CONTROL OF ENDOMETRIAL SECRETION IN CATTLE AND PRODUCTION OF TRANSGENIC SWINE

by

Barry L. Williams

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APPROVED:

F. C. Gwazdauskas, Chairman

R. G. Saacke

R. E. Pearson

H. J. Gerken, Jr.

J. W. Knight

W. H. Velander

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(ABSTRACT)

Endometrial tissue was collected from cows to determine effects of day of the estrous cycle, location of the ovulatory structure and progesterone (P_4) on endometrial protein secretion. Day 0 (estrus) endometrial tissue released more protein than tissue collected on d 9, 14 or 18. Protein synthesis was greater on d 0 and 18 than d 9 and 14. Endometrium from the uterine horn contralateral to the ovulatory stucture synthesized more protein than endometrium ipsilateral to the ovulatory structure. Seventeen protein bands were identified by electrophoresis. Proximity of the ovulatory structure to the uterine horn affected the presence of four proteins. Quantitative release of seven proteins was influenced by day of the estrous cycle and uterine horn. Day of the estrous cycle and location of the ovulatory structure alter endometrial protein secretion and synthetic activity and have effects on individual proteins.

A second project utilized 116 gilts and sows to evaluate estrous synchronization/superovulation schemes. Pronuclear microinjection and zygote culture in excised mouse oviducts also was assessed. Synchronization/superovulation procedures were: 1) sows observed for estrous behavior (NAT), 2) cyclic gilts synchronized with altrenogest (ALT) for 15 to 19 d and superovulated with pregnant mares serum gonadotropin (PMSG) and human chronic gonadotropin (hCG; LALT), 3) gilts between 11 and 16 d of the estrous cycle receiving ALT for 5 to 9 d and superovulated (SALT),

and 4) precocious ovulation induced with PMSG and hCG (PRE). Zygotes from PRE donors received microinjection of buffer, DNA or no microinjection. Ova were cultured in modified Krebs Ringer Bicarbonate medium (KRB) or mouse oviduct (MO) explants. SALT and PRE had higher ovulation rates than LALT (24.7 ± 2.9, 24.3 ± 1.8 vs 11.6 ± 2.7; $\bar{x}$ ± SEM). DNA microinjection resulted in a lower ($P<.05$) cleavage index (CI) than buffer injection or no microinjection (2.16 ± .10 vs 2.80 ± .13 and 2.93 ± .10). MO improved ($P<.01$) CI over KRB. MO culture for 72 h was the most beneficial system ($P<.05$; CI 3.25 ± .12). CI of 2.66 ± .18, 2.79 ± .14 and 2.40 ± .14 were observed from MO for 48, 96 and 120 h, respectively. Transfer of 505 DNA microinjected zygotes into 17 recipients produced seven litters and 50 piglets of which eight were transgenic. Microinjection of DNA, not merely the mechanical procedure, was detrimental to embryo development and culture for 72 h in MO provided optimal CI.

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No one who has attempted to complete a Ph.D. can honestly say that it was a fun and easy undertaking. Parts of it are fun and some things are easier than others, but the only way to survive those "other" times is with the support of your friends, peers, and loved ones. My degree has not been easy. There were many difficult times - times of failure, of self doubt, of fear, and times when I had made the decision to throw in the towel. You cannot survive such times without finding great strength within yourself, but even this will often not be enough. You must draw strength from those around you, those who are your friends and supporters. Only true friends will understand and stand by you during those times when stress and the behavior it causes are pushing away many "fair-weather" friends.

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insight and care for students is only surpassed by his wisdom and knowledge of reproduction. Dr. Pearson has given countless hours of more than just statistical advice, he also took the time to explain why things were done the way they were. That is not an easy task when the student often feels that statistics are a curse that originated when the first researcher, named Adam, altered his data by eating some of his observations in an apple production study.

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my distress and suffered through much of it. Yet she knew it would end and tried to accept things until then. There are not the right words in my vocabulary to tell her what she has and continues to mean to me. She is a wonderful person, she has to be to put up with me.

If a man's greatness was judged by the sum total of his friendships and the qualities of his friends and family, I would be the greatest man on earth. I already know I'm the luckiest.

Thanks to you all. This dissertation is not mine, it is made up of all of you. I hope it is a just tribute.

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SYNCHRONIZATION, SUPEROVULATION, AND COLLECTION OF PORCINE OVA FOR DNA MICROINJECTION, TRANSFER AND CULTURE

Chapter 1

Introduction

In the livestock industry, it would be desirable to have every conception produce a healthy offspring. Sreenan and Diskin (1986) have found conception rate to be 88 and 90% for heifers and cows, respectively. Average calving rate was 55%. Thus 38% of potential offspring were lost after fertilization. It is obvious that embryonic and fetal losses have a significant impact on the economics of cattle production.

Linares (1982) found that repeat breeder cattle did not differ from first service heifers in fertilization rate. However, only 28% of the repeat-breeders had normal embryos by d 7, while 74% of the first service heifers had normal embryo. Repeat-breeders were found to have uterine protein profiles which differed from normal breeding cows (Guise and Gwazdauskas, 1987). These data suggest that an appropriate uterine environment is necessary for embryonic survival.

Uterine secretions changed in a cyclic manner (Fischer and Beier, 1986) and progesterone (P_4) was associated with variation in protein secretion (Anderson et al., 1986). These changes determine if the uterine environment is appropriate to sustain development of an embryo. The ability of the uterine environment to affect embryonic development led to the hypothesis that specific proteins vary during the estrous cycle, and may be under differential control based on site of protein production and hormone concentration. The objectives of this study were to determine the protein profile of endometrial explants and to identify factors that alter the profile of endometrial protein secretion.

Three areas were addresed in the second part of this dissertation, all centering on improvement of production of transgenic swine. The first was the control of the estrous cycle. Estrus in swine can be controlled by progestogen compounds (Baker et al., 1954; Gerrits et al., 1963; Day, 1984; Ebert et al., 1988), prostaglandin (PG) $F_2\alpha$ (Diehl and

Day, 1974) or weaning (Marinat-Boote et al., 1985). The above procedures often resulted in impaired fertility and/or inappropriate timing of estrus. Inappropriate timing of ovulation with synchronization compounds can be overcome with gonadotrophins (Dziuk and Baker, 1962). However, synchronization procedures may produce ova with different viabilities (Pinkert et al., 1989). Therefore in this study we intended to assess methods of synchronization and superovulation.

The second area of investigation was the in vitro control of embryonic development to the prehatching stage. Many factors, including energy and protein source, types of buffer and gaseous atmosphere, affect the ability of an embryo culture system to support development (Wright and Bondioli, 1981; Kane, 1987). Oviductal secretions were beneficial for development of one- and two-cell porcine embryos (Archibong et al., 1989). Krisher et al. (1989b) cultured porcine zygotes in explanted mouse oviducts. They reported that this trans-species system provided the embryos with an appropriate environment for development; however, the in vitro requirements of manipulated ova have not yet been determined. Therefore, in this study porcine embryo development was evaluated at varying times in a mouse oviduct environment.

Thirdly, the aspects of physical manipulation on development of porcine embryos was evaluated. There is the likelihood that certain embryos are more sensitive to environmental changes than are others and the affect of physical manipulation on early embryonic development is still unclear. French et al. (1991) observed small litter sizes following transfer of DNA microinjected porcine ova. The time at which embryonic death occured, resulting in small litters was not known. This suggests that manipulated embryos may be less viable than non-manipulated embryos. Manipulated embryos may need a medium more conducive to in vitro development.

The production of transgenic mice has become a rather routine procedure since 1981, when production of the the first transgenic mouse was reported (Gordon and Ruddle, 1981). The production of transgenic domestic animals is currently very inefficient (cattle - Biery et al., 1988; sheep - Murray et al., 1989; swine - Pursel et al., 1990). Short term in vitro culture and evaluation of manipulated embryos would help determine the timing and extent of decreased viability of these embryos. It also would facilitate testing of embryoss to determine if microinjected genes have been integrated. These evaluation and testing procedures would increase the efficiency of transgenic domestic animal research by providing the researcher with the ability to transfer only viable transgenic embryos.

The objectives of this research were as follows: 1) to evaluate synchronization and superovulation procedures for their overall suitability for the production of transgenic swine; 2) to evaluate the timing and extent of damage caused by pronuclear microinjections; and 3) to assess the effect of mouse oviduct co-culture on embryos receiving different microinjection treatments.

Chapter 2

Review of Literature

Control and Importance of Uterine Protein Secretion

Extensive research has shown that uterine secretions contain unique proteins of endometrial origin. Fluctuations in uterine protein synthesis were correlated with plasma steroid hormone levels. The exact role which unique uterine proteins play in early conceptus development is not fully understood. (Roberts and Bazer, 1988). It is known that interactions between the early conceptus and mother are mediated via these proteins (Malayer et al., 1988). The environment in which the embryo develops influences its survival. If the uterine milieu is inadequate, embryonic death is likely (Fischer and Beier, 1986).

There are significant similarities between the proteins secreted by the bovine endometrium and those in the serum. There are, however, proteins specific to the uterus which are present in minor quantities (Roberts and Parker, 1974). Five to seven uterine specific proteins were observed by three different protein separation procedures (Anderson et al., 1985). The proteins covered a wide range of molecular weights with three to four weighing less than 13,700 and others with weights of approximately 25,000, 60,000 and greater than 160,000. Although there were isolated occurrences of individual proteins being absent at specific stages of the estrous cycle, no apparent trend towards major changes in the protein milieu were observed. There did appear to be a change in the quantitative distribution of the proteins at different stages. In addition to changes in specific proteins, the overall quantity of protein harvested from the uterus by flushing also changed during the estrous cycle. The greatest protein recovery occurred on d 0 and 15, with 18 and 26 mg of protein being recovered. This compares to d 5 and 10 of the estrus cycle with 8 and 9 mg. Differences in protein production during the estrous cycle were observed in the oviduct (Killian et al., 1989). Total protein recovered from

cannulated bovine oviducts was greatest during the non-luteal period. However, protein concentration was lowest during this period because of a substantially higher rate of fluid production which served to dilute the protein present. Differences in uterine proteins also have been described in the pig. Uterine protein production in the pig remained low (< 10 mg) during the first 10 d of the estrous cycle then increased rapidly to approximately 45 mg by d 15. Protein quantity then deceased to the lower levels by d 17 (Murray et al., 1972). Differences were seen in the distribution of the specific proteins present, but more importantly, two specific protein fractions were found only between d 9 and 16. These intermediate size proteins had molecular weights of 45,000 and 20,000. Electrophoresis was used to further describe these protein differences (Squire et al., 1972). The 45,000 MW fraction migrated as a single band under electrophoresis while the 20,000 MW fraction actually consisted of six proteins ranging from 15,000 to 20,000 MW. All of the proteins were luteal phase specific and secretion stopped at d 16 of the estrous cycle. Initiation of secretion varied between d 9 and 14.

The presence of uterine specific secretions and their changes relative to the estrous cycle are obvious. The timing of such changes would suggest that their secretion is under the control of steroid hormones. Using steroid hormone supplemented ovariectomized cows, Anderson et al. (1986) found nine uterine proteins, the presence of which appeared to be dependent on steroid treatment and P_4 concentration. Ovariectomized gilts treated with exogenous steroids were used to evaluate uterine protein secretions in swine (Knight et al., 1973). It was observed that P_4 treatment, with or without estrogen, resulted in a protein profile similar to that of intact control animals and that the lack of supplemental P_4 resulted in the absence of two of five standard porcine uterine secretion fractions. These findings imply that the hormonal changes that control the estrous cycle

also regulate the protein synthetic action of the endometrium. However, this represents a broad systemic control and does not consider local affects and modulation.

It has been observed that the addition of bovine conceptus secretory proteins shifted endometrial synthetic activity from a secretory process to one of synthesis of tissue proteins. The change was somewhat selective in that specific uterine proteins were produced at a higher rate in the presence of conceptus proteins (Gross et al., 1988). The proteins which make up the conceptus secretory proteins appear to have individual actions. Conceptus proteins, in general, enhanced the production of an endometrial protein (14,900 MW) while a component of the conceptus proteins, bovine trophoblastic protein-1, had no effect (Helmer et al., 1989). The specificity of individual conceptus proteins to control uterine secretions provides the opportunity for a wide range of endometrial secretions to respond to changing local conditions.

Various factors have influence on the uterine environment and as such they are involved in early embryonic mortality. These factors include nutrition, external environment, reproductive status and endocrine profile. Under-nutrition in heifers did not influence embryonic mortality but suppressed circulating P_4 (Hill et al., 1970). Later research by Spitzer et al. (1978) contradicted these findings. They observed that nutritional restrictions led to embryonic death but did not alter P_4. Nutritional restriction during the first 10 d of gestation in pigs was found to increase embryo survival (Dyck and Strain, 1980). Nutritional excess increased embryo losses due to changes in the uterine environment (Bazer et al., 1968). Edey (1976) hypothesized that embryo loss due to overfeeding was the result of heat stress, while Wilmut and Sales (1982) postulated that losses were due to the induction of asynchronous embryonic development.

Overall, heat stress decreases fertility in cattle (Sreenan and Diskin, 1983). Biggers et al. (1987) found that the conceptus from heat stressed cattle had a reduced weight on d 17 and suggested this increased the rate of embryo death. Elevated temperature prior to breeding did not effect embryo survival in gilts (Edwards et al., 1968). However, heat stress during the first 15 d of pregnancy resulted in smaller litters at d 30 (Omtvedt et al., 1971).

External environment also alters uterine protein environment. Heat-shock specific bovine endometrial proteins with molecular weights of 72,000 and 90,000 have been reported (Malayer and Hansen, 1990). These proteins were induced in vitro at a temperature of 43°C and were present in endometrial samples of both Holstein and Brahman cows. There was an increase in overall protein secretion due to hyperthermia in both breeds. Additional work by this group using Brahman-cross cows considered the relationship of heat-shock (43°) to day of the estrous cycle and side of the reproductive tract (Malayer et al., 1988). It was observed that hyperthermia reduced the secretion of seven specific proteins in the uterine horn ipsilateral to the active ovary while no alteration in secretion was observed on the contralateral side. In general, secretion of individual proteins tended to be greater on the ipsilateral side under homeothermic conditions with the greatest difference occurring on d 0 and 2 of the estrous cycle. These findings indicate differences in secretion due to proximaty to the active ovary and such control mechanisms are interactive with an external environmental stimuli - heat-shock. In contrast, elevated temperature (42°C) did not effect either protein secretion or tissue protein synthesis in gilts (Gross et al., 1989).

Cyclic changes in the uterine protein environment influence embryonic survival. Primarily through the use of embryo transfer studies, it has been found that placing an

embryo into an uterine environment at a stage different from which it came will often result in the death of that embryo (cattle - Rowson et al., 1969; Gordon, 1982; swine - Pope et al., 1986; sheep - Wilmut et al., 1986; Moore, 1982). The uterus does possess the ability to alter the growth rate of embryos which result from an asynchronous transfer. It was observed in sheep that d 4 embryos placed into a uterine environment at a stage 2 to 3 d earlier were retarded in development and were unable to develop past the early blastocyst stage. When 4 d old embryos were transferred to an environment 2 to 3 d more advanced, embryo development accelerated in an attempt to come into synchrony. However, development past d 12 was retarded resulting in embryo loss (Lawson et al., 1983). In general, synchrony between the embryo and mother must be maintained within 1.5 d in cattle (Seidel, 1980) and 2 d in sheep (Moore, 1982).

It is evident that there is a limited amount of embryo-uterine asynchrony that will allow for continuation of embryonic development and that the uterine environment does have the inherent ability to alter the rate of embryonic development to produce synchrony. However, it is also evident that the embryo is affected by an alteration in the normal rate of development since death often occurs when such changes have been thrust upon it.

Heat and nutritional stress are gross factors be related to embryonic mortality. More subtle changes in reproductive function can also lead to ovum loss. Infertility in repeat breeder cattle has been studied. Linares (1982) found that 28% of embryos recovered from repeat breeder heifers on d 7 had normal morphology compared to 74% in first service heifers. He theorized that the abnormal embryos resulted in increased embryonic death. The uterine protein profile of cows which were classified as repeat-breeders had minor yet significant differences from the protein profiles of cows of normal fertility (Guise and Gwazdauskas, 1987). It is possible that these differences were due to an al-

tered plasma P_4 profile but more importantly, there was strong evidence that embryo loss occurred due to endogenous aberrations in the maternal environment. A difference in steroid hormone profile was found between repeat and normal breeding cows (Ayalon, 1978). Repeat-breeder cows with abnormal embryos had higher estrogen concentrations during the first 4 d post breeding. No difference was seen in P_4. Erb et al. (1976) found elevated estrogen in repeat breeders. Differences in P_4 action may be occurring at the tissue level. Almeida et al. (1987) found that repeat breeders or normal breeding cows carrying an abnormal embryos had elevated P_4 receptors.

Research in swine also reveals embryo death coinciding with alteration of uterine protein environment (Gries et al., 1989). Uterine secretions were altered by estrogen injection on d 9 and 10 of pregnancy. The resulting endometrial protein profile indicated a significant decrease or loss of specific proteins from the milieu. Embryo demise also resulted from this treatment. In neither case, bovine or porcine, can alteration of protein patterns be directly linked to embryonic loss. Asynchrony should not be defined by changes in specific components. It is the overall composition of the uterine luminal fluid and their cyclic changes which determine the appropriateness of the uterine environment (Fischer and Beier, 1986).

Control of the Estrous Cycle in Swine

Systems for estrous synchronization in swine are not as well developed as they are for cattle. However, the development of such procedures would be beneficial to the swine industry for various reasons. These include decreasing the length of the breeding period, more efficient use of artificial insemination, improved use of facilities and decreasing or

eliminating the need for estrous detection, a very time consuming and laborious task. Various procedures have been evaluated for the synchronization of estrus in swine. These include simple management procedures such as group weaning at a specific time, the use of P_4 or other compounds with progestogen activity, as well as the use of prostaglandins, gonadotophins, estrogens or combinations of these compounds. The effectiveness of these procedures in inducing estrus during a specific period and the duration or length of this period is quite variable.

The management practice of weaning a group of sows at the same time to synchronize estrus is commonly used in the industry. Marinat-Botte et al. (1985) found that 89% of multiparous sows were in estrus 3 to 9 d after weaning compared to 76% of primiparous sows. The age of the piglets at weaning had an effect on the precent of sows displaying estrus. Weaning prior to the 15th day of lactation or after the 41st day resulted in a delay in return to estrus. However, an increased lactation period did increase the number of piglets born on the subsequent farrowing.

A number of progestogen compounds have been used to control the estrous cycle and induce estrus. Ulberg et al. (1959) administered P_4 for 15 or 19 d at rates between 12.5 and 100 mg/d. The lower level of P_4 did not inhibit estrus while 25 mg P_4/d or greater inhibited estrus during this period. The fertility of the estrus following P_4 was variable. Baker et al. (1954) found that estrus following P_4 control was infertile at 25 mg P_4 and fertility was low when a 100 mg P_4 was used for 13 to 18 d. This is in contrast to the work by Gerrits et al. (1963) who found that 100 mg P_4/d provided a synchronized estrus with a conception rate to natural service of 82%. When 300 mg of P_4 was given every third day, the conception rate (80%) was similar to the daily P_4 treatment.

The synthetic progestogen, 6-methyl-17-acetoxyprogesterone (MAP) consistently inhibited estrus and ovulation when dosages of 100 to 400 mg were given daily. However, 58% of the animals had one or more cystic follicles. A problem in this study was that it failed to take into account weight differences among animals. When MAP was given at a rate relative to body weight, a dosage of 0.5 mg/lb/d was found to give a high degree of control of estrus and ovulation. Ninety-eight percent of animals treated were in estrus 4 to 5 d later. Seventy-seven percent of these animals conceived to this controlled estrus (Nellor et al., 1961).

The synthetic progestogen, 17β-hydroxy-17αpropadienylestr-4,9-diene-3one (SA-45249) inhibited estrus. Dosages ranging from 3 to 24 mg/d were studied. The 3 mg range produced a high occurrence of cystic follicles, while the higher dosages produced fewer cysts. There appeared to be a dose related effect on the time to synchronized estrus. It was found that a 1 mg increase in dose resulted in an increase to estrus of .1 d. Ninety-three percent of the animals studied showed estrus at a mean interval of 6.5 $\pm$ 1.7 d after the end of SA-45249 treatment (Mayer and Schutze, 1977). The synthetic progestogen, 1αmethylallyl thiocarbamayl-2-methythiocarbamoyl hydrazine (MATCH; ICI-33828), effectively controlled estrus over a wide dosage range when started at various times during the estrous cycle. This compond had no affect on corpra lutea (CL) number, litter size or conception rate (Stratman and First, 1965; Gerrits and Johnson, 1965). This compound was later withdrawn from the U.S. market because of possible teratogenic effects.

The use of 6-chloro-Δ^6-17-acetoxyprogesterone (CAP) was studied at dosages ranging of 3.25 mg to 540 mg/d (Wagner and Seerley, 1961). Estrus was inhibited at 16.5 mg/d but follicular development was not. Dosages of 25 to 50 mg were needed to both inhibit

estrus and follicular development. Dosages in the range of 32 to 50 mg provided optimal synchronization of estrus between 3 to 7 d after withdrawal. Higher dosages were associated with an increased occurence of cystic follicles. The ability of CAP to produce fertile estrus was tested when it was administered in conjunction with diethylstilbestrol (DES). After a 10 d priming period with DES, CAP was administered for 10 d. This resulted in an 87% synchronization rate 6.2 ± 1.2 d later. Seventy-five percent of those animals bred on the controlled estrus conceived. The synthetic progestogen, 17αacetoxy-6-methylpregna-4,6-dien-3,20-dione (AMP) inhibited estrus in 95% of the animals studied when administered at a rate of .66 or 1.12 mg/kg BW for 15 d. However, the lower dosage produced a higher incidence of cystic follicles. When AMP was given in conjunction with the pretreatment of 3-methyl ether of ethynyl estradiol (MEE), 94% of the animals displayed estrus 3 to 10 d after treatment. When bred to this estrus, 85.6% of the animals conceived. This compared favorably to the controls (Pond et al., 1965).

Altrenogest also has been used to control estrus in swine. This compound has been referred to by many names in previous publications including the designations A-35957, RU-2267, allyl trenbolone and the brand name Regu-mate. Altrenogest suppressed follicular development but did not alter the life span of the CL when fed at levels of 15 to 20 mg/d (Day, 1984). Webel (1980) found that when altrenogest was administered at a dosage of 15 or 20 mg/d for 18 d, 82% of all animals treated showed estrus over a 3 d period. Estrus occurred sooner in the 15 mg treatment (5.1 ± 1.4 d) than in the 20 mg treatment (5.7 ± 1.2 d). Similar results were reported by Davis et al. (1980) who found an 87% occurrence of estrus within 2 to 7 d in animals treated with 12.5 mg/d for 19 d. There was no effect on fertilization rate when animals were bred to this estrus. Litter size that resulted from breedings during an altrenogest synchronized estrus was

slightly higher (9.6 piglets/litter) when compared to controls (8.4 piglets/litter; Webel, 1976). This study utilized 15 mg altrenogest/d for 18 d. Animals treated with altrenogest exhibited estrus over a 4 d period ranging from the fourth to seventh day after the end of altrenogest treatment (Pursel et al., 1981). A total of 96% of the animals examined showed estrus during that time, while 76% of the animals exhibited estrus on the fifth or sixth day post-treatment. The presence of cystic follicles in altrenogest treated animals was dependent on the dosage. A dosage of greater than 10 mg/d was needed to prevent of occurrence of cystic follicles. Using a 10 mg dose, Kraeling et al. (1981) found that there was 22% occurrence of cystic follicles. This dose produced the highest ovulation rate (16.4 ± .9) as compared to a 20 mg dose which had no follicular cyst but decreased ovulation rate (15.0 ± .8). This suggests that a dosage in the range of 10 to 20 mg optimizes ovulation rate while decreasing the possibility of cystic follicle occurrence. Davis et al. (1976) considered fertilization rate in animals which had been treated with altrenogest at a rate of 12.5 mg/d for 19 d. Altrenogest treatment did not decreased fertilization rate. A fertilization rate of 99.5% was found in altrenogest treated animals and was comparable to controls (99.6%). These results were confirmed by Knight et al. (1976).

Results of controlled laboratory experiments are not always duplicated in field situations. However, the use of altrenogest in field conditions has been sucessful. In a field trial, 87% of altrenogest treated animals that were bred on the synchronized estrus farrowed with an average litter size of 9.0 piglets. This compared to control animals which had a 59% farrowing rate with 9.6 piglets (Mauleon et al., 1976).

Prostaglandin $F_2\alpha$, which is commonly used to synchronize estrus in cattle, is not widely used in swine because the CL is nonresponsive to this hormone during the early stages

of the luteal phase of the estrous cycle. $PGF_2\alpha$ will not induce luteal regression until approximately d 11 or 12 of the estrous cycle in swine. The CL of early pregnancy is sensitive to $PGF_2\alpha$ regression (Diehl and Day, 1974; Gleeson, 1974; Guthrie and Polge, 1976a; Lindloff et al., 1976). The use of hormone pretreatment increased the effectiveness of $PGF_2\alpha$. The induction of accessory CL with pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) allows for the controlled luteolysis using $PGF_2\alpha$ 12 d after hCG treatment (Guthrie and Polge, 1976b). Using estradiol to extend CL life, followed by $PGF_2\alpha$ injection approximately 5 d later, also serves to increase the efficiency of $PGF_2\alpha$ induced estrous synchronization (Guthrie, 1975; Kraeling and Rampacek, 1977). Day et al. (1965) reported that estrus could be synchronized without $PGF_2\alpha$ simply by using PMSG and hCG to induce accessory CL. These CL will naturally regress an average of 21.9 d later.

Of the synchronization procedures discussed, all resulted in a range in the occurrence of estrus of several days. While the percent of animals exhibiting estrus, fertility and litter size may be equal to that of control or nontreated animals, if the desire is to closely regulate the time of ovulation, these procedures are not sufficient.

Gonadotrophins have been used to more closely regulate the timing of ovulation. Dziuk and Baker (1962) found that 94% of animals ovulated within 48 h after injection of hCG. However, only 4% of these animals had shown estrus during that time. This system utilized a MAP pretreatment for 8 to 10 d. In an attempt to use gonadotrophin releasing hormone (GnRH) to stimulate endogenous gonadotrophin release, Baker et al. (1973) used prepubertal gilts that had been stimulated with PMSG to induce follicular development. They found that 1 mg GnRH resulted in ovulation in only 50% of the gilts within 48 h. No ovulation was seen when .5 mg or less was used. Using a similar pro-

cedure for the induction of fertile estrus in lactating sows, Guthrie et al. (1978) found that fertile estrus could be induced using PMSG and hCG followed by .5 mg of GnRH. Eighty-five percent of the animals treated in this manner ovulated and 83% of the ovulating animals produced ova that were fertilized.

Various combinations of PMSG and hCG have been used to induce a fertile estrus in prepubertal gilts. The use of PMSG or hCG alone are very inefficient in inducing estrus and ovulation in gilts. When PMSG and hCG were administered together 75% of the animals treated showed estrus, while 88% of the animals ovulated. Eight-six percent of those animals that ovulated had fertilized ova (Baker and Rajamahendran, 1974). Using the same treatment of 400 IU PMSG plus 200 IU hCG administered together, Schilling and Cerne (1972) found that 93 to 100% of animals treated showed estrus 3 to 7 d later. Eight-two percent of the animals showing estrus conceived to first service. Guthrie (1977) compared 400 IU PMSG administered alone to the use of PMSG plus 200 IU hCG. Both treatments were followed 72 h later by a single injection of 500 IU of hCG. In this study, the number of animals that exhibited estrus and ovulated was not different between treatments. There was an increase in the number of CL in the PMSG plus hCG followed by hCG treatment (18.5 CL) compared to the PMSG followed by hCG treatment (13.5 CL). In addition, when GnRH was administered instead of the second dose of hCG there tended to be a slight increase in the number of CL present in both treatment situations.

The quantity of ova harvested from prepubertal gilts that had been stimulated by PMSG was considered by Baker and Coggins (1968). They found that increasing dosages of PMSG produced a linear increase in the number of ovulations. There was an increase in the number of immature oocytes and the percent polyspermy after fertilization with

increasing doses of PMSG. Seventy-six percent of the ova collected were considered normal while 6% were polyspermic and 7% immature. Dziuk and Gehlbach (1966) conducted a series of 13 different trials to determine optimal PMSG dosage for induction of ovulation in prepubertal gilts. In dosages ranging from 250 to 1000 IU PMSG, administered either once or twice, no apparent difference was seen in the number of gilts which ovulated. In most trails, 100% of the animals treated ovulated. There was no difference in the mean ovulation rate per gilt regardless of dosage or one or two injection times. Ovulation rate in the study was lower than previous reports ranging from 4.2 to 10.2 ovulations per gilt. Fertilization rate was not different when these gilts were bred during the induced ovulation period.

The development of follicles from immature gilts that were stimulated to precocious ovulation using PMSG and hCG was different from follicular development of naturally cyclic gilts. There was variation in both follicular fluid volume and follicle diameter. In addition to these morphological differences there was a difference in the biochemical development of these follicles. Follicular fluid from induced follicles tended to have a lower estrogen content while having an increased testosterone content as compared to follicles from naturally cycling gilts. This difference was most evident in the population of larger follicles, these being follicles greater than 6 mm in diameter (Wiesak et al. 1990). Pinkert et al. (1989) evaluated ova from immature stimulated gilts for their development in vitro. Ova collected from stimulated prepubertal gilts had an inferior ability to develop in vitro as compared to ova from mature gilts that were cultured under the same environment.

Embryo Culture Systems

Defined vs Undefined Culture Media

Procedures for the culture of mammalian ova date back many years. In 1913, Brachet used in vitro procedures to culture and observe rabbit blastocysts (Brachet, 1913). Culture media based on animal fluids, primarily that of blood and follicular fluid, was used for embryo culture for the next 40 or 50 yr (Wright and Bondioli, 1981). Such natural or complex media showed very poor development of the embryo. By 1957 the use of defined media became a viable alternative when Whitten (1957) cultured two-cell mouse embryos to the blastocysts stage using a simple salt solution supplemented with bovine serum albumin (BSA). In domestic animals, Moore (1970) cultured two- to eight-cell sheep embryos in a basal salt solution. Forty-eight percent of these embryos cleaved at least once. There was no difference in the cleavage rate of the embryos when 1 mg/ml of BSA was added. The addition of 15% sheep serum to the medium resulted in an increased cleavage rate of 66%. In comparison, 56% of the embryos cleaved when sheep serum was used alone. In other domestic animal embryo culture research using defined media, Sreenan et al. (1968) compared the use of the minimal essential medium (MEM) to various media based on biological fluids. These researchers reported that 65% of one- to eight-cell bovine embryos cleaved in culture while no development was observed when bovine serum or a serum-saline medium was used.

Embryo Development in Common Culture Media

Since the introduction of defined or partially defined culture media for mammalian embryos was introduced 20 to 30 yr ago, research has been conducted on the various

chemical constituents and environmental factors associated with these culture media for their consequence on embryo development. The goal of such research was the development of the perfect medium for in vitro development of mammalian embryos. The success of embryo culture is dependent on more than the culture medium alone since different researchers using the same medium and similar procedures will find a great difference in embryo development (Wright and Bondioli, 1981). For the culture of eight-cell mouse embryo, Whittens and Eagles media were found to be superior to Ham's F10 for expansion and hatching. The addition of 10% serum improved results for all media (Biery and Kraemer, 1988). Using Whitten's medium containing 1.5% BSA, Beckmann et al. (1990) found that 89% of one- to two-cell porcine embryo could be cultured to the five-cell or greater stage within 96 h of culture. Tervit et al. (1972) developed a culture medium referred to as synthetic oviduct fluid (SOF). This semi-defined medium was supplemented with BSA and resulted in the development of early sheep embryo to the morula stage 94% of the time. This compared to a 50% development of bovine ova to the morula stage in this medium. When SOF was compared to a simple phosphate buffered saline (PBS) medium enriched with 20% heterologous sheep serum and 10% fetal calf serum, embryo of different ages responded differently. For sheep embryos collected on d 3 post-estrus, SOF resulted in an increased cleavage rate and increased viability of the embryos after transfer compared to the enriched PBS. When embryos were collected on d 5 post-estrus there was no consistent difference between SOF and enriched PBS on in vitro development. SOF resulted in increased pregnancy rates after transfer into recipient ewes. Such a finding indicates that evaluation of embryos in vitro is not representative of their actual viability to produce pregnancy (Tervit and Goold, 1978).

Differences in Media Requirements between Animals

Not only has it been found that different species require different media for optimal development but different strains within a species also require different media for optimal embryonic development. Using a chemically defined media supplemented with BSA, Whitten and Biggers (1968) found that the strain of mouse had a large effect on the development of zygotes to the blastocysts stage. Furthermore, Wright et al. (1978) found that different strains of mice would also react differently to varying levels of BSA within the same basic culture media. These differences in mouse strain requirements may not be consistent throughout the development of the embryo. Strains of mice which exhibit a block of development at the two-cell stage do not develop well in the presence of glucose. Using CZB medium which contains glutamine but not glucose, it was found that the addition of glucose to the medium on d 3 produced optimal development in mice exhibiting the two-cell block. However, when this was compared to mice which do not exhibit two-cell block, optimal development was found when both glutamine and glucose were present in the CZB medium from the onset of culture. The level of glucose did not appear to be a limiting factor since a wide range of glucose added on d 3 resulted in very little difference in development for embryos that normally exhibit the two-cell block of development (Chatot et al., 1990).

There was a significant effect of donor in the ability of porcine embryos to develop past the one- to two-cell stage when exposed to glucose and/or glutamine nutrient systems (Peters et al., 1990), suggesting individual metabolic differences even at this early stage of development. An embryo donor difference also was observed by Pinkert et al. (1989). Embryos from mature gilts developed significantly better from the one- to two-cell stage than embryos collected from stimulated prepubertal gilts. Changes in culture media altered the rate of development of embryos of various species and even various strains. However, culture did not change specific physical characteristics compared to in vivo

embryos at the same stage of development (Lindner and Wright, 1978). The overall diameter was not different between in vitro and in vivo developed embryos. Similarly there was no difference in zona pellucida thickness at any stage of development from one-cell to the blastocyst stage.

Protein Supplementation

It is evident that there are distinct differences in nutrient requirements and metabolic activities of embryos of different species and even from different donors. It is necessary to understand the specific needs of these embryos to allow us to develop culture media that will provide optimal embryo development while cultured in vitro. Extensive research has been conducted on the constituents that make up embryo culture media. Modifications in many of these constituents can change the development of embryos while in culture. The addition of a protein source is common in many media. This protein source is usually a blood-based nutrient, being either BSA or serum derived from many different species in various reproductive states. The addition of varying levels of protein to culture media was beneficial for the development of two-cell mouse embryos. Increasing levels of protein resulting in increased development (Menino et al., 1985). The use of heat treated bovine serum was found to be more beneficial than BSA. The addition of uterine secretions was also examined. Although it might be speculated that the addition of uterine fluid to a culture medium would stimulate embryonic development because this is a product of the normal environment for early embryos, this was not the case. It should be noted that the embryos that were being observed were from the mouse while the uterine secretions were of a bovine source. There is transpecies specificity in such a system.

The use of heat-treated porcine serum was compared to BSA for the culture of porcine zygotes by Menino and Wright (1982). These researchers found that heat-treated porcine serum did not support embryo cleavage as did media supplemented with BSA. In fact, medium supplemented with heat treated porcine serum had a similar cleavage rate as that of medium devoid of a protein source. The impact of varying levels of serum at the chromatin level was considered by Hidekazu et al. (1984). Serum added to medium increased embryonic development as compared to medium without a serum source. However, when serum was added at a level of 10% or greater there was decreased incidence of sister chromatid exchange as compared to a 5% serum level. This exchange is a measure of DNA damage and is a result of mutagenic and carcinogenic agents. It was recommended that 10% serum or greater should be added to media for optimal ovum development.

While it is evident that a protein supplement is necessary to enhance embryo development in vitro, the exact effect this protein source is having is not clear. The influence of individual amino acids on hamster zygotes was studied by Bavister and Arlotto (1990). Phenyalanine, valine, isoleucine, tyrosine, tryptophan, and arginine inhibited development, while glycine, cystine and lysine stimulated development. Such actions led the investigators to suggest that amino acids may be playing a regulatory role in in vitro development. Rosenkrans et al. (1989) conducted a similar study with developing pig embryos. The basic medium was supplemented with lamb serum as well as various individual amino acids. In contrast to the research mentioned previously, these findings indicated that phenyalanine, methionine and isoleucine stimulated ovum development and gave an increased incidence of the initiation of hatching. It was concluded that although individual amino acids can improve development of pig embryos to the blastocysts stage, the additional supplementation of serum is needed for optimal results.

The use of EDTA is possible substitute for protein supplementation (Fissore et al., 1979). Both EDTA and protein supplementation increased blastocysts formation in mice as compared to a medium with no EDTA or protein. The addition of protein to an EDTA supplemented medium did not increase development over EDTA alone. Glycine or alanine could partially substitute for EDTA. The use of citric acid as an alternative chelating agent did not increase blastocysts formation the same as an EDTA supplemented medium. These findings indicate that EDTA is not serving only as a source of fixed nitrogen and chelating agent. The authors suggested that EDTA, as well as protein, is acting as a protective agent.

Energy Source

When considering the composition of culture media for the development of early embryo, energy source is a vital concern. The level at which pyruvate, glucose and lactate are present in mouse oviduct fluid has been evaluated (Gardner and Leese, 1990). These nutrients were present at .37 mM for pyruvate, 3.40 mM for glucose and 4.79 mM for lactate in the oviduct fluid near the cumulus mass. In post-ovulatory animals in which a cumulus mass was not present, the pyruvate level was significantly decreased to .14 mM while glucose had increased to 5.19 mM. This indicated that the presence of the ova and cumulus mass were having an influence on nutrient composition of the oviductal fluid. When comparing zygote development in a standard culture medium (M16) to a modified culture medium containing the nutrients at respective concentrations as were found in the mouse oviduct, development rate to blastocysts was not different between the standard and modified culture systems. Glucose metabolism was different in embryos that were cultured in vitro. Blastocysts which had developed in vitro converted a much greater proportion of the glucose, which they consumed, to

lactate as compared to blastocysts matured in vivo. This switch to aerobic glycolysis may indicate an incapability of the embryo to generate enough ATP by oxidative-phosphorylation, further demonstrating the insufficiency of the in vitro environment. In an attempt to increase development of mouse zygote, Chatot et al. (1989) developed a medium that was referred to as CZB. Glucose was removed from the medium and replaced with the glutamine. This medium greatly increased the number of mouse zygotes that obtained the morula stage after 4 d in culture. Using CZB alone, 63% of the embryos went to the morula stage, when glucose was added back to the media, only 18% of the embryos developed to morula. The authors theorized that glucose actually blocked the central metabolic pathways or that glutamine may be a more desirable nutrient for the very early embryo. Further investigation determined the beneficial mechanism of CZB was an increase in total protein synthesis compared to embryos cultured in Whitten's medium which contains glucose (Poueymirou et al., 1989).

Attempting to produce a single culture medium which would support zygote development to the blastocysts stage may not be an appropriate approach since the embryo's nutrient requirements change throughout its growth cycle (Brackett, 1981). It was found that the murine zygote needs pyruvate or oxaloacetate (OAA) for development. This nutrient requirement changes for the two-cell embryo which can use pyruvate, OAA, lactate or phosphoenolpyruvate (PEP). By the eight-cell stage glucose can be used as an energy source as well as pyruvate, OAA, lactate, PEP, α-ketogluturate or citrate. Blastocysts stage nutrient requirements are similar to that of most adult cells. Boone et al. (1978) found that ovine zygotes could develop to the eight-cell stage with pyruvate and/or lactate. However, this development could not occur if glucose was the only energy source. In contrast to the mouse, it was found that bovine embryos could not be supported from the 16-cell stage to morula when glucose was the only energy substance.

An alternative energy source used for bovine embryos was α-ketogluterate which allowed development to the morula stage.

When considering the energy requirements needed for in vitro culture of developing pig embryos, Pinkert et al. (1989) found media that did not contain pyruvate and lactate were better for development of porcine embryos beyond the one- and two-cell stages than medium containing these nutrients. Peters et al. (1990) evaluated energy sources for the development of one- to two-cell porcine embryos and observed that media supplemented with glucose and glutamine resulted in development to the blastocysts stage. It was determined that glutamine could serve as a sole energy source for in vitro development. However, if the glutamine was absent from the media, glucose was necessary for development. From the four-cell stage or greater, development was significantly improved when pyruvate and lactate were withheld from media and it appeared that pyruvate alone inhibited development. A general assumption from the research was that beyond the four-cell stage nutrient requirements of porcine embryos were minimal since the embryos could develop to blastocysts in simple media containing BSA and glucose as energy sources but they also could develop to the blastocysts stage with BSA alone (Davis and Day, 1978).

Fatty acids also may be acting as energy sources for early developing embryos. The use of a culture medium containing defatted BSA without energy substrate did not allow for embryo development. However, rabbit zygotes could be cultured to the blastocyst stage if fatty acids were added to this medium, as well as if pyruvate was added (Kane and Headon, 1980). When specific fatty acids were added to a basal culture medium containing defatted BSA it was observed that various fatty acids could either support or hinder one-cell rabbit ovum development. The long chain fatty acids myristic, palmitic,

stearic, oleic, and linoleic allowed development of rabbit zygotes to the morula stage. Arachidonic acid appeared to be toxic to these early embryos. Short chain fatty acids, like proprionic and acetic acid, produced moderate growth while butyric and valeric acid did not support rabbit zygote development (Kane, 1979). Waterman and Wall (1988) found that the rabbit embryos sequestered fatty acids from the culture media. Arachidonic and oleic acids were found within the embryo at a concentration of 100 times that of the surrounding medium. Acetate was not concentrated. These findings are somewhat unusual in that arachidonic acid was toxic in the previously mentioned study, while concentration of oleic is a logical occurrence since it did promote ovum development. The majority of the fatty acids that were incorporated into developing embryo went into the production of triglycerides.

Buffer Systems

The addition of buffering compounds to culture media is necessary to regulate the pH of the medium. The most common buffers used are bicarbonate, phosphates and HEPES. The addition of HEPES to culture media will allow for the development of rabbit zygotes to the morula stage. However this buffer will not allow development to the blastocysts stage (Kane, 1975). The use of a bicarbonate system requires an elevated carbon dioxide (CO_2) atmosphere to maintain buffering capacity. This limits the use of a bicarbonate buffer system under standard atmospheric conditions. However, phosphate buffer media are stable under outside atmospheric conditions but their ability to allow for maximal embryo development in vitro is in question. Quinn and Wales (1973) previously reported that developing mouse embryos for the two- to eight-cell stage was decreased in a phosphate buffered medium. However, short term culture of bovine morula in a phosphate buffered system allowed for normal embryo development

(Trounson et al., 1976). This inconsistent action of phosphate buffer systems may be compounded by other components in the media. Serum that is added to a medium brings with it an endogenous bicarbonate component at an approximate concentration of 25 mM. This bicarbonate can combine with hydrogen ions in the medium to result in H_2C0_3 and CO_2, causing an increase in medium pH (Kane 1987). Brackett (1981) suggested that although bicarbonate does serve to buffer a culture medium, it also serves as a carbon source for the developing embryo which would explain the improved level of embryo development in a bicarbonate buffered system.

Inorganic Salts

Inorganic salts make up a large portion of in vitro culture media. Data on the requirements and optimal concentrations of these salts is not clear because of the vast number of salts that can be considered and also species variation. Wright and Bondioli (1981) reported that potassium was necessary for embryo development although development would occur at a wide range of potassium concentrations. Similarly the absence of calcium inhibited cleavage of embryos, while a wide range of calcium levels supported development. It was concluded that the total absence of PO_4, magnesium or SO_4 from the culture medium did not alter mouse embryo development. This was contradicted by Brackett (1981) who concluded that sodium, potassium, calcium, magnesium, chlorine, PO_4, and bicarbonate were necessary in a culture medium for successful mouse embryo development. Also, potassium, calcium, magnesium and phosphate levels for optimal development were similar to those concentrations found in serum.

Kane and Foote (1970) suggested that there was no requirement of trace elements for blastocyst formation in the mouse and rabbit. Kane (1978) suggested that such trace

elements may be supplied as impurities of other major salt constituents that are added to the medium.

Vitamins

In summarizing work conducted on vitamin requirements of developing embryos, Brackett (1981) reported that a mixture of 11 B vitamins was necessary for expansion of rabbit blastocysts as well as for hatching of eight-cell hamster embryos. Inositol, riboflavin, pyridoxine, niacinamide and thiamine were needed for the expansion, while B_{12} concentration that was found in the common culture medium Ham's F10 was toxic to these embryos. The requirement for inositol appeared to be the greatest for the development of the embryos. In contrast to the rabbit and hamster data, Wright and Bondioli (1981) reported that there did not appear to be a vitamin requirement for blastocysts formation in the mouse, bovine, ovine or porcine.

Gas Atmosphere

The gas atmosphere of a culture system affects embryonic development. This was alluded to when considering the buffer requirement, as the need for CO_2 is elevated for a bicarbonate buffer system to maintain an appropriate pH. However, when CO_2 is kept constant at 5%, there is still an influence of other gas constituents of the atmosphere on the developing embryo. The greatest effect being that of oxygen. A reduced oxygen tension of 5% supported embryonic development greater than 20% oxygen as is found in atmospheric air for mice (Whitten, 1971), sheep (Tervit et al., 1972; and Tervit and Rowson, 1974) and swine embryos (Wright, 1977). In contrast, research by Betterbed and Wright (1985) did not a reduced oxygen atmosphere to be beneficial for development of sheep embryos.

Whitten (1966) suggested that this difference in oxygen concentration may be a result of excess oxygen causing oxidative damage to surface membranes. Auerbach and Brinster (1968) went further to determine the minimum oxygen concentration that would allow for development of mouse embryos. There was no depression in the formation of blastocysts from two-cell mouse ova down to an oxygen concentration of 1%. However a decrease in blastocysts formation was seen at .5% and 0% oxygen. These data indicate that while oxygen is needed at a level lower than that found in standard atmosphere, the total elimination of oxygen will not support embryo development indicating that it is a vital nutrient for culture systems.

Osmolarity

Brinster (1965) indicated that osmolarity was not a critical factor in the development of two-cell mouse embryos to the blastocyst stage. He found that development would occur at osmolarities ranging from 200 to 354 mOsm, while 276 mOsm was optimal. This range of osmolarity was not seen for the rabbit. Naglee et al. (1969) found that development of two- or four-cell rabbit embryos to the blastocyst stage was greater at an osmolarity 270 mOsm as compared to osmolarities greater or lower than that value. These researchers hypothesized that although this osmolarity was lower than that normally found in body fluids, there may be other factors in the culture medium influencing the osmotic affect. Possible factors include pH, protein concentrations or other components of the medium.

McKiernan and Bavister (1990) evaluated many of the environmental characteristics of culture media which have been discussed previously. Optimal conditions for the culture of the two-cell hamster embryos to the blastocyst stage consisted of a temperature of 37.5°C with 10% CO_2, 25 mM bicarbonate, 2.0 mM calcium, 0.5 mM magnesium, 3.0

mM potassium, 10% oxygen and osmolarity ranging from 250-300 mOsm. However, they also concluded that species considerations must be made when developing an "optimal" culture medium.

Water Quality

Another factor that must be considered is the variability of results among different laboratories using media of exactly the same chemical constituents. There are many yet unknown factors which will affect an individual researchers' results when culturing embryos of various species even though they are using media that have been reported by other researchers as highly successful. There are other factors which are often of less concern but do have an effect on the development of embryos in vitro. Water is the major component of culture media and water quality varies among laboratories. Whittingham (1971) found that sequential distillation of water in glass increased mouse embryo viability in vitro. No embryos developed to the blastocysts stage when culture media was made with water than had been distilled only once. Using water that had been distilled two and three times to make the media resulted in blastocysts formation in 36 and 93% of the embryos, respectively. This finding not only strongly indicates the importance of quality of water but indicates that minute impurities influence embryo development.

Kane (1987) suggested that water polishing with a system of carbon filtration and high quality ionic exchange in conjunction with membrane filtration produces an extremely high purity water and may improve embryo development. Water quality is probably more important in protein free media since these proteins serve as chelating agents to bind toxic substances which may be carried in with impure water.

Miscellaneous Factors

Schumacher and Fischer, (1988) reported that in rabbits early cleavage stages are more sensitive to visible light than are later cleavage stages, such as the compacted morula. Thus, care should be taken with the handling of embryos outside the incubator.

Kane (1987) reported that the use of parafin oil to cover culture medium may contain toxic substances and suggested that silicone oil be used instead of parafin to decrease the possibility of such toxic affects.

Oviduct or Uterine Secretions

Bavister (1988) discussed the influence of culture systems on the development of embryos in vitro. Not only do cultured embryos have a reduced percent development and decreased viability but there is an increase in generation time for the embryos. Embryo cultured from a very early stage exhibit a block in development in most species studied. This block ranges from the two-cell stage in the mouse to the 8- or 16-cell stage in sheep and cattle. With the exception of rodents, this block occurs at the time when these embryos are normally moving out of the oviduct and into the uterine environment. This would suggest that there is a component of culture media which is absent, as compared to the oviduct environment, that is inhibiting embryo development.

Unique proteins are present in the oviducts of various species including the human (Wagh and Lippes, 1989), mouse (Kapur and Johnson, 1985; Kapur and Johnson, 1986), hamster (Leveille et al., 1987; Robitaille et al., 1988), rabbit (Oliphant and Ross, 1982; Oliphant et al., 1984), sheep (Sutton et al., 1984; Sutton et al., 1986) and pig (Buhi et al., 1989). These unique oviductal proteins have specific associations with oocytes and

early embryos. Oviductal proteins in the hamster are progressively added to the oocyte during oviductal transit (Robitaille et al., 1988). These proteins adhere to the zona pellucida and are produced primarily in the isthmus, but are also secreted from the ampulla and fimbria (Leveille et al., 1987). Unique proteins are further sequested into the perivitelline space in the mouse (Kapur and Johnson, 1986). In the rabbit the production of these oviductal proteins was under the control of estrogen secretion (Oliphant et al., 1984). Estrogen controlled oviductal proteins in the sheep were not found between d 7 and -2 of the estrous cycle. Proteins were only found in a low percentage of animals on either d 5, 6 or -1 of the estrous cycle while 59% of the animals produced these proteins on d 0, 96% on d 1 and 100% on d 2. The percentage of animals secreting these proteins decreased on d 3 and 4. These data indicate the control which estrogen has on the production of these proteins.

The presence of unique, embryo associated proteins and inhibited development at specific stages has led researchers to use media that are supplemented with such unique fluids or which contain oviductal or other tissue to help overcome the inhibition of embryo development.

When one- and two-cell porcine embryos were cultured in oviduct fluid alone, only 5% went to the blastocyst stage compared to 27% which went to the blastocyst stage in the Krebs Ringer Bicarbonate (KRB). However, when the KRB medium was supplemented with oviductal fluid, 73% of the embryos went to the blastocyst stage (Archibong et al., 1989). When embryos were exposed to oviduct fluid for a short period of time (24 h for two-cell, 48 h for zygotes), development was intermediate, at 46.8% blastocyst formation. Embryo cultured in KRB plus oviduct fluid then transferred to KRB alone resulted

in 88.9% blastocyst development. This indicated that the need for the oviduct component is greatest during early development and excessive exposure was detrimental.

Embryo-tissue Co-culture

In general, it has been found that co-culture of early embryos with cellular constituents, most commonly fibroblasts or cells of reproductive tract origin, resulted in an increase in development. When sheep zygotes were co-cultured with either oviductal cells or fibroblasts for 3 d, 95% developed normally compared to 13% in medium alone. When these embryos were transferred, only 33% of the fibroblast co-cultured embryos were viable compared to an 80% viability of those embryos co-cultured with oviductal cells (Gandolfi and Moor, 1987). When the culture period was extended to 6 d, 42% of the embryos co-cultured with oviductal cells developed to the blastocysts stage, whereas only 5% of those embryos in fibroblast co-culture developed in this late stage. While short term culture with oviductal or fibroblast cells may result in similar development, oviductal cells are needed for maximal viability as well as for the survival of embryos in long term culture. Carney and Foote (1990) examined co-culture of rabbit embryos with oviductal cells to determine if this system would eleviate retarded development that is often seen with embryos in vitro. They found that co-culture did provide for improved development over embryos that were cultured in medium alone, but this improved development was still below that of embryos developing in vivo. Such findings reveal that the oviductal cells in culture were performing a beneficial service to the embryo but it was not completely replicating the environment of the oviduct.

The beneficial effect of oviductal cell co-culture has been seen in the culture of one- and two-cell bovine embryos (Ellington et al., 1990a). Fresh oviductal monolayers promoted embryo development more readily than monolayers that had been frozen or medium that

had been conditioned with an oviductal cell monolayer. In a comparison of bovine oviductal epithelial cell monolayer co-culture systems versus oviductal cells that were free floating in the medium, Eyestone and First (1989) found little difference in development to the morula/blastocyst stage (46% and 43%). This finding is very important from an applied view in that the development of oviductal monolayers is a much more tedious procedure than simply adding free floating oviductal cells to culture systems. It was observed that cells cannot be completely removed from the culture system for maximal development This work supported Allen and Wright (1984) who studied development of porcine embryos on either monolayers or conditioned medium. They hypothesized that either fibroblast or endometrial cell projections were penetrating the zona pellucida to allow intimate contact with the embryonic cells. It was suggested that close association between the embryo and co-culture cells may stimulate the release of important factors to aid in embryo development. Such factors would not be found in the supernatant of monolayers or tissue cultured without the presence of embryo.

The use of more than one cell type in co-culture did not increase the development of 2- to 16-cell porcine embryos (White et al., 1989). Co-culture of embryos with either oviductal cells or oviductal cells in conjunction with a fibroblast monolayer resulted in blastocyst formation in 70% and 67%, respectively. This was compared to development to the blastocyst stage of 27% when only the fibroblast monolayer was used. Thus, it is not merely cell contact that is necessary for maximal embryo development, but oviduct epithelial specific constituents are also needed.

Menezo et al. (1990) studied the development of human embryos in culture using vero cells which are epithelial cells derived from monkey kidney. When two- to six-cell human embryos were cultured with vero cells, development was improved (61%) compared

to medium alone (3%). Not only did the co-culture system improve development of human embryos, but that the improved development seen in many species may not be strictly due to the use of reproductive tract tissue. A point to note here is that the vero cells are an epithelial cell whereas many of the previous studies have used fibroblastic cells.

Trophoblastic vessicles are fragments of embryos without the embryonic disk that can be grown in culture. When one- to eight-cell bovine embryos were grown in co-culture with trophoblastic vessicles it was found that development to the morula stage was significantly improved (46%) as compared to embryos that were cultured in the absence of trophoblastic vessicles 18%; (Camous et al., 1984). The ability of trophoblastic vessicles to enable the bovine embryos to pass through the normal block to development in vitro led these researchers to conclude that the vessicles were releasing factors that were normally present in the oviduct that promote the development of the embryo. Such a beneficial effect was not seen in the pig (Pipkin et al., 1990). Co-culture with trophoblastic vessicles or medium that had been conditioned with trophoblastic vessicles did not result in greater development past the four-cell stage. The accuracy of this research must be questioned since none of the various co-culture or conditioned media studied showed embryo development past the four-cell stage at a rate greater than medium alone.

Oviduct Culture of Embryos

Probably the most complex culture system is the removal of the embryos of one animal then replacing them into the oviducts of another living animal and allowing them to develop there. When mouse zygotes were collected and placed into the oviducts of immature mice (Papaioannou and Ebert, 1986), embryos had a lower cell number than

embryos that were transferred to mature pseudopregnant recipients but developmental rate and viability were similar. This led these researchers to suggest that the use of immature mouse oviducts provides a suitable environment for temporary culture of embryos. The beneficial effect of in vivo embryo culture was found in a transpecies experiment that transferred bovine embryos into the oviducts of living rabbits (Hawk et al., 1989). When bovine embryos were transferred into oviducts for 8 or 9 d, the percent of embryos developing to the morula or blastocysts stage was 77 and 67%, respectively. Such a system does have its difficulties. Oviducts must be ligated to minimize loss of embryos during culture and even when utilizing ligation, 42 to 52% of the embryos that are placed in the oviduct for culture could not be recovered. The use of in vivo oviduct culture systems may not be practical. Ellington et al. (1990b) found that development of one- or two-cell bovine embryos was similar between embryos cultured within rabbit oviducts or in a co-culture system using bovine oviduct epithelial cells (83% and 84% morula/blastocyst, respectively). There was no difference in the ability of embryos cultured in either oviduct or co-culture to result in pregnancy upon transfer.

A simpler system than the use of in vivo oviducts is that of removing oviducts and culturing embryos inside these oviducts in an in vitro organ culture system. Ebert and Papaioannou (1989) found that 68% of rabbit embryos cultured in immature mouse oviduct organ culture developed to the morula stage. This was slightly less than development in medium alone (81%). There was difficulty in recovering embryos from the oviduct with a recovery rate ranging from 32 to 87%. This study also evaluated the use of immature mouse oviducts for the culture of porcine embryos. While this system was able to increase the total number of cells in porcine embryos as compared to those held in vitro, it was unable to allow embryo to develop past the four-cell block which was seen within vitro culture systems. This inability to improve development of very early

porcine embryos was disputed by Krisher et al. (1989b) who found that 78% of porcine zygotes placed in mouse oviducts would continue development to the morula or blastocysts stage as compared to 36% of embryos that were held in medium alone. Additional research (Krisher et al., 1989a) found that there was a need for the presence of endogenous oviductal fluid in oviducts maintained in organ culture. There was a lower development of embryos in mouse oviducts which had been flushed (59%) as compared to oviducts which had not been flushed (75 to 77%).

It is obvious that there are many factors required for optimal development of embryos in vitro. Early research focused on trying to produce a chemically defined medium that would replicate the development of ova in vivo. Although success was achieved in producing simple culture systems, researchers were unable to completely replicate the oviductal environment. Developing such a "perfect" system was much more difficult when very early ova were used for culture. More recent research has reverted to the use of oviductal or tissue based media to improve development over defined media, with significant success. Although there are benefits to knowing the exact chemical requirements of developing embryos at all stages, it is equally important to find systems that will give very high development and viability of embryos when they are maintained in cultures whether the chemical constituents of this system are defined or not. For research that involves the manipulation of very early embryos, the use of a culture system that provides maximal development is crucial for success.

Porcine Embryo Transfer

Embryo transfer procedures in a number of laboratory and farm animals have been developed. Such procedures in the pig were developed in the early 1960's (Polge, 1982). In 1962, Hancock and Hovell reported a successful transfer of embryos in pigs. This procedure recovered embryos from the uterus of the donor animals between d 3 and 5 of the estrous cycle. The uterus was flushed by infusing collection medium though the oviduct. The fluid was massaged throughout the uterus and then harvested by making an incision in the uterine wall. A total of 118 embryos were recovered from nine animals. Eighty six were transferred into the uteri of six sows of which three farrowed. The farrowing animals that had received 42 of the embryos that were transferred yielded 31 live births. Embryos were transferred within approximately 2 h of collection. This was the first research that showed a high level of survivability of transferred embryos compared to previous research that resulted in low numbers of offspring born (Kvasnickii, 1951; Pomeroy, 1960).

Unmated recipients were used in Hancock and Hovell's (1962) successful study while research by a Vincent et al. (1964) examined the possibility of using recipients which had been embryo donors. The oviducts of animals were flushed between 2 and 4 d after the onset of estrus. The recipient animals of these embryo transfers were divided into three groups. In the first, donor animals served as their own recipients. In this group, 4 of 7 animals became pregnant and 33% of the embryos transferred survived. Twelve animals were used in a partial reciprocal transfer group in which approximately one-half of donor embryos were transferred to a pair mate, with the remaining half of the embryos going back into the original donor. In this group, 8 animals became pregnant with a 59% embryo survival rate. One animal was used for a complete reciprocal

transfer, however this animal did not become pregnant. It was concluded that the flushing procedure did not leave embryos within the oviduct after the collection even though not all embryos were accounted for when considering CL number. There was no effect on subsequent fertility due to surgical procedure.

In comparison to the success of Vincent et al. (1964), Hunter et al. (1967) evaluated the ability of later stage embryos to develop after collection and transfer. Three hundred twenty-seven blastocysts were transferred into 26 recipient animals. Seven recipients became pregnant. Blastocysts survival, as determined at slaughter, was found to be low. Survival rates ranged from 20 to 50% in 6 of the 7 animals, while the 7th had a survival rate of 80%. Polge and Day (1968) attempted to overcome difficulties often associated with surgical embryo transfer. Embryos ranging from the four-cell to blastocyst stage were transferred into the anterior uterus of recipient animals by a nonsurgical procedure. A total 188 embryos were transferred into 17 recipients using the nonsurgical procedure. Only one was pregnant upon necropsy 17 d later. In this animal, only three living embryos were found. It is evident from this early work that embryo transfer in pigs often resulted in a high embryo mortality. However, results were greatly increased when transfers of very early embryos were made into the oviduct.

The viability of embryos collected by surgery or embryos collected from animals that had been slaughtered was considered by Schlieper and Holtz (1986). Embryos were harvested post-slaughter from animals that either had been stunned electrically or by captive bolt and the genital tracts removed either prior to or after the scalding procedure. There was no difference in the ability of embryos to maintain a pregnancy after transfer from treatments in which embryos were collected by surgery, electrocution with collection prior to scalding or captive bolt stunning with collection prior to scalding (preg-

nancy rate = 82, 60 and 64%). Ova that were collected after scalding yielded a lower pregnancy rate (57%) when compared to the surgery treatment. The scalded group had a decreased occurrence of normal fetuses than non-scalded groups when observed after the first month of gestation. Two possible causes for the decreased ability of embryos collected post-scalding to result in viable fetuses or pregnancies were discussed. The first factor was that the core body temperature in the area of the reproductive tract was increased 1.2°C due to the scalding procedure. The second possible cause was that embryos removal could not be completed until approximately 30 min after stunning as compared to uterine removal and embryos flushing approximately within 4 to 5 min in the nonscalded animals. It was generally concluded that successful embryo transfer could be conducted with embryos collected either by surgery or by slaughter, provided that the reproductive tracts were collected and flushed immediately after slaughter.

Wollenberg et al. (1990) considered the effect of time and temperature on the development of embryos collected after slaughter. They collected four- to eight-cell embryos from gilts either 3 min after slaughter or after the uterus was held for 2 h at either 37° or 20°C. Embryos were then cultured and development was assessed every 24 h. Progressive development was used as a criterion to evaluate embryo viability in culture. By 24 h it was evident that embryos that had been exposed to the 20°C environment and collected 2 h later had a lower developmental ability. Only 18% of these embryos had progressive development, compared to 76 and 70% for embryos that were collected immediately or within 2 h but maintained at 37°C. After 48 h in culture, progressive development for embryos collected immediately or 2 h after slaughter and maintained at 37°C or maintained at 20°C was significantly different in all groups with progressive development occurring in 71, 51 and 3% of the embryos. Such major differences between treatment groups were observed at 72 and 96 h. This study revealed the impor-

tance of rapid collection of embryos following slaughter. This factor is not nearly as important as the need to maintain embryos at an appropriate temperature to minimize developmental losses.

Another factor of importance, when considering embryo transfer procedures, is that of synchrony between the donor and the recipient. Webel et al. (1970) investigated the viability of embryos transferred between synchronous animals and their ability to maintain pregnancy. A pregnancy rate of 20% was observed when embryos collected on d 4 were transferred to recipients which were also on d 4. Pregnancy rate increased to 60% when d 4 embryos were transferred into d 5 recipients. The highest pregnancy rate (100%) was observed when d 5 embryos were transferred into either d 4 or d 5 recipients. Percent pregnancy decreased with older embryos. An 80% pregnancy rate was observed when d 6 embryos were transferred to d 6 recipients and 60% for d 6 embryos transferred to d 4 recipients. No pregnancies were found with the transfer of either d 7 or d 8 embryos. It was concluded that successful transfer could be conducted with donors which were either 1 or 2 d earlier or 1 d later than recipients and that embryos at d 7 or 8 were not viable for transfer.

The cause for differences in pregnancy rate due to asynchronous transfer was partially elucidated by Pope et al. (1986). When d 6 embryos were transferred into d 7 recipients there was more variability in development than when d 7 embryos were transferred to d 6 recipients. When fetal development was examined at d 30, the mortality rate was 42% for d 7 embryos transferred into d 6 recipients as compared to a greater mortality of 62% for d 6 embryos in d 7 recipients. In his review of uterine asynchrony, Pope (1988) hypothesized that the uterus probably does not alter its biological time clock to allow the embryos to reach synchrony with uterine development. However it may be possible that

factors within the uterus will stimulate the embryos to developing either faster or more slowly to allow them to adjust to the uterus' time schedule. Further evidence of the need for appropriate environment for sustained embryo development was reported by Pope and Day (1977). They collected embryos from gilts and subjected them to in vitro culture for either 24 or 48 h. Embryos which were out of the reproductive tract for the longer period of time led to a pregnancy rate of only 15%. However, a pregnancy rate of 83% was observed when embryos were transferred back into recipients after only 24 h of culture.

The influence of embryo number in the reproductive tract may also play a role in embryo survival and maintenance of pregnancy. Dziuk (1968) conducted a study that evaluated a wide range of embryo number within the reproductive tract on fetal survivability. Embryo survival was not altered when uterine space per embryo was above or below what was normally expected. There was a possible effect observed when greater than 14 embryos were present in the uterus. In these cases there was an apparent decrease in embryo survival. This study focused more on over crowding than it did on a low population of embryos. Endogenous embryos, not transferred embryos, were used. Differences in pregnancy and embryo survival rate after transfer of one- to four-cell embryos was considered by Pope et al. (1972). Either 12 or 24 embryos were transferred into recipients. When 24 embryos were transferred, there was a 100% pregnancy rate with a 68% survival at the 25th day of gestation. This compared to a 71% pregnancy rate and 57% survival for recipients which received only 12 embryos. These data can be compared to those from a more recent study (Holtz et al., 1987) in which 12 to 26 embryos were transferred into 97 recipients. These transfers resulted in a 53% farrowing rate with an average of 7.1 piglets being born alive. When the results of transfer studies are compared with the results of studies which used naturally occurring embryos that

there is a reduction in both pregnancy rate and embryo survival for the transferred embryo. When as few as 12 embryos are placed into recipients there is still a high probability of pregnancy being maintained.

When more changes are made in an embryo's environment embryo mortality is greater and there is less chance of pregnancy being maintained. There is also the likelihood that certain embryo are more susceptible to these changes than others. It is still uncertain what impact physical manipulation of the embryo has on later embryo development. In an early report on the viability of splitting porcine blastocysts, Rorie et al. (1985) found that transfer of bisected halves of eight embryos resulted in the birth of four healthy piglets. While this may not provide an adequate representation of a larger gilt population, it does suggest that embryo death in such a situation is high. However, it does not preclude pregnancy.

Another highly invasive procedure being increasingly employed in embryo research is pronuclear microinjection. When an average of 26 pronuclear microinjected embryos from post pubertal donors were transferred into nine recipients, 100% of these animals became pregnant and farrowed. Results were not as good when prepubertal animals were used as donors. In this case, only three of five animals receiving an average of 26 embryos went on to farrow. Not only was pregnancy rate decreased using embryos from prepubertal sources but the litter size also was decreased. Litter size averaged two from embryos of pubertal donors compared to 5.8 for post-pubertal derived embryos (French et al., 1991). Such results indicate that microinjection is having a detrimental action on the ability of embryos to develop once transferred into recipients. However, a larger effect is seen from the source of the embryos. Research evaluating the optimal number of embryos which should be transferred for maximal pregnancy rate and litter size was

conducted by Springmann et al. (1988). Prepubertal gilts were used both as donors as well as recipients. The transfer of less than 40 pronuclear injected embryos resulted in a significantly lower pregnancy rate (25%) as compared to the transfer of greater than 40 injected embryos (48%). Litter size was not different between the groups, being 4.1 and 3.8 piglets for less than and greater than 40 embryos, respectively. It is difficult to assess the impact of microinjection since litter size was not changed by varying numbers of embryos transferred while pregnancy rate was greatly affected by such a change.

Production of Transgenic Animals

The birth of the first transgenic mouse (Gordon and Ruddle, 1981) was the initial step which has led to a significant world wide research effort to produce transgenic farm animals. The blossoming field of transgenic animal research has resulted in the production of transgenic mice, rats, rabbits, chickens, fish, sheep, pigs and cattle. Researchers have been able to target the genes placed in these transgenic animals to be very tissue specific. The application of this new technology to farm animals is leading production agriculture into an exciting future for the propagation and production of animals which are structured for a specific purpose or product (First, 1990). In addition to the development of production farm animals, transgenic procedures may be used to study gene regulation, produce animals for disease models, correct genetic defects, to study the effects of genetic manipulation in developing animals and to produce tissue specific expression of foreign genes (Connelly et al., 1989).

Production of Transgenic Cells

Various procedures have been used in the attempt to make transgenic animals. There are advantages and disadvantages associated with each of these methods as well as variability in the success rate for the production of transgenics. The first procedure for producing transgenic animals that will be discussed is that of retroviral mediated gene transfer which has been thoroughly reviewed by McLachlin et al. (1990). In this procedure, animal viruses are used as vectors to mediate the gene transfer into host animals. Retroviruses are ideally suited for such procedures since they replicate through a DNA intermediate which has been integrated into a host genome. The integrated DNA is present in a defined structure and low copy number but the integration is apparently random relative to site in the host genome.

A major drawback to the use of retroviruses for gene transfer is the limit to the size of the DNA insert which can be transferred. This size ranges from approximately 2 kilobase (kb) to 10 kb. The use of retroviral transfer allows for the attempted manipulation of a large population of cells at one time. However, integration is not 100%, therefore there must be a procedure to identify the transgenic cells and select them for further use. This often is accomplished by linking a marker gene with the transgene of interest. The use of retroviral mediated gene transfer does appear to have promise in the area of gene therapy since viruses can be selected or selectively modified by altering the glycoprotein constituents of the viral gene envelope. The glycoproteins give specificity in the type of cells the virus enters thus allowing specific tissues to be targeted for gene transfer. There is some question as to the effectiveness of such procedures to produce founder lines of animals. This is even more important considering viruses are limited in the size of genes they can carry (McLachlin et al., 1990). Another factor that limits the usefulness of

retrovirus vectors is that cells must be actively replicating at the time of infection to allow for transgene integration (Miller et al., 1990). This can explain some of the lower efficiencies seen in many retroviral infection studies. This knowledge could be used to maximize the production of transgenic cells.

Huckett et al. (1990) reported a procedure which utilized endocytosis for gene transfer in cells. Modified insulin molecules, which carried a predominantly positive charge, were used to covalently link negatively charged ptk NEO and pAL-8 expression vectors. Native insulin receptors recognized this complex and internalized the DNA. Integration frequency was below .006% of all exposed cells. The receptor cells for this study were HepG2. Although success was low in this study, success was achieved and further study needs to be performed to maximize this procedure. It may be a viable method for the production of transgenic cells and animals. Another unique procedure for gene transfer is the use of a liposome vehicle. Cationic liposomes bind DNA and allow for its transport into the cells. Such procedure provides for a noninvasive method of introducing foreign DNA into cell lines (Brigham et al., 1989). The procedure utilized specially synthesized cationic liposomes which had been joined to a plasmid containing the chloramphenicol acetyltransferase (CAT) gene. Injection of this complex into the lung resulted in expression of the gene. However, expression was only transient since expression was observed for a week or less. Integration efficiency was not considered in this study since the lungs of living mice were used to test this system. However production of CAT was present in these animals after transfer. The use of such a localized gene transfer procedure does hold interesting possibilities for human genetic therapy.

Electronic fields can also be used to facilitate gene transfer into cells. In this procedure, cell membranes are made reversibly permeable in the presence of high, yet short duration

electronic pulses. The procedure is generally referred to as electroporation. Successful gene transfer using this procedure has been accomplished in bacteria and yeast as well as plant and animal cells. Electroporation is considered to be relatively simple and easily controlled, however it does possess drawbacks. It is an in vitro procedure which utilizes cell culture, which precludes it from the production of transgenic lines of animals. The efficiency and reliability of gene transfer is also in question since the rate of eletroporation incorporation of transgenes ranges from 3% to as low as .0008% (Forster and Neumann, 1989). While the efficiency of this procedure may be acceptable in tissue culture procedures where there are a vast number of cells to work with, it is questionable if it would ever be feasible for the production of transgenic lines of animals using very early stage embryos.

The procedures described above are useful in the production of transgenic cell lines. However, they are not capable of producing a transgenic animal line for propagation. To produce such an animal would require the insertion of genetic material into a very early stage embryo. Embryonic stem cells are cells which maintain totipotency. These cells are usually derived from blastomeres of early embryos. Such cells can maintain their totipotent characteristic when cultured under well defined conditions (Evans and Kaufman, 1981). Once such a cell line has been developed, DNA transfer/manipulation can be conducted on the stable cell line. Procedures for DNA incorporation into this cell line include electroporation, retroviral mediated gene transfer as well as direct injection of DNA into the nucleus. After gene integration into the embryonic stem cells, these cells can be transferred into the blastocoel of developing blastocysts. These manipulated embryos could then be transferred into a recipient female which would carry the embryos to term. The new animal line which results would be chimeric, which will limit the ability of this animal line to propogate the newly incorporated genetic material.

This procedure does have benefits over previously described procedures in that the transgenic cells are incorporated at a very early age giving a greater probability of integration in large areas of the new animal (Williams, 1990). While this research was conducted in the mouse, there is also similar experimentation being conducted in both pigs and cattle.

A recent report (Evans et al., 1990) described the development of a pluripotent porcine cell line derived from hatched blastocysts. This line was stable in culture for over 1 yr without loss of phenotype leading these researchers to believe that the line was immortal. Under optimal culture conditions, the immortal cells appeared to retain their embryonic nature, while at high densities these cells were able to differentiate to produce muscle, fibroblasts, nerve and endodermal cell types. Stable cell lines were also produced from bovine ova. However, pluripotency had not been confirmed for bovine cells. The authors also stated that they had not yet determined the contribution of the stem cells once they had been transferred into the blastocoel of recipient ova.

A very unique procedure to transfer DNA into mouse embryos utilized the transport of DNA into the cell via sperm (Lavitrano et al., 1989). Spermatozoa were incubated with a foreign DNA construct. This construct was taken up by the sperm. When one-cell ova were in vitro fertilized with these modified sperm, transgenic mice were reported to have been produced. This procedure offered many promising features in that it was a very simple procedure, had a very high efficiency and it also eliminated the need for highly sophisticated ova manipulation procedures. Unfortunately this procedure has not been replicated in the scientific community and its validity is in question.

Nuclear Transfer

It is apparent from previous discussion that the production of a transgenic animal in which all cells contain the new genetic material is of interest. However, production of these animals in sufficient quantities is very difficult. One procedure that could be used to overcome this difficulty is the use of nuclear transfer. This would require the transfer of a nucleus from an embryo or stem cell that contains the new or desired genetic material and placing it into an enucleated cell or embryo. Although amphibian nuclear transfer has been conducted since the early 1950's, it has come into successful practice in mammals only since 1983 (Prather and First, 1990). A very beneficial aspect of nuclear transplantation is that once a nuclear transferred embryo begins to develop it can be recloned, repetitively, to provide an unlimited supply of identical genetic material.

The use of nuclear transfer for the cloning of farm animals has been conducted in the pig (Robl and First, 1985), sheep (Willadsen, 1986) and cattle (Prather et al., 1986). Prather et al. (1987) found that fusion of transferred nuclei in cattle was more successful when nuclei were collected from two- to eight-cell embryos as compared to embryos of later stages. Recipient embryos which were freshly collected had a significantly greater chance of developing after pronuclear transfer than did recipient embryos which had been in vitro matured. This finding suggests that insufficiency of the in vitro matured embryos was not of nuclear origin because these cells were enucleated and had donor nuclei placed in them. Seven pregnancies were achieved from transfers of nuclear transplanted embryos into 13 heifers. Two animals completed pregnancy with the birth of live calves. Use of a nuclear transfer program would be most successful with the use of early cleavage stage embryos. This decreases the overall efficiency of production of multiple copies from a single embryo. However, if repetitive nuclear transfers are made,

it does provide the possibility for a significant number of copies to be made from a single embryo containing identical genetic material.

Prather et al. (1989) examined nuclear transplantation in early pig embryo. They observed that 19% of the pronuclear transferred ova developed to the blastocysts or morula stage when the nuclear component was derived from an eight-cell embryo. This compared to nine and eight percent development to that stage when nuclear material was derived from either a two-cell or four-cell embryo. Eighty-eight nuclear transferred embryos which had been developed in vivo were transferred into nine recipients. One piglet was born from an embryo that had developed after receiving a nucleus from a four-cell embryo. While development of embryos that had been subjected to nuclear transfer and birth rate of nuclear transferred embryos was low, it does indicate the feasibility of multipling the number of animals that have been produced from a single genetic source.

Pronuclear Microinjection

Pronuclear microinjection is probably the best technique for the production of transgenic farm animals at this time. This procedure has shown the greatest probability of producing founder animals which can transmit the desired new genetic material to its progeny and future generations. This procedure is inefficient. Robl and First (1985) summaried microinjection studies in pigs and estimated the following efficiencies. Approximately 60% of the embryos which were injected had immediate survival while 15% of these surviving embryos produced offspring. Integration rate was 24% and only 60% of those animals which carried the gene were able to express the foreign DNA. These figures result in an overall efficiency of approximately one percent. It is obvious, with

such a low efficiency, that economic factors in the production of these animals will play a critical role.

Much of the transgenic research that has been conducted with domestic animals has been directed towards the production of animals with altered growth or meat product characteristics. Transgenic incorporation of human or bovine growth hormone gene has been achieved in swine (Miller et al., 1989). In 18 pigs which integrated the human growth hormone gene and nine pigs which integrated the bovine growth hormone gene, 11 and 8 of these pigs, respectively, expressed the transgene. The number of gene copies which were integrated into these animals varied greatly, ranging from approximately 1 to 490 and similarly, the concentration of the respective growth hormone products in circulation varied greatly. However, there was no correlation between gene copy number in the animal and production level. The presence of either human or bovine growth hormone in the pigs circulation resulted in a suppressed level of native porcine growth hormone indicating significant similarity between the products which resulted in a negative feedback on the native product. These researchers found that the non-native growth hormone product appeared biologically active and was also independent of normal growth hormone regulatory factors.

Pigs that were transgenic for the bovine growth hormone had a 23% faster rate of gain than did their contemporary control animals. In addition to increased rate of gain, these animals had a reduction in subcutaneous fat as well as an increased feed conversion efficiency (Pursel et al., 1989). Unfortunately the insertion of growth hormone genes in pigs did not produce only positive characteristics. The continual excess production of growth hormone in these animals decreased the general health of the animal. Specific health problems included lameness, lethargy and gastric ulcers. More importantly from

a reproductive stand point was that female animals that expressed the foreign growth hormone gene were anestrous. The use of transgenic growth hormone technology in these animals would require very strict control over timing and concentration of the production of growth hormone by the use of additional genetic regulators.

While the productive efficiency of transgenic swine is low, ranging from .3 to 1% transgenics for microinjected zygotes and early embryos (Purcell et al., 1990), 61 to 89% of these transgenic animals did express the gene. Four of five of the growth hormone transgenic expressing boars and four of five nonexpressing transgenic boars and gilts passed the gene to later generations with the percentage of progeny inheriting this transgene ranging from 2 to 73%. Not only was the gene passed to later generations, but the ability to express it was inherited. All transgenic progeny from expressor sires were able to express the gene at concentrations similar to their transgenic founders. Such germ line stability and subsequent expression in later generations is vital for the production of a transgenic line of animals.

Sheep containing transgenic growth hormone genes have been produced using two different growth hormone genes of ovine origin. Production of trangenic lambs ranged from 0 to .74% of ova injected (Murray et al., 1989). A total of seven transgenic sheep were produced. Four of the animals were derived from the sheep growth hormone gene labeled sGH5. None of the animals which contained this transgene expressed the growth hormone product. The researchers could not determine the cause for this lack of expression. It was noted that the gene contained residual viral DNA sequences. Three additional transgenic sheep were made that contained a growth hormone gene labeled sGH9 which had the viral DNA sequences removed. Growth hormone levels at 60 d of age in these growth hormone transgenic animals revealed an increase of circulating

growth hormone by 150 to 1000 times that of the control animals. However, the growth rate in these transgenic animals did not increase as compared to their contemporaries.

Rexroad et al. (1990) reported that the carcass composition of sheep could be altered by a reduction in fat when growth hormone genes were transferred. However, elevated growth hormone resulted in diabetes and death by 1 yr of age. It was suggested that increased effort be placed on the development of promoters with a more precise regulation of transgenes such as growth hormone or that other growth regulating systems be evaluated all together.

Regulation of Gene Expression

The use of a different promotor/regulator was considered by Wieghart et al. (1990). The bovine growth hormone structural gene was inserted into pigs. However, the promoter sequence was from the phosphoenolpyruvate carboxykinase (PEPCK) gene that would lead to primary expression in the liver and regulation by dietary carbohydrate level thus making it a good promoter regulator for a growth promoting gene. One thousand fifty seven porcine ova were microinjected with the PEPCK bovine growth hormone fusion gene. This resulted in 124 piglets being born, seven of possessed the trangene. Five of these transgenic animals had tissue directed expression of the bovine growth hormone in the liver. The production of growth hormone resulted in a beneficial decrease in backfat thickness of 41% compared to litter mate controls. However, the disadvantageous effect of excess growth hormone was not overcome using this promoter. Two of the three boars that were transgenic had reduced libido, which might have been related to abnormal joint development. One female had a delayed estrus that was induced hormonally at eight months of age. Germ line transmission was tested in three of the boars. One animal showed a Mendalian transmission of the transgene while the re-

maining two animals had no transmission. While this procedure did not completely overcome difficulties associated with manipulation of the growth hormone system, it does show promise in the use of tissue directed control of transgenes.

Another tissue directed promoter was discussed by Waldbieser et al. (1990). This group used the promotor for neuropeptide Y to direct the CAT marker gene product for secretion strictly in the nervous sytem. The CAT was produced only in brain tissue. While any direct application of this system to the production of transgenic lines of domestic animals is speculative, it does provide further evidence for the ability to place tight controls on the production of transgenes in animals.

Transgenic Modification of Milk Synthesis

In addition to manipulation of domestic animals by inserting growth related genes for the production of more efficent or modified animal growth, there is also work being conducted to modify milk synthesis. Specific proteins could be synthesized by the mammary gland for collection and use as theurpeutic proteins. One such protein of interest is Factor IX, an essential component of blood coagulation. Hemophilia type B is the result of a deficiency of Factor IX. Clark et al. (1989) reported the production of Factor IX in the milk of ewes that were transgenic for the Factor IX gene. Four Factor IX transgenic sheep were produced, two of which were female. When these animals lambed, they passed the transgene to their offspring as well as secreting the Factor IX in their milk. Mammary specific secretion was targeted using sequences from the beta-lactoglobulin gene. The full beta-lactoglobulin gene is a native sheep gene which is specific for mammary protein production. Although production of the Factor IX was present in the mammary gland, it was at a very low concentration in the milk. The level was approximately 250 fold lower than what was normally found in human plasma. But,

more important than the level of Factor IX was the mammary specific production. This system allowed for the development of a potentially useful foreign protein production system that can be propagated. Additionally, it allowed collection of the protein from a non-native source that was fairly simple to collect. The Factor IX protein produced in milk was active in clotting assays. However, there were differences between the post-translational modifications of the transgenic sheep milk protein and the native human protein (Wilmut et al., 1990).

Another tissue specific DNA promoter is whey acidic protein. This protein has directed secretion to the mammary gland and is a native gene in mice and rats. The whey acidic protein promoter has been used to direct the secretion of human tissue plasminogen activator into the milk of mice. The entire tissue plasminogen activator protein was synthesized and had biological activity (Gordon et al., 1987; and Pittius et al., 1988).

There are numerous advantages to the production of therapeutic protein such as Factor IX and tissue plasminogen activator in the milk of domestic animals. Many therapeutic proteins are presently derived from human blood and therefore caution must be taken to prevent contamination by infectious agents such as the Human Immuno-deficiency Virus (HIV) or hepatitis. Proteins harvested from milk would be potentially free of such infectious contaminants. The production of transgenic domestic animals is currently very expensive due to the inefficiency of integration. Once established, the propagation of lines of these animals would be less expensive than the use of large scale mammalian cell culture systems to produce therapeutic proteins. Direction of production of therapeutic proteins to the mammary gland offers the benefit of relatively simple collection of the carrier fluid, milk, that is often present in large volume. It additionally protects the animal from systemic exposure of such proteins, especially those proteins

that are involved in blood coagulation which could be detrimental to the animal if present in systemic circulation (Wilmut et al., 1990).

Limiting Factors in the Production of Transgenic Animals

There are numerous difficulties in current procedures for the production of trangenic domestic animals. Understanding and overcoming these difficulties is necessary to make procedures more efficient. One such difficulty that which makes microinjection procedures difficult is that of visualization of the embryo pronuclei or nuclei. These structures can be adequately seen in rabbit and sheep embryos by the use of interferance contrast microscopy (Hammer et al., 1986). However, centrifugation in conjunction with interferance contrast microscopy is necessary for visualization of porcine and bovine pronuclei nuclei (Wall et al., 1988).

Another major factor that decreases efficiency of transgenic production is the detrimental effect of the microinjection procedure itself. It has been observed that some zygotes will lyse within a few minutes after injection while others that do not lyse fail to develop in vivo. Hammer et al. (1985) reported that only 10% of 233 sheep embryos that had been microinjected with DNA survived to the blastocyst stage in combined in vitro and in vivo culture. This compared to 26% for non-injected embryos. In pigs, similar results were seen with 23% of 165 microinjected embryos surviving, while 52% of the non-injected survived to the blastocyst stage. The survival rate of non-injected embryos is still below that which is desired and it was speculated that this low development rate may be partially due to embryos being held in culture for 12 h.

Robl and First (1985) reported that the primary embryo loss due to microinjection in mice was due to the mechanical insertion of the microinjection needle into the nucelus.

They observed that 95% of 37 mouse embryos that were not microinjected, but held in vitro, developed to the morula or bastocyst stage. This compared to 64% of 14 embryos surviving to the morula or blastocyst stage after the microinjection needle was inserted in the nucleus but no injection was performed. Seventy percent of 23 embryos which were microinjected with medium developed to the morula blastocyst stage and 62% of 26 embryos which received a DNA construct injection developed to the morula or blastocyst stage. Differences in development between nonmanipulated and microinjected embryos was greater after transfer to recipient animals. Fifty-eight percent of one hundred six non-injected embryos developed to offspring while only 14% of 93 injected embryos developed to offspring. It was speculated that such a loss may be due to chromosome damage which resulted from the microinjection pipette.

Hawk et al. (1989) suggested that bovine embryos may be resistant to the traumatic affect of pronuclear microinjection. One hundred eighteen non-injected embryos and 186 pronuclear injected embryos were cultured for 7 to 9 d in rabbit oviducts. Evaluation of the developing embryos, after removal from the oviduct, showed no difference between the development to the blastocyst or morula stage between the control or injected groups. There was a difference in the rate of development observed after 8 d in culture in that the control embryos tended to develop slightly faster than the injected embryos. Observation of the embryos that were recovered after 9 d revealed a 67 and 68% development to the morula or blastocyst stage by controlled and injected embryos, respectively. Control or injected bovine embryos that had been held in rabbit oviduct for 5 d were transferred to recipient cows. A total of 40% of 15 noninjected embryos and 56% of 25 injected embryos were recovered after 7 d in the recipient. There was no inhibition of development by the foreign DNA either in oviduct culture or after transfer to recipi-

ent cows. However, there was no analysis for the efficiency of integration of the gene, therefore the efficiency of the production of transgenic animals could not be determined.

Brinster et al. (1985) evaluated various other factors which affect integration frequency of microinjected mouse embryos. In considering the effect of microinjection itself, 73% of 60 embryos that were not injected developed into viable fetuses. This compared to 28% of 100 embryos that received a buffer injection only and no DNA. Various DNA concentrations ranging from .01 to 1.0 ng/μl were considered. A 23% survival rate was observed with a DNA injection of .01 ng/μl, 19% survival with .1 ng/ml DNA and 19% for 1 ng/μl DNA injection. Even though survival of embryos injected with 1 ng/μl DNA was low, an increased in integration efficiency made it the most efficient. The overall integration of DNA was 5.9% compared to an integration rate of 3.4 and 1% for microinjection of .1 and .01 ng/μl DNA. Site of DNA microinjection into one- or two-cell mouse embryos had an effect on survival rate and integration frequency. When injection was made into the cytoplasm, survival rate ranged from 17 to 50%, however the integregation frequency ranged from 0 to 1.2%. Nuclear injection of two-cell embryos resulted in a 9.4% survival rate and a 1.3% integration frequency. Integration frequency in fetuses was 17% when injection occurred into the male pronucleus, while a 13% integration rate was observed with injection into the female pronucleus. And finally, it was observed that the breeding of the mouse, from which the ova were collected, had an influence on integration. Hybrid mice yielded a greater integration frequency than did inbred mice.

Cleavage stage at the time of microinjection appears to have an effect on the survivability of porcine embryos. Pursel et al. (1988) reported that 26% of porcine embryos injected at the one-cell stage developed to 16-cell or greater, while 46% of

two-cell injected embryos developed to later stages. Integration frequency for microinjected zygotes was .5%, while two-cell microinjected embryos resulted in a transgenic animals at a rate of 1.0%. The authors stated that there was an increased chance for mosaic integration when two-cell embryos were used (Pursel et al., 1988). These data in swine are in contradiction to published reports in mice which found that survival and integration frequency was better for the microinjection of zygotes when compared to two-cell embryos (Brinster et al., 1985).

From the data presented, there are many factors which must be considered when trying to optimize a transgenic program. The difficulties should not overshadow the success which has occurred in many different species. This gives promise to the future for the efficient production of transgenic animals. Although much work is needed, there is a very bright future for the production of animals that are more efficient in the production of food and fiber as well as the use of animals for the production of necessary and even vital therapeutic proteins.

Homeostatic Mechanisms and Protein C Action

There are three primary mechanisms which control bleeding; 1) constriction of the wall of the damaged vessel, 2) platelet adherence and aggregation at the site of injury, and 3) formation of the fibrin clot. The overall mechanism of hemostasis can be separated into two distinct yet continuous stages. Primary hemostasis refers to the occlusion of the opening in the blood vessel by vessel constriction and platelet aggregation. This mechanism is only of temporary benefit and must be reinforced by fibrin. The secondary mechanism, fibrin formation, gives strength to the platelet plug until healing can be

completed (Saito, 1984). The primary role of the epithelium of the vasculature is to provide a surface which is resistant to clotting. However, epithelial cells contain a number of substances which aid in the clot formation process. These include von Willebrand factor (factor VIII related antigen), plasminogen activator and its inhibitor 1, tissue factor thromboplastin and prostacyclin (PGI_2). In addition, the exposure of the collagen layer beneath these cells stimulates platelet aggregation and the hemostatic process (Giddings, 1988). Adherence of platelets to exposed collagen results in major changes in platelet morphology and substances previously held in dense bodies are released into the cytoplasm and, secondarily, they appear outside the cell. ADP release causes platelets to become sticky which increases adhesion of other platelets and perpetuates the aggregation process. The platelet plug will continue to grow as long as the flow of blood delivers additional platelets or until the procedure is halted by the formation of the fibrin lattice which engulfs the plug (McGilvery, 1983).

Fibrin formation, the secondary stage of hemostasis, is an extremely complex series of reactions involving numerous plasma proteins. The intricacy of this mechanism is evident in that there are 14 well recognized coagulation factors, not to mention their regulatory agents which play a role in coagulation, and other components which are yet unclear (Saito, 1984).

Blood coagulation can be initiated by either intrinsic or extrinsic mechanisms. While these pathways initiate differently, they reach a point of convergence after which the progression of the reaction mechanism is the same. In the intrinsic pathway Factor XII, Prekallikrein and High molecular weight-Kininogen result in the conversion of Factor XI zymogen to the active enzyme. Factor XIa actives Factor IX which can then join Factor VIII and cofactors to activate Factor X. This is the point where the extrinsic

pathway converges with the intrinsic pathway. Factor VII and tissue factor (Factor III) of the extrinsic mechanism can also activate Factor X. Factor X in conjunction with Factor V convert prothrombin to the active thrombin which cleaves the α and β chains of fibrinogen to produce fibrin monomers which can be utilized to form the fibrin fibers of the clot lattice (McGilvery, 1983; Saito, 1984; Giddings, 1988).

There is one area of the coagulation mechanism which needs further detail to serve the purpose of this review. With the complexity of the blood coagulation system and the various coagulating factors which evolve in a cascading mechanism, how is the formation of a clot limited to the needed area and not allowed to spread throughout the body? This is partially due to the trapping of many clotting factors within the clot itself due to binding with fibrinogen and phospholipids. A more important mechanism is the inactivation of the major coagulation Factors V, VIII and X by the serine protease protein C (McGilvery, 1983).

Protein C is a vitamin K dependent coagulation inhibitor which exhibits its strong anticoagulant action only after thrombin (a major late stage coagulation compound) activates the protein C zymogen. The molecular weight of the zymogen is approximately 62,000 of which 23% is carbohydrate. It is made up of a 21,000 MW light chain and a 41,000 MW heavy chain held together by a disulphide bond. The active enzyme has had a segment, approximately 1000 MW, cleaved from the amino terminal of the heavy chain. The active serine site of this protease is on the heavy chain. A somewhat unique and vitally important aspect of the protein C molecule is the presence of gamma carboxylated glutamic acid residues on the light chain. Such modificiations are essential for activity in that they are responsible for the calcium mediated binding of protein C to phospholipids (Giddings, 1988).

The mechanism of action of protein C is a negative feedback loop. Factor VIII and V are involved in activating thrombin. Thrombin, in turn, modifies these componds to increase their activity while simultaneously activating protein C. Protein C then destroys the thrombin activated Factor VIII and V thus slowing the clotting mechanism (Giddings, 1988).

It is evident that the absence of protein C would have profound effects on the inhibition of the clotting mechanism. Such states can include tissue necrosis due to purpura - blood pooling in the skin (Lowe, 1984). Homozygous protein C deficiencies are rare. Without treatment, this state results in thrombotic complications which are fatal to the neonate. Current treatments utilizing blood derivatives, such as fresh frozen plasma or prothrombin complex concentrate, can lead to further complications. These include difficulties associated with catheterization, thrombosis and blood transmitted diseases. Treatment with purified, pasteurized plasma products with high protein C activity or recombinant protein C would be a more desirable method (Marlar and Adcock, 1990).

In a population of American blood donors, it was observed that .3 to .5% of the popuation had a heterozygous deficiency of protein C. However, detrimental effects were not seen even when protein C level was as low as 33% of normal (Miletich et al., 1987). Mann and Bovill (1990) observed that protein C deficiency was an important factor in thrombotic risk, but deficiency alone could not be used to assess the extent of such risk.

Protein C limits platelet-dependent arterial thrombus formation without altering the hemostatic function of platelets (Hanson et al., 1990). Because of this mechanism, protein C may be used for a variety of therapeutic situations. As mentioned previously, it would be effective for treatment of homozygous protein C deficiencies. Other

thrombotic disorders that could be treated with protein C include septic shock and warfarin-induced skin necrosis that results from thrombosis within venules. Heparin-induced thrombocytopenia could be prevented or corrected with the use of protein C therapy instead of heparin for thrombotic disorders such as thrombophlebitis or pulmonary emboli. Protein C also has been indicated as an agent which protects cardiac tissue from damage during ischemia (Comp, 1990).

Chapter 3

The Effect of Day of the Estrous Cycle, Location of Ovulatory Structure and Progesterone on In Vitro Bovine Endometrial Secretions

Barry L. Williams

Abstract

Endometrial tissue was collected by biopsy from mature Holstein cows on d 0 (estrus), 9, 14 and 18 of the estrous cycle to determine the effects of day of the cycle, ovulatory structure (mature follicle or CL) and progesterone (P_4) on endometrial protein secretion. Tissue was incubated for 24 h in supplemented media containing ^{14}C amino acids and either 0.0, 4.7 or 47 ng P_4/ml. Medium was analyzed for total protein, radiolabelled protein and profile of protein released during incubation. Day 0 endometrial tissue released more protein than did tissue collected on all other days ($P < 0.05$). ^{14}C labelled proteins were greater on d 0 and 18 than d 9 and 14. Endometrium from the uterine horn contralateral to the ovulatory structure. released a greater amount of radiolabelled protein than endometrium from the uterine horn ipsilateral to the ovulatory structure. Seventeen protein bands were identified by electrophoresis. Proximity of the ovulatory structure to the uterine horn affected the presence of four specific proteins (MW 21.4, 55.0, 74.6 and 88.1 x 10^3). The proportion of released proteins which was represented by proteins MW 12.7, 17.0, 19.1, 21.4, 32.0, 40.3 and 66.5 x 10^3 was affected either by day of the estrous cycle, relationship of the uterine horn to the ovulatory stucture or P_4 concentration. The results show that day of the estrous cycle and location of the ovulatory structure not only alter overall endometrial protein secretion and synthetic activity but also have specific effects on individual proteins.

Introduction

It is well established that variation occurs in uterine protein production during the estrous cycle as well as during pregnancy (Roberts and Parker, 1974). The importance of this changing uterine environment, which results from uterine secretions, on the development of the embryo has been documented by Wilmut et al. (1986). Fischer and Beier (1986) reviewed the functions of uterine secretions which change in a cyclic manner. These changes result in a synchronous environment that meets the changing demands of the preimplantation embryo. These phenomena have best been demonstrated by the decreased ability to maintain pregnancy when embryo are transferred to nonsynchronous females (Seidel, 1980).

Embryonic secretions are known to have an effect on secretion of specific uterine proteins during the period of maternal recognition of pregnancy (Gross et al., 1988). However, factors which control changes in uterine protein secretion prior to pregnancy recognition or during the estrous cycle are less clear. Plasma hormone profiles have been shown to be associated with variation in uterine protein secretion (Anderson et al., 1986; Guise and Gwazdauskas, 1987). During the first 8 d of the cycle, Malayer et al. (1988) and Malayer and Hanson (1990) found that heat stress, day of the estrous cycle and local ovarian influence had an effect on endometrial protein secretion. It is not known whether these or other factors, independent of the embryo, prepare or allow for maternal recognition of the embryonic signal which is critical for pregnancy maintenance.

Kickerbocker and Niswender (1989) found that changes in the ovine trophoblastic protein-1 (oTP-1) system were similar in both cyclic and pregnant ewes during the first 12 d following estrus. Endometrial receptors for this protein increased in both number

and affinity as the endometrium was prepared for embryonic signaling. Although this report was from the ovine species, the process of pregnancy recognition in the bovine and ovine are believed similar due to the similarity of the bovine trophoblastic protein-1 (bTP-1) and oTP-1 systems (Helmer et al., 1987). The finding of this maternal preparative change in sheep and cattle is one example of the types of maternal changes which might occur. The current study was conducted to determine if day of the estrous cycle, location of ovulatory structure or progesterone (P_4) control in vitro synthesis and secretion of endometrial proteins.

Materials and Methods

Tissue Incubation

Uterine biopsy forceps (Jorgensen Laboratories, Inc., Loveland, CO) were used to collect tissue from each uterine horn of mature Holstein cows on d 0 (estrus), 9, 14 and 18 of the estrous cycle. These sample times represented estrus, early luteal phase, mid-luteal and luteal regression. Tissues were collected from each uterine horn for the days sampled. Sixteen estrous cycles from 15 cows were represented (Fig. 1). After collection, tissue was washed twice in Medium 199 (Gibco, Grand Island, NY) to remove blood and other extraneous material. The endometrium from uterine tissue (approximately 84 mm^3) was isolated and cut into 1.5 mm^3 or smaller pieces. Two to seventeen mg of endometrial tissue were aliquoted into 1.6 ml of Medium 199 supplemented with an antibiotic/antimycotic (100 units penicillin, 100 μg streptomycin, 0.25 μg amphotericin B/ml; Gibco), ^{14}C amino acids (5 μCi; Amersham, Arlington Heights, IL) and either 0.0,

4.7 or 47 ng P_4/ml. Tissues were incubated for 24 h at 37°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Media collected at 24 h for protein analysis.

Quantitative Protein Analysis

Protein was precipitated from enriched medium using trichloroacetic acid (TCA). The procedure involved adding 2 ml of 10% TCA (4°C) to 0.6 ml of medium followed by 30 min incubation at 4°C and centrifugation at 2000 x g for 10 min. The supernatant was removed and the pellet washed three times using 0.5 ml 5% TCA (4°C) with centrifugation at 2000 x g for 5 min. The final pellet was resuspended in 0.5 ml of 0.2 M NaOH. Spectrophotometric determination of total protein utilized the Coomassie brilliant blue protein dye technique as described by Bradford (1976). This procedure measured all protein released into the media. Reference protein concentration standards were bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO.).

Measurement of radiolabelled amino acid presence in the precipitated proteins was by liquid scintillation spectroscopy of 50 μl of resuspended protein. Analyzing the precipitated protein for ^{14}C provided a measure of protein synthesis and tissue activity during incubation. ^{14}C disintegrations per minute (DPM) were corrected for NaOH quench, while the level of protein was found to have no quench effect. Wet weights of the tissues after incubation were recorded for standardization of data on a per mg tissue basis.

Qualitative Protein Analysis

Samples within day by uterine horn by P_4 supplementation level (n = 141) were randomly paired and pooled. This provided for an increase in the protein quantity to facilitate SDS-PAGE analysis of qualitative differences between the protein samples.

Protein recovery from incubation medium for this procedure also utilized TCA precipitation with the modification that the pellet was washed twice with diethyl ether to remove residual TCA and dried. The protein was resuspended in 50 μl of reducing SDS-PAGE sample buffer (0.625 M Tris, 10% glycerol, 0.01% w/v bromophenal blue, and 9% v/v 2-mercaptoethanol at pH 6.8). Protein samples were separated by Laemmli (1970) SDS-PAGE procedures as described by Hoefer Scientific Instruments (1988-1989) using 4% stacking gels with 10% separating gels. Gels were stained using 0.1% Coomassie brilliant blue in 41.7% methanol and 16.7% acetic acid, destained with 3 changes of 30% methanol and stored in 10% acetic acid for densitometric scanning. Denatured protein standards used for molecular weight (MW) estimates ranged from 97.4 x 10^3 to 14.2 x 10^3 (Sigma Chemical Co., St. Louis, MO). Gels were run in a cooled buffer, 14 x 16 cm vertical slab gel apparatus (Hoefer Scientific Equipment, Model SE 600).

Statistical analysis

Quantitative differences in total protein and presence of radiolabelled amino acid in the protein were determined using GLM and Tukey's mean separation test (SAS, 1985). The dependent variables were tested for variation due cow, relationship of the uterine horn to the ovulatory structure, day of the estrous cycle and P_4 supplementation level. Qualitative differences in endometrial protein were determined by two methods. The presence or absence of specific protein bands was tested by Fisher's Exact Test as well as Chi-square analysis with comparable results. Variation in the individual protein percent of the total protein was analyzed using GLM procedures (SAS, 1985). The model included polyacrylamide gel on which proteins were seperated, day of the estrous cycle,

proximity of ovarian structure (uterine horn), P_4 level in culture and all two-and three-way interactions.

Results

Quantitative Protein Analysis

Least-squares means for total protein in the medium (μg/mg tissue) and presence of ^{14}C labelled protein (DPM/mg tissue) for day of the estrous cycle, uterine horn relative to most recent ovulatory structure and P_4 supplementation are presented in Tables 1 - 3. Day significantly affected ($P<0.01$) both total and labelled protein. Total protein was greater on d 0 than all other days while d 14 protein level was higher than d 9 (Table 1).

No difference was seen in total protein produced due to the proximity of the ovulatory structure. However, labelled protein was greater ($P<0.05$) from the contralateral uterine horn (Table 2). Varying amounts of P_4 in vitro had no effect on total or labelled protein (Table 3) and no interaction effects between day, uterine horn and P_4 concentration were detected.

Qualitative protein analysis

Seventeen protein bands identified by SDS-PAGE were considered when evaluating the possible effects of day of the estrous cycle, uterine horn and P_4 level on protein profile. Densitometric scans of the electrophoresed samples allowed comparison of treatment groups and identification of differences present (Fig. 2). The estimated MW, relative

mobility to albumin (Ra) and mean percentage that each protein represented as a proportion of total are presented in Table 4.

Fisher's Exact t-Test for differences in the presence of individual protein bands, without regard to the quantity present, indicated no effect due to in vitro P_4 concentration. The presence of proteins MW 21.4 x 10^3 and 88.1 x 10^3 was affected by uterine horn ($P<0.05$, data not shown).

Uterine horn relative to ovulatory structure had a significant effect ($P<0.05$) on the profile of media proteins, as well as day of the estrous cycle ($P<0.10$). In vitro P_4 concentration and interactions between main affects did not significantly affect overall protein profile ($P>0.10$). Further analysis identified specific proteins which were responsible for these overall effects. The percentage of the total protein which a specific band represented revealed that the distribution of seven proteins was affected ($P<0.10$) by one or more of the main effects. The proportion of proteins MW 21.4 x 10^3 and 32.0 x 10^3 was affected by uterine horn ($P<0.05$), having a greater presence in the contralateral uterine horn. Protein MW 66.5 x 10^3 (albumin) also was affected by uterine horn ($P<0.10$), however its quantity was greater in the ipsilateral uterine horn (Table 5). In addition to the uterine horn effect, protein MW 32.0 x 10^3 was affected by the interactions between uterine horn and P_4 ($P<0.05$; Table 6) and uterine horn and day of the cycle ($P<0.01$; Table 7). Day of the estrous cycle was the only factor affecting proteins MW 40.3 x 10^3 ($P<0.10$) and 12.7 x 10^3 ($P<0.01$; Table 8). As stated previously, P_4 concentration had no effect when considering the overall protein profile. However, P_4 concentration (Table 9) and uterine horn by P_4 interaction (Table 6) were responsible for variation in individual proteins MW 19.1 x 10^3 and 17.0 x 10^3 ($P<0.10$). Addi-

tionally, protein MW 17.0 x 10^3 ($P<0.10$) was affected by day of the estrous cycle (Table 8).

Proteins MW 66.5 x 10^3 and 59.4 x 10^3 were the most prevalent representing 32.8 and 22.8% of the total protein while eight proteins contributed less than one percent of the total protein present (Table 4). The amounts of individual proteins that were different due to uterine horn and day of the cycle are presented in Tables 5 and 8, respectively.

Discussion

Quantitative protein analysis

At the time of explant preparation, gross observation of d 0 tissue revealed an edematous state. This condition is consistent with the estradiol predominant environment during that period (Asdell et al. 1949; Weber et al., 1948). Such a condition could result in greater release of proteinaceous tissue components as transudate into the medium and would explain the higher total protein amounts on d 0. This finding is similar to that of Moffatt et al. (1987) who found that estrone increased total uterine protein secretion in pregnant ewes when measured in vivo. The cause of the difference between d 9 and 14 protein levels in the current study is less apparent. A possible explanation is the initiation or acceleration of protein secretion between d 9 and 14 to provide nutrients for the possible presence of a developing embryo.

During the luteal phase of the estrous cycle, endometrial synthesis and secretion of protein (Table 1) does not appear to be increased to support possible embryonic devel-

opment prior to the maternal recognition of pregnancy. This finding would suggest that if an alteration in endometrial synthetic activity does occur during the luteal phase, it may result in a qualitative redistribution of the proteins synthesized rather than a simple increase or decrease in total protein. This is supported by the qualitative data.

Embryo transfer research in cattle has shown a significant decrease in pregnancy rate when the transferred embryo is placed in the uterine horn contralateral to the CL (Greve and Del Campo, 1986; Sreenan and Diskin, 1987). Our data do not support a quantitative local synthetic activity in the ipsilateral uterine horn to supply nutrients to the developing ovum if present. However, the greater amount of labelled protein in the contralateral uterine horn does suggest a difference in the biosynthetic mechanisms. The present finding of no in vitro P_4 effect is in agreement with previous work (Moffatt et al., 1987) that found no significant effect on total in vitro endometrial protein production from tissue of ovariectomized P_4 supplemented ewes. However, these workers found that protein synthetic activity was enhanced. The absence of a P_4 effect in vitro is in contrast to the known association of varying endogenous P_4 levels with uterine protein production in vivo in cattle (Anderson et al., 1986; Guise and Gwazdauskas 1987) and swine (Knight et al., 1973). The importance of appropriate P_4 stimulation of the endometrium must not be overlooked. Evidence of decreased fertility due to an abnormal number of endometrial P_4 receptors has been found (Almeida et al., 1987). In vitro duplication of endogenous P_4 activity has not been accomplished in this study but developing such a model would be beneficial in future research on endometrial secretions.

Qualitative protein analysis

The effect of specific uterine proteins on the developing embryo is not understood. However, it is evident from the electrophoretic analysis of proteins that variation between day of the cycle and changes in the physiological status of the endometrium are expressed in secretion of proteins. Malayer et al. (1988) reported differences in specific proteins relative to the location of the developing CL especially early in the estrous cycle. One such protein, 35 x 10^3 MW, which these investigators reported as being affected by side of the CL may be the same as the 32.0 x 10^3 MW protein reported here. However, the previous work indicated a greater presence in the ipsilateral uterine horn which is in contrast to the findings discussed above. It is of interest that a protein resembling serum albumin (MW 66.5 x 10^3; Table 5) was found to represent a greater proportion of the proteins released into the ipsilateral uterine horn. The significance of this is unclear. However, it does show that protein differences between uterine horns may not be due only to synthetic mechanisms but to selective secretion of specific cellular and serum proteins.

The interaction between uterine horn and P_4 provides evidence of a local ovarian control mechanism. Although ipsilateral and contralateral tissues were exposed to the same level of hormone in vitro, local mechanisms prior to tissue collection could have prepared or directed the tissue action in vitro (Pope et al., 1982). The changing presence of proteins suggests that specific mechanisms vary due to changes in the estrous cycle. Varying P_4 in medium affected presence of MW 17.0 x 10^3 and 19.1 x 10^3 proteins. An inverse relationship existed between these proteins and P_4 alone (Table 9) and based on location of the ovulatory structure (Table 6).

The presence of two protein bands (MW 21.4, and 88.1 x 10^3) was affected by uterine horn. Since the majority of the observed proteins were present without regard to the main effects that were considered, it would appear that changes in the uterine protein profile are changes in quantitative protein not qualitative protein. The observation of variation in the proportion of individual proteins relative to the uterine horn (Tables 5-7) supports the assumption of local control of uterine secretions by ovarian structures. Interestingly, bTP-1 has been reported to have a MW of 21.2 x 10^3 (Lifsey et al., 1989) and 22 to 24 x 10^3 (Godkin et al., 1988). It is not known whether the protein MW 21.4 x 10^3 (Table 5) from the current study shares any characteristics with bTP-1 other than MW. The possibility of similarity between this embryonic signal and an endometrial protein regulated locally by ovarian structures needs further investigation.

While the statistical analysis indicated that 7 of the 17 protein bands were affected by day of the cycle, uterine horn, P_4 and/or their interactions, only protein MW 32.0 x 10^3 had more than one factor resulting in a significant effect. This protein represented 14.4% of the total media protein. These results demonstrate that production of this protein is subject to complex control mechanisms. Such intricate regulation suggests that this protein may be especially important in the changing uterine environment that is involved in the early development of pregnancy. Multiple interactive control mechanisms are consistent with the report of Malayer et al. (1988) who described the apparent interaction of local ovarian influence and d of the estrous cycle with heat shock alteration of endometrial secretion. Gross et al. (1988) reported that a 13.0 x 10^3 MW protein was secreted from both cyclic and pregnant 17 d endometrium under the influence of bovine conceptus secretory proteins. The present work identified a MW 12.7 x 10^3 protein which was less prevalent on d 18 than during d 9 and 14 of the luteal phase. It is uncertain if these are similar proteins. If these uterine proteins are the same, this

would suggest that the embryonic secretions are stimulating maternal factors which are present during the luteal phase even if pregnancy has not occurred.

Conclusions

The results of this study indicate that day of the estrous cycle and relative location of the ovulatory structure affect not merely the quantity of protein secretion and synthetic activity of the endometrium but also have specific effects on individual proteins released in vitro. Better understanding of the normal uterine protein profile, as it changes throughout the estrous cycle and especially during the period of changes necessary for pregnancy maintenance, will facilitate the determination of factors obligatory for support of early pregnancy.

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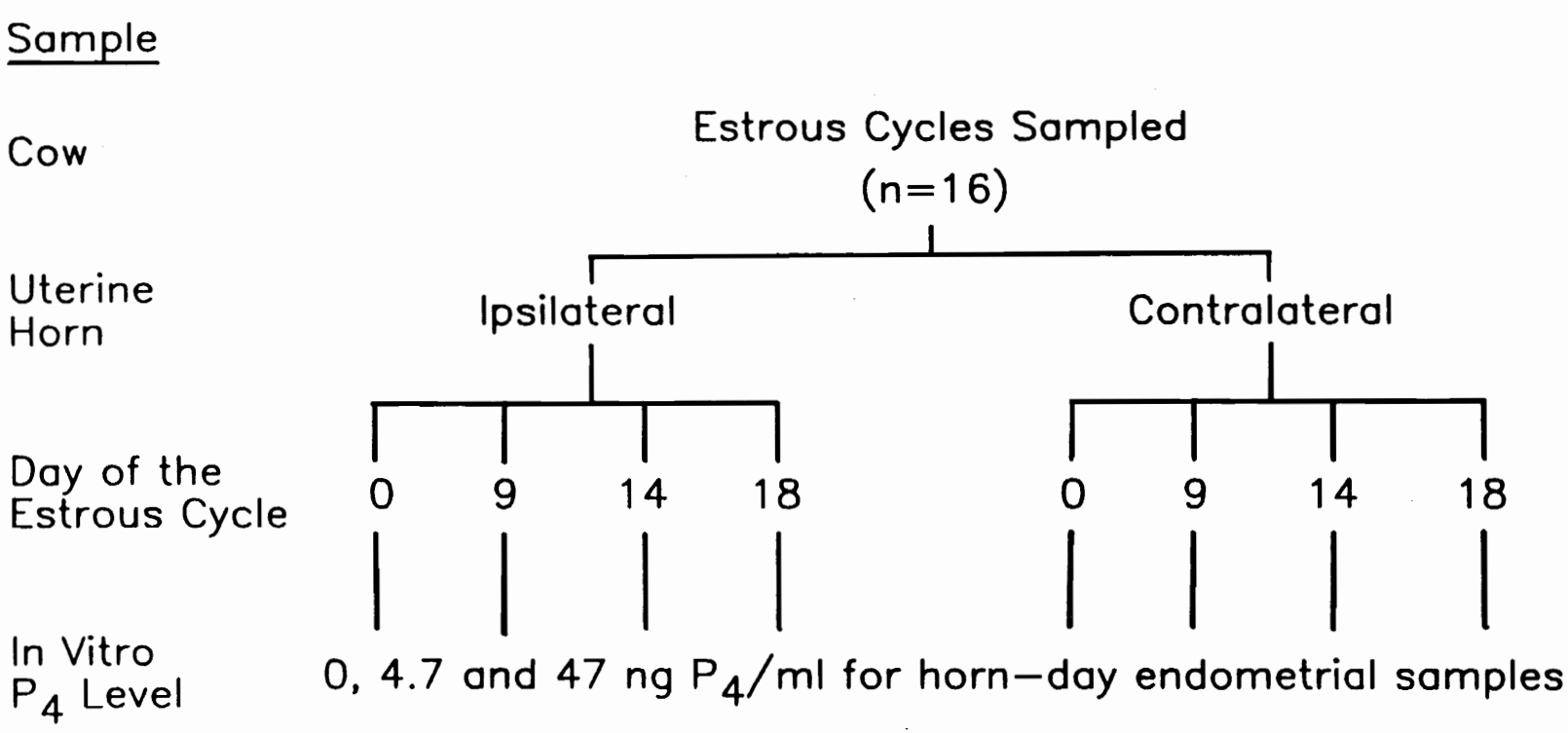

Figure 1. Schematic representation of the experimental design.

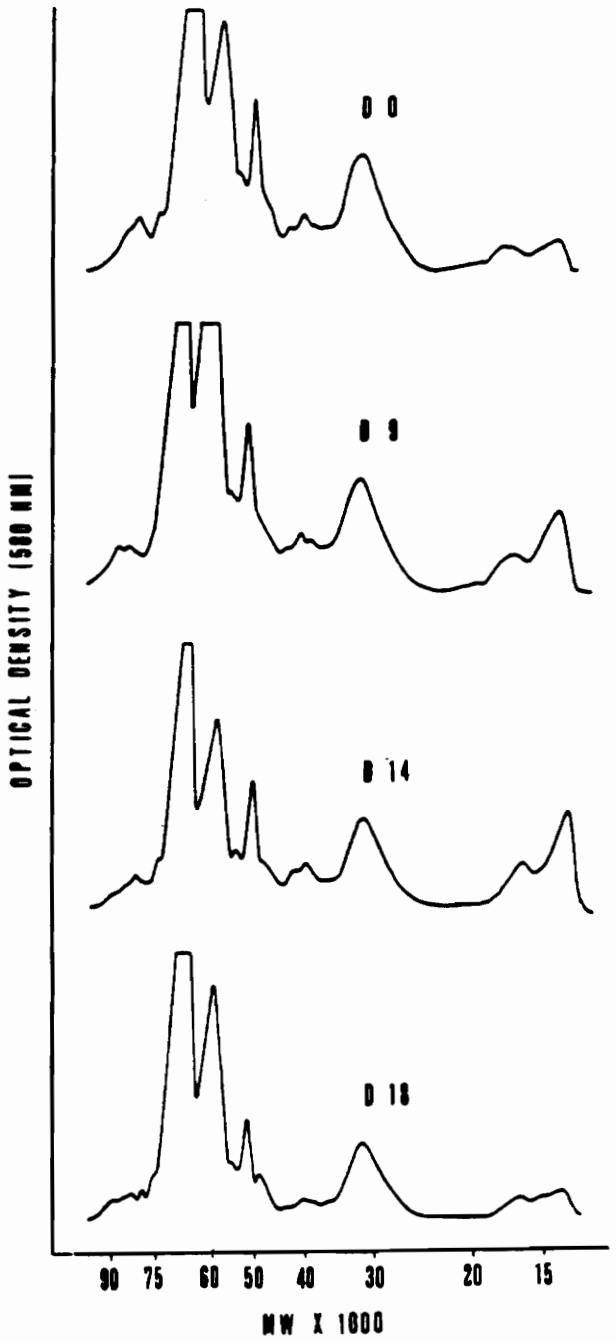

Figure 2. Representative SDS-PAGE densitometric profiles of media proteins from explanted bovine endometrium obtained on d 0 (D0 estrus), 9 (D9), 14 (D14), and 18 (D18) of the estrous cycle.

Table 1. Total and radiolabelled protein released from endometrial tissue collected on different day of the estrous cycle.

Day	Total media protein[a] (µg/mg tissue)	^{14}C Labelled protein[a] (DPM/mg tissue)
0	11.6 ± 0.8[b,c]	76.8 ± 4.1[c]
9	6.8 ± 0.9[d,e]	34.4 ± 4.6[d]
14	8.0 ± 0.9[d,f]	40.4 ± 4.5[d]
18	6.8 ± 0.9[d]	47.6 ± 4.3[c]

[a] Day effect was significant $P < 0.01$.
[b] Least squares $\bar{X}$ ± SEM.
[c,d] Values with different superscripts within columns differ $P < 0.05$.
[e,f] Values with different superscripts within columns differ $P < 0.05$.

Table 2. Total and radiolabelled protein released from endometrial tissue collected from different uterine horns.

Uterine Horn	Total media protein (μg/mg tissue)	^{14}C Labelled protein (DPM/mg tissue)
Ipsilateral[a]	7.8 ± 0.5[b]	45.9 ± 2.4[c]
Contralateral	8.8 ± 0.5	53.7 ± 2.8

[a] Side adjacent to ovulatory structure.
[b] Least squares $\bar{X}$ ± SEM.
[c] Uterine horn effect was significant ($P < 0.05$).

Table 3. Total and radiolabelled protein released from endometrial tissue exposed to different concentrations of progesterone (P_4) in vitro.

P_4 ng/ml	Total media protein (μg/mg tissue)	^{14}C Labelled protein (DPM/mg tissue)
0.0	8.4 ± 0.6[a]	50.7 ± 3.1
4.7	8.6 ± 0.6	49.1 ± 3.1
47.0	7.9 ± 0.6	49.5 ± 3.1

[a] Least squares $\bar{X}$ ± SEM.

Table 4. Estimated molecular weight (MW), relative mobility to albumin (Ra) and percentage of observed protein bands.

MW x 10^3	Ra	%	MW x 10^3	Ra	%
88.1	.66	0.8	42.2	1.55	0.9
82.2	.74	2.8	40.3	1.61	0.7
74.6	.86	0.8	32.0	1.88	14.4
66.5	1.00	32.8	30.2	1.95	0.7
59.3	1.14	22.8	21.4	2.37	0.3
55.0	1.23	2.3	19.1	2.51	0.7
51.3	1.32	9.2	17.0	2.65	2.7
49.8	1.35	1.5	12.7	2.89	6.3
44.4	1.49	0.3			

Table 5. Percent of total protein present as specific proteins as affected by uterine horn.

MW x 10^3	Uterine Horn: Ipsilateral[d]	Contralateral
66.5[b]	34.8 ± 1.42[a]	30.7 ± 1.21
32.0[c]	13.0 ± 1.41	15.7 ± 0.85
21.4[c]	0.1 ± 0.05	0.2 ± 0.02

[a] $\bar{X}$ ± SEM.
[b] Difference between uterine horns was significant ($P < 0.10$).
[c] Difference between uterine horns was significant ($P < 0.05$).
[d] Ipsilateral - side adjacent to the ovulatory structure.

Table 6. Effect of uterine horn by progesterone (P_4) interaction on percentage of protein distribution.

MW x 10^3	Uterine Horn[d]	P_4 level 0 ng	4.7 ng	47 ng
		%		
32.0[b]				
	ipsilateral	9.5 ± 4.8[a]	14.8 ± 1.0	13.9 ± 1.1
	contralateral	15.5 ± 0.5	15.0 ± 0.9	16.5 ± 2.6
19.1[b]				
	ipsilateral	0.2 ± 0.2	2.5 ± 0.8	0.0 ± 0.0
	contralateral	0.8 ± 0.4	0.2 ± 0.1	0.3 ± 0.1
17.0[c]				
	ipsilateral	2.9 ± 1.2	0.9 ± 0.3	3.7 ± 0.7
	contralateral	2.3 ± 1.1	3.0 ± 0.9	3.5 ± 0.8

[a] $\bar{x}$ ± SEM.
[b] Interaction effect significant ($P < 0.05$).
[c] Interaction effect significant ($P < 0.10$).
[d] Side adjacent to or opposite ovulatory structure.

Table 7. Effect of day by uterine horn interaction on protein MW 32.0 x 10^3 distribution.

Uterine Horn[a]	Day[b]			
	0	9	14	18
Ipsilateral	10.4 ± 5.3	14.1 ± 1.8	14.2 ± 0.4	13.8 ± 0.9
Contralateral	16.0 ± 0.8	14.4 ± 1.2	14.1 ± 1.4	18.2 ± 2.6

[a] ($P < 0.01$).
[b] % $\bar{X}$ ± SEM.

Table 8. Percent of total protein present as specific proteins as affected by day of the estrous cycle.

	Day			
MW x 10^3	0	9	14	18
40.3[b]	0.6 ± 0.1[a]	0.7 ± 0.2	0.9 ± 0.4	0.9 ± 0.3
17.0[b]	1.2 ± 0.3	3.4 ± 0.8	4.2 ± 0.8	2.3 ± 0.4
12.7[c]	3.2 ± 0.3	9.1 ± 1.0	9.5 ± 1.3	3.8 ± 0.7

[a] $\bar{X}$ ± SEM.
[b] Difference between day of the estrous cycle was significant ($P < 0.10$).
[c] Difference between day of the estrous cycle was significant ($P < 0.01$).

Table 9. Percent of total protein present as specific proteins as affected by concentration of progesterone (P_4).

	P_4[a]		
MW x 10^3	0 ng	4.7 ng	47.0 ng
19.1[b]	0.5 ± 0.3[a]	1.4 ± 0.6	0.1 ± 0.1
17.0[b]	2.6 ± 0.7	2.0 ± 0.6	3.6 ± 0.5

[a] $\bar{x}$ ± SEM.
[b] Difference between P_4 concentrations was significant ($P < 0.10$).

Chapter 4

Summary of Bovine Endometrial Secretions

Day of the estrous cycle, proximity of the ovulatory structure and P_4 were found to affect bovine endometrial protein secretions. Increased total protein release into the media from tissues of estrous cows and increased release of proteins synthesized while in culture from estrous and proestrous tissues parallel the changing endocrine profiles involved in regulating endometrial activity. These variables did not assess possible variation in intracellular protein accumulation or depletion. Evaluation of intracellular protein may provide further insight into the physiological changes occurring in the endometrium during the estrous cycle.

At the onset of the study it was theorized that differences in protein production occurred relative to the side of the ovulatory structure. These differences could result in an increased protein level in the ipsilateral uterine horn. While the data indicated differences in synthetic activity between uterine horns, protein synthesis and release was greater in the contralateral horn. The importance of this shift is unclear. Testing specific proteins that were major components of the uterine horn difference on developing embryo may provide further insight into the physiological consequences of local changes in uterine protein secretion. The action of all proteins in the uterine secretions should be considered. This is suggested because of the observation of a uterine horn specific secretion pattern of a protein with characteristics resembling serum albumin.

Changes in the presence or level at which specific uterine proteins occur help delineate the control mechanisms of endometrial protein secretion. The results allude to the complexity of such controls. Understanding the importance of such changes is necessary to more completely define the role of uterine secretions and their control mechanisms. Future research considering the uterine protein environment should evaluate the effect of proteins which fluctuate during the estrous cycle. The three proteins in this exper-

iment that were influenced by day of the estrous cycle may have specific actions that are involved in determining synchrony of the uterine environment with the early embryo or may control embryonic development. Determining which proteins are beneficial or detrimental to embryonic development is essential. The development of a map of appropriate uterine environment would be useful as a test to evaluate the reproductive capacity of an animal.

Chapter 5

Synchronization, Superovulation, and Collection of Porcine Zygotes for DNA Microinjection, Transfer and Culture

Barry L. Williams

Abstract

One hundred sixteen gilts and sows were used in a series of studies to evaluate estrous synchronization/ superovulation schemes for the collection of zygotes for DNA microinjection and transfer. Additionally, the effect of pronuclear microinjection of DNA and culture on zygote development was assessed in explanted mouse oviducts. Four synchronization/superovulation procedures were used: 1) sows were observed for estrous behavior; 1000 IU human chorionic gonadotropin (hCG) was administered at the onset of estrus. The sows were bred and zygotes collected 36 to 96 h after hCG (NAT); 2) cyclic gilts were synchronized with 17.6 mg altrenogest (ALT)/d for 15 to 19 d followed by superovulation with 1500 IU pregnant mares serum gonadotropin (PMSG) and 500 IU hCG. The animals were bred and embryos were collected 48 to 96 h after hCG (LALT); 3) gilts between 11 and 16 d of the estrous cycle received 17.6 mg ALT for 5 to 9 d. PMSG and hCG were used to induce superovulation. Gilts were bred and embryos collected 48 to 56 h after hCG (SALT) and 4) precocious ovulation was induced in prepubertal gilts with PMSG and HCG. Gilts were bred and embryos collected 52 to 57 h after hCG (PRE). Zygotes from PRE donors received either pronuclear microinjection of buffer alone, DNA construct or no microinjection. Zygotes were cultured in vitro in either modified Krebs Ringer Bicarbonate medium (KRB) for 144 h or in mouse oviduct (MO) explant culture with KRB for the initial 48, 72, 96 or 120 h with KRB for the remainder of the 144 h. NAT sows had fewer ovulatory structures (corpora hemorragica, corpora lutea and unovulated follicles) than all other procedures ($P < .05$). Synchronization treatments with PMSG did not differ ($P>.05$) in the number of ovulatory structures but SALT and PRE had a higher ovulation rate than LALT (24.7 $\pm$ 2.9, 24.3 $\pm$ 1.8 vs 11.6 $\pm$ 2.7 ovulations; $\bar{x}$ $\pm$ SEM). SALT and PRE provided 12.3

± 2.6 and 17.7 ± 1.7 zygotes. Pronuclear microinjection of DNA resulted in a lower (P<.05) cleavage index (CI) than buffer or no microinjection (CI 2.16 ± .10 vs 2.80 ± .13 and 2.93 ± .10). Loss was greatest for DNA injected zygotes at the two-cell stage of development. Coculture of zygotes in MO improved (P<.01) CI over culture in KRB. Culture in MO for 72 h was the most beneficial system compared to MO for 48, 96, or 120 h (P<.05; CI 3.25 ± .12 vs 2.66 ± .18, 2.79 ± .14 and 2.40 ± .14, respectively). A total of 505 DNA microinjected zygotes and embryos transferred into 17 recipients have produced 7 litters and 50 piglets of which 10 were transgenic. Microinjection of DNA, not merely the mechanical procedure, was detrimental to early embryo development and that culture for 72 h in MO provides for optimal CI.

Introduction

Production of transgenic animals by microinjection requires the use of early embryos. The overall efficiency of producing transgenic swine is about one percent due to a 40% ova degeneration rate within a few minutes following microinjection (Robl and First, 1985). In mice, the zygote is more likely to survive pronuclear microinjection and result in DNA integration than the nuclear injected two-cell embryo (Brinster et al., 1985). In contrast, two-cell porcine embryos are more likely to develop after microinjection when compared to zygotes. However, the use of two-cell embryos results in a greater probability of mosaic integration (Pursel et al., 1988). The need to obtain embryos of a specific developmental stage requires precise control of synchronization of ovulation and estrus.

Various approaches have been used to synchronize estrus in swine. These include the use of progestogen compounds that often resulted in impaired fertility and/or variation in time of estrus incompatible with successful collection of zygotes (Baker et al., 1954; First et al., 1963; Mayer and Schultze, 1977 and Webel, 1980). Use of prostaglandin (PG) $F_2\alpha$ or its analogs is not an efficient method to synchronize swine since the porcine corpora lutea (CL) are non-responsive to $PGF_2\alpha$during the first half of the estrous cycle (Diehl and Day, 1974).

Variability in expression of estrus and ovulation in swine may be reduced by the use of exogenous gonadotropins. Dziuk and Baker (1962) reported that 94% of animals receiving human chorionic gonadotrophin (hCG) ovulated within 48 h of the injection. Various combinations of pregnant mares serum gonadotrophin (PMSG) and hCG have been used to induce superovulation and estrus in prepubertal gilts. Guthrie (1977) reported that injection of PMSG followed by hCG 72 h later or PMSG/hCG co-injection followed by hCG 72 h later resulted in a similar number of animals exhibiting estrus and ovulation. However, there was a beneficial effect on ovulation rate with the PMSG/hCG co-administration (18.5 vs 13.5 ovulations). More recent work has shown that the combined use of a progestogen and PMSG/hCG allows for controlled synchronization and superovulation (Ebert et al., 1988; and Miller et al., 1989).

Research directed toward production of transgenic animals, is hampered by the inadequate in vitro development of early embryos. Recent studies have shown that the use of media supplemented with porcine oviductal epithelial cells results in a greater proportion of two-cell porcine embryos developing to blastocyst than those in medium alone (62 vs 0%; White et al., 1989). A more complex coculture system (Krisher et al., 1989) used in vitro culture of one-cell porcine embryos for 6 d in explanted mouse

oviducts. With this procedure, 78% of the embryos reached the morula or blastocyst stage compared to 36% in medium alone. While complex systems do allow for increased in vitro development of early embryos, the time that embryos must remain in such systems for improved development is not known.

Our object was to evaluate several synchronization and superovulation procedures for production of zygotes suitable for pronuclear microinjection. Culture procedures and incubation times were also evaluated for zygote development to the blastocyst stage.

Materials and Methods

Estrous and ovulation synchronization

Crossbred gilts and sows (n = 116) were used to compare four estrous synchronization/superovulation procedures. The first procedure used nonmanipulated estrous cycles of four multiparous sows (NAT). Sows were observed for estrous behavior two to three times daily. One thousand IU hCG was administered i.m. at the initial observation of standing estrus. Each sow was then bred by natural service two to four times prior to embryo collection. Collection of embryos and ovarian observation was accomplished by midventral laporatomy at decreasing time intervals (96 to 36 h) after hCG injection.

In the second study, the estrous cycles of 28 cyclic gilts between 205 and 265 d of age were synchronized by oral administration of 17.6 mg altrenogest in approximately 1.8 kg of feed. The synthetic progestogen was fed for 15 to 19 d and was initiated without

regard to stage of the estrous cycle (LALT). The initial 6 d regimen for superovulation utilized 1500 IU PMSG 24 h after the last feeding of ALT with 500 IU hCG 72 h later. Gilts were bred at 24 and 36 h after hCG by either natural service or artificial insemination (AI) Surgery was performed 48 to 53 h after hCG (6 d after last ALT feeding). Difficulties, which will be detailed below, required modification of the initial timetable for superovulation to a 7 and 8 d regimen. The 7 d schedule extended the breeding period to 60 h instead of the 36 h in the 6 d regime. The 8 d schedule also maintained the ALT, PMSG and hCG intervals but further extended the interval from hCG to surgery to 96 h and incorporated an additional hCG injection during that period in 81% of the animals treated. The time of the second hCG injection ranged between 12 and 48 h after the initial hCG injection.

In the third procedure, cyclic gilts (n = 24; 265 to 280 d of age) between 11 and 16 d of the estrous cycle were fed 17.6 mg ALT as previously described for 5 to 9 d (SALT). One thousand five hundred IU PMSG was injected 24 h after the last altrenogest feeding with 500 IU hCG injected 68 to 72 h later. Ovarian observation and ova collection occurred 48 to 56 h after hCG. Gilts used for ova collections were bred at 24 and 36 h after hCG.

In the final synchronization group, precocious ovulation was stimulated in 60 prepubertal gilts (PRE) 132 to 197 d of age. These females were classified as prepubertal by age, weight, external genitalia observation and herd history. Ovulation was synchronized by administration of 1500 IU PMSG followed by 500 IU hCG 68 h later. Gilts were bred by AI at 24 and 36 h post hCG followed by laparotomy 52 to 57 h post hCG.

Ovarian observation and embryo collection.

Surgical anesthesia was initiated with 1.5 g Pentothal and maintained with halothane and nitrous oxide. Ovarian examination was accomplished by exteriorizing the ovaries following midventral laparotomy. The number of corpora hemorrhagica (CH), CL and unovulated follicles by diameter were recorded for each ovary. The oviducts of animals serving as ova donors were flushed by inserting a silastic cannula through the ostium of the infundibulum reaching approximately 6 to 8 cm into the ampulla of the oviduct. Two 10 ml volumes of phosphate buffered saline (PBS) supplemented with 0.4% albumin, 10% newborn calf serum and antibiotic (.025 g/l) were flushed through the oviduct by inserting a 20 gauge hypodermic needle into the uterine lumen 0.5 cm posterior to the utero-tubal junction (UTJ) then passing the needle through UTJ or the isthmus immediately anterior to the UTJ. The flushing fluid was collected into sterile test tubes and transported to the laboratory at 37°C. A two piece collection cannula constructed of 1.98 mm ID, 3.18 mm OD tubing with a heat flared funnel of 1.57 mm ID, 2.08 mm OD tubing fitted to the inserted end of the larger tubing was used to flush the oviducts.

Microinjection and embryo transfer.

Zygotes were held in PBS during all manipulation and transfer procedures. To allow for visualization of pronuclei and nuclei, embryos were centrifuged at 15,000 x g for 8 min (Wall et al., 1985). After centrifugation, embryos were examined for presence of accessory sperm and pronuclear formation using Hoffman modulation optics (200 X). Zygotes with no visible pronuclei were classified as unfertilized ova (UFO). A portion of zygotes from PRE donors were randomly assigned to treatments of either no injection, buffer injection, or DNA injection. Buffer and DNA microinjection was ac-

complished by inserting a glass injection pipette (outside tip diameter 1.0 to 1.5 μm) into the most visible pronucleus while the zygote was held stationary with suction from a blunt polished holding pipette. One to two pl of buffer or DNA construct was precisely delivered using an automated microinjector. The remaining zygotes and embryos containing two or more blastomeres were used only for DNA injection. Two nuclei of embryos were injected. Zygotes and embryos to be transferred to recipients were placed into 1.57 mm ID, 2.08 mm OD polyethylene tubing. Recipients were surgically prepared as described previously. Recipients had "native" embryos flushed from the oviducts as previously discribed. Oviductal transfer was accomplished by inserting the tubing through the ostium of the infundibulum so that the end of the tubing was in the region of the ampullary-isthmic junction. Ova were expelled from the tubing using 0.5 to 1.0 ml PBS.

Construct preparation and analysis for incorporation of construct.

Plasmid DNA was isolated using a variation of Birnboim and Doty procedure (Birnboim and Doty, 1979). The DNA construct consisted of 1.5 kilobase (kb) of cDNA coding for human protein C inserted into the first exon of the murine Whey Acidic Protein gene (WAP; Hennighausen et al., 1984). Large scale purification of the DNA construct was done by digesting 200 μg of plasmid DNA with the restriction enzyme EcoRI (the WAP gene is flanked by restriction sites). The digested DNA was precipitated with isopropanol, and resuspended in TE buffer at 0.3 μg/ml. Fragment purification was performed using a Waters GEN FAX PAC high performance liquid chromatography (HPLC) column. The column was run isocratically using a buffer consisting of 25 mM Tris-HCl (pH 7.5), 1 mM sodium EDTA, and 0.63 M NaCl. This was the minimum NaCl concentration that would elute the large construct fragment and resulted in the

best seperation from the smaller vector fragment, which eluted just prior to the construct fragment. About 15 μg of digested DNA was loaded on the column. The construct fragment samples from all chromatographic runs were pooled, reprecipitated, and passed through the column a second time.

Methodologies have been developed for the rapid isolation of DNA from pig tails. The procedure used was a variation of the Marmur procedure (Marmur, 1959). Important features include: 1) Freshly cut tails were plunged into liquid nitrogen and stored until they were placed in lysing solution. This increased the rate at which the tissue was lysed and led to a reduced nuclease activity. 2) After an initial chloroform extraction, an aliquot was removed, precipitated and screened by Polymerase Chain Reaction (PCR) analysis. Samples from negative animals were discarded and pure DNA was isolated only from positive animals. 3) The first alcohol precipitation step was with 0.6 vol of isopropanol, which lowered the level of polysaccharide contamination. 4) The RNA was digested using both RNase A and T_1 RNase, which resulted in nearly RNA-free DNA preparations.

The general reaction mixture described by Saiki et al. (1988) was used in all PCR analyses. The oligonucleotide primer concentration was reduced from 1.0 μM to 0.5 μM. Cycle temperatures and times were 95.5°C for 1 min for denaturation, 58°C for 2 min for annealing, and 72°C for 2 min for elongation. Typically, 40 amplification cycles were run. Initially, 100 ng of purified DNA (about 10,000 genome copies) were used as template in the survey experiments. More recently, unmeasured, partially purified template DNA was used so that there would be more variation in template copy number. Therefore, two sets of PCR primers were included, one specific for the transgene and another specific for an indigenous gene (lactic dehydrogenase) as an internal control.

Embryo culture.

Zygotes recovered from 20 PRE gilts were used in each of two replicates to evaluate the development of zygotes maintained in mouse oviduct (MO) organ culture. Oviducts were collected from immature CD1 (21 to 28 d old) mice which were superovulated by injection of 7 IU PMSG followed by 7 IU hCG 48 h later. Each female was placed with one male at the time of hCG injection. Mice which exhibited a vaginal plug the following morning were used. Oviducts were removed 20 to 22 h post hCG and prepared in the manner described by Krisher et al. (1989). Zygotes that were either noninjected, buffer injected, or DNA injected were transferred into mouse oviducts using a fine glass pipette or were maintained as controls in modified Krebs Ringer bicarbonate medium (Davis and Day, 1978) without lactate and pyruvate (KRB). Three to 13 zygotes were transferred into the swollen ampullary region of each oviduct. Oviducts were placed on a raft of .20 μm filter paper suspended in KRB. The organ culture was held at 37.5°C in a humidified atmosphere of 5% CO_2 in air. Zygotes classified as UFO were not microinjected, but were cultured in KRB.

Porcine embryos within MO were removed after either 48, 72, 96, or 120 h of culture and placed in KRB for the remainder of the culture period. These treatments were identified as MO 48, MO 72, MO 96 and MO 120. Development of embryos cultured in KRB was assessed at 24 h intervals, while embryos cultured in MO were assessed upon termination of organ culture and at 24 h intervals thereafter. Duration of culture was 144 h. Embryo development was scored using a cleavage index (CI) where two-cell embryos equal CI of 1, three to four-cell = 2, five to eight-cell = 3, nine to 16-cell = 4, morula = 5 and blastocyst = 6.

Statistical analysis.

Statistical analysis of differences in synchronization, ovulation and superovulation response was performed using Duncan's mean separation test (SAS, 1985). Variation in development of cultured embryos was tested by two methods. Chi square analysis was used to determine differences in the distribution of embryo CI due to microinjection or culture treatments. CI was also analyzed using GLM with a model that included microinjection, culture system, microinjection x culture and replicate. Non-orthogonal contrasts were used to compare differences between pairs of microinjection and culture system treatments and were tested using the Improved Bonferroni F statistic (SAS, 1985).

Results

Synchronization of ovulation.

There were no difference among treatments ($P>.05$) in ovulatory structures (Table 1). However, animals which did not receive PMSG had less ($P<.05$) ovarian activity. Ovulation response did not differ ($P>.05$) between the SALT or PRE treatments. Although not significant ($P>.05$), the PRE group supplied 43.9% more zygotes than SALT. A sub-sample of the ova classified as UFO from the SALT ($n=136$) and PRE ($n=177$) groups were placed into culture. A total of 30.9% of the ova from the SALT group cleaved at least once in culture, compared to 43.5% from PRE treatment group. Only 37.5 and 75% of the gilts in the LALT treatment ovulated with the 6 and 7 d regimen prior to surgery compared to 93.8% ovulation for animals on the 8 d regimen.

Pronuclear microinjection.

Four hundred thirty six (72.9%) of 598 pronuclear microinjected zygotes that were cultured in vitro cleaved. Neither pronuclear microinjection nor culture method affected the proportion of zygotes that cleaved to two-cell (P>.05). Only cultured embryos that achieved a CI of 1 or greater were included in further statistical analysis of maximum CI. Embryos that were initially classified as UFO and attained a CI of 1 were treated identically to nonmicroinjected embryos cultured in KRB. A total of 514 embryos with a CI of 1 or greater were included in the final data set.

Microinjection affected (P<.01) CI attained in vitro. At 144 h, CI for non-injected, buffer or DNA microinjected embryos was 2.93 ± .10, 2.80 ± .13 and 2.16 ± .10, respectively

($\bar{x}$ ± SEM, Figure 1). The DNA injected group had a lower CI (P<.01) than the buffer injected group, while the non-injected group had a significantly greater CI (P<.01) than the DNA injection treatment, but not the buffer group. However, the effect of DNA microinjection was largely manifested by inhibition of development at the two-cell stage (Table 2).

Culture system.

Embryo development differed (P<.01) among culture systems. In vitro culture in excised MO for 72 h resulted in the highest CI (3.25 ± .12) while maintaining ova in KRB for the entire period produced the lowest CI (2.04 ± .09; Figure 2). Culture in excised MO for 48, 96 or 120 h resulted in CI of 2.66 ± .18, 2.79 ± .14 and 2.40 ± .14, respectively. Non-orthogonal contrasts showed that CI attained in KRB (2.04 ± .09) was lower (P<.01) than the CI in MO (2.77 ± .07). Cleavage index for MO 72 was greater

(P<.05) than MO 48 and MO 96 (P<.05). The interaction (P < .01) of microinjection by time in oviduct culture showed that maximum CI was attained at 72 h for non-injected and DNA injected embryos, whereas buffer injected embryos development was greatest for 96 h in MO (Figure 3).

Parturition and transgenic animal production.

An average of 10.3, 26.4, 35.7 and 45.3 microinjected embryos were transferred to a single oviduct of 17 recipients that had been synchronized using either the NAT, LALT, SALT or PRE protocols, respectively. Transfer of embryos from LALT synchronized donors resulted in no pregnancies, while ova transferred from SALT gilts yielded 2 pregnancies from 3 transfers. The proportion of offspring that incorporated the gene construct was similar between SALT and PRE groups (28 and 38%; Table 3).

Discussion

A problem was encountered when naturally cycling sows in the NAT group were used as zygote donors. Timing of the onset of estrus was difficult to predict and timing of ovulation in relation to surgical collection of embryos was not precise enough to provide adequate numbers of zygotes (Table 1). Transfer of ova recovered from the NAT group yielded the greatest proportion of pregnancies (Table 3). This may be because the majority of embryos being injected were beyond the zygote stage. Damage to a single cell of such embryos would have a lower probability of resulting in the death of the entire embryo (Pursel et al., 1988).

The finding that 31 to 44% of the embryos classified as UFO cleaved in culture indicates an underestimation of fertilization rate. When the percent pronuclear zygote value (Table 1) is adjusted for cleavage of UFO, the overall fertilization rate is estimated to have been 75.3 and 90.2% ($\bar{x}$ = 14.5 and 19.3 zygotes) for SALT and PRE groups. Wall et al. (1985) observed that pronuclei or nuclei could not be observed in 15% of one- and two-cell embryos, while Brem et al. (1990) observed that approximately one-third of the zygotes had no visible pronuclei.

The finding that injection of DNA impaired development when compared to buffer injection or no injection suggests that DNA, not merely the mechanical procedure of pronuclear injection, was either altering the pronuclear environment preventing normal development, or perhaps the DNA construct was often being integrated into the chromatin in such a way that development was inhibited. Hammer et al. (1986) reported that DNA microinjection decreased embryo survival to the blastocyst stage by approximately 50% in both sheep and swine compared to non-injected controls. Robl and First (1985) found that development of pronuclear microinjected mouse zygotes to the morula or blastocyst stage was not different after DNA injection, buffer injection or after inserting the tip of the pipette into the pronucleus with no injection. However, development in those treatments was lower than no manipulation. The exact mechanism(s) by which DNA microinjection caused embryonic death in the present study is not known. Surprisingly, the microinjection event alone did not inhibit in vitro development of porcine embryos when compared to controls. These results contrast with findings in mice (Brinster et al., 1985) that have shown that injection of buffer alone decreases in vitro embryo development similar to DNA injection, suggesting that porcine zygotes may be more resiliant than mouse zygotes.

Bavister (1988) noted that in non-rodent species, developmental block of embryos maintained in vitro tended to occur at the stage at which the embryo moves out of the oviduct and into the uterus in vivo. Our data show that the culture of porcine embryos in the oviduct of a mouse for as little as 48 h allowed development past the four-cell block which was not attained by culture in KRB alone (Fig 3). However, coculture within MO for 72 h was necessary for optimal embryo development. Development of embryos held in MO for 96 and 120 h was less than optimal, suggesting the oviduct culture environment was not adequate for later stages of porcine embryo development. Low development could be due to either an inappropriate environment present in MO that cannot support porcine embryos or due to tissue breakdown during long term culture. Maximum devolopment of the embryos was lower than has previously been reported (Krisher et al., 1989). Embryos in the previous study received far less manipulation which could account for the differences observed.

Data are not sufficient to determine if there is a difference in the ability of embryos derived from PRE or SALT donors to produce a pregnancy. In vitro study of porcine embryos from similar sources has shown that embryos from superovulated prepubertal gilts do not have equivalent developmental capacity to those obtained from mature gilts (Pinkert et al., 1985; Wiesak et al., 1990; French et al., 1991). Our data show that five transfers of embryos from PRE donors yielded a single pregnancy, while three transfers of embryos recovered from SALT donors produced two pregnancies. Embryos from PRE donors have produced five transgenic offspring, while five transgenic piglets have been born from SALT embryos (Table 3). No abnormal health problems have been observed in the transgenic animals. In addition, two of the transgenic gilts have farrowed normal litters and express human protein C in their milk. The F_1 litters carry the transgene at rates of 78 and 86%. Normal lactations have been observed. No adverse

effects of cosumption of human protein C containing milk have been observed in the piglets.

Conclusions

The SALT and PRE systems provide adequate stimulation and synchronization of ovulation for the efficient collection of zygotes. Maintaining these zygotes within explanted MO for as little as 48 h allows for significantly improved development when compared to culture in medium alone. When considering the detrimental effect of pronuclear microinjection, the impairment to development was found to be due to the injection of DNA and not the mechanical process of microinjection itself. Finally, normal pigs containing the WAP-PC gene construct can be produced by pronuclear microinjection.

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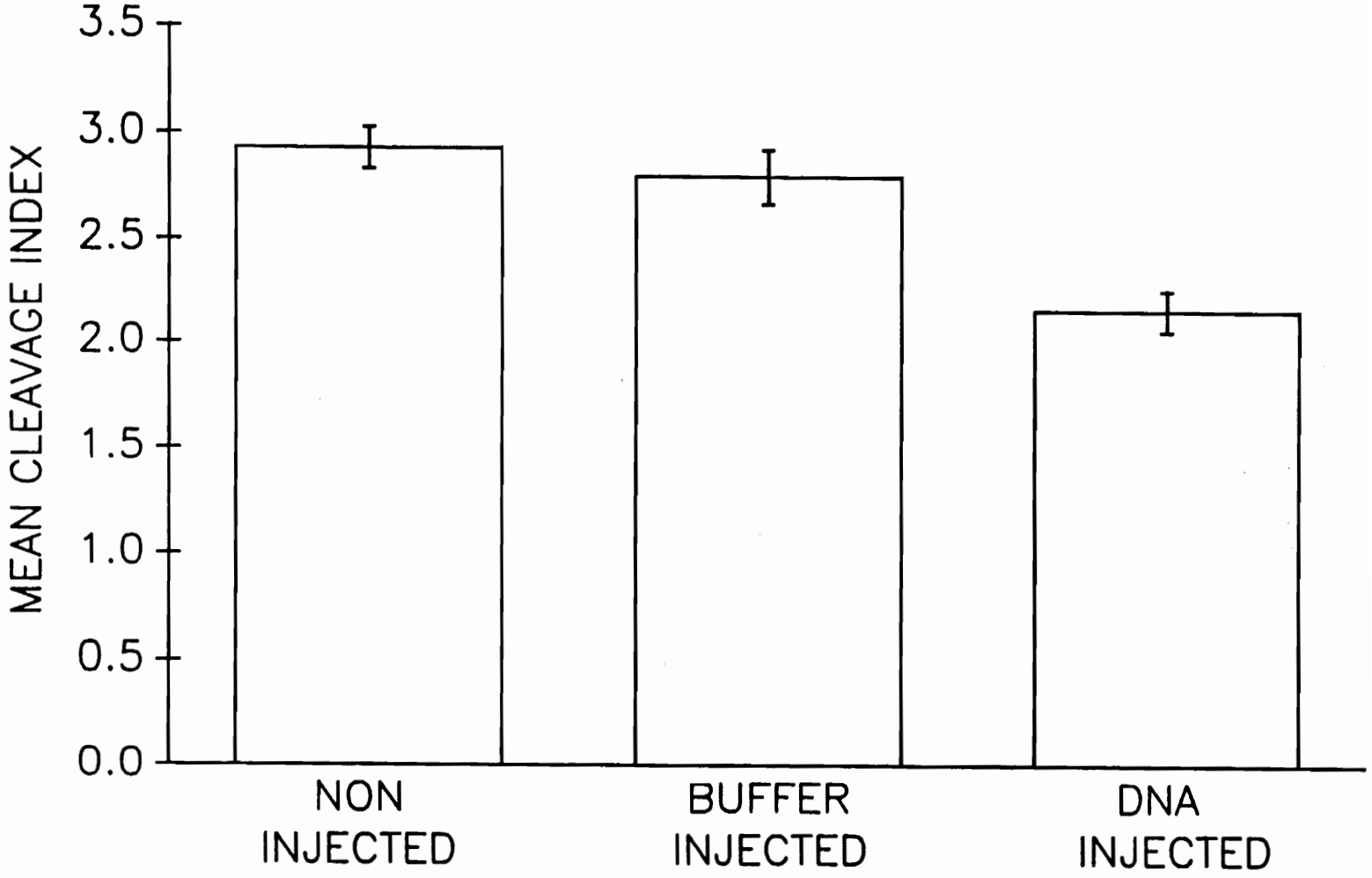

Figure 1. Mean cleavage indices attained by embryos receiving no microinjection, buffer microinjection or DNA microinjection treatments. SEM are indicated by vertical bars.

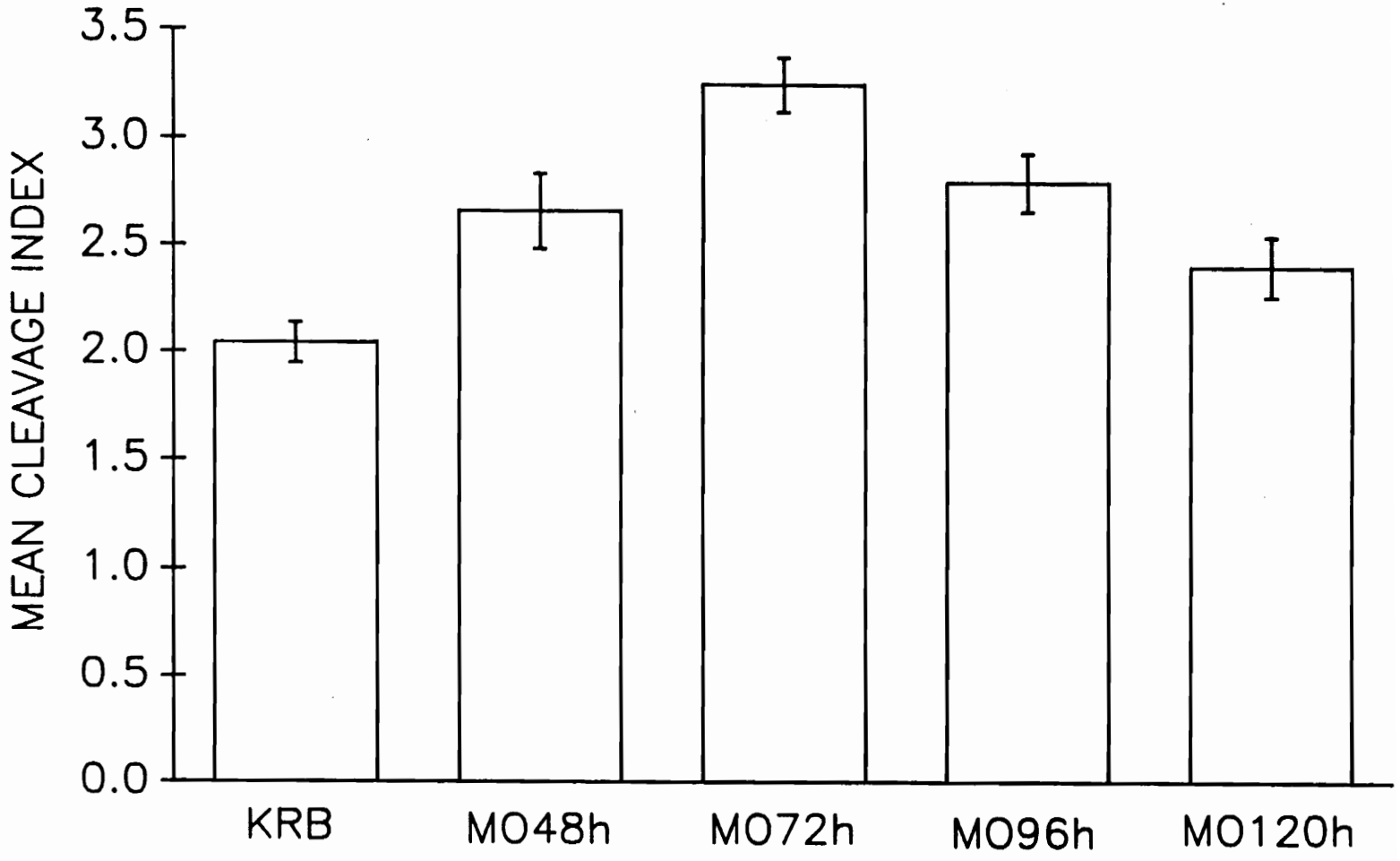

Figure 2. Mean cleavage indices attained by embryos at 144 h with KRB alone or preceded by co-culture in mouse oviducts (MO) for 48, 72, 96 or 120 h. SEM are indicated by vertical bars.

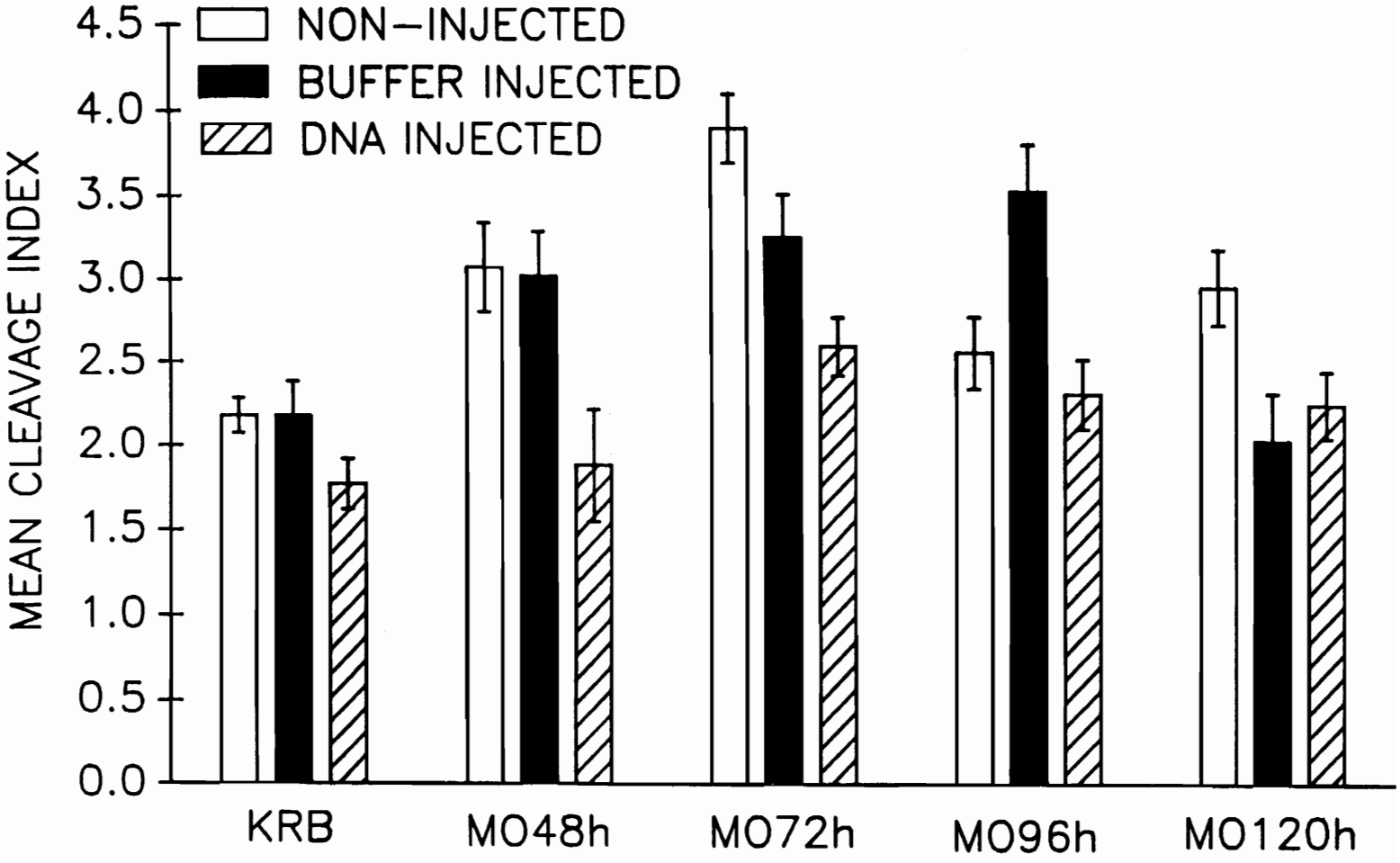

Figure 3. Mean cleavage indices for embryos receiving no microinjection, buffer microinjection or DNA microinjection treatments cultured in KRB alone for 144 h or with initial culture in mouse oviduct (MO) co-culture (48, 72, 96 or 120 h). Microinjection by mouse oviduct interaction was significant ($P < .01$). SEM are indicated by vertical bars.

Table 1. Synchronization and ovulation response for female swine receiving natural estrus (NAT), long term altrenogest (LALT), short term altrenogest (SALT) and precocious ovulation of prepubertal gilts (PRE) synchronization procedures.

Treatment	n	% Ovulating	Ovulatory[a] Structures	Corpora Hemorrhagica	Embryos Collected	Number Zygotes	Number UFO[b]
NAT	4	100	14.5(6.7)[c,d]	14.5(7.1)[d,e]	13.0(7.6)[d,e]	0.3(6.5)[d]	0.3(3.3)[d]
LALT	28	78.6	26.0(2.5)[e]	11.6(2.7)[e]	6.6(4.6)[d,f]	g	g
SALT	24	87.5	28.5(2.7)[e]	24.7(2.9)[d]	19.2(3.1)[d,e]	12.3(2.6)[d,e]	6.8(1.3)[d]
PRE	60	95.0	29.1(1.7)[e]	24.3(1.8)[d]	21.4(2.0)[e]	17.7(1.7)[e]	3.4(0.8)[d]

[a] Corpora hemorrhagica, corpora lutea, unovulated follicles.
[b] UFO - unfertilized ova.
[c] $\bar{x}$(SEM).
[d,e] Values with different superscripts within column differ $P < .05$.
[f] n=11.
[g] Atypical ova morphology prevented accurate classification.

Table 2. Distribution of porcine embryos reaching specific developmental stages following 144 h of in vitro culture.[a]

Maximum Development	CI[b]	Non-Injected	Buffer Injected	DNA Injected
2-cell	1	10.4 (24)[c]	8.3 (9)	30.9 (54)
3-to 4-cell	2	47.6 (110)	41.7 (45)	40.6 (71)
5-to 8-cell	3	26.6 (62)	36.1 (39)	20.0 (35)
9-to 16-cell	4	3.6 (13)	0.9 (1)	3.4 (6)
Morulae	5	2.2 (5)	4.6 (5)	0.6 (1)
Blastocysts	6	7.4 (17)	8.3 (9)	4.6 (8)
Total		(231)	(108)	(175)

[a] Chi square $P < .01$.
[b] Cleavage index.
[c] % (n).

Table 3. Pregnancy and gene construct[a] integration rate for embryo transfers from natural estrus (NAT), long term altrenogest (LALT), short term altrenogest (SALT) and precociously ovulating prepubertal gilt (PRE) embryo donors.

Donor Treatment	Ova/recipient ($\bar{X}$ ± SEM)	Number Recipients	Number Pregnant[b]	Offspring Born	Number Transgenic
NAT	10.3 ± 1.3	4[c]	4	24	0
LALT	26.4 ± 4.9	5[d]	0	0	0
SALT	35.7 ± 4.3	3[e]	2	18	5
PRE	45.0 ± 1.1	5[e]	1	8	5

[a] Whey acidic protein - Protein C mammary specific fusion gene. Positively determined by PCR at day 2.
[b] As determined at parturition.
[c] Recipient served as its own donor.
[d] Recipient synchronized by LALT procedure.
[e] Recipient synchronized by SALT procedure.

Chapter 6

Summary of Porcine Zygote Manipulation

The second area of study delineated various factors affecting the harvest and development of porcine zygotes. The use of the SALT and PRE procedures were suitable for synchronization of ovulation. Difficulties in synchronization associated with long-term exposure to ALT may be due to residual effects of the progestogen. This procedure might provide acceptable results if modifications were made in dosage and/or time intervals for gonadotropin stimulation after ALT withdrawal. A significant research effort would be necessary to optimize new treatments. Such work may be unnecessary since the use of SALT or PRE synchronized animals produced similar acceptable results for embryo collection. Differences in the ability of SALT and PRE procedures to produce viable pregnancies needs to be determined. Further investigation is necessary and planned to evaluate differences in the viability of embryos derived from SALT and PRE treated animal. The first factor which should be considered is that of time for follicular development using PMSG prior to the induction of ovulation with hCG in prepubertal gilts.

In vitro culture treatments affected subsequent embryonic development with 72 h in MO providing the best CI. It was not surprising that the more complex mouse oviduct culture system improved development in comparison to KRB. The point of greatest interest was the influence of the length of time the ova were held in the oviduct. Improved ova development with increasing resident times in MO (up to 72 h) suggests there is a range of time when complex culture environments are needed to enhance development of early stage ova. The cause of decreased development at periods in oviduct culture greater than 72 h is less clear. Two possible explanations are likely. Either oviductal tissue undergoes demise after longer periods in culture and is producing an environment that is detrimental to the embryo or the oviduct is functioning properly but its products

are no longer appropriate for later stage embryos. Biochemical and histological evaluation of oviducts would provide evidence to answer this question.

A detrimental action of pronuclear microinjection of DNA was not unexpected. It was surprising that impaired embryonic development was due to the transfer of DNA into the pronucleus and not the mechanical action of microinjection. The pronucleus was capable of withstanding a 3.4 fold increase in volume while alteration of the endogenous genetic component was not compatible with future development. While microenvironmental alteration or physical hinderance due to the injected DNA may play a role in this detrimental effect, the more likely cause is the incorporation of the injected DNA in such a manner as to alter or disrupt critical genetic sequences that are necessary for embryonic survival.

Efficiency of transgenic animal production was low. The efficiency of production of transgenic pigs using the NAT, LALT, SALT, and PRE procedures was 0, 0, 4.7, and 1.4 %. Improved efficiency is critical to allow this new technology to be used for the benefit of mankind. Such improvements include procedures to overcome the detrimental effects of DNA injection. This includes targeting integration sites or adding enzymes to increase the probability of integration into acceptable positions. Further work to improve embryo culture systems also is needed to allow for genetic testing of embryos so that only viable embryos containing the inserted gene can be transferred to recipients. Finally, procedures to maximize ova collection and pregnancy are critical to overcome the current wastage of animals because of the inherent inefficiencies.

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Vita

Barry Lee Williams

The author, son of Bruce and Carol Williams, was born on June 5, 1962 and was raised in Baird, Texas. After graduation from Baird High School in 1980 he attended Texas Tech University where he received a B.S. degree in Animal Science with emphasis in Beef Production in 1984. At that point he entered Washington State University and received a M.S. degree in Animal Science/Reproductive Physiology in 1986. He then continued his training at Virginia Polytechnic Institute and State University where he received a Ph.D. in Animal Science in 1991.

The author has been inducted into Phi Eta Sigma, Gamma Sigma Delta, and Sigma Xi honor societies and is a member of the Society of Animal Science and the Society for the Study of Reproduction.

Barry L. Williams

Publications

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