozen-thawed semen (Control)</td>
<td>No</td>
</tr>
</tbody>
</table>

*Three replicates per treatment.
for fresh sperm cell preparation. Seminal plasma was then removed from the fresh semen according to the specific treatment by washing with B-O medium and centrifuging at 600 x g for 10 minutes. Sperm cell concentration was adjusted to 1 x $10^8$ sperm/ml in B-O medium. A volume of 100 μl of semen was allotted to treatments (three replicates).

**Incubation of Semen Sample with Radio-Labelled Plasmid pCMVB**

After sperm samples were prepared for respective treatments, radio-labelled plasmid pCMVB was added to each treatment for a final concentration of 4 μg foreign DNA/ml of the semen sample. After 1 hour of incubation at room temperature (22°C), sperm samples were washed with B-O medium up to 10 times to remove free foreign DNA molecules and centrifuged at 600 x g for 10 minutes. Radioactivity was measured in the supernatant and sperm pellet by dissolving them in soluene, diluted in 5 ml toluene-based scintillation cocktail, and counted in a scintillation counter (LKB, Wallac, Sweden). A sperm sample without foreign DNA was used as a blank control during scintillation counting. The amount of foreign DNA left in each sperm sample was calculated per sperm pellet as described by Castro et al. (1990).

**Statistical Evaluation**

Statistical evaluation was made with a Student's t-test with the detection limits set at the P<0.05 level. Within each treatment, a general purpose regression analysis was used to compare the amount of foreign DNA left in the sperm sample after each post-washing.

**Results**

**Experiment 3.1**

A summary of fresh goat sperm cell binding of the radio-labelled plasmid pCMVB is presented in Table 3. In this experiment, after 10 extensive washings following goat sperm cell co-incubated with foreign DNA, the foreign DNA was
Table 3. The amount of foreign DNA in fresh caprine sperm pellets after a series of post-washings

<table>
<thead>
<tr>
<th>No. of post-washings&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pre-washing treatment&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
</tr>
<tr>
<td>1</td>
<td>234±91.4</td>
</tr>
<tr>
<td>2</td>
<td>208±95.3</td>
</tr>
<tr>
<td>3</td>
<td>199±92.9</td>
</tr>
<tr>
<td>4</td>
<td>188±95.3</td>
</tr>
<tr>
<td>5</td>
<td>179±98.5</td>
</tr>
<tr>
<td>6</td>
<td>168±103.0</td>
</tr>
<tr>
<td>7</td>
<td>157±101.3</td>
</tr>
<tr>
<td>8</td>
<td>146±102.6</td>
</tr>
<tr>
<td>9</td>
<td>138±98.2</td>
</tr>
<tr>
<td>10</td>
<td>132±95.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three replicates per treatment.

<sup>b</sup>F=fresh semen without pre-washing; F1W=fresh semen once pre-washed; F2W=fresh semen twice pre-washed.

<sup>c</sup>Mean ± SE of foreign DNA (ng) calculated per sperm sample (1 x 10<sup>7</sup> cells).
found to be retained in the fresh sperm pellets in each treatment. After 10 post-washings, there was no significant difference in the amount of foreign DNA left in the sperm pellets among the three treatments.

General purpose regression analysis was performed within each treatment (each column of Table 3). In the fresh goat semen without pre-washing treatment, the first three post-washings removed significantly more of the extra foreign DNA material from the sperm sample than subsequent post-washings. After the third post-washing, there was no significant difference of foreign DNA in the sperm pellet among the subsequent post-washings. The regression line for this treatment was $Y = 234.4 - 10.8X$ (Y represents the amount of foreign DNA left in sperm pellet after each post-washing, X represents the number of post-washing), with an $r$ value of -0.36.

In the fresh goat semen once pre-washed treatment, there was no significant difference of the amount of foreign DNA left in sperm pellet among the one to ten post-washings. The regression line for this treatment was $Y = 206.6 - 9.4X$, with an $r$ value of -0.34.

In the fresh goat semen twice pre-washed treatment, the first two post-washings removed significantly more of the extra foreign DNA material from sperm sample than subsequent post-washings. After the second post-washing, there was no significant difference of amount of foreign DNA left in sperm pellet among post-washings until the seventh post-washing. After the seventh post-washing, the amount of foreign DNA left in sperm pellet was significantly lower than that after the sixth post-washing. The regression line for this treatment is $Y = 144.8 - 8.5X$, with an $r$ value of -0.94.

Experiment 3.2

A summary of frozen-thawed goat sperm cell binding of the radio-labelled plasmid pCMVβ is presented in Table 4. After 10 extensive washings following goat sperm cell co-incubation with foreign DNA, the sperm pellets in each treatment still
Table 4. The amount of foreign DNA in frozen-thawed caprine sperm pellet after a series post-washings

<table>
<thead>
<tr>
<th>No. of Post-washings&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pre-washing treatment&lt;sup&gt;bc&lt;/sup&gt;</th>
<th>T</th>
<th>T1W</th>
<th>T2W</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>127±22.7</td>
<td>157±42.2</td>
<td>194±95.8</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>49±11.7</td>
<td>80±42.1</td>
<td>138±106.4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>27±13.7</td>
<td>60±41.4</td>
<td>121±103.0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>21±15.0</td>
<td>54±41.8</td>
<td>113±100.2</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>19±15.0</td>
<td>51±40.3</td>
<td>108±96.7</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>18±14.5</td>
<td>49±39.4</td>
<td>102±93.6</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>17±14.1</td>
<td>47±37.5</td>
<td>96±89.1</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>16±13.1</td>
<td>44±35.9</td>
<td>92±85.9</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>15±12.6</td>
<td>42±33.5</td>
<td>85±78.0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>14±11.6</td>
<td>38±30.3</td>
<td>78±71.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three replicates per treatment.

<sup>b</sup>T=frozen-thawed goat semen without pre-washing;
T1W=frozen-thawed goat semen once pre-washed;
T2W=frozen-thawed goat semen twice pre-washed;

<sup>c</sup>Mean ± SE of foreign DNA (ng) calculated per sperm sample (1 x 10⁷ cells).
retained foreign DNA. There was no significant difference in the amount of foreign DNA bound to the sperm pellets across the three treatments (P>0.05) after 10 post-washings. However, pre-washing of frozen-thawed semen before incubation with foreign DNA tend to increase the ability of sperm cells to bind foreign DNA.

Within each treatment, general-purpose regression analysis was performed. In the frozen-thawed goat semen without pre-washing treatment group, the first three post-washings removed significantly (P<0.05) more of the foreign DNA from the sperm samples than subsequent post-washings. After the third post-washing, there was no significant difference in the amount of DNA retention among subsequent post-washings (P>0.05). The regression line for this treatment was $Y = 76.1 - 8X$, with an r value of -0.65.

In the frozen-thawed goat semen once pre-washed treatment group, the first two post-washings removed significantly more foreign DNA from the sperm samples than subsequent post-washings (P<0.05). After the second post-washing, there was no significant difference among post-washings (P>0.05). The regression line of this treatment was $Y = 110.3 - 8.8X$, with an r value of -0.55.

In the frozen-thawed goat semen twice pre-washed treatment group, there was no significant difference among the post-washings. The regression line for this treatment was $Y = 166.9 - 9.8X$, with an r value of -0.55.

Discussion

The results from this study indicate that both fresh and frozen-thawed goat sperm cells bind foreign DNA. Furthermore, the interaction between foreign DNA and sperm cells appeared very stable, since after 10 post-washings, the foreign DNA remained bound to the sperm cells within the pellets. Castro et al. (1990) found that goat sperm cells could retain foreign DNA after five post-washings. In current study, results indicate that three post-washings can remove most unbound foreign DNA.
In the present study, removing seminal plasma from a fresh ejaculate before co-incubation with foreign DNA had no significant effect (P>0.05) on sperm cell binding of foreign DNA. These results contrast with findings of other studies with mice (Lavitrano et al., 1992a; Lavitrano et al., 1992b; Lavitrano et al., 1992d). In later studies, when seminal plasma was added to the sperm mixture, it prevented mouse sperm cell from binding of foreign DNA (Lavitrano et al., 1992a; Lavitrano et al., 1992b; Lavitrano et al., 1992d). There are three potential explanations for the different results. First, different animal species were used. In the present experiment, goat sperm cells were used while the other studies involved mouse sperm cells (Lavitrano et al., 1992a; Lavitrano et al., 1992b; Lavitrano et al., 1992d). Species have been shown to differ in the abilities of their sperm cells to bind foreign DNA (Castro et al., 1990).

Secondly, in the present study semen was diluted 30 times to adjust the sperm cell concentration to $1 \times 10^8$ sperm/ml before foreign DNA was added to the samples. While in other studies, extra undiluted seminal fluid was added to the sperm mixture (Lavitrano et al., 1992a; Lavitrano et al., 1992b; Lavitrano et al., 1992d). So in the later studies adding extra seminal fluid may provide high concentrations of certain components in seminal plasma which can prevent sperm cells from binding of foreign DNA. Seminal plasma may not be the only factor that interfered with sperm cell binding of foreign DNA. This possibility was confirmed by results from the frozen-thawed sperm cells in Experiment 3.2. These results showed that washing frozen-thawed sperm cells before co-incubation with foreign DNA improved the capacity of goat sperm cells to bind foreign DNA. Extender used in freezing sperm cells usually contain many large macro molecules from egg yolk. These molecules from the extender could have been responsible for interfering with the binding of foreign DNA to sperm cells. This suggests that large molecules can interfere with the ability of sperm cell to bind foreign DNA. It has been shown that mouse sperm cells were
prevented from binding of foreign DNA when 10 μg/ml of heparin (a positively charged molecule) were added to the sperm mixture (Camaioni et al., 1992).

Thirdly, DNase may be present in the extender used in semen freezing. In the current study, results showed that pre-washing frozen-thawed semen before co-incubation with foreign DNA improved the capacity of the sperm cell to bind foreign DNA. These results may be explained on the basis that, without pre-washing, a high concentration DNase in the extender degraded foreign DNA molecules; after pre-washing once or twice before the addition of foreign DNA, the concentration of DNase could have been diluted and subsequently less foreign DNA was degraded.

In summary, both fresh and frozen-thawed goat sperm cells are capable of binding of foreign DNA (as the plasmid pCMVβ). In this study, the association between foreign DNA and goat sperm cells appeared to be very stable; even after 10 post-washings, foreign DNA remained associated with the sperm cells. Factors present in the extender interfere with the frozen-thawed sperm cell’s ability to bind foreign DNA, and pre-washing frozen-thawed goat semen to remove the extender tended to increase the amount of foreign DNA bound to sperm cells.
CHAPTER 4

EVALUATION OF CAPRINE SPERM CELL BINDING OF FOREIGN DNA USING CONFOCAL MICROSCOPY

Introduction

Lavitrano et al. (1989) reported that transgenic mice could be produced by in vitro fertilization (IVF) of mouse ova with sperm cells co-incubated with foreign DNA. Furthermore, 30% of the offspring were transgenic and the transgene was transmittable to the progeny. This approach is simpler and more efficient than oocyte microinjection. Subsequently, in vitro fertilization experiments were conducted in an effort to verify these findings. However, the results were negative, and thus, much skepticism relating to sperm-mediated gene transfer surfaced in the scientific community (Brinster, 1989; Barinaga, 1989). However, during recent years, it has again been reported that sperm cells from various species are capable of transporting foreign DNA into ova during fertilization, and in some cases, the transgene was detected in their offspring (Sun et al., 1994; Scheduler et al., 1995). However, the efficiency (2.5% to 3%) of production of transgenic sheep and cattle in these studies with sperm cell mediated DNA gene transfer has been much lower than the efficiency (30%) reported for mice by Lavitrano et al. (1989). Sperm-mediated gene transfer is an intriguing method to produce transgenic offsprings, but many factors may affect the capacity of sperm cells to bind foreign DNA.

Castro et al. (1990) reported that sperm cells from different species had different capacities in binding of foreign DNA. Further results suggested that not all sperm cells could bind foreign DNA. For example, only 30 to 35% of rabbit sperm cells were detected binding of foreign DNA in an earlier study (Brackett et al., 1971). In situ hybridization studies showed that 30 to 70% of the motile porcine sperm carried foreign DNA on the post-acrosomal region of the sperm (Horan et al., 1991),...
and this percentage was increased 10% with electroporation (Horan et al., 1992a). In another study, 13 to 20% of sheep sperm cells were detected to bind foreign DNA (Sun et al., 1994). As many as 85 to 90% of mouse sperm cells were identified as binding of foreign DNA (Wu et al., 1990; Francolini et al., 1993). Finally, 39 to 47% of bull sperm cells were capable of binding of foreign DNA (Atkinson et al., 1991; Camaioni et al., 1992). In the chicken, the proportion of the sperm cell binding of foreign DNA has been reported to be very low (6.3%) (Nakanishi and Iritani, 1993). However, after electroporation or lipofectin treatments, the percentage of chicken sperm cell binding of foreign DNA increased to ~ 60% (Nakanishi and Iritani, 1993).

It is important to determine which part of the sperm cell is responsible for binding of foreign DNA. Light microscopic autoradiography of sperm cells showed that binding occurred mainly at the post-acrosomal region of the rabbit (Brackett et al., 1971), the mouse (Lavitrano et al., 1992b; Wu et al., 1990; Camaioni et al., 1992), the bull (Atkinson et al., 1991) and the pig (Horn et al., 1992a) sperm head, but not on the sperm tail. Other findings indicate that the sites of uptake of foreign DNA fragments are the post-acrosomal and equatorial regions of the mouse sperm head (Francolini et al., 1993). Nakanishi and Iritani (1993) reported that chicken sperm cells had three foreign DNA binding patterns: the anterior head, the posterior head or the whole head. These results indicated that different foreign DNA binding patterns of sperm cells may occur in different species. It is not clear which binding pattern is more important for successful sperm-mediated gene transfer.

During fertilization, sperm cells must penetrate the zona pellucida, complete the acrosome reaction and fuse to the oolemma. Therefore, it seems reasonable that it would be more effective to transfer foreign DNA into the ovum by inserting the DNA into the sperm nucleus. Electron microscopic autoradiography showed that $^{3}$H-end-labelled plasmids were found over the nuclear region of some mouse sperm cells (Francolini et al., 1993; Lavitrano et al., 1992b; Camaioni et al., 1992). These
observations suggest that plasmids are able to penetrate into the sperm cell head. However, the majority of the foreign DNA molecules appear to be localized externally on the membrane surface (Franncolini et al., 1993; Lavitrano et al., 1992b; Camaioni et al., 1992). A portion of foreign DNA molecules were protected by mouse sperm cells from digestion with DNase I (Castro et al., 1990; Atkinson et al., 1991; Nakanishi and Iritani, 1993) also suggesting that a portion of foreign DNA may penetrate the sperm cells.

A preliminary study indicated that goat sperm cells could bind foreign DNA (Castro et al., 1990). Our previous studies (Chapter 3) further showed that both fresh and frozen-thawed goat sperm cells were able to bind foreign DNA, and that there were likely factors in the extender affecting frozen-thawed goat sperm cell binding of foreign DNA. However, there was no study on foreign DNA binding patterns on goat sperm cells and it was undetermined whether foreign DNA could penetrate the goat sperm nucleus. Before any further goat in vitro fertilization experiments were conducted, it thought advisable to investigate the foreign DNA binding patterns on goat sperm cells, and to determine whether foreign DNA could penetrate the goat sperm cell.

The specific objectives of this study were to evaluate the patterns of foreign DNA binding on goat sperm cells, the percentage of sperm cells with foreign DNA, the difference between fresh and frozen-thawed goat sperm cells in the percentage of sperm cells with foreign DNA, and to determine whether foreign DNA was present in the sperm nucleus after co-incubation with foreign DNA.

Materials and Methods

Experimental Design

Experiment 4.1. Evaluation of Goat Sperm Cell Binding of Foreign DNA Using Confocal Microscopy
This experiment was designed to localize the foreign DNA binding position on goat sperm cells, and to evaluate foreign DNA binding patterns on these sperm cells. Negative and positive controls were used to distinguish the foreign DNA from background of the sperm and other chemical agents used in the experiments. The experimental design is outlined in Table 5.

Experiment 4.2. Comparison of Fresh and Frozen-Thawed Goat Sperm Cell Binding of Foreign DNA Using Confocal Microscopy

This experiment was designed to evaluate whether all goat sperm cells from a sample population could bind foreign DNA, and if not, what percentage of goat sperm cells could bind foreign DNA. The difference between fresh and frozen-thawed sperm cells on binding foreign DNA was also evaluated based on total 1,000 sperm cells in three replicates of each treatment. The experimental outline is shown in Table 6.

Experiment 4.3. Longitudinal Sections of Goat Sperm Cells Bound to Foreign DNA Using Confocal Microscopy

This experiment was designed to evaluate whether foreign DNA was only on the sperm membrane or would be able to penetrate into the sperm nucleus. Longitudinal sections of the goat sperm cells with confocal microscope at 0.4 μm intervals were performed through the representative sperm. Negative control was used to eliminate the background of the sperm and positive control was used to distinguish the foreign DNA from fluorescein-11-dUTP and other chemical agents used for labelling foreign DNA.

Foreign DNA

pCMVβ plasmids (Clontech, Palo Alto, CA), size of which is 7.2 kb, were used in Experiments 4.1, 4.2 and 4.3. The plasmid is a mammalian reporter vector designed for the expression of β-galactosidase in mammalian cells as transcribed by
Table 5. Experimental design for Experiment 4.1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation with foreign DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>sperm + buffer</td>
</tr>
<tr>
<td>Positive control</td>
<td>sperm + fluorescein-11-dUTP</td>
</tr>
<tr>
<td>DNA control</td>
<td>sperm + nonlabelled pCMVβ plasmids</td>
</tr>
<tr>
<td>Sperm + foreign DNA</td>
<td>sperm + fluorescein-11-dUTP labelled pCMVβ plasmids</td>
</tr>
</tbody>
</table>

*aThree replicates per treatment.*
Table 6. Experimental design for Experiment 4.2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of sperm cells to be evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh sperm + foreign DNA</td>
<td>1,000</td>
</tr>
<tr>
<td>Frozen-thawed sperm + foreign DNA</td>
<td>1,000</td>
</tr>
</tbody>
</table>

*Three replicates per treatment.*
the cytomegalovirus promoter (MacGregor and Caskey, 1989). It was stored at 1 μg/μl in TE buffer at -20 °C.

**Preparation of Fluorescein-Labelled DNA**

Prior to use, the pCMVβ plasmid was randomly labelled with non-radioactive isotope fluorescein-11-dUTP with random primers (Amersham Corp., Arlington Heights, IL). The labelling protocol used was the Amersham Multiprimer DNA labelling system. After labelling, extra free fluorescein-11-dUTP was removed with Bio-Spin Chromatography Columns (Bio-Red Laboratories, Richmond, CA).

**Sperm Cell Preparation**

Goat semen was collected from a healthy Spanish buck with an artificial vagina, and then used as fresh or frozen-thawed samples. Sperm cell concentration was adjusted to approximately 3 x 10^8 cells/ml in an egg yolk-citrate glycerol diluent and stored under liquid nitrogen. French straws (0.25 ml each) of sperm cells were thawed at 37°C for 1 minute and the sperm samples were processed as described for fresh goat sperm cell preparation. With fresh semen samples, the seminal plasma was removed by washing with Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975) and centrifuged at 600 x g for 10 minutes. The final sperm cell concentration was adjusted to 1 x 10^8 cells/ml in B-O medium and a volume of 100 μl of semen was used in each treatment (3 replicates).

**Incubation of Goat Sperm Cells with Foreign DNA**

Fresh and frozen-thawed goat semen were washed twice with B-O medium and centrifuged at 600 x g for 10 minutes. Sperm concentration was adjusted to 1 x 10^7/ml, and fluorescein-11-dUTP labelled pCMV plasmids were added to the sperm mixture at 2 μg/ml of semen and incubated for 30 minutes. The samples were then washed twice with B-O medium to remove the unbound foreign DNA pCMVβ plasmids. After washing, sperm cells were fixed with 2% glutaradehyde in phosphate-buffered saline (PBS) and a smear was made on a clean slide with a drop of the sperm
mixture. A cover slide was then applied and the slide was sealed with nail polish. The slides were evaluated using confocal microscopy. Sperm samples from control group were processed in a similar manner and also evaluated with confocal microscopy.

Single sperm cells were longitudinal sectioned at 0.4 μm intervals with confocal microscopy to evaluate whether foreign DNA was inside the sperm cell (Fisher et al., 1993).

Statistical Analysis

The difference in the number of sperm cell binding of foreign DNA between fresh and frozen-thawed goat sperm cells was tested using Chi square analysis. The P-value of <0.05 was the level set for defined as statistically significance in this study.

Results

Experiment 4.1

A direct evaluation procedure for viewing goat sperm cell binding of foreign DNA under a confocal microscope was developed in this experiment. As shown in Figure 1, fluorescein-11-dUTP labelled pCMV plasmids had binding patterns over the sperm cells (Figure 1d) while negative control sperm cells (Figure 1a) or positive control fluorescein-11-dUTP did not produce these patterns (Figure 1c). Results also showed that there were two patterns of foreign DNA binding to goat sperm cells (Figure 1d). One binding pattern was restricted to the posterior head of sperm cells, and the other binding pattern was extended to both anterior head and posterior head positions of sperm cells as shown in Figure 1d.

Experiment 4.2

With the method for viewing goat sperm cell binding of foreign DNA established in Experiment 4.1, a comparison was made between fresh and frozen-thawed goat sperm cell on binding of foreign DNA. Results are summarized in Table 7. In this study, over 2,200 goat sperm cells were evaluated in two treatment groups.
Figure 1. Goat sperm cell binding of foreign DNA. (a) sperm cells as a negative control. (b) sperm cells co-incubated with nonlabelled plasmids, as a DNA control. (c) sperm cells co-incubated with fluorescein-11-dUTP, as positive control. (d) sperm cells co-incubated with fluorescein-11-dUTP labelled plasmids showing typical binding patterns of goat sperm cells with foreign DNA. (Magnification of a, b, c at 200X and d at 400X).
Table 7. Fresh and frozen-thawed caprine sperm cell binding of plasmids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. of sperm evaluated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sperm binding of plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>Fresh sperm + plasmids</td>
<td>1,205</td>
<td>402</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen-thawed sperm + plasmids</td>
<td>1,015</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Three replicates per treatment.

<sup>b</sup>Mean ± SE.
Figure 2. Longitudinal confocal microscope sections of a sperm cell with bound foreign DNA. From top left to top right then from bottom left to bottom right there are six longitudinal sections of a sperm cell bound to fluorescein labelled plasmids (at intervals of 0.4 μm) showing evidence of foreign DNA in all sections. (Magnification at 600X).
over three replicates. In summary, 37% of the fresh goat sperm cells and 33% of the frozen-thawed goat sperm cells bound fluorescein-11-dUTP labelled pCMV plasmids. There was no significant difference between fresh and frozen-thawed goat sperm cells on binding of foreign DNA.

Experiment 4.3

This experiment was continuation of Experiment 4.2. Longitudinal sections of single sperm cell at 0.4 μm intervals with confocal microscopy were evaluated to determine whether foreign DNA was inside the sperm cell (Figure 2). In this study, after the single sperm cell was longitudinal sectioned at 0.4 μm interval and a total of six sections were made, all sections showed fluorescein-11-dUTP labelled pCMV plasmids. This internal fluorescence indicates that a portion of foreign DNA molecules had penetrated into the goat sperm nucleus in some of the sperm cells. Results also indicated that some sperm cells had foreign DNA only on their surface but not inside the nucleus.

Discussion

In the present studies, a direct evaluation of sperm cell binding of foreign DNA was developed. By labelling foreign DNA with fluorescein-11-dUTP, sperm cell binding of foreign DNA can be evaluated directly under a confocal microscope. With this procedure, goat sperm cells were found to be able to bind foreign DNA. These results were in agreement with results when radio-labelled foreign DNA was used (Chapter 3). These results may further indicate that binding of foreign DNA is a general feature of sperm cells at least in animal species studied.

There have been previous reports suggesting that there are unique patterns of sperm cell binding of foreign DNA. Foreign DNA binding on sperm cells has been noted to occur in the posterior head of sperm cells, and other sperm cell binding patterns were found to extend to the anterior head of sperm in the rabbit (Brackett et
al., 1971), mouse (Lavitrano et al., 1992b; Wu et al., 1990; Camaioni et al., 1992), cattle (Atkinson et al., 1991) and pig (Horn et al., 1992a). In the present study, there were also two binding patterns observed. Our results are in agreement with studies on multiple binding patterns of other animal species, such as rabbits (Brackett et al., 1971), mice (Lavitrano et al., 1992b; Wu et al., 1990; Camaioni et al., 1992), cattle (Atkinson et al., 1991) and pigs (Horn et al., 1992b).

In the present study, 37% of fresh goat sperm cells and 33% frozen-thawed goat sperm cells bound foreign DNA after 1 hour of co-incubation. This percentage of binding was similar to the percentage reported in rabbits (30 to 35%) (Brackett et al., 1971), in pigs (30 to 70%) (Horan et al., 1991) and in cattle (39 to 47%) (Atkinson et al., 1991; Camaioni et al., 1992) and much lower than the percentage in mice (85 to 90%) (Francolini et al., 1993; Wu et al., 1990). This variation is likely due to the different animal species evaluated. It is still unknown why some sperm cells can bind foreign DNA more easily than others. The binding pattern in sperm cell most effective transport of foreign DNA into oocytes has not been identified.

Previous studies have indicated that at least some foreign DNA molecules were able to penetrate to the nuclei of the mouse sperm cells (Castro et al., 1990; Atkinson et al., 1991; Lavitrano et al., 1992b; Camaioni et al., 1992; Francolini et al., 1993; Nakanishi and Iritani, 1993). In the present study, results showed that all six longitudinal sections of the goat sperm with confocal microscopy had retained labelled DNA. This result suggest that goat sperm cells have a similar membrane structure found in other animal species, which permits foreign DNA penetration into the nucleus.

There was no report on how foreign DNA binding patterns affect the results of sperm-mediated gene transfer. It also remains unknown whether foreign DNA molecules must penetrate sperm nuclei for sperm-mediated gene transfer. It is known that to fertilize an oocyte, sperm cells must pass through the cumulus cell layer, finish
the acrosome reaction, penetrate the zona pellucida and the membrane overlying the post-acrosomal region or the plasma membrane of the equatorial segment of the sperm fuse with the oocyte plasma membrane (Yanagimachi, 1977; Bedford and Cooper, 1978). During this process it seems reasonable that foreign DNA contained inside the sperm nuclei has a better chance for transfer into oocytes than those loosely attached on the sperm surface.

In summary, in this study we found that goat sperm cells could bind foreign DNA and that there were two patterns of sperm cell binding of foreign DNA. One binding pattern was restricted to the posterior head of sperm cells and the other binding pattern was extended to both anterior head and posterior head of sperm cells.

In this study, 37% of fresh goat sperm cells could bind foreign DNA while 33% of frozen-thawed goat sperm cells could bind foreign DNA. Furthermore, longitudinal sections of sperm cells showed that some foreign DNA molecules had penetrated into goat sperm nuclei. Further studies are needed to evaluate whether goat sperm cells can introduce foreign DNA into oocytes during fertilization.
CHAPTER 5

IN VITRO FERTILIZATION OF GOAT OOCYTES WITH SPERM CELLS CO-INCUBATED WITH FOREIGN DNA

Introduction

Chang (1951) and Austin (1951) independently showed that sperm cells must undergo capacitation to achieve the capacity to penetrate oocytes, thus making in vitro fertilization (IVF) a reality. In vitro fertilization has become an alternative method for producing pronuclear oocytes for microinjection of foreign genes to produce transgenic animals. This method is especially attractive to large farm animals producers because of animal cost and of limited access to a large number of oocytes. Also, in farm animals, there is greater variability in the development stage of embryos making fewer oocytes available for microinjection. Over the last 20 years, modifications in bovine IVF methodology has made marked improvement in in vitro calf production (Lu et al., 1987; Goto et al., 1988; Eyestone and First, 1989; Zhang et al., 1992). Fertilized oocytes derived from IVF have been used to produce a few transgenic cattle (Bowen et al., 1994).

Until recently, relatively little success has been reported for in vitro fertilization of goat oocytes. Hanada (1985) first reported the birth of live goat offsprings derived from in vitro fertilization of in vivo matured oocytes. Song and Iritani (1988) subsequently developed goat IVF procedures using in vitro matured oocytes, and approximately 50% cleavage was achieved. Chauhan and Anand (1991) also reported a fertilization rate of 63% for goat oocytes matured in vitro. The live birth of goat offspring from in vitro matured and in vitro fertilized embryos was reported by Crozet et al. (1993). Recently, a series of studies were performed on goat oocyte in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) in this laboratory (Han et al., 1996). The first offspring from IVM/IVF/IVC and
frozen-thawed goat embryos was reported in 1996 (Han et al., 1996). The major problem in goat in vitro fertilization is a low cleavage rate (overall 40-50%) compared with the in vitro fertilization rate in other mammals (86% in bovine; Zhang et al., 1992). More research is needed to enhance capacitation of sperm cells in order to achieve a higher fertilization rate in goat in vitro fertilization.

It has been well established that a Ca\(^{2+}\) flux is required to induce capacitation and the acrosome reaction in sperm cells (Yanagimachi and Usui, 1974; Yanagimachi, 1982). Efforts have been made to capacitate sperm cells by limited exposure of un-capacitated sperm cells to the Ca\(^{2+}\) ionophore A23187. Ca\(^{2+}\) ionophore A23187 has been successfully used to induce sperm capacitation and the acrosome reaction in murine (Suarez et al., 1978), porcine (Smith et al., 1983), human (Atiken et al., 1984), ovine (Shams-Borhan and Harrison, 1981) and bovine (Byrd, 1981; Takahashi and Hanada, 1984; Bird et al., 1989) sperm cells. The mechanism by which Ca\(^{2+}\) ionophore A23187 induces the capacitation and acrosome reaction is not clear.

Talbot et al. (1976) proposed that Ca\(^{2+}\) ionophore A23187 can form a lipophilic complex with calcium ion and will effectively move extracellular Ca\(^{2+}\) across the plasma membrane, resulting in sperm capacitation and the acrosome reaction. It was suggested that Ca\(^{2+}\) ionophore A23187 was able to stimulate sperm motility, acrosome reactions and fertilization ability by increasing sperm intracellular Ca\(^{2+}\) (Fraser, 1982). Calcium ionophore A23187 has been used successfully to capacitate bull sperm for in vitro fertilization with cattle (Byrd 1981; Aoyagi et al., 1990; Zhang et al., 1992). The first IVF goat was also produced by capacitation of goat sperm cells with Ca\(^{2+}\) ionophore A23187 (Hanada, 1985). Further studies are needed on capacitation of goat sperm cells with certain levels of Ca\(^{2+}\) ionophore A23187 to improve the goat in vitro fertilization and cleavage rates.

Recently, reports have indicated that sperm cells can be used as vectors to introduce foreign DNA into oocytes during the fertilization process. Sea urchin sperm
have been shown to introduce pSV-CAT and pRSV-CAT plasmids into sea urchin eggs during fertilization. Furthermore, chloramphenicol acetyltransferase (CAT) enzyme activity was detected at the swimming blastula stage in the sea urchin (Arezzo, 1989).

Perez et al. (1991) have reported that bovine sperm cells were capable of transferring foreign DNA into oocytes, and the resulting blastocysts containing foreign DNA. Gagné et al. (1991) have studied the effect of electroporation of bovine sperm cells co-incubated with foreign DNA and found that electroporation enhanced foreign DNA binding and foreign DNA could be detected in 5-day-old bovine embryos after fertilization with sperm electroporated with foreign DNA. In this study, foreign DNA was found in up to 22% of bovine blastocysts derived from in vitro fertilization. Perez et al. (1991), de la Fuente et al. (1991) and Hochi et al. (1992) have shown that the earliest stage mouse embryos retained foreign DNA after sperm-mediated gene transfer. In a recent study, Chan et al. (1995) reported that human sperm cells could deliver foreign DNA into mouse blastocysts.

In previous studies in this laboratory (Chapters 3 and 4), it was shown that 37% of fresh goat sperm cells and 33% frozen-thawed goat sperm cells could bind foreign DNA. Furthermore, results indicated that some foreign DNA had penetrated into sperm nuclei. Thus, it became necessary in this series of experiments to evaluate whether these goat sperm cells could introduce foreign DNA into oocytes during fertilization.

The objectives in present study were to evaluate whether foreign DNA could be introduced into goat oocytes by sperm cells during the in vitro fertilization process. The effect of DNA-treated fresh and frozen-thawed sperm cells on cleavage rates of IVF goat oocytes will also be evaluated. Since a primary goal in goat IVF is to improve the cleavage rate, another aim was to evaluate Ca\(^{2+}\) ionophore A23187 on capacitation of goat sperm cells during in vitro fertilization.
Materials and Methods

Experimental Design

This experiment was designed to evaluate whether foreign DNA can be introduced into goat oocytes by sperm cells during fertilization and to compare the effects of fresh and frozen gametes on sperm-mediated gene transfer. Different levels of Ca\(^{2+}\) ionophore A23187 (range from 20 to 100 nM) were evaluated for their ability to capacitate goat sperm cells in an effort to maximize the cleavage rate of goat in vitro fertilized embryos. The experimental design is outlined in Table 8.

Foreign DNA

The pCMVβ plasmids (Clontech, Palo Alto, CA) were used in this study. The pCMVβ plasmid is a mammalian reporter vector designed for the expression of β-galactosidase in mammalian cells, as transcribed by the cytomegalovirus promoter (MacGregor and Caskey, 1989). Detection of this gene can be made by monitoring the expression of β-galactosidase, in which early stage embryos can be histochemically stained with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) and β-galactosidase can cleave off the substrate X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside). The embryos expressing the gene will stain blue.

Sperm Cell Preparation

The semen was collected from fertile Spanish bucks with an artificial vagina. Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975) was used to remove seminal plasma from fresh semen by washing twice and centrifuging at 600 x g for 10 minutes. The concentration of sperm cell was adjusted to 1 x 10^8/ml in B-O medium. pCMVβ plasmids were added to the sperm mixture to obtain a final concentration of 8 μg/ml of sperm mixture and incubated for 1 hour at room temperature (22°C).

Frozen semen used in the experiments was similarly collected from healthy Spanish bucks. The concentration of sperm samples was adjusted to approximately 3 x 10^8 cells/ml and an egg yolk-citrate glycerol diluent was used to freeze the sperm.
Table 8. Experimental design for Experiment 5

<table>
<thead>
<tr>
<th>Semen source</th>
<th>Sperm incubation with pCMVβ</th>
<th>Capacitation with A23187 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>No (negative control)</td>
<td>20</td>
</tr>
<tr>
<td>Fresh</td>
<td>Yes</td>
<td>20</td>
</tr>
<tr>
<td>Fresh</td>
<td>Yes</td>
<td>40</td>
</tr>
<tr>
<td>Fresh</td>
<td>Yes</td>
<td>100</td>
</tr>
<tr>
<td>Frozen-thawed</td>
<td>Yes</td>
<td>20</td>
</tr>
<tr>
<td>Frozen-thawed</td>
<td>Yes</td>
<td>40</td>
</tr>
<tr>
<td>Frozen-thawed</td>
<td>Yes</td>
<td>100</td>
</tr>
</tbody>
</table>
samples. After freezing in French straws (0.25 ml each), sperm samples were stored under liquid nitrogen. Frozen straws were thawed for 1 minute at 37°C and the sperm samples were washed twice with B-O medium and centrifuged at 600 x g for 10 minutes. The concentration of sperm cells was adjusted to 1 x 10⁸ sperm/ml in B-O medium. pCMVβ plasmids were added to the sperm mixture to obtain a final concentration of 8 µg/ml of sperm mixture and incubated for 1 hour at room temperature (22°C).

Following incubation with pCMVβ plasmids, sperm cells were treated with Ca²⁺ ionophore A23187 for 10 minutes at a final concentration of 20 nM, 40 nM or 100 nM. Sperm cells were then washed once with B-O medium to remove Ca²⁺ ionophore A23187.

**Oocyte Collection and Maturation**

The oocytes used in this study were collected from ovaries of Spanish-type crossbred goats at an abattoir in San Angelo, Texas. After collection, the ovaries were transported in 0.9% saline solution (room temperature) supplemented with 200 units/ml penicillin and 200 µg/ml streptomycin to the St. Gabriel Laboratory within 12 hours after collection. The ovaries were then washed with phosphate-buffered saline (PBS) twice and oocytes were aspirated using a 18-gauge needle attached to a 3-ml sterile syringe from follicles with a diameter between 2 and 7 mm.

Oocytes were collected and washed twice in PBS containing 0.1% (g/v) polyvinyl alcohol (PVA) and evaluated morphologically. Cumulus-oocyte complexes were evaluated based on the conditions of the surrounding cumulus cells and the consistency of ooplasm.

Only oocytes with intact, compact cumulus cells and homogenous ooplasm were randomly assigned to the experiment. After evaluation, the oocytes were washed three times in the maturation medium and incubated in groups of 20 to 30 oocytes in 500 µl maturation medium in individual wells of 4-well tissue culture plates (Nunc®,
France) at 39°C in an atmosphere with 5% CO$_2$ in humidified air for 27 hours. The medium used for maturation was TCM-199 (GIBCO BRL, Cat. No. 12340-014) supplemented with 10% heat-inactivated fetal bovine serum (Hycolone, Cat. No. A-1114-D) (Han et al., 1996).

**In Vitro Fertilization**

Fertilization medium was B-O medium supplemented with 20% FBS and 7.75 mM calcium lactate (Han et al., 1996). After maturation for 27 hours, oocytes were washed in fertilization medium twice and were placed in 50 μl droplets (8 to 10 oocytes/droplet) and then 50 μl of sperm suspension was added to the oocyte droplet to make a final sperm concentration of 2.5 x 10$^6$ sperm/ml. Insemination was conducted at 39°C in an atmosphere with 5% CO$_2$ in humidified air for 18 hours.

**Early Stage Embryos Co-culture**

Oocytes were removed from extra sperm cells after 18 hours insemination and oocytes were washed twice with the TCM-199 supplemented with 10% heat-inactivated fetal bovine serum and then returned to the original maturation dishes for another 30 hours of incubation. At 48 hours post-insemination, the cleaved embryos were co-cultured on a bovine oviduct epithelial cell monolayer. At 48-hour intervals, embryo culture medium was replaced with fresh culture medium. After 8 days of culture, embryos were stained to evaluate β-galactosidase gene expression.

**β-Galactosidase Staining**

Embryo preparation for β-galactosidase staining was performed as described previously (Dannenburg and Suga, 1981), with minor modification. Embryos were fixed for 5 minutes at 4°C in 2% formaldehyde plus 0.2% glutaraldehyde in PBS. Embryos were then washed with PBS and overlaid with a histochemical reaction mixture containing 1 mg/ml X-Gal (5-bromo-4chloro-3-indoly|β-D-galactoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl$_2$ in PBS.
Incubation was at 39°C for 18 to 24 hours. β-galactosidase positive embryos were identified by a characteristic blue color.

Results

In this experiment, different levels of calcium ionophore A23187 were evaluated to induce capacitation of fresh and frozen-thawed goat sperm cells using an IVF procedure. Results of this study are summarized in Table 9.

Using fresh goat sperm cells, 55 to 70% cleavage rates were achieved among different levels of calcium ionophore A23187 (ranging 20 to 100 nM) when foreign DNA was added to the sperm mixture, on the other hand, 70% cleavage rate was achieved with control group when foreign DNA was not added to the sperm mixture. Cleavage rates in treatment groups and control group are not significant different (P>0.05). For fresh sperm cells, there was no significant difference among different levels of calcium ionophore A23187 ranging from 20 nM to 100 nM on morula and blastocyst formation of goat oocytes either (P>0.05).

Using frozen-thawed goat sperm cells, 53 to 94% cleavage rates were achieved among different levels of calcium ionophore A23187. Furthermore, cleavage rate was significantly greater with the lowest levels of A23187 (20 nM) than with higher concentration of A23187 (100 nM). Treatment group with 40 nM calcium ionophore A23187 was significant higher on the formation of morula that other treatment groups with 20 nM and 100 nM A23187 (Table 9).

The effect of foreign DNA on IVF was evaluated in fresh goat sperm cells at a calcium ionophore level of 20 nM. Both control group and treatment group had same cleavage rate (70%) and similar morula rate (40% in control group and 35% in treatment group). Results showed that there was no significant difference in cleavage rate and early embryonic development between adding and not adding foreign DNA to the sperm mixture.
Table 9. In vitro fertilization with calcium ionophore-treated fresh and frozen-thawed caprine sperm cells co-incubated with pCMVB plasmid

<table>
<thead>
<tr>
<th>Addition of semen</th>
<th>A23187 pCMVB (nM)</th>
<th>Oocytes</th>
<th>X-Gal stain</th>
<th>Total</th>
<th>% CLEV</th>
<th>MORL/ CLEV</th>
<th>BLST/ CLEV</th>
<th>positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh No</td>
<td>20</td>
<td>37</td>
<td>70&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh Yes</td>
<td>40</td>
<td>33</td>
<td>55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>31</td>
<td>65&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen-thawed Yes</td>
<td>20</td>
<td>18</td>
<td>94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>15</td>
<td>87&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>80&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15</td>
<td>53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>26</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CLEV=cleaved; MORL=morula; BLST=blastocyst.
<sup>bc</sup>Different superscripts with in a column across treatment groups are significantly different (P<0.05).
Figure 3. In vitro fertilization of goat oocytes with sperm cells co-incubated with foreign DNA (pCMVβ plasmids). (a) Immature goat oocytes used in the experiments, (b) early cleaved goat IVF embryos, (c) goat IVF embryos before X-Gal staining and (d) a positive partial blue stained embryo after X-Gal staining.
Embryos were stained to evaluate expression of β-galactosidase at 8 days of culture after fertilization. With foreign DNA-treated sperm cells, a total of 104 cleaved embryos resulted from 152 in vitro matured oocytes, for a cleavage rate of 68%. A total of 3 X-Gal positive embryos resulted from 104 cleaved embryos after X-Gal stain (3% efficiency) compared with 0 of 25 in the control group. Among the 3 X-Gal positive embryos, all cells from one embryo (hatched blastocyst) were blue, while only random cells from the other two embryos exhibited a blue color after X-Gal stain (Figure 3d), indicating that mosaicism had occurred.

Discussion

Calcium ionophore A23187 at a level of 100 nM concentration exposed for 1 minute has been used successfully for bovine in vitro fertilization (Byrd 1981; Aoyagi et al., 1990; Zhang et al., 1992). The first IVF goat was also produced by capacitation of goat sperm cells with calcium ionophore A23187 (1 minute) at the 100 nM level (Hanada, 1985). In the present study, exposure of goat sperm cells to low levels of calcium ionophore A23187 (20 or 40 nM) for a longer interval (10 minutes) resulted in a 55 to 70% cleavage rate for fresh sperm cells. The lower level of calcium ionophore A23187 (20 nM) tended to give a higher cleavage rate (70%) than the higher levels of calcium ionophore A23187 (40 and 100 nM). Using frozen-thawed sperm cells, 53 to 94% cleavage was detected. Even though foreign DNA was added together with calcium ionophore in most sperm cell treatments, the results indicate foreign DNA treated sperm cells had no significant effect on cleavage rate, when fresh sperm cells were used in this study. These results were in agreement with the other results when mouse IVF was performed (Hochi et al., 1990).

A high level of calcium (7.75 mM calcium lactate) was used in the fertilization medium. Since Ca²⁺ flux in required to induce sperm capacitation and acrosome reaction, an increased cleavage rate may be caused by a synergistic effect of calcium
ionophore A23187 and a high calcium level in this study. Han et al. (1996) achieved ~50% cleavage rate in caprine IVF when sperm cells were exposed to B-O medium containing high calcium (7.75 mM) and 20% FBS for 3 hours without calcium ionophore A23187 before fertilization. It has been shown that high levels of A23187 (10 μM) are detrimental to mouse sperm cells and low levels of A23187 (30 nM) give high penetration rates in mouse IVF (Tanphaichitr and Hansen, 1994).

Shams-Borhan and Harrison (1981) reported that higher concentration of ionophore had a detrimental effect on ram sperm motility. Adding serum albumin shortly after the addition of the calcium ionophore, however, preserved motility, while the acrosome reaction occurred as usual. Calcium ionophore A23187 is a very effective agent for sperm cells capacitation and acrosome reaction. However, sperm cells from different animal species apparently require different levels of A23187 (Suarez et al., 1978; Smith et al., 1983; Atiken et al., 1984). It has been shown that there is a species difference in the requirement of physicochemical conditions, such as the time of exposure and the type of energy source for in vitro sperm capacitation (Chang and Hunter, 1975; Rogers and Yanagimachi, 1975). Differences in chemical characteristics of the sperm plasma membrane across species may account for this disparity of capacitation in different animal species (Yanagimachi, 1977). The optimum level of A23187 for capacitation of sperm cells still needs to be identified to achieve maximize efficiency of in vitro fertilization in the goat.

Sperm cells from some species have been shown to possess the ability to transfer foreign DNA into oocytes during fertilization in vitro (sea urchin, Arezzo, 1989; cattle, Perez et al., 1991; pig, Gagné et al., 1991; mouse, Lavitrano et al., 1989; Perez et al., 1991; de la Fuente et al., 1991 and Hochi et al., 1992). The present study showed that goat sperm cells, serving as vectors, could introduce foreign DNA into oocytes and subsequent embryos during in vitro fertilization. Three percent efficiency was achieved in this preliminary study, fairly low compared with results (22%)
achieved with bovine IVF (Gagné et al., 1991). Castro et al. (1990) reported that sperm cells from different species had different abilities to bind foreign DNA. This likely accounts for this across species variability.

In present study, the addition of foreign DNA in the sperm sample did not have a significant effect on the in vitro fertilization ability of goat sperm cells. This finding agrees with the results from a similar study on sperm-mediated gene transfer in the mouse (Hochi et al., 1990). However, our findings contrast with the IVF results during an attempt at sperm-mediated gene transfer in the cattle (Gagné et al., 1991). These different results may be explained by the conditions being used for sperm and DNA co-incubation. In the latter, the sperm cells were electroporated before the in vitro fertilization process. Electroporation has been proposed to affect the sperm cell motility, and this may be the major reason why the fertilization rate was very low in their study. In the present study, sperm cells were co-incubated with foreign DNA without electroporation, thus the sperm motility was not affected.

Results of a previous study in this laboratory showed that 37% fresh sperm cells and 33% frozen-thawed sperm cells could bind foreign DNA (Chapter 4). In the present study, only 3% of the in vitro fertilized embryos had cell level foreign DNA, as indicated by X-Gal staining. The reason for this difference in these findings is unknown. However, there were several possible explanations: 1) sperm cells bound to foreign DNA had less fertilizing ability than sperm cells without foreign DNA, 2) after fertilization, foreign DNA did not integrate into goat genomic DNA, and was later degraded by endogenous enzymes in the embryos, and 3) different techniques used to detect foreign DNA may vary in accuracy.

In other sperm-mediated gene transfer studies, foreign DNA was detected in embryos with the PCR technique (Hochi et al., 1990; Gagné et al., 1991). It has now been established that this technique results in false positives for gene incorporation (Ninomiya et al., 1989; Burdon and Wall, 1992; Bowen et al., 1994). In the present
study, β-galactosidase staining was used to detect foreign DNA in embryos. Even though some mammalian cells possess endogenous β-galactosidase, false positive staining can be minimized by staining at pH 7.5 to 8 and using a negative control. In this study, control embryos showed no blue color after X-Gal staining, while three positive embryos were noted in the DNA treated sperm groups.

Two of 3 pCMVβ positive embryos stained only partially blue after X-Gal staining. This may indicate that these two embryos were mosaic for pCMVβ. Previous reports showed that Approximately 70% of transgenic mice produced by microinjection carried the transgene in all cells, and the remaining 30% were mosaic (Wilkie et al., 1986; Palmiter and Brinster, 1986). A more recent study reported that ~62% of transgenic mouse embryos produced by the microinjection approach were mosaic (Bruce et al., 1993). In another study, none of the 8-cell mouse embryos had transgenes in more than 4 blastomeres (Burdon and Wall, 1992), while other results showed that as mouse embryos developed, the incidence of mosaicism significantly increased from 34.9% at 21 hours after microinjection to 60.7% at 67 to 69 hours post-microinjection (Lewis-Williams et al., 1996).

Mosaicism also appear to be a problem with the production of transgenic farm animals. Pursel et al. (1990) reported that 20% of founder transgenic pigs and sheep fail to transmit the gene to progeny, and another 20 to 30% transmit the transgene to <50%, which was due to mosaicism in the germ cells. The mechanisms that govern gene integration are unknown. At this stage it seems to be a random event. Further transgenic research is needed to reduce mosaicism to improve the efficiency of transgenic animal production.

In this study, low levels of calcium ionophore A23187 (20 nM) for a longer interval (10 minutes) capacitated goat sperm cells and tended to give a higher cleavage rate than a high level of calcium ionophore A23187 (100 nM) for both fresh and frozen-thawed sperm cells during in vitro fertilization. In this study, the best cleavage
rate (94%) came from the treatment group when 20 nM calcium ionophore A23187 was used with frozen-thawed sperm cells. Foreign DNA was transferred by goat sperm cells into goat oocytes during fertilization. Furthermore, foreign DNA was expressed in early stage embryos as indicated by expression of β-galactosidase. In the present study, 3% of the fertilized embryos were positive for pCMVβ with X-Gal staining. Further studies are needed to examine the mechanism of sperm cell binding of foreign DNA and to increase the percentage of sperm cell binding of foreign DNA. This could result in DNA-treated sperm cells having a higher chance of fertilizing oocytes and thus increasing the efficiency of sperm-mediated gene transfer.
CHAPTER 6

MECHANISM OF GOAT SPERM CELL BINDING OF FOREIGN DNA

Introduction

The transfer of foreign DNA into the genome of cells has been a hurdle for animal biotechnology for two decades. Mammalian cells can be transformed through chemical methods, such as DNA co-precipitation with calcium phosphate and complex formation with high-molecular-weight polycations for incorporation into liposomes. The efficiency of these methods are very low and have a multitude of limitations. Neumann et al. (1982) pioneered an alternative approach with electric field pulses on cell membranes to increase the efficiency of transfer of foreign DNA into mammalian cells. Electroporation has been used to obtain stable transformation in mammalian cell lines (Neumann et al., 1982; Evens et al., 1984; Knutson and Yee, 1987). Electroporation is now used to transform a broad and a large volume of cells (Potter, 1992; Rols et al., 1992). The molecular processes involved in the electroporation mechanisms of creation, stabilization, and annihilation of the transient structures responsible for permeation are still poorly understood. It has been suggested that foreign DNA crosses the cell membrane during the pulse due to the induction of an electrophoremeabilized state under the effects of electrophoretic forces (Klenchin et al., 1991; Sukharev et al., 1992).

There are many parameters affecting electrotransformation of mammalian cells, including cell types, electroporation medium (Knutson and Yee, 1987), foreign DNA form (circular or linear), concentration and even temperature (Potter et al., 1984). However, the most important parameters in electroporation are the intensity of the electroporation field and pulse duration. Effective transformation of mammalian
cells requires that electroporation voltage must be higher than threshold to make the cell membrane permeable (Wolf et al., 1994).

In previous studies (Chapter 4 and 5), results showed that 30 to 40% of the goat sperm cells were able to bind foreign DNA. However, only 3% of the IVF-derived embryos contained foreign DNA. One reason for the variation in results may be the low percentage of sperm cell binding of foreign DNA resulting in a low chance for these sperm cells to have an opportunity to fertilize ova. Thus, increasing the percentage of goat sperm cell binding of foreign DNA may allow these sperm cells with foreign DNA have a greater chance to fertilize the oocyte during in vitro fertilization process.

In a preliminary study, Gagné et al. 1991 indicated that electroporation could increase bovine sperm cell binding of foreign DNA. After oocytes were fertilized with electro-transformed sperm cells, ~20% of the embryos at the blastocyst stage retained foreign DNA compared with only 12% in the control group, without electroporation. In other studies, it was reported that electroporation of both pig sperm cells (Horan et al., 1992a) and chicken sperm cells (Nakanishi and Iritani, 1993) could increase the amount of foreign DNA bound by these haploid cells.

The sperm membrane may play a crucial role in regulation of sperm cell binding of foreign DNA. A protein of 30 to 35 kd in the sperm head was found to be a potential substrate for foreign DNA binding in mice (Lavitrano et al., 1992c). Wu et al. (1990) showed that the site of mouse sperm binding of foreign DNA was characterized by a complex structure of MHC Class-II molecules localized at posterior region of the sperm head.

Another possible reason for the fact that 30 to 40% of the goat sperm cells can bind foreign DNA, but only 3% of the fertilized embryos contained foreign DNA was that some foreign DNA molecules may be degraded before they were integrated into the genomic DNA. It has been reported that foreign oligonucleotide molecules rapidly
degrade (within hours) after they are microinjected into the nuclei of live cells (Fisher et al., 1993). This degradation is caused by nucleases, which are present in the cytoplasm and nuclei of live cells (Fisher et al., 1993). Whether these enzymes are present in the goat sperm cells remains to be determined.

Bull seminal plasma has been found to contain relatively high levels of 5'-nucleotidase (Mann, 1954) and ribonuclease (d'Alessio et al., 1972). It is reasonable to assume that 5'-nucleotidase serves as a guard to protect sperm cells from contamination of foreign DNA. This protection may allow genomic DNA of animal species to remain stable over generations. Thus, it became necessary to ascertain whether foreign DNA was degraded after being bound to sperm cells, and if so, to examine ways inhibit or slow this degradation process.

The objectives of this study were to evaluate the effects of electroporation on goat sperm cell binding of foreign DNA, to evaluate the role of the sperm cell membrane in sperm cell binding of foreign DNA and to study the fate of foreign DNA once bound to sperm cells.

Materials and Methods

Experimental Design

Experiment 6.1. The Effect of Electroporation on Fresh Goat Sperm Cell Binding of Foreign DNA

This experiment was designed to evaluate the effect of electroporation voltage on goat sperm cell binding of foreign DNA and on goat sperm motility. In this experiment, five electroporation voltage levels were evaluated. Voltage started with 0 volts/cm as a control and increased at 300 volt intervals to a highest voltage of 1,200 volts/cm (0, 300, 600, 900 and 1,200 volts/cm). After electroporation, total 1,000 sperm cells in three replicates from each treatment were evaluated for sperm cell
binding of foreign DNA using confocal microscopy. The experimental design for this study is shown in Table 10.

Experiment 6.2. The Role of the Sperm Membrane on Sperm Cell Binding of Foreign DNA

This experiment was designed to evaluate sperm membrane effect on goat sperm cell binding of foreign DNA. Sperm cells were subjected to different treatments as follows: 1) sperm cells co-incubated with foreign DNA without any additional treatment, serving as a control, 2) electroporation was applied to permeabilize the sperm membrane after co-incubation with foreign DNA, 3) electroporation was applied before co-incubation with foreign DNA, 4) lysolecithin was used to permeabilize the sperm membrane before sperm cell co-incubation with foreign DNA, 5) sperm cells were frozen in liquid nitrogen without cryoprotectant before sperm cells co-incubation with foreign DNA (low temperature effect), and 6) sperm cells were subjected to 65°C for 30 minutes before sperm cells co-incubation with foreign DNA (high temperature effect). The design for this experiment is presented in Table 11.

Experiment 6.3. The Fate of Foreign DNA in Sperm Cells After Sperm and Foreign DNA Co-incubation.

This experiment was designed to evaluate whether foreign DNA was degraded after goat sperm cells were co-incubated with foreign DNA. Different treatments of sperm cells were evaluated as follows: 1) incubation of sperm cells with foreign DNA without other treatments, 2) electroporation after sperm cell co-incubation with foreign DNA and 3) heating sperm cells to 65°C for 30 minutes to inactivate enzymes in sperm cells; afterward, foreign DNA was added to the sperm mixture. The experimental outline is shown in Table 12.

Foreign DNA

The pCMVβ plasmids (Clontech, Palo Alto, CA) were used in Experiment 6.1, 6.2 and 6.3. This 7.2 kb plasmid has a single cutting site for restriction enzyme Sal I.
Fluorescein-Labelling pCMVβ Plasmids

For Experiments 6.1 and 6.2, the plasmid construct was randomly labelled with fluorescein-11-dUTP according to the manufacturer’s recommended protocols (Behringer Mannheim; Indianapolis, IN). After labelling, free fluorescein-11-dUTP was removed using Bio-Spin Chromatography Columns (Bio-Red Laboratories, Richmond, CA).

Linearization of pCMVβ Plasmids

In Experiment 6.3, the plasmid pCMVβ construct was linearized with restriction enzyme Sal I and then incubated at 65°C for 30 minutes to inactivate the Sal I. The linearization was further verified with 0.8% agarose gel electrophoresis (Maniatis et al., 1982).

Sperm Cell Preparation

Fresh goat semen was collected from a healthy Spanish buck with an artificial vagina. The seminal plasma was removed from fresh semen by washing with Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975) and centrifuging at 600 x g for 10 minutes. Sperm cell concentration was adjusted to 1 x 10^8 sperm/ml with B-O medium. An aliquot of 100 µl sperm mixture was used for each treatment.

Electroporation of Sperm Cells

Foreign DNA pCMVβ plasmids labelled with fluorescein-11-dUTP (in Experiment 6.1) or linearized with Sal I (in Experiment 6.3) were added to the sperm mixture at 2 µg/ml and incubated for 30 minutes at room temperature (22°C). At the end of incubation period, 0.4 ml of the sperm mixture was transferred into a 1.4 ml Gene Pulser Cuvette (Bio-Red Laboratories, Richmond, CA) with a distance of 4 mm between electrodes. Electroporation was accomplished with different voltage (0, 300, 600, 900, 1,200) for Experiment 6.1 and a voltage of 1,200 for Experiments 6.2 and 6.3 at the same capacitance (25 µFarads) at room temperature using a Gene Pulser Apparatus. The motility of sperm cells was evaluated before and after electroporation.
Table 10. Experimental design for Experiment 6.1

<table>
<thead>
<tr>
<th>Treatment group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Electroporation voltage (v/cm)</th>
<th>No. of sperm evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (control)</td>
<td>0</td>
<td>1,000</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>1,000</td>
</tr>
<tr>
<td>3</td>
<td>600</td>
<td>1,000</td>
</tr>
<tr>
<td>4</td>
<td>900</td>
<td>1,000</td>
</tr>
<tr>
<td>5</td>
<td>1,200</td>
<td>1,000</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three replicates per treatment.
Table 11. Experimental design for Experiment 6.2

<table>
<thead>
<tr>
<th>Treatment group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Step 1</th>
<th>Step 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (control)</td>
<td>sperm + foreign DNA</td>
<td>buffer</td>
</tr>
<tr>
<td>2</td>
<td>sperm + foreign DNA</td>
<td>electroporation</td>
</tr>
<tr>
<td>3</td>
<td>sperm + electroporation</td>
<td>foreign DNA</td>
</tr>
<tr>
<td>4</td>
<td>sperm + lysolecithin</td>
<td>foreign DNA</td>
</tr>
<tr>
<td>5</td>
<td>sperm + liquid nitrogen</td>
<td>foreign DNA</td>
</tr>
<tr>
<td>6</td>
<td>sperm + 65°C for 30 min</td>
<td>foreign DNA</td>
</tr>
</tbody>
</table>

Three replicates per treatment.
Table 12. Experimental design for Experiment 6.3

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Step 1</th>
<th>Step 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (negative control)</td>
<td>sperm + buffer</td>
<td>buffer</td>
</tr>
<tr>
<td>2</td>
<td>sperm + foreign DNA</td>
<td>buffer</td>
</tr>
<tr>
<td>3</td>
<td>sperm + foreign DNA</td>
<td>electroporation</td>
</tr>
<tr>
<td>4</td>
<td>sperm + 65 °C for 30 min</td>
<td>foreign DNA</td>
</tr>
</tbody>
</table>

aThree replicates per treatment.
Changing Sperm Membrane Properties by Freezing Sperm Cells

A aliquot of 100 µl sperm solution in a 1.5 ml Eppendorf tube was dipped into liquid nitrogen for 10 minutes, then thawed at room temperature for 30 minutes and followed by co-incubation with foreign DNA pCMVβ plasmids labelled with fluorescein-11-dUTP at 2 µg/ml sperm solution for 30 minutes at room temperature.

Inactivation of Potential Enzymes in Sperm Cells by Heating Sperm Cells

An aliquot of 100 µl sperm solution in a 1.5 ml Eppendorf tube was heated at 65°C for 30 minutes. Foreign DNA pCMVβ plasmids labelled with fluorescein-11-dUTP (in Experiment 6.2) or linearized with Sal I (in Experiment 6.3) were added to the sperm solution at 2 µg/ml and co-incubated for 30 minutes at room temperature.

Permeabilization of Sperm Cell Membrane with Lysolecithin

Sperm cells were permeabilized with lysolecithin at 100 µg/ml for 10 minutes, followed by co-incubation for 30 minutes at room temperature with foreign DNA pCMVβ plasmids labelled with fluorescein-11-dUTP at 2 µg/ml sperm sample.

Evaluation of Sperm Cells Using Confocal Microscopy

After sperm cells were co-incubated with pCMVβ plasmids labelled with fluorescein-11-dUTP, sperm cells were washed twice with B-O medium to remove the free foreign DNA. After washing, sperm cells were fixed with 2% glutaradehyde in phosphate-buffered saline. And a smear was made with a drop of the sperm mixture, a cover slide was applied and the sample was sealed with clear nail polish oil. The slides were then evaluated using confocal microscopy (Fisher et al., 1993).

Extraction of Goat Sperm Genomic DNA

After co-incubation with pCMVβ plasmids linearized with Sal I, goat sperm cells were lysed and genomic DNA was extracted according to Crouse's procedure (Crouse et al., 1993), but without sonication. Following sperm/foreign DNA incubation, sperm samples were washed thoroughly with B-O medium by centrifugation (6,000 rpm) for 5 minutes in a microcentrifuge. Sperm cells were lysed
in 4% instaGene Matrix (Bio-Red Laboratories, Richmond, CA), 50 mM DTT. 0.3 mg/ml proteinase K at 56°C for 2 hours. Purification of sperm DNA included a single phenol/chloroform:isoamyl alcohol extraction followed by ethanol precipitation (Manitas et al., 1982).

**Southern Blotting Procedure**

The probe being used to detect foreign DNA in samples was plasmid pCMVβ. This plasmid was randomly labelled with α-32P-dATP and free α-32P-dATP was removed with Bio-Spin Chromatography Columns (Bio-Red Laboratories, Richmond, CA). The sperm genomic DNA was electrophoresed on 0.8% agarose gel in TBE buffer at 80 volts for 2 hours. Then sperm DNA was transferred from the gel onto Zeta-probe® GT blotting membranes with a standard protocol outlined in the Zeta-probe® GT blotting membranes instruction manual (Bio-Red Laboratories, Richmond, CA). For Southern blotting, the procedures for hybridization protocol were standard protocols outlined by Zeta-probe® GT blotting membranes instruction manual (Bio-Red Laboratories, Richmond, CA). The blotted membranes were both exposed to X-ray film for autoradiography and were analyzed with phosphor-imaging system.

**Statistical Analysis**

The results were analyzed with SAS ANOVA procedure (SAS institute, Gary, NC) and a general purpose regression analysis was also used on the relationship between the voltage of electroporation and the percentage of sperm cell binding of foreign DNA and on the relationship between the voltage of electroporation and the sperm motility.

**Results**

**Experiment 6.1**

In this experiment the effect of electroporation on goat sperm cell binding of foreign DNA was evaluated. The results, summarized in Table 13, showed that as the
Table 13. The effect of electroporation (voltage) on caprine sperm cell binding of foreign DNA

<table>
<thead>
<tr>
<th>Electroporation (voltage/cm)</th>
<th>Total no. sperm cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sperm cell binding of plasmid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of sperm binding plasmid</th>
<th>Percent of total sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1,050</td>
<td>347</td>
<td>33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±2.6</td>
</tr>
<tr>
<td>300</td>
<td>1,100</td>
<td>715</td>
<td>65&lt;sup&gt;d&lt;/sup&gt;</td>
<td>±3.2</td>
</tr>
<tr>
<td>600</td>
<td>1,027</td>
<td>934</td>
<td>91&lt;sup&gt;e&lt;/sup&gt;</td>
<td>±13.8</td>
</tr>
<tr>
<td>900</td>
<td>1,045</td>
<td>941</td>
<td>90&lt;sup&gt;e&lt;/sup&gt;</td>
<td>±2.6</td>
</tr>
<tr>
<td>1,200</td>
<td>1,055</td>
<td>1,055</td>
<td>100&lt;sup&gt;f&lt;/sup&gt;</td>
<td>±0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three replicates per treatment.

<sup>b</sup>Mean ± SE.

<sup>cdef</sup>Different superscripts within a column across treatment group are significantly different (P<0.05).
voltage of electroporation increased, the percentage of sperm cell binding of foreign DNA also increased. The percentages of sperm cell binding of foreign DNA was 33% without electroporation and 65, 91, 90 and 100% for sperm cell binding of foreign DNA at 300, 600, 900 and 1,200 volts/cm, respectively. All treatment groups, showed a significantly (P<0.05) greater binding rate than the control group where sperm cells were not exposed to electroporation. A linear regression was developed for the relation of electroporation voltage to the percentage of sperm cell binding foreign DNA. The regression line was $Y = 0.43 + 0.0005X$ (Y represents the percentage of sperm cells binding foreign DNA, X represents the voltage of electroporation); $r^2 = 0.8449$.

The effect of electroporation on goat sperm cell motility was also evaluated in the Experiment 6.1. Results on sperm motility are presented in Table 14. In this study, as the voltage of electroporation increased, progressive sperm cell motility decreased. Sperm cell motility was 55% without electroporation, after electroporation, sperm motility decreased to 53%, 25% and 13% for 300, 600 and 900 volts/cm, respectively. When the voltage level reached 1,200, all sperm cells became immotile. At low voltage of electroporation (300 volts), sperm motility apparently was not affected. The motility was significantly (P<0.05) reduced after electroporation at 600, 900 and 1,200 volts/cm. A linear regression was developed for the relation of electroporation voltage to sperm cell motility. The regression line was $Y = 0.58 - 0.0005X$ (Y represents the sperm motility after electroporation, X represents the voltage of electroporation), $r^2 = 0.8917$.

Experiment 6.2

In this experiment, treatments were used in an attempt to change the sperm membrane properties. After sperm cells were co-incubated with foreign DNA without any additional treatment, only 33% sperm cells bound foreign DNA.
Table 14. The effect of electroporation (voltage) on caprine sperm motility

<table>
<thead>
<tr>
<th>Electroporation (voltage/cm)</th>
<th>Sperm motility&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>before</th>
<th>after</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>55% (±2.9)</td>
<td>52% (±2.9)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>55% (±2.9)</td>
<td>53% (±2.9)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>55% (±2.9)</td>
<td>25% (±7.6)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>900</td>
<td>55% (±2.9)</td>
<td>13% (±2.8)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>1,200</td>
<td>55% (±2.9)</td>
<td>0 (±0)&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Three replicates per treatment.

<sup>b</sup> Mean ± SE.

<sup>cd</sup> Different superscripts within a column are significantly different (P<0.05).
Figure 4. Caprine sperm membrane effect on sperm cell binding of foreign DNA. (a) goat sperm cells subjected to electroporation after co-incubation with foreign DNA, (b) goat sperm cells incubated with lysolecithin then co-incubated with foreign DNA, (c) goat sperm cells co-incubated with foreign DNA after being frozen in liquid nitrogen without cryoprotectant and thawed at 37°C, (d) goat sperm cells co-incubated with foreign DNA after being subjected to 65°C for 30 minutes. Showing all goat sperm cells bound foreign DNA following each treatment, except for the lysolecithin treatment. (Magnification 200X).
Figure 5. Southern blotting of goat sperm genomic DNA. The corresponding lanes are as follows: positive control (lane 1, pCMV plasmids), sperm DNA as negative control (lane 2), sperm co-incubated with plasmids (lane 3), sperm cells subjected to electroporation after co-incubation with plasmids (lane 4), goat sperm cells co-incubated with plasmids after being subjected to 65°C for 30 minutes (lane 5). Plasmids were degraded in all treatments, except in the treatment in which sperm cells were subjected to 65°C for 30 minutes.
Electroporation treatment (both before and after sperm co-incubation with foreign DNA) effectively increased sperm cell binding of foreign DNA. At 1,200 volts/cm, all sperm cells bound foreign DNA in both treatments (electroporation was applied to sperm cells before co-incubation or after co-incubation with foreign DNA) (Figure 4a). After sperm cells were permeabilized with lysolecithin, no foreign DNA could be detected in any sperm cells in this study (see figure 4b). Heating sperm cells at 65°C for 30 minutes or freezing sperm cells in liquid nitrogen both effectively increased sperm cell binding of foreign DNA. In this study, all sperm cells bound foreign DNA after both treatment with liquid nitrogen and heating (Figure 4c,d).

Experiment 6.3

This experiment was used to evaluate the fate of foreign DNA after co-incubation with sperm cells. Because circular plasmids could have multiple forms and are difficult to judge with Southern blotting, plasmids were first linearized with Sal I. Sperm DNA was analyzed using the Southern blotting method (Figure 5). The standard size for plasmid pCMVβ was 7.2 kb. Results showed that some plasmid pCMVβ had been degraded after the plasmid pCMVβ was co-incubated with sperm cells without electroporation (Figure 5, lane 3) or with electroporation (Figure 5, lane 4). When sperm cells were first subjected to a heating treatment at 65°C for 30 minutes before plasmid pCMVβ was added to the sperm mixture, plasmid pCMVβ degradation was reduced (Figure 5, lane 5).

Discussion

Recent data show that sperm cells can introduce foreign DNA into goat oocytes during fertilization (Chapter 5). In our previous study (Chapter 5), 3% of fertilized oocytes contained foreign DNA after in vitro fertilization. In other studies, 2.5% of lambs were transgenic after ewes were inseminated with sperm cells co-incubated with foreign DNA (Sun et al., 1994). 2.4% of calves retained foreign DNA when artificial
insemination was performed with sperm cells co-incubated with foreign DNA (Schellander et al., 1995).

In vitro studies showed that not all goat sperm cells can bind foreign DNA (Chapter 4). This may indicate a low percentage of sperm cell binding of foreign DNA, resulting in a low percentage of sperm cell binding of foreign DNA fertilizing the oocytes. Increasing the percentage of sperm cell binding of foreign DNA may increase the chance of these sperm cells attempting to fertilize the oocytes.

Electroporation has been shown to increase bovine sperm cell binding of foreign DNA (Gagné et al., 1991). After in vitro fertilization with electrotransformed sperm cells, ~20% of the bovine embryos at blastocyst stage retained foreign DNA compared with only 12% retained foreign DNA in the control embryos. Also, electroporation of porcine sperm cells increased the amount of foreign DNA bound by the sperm cells (Horan et al., 1992a). In addition, electroporation significantly increased the percentage of rooster sperm cell binding of foreign DNA (Nakanishi and Iritani, 1993). In the present study, results indicated that electroporation could increase the percentage of goat sperm cell binding of foreign DNA, and that the efficiency was related to the electroporation voltage. When voltage of electroporation was 1,200, 100% sperm cells bound foreign DNA in our study.

Effective transformation of mammalian cells requires that electroporation voltage must be higher than threshold to permeabilize the cell membrane (Wolf et al., 1994). In our study, it was found that the percentage of sperm cell binding of foreign DNA was positively related to the electroporation voltage used during the procedure.

The molecular processes involved in the mechanisms of creation, stabilization and annihilation of the transient structures of cell membranes responsible for permeation by electroporation are still not clear. It was suggested that electroporation changes the permeability of the membrane and foreign DNA crosses the membrane during the pulse due to the induction of an electroporemeabilized state under the effects...
of electrophoretic forces associated with the external field (Klenchin et al., 1991; Sukharev et al., 1992).

In the present study, electroporation of sperm cells before co-incubation with foreign DNA was as effective as electroporation of sperm cells after co-incubation with foreign DNA. This may indicate that, once the state of sperm membrane permeability is changed, it takes an extended interval for it to recover. This finding agrees with another study showing that electroporation could effectively transform Chinese hamster cells (Wolf et al., 1994) when electroporation was applied both before and after adding foreign DNA into Chinese hamster cell samples.

In the present study, freezing and heating the sperm cells were also applied to disrupt the sperm membrane. The results showed that after these varied temperature treatments, all sperm cells could bind foreign DNA. However, once sperm cells were treated with lysolecithin no foreign DNA was found in the sperm cells. It is possible that the pores in the sperm membrane created by lysolecithin was too large and foreign DNA was easily washed away by the experimental procedure. Leno et al. (1992) showed that sperm nuclear membrane can be permeabilized with lysolecithin allowing large molecule markers like TRITC-IgG to diffuse through the cell membrane. These observations further verified that the sperm membrane plays a crucial role in retaining foreign DNA molecules.

It is possible that the low percentage of sperm cell binding of foreign DNA was not the only reason for the low efficiency of sperm-mediated gene transfer reported in recent studies (Chapter 5). If each sperm cell has an equal chance of fertilizing the ovum, the efficiency would be much higher than currently reported (Chapter 5). Previously, it has been reported that sperm cells treated with foreign DNA do not affect the fertilization rate (Lavitrano et al., 1989; Hochi et al., 1990; Chapter 5), suggesting that foreign DNA may have been degraded after fertilization before it integrated into the genomic DNA.
Unexpected DNA fragments have been found in the offsprings of chicken and cattle produced from sperm-mediated gene transfer (Bachiller et al., 1991; Schellander et al., 1995). Alteration of integrated foreign DNA has also been reported with the microinjection gene transfer procedure in mice (Gordon et al., 1980; Wagner et al., 1981; Ross and Solter, 1985; Strojek, 1986). It has been suggested that before foreign DNA was integrated into the genome, deletions and recombinations of foreign DNA were facilitated by DNA-active enzymes and/or circular DNA was linearized at different sites before integration (Roschlau et al., 1989). It is not known whether these enzymes are present in sperm cells. Southern blotting results in our experiments further demonstrate that most foreign DNA molecules were degraded after co-incubation with sperm cells.

These observations may provide a clue to how the genomic DNA of mammalian species has remained stable for a relatively long period. Bull seminal plasma has been found to contain a relatively high amount of 5'-nucleotidase (Mann, 1954) and ribonuclease (d’Alessio et al., 1972), and the seminal vesicles have been identified as the source of these enzymes (Shivaji et al., 1990). Recently, 5'-nucleotidase was found in both epididymal and ampullary bovine sperm cells (Schiemann et al., 1994). The 5'-nucleotidase is essential in the regulation of extracellular adenosine concentration (LeHir and Kaissling, 1993). The precise functions of 5'-nucleotidase in sperm cells are still unknown. However it thought to be reasonable to regard 5'-nucleotidase as a guard to protect sperm cells from contamination by foreign DNA. This may be a primary way of keeping the genomic DNA of higher order animals stable.

For example, during the in vivo fertilization process the fertilization environment is protected, however, sperm cells still may be exposed to foreign DNA molecules. These DNA molecules are likely from degradation of cells in the female reproductive tract, which could be DNA from the same species as the sperm cells.
Apparently, these DNA molecules are not "foreign DNA", and not cause transformation. During the early evolution process, however, fertilization occurred in an open environment (e.g. lake, ocean). Sperm cells were likely surrounded by foreign DNA molecules and foreign DNA could have been introduced into the genomic DNA during fertilization, thus facilitating the evolutionary process.

During in vitro maturation and fertilization, the media contains serum, BSA and agents that are the sources of nucleases. These nucleases may be one of the primary reasons for low efficiency of mammalian cell transformation. Nucleases are also present in the cytoplasm and nuclei of live cells. These nucleases were regarded as the cause of the rapid degeneration of foreign oligonucleotide molecules after they were microinjected into the nuclei of live cells (Fisher et al., 1993).

If foreign DNA were degraded once it is bound to sperm cells, experimental measures would need to inhibit or slow this process. In the present study, foreign DNA had been degraded after being bound to sperm cells. However, after sperm cells are exposed to 65°C for 30 minutes, foreign DNA degradation was reduced (Figure 5, lane 5).

Results of our study (Experiment 6.1 and 6.2) suggest that sperm motility is not related to the ability of sperm cell binding of foreign DNA. This inverse relationship between motility and foreign DNA binding is in agreement with the results of bull sperm cells reported by Bird et al. (1992) and Atkinson et al. (1991). However, these results contrast with most other observations of mouse sperm cell binding of foreign DNA (Lavitrano et al., 1989; Castro et al., 1990; Clausen et al., 1991), which indicate that only motile sperm cells can bind foreign DNA. Our experiments indicate that after immobilization of sperm cells with electroporation, deep freezing or heating, goat sperm cells remain capable of binding foreign DNA.

It is known that sperm motility is regulated by mitochondria located in the sperm tail. The positions for binding of foreign DNA are located in the sperm head.
Thus it seems that sperm motility should not affect sperm cell binding of foreign DNA. However, only motile sperm cells can fertilize oocytes during in vivo fertilization and in vitro fertilization. Even though electroporation could make 100% of the sperm cell binding of foreign DNA, sperm cells became immotile after electroporation procedure. Therefore, possibility of sperm-mediated gene transfer by artificial insemination or in vitro fertilization was all but eliminated after these experiments.

Goto et al. (1990) have reported that immotile bovine sperm cells could fertilize when sperm cells were microinjected into cattle oocytes and live offsprings could be produced. Human sperm cells have also been immobilized and microinjected into oocytes. This microfertilization protocol has become successful in treating male infertility (Palermo et al., 1992). Yanagida et al. (1991) have reported mouse sperm cells treated with high temperature (90°C) retain the ability to decondense after they are injected into the cytoplasm of matured oocytes. Only extremely high temperature (120°C steam) completely eliminated the fertilizing ability of sperm cells as indicated by their failure to undergo decondensation after they were injected into mouse oocytes. In present study, sperm cells were exposed to 65°C for 30 minutes and this treatment may only immobilize the sperm cells while the fertilizing ability of the sperm cells is retained.

Since the microinjection gene transfer method is still problematic and reliable stem cell lines have not yet established for farm animals, we felt it was worthwhile to microinject transformed immotile sperm cells into oocytes to further evaluate sperm-mediated gene transfer.
CHAPTER 7

GOAT OOCYTE ACTIVATION AND INTRACYTOPLASMIC SPERM INJECTION

Introduction

Sperm injection and subsequent oocyte activation have been used for many years as a tool in echinoderm (Hiramoto, 1962) and amphibian fertilization research (Brun, 1974). The first sperm injection experiments with mammals were conducted with hamster by Uehara and Yanagimachi (1976). Decondensation of the sperm head, formation of pronuclei and subsequent early cleavage has been achieved in the hamster (Uehara and Yanagimachi, 1976). Over the years, live births have been reported with injection of sperm cells into the cytoplasm of oocytes (ICSI) in mice (Roknabadi et al., 1994), rabbits (Hosoi et al., 1988), cattle (Goto et al., 1990) as well as in humans (Palermo et al., 1992). Now ICSI is being used successfully as an effective technique for treating couples with infertility. One advantage of ICSI over other assisted fertilization techniques is that sperm cells need not to be motile with ICSI (Goto et al., 1990; Palermo et al., 1992). ICSI may be especially useful in farm animals in cases where a premium male has a physical injury so testicular sperm can be collected and used for sperm injection.

Hiramoto (1962) has shown that when live sea urchin sperm cells were injected into sea urchin oocytes and the sperm nucleus did not decondense in the ooplasm. However, when the sperm-injected egg was stimulated by insemination with extra sperm, the injected sperm nuclei could decondense. This experiment indicated only that injected sperm cells could not activate the oocyte and exogenous stimulation was needed to activate of the sea urchin oocyte and to decondense the injected sperm nucleus. In contrast to sperm injection in the sea urchin, frog eggs can be activated by injected frog sperm without extra activation (Graham, 1966; Brun, 1974).
Hosoi et al. (1988) reported the birth of live rabbit offspring following transfer of ova fertilized by injection of a live sperm cell into the ooplasm without exogenous activation. Goto et al. (1990) reported that the birth of live calves after intracytoplasmic injection of frozen-thawed bovine sperm cells followed by subsequent activation of oocytes with exogenous calcium ionophore A23187. These results indicate that species differences in the need for exogenous stimuli after sperm are injected into cytoplasm of the oocyte to activate the oocyte.

During fertilization, oocyte activation is initiated after the sperm cell membrane fuses with the membrane of the ovum. The sperm receptor on the oocyte surface interacts with an oocyte-binding protein located on the sperm surface to form a complex resulting in species-specific adhesions between the sperm cell and the oocyte (Kinsey et al., 1980). Intracytoplasmic sperm injection procedure, however, bypasses a primary sequence of the fertilization event.

It has been suggested that oocytes may be activated by the injection of high-calcium medium during intracytoplasmic sperm injection (Edwards and Van Steirteghem, 1993). However, recent results indicate that oocyte activation is started after a considerable lag period following sperm injection. This activation is probably caused by a soluble factor released from the exogenous sperm cell after it enters the ooplasm (Tesarik et al., 1994). The absence of human oocyte activation is the cause of fertilization failure in most cases when intracytoplasmic sperm injection (ICSI) fails (Tesarik and Sousa, 1995). Exogenous calcium A23187 can activate sperm-injected human oocytes (Tesarik and Sousa, 1995).

Electroporation is a very effective way to increase the efficiency of goat sperm cell binding of foreign DNA. After electroporation at 1,200 volts/cm, 100% of goat sperm cells bound foreign DNA, however, all sperm cells became immobilized (Sun et al., 1996). ICSI is the next logical step to evaluate whether these immobilized sperm cells have the ability to fertilize the oocyte and whether the efficiency of sperm-
mediated gene transfer can be increased using an electroporation procedure. Immotile sperm cells from humans, bulls and rabbits can fertilize oocytes when sperm cells are injected into the ooplasm of oocytes rabbits (Hosoi et al., 1988; Goto et al., 1990; Palermo et al., 1992), which suggests that immotile goat sperm cells also could fertilize oocytes once injected into the oocyte cytoplasm. Unfortunately, there is no information on goat intracytoplasmic sperm injection presently available. Furthermore, it is not clear whether goat oocytes need exogenous stimulation to be activated after sperm are injected into oocyte cytoplasm.

The objectives of this study were to evaluate sham-injected goat oocyte activation and to evaluate the injection of immobilized goat sperm cells into oocytes to activate oocytes and to evaluate subsequent cleavage and development in vitro.

**Materials and Methods**

**Experimental Design**

Experiment 7.1. Calcium Ionophore A23187 Activation of Sham-Injected Goat Oocytes

This experiment was designed to evaluate if the act of injection is enough to activate the goat oocyte and secondly to titrate the concentration of calcium ionophore A23187 required to activate goat oocytes. After oocytes were matured for 27 hours, sham-injection was performed using the same procedure as for sperm injection, but without injection of a sperm cell. After injection, goat oocytes were activated with different levels of A23187 after randomly assigned to the treatments shown in Table 15. Activated oocytes were further cultured on buffalo rat liver (BRL) cell monolayer and subsequent development of injected oocytes was evaluated.
Table 15. Experimental design for Experiment 7.1

<table>
<thead>
<tr>
<th>Calcium ionophore concentration (µM)</th>
<th>No. oocytes</th>
<th>sham injected&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Four replicates per treatment.
Experiment 7.2. Intracytoplasmic Injection of Goat Oocytes with Immotile Sperm Cells

This experiment was designed to evaluate goat oocyte activation and early cleavage after injection of immotile goat sperm cells, to evaluate the effect of electroporation on sperm cells related to oocyte activation and early cleavage, and to compare two levels of A23187 (10 and 50 μM) on activation of sperm-injected oocytes. The experimental outline is presented in Table 16.

Oocyte Collection and Maturation

The goat ovaries were harvested from an abattoir in San Angelo, Texas. Following collection, the ovaries were transported in a thermos containing 0.9% saline solution (room temperature) supplemented with 200,000 units penicillin and 200,000 μg streptomycin/L to the St. Gabriel Laboratory within 12 hours. The ovaries were then washed thoroughly with phosphate-buffered saline (PBS) and oocytes were aspirated using a 18-gauge needle attached to a 3-ml sterile syringe. Oocytes were collected from follicles with diameter between 2 and 7 mm and washed twice in PBS containing 0.1% (g/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes were evaluated based on the layer of cumulus cells and the consistency of ooplasm. Only good quality oocytes with intact, compact cumulus cells and homogenous ooplasm were randomly allotted to the experiments. Oocytes were washed twice and matured in TCM-199 (GIBCO BRL, Cat. No. 12340-014) with 10% heat-inactivated fetal bovine serum (Hycolone, Cat. No. A-1114-D) (Han et al., 1995). Groups of 20 to 30 oocytes were placed in 500 μl maturation medium in each individual well of 4-well tissue culture plates (Nunc®, France) at 39°C in an atmosphere of 5% CO₂ in humidified air for 27 hours.

Preparation of Oocytes for Injection

Matured cumulus-oocyte complexes were briefly (1 minute) treated with hyaluronidase (1 mg/ml in TCM-199) and the cumulus cells surrounding the oocytes
Table 16. Experimental design for Experiment 7.2

<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>Oocyte no.(^a)</th>
<th>Oocyte activation A23187 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Sperm</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>Sperm + electroporation</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Sperm + electroporation</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>Sham control</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Sham control</td>
<td>30</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^a\)Four replicates per treatment.
were partially removed by pipetting. The oocytes were then washed in PBS + 10% FBS. The injection window was set at 27 to 33 hours after starting maturation of oocytes.

Frozen semen from a fertile Spanish buck was used during the experiment. French straws (0.25 ml each) of sperm cells were thawed for 1 minute at 37°C, then the extender was removed from frozen-thawed semen by washing twice with Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975) and centrifuging at 600 x g for 10 minutes. Sperm cells were washed once with an electroporation buffer composed of 260 mM sucrose, 5 mM HEPES, pH 7.3-7.4, 50 μg/ml polyvinyl alcohol (125,000 Kda) and 5 mM CaCl₂·2 H₂O (Tomkins and Houghton, 1988).

**Induction of Acrosome Reaction**

After sperm cells were washed in electroporation buffer, sperm cell concentration was adjusted to 1 x 10⁷ sperm/ml. Calcium ionophore A23187 was added to the sperm mixture to a final concentration of 1 μM and incubation at 37°C for 30 minutes to induce the acrosome reaction (Watson et al., 1991).

**Electroporation of Sperm Cells**

After induction of the acrosome reaction, sperm cell concentration was adjusted to 1 x 10⁶ sperm/ml in the electroporation buffer. For electroporation, 0.4 ml of the sperm mixture was transferred to a 1.4 ml Gene Pulser Cuvette (Bio-Red Laboratories, Richmond, CA). The distance between electrodes was 4 mm. Electroporation was applied with a voltage of 1,200 volts/cm at a capacitance of 25 μF at room temperature using a Gene Pulser Apparatus (Bio-Red Laboratories, Richmond, CA). Sperm suspension was mixed (1:10) with 10% polyvinypyrrolidone in PBS prior to injection.

**Preparation of Oocyte-Holding and Sperm-Injection Pipettes**

The holding and injection pipettes were made by drawing glass capillary tubes with a pipette puller and were further processed on a microgrinder and a microforge.
The outer and inner diameters of the pipettes were 100 and 20 μm and 10 and 6 μm for the holding and injection pipettes, respectively. The injection pipettes were beveled at angle of 50°.

**Intracytoplasmic Sperm Injection**

Intracytoplasmic sperm injection was conducted on the heated stage of an inverted microscope (Nikon, Surrey, United Kingdom). The pipettes were mounted to micromanipulators. The dishes used for injection were cell culture chamber slides (Nunc®, France). One elongated drop was made of PBS with 10% heat-inactivated FBS and oocytes were placed at one end of the droplet (6 to 10 oocytes/droplet). Then 2 μl of sperm-PVP mixture was placed next to the oocyte droplet on the slide. These two drops were covered with mineral oil (medical grade) to prevent evaporation during injection.

First, an immotile sperm cell was aspirated tail first into the injection pipette. The injection pipette was raised and the stage was moved to locate the oocyte droplet. Suction was applied to hold the oocyte onto the holding pipette (see Figure 6b), the injection pipette was then pushed through the zona pellucida and the oolemma at the opposite pole to the holding pipette. A single sperm was injected into the cytoplasm of oocyte. Because goat oocyte cytoplasm is very opaque, injection was performed under 800X magnification to make sure the whole sperm was completely injected into the ooplasm. This was done by observing the sperm tail pass through the oocyte plasma membrane.

Sham-injection was performed using the same procedure as for sperm injection, but without the inclusion of the sperm cell. Once all oocytes were injected, oocytes were washed in PBS and activated in calcium ionophore A23187 at a final concentration according to each treatment (See experiment design). After activation for 10 minutes, oocytes were washed in PBS containing 0.3% BSA to stop the action
of A23187. Then oocytes were cultured on a monolayer of buffalo rat liver cells for further development observation (Han et al., 1996).

**Statistical Analysis**

The criteria for evaluation included the percentage of oocytes that degenerated and the percentage of oocytes that cleaved. The results were analyzed with SAS ANOVA procedure (SAS institute, Gary, NC). The relationship between the levels of calcium ionophore for activating goat oocytes and the cleavage rate of goat oocytes after activation was analyzed with linear regression. A P value of < 0.05 was regarded as statistically significant. The differences among individual treatments were assayed by developing 95% confidence intervals.

**Results**

**Experiment 7.1**

In this experiment, the effect of exogenous calcium ionophore on activation of sham-injected goat oocytes was evaluated. Oocyte degeneration and cleavage results are shown in Table 17. In the present study, goat oocytes were not activated by sham-injection action. Furthermore, low levels of A23187 (0.1 to 1 μM) did not activate sham-injected oocytes. When the concentration of calcium ionophore was raised to 10 μM to activate the oocytes, a very low percentage (3%) of sham-injected goat oocytes was activated and performed the first cycle of cleavage. As the A23187 concentration increased, more sham-injected goat oocytes were activated. The highest cleavage rate (66%) was achieved when 200 μM A23187 was used to activate sham-injected goat oocytes. The linear regression between the cleavage rate and the concentration of calcium ionophore A23187 was $Y = -0.0027 + 0.0038X$ (Y represents the cleavage rate of sham-injected goat oocytes and X represents the concentration of A23187 in μM); $r^2 = 0.873$. In this experiment, ~10% of the goat oocytes degenerated after sham-
injection. There was no significant difference of oocyte degeneration among different levels of calcium ionophore.

**Experiment 7.2**

Electroporation effect on sperm cells related to oocyte cleavage was evaluated and the effect of two concentrations of calcium ionophore A23187 on activation of sperm injected goat oocytes was compared. The oocyte degeneration and cleavage rates of each treatment are shown in Table 18. Pronuclei were detected in sperm-injected oocytes (Figure 6c). Cleavage was observed in all treatments with injected sperm cells or sham control without injected sperm cells after activation with calcium ionophore. In this study, cleavage was not significantly different between sperm-injected oocytes and sham-injected oocytes after oocytes were activated at either 10 μM or 50 μM of calcium ionophore when electroporation was not used in the procedure.

With electroporation, when oocytes were activated at 10 μM A23187, there was a substantial difference between cleavage of electroporated sperm-injected oocytes and sham-injected oocytes. When oocytes were activated with 50 μM calcium ionophore, cleavage was also substantially greater (37%) in electroporated sperm-injected oocytes than in sham-injected controls (18%) (P=0.10). The cleavage rate in oocytes injected with electroporation treated sperm cells was substantially greater than that in oocytes injected with nonelectroporated sperm cells (P=0.18).

Without electroporation, there was no difference in cleavage between sperm-injected oocytes or sham-injected oocytes when oocytes were activated at either 10 μM or 50 μM level of calcium ionophore A23187.

Degeneration of some oocytes was observed in all treatment groups. There was a significantly greater (P<0.05) degeneration rate (16%) in sperm injected oocytes than in sham injected oocytes (3.6%). There was no difference in degeneration rate among sperm-injected oocytes whether electroporation or different A23187 concentrations were used for oocyte activation.
Table 17. Calcium ionophore A23187 activation of sham-injected caprine oocytes

<table>
<thead>
<tr>
<th>A23187 (μM)</th>
<th>No. oocytes sham injecteda</th>
<th>At 48 h after injection</th>
<th>Degenerated</th>
<th>2- to 4-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24</td>
<td>4 (16.7%)</td>
<td>0d</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>24</td>
<td>3 (12.5%)</td>
<td>0d</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>3 (12.5%)</td>
<td>0d</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>3 (10.0%)</td>
<td>1 (3.3%)d</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>25</td>
<td>3 (12.0%)</td>
<td>4 (8.0%)ad</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>23</td>
<td>2 (8.7%)</td>
<td>8 (34.8%)e</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>24</td>
<td>3 (12.5%)</td>
<td>16 (66.7%)b</td>
<td></td>
</tr>
</tbody>
</table>

aFour replicates per treatment.

bcDDifferent superscripts within a column are significantly different (P<0.05).
Figure 6. Goat intracytoplasmic sperm injection. (a) a morphologically mature goat oocyte, (b) intracytoplasmic sperm injection, (c) Two pronuclei formed as a result of ICSI, (d) blastocysts derived from ICSI. (Magnification 400X).
<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>Oocyte activation</th>
<th>No. oocytes injected</th>
<th>48 hours post injection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A23187 (μM)</td>
<td></td>
<td>Degenerated</td>
<td>2 to 4-cell</td>
</tr>
<tr>
<td>Sperm</td>
<td>10</td>
<td>28</td>
<td>5 (17.9%)</td>
<td>5 (17.9%)</td>
</tr>
<tr>
<td>Sperm</td>
<td>50</td>
<td>30</td>
<td>4 (13.3%)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2 (6.7%)</td>
</tr>
<tr>
<td>Sperm + electroporation</td>
<td>10</td>
<td>30</td>
<td>6 (20.0%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9 (30.0%)</td>
</tr>
<tr>
<td>Sperm + electroporation</td>
<td>50</td>
<td>30</td>
<td>4 (13.3%)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>11 (36.7%)</td>
</tr>
<tr>
<td>Sham control</td>
<td>10</td>
<td>27</td>
<td>1 (3.7%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 (3.7%)</td>
</tr>
<tr>
<td>Sham control</td>
<td>50</td>
<td>28</td>
<td>1 (3.6%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5 (17.9%)</td>
</tr>
</tbody>
</table>

*Four replicates per treatment.

<sup>b,c</sup>Different superscripts within a column are significantly different (P<0.05).
Discussion

In all mammalian species studied so far, it has been well established that for successful fertilization, sperm cells must activate the oocyte. Sperm trigger the oocyte repetitive intracellular rises in the Ca\(^{2+}\) after gamete fusion, which occur at regular intervals for several hours in the mouse (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992), the hamster (Igusa and Miyazaki, 1986; Miyazaki, 1990), cattle (Fissore et al., 1992; Sun et al., 1994), pig (Sun et al., 1992), and human (Taylor et al., 1992). It has been proposed that these oscillations of intracellular free calcium Ca\(^{2+}\) lead to oocyte activation, DNA synthesis and further embryonic development (Kline and Kline, 1992). It has been recently suggested that the activation factors originate from sperm cells. Swann (1990) has demonstrated that injection of sperm extract causes repetitive transients of free calcium Ca\(^{2+}\) in hamster oocytes. Kimura and Yanagimachi (1995) have concluded that these factors are formed during sperm maturation.

Mammalian oocytes can also be activated with exogenous stimuli (Steinhardt et al., 1974). Injecting Ca\(^{2+}\) directly into oocytes, electroporation in the presence of Ca\(^{2+}\), or addition of calcium ionophore A23187 all trigger exocytosis of cortical granules, completion of meiosis and early development in mouse, hamster and pig oocytes (Steinhardt et al., 1974; Fulton and Whittingham, 1978; Sun et al., 1992). In the present study, sham-injected goat oocytes were also activated when calcium ionophore A23187 was added to the culture medium for a short interval (10 minutes). Results from the present study also indicate that a minimum level of ionophore A23187 is required to activate sham-injected goat oocytes. The addition of 1 μM of ionophore A23187 started activation in a few goat oocytes (3%) while maximum cleavage was achieved on similar oocytes with 200 μM of ionophore A23187.

Calcium ionophore also has been successfully used to activate sperm-injected bovine oocytes. Goto et al. (1990) reported that intracytoplasmic sperm-injected
bovine oocytes could be activated with either 50 μM or 100 μM A23187. Furthermore, calcium ionophore A23187 activation of sperm-injected bovine oocytes did support full development to term and one live calf was obtained. Tesarik and Sousa (1995) have reported a 90% fertilization rates with sperm-injected human oocytes after activation with 10 μM A23187. These results indicate that various levels of A23187 can activate sperm-injected oocytes and allowing a normal fertilization process. It appears that once sperm are injected into the ooplasm, exogenous stimuli such as A23187 function differently from those actions on oocytes without the presence of sperm cells. Perhaps some factor(s) from sperm cells can synergize with exogenous stimuli such as A23187 to activate sperm-injected oocytes and to aid the normal fertilization process.

Kimura and Yanagimachi (1995) demonstrated that mouse oocytes injected with testicular and epididymal sperm cells did not need exogenous stimuli for oocyte activation, while spermatids or spermatocytes were unable to activate oocytes. Furthermore, oocytes containing injected spermatids or spermatocytes had to be activated artificially to support normal fertilization and full term development. These results demonstrate that sperm factors exist in mouse sperm cells and these factors are formed during sperm maturation, and that artificial stimulation can support full development of mouse oocytes injected with spermatids or spermatocytes.

Tesarik and Testart (1994) showed that treatment of sperm-injected human oocytes with A23187 supported the development of calcium oscillations. It is known that calcium oscillations are found in fertilized oocytes while parthenogenetic activation does not produce such oscillations (Cuthbertson et al., 1981). In the present study, 10 or 50 μM A23187 was used for 10 minutes to activate sperm-injected goat oocytes. Even though we could not distinguish normally fertilized oocytes from possibly parthenogenetic cleaved oocytes, the results show that a higher cleavage rate was achieved with oocytes injected with electroporated sperm cells than with non-
electroporated sperm cells or sham control treatment. This finding indicated that factors from sperm cells could be released more easily after electroporation, and that these factors could stimulate with calcium ionophore to activate oocytes. More blastocysts resulted from electroporated sperm cells than from nonelectroporated sperm cells (see Figure 6d), while sham-injected oocytes never developed beyond the 32-cell stage.

In the present study, the combination of electroporation of sperm cells with 50 μM A23187 activation of sperm-injected goat oocytes resulted a 37% cleavage rate. This further supports the view that factor(s) from sperm cells can act with exogenous stimuli such as A23187 to activate sperm-injected oocytes and perform fertilization and early embryo development. Whether these cleaved goat embryos could develop to full term remains to be determined. The best quality blastocysts derived from ICSI (n=6) were frozen stored in liquid nitrogen and will be used for embryo transfer at a late date. Seven blastocysts were transferred into 3 recipients and one pregnancy was obtained. This pregnancy was lost at 2 month of pregnancy from unknown causes.

It should be noted that goat ovaries used in this study had been transported via airline for up to 8 hours prior to oocyte aspiration in our laboratory. The goat oocyte maturation system resulted in only a 70% maturation rate (Han et al., 1996) compared with a 90% maturation rate for bovine oocytes in our laboratory (Zhang et al., 1992). Furthermore, we did not identify the first polar body before the ICSI procedure was performed, because the cumulus cells were only partially removed from the oocytes. Without complete removal of cumulus cells, it was very difficult to detect the first polar body. It should not be overlooked that the cumulus cells support maturation and early stage embryo development in farm animal embryos (Zhang et al., 1995).

Experience performing ICSI was definitely another factor that may have affected the results. At the beginning of this trial, it took 3 to 4 minutes to perform one ICSI procedure. This extended time interval may explain the higher oocyte
degeneration rate with injection of sperm cell than with sham-injection control procedure.

In this study, immotile goat sperm cells were used for intracytoplasmic sperm injection. Results indicate that goat sperm motility was not required for fertilization with the ICSI procedure. This result agrees with results from the human (Palermo et al., 1992) and the cow (Goto et al., 1990). In contrast, it has been reported that motile mouse sperm are required to fertilize the ova, while immotile sperm fail to fertilize when the sperm are injected under the zona (Kobayashi et al., 1992), suggesting that motile sperm function is required for sperm penetration of zona and fusion with ovum cytoplasm membrane. If motile mouse sperm cells were injected into the cytoplasm of the oocyte, it was detected that they kept moving within the ooplasm for as long as 20 minutes before they became motionless (Kimura and Yanagimachi, 1995). Results further indicated that continuous movement of sperm cells within the cytoplasm may disorganize the oocyte structural elements and/or disturb the wound healing of oolemma (Kimura and Yanagimachi, 1995).

In summary, a preliminary goat intracytoplasmic sperm injection procedure with immotile sperm cell was established in this study. Overall, 37% cleavage rate was achieved with sperm injected goat oocytes activated with 50 µM calcium ionophore A23187 for 10 minutes and with electroporation of sperm cells prior to microinjection. Furthermore, it was noted that only sperm-injected goat oocytes developed to the blastocyst stage in this study, while the sham-injected oocytes never developed beyond the 32-cell stage.
CHAPTER 8

SPERM-MEDIATED GENE TRANSFER VIA INTRACYTOPLASMIC SPERM INJECTION

Introduction

It has been shown that goat sperm cells can bind foreign DNA (Sun et al., 1996). In goats, 33 to 37% of sperm cells can bind foreign DNA after a short period of co-incubation of sperm cells with foreign DNA (Sun et al., 1996). However, after in vitro fertilization of goat oocytes with sperm cells co-incubated with foreign DNA, only 3% fertilized oocytes had incorporated foreign DNA (Chapter 5). Why 33 to 37% sperm cells having foreign DNA only produced 3% of the embryos expressing foreign DNA is not clear. One reason for this may be that those sperm cells with foreign DNA had a lesser ability to fertilize the oocyte than those sperm cells without foreign DNA. To test this hypothesis, individual sperm cells could be injected into goat oocytes to verify that 33% cleavage embryos could incorporate foreign DNA.

To increase the percentage of sperm cell binding of foreign DNA, electroporation was conducted and at 1,200 volts/cm and 100% of sperm cells bound foreign DNA. In this study, all sperm cells, however, were immobilized after electroporation (Sun et al., 1996). It has been shown that immotile sperm cells can fertilize oocytes once injected inside the ooplasm (Goto et al., 1990; Palermo et al., 1992). To further evaluate sperm-mediated gene transfer, it is proposed that intracytoplasmic sperm injection methodologies as a part of the experimental procedure.

In our previous study (Chapter 7), a preliminary goat ICSI procedure was established. Furthermore, results showed that when sperm cells were electroporated at 1,200 volts/cm more cleaved embryos were obtained. At this voltage, 100% of the goat sperm cells were found to bind foreign DNA. This finding suggested that this
voltage of electroporation could be used both to activate sperm cells and to enhance sperm cell binding of foreign DNA. Injection of these electroporated sperm cells into oocytes would likely increase the efficiency of sperm-mediated gene transfer, since all sperm cells bound foreign DNA after electroporation.

The objective of this study was to further evaluate sperm-mediated gene transfer using the intracytoplasmic sperm injection (ICSI) technique. A comparison between electroporated sperm cells co-incubated with foreign DNA and nonelectroporated sperm cells co-incubated with foreign DNA will be made to evaluate the electroporation effect on the efficiency of sperm-mediated gene transfer. Fluorescent in situ hybridization (FISH) will be used to verify integration of foreign DNA in goat embryos. Since the final goal is to produce transgenic offspring, the developmental competence of ICSI embryos was further evaluated by co-culture with a somatic monolayer.

Materials and Methods

Experimental Design

Experiment 8.1. Intracytoplasmic Injection of Oocytes with Sperm Treated with BC31 Plasmid

This experiment was conducted using the following treatments: 1) transgenic goat sperm cells were used as a control group, 2) foreign DNA (BC31) incubated with sperm cells without electroporation, 3) sperm cells co-incubated with foreign DNA (BC31), and electroporation applied to the sperm mixture and 4) sham-injected oocytes serving as an experimental procedure (injection) control. The outline of treatments for this experiment is shown in Table 19.
Table 19. Experimental design for Experiment 8.1

<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>No. oocytes injected$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic sperm (control)</td>
<td>60</td>
</tr>
<tr>
<td>Sperm + foreign DNA</td>
<td>60</td>
</tr>
<tr>
<td>Sperm + foreign DNA + electroporation</td>
<td>60</td>
</tr>
<tr>
<td>Sham control</td>
<td>30</td>
</tr>
</tbody>
</table>

$^a$Five replicates per treatment.
Foreign DNA

Plasmid BC31 was used in this experiment and was generously provided by Genzyme Transgenic Cooperation (Framingham, MA). The size of the plasmid is 24 kb and it has goat casein promoter fused with human antithrombin III structural gene.

Sperm Cell Preparation

French straws (0.25 ml each) of sperm cells were thawed for 1 minute at 37°C, Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975) was used to remove extender from frozen-thawed semen by washing twice and centrifuging at 600 x g for 10 minutes. Sperm cells were washed once with electroporation buffer that was composed of 260 mM sucrose, 5 mM HEPES (pH 7.3-7.4), 50 µg/ml polyvinyl alcohol (mw 125,000) and 5 mM CaCl₂·2H₂O (Tomkins and Houghton, 1988).

Co-incubation of Sperm Cells with Plasmid BC31

After washing, sperm concentration was adjusted to 1 x 10⁷ sperm/ml with electroporation buffer. The plasmids (BC31) were added to the sperm mixture to a final concentration 4 µg/ml and co-incubated for 1 hour at room temperature (22°C).

Induction of Acrosome Reaction

After sperm cells were co-incubated with plasmid BC31, calcium ionophore A23187 was added to the sperm mixture at a final concentration of 1 µM and sperm were incubated at 37°C for 30 minutes to induce an acrosome reaction (Watson et al., 1991).

Electroporation of Sperm Cells

After induction of the acrosome reaction, sperm cell concentration was adjusted to 1 x 10⁶ cells/ml in the electroporation buffer. In preparing for electroporation, a 0.4 ml sperm mixture was transferred into a 1.4 ml Gene Pulser Cuvette (Bio-Red Laboratories, Richmond, CA). The distance between electrodes was 4 mm. Sperm mixture was subjected to electroporation with a voltage of 1,200 volts/cm at a capacitance of 25 µFarads at room temperature using a Gene Pulser.
Apparatus (Bio-Red Laboratories, Richmond, CA). Sperm suspension was mixed (1:10) with 10% polyvinpyrrolidone in phosphate-buffered saline (PBS) prior to injection.

Semen from a transgenic buck (with transgene BC31) was generously provided by Genzyme Transgenic Cooperation. The procedure used to treat transgenic sperm cells was the same as the above procedure without adding foreign DNA (BC31).

**Oocyte Collection and Maturation**

The ovaries used in the experiments were collected from Spanish-type crossbred goats at an slaughterhouse in San Angelo, Texas. Within 12 hours after collection, the ovaries were transported via air in a container containing 0.9% saline solution (room temperature) supplemented with 200 units/ml penicillin and 200 μg streptomycin/ml and transported to the St. Gabriel Laboratory. The ovaries were then washed twice with PBS and oocytes were aspirated using an 18-gauge needle attached to a 3-ml sterile syringe from follicles with diameters between 2 and 7 mm. Oocytes were collected and washed twice in PBS containing 0.1% (g/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes were graded based on the conditions of the surrounding cumulus cells and the consistency of ooplasm. Only first and second grade oocytes with intact, compact cumulus cells and homogenous ooplasm were randomly allotted to the experiments.

The maturation medium was TCM-199 (GIBCO BRL, Cat. No. 12340-014) with 10% heat-inactivated fetal bovine serum (Hycolone, Cat. No. A-1114-D) (Han et al., 1996). The oocytes were washed in the maturation medium three times and incubated in groups of 20 to 30 oocytes in a 500 μl maturation medium drop in each individual well of 4-well tissue culture plates (Nunc®, France) at 39°C in an atmosphere of 5% CO₂ in humidified air for 27 hours.
Preparation of Oocytes for Injection

Matured cumulus-oocyte complexes were treated with hyaluronidase (1 mg/ml in TCM-199) for up to 1 minute and the cumulus cells surrounding the oocytes were partially removed by pipetting. The oocytes were then washed in PBS supplemented with 10% FBS. The injection window was set for 27 to 33 hours after starting maturation of oocytes.

Preparation of Oocyte-Holding and Sperm-Injection Pipettes

Glass capillary tubes were pulled with a puller to make holding and injection pipettes. Injection pipettes were further processed on a microgrinder and a microforge. The outer and inner diameters of the pipettes were 100 and 20 µm for holding pipettes and 10 and 6 µm for injection pipettes. The injection pipettes had a bevel angle of 50 degrees.

Intracytoplasmic Sperm Injection

A heated stage of an inverted microscope (Nikon UK Ltd., Surrey, United Kingdom) was used to conduct the intracytoplasmic sperm injection procedure. Both injection and holding pipettes were mounted to micromanipulators. Cell culture chamber slides (Nunc®, France) were used as an injection plate. One elongated droplet was made of PBS supplemented with 10% heat-inactivated FBS for holding the oocytes, oocytes were placed at one end of the droplet (6 to 10 oocytes / droplet. Another droplet was made of 2 µl of sperm PVP mixture and placed next to the oocyte drop. These two drops were covered with mineral oil to prevent evaporation during the injection procedure.

First, an immotile sperm cell was aspirated with tail first into the injection pipette, the injection pipette was raised and the stage was moved to locate the oocyte droplet. Suction was applied to hold one oocyte onto the holding pipette, the injection pipette was then pushed through the zona pellucida and the oolemma at the opposite pole to the holding pipette. A single sperm was injected into the cytoplasm of the
oocyte. Injection was performed under 800X magnification to observe the sperm tail pass through the oocyte plasma membrane.

Injected oocytes were transferred to the other end of the oocyte droplet to avoid repeated injection of the same oocytes. The same procedure was performed for sham-injection without injection of the sperm cell. Once all oocytes were injected, oocytes were washed in PBS and activated in calcium ionophore A23187 at a final concentration 50 μM. After activation for 10 minutes, oocytes were washed in PBS supplemented with 0.3% BSA to stop the action of A23187 (Goto et al., 1990). Oocytes were then cultured in BRL monolayer for further development and observation.

In Vitro Co-culture Early Stage ICSI Embryos

The cleaved oocytes were co-cultured with a monolayer of BRL cells. These cells were obtained from a commercial source (BRL 3A, American Type Culture Collection, Rockville, MD). Embryos were co-cultured in vitro with BRL cells for up to 10 days. During the period of co-culture, the culture medium was replaced with fresh TCM-199 plus 10% inactivated FBS at 48-hour intervals.

Embryo Biopsy

After 10 days of co-culture, randomly selected embryos were evaluated for foreign DNA integration. The zona was removed by digestion with 0.5% pronase in TCM-199 for approximately 2 minutes while under continuous observation under the stereomicroscope. As soon as the zona was dissolved, the embryos were transferred into calcium-free, magnesium-free PBS and incubated the embryos for 15 minutes. The individual cells of the embryo were disaggregated by pipetting through a flame-polished glass pipette. Five to 10 embryonic cells were used for foreign DNA evaluation.
Detection of Foreign DNA with Fluorescent In Situ Hybridization (FISH)

The blastomeres were hybridized with a digoxigenin-labelled goat genomic probe as an internal control, as well as, a biotin labelled probe recognizing the human AT III transgene (BC31). Hybridization was then visualized with Cy3-Streptavidin and FITC-anti digoxigenin using DAPI/antifade as a cell locator (Lewis-Williams et al., 1996).

Statistical Analysis

The criteria for evaluation included the number of oocytes that degenerated and the number that cleaved across treatment groups. The results were analyzed with SAS ANOVA (SAS institute, Gary, NC). A P-value of <0.05 was set as statistically significant for this experiment. The differences among individual treatments were assayed by developing 95% confidence intervals.

Results

The development of cleaved goat embryos from sperm-injected oocytes is shown in Table 20. Overall, a 41% cleavage rate was achieved with sperm-injected oocytes in this experiment. Similarly, the sham-injected controls had a 40% cleavage rate. There was no significant difference between treatment groups and control group. When cleaved embryos were further cultured to evaluate developmental competence, there was not a significant difference between sperm-injected treatment groups and sham-injected control at the 8- to 16-cell stage. While ~28% of sperm-injected oocytes developed to morula stage, no sham-injected oocyte developed into the morula stage. Furthermore, 3.3 to 16.7% of sperm-injected treatment groups developed to the blastocyst stage during 10 days co-culture. At the blastocyst stage, significantly (P<0.05) more blastocysts resulted from electroporated sperm cells than from nonelectroporated DNA-treated sperm cells.
Table 20. Intracytoplasmic injection of caprine oocytes with sperm cells co-incubated with foreign DNA

<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>No. of oocytes injected&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Embryo development during in vitro co-culture&lt;sup&gt;b&lt;/sup&gt;</th>
<th>2-4 cell</th>
<th>8-16 cell</th>
<th>Morula</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic sperm</td>
<td>60</td>
<td>29(48.3%)</td>
<td>28(46.7%)</td>
<td>17(28.3%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4(6.7%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Sperm + DNA</td>
<td>60</td>
<td>19(31.7%)</td>
<td>18(30.0%)</td>
<td>12(20.0%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2(3.3%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Sperm + DNA + electroporation</td>
<td>60</td>
<td>28(46.7%)</td>
<td>28(46.7%)</td>
<td>21(35.0%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10(16.7%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Sham control</td>
<td>30</td>
<td>12(40.0%)</td>
<td>9(30.0%)</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Five replicates per treatment.

<sup>b</sup> Co-cultured with Buffalo Rat Liver cell monolayer for 10 days.

<sup>cd</sup> Different superscripts within a column are significantly different (P<0.05).
Table 21. Detection of transgenes in caprine embryos derived from intracytoplasmic injection of oocytes with sperm cells co-incubated with foreign DNA by fluorescent in situ hybridization

<table>
<thead>
<tr>
<th>Sperm treatment</th>
<th>Embryos biopsied</th>
<th>Embryo responding to FISH</th>
<th>FISH positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic sperm (control)</td>
<td>14</td>
<td>7 (50%)</td>
<td>0</td>
</tr>
<tr>
<td>Sperm + foreign DNA</td>
<td>9</td>
<td>7 (77.8%)</td>
<td>1(^a)(14%)</td>
</tr>
<tr>
<td>Sperm + foreign DNA + electroporation</td>
<td>10</td>
<td>8 (80.0%)</td>
<td>2(^b)(25%)</td>
</tr>
</tbody>
</table>

\(^{a}\)Diffuse signal detected for transgene but not integrated.

\(^{b}\)Multi-copy signal for transgene but only in some of biopsied cells from each embryo.
Figure 7. Detection of foreign DNA in embryos with fluorescent in situ hybridization. (a) the green color shows total goat DNA in one cell staining with digoxigenin, (b) the red color shows transgene with biotin labelled probe, indicating multi-copy foreign DNA was integrated into goat genomic DNA, (c) the blue is a DAPI/antifade counterstain.
The results of fluorescent in situ hybridization are presented in Table 21. In the control group, a total 14 embryos were biopsied, however, only 7 of these embryos responded to the FISH procedure. Unfortunately no positives were found. When sperm cells were co-incubated with BC31 in the nonelectroporation group, 9 embryos were biopsied and 7 embryos responded to the FISH procedure. One of the embryos (14%) showed a diffused signal of foreign DNA BC31 in some cells, which indicated that foreign DNA was not integrated into the genomic DNA.

In electroporation group, 10 embryos were biopsied and 8 of these embryos responded to the FISH procedure. Two of 8 embryos (25%) showed a very strong spot of signal for foreign DNA BC31, which indicated that multicopy foreign DNA was integrated into goat genomic DNA (see figure 7b). Results of that procedure also indicated that these two embryos were mosaic transgenic embryos.

Discussion

Intracytoplasmic sperm injection of goat oocytes was evaluated in this experiment. Unexpectedly, when calcium ionophore A23187 was used to activate the oocyte, regardless of whether a sperm cell was present inside the oocyte after activation, oocytes could divide and go through early cleavage stages. Results show that parthenogenetic activated oocytes can develop only for a relatively short period (in this study to the 8- to 16-cell stage). Furthermore, once the sperm cell was present inside the goat oocyte, the cleaved embryos developed further than parthenogenetic oocytes. These results further verify that sperm factors are likely involved in later development of cleaved embryos in goat (Chapter 7). The results of this study show that more blastocysts were obtained from injection of electroporated sperm cells than nonelectroporated sperm cells. This further supports the idea that sperm factors are required to support embryo development. The results of these experiments also show
that electroporation could enhance factors release from sperm cells after the sperm were injected into the oocyte.

In our previous study (Chapter 5), 3% of cleaved IVF-derived goat embryos were able to express foreign DNA. Whether foreign DNA had integrated into the genomic DNA was not verified. Current results indicate that foreign DNA can integrate into goat genomic DNA with sperm-mediated gene transfer. Furthermore, with electroporation of sperm cells after co-incubation with foreign DNA, 25% integration efficiency was achieved in this study.

Others have reported that up to 22% bovine embryos had foreign DNA when oocytes were inseminated with electroporated sperm cells co-incubated with foreign DNA (Gagné et al., 1991). However, PCR as the procedure was used to detect foreign DNA in the embryos in that study. PCR was not able to distinguish truly integrated foreign DNA from free copies in the embryos and resulted in a very high false positive error rate (Ninomiya et al., 1989; Lo et al., 1989).

In the present study, FISH technique was considered to be a more accurate procedure detecting foreign DNA in goat embryos. The FISH technique had an additional advantage over PCR or marker gene expression (such as X-Gal stain) in detection of transgenic embryos. The latter two could not distinguish integrated foreign DNA from unintegrated foreign DNA while FISH can distinguish truly integrated foreign DNA from unintegrated residual DNA. In this study that was 25% integration efficiency was without any false positive readings.

Integration efficiency may be affected by the transgene structure (Brinster et al., 1985). It has been shown that linear DNA integrates more easily than circular DNA, and large DNA is less likely to be integrated than a small fragment (Brinster et al., 1985). In our study, a circular plasmid was used, and the size of this plasmid was very large ~24 kb. These two factors must be considered when results are discussed.
and comparisons are made with other results which linear and smaller size foreign DNA was used for interpretation of the efficiency of integration.

Palmiter et al. (1986) have suggest that DNA integration is likely the result of damage to the chromosomes during injection and then the gene incorporates into one of these sites during DNA repair. It has also been suggested that transgene integration happens during DNA replication (Bishop and Smith, 1989). In mice, the frequency of integration of the transgene is affected by factors such as transgene buffer composition, conformation of the transgene and concentration of transgene used for microinjection (Brinster et al., 1985). Pronuclear injection can generate a hemizygous embryo or a mosaic embryo with respect to the transgene, depending on when the injected transgene integrates into the genomic DNA (Wilkie et al., 1986). Hemizygous transgenic animals will be produced if the integration event occurs before the first round of DNA replication after pronuclear microinjection. Correspondingly, mosaic embryos will be produced if the integration event occurs after this period.

In one study, ~62% of transgenic mouse embryos produced by microinjection were mosaic (Bruce et al., 1993). In other studies, ~30% of transgenic mice produced by microinjection were mosaic (Wilkie et al., 1986; Palmiter and Brinster, 1986). In another report, none of the 8-cell mouse embryos had transgenes in more than 4 blastomeres (Burdon and Wall, 1992). Other results showed that as mouse embryos developed, the incidence of mosaicism significantly increased from post microinjection at 21 hours 34.9 to 60.7% at 67 to 69 hours post microinjection (Lewis-Williams et al., 1996).

Pursel et al. (1990) reported that 20% of founder transgenic pigs and sheep fail to transmit the gene to progeny and another 20 to 30% transmit the transgene to less than 50%. It was proposed that this is due to mosaicism in the germ cell line. In our study, mosaicism was also detected in goat embryos. It indicated that even though
foreign DNA had bound to sperm cells, integration still happened after the first round of DNA replication of the fertilized ovum.

In summary, with sperm-mediated gene transfer foreign DNA could be integrated into goat genomic DNA at an early stage of embryonic development. With injection of electroporated sperm cells co-incubated with foreign DNA, a 25% integration efficiency was achieved with sperm-mediated gene transfer in the goat.
CHAPTER 9

CONCLUSION

Transgenic animal biotechnology has developed slowly over the last 15 years. Now genes can be designed and constructed in the laboratory and subsequently these transgenes can be transferred into genomic DNA of laboratory and farm animals. However, the major gene transfer method (pronuclear microinjection) is still problematic for large farm animals. An alternative gene transfer method, such as sperm-mediated gene transfer, is now being developed. Unfortunately, a lot of factors affecting this method are still not clear. To evaluate factors affecting the success of sperm-mediated gene transfer, a series of experiments were conducted over the last 3 years at this laboratory.

The goat sperm cell was used as a model in these experiments. First, experiments were conducted to evaluate whether both fresh and frozen-thawed goat sperm cells could bind foreign DNA. Results showed that both fresh and frozen-thawed sperm cells could, to some degree, bind foreign DNA. The binding was very stable after a series of washings. After 10 washings, it was noted that foreign DNA was still bound to the fresh and frozen goat sperm cells. There appears to be factors in the extender of frozen-thawed semen, that negatively affecting sperm cell binding of foreign DNA. Also two foreign DNA binding patterns were detected on goat sperm cells. One pattern was restricted to only the posterior head of sperm cells and the other binding pattern was located at both anterior head and posterior head positions of sperm cells. Longitudinal sections of sperm cells with confocal microscopy showed that some foreign DNA molecules had penetrated into goat sperm nuclei. However, not every sperm cell was found to bind foreign DNA, with only 37% of fresh goat sperm and 33% of frozen-thawed goat sperm cells did bind foreign DNA in the present study.
In vitro fertilization experiments were conducted to evaluate whether goat sperm cells could transfer foreign DNA into goat oocytes during fertilization. Oocytes were harvested from abattoir ovaries and were matured in hormone-free medium for 27 hours. Fresh or frozen-thawed sperm cells were washed, co-incubated with foreign DNA (pCMVβ) plasmid for 30 minutes, and treated with different levels of calcium ionophore A23187 for sperm maturation. After ionophore treatments, sperm cells were used to inseminate matured oocytes in vitro. Cleaved oocytes were further co-cultured on bovine oviduct epithelium cell monolayers for 9 days. Embryos were stained with X-Gal for identifying the expression of foreign DNA pCMVβ in each individual embryo. Results showed that goat sperm cells could transfer foreign DNA during fertilization. A total of 3% of the cleaved embryos expressed the foreign DNA. Results also indicated that treatment of sperm cells with lower levels of calcium ionophore A23187 (20 to 40 nM) resulted in a higher fertilization rate and a higher ratio of blastocyst formation for frozen-thawed semen.

To improve the efficiency of sperm-mediated gene transfer, electroporation was applied to sperm cells to increase goat sperm cell binding of foreign DNA. At a voltage of 1,200 volts/cm, 100% of the goat sperm cells bound foreign DNA with electroporation. The electroporation voltage plays a crucial role on sperm cell binding of foreign DNA. However, sperm cells were not motile after electroporation in this study.

Further studies verified that the sperm plasma membrane plays a very important role in sperm cell binding of foreign DNA. After deep freezing in liquid nitrogen or heating sperm cells to 65°C for 30 minutes, all sperm cells were able to bind foreign DNA. However, after sperm cells membrane were permeabilized with lysolecithin (allowing large molecules pass through the membrane), sperm cells lost their ability to retain foreign DNA. In this study, it was also found that even though
sperm genome was not activated, some enzymes in sperm cells could degrade foreign DNA.

Even though 100% of the sperm cells could bind foreign DNA after electroporation, unfortunately, all sperm cells were immotile after the procedure. To further evaluate the potential of sperm-mediated gene transfer, intracytoplasmic injection of goat oocytes with immotile sperm was evaluated in a subsequent experiment. Results showed that goat oocytes need exogenous stimuli, such as calcium ionophore A23187 for oocyte activation. Further observations supported the concept that there were male factors worked synergistically with calcium ionophore to activate oocytes and subsequently support early caprine embryo development to the blastocyst stage. Electroporation not only enhances sperm cell binding of foreign DNA, but also appears to enhance the release of male factors to support embryo development. A low percentage of sperm-injected oocytes developed to the blastocyst stage (7 to 17%) while sham-injected oocytes only developed to the 16- to 32- cells stage.

Sperm-mediated gene transfer was further evaluated with the established goat intracytoplasmic sperm injection methodology. Goat sperm cells were first co-incubated with foreign DNA (BC31), then sperm cells subjected to electroporation. After treatment, individual sperm cells were randomly selected and injected into the cytoplasm of in vitro matured goat oocytes. After 9 days, individual embryos were biopsied and individual cells fixed and hybridized with probes for BC31 with FISH procedures.

Results showed that, without electroporation of goat sperm cells, only 14% of the cleaved embryos contained foreign DNA, while 25% cleaved embryos contained foreign DNA with electroporation. FISH results also indicated that foreign DNA could be integrated into the goat genomic DNA with the sperm-mediated gene transfer method. Unexpectedly, mosaic embryonic cells resulted in the embryos with this
approach. Results of these experiments, also showed that electroporation of sperm cells not only increased the efficiency of sperm cell binding of foreign DNA, but also appears to have enhanced the development of the cleaved ICSI embryos to blastocyst stage.

In summary, this study demonstrated that goat sperm cells can bind foreign DNA, and can transport foreign DNA into oocytes during fertilization. Foreign DNA can be integrated into the genomic chromosome at an early stage of embryonic development. In these experiments, foreign DNA size (ranged from 7 to 24 kb), which is likely beyond the limitation of retrovirus mediated gene transfer.

Sperm-mediated gene transfer eliminated the need for localization of the pronucleus, a requirement of the pronuclear microinjection gene transfer method. Stem cells technology is an ideal gene transfer method but this method is not yet available for large farm animals. However, sperm-mediated gene transfer offers an alternative method, and this method needs to be studied further to improve the efficiency of transgenic animal production.


Fraser, L. R. 1982. Ca\textsuperscript{2+} is required for mouse sperm capacitation and fertilization in vitro. J. Androl. 3:412-419.


Sci. 28:269-276.

mammalian embryos. In: M. M. Seidel, J. Bernstein, A. A Keisling and S.
Levin (Eds.) Infertility in the 1990's Technical Advances and Their

transformation of genes injected into mouse pronuclei. Science 214:1244-
1246.

Genetic transformation of mouse embryos by microinjection of purified DNA.

Pregnancies after co-culture of cumulus cells with bovine embryos derived
from in-vitro fertilization of in-vitro matured follicular oocytes. J. Reprod.
Fertil. 83:753-758.

Goto, K., Y. Kinoshita, and K. Ogawa. 1990. Fertilization of bovine oocytes by the

Graham, C. F. 1966. The regulation of DNA synthesis and mitosis in multinucleate

Graham, J. K., R. H. Foot and J. J. Parrish. 1986. Effect of dilauroyl-
phosphatidylcholine on the acrosome reaction and subsequent penetration of
bull spermatozoa into zona-free hamster oocytes. Biol. Reprod. 35:413
(Abstr.).

of sperm DNA: Implications for activation of the paternal genome. Science
228:1061-1068.

Gundersen, G. G., L. Medill and B. M. Shapiro. 1986. Sperm surface proteins are
incorporated into the egg membrane and cytoplasm after fertilization. Dev.
Biol. 113:207-217.

Gwatkin, R. B. L. 1977. Fertilization Mechanism in Man and Mammals. New York:

Hammer, R. E., V. G. Pursel, C. E. Rexroad, Jr., R. J. Wall, D. J. Bolt, K. M. Ebert, R.


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