FACTORS AFFECTING THE EFFICIENCY OF GENE TRANSFER IN MICE

by

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(Abstract)

In order to optimize the overall efficiency of pronuclear microinjection, we designed experiments to: 1) test the best developmental stage for transferring injected embryos to obtain pregnancies and transgenic pups; 2) determine the optimum number of injected embryos transferred to obtain pregnancies and transgenic pups; 3) investigate whether addition of non-injected embryos with injected embryos increased pregnancy rate (PR) and transgenic pups; and 4) establish the time during pregnancy of highest embryonic or fetal loss. Mice (CD1; 3 to 4 wk old),were superovulated with 10 iu PMSG and 5 iu hCG 48 h apart. One-cell embryos were collected for microinjection 20 to 24 h after hCG. The gene used was the whey acidic protein promoter linked to a coding sequence of the human protein C gene (WAP-hPC). Embryos were cultured in CZB at 37°C in 5% CO_2 in air. All the live pups born and embryos and fetuses recovered were analyzed by the polymerase chain reaction to detect the presence of the transgene. Experiment one consisted of nine transfer treatments (TRT) which included all the combinations of three developmental stages (1-cell, 2-4 cell and morula/blastocyst) with three quantities of embryos per transfer (15-24, 25-34 and 35-44). Ten transfers were performed for each TRT. The highest PR and total pups born (TOTP) were obtained after transferring 25 to 34 2-4 cell embryos (PR=90% TOTP=3.5/ pregnancy). However, overall analysis indicated that the percentage of transgenic pups born (%TRS) was highest using 1-cell embryos [33.9%, 20.0% and 11.1% for 1-cell, 2-4 cell and morula/blastocyst (mor/bl), respectively]. The second experiment consisted of six transfer TRT: 20-0, 16-4, 12-8, 30-0, 26-4 and 22-8 injected - non-injected embryos, respectively (10 transfers /TRT). Data showed that PR and TOTP can be improved by addition of non-injected embryos. However, the percentage of transgenic pups was significantly ($p < .05$) higher when all the embryos transferred were injected (53.6 % vs 46.4 % for transfers without and with non-manipulated embryos, respectively). Additionally, 30 embryos per transfer yielded a significantly higher percentage ($p < .05$) of transgenic pups than 20 embryos per transfer (67.9 % vs 32.1 % for 30 and 20 embryos per transfer, respectively). In experiment three,

45 transfers of microinjected embryos were performed (30 embryos per transfer). Fifteen recipients were sacrificed on day 4, 12 and 18 of gestation. On each day all embryos and fetuses were counted and analyzed for the presence of the transgene. The percentage of transgenic embryos or fetuses was not statistically different at any recovery day (45.8%, 35.5% and 34.6% for days 4, 12 and 18, respectively). However, the number of viable embryos at day 4 was significantly greater than the number of viable fetuses on days 12 or 18 (10 ± 1.1, 5.1 ± 1.6, and 2.4 ± 1.3 for days 4, 12 and 18, respectively). Collectively, the results indicate that: 1) transfer of 20 to 30 1-cell embryos was the best method to obtain transgenic mice, 2) addition of non-injected embryos decreased the number of transgenic pups obtained per pregnancy, and 3) although most of the embryonic losses after microinjection happen before day 4 of gestation, additional losses occurred between days 4 and 18 of pregnancy.

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CHAPTER I

INTRODUCTION

Recombinant DNA technology has made it possible to isolate and clone many of the genes of higher eukaryotes. Using the system of pronuclear microinjection, it has been demonstrated by many groups that transfected genes can be incorporated into the host genome and can be transmitted sexually through numerous generations. It also has been shown that transfected genes are expressed in many instances. This technology has been available since 1980 (Gordon et al.). Animals that have integrated a cloned gene into the genome are called transgenic.

Integration of genes into mice has become extraordinarily useful for studies of gene function, developmental biology, physiology and immunology. More recently, several research groups have been using transgenic animals as bioreactors to produce pharmaceuticals. In order to produce human proteins on a large scale the optimal system must: 1) be able to yield substantial quantities of the protein of interest, 2) the protein produced must be biologically active and be identical or nearly identical to its human counterpart, and 3) the cost of production must be relatively low in relation to the eventual cost of the protein. Transgenic animals seem to fill all these requirements. In fact, transgenic laboratory and farm animals which express pharmaceuticals in their milk have been produced (Clark et al., 1987 and 1989; Simons et al., 1987 and 1988; Wright et al., 1991; Ebert et al., 1991; Velander et al., 1992).

Several researchers have analyzed some of the factors associated with the efficiency of producing transgenic mice. They include: concentration and shape of the DNA injected, buffer composition, mouse strain (Brinster et al., 1985), size of the injection pipettes, timing of injection in relation to first cleavage (Walton et al., 1987), intensity of the microscope light, quality of the injection pipette and the gene that is injected (Voss et al., 1990). In spite of the amount of research currently being conducted with transgenic mice, some basic questions remain to be answered. For example, typically 20 to 30 embryos are transferred shortly after microinjection (Hogan et al., 1986). Other researchers prefer to wait from 24 to 96 hours before making the transfer. In addition, previous reports mention the use of non-manipulated embryos transferred

along with injected embryos to help maintain pregnancy. To date we have been unable to find a systematic study that analyzed all of these factors.

Microinjected embryos usually have low viability after transfer (Voss et al., 1990). Some investigators have reported different levels of pre- and post-implantation embryonic mortality (Burki and Ulrich, 1982; Brinster et al., 1983). However, whether this is caused by DNA integration or not remains to be elucidated.

The objectives of this study were: 1) to produce transgenic mice with the WAP-hPC construct; 2) to establish the optimum developmental stage of transfer to produce pregnancies with transgenic mice; 3) to establish the optimum number of embryos per transfer to produce pregnancies with transgenic mice; 4) to determine whether the addition of non-manipulated embryos improves the efficiency of gene transfer in mice; and 5) to investigate embryonic and fetal losses after transfer of microinjected embryos.

CHAPTER II
LITERATURE REVIEW

TRANSGENIC ORGANISMS

The ability to introduce foreign genes into the germ line of mammals is one of the major technological advances in recent years. By definition, transgenic organisms are those that have integrated foreign DNA into their germ line as a consequence of experimental introduction of DNA (Palmiter and Brinster, 1985). The first animals carrying experimentally introduced foreign genes were produced by microinjection of simian virus 40 (SV40) DNA into the blastocoel cavity of early embryos (Jaenisch and Mintz, 1974). However, integration of the viral DNA into the germ line was not demonstrated. Germ line transmission of foreign DNA was detected in subsequent studies when mouse embryos were exposed to infectious Moloney leukemia retrovirus (M-MuLv), which resulted in the generation of the first transgenic mouse strain (Jaenisch, 1976).

METHODS OF TRANSGENESIS

Currently, the most used methods to introduce genes into animals include pronuclear microinjection, retroviral infection and embryonic stem cells. Microinjection of cloned DNA directly into the pronucleus of a fertilized egg has been the most widely and successfully used method for generating transgenic mice. Using this method, the microinjected DNA usually integrates at one random site prior to cleavage, such that all cells of the embryo and extraembryonic tissues carry the foreign DNA. One disadvantage of this method is that it can not be used to introduce genes into cells at later developmental stages. Moreover, the cloning of the chromosomal insertion site may be difficult because of the multiple copy inserts and host sequence rearrangements (Jaenisch, 1988).

Retroviral infection involves exposure of preimplantation stage embryos to concentrated virus stocks (Jaenisch, 1976), or co-culture of embryos with monolayers of virus producing cells (Jaenisch, 1988). In contrast to microinjected DNA, retroviruses integrate by a very precise mechanism. Only a single proviral copy of

DNA is inserted at a given chromosomal site and no rearrangements of the host genome are induced apart from a short duplication of host sequences at the site of integration (Varmus, 1982). The main advantage of this method is the technical ease of introducing viruses into the embryos at various developmental stages. Furthermore, it has proved much easier to isolate the flanking host sequences of a proviral insert than those flanking a DNA insert derived from pronuclear microinjection. A big disadvantage of using retroviruses for gene transfer is the size limitation for transduced DNA.

Embryonic stem (ES) cells also have been used to produce transgenic animals. ES cells are typically established in vitro from explanted blastocysts. Then, genes are introduced into the ES by retrovirus-mediated transduction. Cell clones selected for the presence of foreign DNA are then injected into host blastocysts, where they colonize the embryo and contribute to the germ line of the resulting chimeric animal (Jaenisch, 1988).

ANIMAL MODELS

Research applications for transgenic animals are numerous, they include tissue specific control of gene expression, study of the immune system, genetic diseases, oncogenes (Camper, 1987), and production of human therapeutic proteins (Clark et al., 1987).
Some mammalian species used as models to create transgenics include: the mouse, pig, sheep, goat and cow.

Transgenic Mice

The mouse is the most common animal model for transgenics. Many different genes have been tested in mice with varying degrees of success. A few examples follow.

Growth Hormone - In a classic paper, Palmiter et al. (1982) reported the dramatic growth of mice that develop from one-cell microinjected embryos with the mouse metallothionein - I (mMt) gene fused to the structural gene of rat growth hormone (rGH). Furthermore, they found that several of these transgenic mice had high levels of the fusion mRNA in their liver and growth hormone in their serum.

Human growth hormone (hGH) gene also has been inserted into fertilized one-cell mouse embryos. Brem et al. (1989) created transgenic mice carrying a mouse mMt-hGH fusion gene. Although these mice had significantly higher weights than control mice, they also had reduced fertility, shortened life-span and pathological lesions in kidney, liver and heart. In spite of the adverse effects of the growth hormone gene, transgenic animals are still considered a valuable tool to study gene expression. For example, Chen and coworkers (1990) investigated the expression of a mutated bovine growth hormone (bGH) gene in transgenic mice. They altered the third alpha-helix of bGH by changing glutamate 117 to leucine, glycine 119 to arginine and alanine 122 to aspartate. Transgenic mice carrying this construct showed a significant growth suppressed phenotype that was related to serum levels of the altered bGH molecule.

Immunology - This is an area in which transgenic mice technology has been widely used. Early work by Brinster et al. (1983) involved the production of transgenic mice by pronuclear microinjection into fertilized one-cell mouse embryos of a rearranged functional immunoglobulin kappa gene. After mating the resulting transgenic mice, they observed expression of the transgene in the spleen but not in the liver of mice which inherited this gene. They concluded that the immunoglobulin kappa gene was a good example of a natural gene that is expressed in a highly tissue-specific manner apparently retaining this tissue specificity in transgenic mice.

Oncogenes - Another use for transgenic mice is in cancer research. The reports of transgenic mice carrying tumors are numerous (Adams et al., 1982; Hanahan 1985; Quaife et al., 1987; Stewart et al., 1984; Suda et al., 1987). More recently, malignant tumors have been found in transgenic embryos. Katsuki et al. (1989) attempted to create transgenic mice carrying activated human c-Ha-ras genes. They were unable to obtain any transgenic pups which developed to term because all transgenic embryos were malformed, became developmentally arrested conceptuses or developed embryonic tumors during ontogenesis.

Transgenic Domestic Animals

One of the first reports on transgenic farm animals was that of Hammer et al. (1985). These investigators microinjected the fusion gene mMt-hGH into one-cell embryos and were able to obtain integration in rabbit sheep and pigs. However, expression of the transgene was only observed in rabbits and pigs. More recently, Pursel et al. (1990) reported that expression of mMt-bGH transgene in pigs caused a marked repartitioning of nutrients from subcutaneous fat into other carcass components, including skin, bone and certain organs. However, the persistent excess of bGH in transgenic pigs was detrimental to general health since it caused lameness, lethargy, gastric ulcers and anestrus in transgenic females. Their results suggested that a controlled expression of this gene had to be achieved before it could be used on a commercial level.

Nancarrow et al. (1988) investigated the controlled production of sheep growth hormone (sGH) in transgenic sheep. They microinjected the ovine metallothionein-growth hormone (oMt-sGH) fusion gene into one-cell embryos. Of twenty live lambs born three were shown to have integrated this gene. Moreover, these three lambs had a plasma growth hormone level three times greater than control lambs. However, growth rates of the three expressing lambs were in the normal range.

Similar results were obtained by Murray et al. (1989). These researchers produced seven transgenic merino sheep with two different oMt-sGH fusion genes. Four transgenic sheep that contained the gene oMt-sGH5 did not express the transgene. In contrast, three sheep carrying the oMt-sGH9 gene expressed the gene at high levels in a variety of tissues and had elevated blood levels of ovine growth hormone. However, the growth levels of these three lambs did not appear to be increased by the high levels of growth hormone. They concluded that the sheep model was inappropriate for the study of these genes, and that different fusion genes may be needed to obtain a productive response.

In addition, Wright et al. (1991) were able to obtain high level expression of active human α-1-antitrypsin in the milk of transgenic sheep. These researchers used the fusion of the ovine b-lactoglobulin gene promoter to the human α-1- antitrypsin to generate five transgenic sheep. Analysis for expression of this protein in the milk of

three females showed that all expressed at levels greater than 1 g/l. Furthermore, in one case, initial levels of expression exceeded 60 g/l, and stabilized at 35 g/l as lactation progressed.

The first report on successful production of transgenic goats that expressed a heterologous protein in their milk was that of Ebert et al. (1991). They obtained 29 goats of which two were transgenic for the whey acidic protein promoter linked to the cDNA of a modified version of human tissue plasminogen activator (WAP-LAtPA). The first lactating transgenic female produced ezymatically active LAtPA at a concentration of 3 mg/ml.

Kraemer et al. (1985) microinjected herpes simplex virus thymidine kinase (HSV-TK) genes into the male pronuclei of eighty five one-cell bovine embryos. Forty one of these embryos were transferred to the oviducts of 11 recipients. Two pregnancies were obtained that produced three male calves. However, data on integration rate was not reported. This was one of the first studies to show that bovine one-cell embryos can survive pronuclear microinjection and sustain pregnancy to term.

In 1987, Bieary et al. were able to produced transgenic 60-day old bovine fetuses carrying a construct of a chloramphenicol acetyl transferase gene fused to a Rous sarcoma virus promoter (RSVCAT). They reported that although integration efficiency was low (.83 to 1.67%), the RSVCAT gene can be successfully incorporated and expressed in the bovine genome.

Roschlau et al. (1989) found that gene integration in 14-day old bovine embryos was between .8 and 6.9% depending on the gene used. In this study they used bovine papilloma virus DNA, the alcohol dehydrogenase gene of Drosophila melanogaster, and the human or the bovine growth hormone genes. After transfer of 43 embryos into 23 recipients they obtained one transgenic calf that carried the bGH gene. More recently, McEvoy and Sreenan (1990) investigated the efficiency of production, centrifugation, microinjection and transfer of one- and two-cell bovine embryos in a gene transfer program. Three gene constructs were used. All three constructs contained a promoter region for the mouse metallothionein gene. The pMt-rGH construct had the structural gene for rat growth hormone fused to this promoter. The pMt-rGH-BE was similar but

also contained enhancer sequences from viral DNA. The pMt-Bgal construct contained the structural gene for beta-galactosidase, isolated from bacteria. Pronuclei were visible in 81 of 119 (68%) centrifuged one-cell embryos and nuclei were visible in 112 of 140 (80%) two-cell embryos. Of 152 microinjected embryos, 31 (20%) were lysed after microinjection. After transfer of 66 embryos into 26 recipients, 14 (54%) recipients were pregnant at day 55 with 26 (39%) viable fetuses. Eleven (42%) of these recipients delivered 21 (32%) calves. However, none of these were transgenic. This is an example of how costly and time consuming gene transfer can be in farm animals.

More recently, Krimpenfort et al. (1991) combined gene transfer with in vitro embryo production technology to produce transgenic cattle. Using this approach, 19 calves were produced of which 2 were transgenic for the bovine casein-human lactoferrin gene. Southern blot analysis indicated that the transgene sequences were present in blood ,ear tissue and placenta from one calf (male), whereas the other calf (female) was mosaic since only placental tissue was transgenic.

MEDICALLY IMPORTANT PROTEINS

In recent years, several genes encoding medically important proteins have been cloned including insulin, plasminogen activator, α-1-antitrypsin, coagulation factors VIII and IX, and human protein C (hPC). Purification of these proteins from blood and tissue is an expensive and time consuming process and carries the risk of transmitting infectious diseases such as the Human Immuno Defficiency Virus. Therefore, another source to obtain these proteins would be very valuable. The expression of genes encoding for these proteins in microbial hosts very often proves unsatisfactory because the foreign proteins are not correctly processed. Additionally, expression of these proteins in mammary tissue culture, though a viable strategy, is both expensive and technically demanding.

An alternative approach has been the utilization of transgenic animals as bioreactors. By combining regulatory elements from one gene with the coding sequence of another it is possible to direct the synthesis of a protein to a particular body tissue in a transgenic animal (Clark et al., 1987).

Pancreas Expression

Bucchini et al. (1989) were able to obtain transgenic mice which specifically expressed the human insulin gene in the pancreatic beta cells. Furthermore, by fusing the human insulin gene to a SV40 gene (oncogene), they were able to create transgenic mice which developed tumors of the pancreas. These investigators suggested that the possibility of directing expression of any gene to β cells of transgenic mice allows the creation of animal models to study various pathologies.

Liver Expression

The specific expression of transgenes in the liver of transgenic animals has also been the topic of several reports. In 1989, Iwanaga et al. investigated the liver specific and high level expression of human serum amyloid P component in transgenic mice. Serum amyloid P component (SAP) is synthesized by hepatocytes of various mammalian species and is one of the major acute phase reactants that are induced by acute inflammation. Seven transgenic mice were produced carrying a 3.3 kb fragment of the human SAP gene that was expressed only in the liver. Thus, they were able to study the mechanism of SAP gene expression and the role of SAP in the development of various amyloidosis.

Another gene expressed in the liver of transgenic mice is the human protein C gene (hPC). Vitale et al.(1990) used the liver specific promoter/enhancer for mouse albumin gene fused to the cDNA for hPC to create transgenic mice. Human protein C is a therapeutic of clinical significance for the prevention of blood clots. All their lines of transgenic mice had human protein C in their blood. Furthermore, the secreted protein C, when analyzed by Western blotting, co-migrated with native hPC indicating that the protein was probably properly made.

Mammary Gland Expression

The production of human proteins in transgenic animals has been directed to body fluids rather than solid tissues because first, fluids are to some extent renewable, and second, most proteins of biomedical importance are in fact secreted into body fluids.

As mentioned above, secretion of proteins into the bloodstream is possible by targeting expression to liver, kidney, or circulating lymphocytes. Nevertheless, the volume of blood from each producing animal is limited and continuous collection may be prejudicial to health. Furthermore, high circulating levels of biologically active proteins may prove detrimental to the animal. On the other hand, biosynthesis of the protein in the mammary gland and secretion into the milk is a promising alternative. Milk is more readily collected and in larger quantities than blood.

The mammary gland, appears as an attractive model to direct expression of foreign proteins, and collect its secretion in the milk. Promoter sequences for milk proteins used for this purpose include sheep beta-lactoglobulin in mice (Simons et al., 1987), and sheep (Simons et al., 1988; Clark et al., 1989), murine whey acidic protein (WAP) (Gordon et al., 1987; Pittius et al., 1988), and bovine alpha-lactalbumin in mice (Vilotte et al., 1989). Some of these promoters have been hybridized to coding sequences for human blood proteins and some of the resulting transgenic animals secreted these human proteins in their milk.

Clark et al. (1989) microinjected a hybrid gene, sheep beta lactoglobulin promoter - human factor IX, into one-cell sheep embryos. They obtained two transgenic ewes each carrying about ten copies of the foreign gene. Furthermore, both animals expressed human factor IX RNA in the mammary gland and secreted the corresponding protein into their milk. Another human protein secreted in the milk of transgenic animals was tissue plasminogen activator (tPA). Westphal (1989) isolated the promoter sequence from the gene of whey acidic protein (WAP), a major constituent of milk in the mouse, and annealed it to the coding sequence of the human tPA. After injecting this construct into one-cell mouse embryos they obtained several lines of transgenic mice expressing the WAP-tPA gene in their lactating mammary glands.

Pigs also have served as models for mammary gland expression of transgenes. Wall et al. (1991) introduced WAP gene into the genome of swine. They analyzed three lines of transgenic swine and were able to detect mouse WAP in milk from all lactating females at concentrations of about 1 g/l. Moreover, expression of the corresponding RNA was specific to the mammary gland. Since mouse WAP accounted for about 3% of the total milk proteins in transgenic pigs, they concluded that it was possible to

produce high levels of a foreign protein in the milk of farm animals. Other pharmaceutical proteins expressed in mammary gland include α-1-antitrypsin in sheep (Wright et al., 1991) and tissue plasminogen activator in goats (Ebert et al., 1991).

More recently, Velander et al. (1992) microinjected a fusion gene consisting of the cDNA for human protein C inserted into the first exon of the mouse whey acidic protein gene into the pronuclei of 1-cell swine embryos. These researchers were able to generate transgenic pigs that produced human protein C at up to 1 g/liter. Furthermore, the biological activity of this protein C (38% of 380 μg/ml) was equivalent to that of protein C derived from human plasma.

PROTEIN C

Protein C, a 62,000 mw glycoprotein is a serine protease zymogen with a central role in one of the major regulatory mechanisms of hemostasis. Human protein C is an inhibitor of coagulation by inactivating factors V and VIII. Human protein C interferes with the cofactors for the rate limiting step of coagulation. Protein C also potentiates fibrinolysis by a complex mechanism resulting in the elevation of plasminogen activator in plasma (Marlar, 1987). The normal range of hPC is 70 to 130% of a normal plasma pool. The plasma concentration of hPC is 4 mg/ml. There is no difference in hPC levels of various age groups nor between males and females. However, significantly lower levels are found in normal newborn infants (16 to 47% of a normal plasma pool; Marlar, 1987).

Heterozygous or half-normal levels of hPC have been reported in individuals with thromboembolic complications. Marlar (1987) diagnosed over 60 patients with hereditary hPC deficiency from sixteen families and found a range in concentrations in these heterozygous patients from 12 to 65% of a normal plasma pool. Forty-five percent of these individuals had thromboembolic complications. The initial thrombotic event usually does not occur until 16 to 22 yr of age. However, some deficient patients over 50 years of age never developed thromboembolic complications. The major symptoms are deep vein thrombosis, pulmonary embolism, superficial thrombosis, mesenteric venous thrombosis and rarely, arterial thrombosis.

Homozygous deficiencies of hPC have been described in several cases. Infants with homozygous hPC deficiency develop purpura fulminans which is characterized by a massive superficial thrombosis in the microvasculature of the skin. Large dark-blue lesions develop over the skin which are painful and swollen. If the child lives long enough, these lesions become necrotic with excessive scarring. It is currently possible to treat these infants with factor IX which contains significant amounts of hPC. The half life of hPC in vivo is 6 to 8 h and infants with homozygous hPC deficiency are treated every 48 h. According to Marlar (1987), before 1987 no recurrence of purpura fulminans-like abnormalities occurred in a factor IX concentrate treated individual. Currently, the demand for hPC in the U.S. is over 100 kg/year (W. Drohan, American Red Cross, personal communication). Only 10% of this amount can be obtained from human plasma, so recombinant methods are needed to provide the other 90%.

Transgenic mice expressing hPC have already been produced. Vitale et al. (1989) microinjected the liver specific promoter for mouse albumin gene fused to hPC cDNA into one-cell mouse embryos. They were able to generate several lines of transgenic mice with various levels of hPC in their blood. This approach, however, may not be very practical because the amount of blood that can be harvested from any animal is limited. The best animal model for lactational expression of proteins would be the bovine. However, in general, as mentioned above, gene transfer experiments in cattle have been low in efficiency and cost ineffective (Hawk et al.; 1989, McEvoy and Sreenan, 1990; Roschlau et al., 1989).

EARLY DETECTION OF TRANSGENE INTEGRATION

Ninomiya et al. (1989) reported the use of a polymerase chain reaction (PCR) system to detect transgenes in mouse preimplantation embryos so that embryos could be selected before they were transferred to recipient mice. This selection system involved bisection of morulae, identification of the half morulae containing the target sequences within 7 h and transfer of positive sister half morulae. First, they tested morulae derived from mice they knew were transgenic. Five of 41 implanted embryos tested as positive were negative, only 1 of 28 embryos that tested negative was positive. When they tested morulae derived from microinjected embryos, 7 implanted embryos tested as negative contained no detectable amount of the transgene, and 1 of 2 embryos tested as positive

was transgenic. New techniques in embryo culture and biopsy make this approach a promising alternative to increase gene transfer efficiency in farm animals.

REPRODUCTIVE DILEMMAS

For techniques like this to work, it is necessary to establish how culture periods of varying lengths affect embryo viability. Of particular interest is the effect of culturing microinjected embryos for different periods of time on pregnancy rates in mice. Routinely, in studies involving pronuclear microinjection in mice, the surviving embryos are transferred shortly after they have been microinjected (Hogan et al., 1986). On the other hand, some investigators prefer to culture embryos to the 2-cell stage or to the morula/blastocyst stage prior to transfer into the uteri of foster mothers (Voss et al., 1990; Burki and Ulrich, 1982; Wagner et al., 1981).

Additionally, although most researchers transfer 20 to 30 microinjected embryos into a recipient, the threshold number of microinjected embryos to produce a pregnancy in the mouse has not been systematically studied. Another factor influencing the production of transgenic mice is the reduced viability of microinjected embryos. Some investigators have suggested that pregnancy rates can be improved by transferring some non-manipulated carrier embryos along with the microinjected embryos (Wagner et al., 1983).

The overall efficiency of gene transfer via pronuclear microinjection is low, varying from 0.2 to 6.0 % (Burki and Ulrich, 1982; Frels et al., 1985). Early embryonic (pre-implantation) death rates of 38.5 to 78.7 % have been reported (Burki and Ulrich, 1982; Gordon et al., 1987), and post implantation embryonic losses vary between 27.0 and 94.3 % (Frels et al., 1985; Brinster et al., 1983). However, a complete study on the effect of DNA integration on pre- and post- implantation losses has not been conducted.

CHAPTER III

EFFECT OF DEVELOPMENTAL STAGE AND NUMBER OF EMBRYOS PER TRANSFER ON THE EFFICIENCY OF GENE TRANSFER IN CD1 MICE

ABSTRACT

Our objective was to establish a combination of developmental stage and number of microinjected embryos per transfer that was optimal to obtain pregnancies and transgenic CD1 mice. A total of 423 immature mice were superovulated with 10 iu PMSG and 5 iu hCG 48 h apart. Twenty to 24 h after hCG, 6,966 embryos were recovered. Of these, 5,660 were fertilized. All fertilized embryos were microinjected with the whey acidic protein promoter - human protein C (WAP-hPC) gene construct. One hour after microinjection the surviving embryos (4,872;86%) were transferred to pseudopregnant recipients or cultured in CZB medium at 37°C in 5% CO_2 in air for 24 to 48 h (2 to 4-cell stage) or 72 to 96 h (morula/blastocyst stage; M/B) before transfer. There were three treatments of varying embryo numbers: 15-24, 25-34 and 35-44 for 1-cell, 2 to 4-cell and M/B embryos (10 transfers each). DNA from all pups born was analyzed by the polymerase chain reaction for detection of the transgene. Analysis of variance indicated that the highest pregnancy rate (PR) and total pups born (TOTP) was obtained after transferring 25 to 34 2-4 cell embryos (PR=90% TOTP=3.5/ pregnancy). This may be an artifact resulting from an inability to distinguish viable embryos and including some dying 1-cell embryos when no culturing was included, because even 1 h after microinjection some 1-cell embryos may not be viable even though they appear morphologically intact. Therefore, they are incapable of sustaining a pregnancy. However, if the embryos are cultured for 24 to 48 h after microinjection, one can better judge embryo viability and transfer only morphologically normal embryos. Pregnancy rate and total pups born for 1-cell embryos was intermediate but not different from the other treatments. Overall analysis indicated that the percentage of transgenic pups born was highest using 1-cell embryos (33.9%, 20.0% and 11.1% for 1-cell, 2- to 4- cell and Mor/Bl respectively). This finding suggests that culturing the embryos for 72 to 96 h may be detrimental for subsequent embryonic development in utero. We conclude that microinjected embryos should be transferred to recipients at the 1-cell stage to obtain the highest number of transgenic pups possible with CD1 mice.

INTRODUCTION

The optimum number and embryonic developmental stage at transfer to produce pregnancies with microinjected mouse embryos has not been well-established. Routinely, in studies involving DNA pronuclear microinjection in mice, the surviving embryos are transferred shortly after they have been microinjected (Hogan et el., 1986). However, some investigators prefer to culture the embryos to the 2-cell stage or to the morula/blastocyst (mor/bl) stage prior to transfer (Voss et al., 1990; Burki and Ulrich, 1982; Wagner et al., 1989). Although many researchers transfer 20 to 30 embryos per recipient, the threshold number of microinjected embryos to produce a pregnancy in mice has not been systematically studied. Hogan et al. (1986) recommends transferring 20 to 30 embryos per recipient. Whereas, Castro and Aguilar (1992) found that 10 microinjected embryos per transfer was sufficient to obtain high pregnancy rates and pups born per litter.

The objective of this study was to evaluate the effect of transferring microinjected embryos at three developmental stages (1-cell, 2 to 4-cell and mor/bl) in three quantities (15 to 24, 25 to 34 and 35 to 44 embryos per transfer) on pregnancy rate, total number of pups born and transgenic pups.

MATERIALS AND METHODS

Superovulation

The procedures for superovulation, embryo recovery, microinjection and transfer were similar to those described by Hogan et al. (1986). Female CD-1 mice 3 to 4 wk of age were given 10 i.u. of PMSG (Diosynth Inc., Chicago, IL) i.p. Forty-eight hours later, 5 i.u. of hCG (Sigma, St. Louis, MO) were administered i.p. Females were placed with intact males for matings. At this point, adult (8 weeks or older) CD-1 females were placed with vasectomized males to be used as pseudopregnant recipients.

Embryo Recovery

Twenty to twenty-four h after the hCG injection, females showing a copulatory plug were sacrificed by cervical dislocation. The ovaries and oviduct were excised and placed in a Petri dish in M2 media. The ampulla of each oviduct was located under a stereomicroscope at 10X and ruptured with watch maker's forceps to release the 1-cell embryos from the oviduct. The embryos were then washed in M2 containing 100 mg/ml hyaluronidase to dislodge the granulosa cells. After the embryos were completely clean, they were kept in M2 at 37°C in a dry bath.

Microinjection

The embryos were placed in a 100 μl drop of M2 on the lid of a 100 mm diameter Petri dish covered with silicone oil. The injection chamber containing the embryos was then placed on the heated stage of an inverted microscope (Zeiss, model ICM 405; Eastern Microscopes, Raleigh, NC). The microinjections were conducted under Hoffman modulation optics at 200x with the aid of a Leitz micromanipulator and an Eppendorf (Eppendorf model 5242; Eastern Microscopes, Raleigh, NC) automatic microinjector. A finely drawn glass pipette about 70 microns tip diameter (holding pipette) was connected to a syringe with a mechanism for fine movement and placed on one arm of the micromanipulator. The other arm held the injection pipette (1-2 micron tip diameter), which was loaded with 1 to 2 μl of the DNA construct solution and connected to the microinjector. Once the embryos were located under the microscope, an embryo was held by suction with the holding pipette and the most visible pronucleus injected with 2-3 pl of DNA. Between 20 and 30 embryos were injected at one time. After all the embryos were injected, they were held in M2 in the incubator for 30 to 60 min until they were transferred to pseudopregnant recipients. Embryos transferred at more advanced developmental stages were cultured in CZB (Chatot et al., 1989) in 25 μl drops covered with silicone oil. Once they reached the desired developmental stage, these embryos were placed in M2 medium and transferred to the oviducts of a day 1 pseudopregnant recipient (Bronson and McLaren, 1970), or to the uterus of a day 2.5 recipient depending on the embryonic developmental stage.

Embryo Transfers

Oviductal Transfers - A capillary tube was finely drawn under a flame and cut to a final diameter of about 120 microns. This tube was polished using a microforge (Narishige model MF-83; Medical Systems Corp., Greenvale, NY) and used as a transfer pipette. The transfer pipette was connected to a mouth piece for fine suction control. The embryos were loaded in a small amount of fluid (1-2 μl). Then, the pipette with the embryos was placed on a piece of clay undisturbed until the recipient was ready to receive the embryos.

A female mated by a vasectomized male (pseudopregnant) was anesthetized with 0.4 mg/g BW i.p. sodium pentobarbital (Barber Veterinary Supply Co. Richmond, VA). After cleaning the mouse's back with 70% alcohol, an incision was made about 1 cm to the left of the spinal cord, at the level of the last rib. The left ovary was located by sliding the skin toward the left side of the mouse. Then, an incision was made through the body wall and the fat pad picked up and pulled out with blunt forceps so that the ovary, oviduct and left uterine horn were exposed. A serafine clamp was attached to the fat pad and laid over the mouse's back to prevent the uterus from retracting.

At this point 1 drop of epinephrine (1:1000; Anpro Pharmaceutical, Arcadia, CA) was applied to the ovarian bursa to prevent excessive bleeding. Using watchmaker's forceps the bursa was torn apart and tucked under the ovary. The transfer pipette containing the embryos was then inserted down into the ampulla and the embryos discharged from the pipette. A small air bubble entering the oviduct signified that the expulsion was complete. After this, the serafine clamp was removed, the ovary and oviduct placed back inside the body wall and the incision closed with a wound clip. Nineteen to 20 days later, the recipients were checked for the presence of pups. If on day 20 a recipient looked pregnant but had not delivered any pups, a cesarean section was performed. Any pups obtained in this manner were permanently identified and placed with a foster mother.

Uterine Transfer - The procedures to expose the uterus were similar to that of the oviductal transfer. A 25 gauge needle was used to make a small incision in the right uterine horn. Then, a transfer pipette containing the embryos was introduced through this incision and into the uterine lumen where the embryos were deposited.

Gene Construct

The gene construct used in this study was a 9.5 kb cDNA clone of human protein C (hPC) that was cloned into the whey acidic protein (WAP) gene. The WAP gene was an EcoRI fragment cloned into the plasmid. The hybrid gene containing the promoter sequence from the WAP gene and the coding sequence from the hPC gene was purified by digesting plasmid DNA with restriction endonuclease EcoRI. The WAP-hPC construct was purified from the plasmid DNA using a GEN PAC FAX (Millipore Corp., Milford, MA) high performance liquid chromatography (HPLC) column. The elution conditions for the WAP-hPC product were determined by gradient chromatography over the range 0.5 to 1.0 M NaCl. An isocratic elution condition was chosen (25 mM Tris-HCl, 1.0 mM EDTA, and 0.63 M NaCl, pH 7.5). Approximately 15 to 20 μg of digested DNA was injected per run, and eluents containing the WAP-hPC fragment from each injection were pooled, precipitated and processed by HPLC a second time. The purity and concentration of WAP-hPC were determined on a 1% agarose gel stained with ethidium bromide.

DNA Extraction

Tail tissue was biopsied from pups which were approximately 20 days old and DNA was isolated from the tissue by a modification of the procedure developed by Marmur (1961). Briefly, 840 μl of lysing solution (50 mM Tris-HCl, 0.15 M NaCl, 1M Na_2ClO_4, 10 mM EDTA, 1% sodium duodecyl sulfate, 1% 2-mercaptoethanol, 100 μg/ml Proteinase K, pH 8.0) was added to each tube containing a tissue sample which had been previously frozen with liquid nitrogen. The tubes were incubated overnight at 50°C and then extracted with 250 μl of chloroform: isoamyl alcohol (25:1) by mixing for 10 to 15 sec on a "Mini Bead-Beater" (Biospec Products, Bartlesville, OK) followed by centrifugation for 10 min at 15,000 x g DNA was precipitated by adding 50 μl of isopropyl alcohol to 83 μl of the aqueous supernatant, then centrifuged and washed with 80% ethanol. The pellets were dried at 37°C and resuspended in 50 μl TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) and stored at -20°C until assayed by Polymerase Chain Reaction.

Polymerase Chain Reaction Analysis

Analysis was done by the general method of Saike et al.(1989). One μl of DNA solution was used as template in 25 μl reaction volumes [1X Taq buffer, 2.5 mM dNTP's, 0.5 mM oligonucleotide primers, 0.625 units Taq polymerase (Promega Corp., Madison, WI)]. Primers used to amplify a 402 bp target sequence in the transgene were: WAP-specific sense 5'-GTG GCC AAG AAG GAA GTG TTG, and hPC-specific antisense 5'- GTG CTT GGA CCA GAA GGC CAG. The WAP-specific antisense primer 5'-GAC TTG TTC CTC TAG GTT CTG was also used to amplify a 222bp fragment contained within the endogenous WAP gene. Initial denaturation was performed at 96°C for 1 min, followed by 40 cycles of: 55°C annealing for 2 min, 77°C elongation for 75 sec, and 96°C denaturation for 15 sec. Amplification products for mouse tail DNA were run concurrently with those from plasmid DNA on 1% agarose gels stained with 0.5 μg/ml ethidium bromide.

Determination of Expression

Mouse Milk Collection and Preparation

Lactating females were anesthetized with Metofane (methoxyfluoromethane; Pitmann-Moore, Washington Crossing, NJ) and induced to let down milk by intramuscular administration of 5.0 i.u. oxytocin (Vedco Inc, St Joseph, MO). Milk was collected in 1.8 ml screw cap microcentrifuge tubes using capillary tubes (Kimax brand, 2.0 mm I.D.) that were flame polished to prevent tissue damage. The capillary was partially inserted into a stoppered hand held receiving chamber containing the microfuge tube while operating the receiving chamber at 12 cm H_2O vacuum. Upon collection of 150 to 500 μl of milk, the tubes were stored at -90°C until the final whey preparation stage.

To maximize recovery of whey-soluble proteins, the whole milk was diluted with 3 volumes of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.2). Individual samples were ultracentrifuged at 115,000 x g for 30 min at 4°C. The buffer expanded whey phase was pipetted from the heavy pelleted precipitate and lighter lipid layer, placed in a clean tube and immediately frozen to -90°C. Diluted control mouse whey was identically prepared.

Protein C ELISA

Standard curves and milk samples were exposed to microtiter plates previously coated with monoclonal antibodies (7D7 or HPC4) against hPC for 2 to 3 h. Then, the plates were washed. Assera C (IgG against PC) was added and allowed to incubate for another 2 to 3 h. The plates were washed again and Antirabbit IgG horseradish peroxidase conjugate were added. Two or 3 h later, horseradish peroxidase substrate was added. Three to 5 h later, the reaction was stopped with sulfuric acid and absorbance read in a spectrophotometer at 492 nm wave length. This yielded μg of antigenic hPC/ml of milk (**presence** of hPC in milk).

Experimental Design

All embryos were microinjected at the one-cell stage. Embryos were transferred either immediately after microinjection, at the 2 to 4 cell stage or at the mor/bl stage. Treatments were as follows:

Treatment # 1:	1-cell embryos	15 to 24 per transfer
Treatment # 2:	" "	25 to 34 "
Treatment # 3:	" "	35 to 44 "
Treatment # 4	2 to 4 cell embryos	15 to 24 "
Treatment # 5	" "	25 to 34 "
Treatment # 6	" "	35 to 44 "
Treatment # 7	Morula/Blastocyst	15 to 24 "
Treatment # 8	" "	25 to 34 "
Treatment # 9	" "	35 to 44 "

Ten transfers per treatment were performed. Pregnancy rates, number of pups born dead, alive, sex of the pups and percentage of transgenic pups were recorded. Analysis of variance was used to determine differences among treatments. The independent variable was treatment, whereas pregnancy status, total pups born and number of transgenic pups served as dependent variables.

RESULTS

Seventy-four percent of the superovulated females were mated. Of the ova recovered 7.9% were unfertilized, 10.3% degenerated and 81.2% fertilized. Eighty-six percent of the embryos injected were morphologically normal 1 h after microinjection. A total of 2,599 embryos was transferred (Table 1).

Figure 1 shows the effect of developmental stage of DNA microinjected embryos on the number of pups per pregnancy. Culturing the embryos for 72 to 96 h (mor/bl stage) caused a significant decrease ($p<.05$) in the number of pups per pregnancy. The number of pups born was greater ($p<.05$) when 2 to 4-cell embryos were transferred than when mor/bl were transferred (Table 2). Pregnancy rate was highest for 2 to 4-cell embryos transfers. The percentage of transgenics was greater for 1-cell transfers.

Overall analysis of all combinations of developmental stage and number of embryos per transfer indicated that the highest PR was attained when 25 to 34 2- to 4-cell stage embryos were transferred (90%) and the highest number of total pups per treatment was obtained when 25 to 44 2- to 4-cell stage embryos were transferred (Table 3). However, the highest number of pups per pregnancy was for the treatment of 35 to 44 morula / blastocyst stage embryos were transferred (5.0±.09; Table 3). The level of expression of hPC in the transgenic mice tested is presented in Table 4 Antigenic hPC varied from .03 to 1.92 μg/ml of mouse milk.

Table 1. Superovulation response for experiment 1

Variable	Number	(%)
Superovulated Mice	423	
Mated	314	(74.2)
Total Embryos	6,966	
Unfertilizad Ova	556	(7.9)
Degenerated	720	(10.3)
DNA Microinjected	5,660	(81.2)
Survived	4,872	(86.1)
Transferred	2,599	

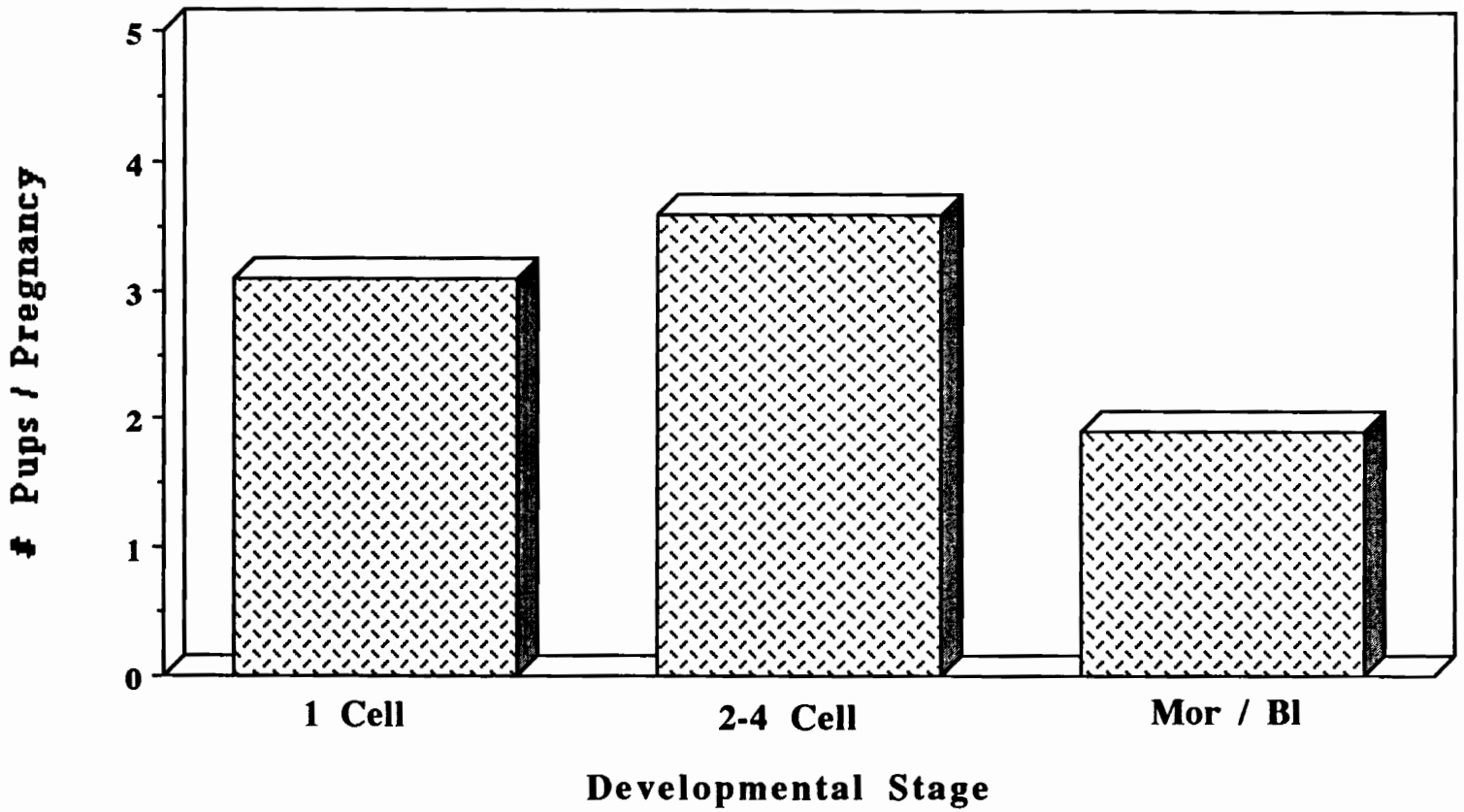

Figure 1. Effect of developmental stage of DNA injected embryos before transfer on number of pups per pregnancy.

Table 2. Effect of developmental stage of microinjected embryos on pregnancy rate, number of pups and percentage of transgenic mice.

Development Stage	Pregnancy Rate (%)	# of Pups Born	Percentage Transgenic
1 - cell	63	$3.1 \pm .5^{a,b}$	33.9
2 - to 4 - cell	83	$3.6 \pm .4^{a,}$	20.0
Morula/Blast	43	$1.9 \pm .6^{b}$	11.1

^{a,b} Means with different superscripts are different ($p<.05$)

Table 3. Effect of embryonic development stage and number of embryos per transfer on pregnancy rate, number of pups born per transfer and total number of pups born.

Development Stage	Number of Embryos per Transfer	Pregnancy Rate (%)	Mean Number of Pups per Transfer	Total Number of Pups
1 - cell	15 - 24	50	4.8 ± 0.9 [a]	24
1 - cell	25 - 34	80	3.4 ± 0.7 [a,b]	27
1 - cell	35 - 44	60	2.0 ± 0.8 [b]	12
2 - to 4 - cell	15 - 24	80	3.1 ± 0.7 [a,b]	25
2 - to 4 - cell	25 - 34	90	3.9 ± 0.7 [a]	35
2 - to 4 - cell	35 - 44	80	4.4 ± 0.7 [a]	35
Morula/Blast	15 - 24	50	1.4 ± 0.9 [b]	7
Morula/Blast	25 - 34	30	1.3 ± 1.1 [b]	4
Morula/Blast	35 - 44	50	5.0 ± 0.9 [a]	25

[a,b] Means with different superscripts are different ($p < .05$)

Table 4. Expression level of human protein C in transgenic mice.

Founder Mouse Number	Expression level μg/ml
3	0.31
4	0.13-0.20
5	0.67-1.55
11	0.11-0.51
12	0.03-0.08
13	0.26
15	0.22
29	0
32	0.17
34	1.92

DISCUSSION

The fertilization rate observed in this study (81.2%) was consistent with that of the current literature (Hogan et al. 1986). The percentage of embryos that survived microinjection was close to the range of 50 to 80% published by Hogan et al. (1986) but higher than the 43.2% obtained by Voss et al. (1990).

Hogan et al. (1986) reported that although microinjected embryos can be transferred at the blastocyst stage, is better to transfer them either shortly after microinjection or at the 2-cell stage (24 h later). Our results generally agree with this observation. We obtained lower PR with morula/blastocyst stage embryos than with 1- or 2-4-cell stage embryos. There are at least two ways to explain this finding. First, it has been known that long periods of in vitro culture can be detrimental to embryonic development after transfer (Bronson and McLaren, 1970). Second, blastocyst stage embryos show a reduced survival in utero compared to morulae stage embryos (Hoppe and Coman, 1983). This is because that embryos developing from morulae lie deep within the decidua where they are surrounded by numerous and large blood vessels. However, embryos developing from transferred blastocysts implant more distally to the maternal blood vessels and only a few blood islands surround the embryos. In addition, Hoppe and Coman (1983) found that although the implantations from transferred morulae and blastocysts were similar (42% and 47%, respectively) significantly more implantations were resorbed after transfer of blastocysts (78%) as compared with morulae (15%). In our study, about half of the embryos transferred at the mor/bl stage were blastocysts. Therefore, it is possible that problems with implantation may have contributed to the lower pregnancy rate and number of pups born obtained with this treatment..

It was interesting that the percentage of transgenic pups born decreased as developmental stage of the embryos before transfer advanced. This suggests that an interaction between culture period and DNA microinjection may have a negative effect on post transfer embryonic development. On the other hand, Walker et al, (1990) found that in vitro culture of sheep embryos for up to 3 days after pronuclear microinjection was not detrimental to viability after transfer. Furthermore, they recommend 3 days of culture to discard non-viable embryos before transfer. In a similar study, Rexroad et al. (1990), utilized a co-culture system as a method for selecting viable microinjected

sheep embryos before transfer. Unlike the report of Walker et al. (1990) they found that very little selection was possible because a relatively high proportion (80%) of the microinjected embryos developed to at least the 8-cell stage during culture.

The reason for the higher viability of embryos from different species that survive microinjection could be due to several factors. It is possible that the mechanical damage caused by microinjection is not as critical for the larger sheep embryo compared to the mouse embryo (Walton et al., 1987). In addition, when comparing the gestation length of both species (150 d vs. 21 d for sheep and mice, respectively) one realizes that 72 h of culture is a large proportion of gestation for mouse embryos to be out of their natural environment. Walker et al. (1988) reported that when the culture period was extended from 3 to 5 days the viability of the 1-cell embryos declined, suggesting that long term culture can also be detrimental for sheep embryos.

The ranges of embryo numbers per transfer tested in this study did not affect pregnancy rate or the number of pups born per litter (Table 3). This result is consistent with those of Castro and Aguilar (1992). These investigators transferred 10, 15 or 20 microinjected 1-cell mouse embryos per recipient and found that 10 embryos was
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The ranges of embryo numbers per transfer tested in this study did not affect pregnancy rate or the number of pups born per litter (Table 3). This result is consistent with those of Castro and Aguilar (1992). These investigators transferred 10, 15 or 20 microinjected 1-cell mouse embryos per recipient and found that 10 embryos was enough to attain the high pregnancy rate and maximum number of pups per litter. Contrasting results were published by Springmann et al (1988) who found that in pigs, 40 1-cell microinjected embryos have to be transfer to reach acceptable pregnancy rates (< 40 embryos = 25 % PR; > 40 embryos = 48 % PR). This could reflect species variation between pigs and mice. Similarly, Wang et al. (1992) transferred different numbers of microinjected embryos in pigs. They found that the pregnancy rate was higher when more embryos were transferred (15 emb/trs=50% PR, 24 emb/trs=69% PR and 34 emb/trs=75% PR).

Finally, we were able to obtain expression of a complex protein that was biologically active from the milk of most of our transgenic mice (Velander et al., 1992).

In summary, transfer of 2- to 4-cell embryos yielded the highest PR and total pups. However, about 50% of the microinjected embryos fail to cleave or fragmented when cultured in vitro. Therefore, the most efficient method to produce transgenic mouse pups was using 1-cell embryos shortly after microinjection.

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CHAPTER IV
EFFECT OF TRANSFERRING NON-MANIPULATED EMBRYOS ALONG WITH MICROINJECTED EMBRYOS ON GENE TRANSFER EFFICIENCY

ABSTRACT

The objective of this study was to investigate the effect of addition of non-microinjected embryos along with injected embryos on pregnancy rate (PR) and transgenic pup production. One hundred and thirty six mice (CD1; 3 to 4 wk old) were superovulated with 10 iu PMSG and 5 iu hCG 48 h apart. One-cell embryos (2,230) were collected 20 to 24 h after hCG. Those fertilized (1,764) were microinjected with the whey acidic protein promoter - human protein C (WAP-hPC) gene construct. One hour after microinjection the surviving embryos (1,541; 87.4%) were evaluated and 1,260 of them were transferred to pseudopregnant recipients. In order to be able to differentiate pups born from microinjected or non-manipulated embryos, females were mated to C57Bl/6 males and these embryos were non-manipulated and transferred with injected embryos. There were six transfer treatments (TRT): 20-0, 16-4, 12-8, 30-0, 26-4 and 22-8 injected - non-injected embryos, respectively (10 transfers/TRT). All the live pups born from microinjected embryos (white pups) were analyzed by the polymerase chain reaction to detect the presence of the transgene. Pregnancy rate and the total number of pups born were improved ($p<.05$) by addition of non-injected embryos. However, the highest percentage of transgenic pups obtained was when 20 embryos were transferred (25.0%) compared to transfer of 30 embryos (19.8%). There was 32% transgenic pups when 0 non-injected embryos were transferred compared with 15.1% transgenic pups when 4 or 8 non-injecte embryos were added to the transfers.

Our results indicate that addition of non-injected embryos although it increased pregnancy rate and the number of pups born from microinjected embryos, actually decreased the number of transgenic pups obtained per pregnancy.

INTRODUCTION

The use of non-manipulated embryos in conjunction with DNA injected embryos to help maintain pregnancy rate was reported in the literature almost 10 years ago.

Wagner et al. (1983) were some of the first researchers to mention transferring non-manipulated embryos along with microinjected embryos to produce transgenic mice. However, their objective was to produce transgenic mice, and they did not report specific data on embryo transfer itself. Non-manipulated embryos also have been used in gene transfer programs with other species. Pursel et al. (1988) transferred either 1- or 2-cell porcine microinjected embryos to test which developmental stage had the highest survival rate. They included 3 to 6 non-manipulated embryos per transfer to help maintain pregnancy. More recently, Wall et al. (1991) introduced the mouse whey acidic protein (WAP) gene into pig embryos. Similar to the previous report, they mentioned adding 2 to 4 non-manipulated embryos to some of their recipients. However, neither of these two studies were designed specifically to test the effect of non-manipulated embryos on pregnancy rate or number of piglets and transgenic piglets obtained per litter. The objective of this experiment was to investigate the effect of addition of non-injected embryos along with injected embryos on pregnancy rate (PR), the number of pups born from microinjected embryos, and the number of transgenic pups born.

MATERIALS AND METHODS

Superovulation

The procedures for superovulation, embryo recovery, microinjection and transfer were similar to those described by Hogan et al. (1986). Female CD-1 mice 3 to 4 wk of age were given 5 i.u. of PMSG (Diosynth Inc., Chicago, IL) i.p. Forty-eight h later, 5 i.u. of hCG (Sigma, St. Louis, MO) were administered i.p. Females were placed with intact males for matings. In order to identify the pups born from non-injected transfers, some superovulated females were placed with C57BL/6 males. Adult CD-1 females (8 wk or older) were placed with vasectomized males to be used as pseudopregnant recipients.

Embryo Recovery

Twenty to twenty-four h after the hCG injection, superovulated females showing a copulatory plug were sacrificed by cervical dislocation. The ovaries and oviduct were excised and placed in a Petri dish in M2 media. The ampulla of each oviduct was

located under a stereomicroscope at 10X and ruptured with watch maker's forceps to release the 1-cell embryos from the oviduct. The embryos were then washed in M2 containing 100 μg/ml hyaluronidase to dislodge the granulosa cells. After the embryos were completely clean, they were kept in M2 at 37^{o}C in a dry bath.

Microinjection

The embryos were placed in a 100 μl drop of M2 on the lid of a 100 mm diameter Petri dish and covered with silicone oil. The injection chamber containing the embryos was then placed on the heated stage of an inverted microscope (Zeiss, model ICM 405; Eastern Microscopes, Raleigh, NC). The microinjections were conducted under Hoffman modulation optics at 200x with the aid of a Leitz micromanipulator and an Eppendorf (Eppendorf model 5242; Eastern Microscopes, Raleigh, NC) automatic microinjector. A finely drawn glass pipette about 70 microns tip diameter (holding pipette) was connected to a syringe with a mechanism for fine movement and placed on one arm of the micromanipulator. The other arm held the injection pipette (1-2 micron tip diameter) which was loaded with 1 to 2 μl of the DNA construct solution and connected to the microinjector. Once the embryos were located under the microscope, an embryo was held by suction with the holding pipette and the most visible pronucleus injected with 2-3 pl of DNA. Between 20 and 30 embryos were injected at one time. After all the embryos were injected, they were held in M2 in the incubator for 30 to 60 min until they were transferred to pseudopregnant recipients.

Embryo Transfers

One h after microinjection, lysed embryos were discarded. Morphologically normal embryos were assigned to the transfer treatments. Every group of embryos for each transfer was placed in a separate petri dish. A capillary tube was finely drawn under the flame and cut to a final diameter of about 120 microns. This tube was polished using a microforge (Narishige model MF-83; Medical Systems Corp., Greenvale, NY) and used as a transfer pipette. The transfer pipette was connected to a mouth piece for fine suction control. The embryos were loaded in a small amount of fluid (1-2 μl). Then, the pipette with the embryos was placed on a piece of clay undisturbed until the recipient was ready to receive the embryos.

A female mated by a vasectomized male (pseudopregnant) was anesthetized with 0.4 mg/g BW i.p. sodium pentobarbital (Barber Veterinary Supply Co., Richmond, VA). After cleaning the mouse's back with 70% alcohol, an incision was made about 1 cm to the left of the spinal cord, at the level of the last rib. The left ovary was located by sliding the skin toward the left side of the mouse. Then, an incision was made through the body wall and the fat pad picked up and pulled out with blunt forceps so that the ovary, oviduct and left uterine horn were exposed. A serafine clamp was attached to the fat pad and laid over the mouse's back to prevent the uterus from retracting.

At this point 1 drop of epinephrine (1:1000, Anpro Pharmaceutical; Arcadia, CA) was applied to the ovarian bursa to prevent excessive bleeding. Using watchmaker's forceps the bursa was torn apart and tucked under the ovary. The transfer pipette containing the embryos was then inserted down into the ampulla and the embryos discharged from the pipette. A small air bubble entering the oviduct signified that the expulsion was complete. After this, the serafine clamp was removed, the ovary and oviduct placed back inside the body wall and the incision closed with a wound clip. Nineteen to 20 days later, the recipients were checked for the presence of pups. If on day 20 a recipient looked pregnant but had not delivered any pups, a cesarean section was performed. Any pups obtained in this manner were permanently identified and placed with a foster mother.

Gene Construct

The gene construct used in this study was a 9.5 kb cDNA clone of human protein C (hPC) that was cloned into the whey acidic protein (WAP) gene. The WAP gene was an EcoRI fragment cloned into the plasmid. The hybrid gene containing the promoter sequence from the WAP gene and the coding sequence from the hPC gene was purified by digesting plasmid DNA with restriction endonuclease EcoRI. The WAP-hPC construct was purified from the plasmid DNA using a GEN PAC FAX (Millipore Corp., Milford, MA) high performance liquid chromatography (HPLC) column. The elution conditions for the WAP-hPC product were determined by gradient chromatography over the range 0.5-1.0 M NaCl. An isocratic elution condition was chosen (25 mM Tris-HCl, 1.0 mM EDTA, and 0.63 M NaCl, pH 7.5). Approximately 15 to 20 μg of digested DNA was injected per run, and eluents containing the WAP-

hPC fragment from each injection were pooled, precipitated and processed by HPLC a second time. The purity and concentration of WAP-hPC were determined on a 1% agarose gel stained with ethidium bromide.

DNA Extraction

Tail tissue was biopsied from pups which were approximately 20 days old and DNA was isolated from the tissue by a modification of the procedure developed by Marmur (1961). Briefly, 840 μl of lysing solution (50 mM Tris-HCl, 0.15 M NaCl, 1M Na_2ClO_4, 10 mM EDTA, 1% sodium duodecyl sulfate, 1% 2-mercaptoethanol, 100 μg/ml Proteinase K, pH 8.0) was added to each tube containing a tissue sample which had been previously frozen with liquid nitrogen. The tubes were incubated overnight at 50°C and then extracted with 250 μl of chloroform: isoamyl alcohol (25:1) by mixing for 10 to 15 sec on a "Mini Bead-Beater" (Biospec Products, Bartlesville, OK) followed by centrifugation for 10 min at 15,000 x g DNA was precipitated by adding 50 μl of isopropyl alcohol to 83 μl of the aqueous supernatant, then centrifuged and washed with 80% ethanol. The pellets were dried at 37°C and resuspended in 50 μl TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) and stored at -20°C until assayed by Polymerase Chain Reaction.

Polymerase Chain Reaction Analysis

Analysis was done by the general method of Saike et al.(1989). One μl of DNA solution was used as template in 25 μl reaction volumes [1X Taq buffer, 2.5 mM dNTP's, 0.5 mM oligonucleotide primers, 0.625 units Taq polymerase (Promega Corp. Madison, WI)]. Primers used to amplify a 402 bp target sequence in the transgene were: WAP-specific sense 5'-GTG GCC AAG AAG GAA GTG TTG, and hPC-specific antisense 5'- GTG CTT GGA CCA GAA GGC CAG. The WAP-specific antisense primer 5'-GAC TTG TTC CTC TAG GTT CTG was also used to amplify a 222 bp fragment contained within the endogenous WAP gene. Initial denaturation was performed at 96°C for 1 min, followed by 40 cycles of: 55°C annealing for 2 min, 77°C elongation for 75 sec, and 96°C denaturation for 15 sec. Amplification products for mouse tail DNA were run concurrently with those from plasmid DNA on 1% agarose gels stained with 0.5 μg/ml ethidium bromide.

Experimental Design

Transfer treatments were as follows:
Treatment # 1: 20 microinjected + 0 non-manipulated embryos.
Treatment # 2: 16 microinjected + 4 non-manipulated embryos.
Treatment # 3: 12 microinjected + 8 non-manipulated embryos.
Treatment # 4: 30 microinjected + 0 non-manipulated embryos.
Treatment # 5: 26 microinjected + 4 non-manipulated embryos.
Treatment # 6: 22 microinjected + 8 non-manipulated embryos.

Ten transfers per treatment were performed. Non-manipulated embryos were obtained from superovulated CD1 females mated by C57BL/6 males. Therefore, any pups born from non-manipulated embryos were black. Analysis of variance was used to determine differences among treatments. The independent variable was treatment and the dependent variables were total pups born, number of pups born from microinjeted embryos and number of transgenic pups born. The overall effect of treatments was also tested by Chi Square using the statistical analysis system (SAS Institute, Inc., North Carolina).

RESULTS

Table 5 illustrates the superovulation data for females placed with CD1 and C57BL/6 males. More of the females placed with CD1 males were mated (136 and 76% vs 59 and 53%) and yielded a higher percentage of fertilized embryos (79.1% vs 65.6%) and a lower percentage of unfertilized and degenerated embryos (6.9% vs 22.5 % and 4.9 % vs 11.8 %, respectively) than those placed with C57BL/6 males. We transferred 1,260 injected and 240 non-injected embryos to complete this experiment. The addition of non-manipulated embryos increased pregnancy rate (Figure 2). When 20 embryos were transferred per recipient, the pregnancy rate improved from 40% to 50% and 80% with 0, 4 and 8 of the embryos being non-manipulated. Similarly, In the group of 30 embryos per transfer, pregnancy rate increased from 60% to 90% and 100% for 0, 4 and 8 non-manipulated embryos, respectively.

Table 5. Superovulation response for experiment 2.

	C57BL/6 Male		CD 1 Male	
Variable	Number	(%)	Number	(%)
Superovulated Mice	59		136	
Mated	31	(52.5)	104	(76.4)
Total Embryos	786		2,230	
Unfertilizad Ova	177	(22.5)	156	(6.9)
Degenerated	93	(11.8)	110	(4.9)
Fertilized	516	(65.6)	1,764	(79.1)
DNA Microinjected	n a*		1,764	(79.1)
Survived	n a		1,541	(87.4)
Transferred	240		1,260	

* na not-applicable

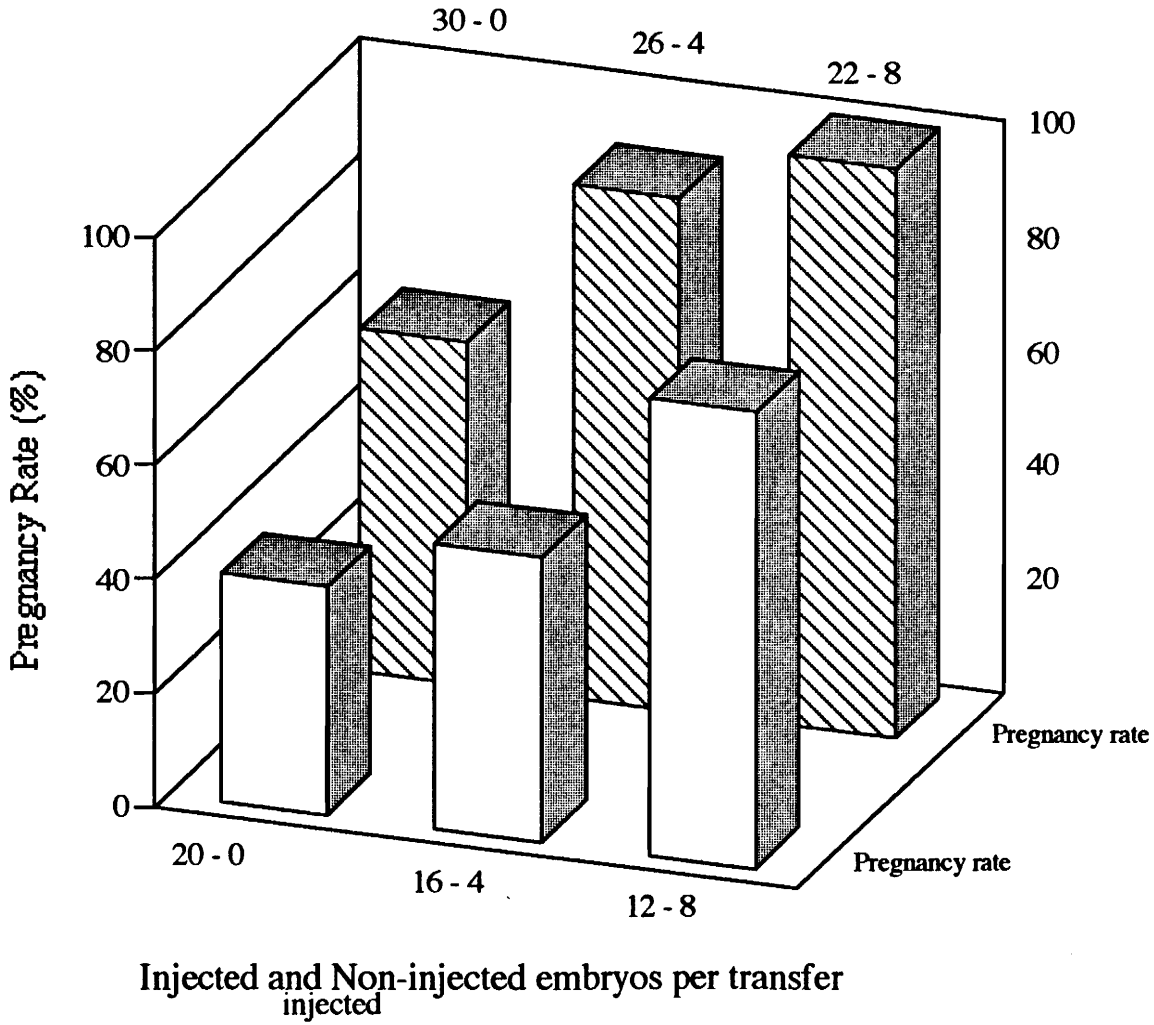

Figure 2. Effect of the number of injected and non-injected embryos per transfer on pregnancy rate.

The number live pups was higher ($p<.05$) when non-manipulated embryos were included in the transfer treatments (from 3.7 ± 1.4 to 5.6 ± 1.0 for 0 and 8 non-manipulated embryos, respectively, at 20 embryos per transfer and from 5.2 ± 1.1 to 7.8 ± 0.9 for 0 and 8 non-manipulated embryos, respectively at 30 per transfer; Table 6). Additionally, the number of pups born from non-manipulated embryos was not significantly different between 4 and 8 non-manipulated embryos at 20 embryos per transfer (2.4 ± 0.6 and 3.9 ± .04 for 4 and 8 non-manipulated embryos, respectively), but it was significantly ($p<.05$) different at 30 embryos per transfer (2.4 ≠ .04 and 4.5 ± .04 for 4 and 8 non-manipulated embryos, respectively; Table 6).

Chi square analysis revealed that the total number of pups born transgenic, non-transgenic and the number of embryos that did not develop into live pups were not independent from treatment ($p<.05$; Table 7). In addition, Chi square analysis of combined treatments (20 vs 30 embryos per transfer and 0 vs 4 and 8 non-injected embryos per transfer) also showed that the total number of pups born transgenic, non-transgenic and the number of embryos that did not develop into live pups were significantly affected by treatment ($p<.05$; Tables 8 and 9). However, the highest percentage of transgenic pups obtained was when 20 embryos were transferred (25.0%) compared to transfer of 30 embryos (19.8%). Additionally, there was 32% transgenic pups when 0 non-injected embryos were transferred compared with 15.1% transgenic pups when 4 or 8 non-injecte embryos were added to the transfers.

Table 6. Least square means for total pups, pups from injected and non-injected embryos (pregnant recipients)

Embryos per Transfer		Number of Total pups born	Number of Pups from injected embryos	Number of Pups from non-injected embryos
Injected	Non-injected			
20	0	3.7 ± 1.4^{b}	3.7 ± 1.4	n a*
16	4	3.8 ± 1.2^{b}	1.4 ± 1.2	2.4 ± 0.6^{b}
12	8	$5.6 \pm 1.0^{a,b}$	1.7 ± 1.0	$3.9 \pm 0.4^{a,b}$
30	0	$5.2 \pm 1.1^{a,b}$	5.2 ± 1.1	n a
26	4	$6.0 \pm 0.9^{a,b}$	3.5 ± 0.9	2.4 ± 0.4^{b}
22	8	7.8 ± 0.9^{a}	3.3 ± 0.9	4.5 ± 0.4^{a}

^{a,b} Values with different superscripts different ($p<.05$)
* na not-applicable

Table 7. Effect of the number of embryos injected and non-injected per transfer on the number of pups born non transgenic,transgenic and embryos not developing to term.

Embryos per Transfer		Number of pups non - transgenic	Number of pups transgenic	Number of embryos not developing to term
Injected	Non-injected			
20	0	8* (16.5)**	7 (4.4)	185 (179.0)
16	4	6 (13.2)	1 (3.5)	153 (143.2)
12	8	13 (10.0)	1 (2.7)	106 (107.5)
30	0	23 (24.8)	8 (6.7)	269 (268.5)
26	4	25 (21.4)	7 (5.8)	228 (232.8)
22	8	29 (18.2)	4 (4.9)	187 (196.9)

* Observed values
** Expected values
Chi square = 23.0; $p<.05$

Table 8. Effect of transferring microinjected embryos with and without non-injected embryos on the number of pups not transgenic, pups transgenic and embryos that did not develop into pups.

Number of non-injected embryos	Pups non transgenic (%)	Pups transgenic (%)	Pups not born (%)
0	31* [41.0]** (67.4)	15 [11.1] (53.6)	454 [447.6] (40.2)
4 or 8	73 [72.8] (84.9)	13 {16.9} (46.4)	674 [680.4] (59.8)

* Observed value
** Expected value
Chi square = 6.6 ($p<.05$)

Table 9. Effect of the total number of embryos per transfer on the number of pups not transgenic, pups transgenic and embryos that did not develop into pups.

Number of embryos transferred	Pups non transgenic (%)	Pups transgenic (%)	Pups not born (%)
20	27* [39.6]** (75.0)	9 [10.7] (32.1)	444 [429.7] (39.3)
30	77 [64.4] (80.2)	19 [17.3] (67.9)	684 [698.2] (60.7)

* Observed value
** Expected value
Chi square = 7.6 ($p<.05$)

DISCUSSION

Literature concerning addition of non-manipulated embryos to improve efficiency of pronuclear microinjection is scarce (Wagner et al., 1983; Pursel et al.,1988; Wall et al., 1991). However, none of these three studies were designed specifically to test the effect of non-manipulated embryos on pregnancy rate or number of offspring and transgenic offspring obtained per litter.

In our study, the addition of non-manipulated embryos was beneficial in almost every aspect of embryo transfer. Pregnancy rate, total number of pups born, number and percentage of pups from microinjected embryos, were improved by adding 4 or 8 non-manipulated embryos per transfer. The number of transgenics, the number of non-transgenics, and the number of non-developing embryos were not independent of treatment. Nevertheless, the number of transgenic pups obtained per pregnancy decreased as the number of non-manipulated embryos increased (Table 6). The highest number of transgenic pups (Table 7) was obtained when 30 microinjected embryos were transferred (8 pups) whereas, the lowest obtained was for the treatments of 16 and 12 microinjected embryos with 4 and 8 non-manipulated embryos per transfer (1 pup). This is an indication that microinjected embryos are less competitive than non-manipulated embryos in utero. Furthermore, even when almost three times as many microinjected embryos were transferred as non-manipulated embryos (22-8) per recipient, the number of pups from non-manipulated embryos was still higher than that of microinjected embryos (4.5±.4 vs. 3.3±.9 respectively; Table 6). Brem et al. (1990) analyzed some factors affecting the success of transgenic pig programs. Similar to our findings, they reported that addition of non-manipulated embryos improved pregnancy rate and the number of piglets per transfer and per litter. However, in that report the authors failed to mention how many non-manipulated embryos were added per transfer, and whether or not they were able to increase the number of transgenic piglets per transfer.

In conclusion, our study demonstrates that transferring non-manipulated embryos along with microinjected embryos increases pregnancy rate and total number of pups per litter, but decreases the number of transgenic pups born. Therefore, it is best to

transfer microinjected embryos alone in order to maximize the efficiency of gene transfer after pronuclear microinjection in mice.

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CHAPTER V

EFFECT OF PRONUCLEAR DNA MICROINJECTION ON EMBRYONIC AND FETAL SURVIVAL OF CD1 MICE.

ABSTRACT

Litter size of DNA microinjected zygotes is lower than for non-manipulated zygotes. The objectives of this study were to determine: 1) the rate of embryonic and fetal survival in early, mid and late gestation; and 2) whether DNA integration was the cause of embryonic losses. One hundred and twenty-nine immature CD1 mice were superovulated with 10 iu PMSG and 5 iu hCG 48 h apart. One-cell embryos (1,765) were collected 20 to 24 h after hCG. Those fertilized (1,593) were microinjected with the whey acidic protein promoter - human protein C (WAP-hPC) gene construct. One h after microinjection the surviving embryos (1,385;90.6%) were transferred to pseudopregnant recipients (45 transfers of 30 embryos each). Fifteen recipients were sacrificed on day 4, 12 and 18 of gestation. On each day all embryos and fetuses were counted and analyzed for the presence of the transgene. The percentage of transgenic embryos or fetuses was not significantly different at any day (45.8%, 35.5% and 34.6% for days 4, 12 and 18, respectively). However, the number of viable embryos at day 4 was significantly greater than that of viable fetuses on days 12 or 18 (10 $\pm$1.1, 5.1 $\pm$1.6, and 2.4 $\pm$1.3 for days 4, 12 and 18, respectively). In addition, a high degree of mosaicism was observed in day 18 fetuses and placentae recovered (34.6% and 56.0% transgenics for fetuses and placentae, respectively). In summary, a high degree of embryonic and fetal mortality occurs among microinjected embryos. Furthermore, since the percentage of transgenesis did not change throughout pregnancy, DNA integration can not account for all of the embryonic losses. Other factor(s) related to the microinjection procedure may be involved in the embryonic and fetal failure of microinjected embryos.

INTRODUCTION

Most reports on transgenic animals agree that microinjected embryos have a lower survival rate throughout pregnancy than normal embryos. Burki and Ulrich (1982) and

Gordon et al. (1987) reported that the rate of early embryonic death (preimplantation) varies from 38.5% to 78.7%. Additionally, postimplantation embryonic losses range between 27.0% and 94.3% (Frels et al., 1985; Brinster et al., 1983). However, factors which reduce survival remain to be elucidated. The objectives of this study were to: 1) determine pre- and post-implantation rate of CD1 DNA microinjected embryos; and 2) determine if DNA integration was responsible for embryonic and fetal losses.

MATERIALS AND METHODS

Superovulation

The procedures for superovulation, embryo recovery, microinjection and transfer were similar to those described by Hogan et al. (1986). Female CD-1 mice 3 to 4 wk of age were given 5 i.u. of PMSG (Diosynth Inc., Chicago, IL) i.p. Forty-eight h later, 5 i.u. of hCG (Sigma, St. Louis, MO) were administered i.p. Females were placed with intact males for matings. Also, adult CD-1 females (8 wk or older) were placed with vasectomized males to be used as pseudopregnant recipients.

Embryo Recovery

Twenty to twenty-four h after the hCG injection, females showing a copulatory plug were sacrificed by cervical dislocation. The ovaries and oviduct were excised and placed in a Petri dish in M2 media. The ampulla of each oviduct was located under a stereomicroscope at 10X and ruptured with watch maker's forceps to release the 1-cell embryos from the oviduct. The embryos were then washed in M2 containing 100 μg/ml hyaluronidase to dislodge the granulosa cells. After they were completely clean, they were kept in M2 at 37^{o}C in a dry bath.

Microinjection

The embryos were placed in a 30 μl drop of M2 on the lid of a 100 mm diameter Petri dish and covered with silicone oil. The injection chamber containing the embryos was then placed on the heated stage of an inverted microscope (Zeiss, model ICM 405; Eastern Microscopes, Raleigh, NC). The microinjections were conducted under

Hoffman modulation optics at 200x with the aid of a Leitz micromanipulator and an Eppendorf (Eppendorf model 5242; Eastern Microscopes, Raleigh, NC) automatic microinjector. A finely drawn glass pipette about 70 microns tip diameter (holding pipette) was connected to a syringe with a mechanism for fine movement and placed on one arm of the micromanipulator. The other arm held the injection pipette (1 to 2 micron tip diameter) which was loaded with 1 to 2 μl of the DNA construct solution and connected to the microinjector. Once the embryos were located under the microscope, an embryo was held by suction with the holding pipette and the most visible pronucleus injected with 2 to 3 pl of DNA. Between 20 and 30 embryos were injected at one time. After all the embryos were injected, they were held in CZB in an incubator at 37^{o}C in 5% CO_2 in air for 30 to 60 min until they were transferred to pseudopregnant recipients.

Embryo Transfers

A capillary tube was finely drawn under the flame and cut to a final diameter of about 120 microns. This tube was polished using a microforge (Narishige model MF-83; Medical Systems Corp., Greenvale, NY) and used as a transfer pipette. The transfer pipette was connected to a mouth piece for fine suction control. The embryos were loaded in a small amount of fluid (1 to 2 μl). Then, the pipette with the embryos was placed on a piece of clay undisturbed until the recipient was ready to receive the embryos.

A female, mated by a vasectomized male (pseudopregnant), was anesthetized with 0.4 mg/g BW i.p. sodium pentobarbital (Barber Veterinary Supply Co., Richmond, VA). After cleaning the mouse's back with 70% alcohol, an incision was made about 1 cm to the left of the spinal cord, at the level of the last rib. The left ovary was located by sliding the skin toward the left side of the mouse. Then, an incision was made through the body wall and the fat pad picked up and pulled out with blunt forceps so that the ovary, oviduct and left uterine horn were exposed. A serafine clamp was attached to the fat pad and laid over the mouse's back to prevent the uterus from retracting.

At this point 1 drop of epinephrine (1:1000, Anpro Pharmaceutical; Arcadia, CA) was applied to the ovarian bursa to prevent excessive bleeding. Using watchmaker's forceps

the bursa was torn apart and tucked under the ovary. The transfer pipette containing the embryos was then inserted down into the ampulla and the embryos discharged from the pipette. A small air bubble entering the oviduct signified that the expulsion was complete. After this, the serafine clamp were removed, the ovary and oviduct placed back inside the body wall and the incision closed with a wound clip.

Embryo and Fetal Recovery

Day 4 Embryos - On day 4 of gestation the embryos were recovered following the procedure of Hogan et al. (1986). The recipients were sacrificed by cervical dislocation. The uterus, oviducts and ovaries were dissected and placed in a 100 mm diameter plastic petri dish. At this point, the ovaries and oviducts were cut from the uterus and both uterine horns were also separated. Using watch maker's forceps, a 5 cc syringe and a 25 g needle, a uterine horn was held with the ovarian side up, and flushed with 5 ml of M2 medium. Then, the other horn was flushed using the same procedure. After this, both sets of ovaries and oviducts were placed in a 35 mm diameter plastic petri dish. The oviducts were separated from the ovaries and then cut into several smaller pieces. Each piece was then squeezed with watchmaker's forceps to force any remaining embryos to come out of the oviducts. Once all the dishes were searched, the embryos were washed three times in M2 medium. Utilizing a fire polished Pastur pipette, each embryo was held with approximately 2 to 3 ml of M2 medium and placed in a microfuge tube containing 5 ml of embryo lysing buffer (20mM Tris-HCl, pH 8.3, .9%. Tween 20, .9% Nonidet and 400 μg/ml Proteinase K). At this point, the embryos were covered with 25 ml of paraffin oil and stored at -87°C.

Day 12 Fetuses - The recipients were sacrificed by cervival dislocation. The uteri were dissected out and separated from the ovaries and oviducts. Using small scissors each fetus was dissected from the uteri. At this point, each fetus was held with watch-maker's forceps on top of a 100 mm diameter a Petri dish and rinsed with 20 ml of sterile saline. In between fetuses the forceps and scissors were rinsed with 70% ethanol and wiped off with sterile gauze. Each fetus was then placed in a sterile microfuge tube and stored at -87°C.

Day 18 Fetuses - The procedure was the same as the one used for day 12 fetuses, except that each fetus and placenta was rinsed separately. Also, only about 1/3 of each fetus and 1/2 of its placenta were frozen for DNA extraction and analysis.

Gene Construct

The gene construct used in this study was a 9.5 kb cDNA clone of human protein C (hPC) that was cloned into the whey acidic protein (WAP) gene. The WAP gene was an EcoRI fragment cloned into the plasmid. The hybrid gene containing the promoter sequence from the WAP gene and the coding sequence from the hPC gene was purified by digesting plasmid DNA with restriction endonuclease EcoRI. The WAP-hPC construct was purified from the plasmid DNA using a GEN PAC FAX (Millipore Corp., Milford, MA) high performance liquid chromatography (HPLC) column. The elution conditions for the WAP-hPC product were determined by gradient chromatography over the range 0.5-1.0 M NaCl. An isocratic elution condition was chosen (25 mM Tris-HCl, 1.0 mM EDTA, and 0.63 M NaCl, pH 7.5). Approximately 15 to 20 μg of digested DNA was injected per run, and eluents containing the WAP-hPC fragment from each injection were pooled, precipitated and processed by HPLC a second time. The purity and concentration of WAP-hPC were determined on a 1% agarose gel stained with ethidium bromide.

DNA Extraction

Tail tissue was biopsied from pups which were approximately 20 days old and DNA was isolated from the tissue by a modification of the procedure developed by Marmur (1961). Briefly, 840 μl of lysing solution (50 mM Tris-HCl, 0.15 M NaCl, 1M Na_2ClO_4, 10 mM EDTA, 1% Sodium duodecyl sulfate, 1% 2-mercaptoethanol, 100 μg/ml Proteinase K, pH 8.0) was added to each tube containing a tissue sample which had been previously frozen with liquid nitrogen. The tubes were incubated overnight at 50°C and then extracted with 250 μl of chloroform: isoamyl alcohol (25:1) by mixing for 10 to 15 sec on a "Mini Bead-Beater" (Biospec Products, Bartlesville, OK) followed by centrifugation for 10 min at 15,00 x g. DNA was precipitated by adding 50 μl of isopropyl alcohol to 83 μl of the aqueous supernatant, then centrifuged and washed with 80% ethanol. The pellets were dried at 37°C and resuspended in 50 μl TE (10 mM

Tris-HCl, 1.0 mM EDTA, pH 8.0) and stored at minus 20°C until assayed by Polymerase Chain Reaction.

Polymerase Chain Reaction Analysis

DNA analysis was done by the general method of Saike et al.(1989). One μl of DNA solution was used as template in 25 μl reaction volumes [1X Taq buffer, 2.5 mM dNTP's, 0.5 mM oligonucleotide primers, 0.625 units Taq polymerase (Promega Corp. Madison, WI)]. Primers used to amplify a 402 bp target sequence in the transgene were: WAP-specific sense 5'-GTG GCC AAG AAG GAA GTG TTG, and hPC-specific antisense 5'- GTG CTT GGA CCA GAA GGC CAG. The WAP-specific antisense primer 5'-GAC TTG TTC CTC TAG GTT CTG was also used to amplify a 222 bp fragment contained within the endogenous WAP gene. Initial denaturation was performed at 96°C for 1 min, followed by 40 cycles of: 55°C annealing for 2 min, 77°C elongation for 75 sec, and 96°C denaturation for 15 sec. Amplification products for mouse fetus DNA were run concurrently with those from plasmid DNA on 1% agarose gels stained with 0.5 μg/ml ethidium bromide.

The temperature sequence for PCR analysis of embryos was as follows: 1) 55°C for 30 min to allow proteinase K to digest the embryo; 2) 98°C for 10 min to cause DNA denaturation and also to inactivate proteinase K; 3) 85°C for 10 min to add the PCR reaction, and 4) 45 cycles of 96°C for 15 sec (denaturation), 55°C for 2 min (annealing) and 75°C for 30 sec (elongation).

Experimental Design

A total of 45 transfers were performed (30 embryos per transfer). Fifteen recipients were sacrificed on day 4, 12 and 18 of gestation (Figure 3). All viable embryos and fetuses recovered were analyzed for the presence of the transgene by polymerase chain reaction. Differences among treatments were analyzed by analysis of variance. The independent variable was day of recovery and the dependent variables were pregnancy status, number of viable embryos or fetuses and number of transgenic embryos or fetuses.

RESULTS

Ninety-two of 129 females (71.3%)were mated (Table 10). Fertilization rate of 1,765 zygotes was 90.2%. There were 4.2% unfertilized ova and 5.5% degenerate ova.Ninety-six percent of the zygotes were microinjected with WAP-hPC and 90.6% survived the microinjection procedure before transfer.

Figure 4 shows the developmental stage of microinjected embryos after 4 days of in vivo culture. We were able to recover 61.6% (277/450) of the embryos. Of these, 43.3% (120) were morulae or blastocyst, 22.7% (63) two to four-cell and 33.9% (94) had degenerated. Also recovered were 87 unfertilized ova that were from the recipients and accounted for 23.9 % of all the embryos recovered (87 of 364).

The pregnancy rate was highest at day 4 of gestation (80 %; Table 11), but dropped by day 12 and remained constant through the rest of gestation (60% and 66% for days 12 and 18, respectively). Similarly, the number of viable embryos or fetuses was significantly higher at day 4 of gestation , than at day 12 or day 18 of gestation (10±1.1, 5.1±1.and 2.4±1.3 for days 4, 12 and 18, respectively; $p<.05$). We did not detect a significant difference in the percentage of transgenic embryos or fetuses at each recovery day (Figure 5). However, a high degree of mosaicism was observed on day 18 since 34.6% of the fetuses were transgenic but 56% of the placentae were transgenic.

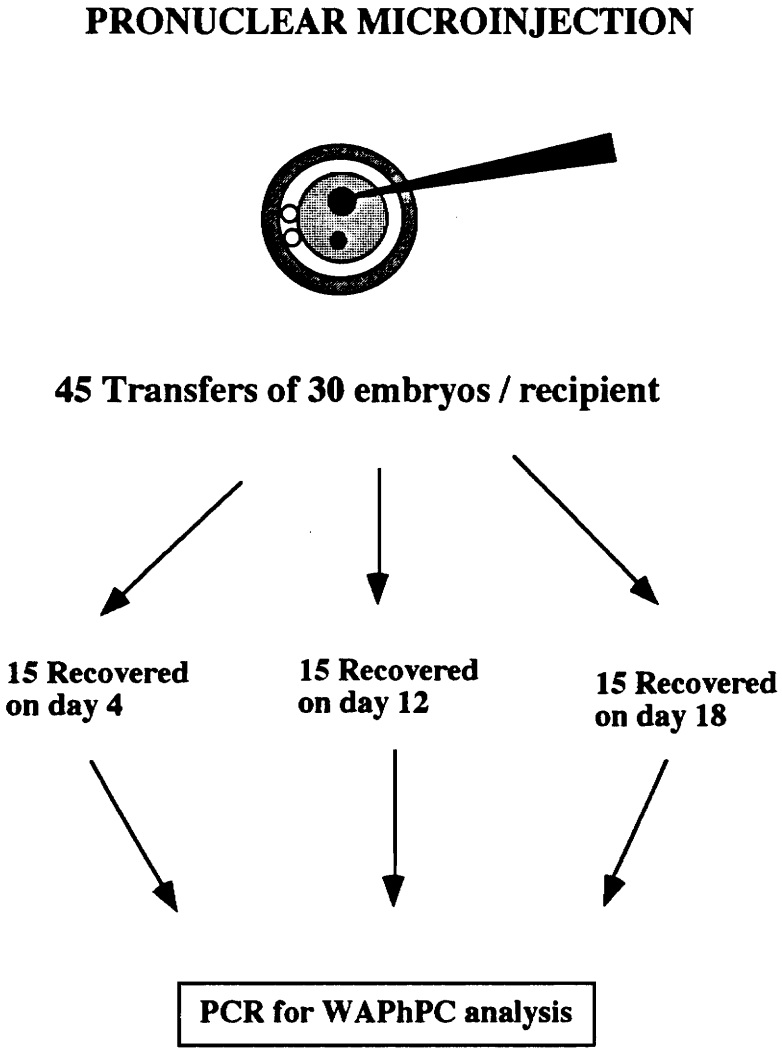

Figure 3. Outline for experiment 3.

Table 10. Superovulation response for experiment 3..

Variable	Number	(%)
Superovulated Mice	129	
Mated	92	(71.3)
Total Embryos	1,765	
Unfertilizad Ova	75	(4.2)
Degenerated	97	(5.5)
Fertilized	1,593	(90.2)
DNA Microinjected	1,528	(95.9)
Survived	1,385	(90.6)
Transferred	1,350	

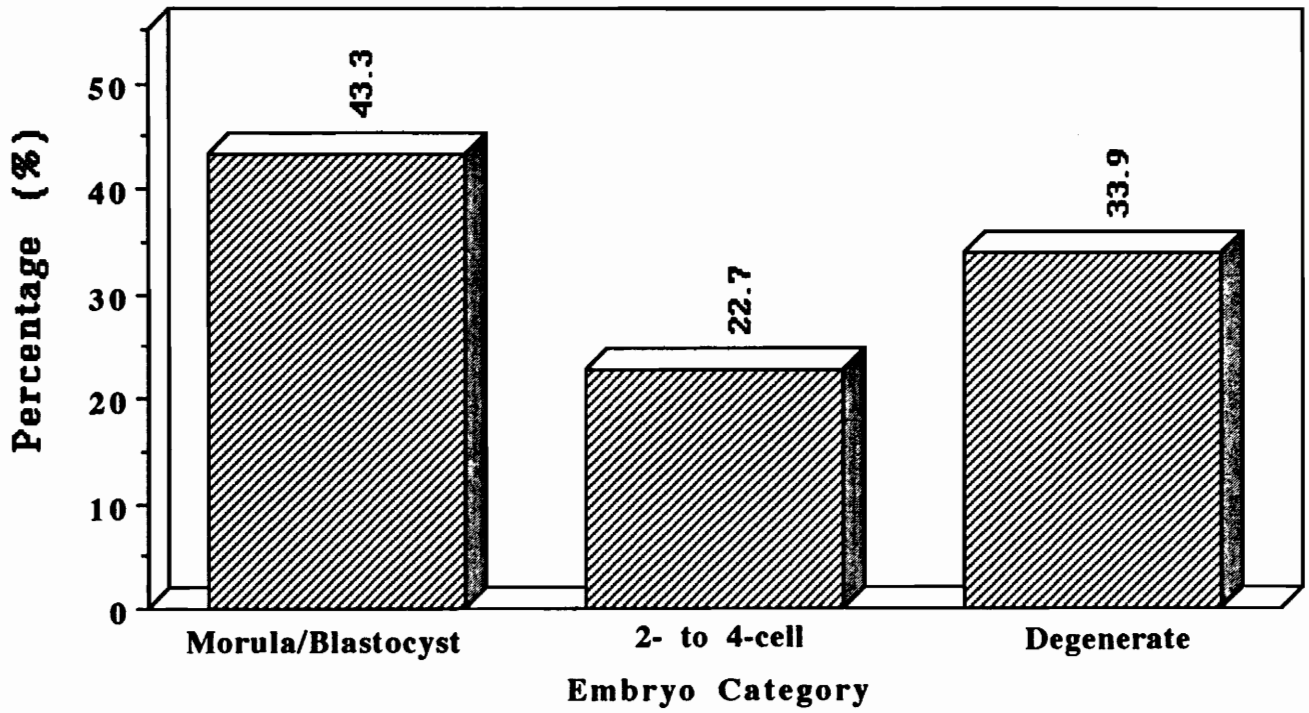

Figure 4. Developmental stage of microinjected embryos recovered 4 days after transfer

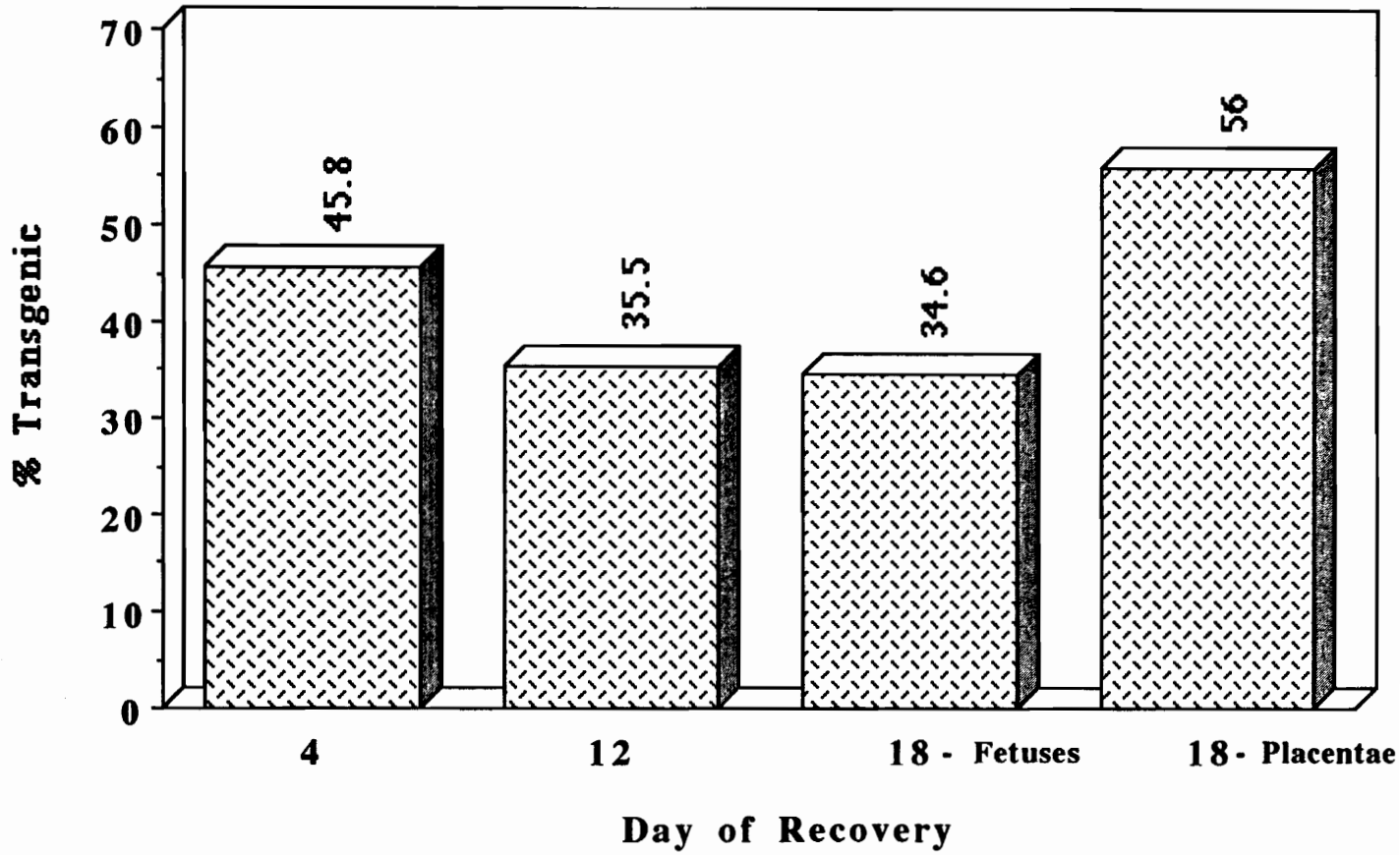

Figure 5. **Percentage of transgenic embryos or fetuses during pregnancy.**

Table 11. Pregnancy rate and number of viable embryos or fetuses recovered on day 4, 12 or 18 of gestation.

Day of Recovery†	4	12	18
Pregnancy Rate (%)	80	60	66
Number of Viable Embryos/Fetuses*	10 ± 1.1^{a}	5.1 ± 1.6^{b}	2.4 ± 1.3^{b}

† 15 Transfers per day of recovery 30 embryos / transfer.
* Least squares means ± SE.
a,b Numbers with different superscripts differ (P<.05).

DISCUSSION

The percentage of embryos recovered at day 4 in our study (61.6%) was similar to that obtained by Brem et al. (1989) after the transfer of centrifuged and microinjected pig embryos (75%) Nevertheless, their survival rate was considerably lower than what we obtained with mouse embryos (12% vs 43.3%). This may reflect a species difference to withstand microinjection and/or DNA integration. Porcine embryos need to be centrifuged to visualize the pronuclei. This additional embryo manipulation may lead to lower viability. However, the studies published on the effect of centrifugation on viability of porcine embryos are contradictory. Some researchers report that centrifugation does not have a significant detrimental effect on embryo viability (Wall et al., 1985), while others have found the opposite effect (Pivko et al., 1992). Moreover, in the report by Brem et al. (1990) embryos that were only centrifuged but not injected with DNA had a lower survival rate by day 4 of gestation than control embryos (47 and 57%, respectively).

The pregnancy rate at day 18 (66%) in this study was similar to that obtained by Brinster et al. (1985) with DNA microinjected hybrid embryos (51/85; 60%). However, they obtained a higher percentage of pups born than with C57BL/6 X SJL mice (11.5% and 5.5%, respectively). A mouse strain effect could account for these differences. Brinster et al. (1985) obtained 7.5% pups born from DNA microinjected embryos using inbred strains. This is similar to our results (5.5%). In addition, the gene construct injected could influence embryo viability after transfer (Voss et al., 1990).

The rate of embryonic loss observed in this experiment falls within the ranges published elsewhere (38.5% to 78.7% for preimplantation embryonic loss; Burki and Ulrich; 1982, Gordon et al., 1987; and 27.0% to 94.3% for post implantation embryonic death; Frels et al., 1985; Brinster et al., 1983). The reason for this is not clear. It has been documented that after DNA microinjection some cellular DNA rearrangements can cause early developmental arrest and post implantation embryo mortality (Covarrubias et al., 1986; 1987). In our study, the percent of transgenic embryos or fetuses was not significantly different during the stages of gestation tested. Therefore, DNA integration can not account for most of the embryonic losses at least

during the gestation periods tested in this experiment. It is possible that other factor(s) associated with microinjection itself may be responsible for the high degree of embryonic losses observed.

The degree of mosaicism observed in this experiment, 35.7% (of 25 pair of fetus and placenta, 9 pair were transgenic and 5 placentae but not the fetuses were transgenic), is higher than that reported in the literature (10 to 20%, Hogan et al., 1986). That may be because most researchers test for mosaicism by analyzing the offspring of all transgenic mice born. Wilkie et al. (1986) reported that after analyzing the offspring of 262 transgenic founders, 25% of them transmitted the transgene to significantly less than half of their offspring. However, when they restricted the analysis to only those founders that had 10 or more offspring, 63 of 171 mice (36%) were mosaic. These researchers also reported that out of 12 pairs of fetuses and placentae tested, 7 were transgenic in both, 1 was transgenic in the fetus only and 1 was transgenic in the placenta only. Wilkie et al. (1986) hypothesized that mosaicism could arise by integration occurring in one of the early cleavage stage cells, followed by unequal segregation of transgenic cells to the trophoectoderm and inner cell mass of the blastocyst. This could cause segregation of the transgene to the fetus or placenta only.

In conclusion, using outbred (CD1) mice, a high degree of embryonic and fetal losses was observed after the transfer of DNA microinjected embryos, and this losses can not be attributed soley to DNA integration.

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CHAPTER VI

GENERAL CONCLUSIONS

In general, the results from these experiments provide more insight on how to improve the efficiency of gene transfer in mice. Whether or not our findings can be applied to farm animals remains to be seen. Previous work by Rexroad et al. (1990) and Walker et al. (1990) suggest that microinjected sheep embryos can be cultured for at least 3 days without decreasing viability significantly. We found the opposite in CD1 mice. Comparing the gestation length of these two species is easy to see that while 3 days is not a large portion of gestation in sheep, it is almost a seventh of the entire gestation period in mice. Usually, culturing the embryos for long periods is detrimental to post-transfer viability (Hogan et al., 1986). It is possible that the interaction of microinjection and prolonged cultured is responsible for the low viability of morula/blastocyst stage embryos that we observed in our study.

Although the numbers of embryos per transfer tested in experiment 1 were not different in their ability to produce pregnancies, we saw a tendency to get more pups when we transferred more embryos. This finding disagrees with that of Castro et al. (1992) who found that 10 microinjected embryos was sufficient to obtain high pregnancy rates. However, these investigators only compared 10, 15 and 20 embryos per transfer which may have not been a wide enough range to detect an effect. We also found from our second experiment that addition of non-manipulated embryos increased pregnancy rate and the percentage of pup born from microinjected embryos However, overall analysis indicated that the number of transgenic pups born decreased as the number of non-manipulated embryos per transfer increased. Therefore, we do not recommend using non-manipulated embryos in gene transfer programs. Brem et al. (1990) found that in pigs, addition of non-manipulated embryos increased pregnancy rate and number of piglets per litter. However, they did not evaluate whether that improved their efficiency to produce transgenic pigs or not.

Finally, the embryonic and fetal survival found in our experiment falls within the ranges reported in the literature for DNA microinjected embryos. Since the percentage of transgenic embryos was not different on day 4 from that of fetuses on days 12 and

18, we conclude that this embryonic mortality is not due, at least totally, to DNA integration. In addition, we found about 35.7% mosaicism on day 18 fetuses and placentae. That is somewhat higher than the 10 to 20% reported by other groups (Hogan et al., 1986; Brinster et al., 1985). A report by Wilkie et al. (1986) is consistent with our results. They mentioned that out of 171 transgenic founders with 10 or more offspring, 63 (36%) were mosaic. The reason that we observed more transgenic placentae than fetuses is unclear.

According to Brinster et al. (1985), microinjector experience is one of the factors that affects the efficiency of gene transfer. In order to evaluate whether we were consistent with the microinjection technique, we compared the survivability rate for the three experiments. The data can be seen in Figure 6. We did not find a significant difference in the percentage surviving microinjection among the three treatments (86.1, 87.4 and 90.6% for experiments 1, 2 and 3, respectively), which gives us confidence with our technique for microinjection

In conclusion, our results indicate that: 1) transfer of 20 to 30 1-cell embryos was the best method to obtain transgenic mice. 2) addition of non-injected embryos decreased the number of transgenic pups obtained per pregnancy, and 3) although most of the embryonic losses after microinjection happen before day 4 of gestation, an additional loss occurs between days 4 and 18 of pregnancy.

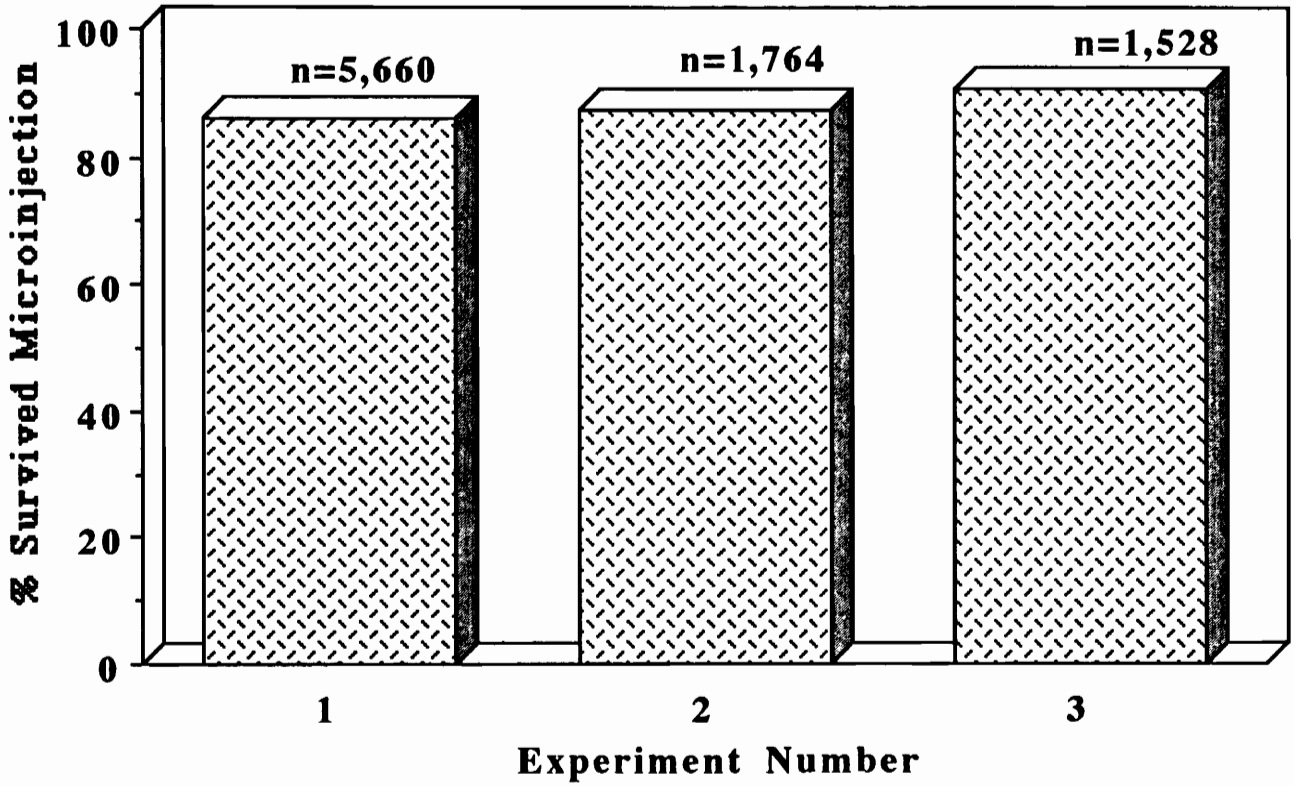

Figure 6. Percentage of 1-cell mouse embryos surviving DNA pronuclear microinjection on each experiment.

CHAPTER VII

SYSTEMS TO IMPROVE THE EFFICIENCY OF GENE TRANSFER

Pronuclear microinjection is the most widely used technique to create transgenic animals, but it requires expensive equipment, technical skill and its efficiency is low (.2 to 10%; Voss et al., 1990). Due to these factors, researchers are always looking for other ways to deliver foreign genes to mammalian embryos. One way to do that is through the use of cytoplasmic injections. This would reduce the mechanical damage that usually kills 20 to 30% of the embryos shortly after microinjection and would allow for better use of the embryos harvested. In addition, embryos from farm animals have dense cytoplasm. In order to visualize pronuclei in these embryos they must be centrifuged. This decreases embryo viability.

Early research by Brinster et al. (1985) indicated that cytoplasmic injection of DNA was extremely inefficient for gene transfer. However, recent research is promising. Powell et al. (1992) investigated the fate of DNA injected into mammalian oocytes and zygotes at different stages of the cell cycle. They reported that in sheep and bovine cells, DNA metabolizing activities appear to increase during oocyte maturation and fertilization. However, DNA metabolism was decreased in the cytoplasm of the metaphase oocyte and zygote. More interesting was the fact that they claimed having preliminary data which shows that foreign cytoplasmic DNA can integrate into the ovine genome. They expressed their intention to pursue experiments injecting DNA at various stages of oocyte maturation and fertilization and at various concentrations to determine the frequency of integration after cytoplasmic injection.

Similarly, preliminary data in our laboratory, seem to indicate that complexing the DNA with some compounds like polylysine increases the residence time of the DNA in the embryo and allows for more chances of integration even if it is injected in the cytoplasm (Ray Page, personal communication). Future research in this area is likely to make cytoplasmic injection a good alternative for producing transgenic animals, especially since it does not compromise embryo viability as much as pronuclear microinjection does.

Another system that may prove very beneficial in the future is the use of embryonic stem cells. Embryologists have been fascinated for a long time with the complex mechanisms underlying the developmental program of multicellular organisms. In mouse embryology, in vitro models have been a prerequisite for the understanding of the molecular processes during development. One major outcome has been the development of embryonic stem cells (ES cells). Embryonic stem cells retain their embryonic properties when cultured properly (Baribault and Kemler, 1989). These cells can then be transfected by retroviruses and injected into a blastocyst. The resulting chimeric animal can then give raise to transgenic offspring. This technique is not new in mice. Nevertheless, ES cell lines have proven difficult to develop in farm animals (Gary Anderson, personal commuication).

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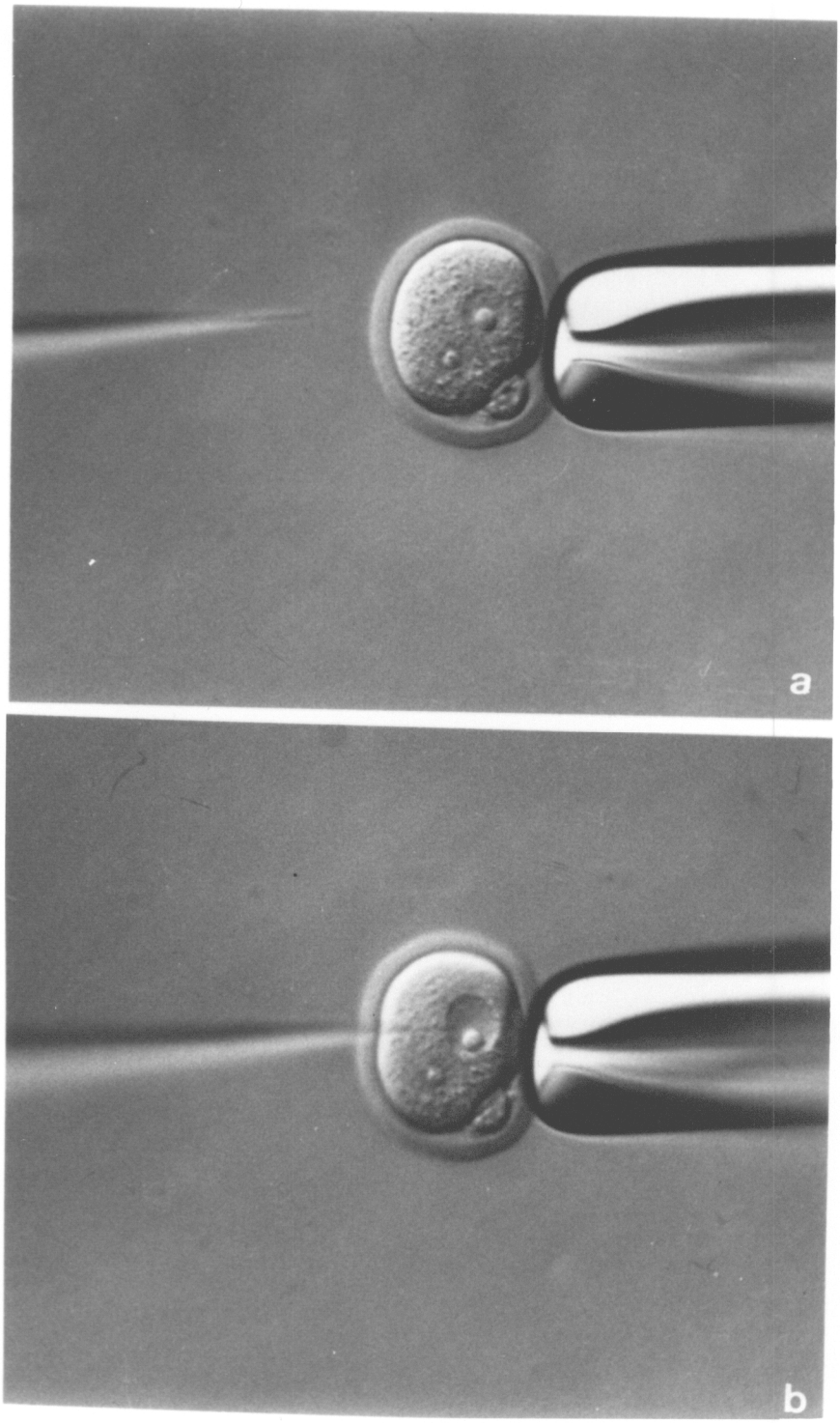
APPENDIX

Figure 7. **a. 1-cell mouse embryo before microinjection.**

b. 1-cell mouse embryo during microinjection.


a
b

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