THE EFFECT OF UTERINE FLUSHINGS ON EARLY BOVINE EMBRYO DEVELOPMENT IN VITRO

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(Abstract)

Bovine morulae (day 6; n=328) were obtained to evaluate the effect of uterine flushings (UF) obtained from ovariectomized progesterone (P) or estrogen (E) treated cows or from previously superovulated cows on day 6, 10, or 15 post insemination on early embryo development. Ninety-eight cows were superovulated with follicle stimulating hormone. Embryos were collected by non-surgical flushing procedures and morphologically evaluated and randomly assigned to culture. Embryos were cultured in Ham's F-10 containing 10% steer serum (SS), 10% P or E concentrated UF, 10% SS plus 1% UF from day 6, 10, or 15, or different concentrations of UF from day 6, 10, or 15 (10%, 1%, .1%, .01%). Embryos were cultured up to the hatched blastocyst stage or degeneration and evaluated at 12 h intervals. Treatment differences were evaluated by analysis of variance after assigning a value of 0 to 5 to each embryo representing its final stage of development. Overall analysis revealed that embryo development was significantly affected by supplement, but not affected by initial quality. Final developmental score was significantly higher

in SS than in all other supplements. Combination of 1% UF and 10% SS resulted in enhanced embryo development compared to all single UF protein treatments. Time to the hatching blastocyst and hatched blastocyst developmental stages was reduced ($P<.05$) in 1% day 15 UF compared to 1% day 6 and day 10 UF plus 10% SS, or 10% SS. These results suggest that day 15 UF may contain a substance which may shorten the time of embryo hatching *in vitro*.

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Chapter I

INTRODUCTION

The primary cause of reproductive failure in dairy cattle today is the condition of early embryonic death. This major problem leads to increases in days open and longer calving intervals which result in large economic losses for the dairy farmer due to decreased milk production and higher insemination and veterinary costs. When cattle are properly inseminated with high quality semen fertilization rates generally range from 90 to 100%. However, calving rates to a single insemination are closer to 50%. The difference between the fertilization rate and the calving rate is probably due to early embryonic death. Previous research suggests that the highest percentage of embryonic death occurs somewhere between days 6 to 16 of pregnancy. During this time period the embryo is free floating inside the uterine environment and depends on uterine secretions for survival and further development.

Previous work with the culture of bovine embryos *in vitro* has determined that nutritive substrates, ions, macromolecules, atmosphere, temperature, osmolarity, and pH, must be optimal for successful early embryonic development. Recent work has investigated the relationship between the em-

bryo and the uterus and its secretions. First in laboratory animals and later in pigs, sheep, and cattle, specific uterine proteins have been found in collected uterine flushings. Although the precise roles of uterine specific proteins are unknown, it has been suggested that they are needed for proper early embryo development. The addition of rabbit uterine specific proteins to culture media has resulted in increased growth and development of rabbit ova. Also, limited studies in the bovine have reported increases in survival and growth of embryos cultured *in vitro* when supplemented with uterine secretions. Previous work in our laboratory has identified uterine specific proteins collected from ovariectomized cattle subjected to varying progesterone-estrogen replacement regimes and has cultured bovine morulae in these secretions.

It is postulated that if production of uterine specific proteins are necessary for normal embryo development *in vivo* then supplementation of these substances to embryo culture systems will aid in promoting early bovine embryo development *in vitro*.

The objective of this study was to test the capabilities of several sources of uterine proteins as supplements to culture media for their ability to promote *in vitro* development of bovine morulae. In this way we hope to obtain

an optimal nutritive substrate for use in bovine embryo culture.

Specific objectives were: 1) to test concentrated uterine flushings obtained from ovariectomized estrogen or progesterone treated cows; 2) to evaluate 10%, 1%, .1%, and .01% concentrated uterine proteins obtained from cows flushed on day 6, 10, or 15 following superovulation; and 3) to determine the effects of combining steer serum with 1% uterine proteins obtained from day 6, 10, or 15 of the estrous cycle on early embryo development; and 4) to assess development of fair and poor embryos in culture; and finally 5) to study factors affecting superovulation and estrus synchronization.

Chapter II

LITERATURE REVIEW

RESEARCH IN BOVINE EMBRYO CULTURE

Early work involving *in vitro* culture of mammalian embryos dates back to the end of the nineteenth century. It was only during the last 20 to 30 years that systematic studies were conducted to establish suitable culture methods to study embryo development. Most of the early work in mammalian embryo culture was conducted with laboratory species such as mice, rats, and rabbits (Brinster, 1963; Whitten, 1957; Kane and Foote, 1970). These animals naturally produce many embryos and feeding and maintenance cost are relatively inexpensive when compared to larger species.

Early research with bovine embryo culture was limited due to their monotocous nature combined with the relatively high feed and maintenance costs associated with this specie. The innovation of superovulation combined with the added knowledge and improvements made in the non-surgical uterine flushing techniques has helped lower the cost associated with bovine embryo culture and has helped both to supply greater numbers of ova and facilitate the ease in which these ova are obtained. Before the perfection of surgical and non-surgical flushing techniques, cows usually had to be

slaughtered in order to obtain ova. Today almost all large embryo transfer operations use non-surgical flushing procedures to obtain their embryos for transfer (Greve, 1980; Tervit et al., 1980; Sreenan, 1983).

The requirements for culturing cow ova in vitro have not been systematically examined as have been reported for mice. Early attempts to culture ova used various natural media such as blood serum (Brock and Rowson, 1952) and follicular fluid (Sreenan et al., 1968b).

One of the first bovine embryo culture experients was conducted by Dowling (1949) using an egg-saline medium developed for mouse embryo culture. He found that only one of 14 eight-cell bovine embryos developed to the 16 cell stage. Pincus (1951) utilized bovine serum as a culture media for one- to two-cell embryos and reported an average of .8 divisions per embryo. Brock and Rowson (1952) reported similar poor results with bovine serum as a culture medium because only three of four 4-cell embryos divided once. Also, Wintenberger et al. (1953) found an average of 1.2 divisions per embryo when eight- to 12-cell embryos were cultured in bovine serum. These low success rates in bovine serum may have been due to the failure to heat-treat the serum to remove complement, which was reported to be embryotoxic (Chang, 1949).

in 1963, Hafez cultured premorula embryos in a serum-saline mixture and found that four of 97 divided once. Although development was still limited this was the first report of any bovine embryo development after 48 h in culture. Thibault (1966) cultured bovine ova in amniotic fluid, Locke's Medium with serum, aqueous humor, and fluid from Graafian follicles. Amniotic fluid was highly toxic even after heating at 58 to 60 C for 20 min. Except, for follicular fluid, the other media did not support cleavage. Conversely, nine of 27 four-cell embryos developed to morulae in follicular fluid after 119 h in culture. This was the first in vitro observation of more than 2 complete divisions per embryo. Six- to 10-cell bovine ova have been shown to continue normal cleavage to early blastocyst stages after transfer to the uterine tubes of pseudopregnant rabbits (Sreenan and Scanlon, 1968a). These investigators also cultured bovine eggs in a medium containing homologous serum and sodium chloride (50:50), follicular fluid, minimal essential medium (MEM; Eagle, 1956), bovine fetal serum, and chicken embryo extract. Cleavage was not obtained in the homologous serum. Limited development was observed in both the follicular fluid and the MEM with 47 of 67 embryos dividing once in the follicular fluid and 11 of 17 dividing once in the MEM. Although these results were not optimal,

MEM was found to be comparable to any biological fluid tested. This marked a change in media selection. For the first time, a defined media, MEM, was utilized for bovine embryo culture. This led to the future testing of many other defined media for their ability to promote early bovine embryo development.

In 1971, Seidel et al. tested tissue culture media-199 (TCM-199) against modified Ham's F-10 (Ham, 1963) for their ability to promote development of two- to three-cell embryos; both were supplemented with 1.5% bovine serum albumin (BSA). The modified Ham's F-10 exhibited slightly better results then TCM-199, but neither medium supported embryonic development well, averaging 1.3 and .7 divisions per embryo, respectively. In these studies, embryos were cultured under paraffin oil in an atmosphere of 5% CO_2-in air. Tervit et al. (1972) cultured bovine embryos in stoppered test tubes flushed with different atmospheres utilizing several defined media. Neither TCM-199 nor Whittingham's Medium (Whittingham, 1971) supported embryo development while encouraging results were obtained using synthetic oviduct fluid (SOF). This medium developed by Restall and Wales (1966) was based on the composition of oviduct fluid from sheep. They obtained considerable development after culturing 10 one-cell embryos for 4 days, and two pregnancies resulted from the

transfer of four embryos that had been cultured for 4 days. An atmosphere of 5% CO_2, 5% O_2, and 90% N_2 was superior to 5% CO_2-in air in promoting embryo development.

Brinster's Ova Culture Media (BMOC-3), a variation of mouse culture media (Brinster, 1963), was tested with only 14 of 54 eight- to 12-cell ova developing to morulae. In 1973, McKenzie and Kenny cultured bovine embryos in BMOC-3 under paraffin oil in a 5% CO_2-in air atmosphere. No improvement over earlier work was noted with 25 of 108 one- to eight-cell ova exhibiting at least one division. Seidel (1974) compared modified Ham's F-10 to TCM-199 containing 35mM $NaHCO_3$ buffered with 25 mM HEPES and supplemented with 5% heat-treated bovine serum for storing embryos for one to 10 h between collection and transfer. The TCM-199 was in covered petri dishes in air and the Ham's F-10 was under paraffin oil in an atmosphere of 5% CO_2-in air. Following transfer, 47 of 88 embryos (53%) maintained in TCM-199 resulted in pregnancies, compared with 44 of 102 embryos (43%) in modified Ham's F-10.

Shea et al. (1974) compared BMOC-3 and HEPES-buffered SOF, with and without agitation during the culture period, in stoppered test tubes with a mixture of 5% CO_2, 5% O_2, and 90% N_2. Agitation was found to have no effect on embryo development. When eight- to 12-cell embryos were cultured for

72 h, 26% (14/54) developed to morulae in BMOC-3, compared with 57% (28/49) in the SOF. Earlier staged embryos were also cultured in SOF with only 6% of the one-cell and 26% of the two-cell embryos developing beyond the eight- to 12-cell stage. However, 12 of 18 four-cell embryos developed to the 12-cell stage and two of 18 became morulae. They concluded that embryos needed to be at more than the eight-cell stage at the start of culture in order to obtain sufficient development.

In 1975, Boland et al. transferred bovine embryos non-surgically after storage in TCM-199 or phosphate-buffered saline (PBS) with a supplement of 15% fetal calf serum (FCS). Four of 12 embryos stored in PBS resulted in pregnancies compared with two of 12 in the TCM-199. Bowen et al. (1975) compared factorial combinations of three media (SOF, modified Ham's F-10, and SOF supplemented with several amino acids), two osmolarities (270 and 300 mOsm), and two atmospheres (5% CO_2-in air and a mixture of 5% CO_2, 5% O_2, and 90% N_2). Embryos were recovered 3 or 6 days after breeding. Atmospheres or media were found to have no significant effects on embryo development. However, an osmolarity of 270 mOsm proved significantly better than an osmolarity of 300 mOsm for both 3-day and 6-day embryos. Eight of nine and 16 of 20 morulae developed to blastocysts in Ham's

F-10 and SOF, respectively at 270 mOsm, compared to five of eight and eight of 15 at 300 mOsm. Thus, as in culturing embryos from several laboratory species, an osmolarity slightly lower than blood serum was recommended. Kanagawa et al. (1975) cultured eight- to 32-cell embryos in SOF and BMOC-3 without any gaseous atmosphere. No significant differences in embryonic development were found among the treatments, as over 65 to 85% of the embryos developed to the blastocyst stage. Sreenan et al. (1975) compared TCM-199 with Earle's salts to HEPES-buffered TCM-199 with Hank's salts for storing embryos between collection and transfer. Results were encouraging with 35 of 58 and 30 of 52 embryos, respectively, developing into fetuses. However, they did observe a decline in pregnancy rates for embryos stored in TCM-199 alone for up to 2 h before their transfer.

In 1976, Gordon compared embryo storage at 30 C in either TCM-199 or PBS supplemented as described by Whittingham (1971). Both media contained 15% FCS. Only 5% of the embryos cultured in TCM-199 continued development, but 60% did so in PBS. Similar favorable results in PBS were obtained by Trounson et al. (1976). They cultured day 6 to 7 morulae in PBS supplemented with 20% FCS for 24 to 96 h in air at 37 C prior to transfer to recipients. Of the 26 embryos which developed normally during 48 h in culture, 50%

resulted in pregnancies. Also, they reported that FCS did not appear to be essential, as substitution of heat-inactivated sheep serum also consistently allowed early bovine embryo development. The encouraging results obtained with phosphate instead of bicarbonate as a hydrogen-ion buffer, along with results indicating that TCM-199 was not beneficial for bovine embryo culture (Gordon, 1976; Wright et al. 1976a), led to widespread substitution of modified PBS for TCM-199 as the media of choice for routine embryo transfers (Wright and Bondioli, 1981).

Besides the better results obtained with PBS, the phosphate-buffered media has no special atmosphere requirements for pH maintenance unlike TCM-199, which requires carbon dioxide to maintain pH. Wright et al. (1976a) tested several media (Ham's F-10, MEM, TCM-199, BMOC-3, SOF, Whitten's Medium) in microdrops under paraffin oil for their ability to promote development of two to eight-cell bovine embryos. All media were supplemented with either 1 to 4 g BSA/liter or with 10 to 50% FCS. Ham's F-10 supplemented with 10 or 50% FCS was significantly better than all other media. Embryos cultured in Ham's F-10 averaged 2.3 divisions per embryo (n=22), with one developing to the hatched blastocyst stage after 160 h in culture. This was the first report of a bovine embryo hatching <u>in vitro</u>. The research-

ers also tested the effects of two different atmospheres (5% CO_2, 5% O_2, 90% N_2 and 5% CO_2-in air). They concluded that the 5% O_2 atmosphere was significantly superior to the 20% O_2 atmosphere. Utilizing the results of their first experiment, Wright et al. (1976b) compared BSA with FCS for supplementation to Ham's F-10. With FCS, development to the expanded blastocyst stage occurred in three of 14 one- to two-cell embryos tested and 11 of 20 eight-cell embryos with six hatching from the zona pellucida. Results with the BSA were poor, with only four of 28 one- to eight-cell embryos developing to blastocysts. Although small numbers of embryos were involved, this was the first report of one- to two-cell embryos reaching the expanded blastocyst stage *in vitro*.

Renard et al. (1976) compared BMOC-3 supplemented with BSA (5g/liter) and Menezo's Medium B2 (Menezo, 1976) for promotion of development of day 6 and day 7 embryos. Medium B2 was superior, with embryos exhibiting a hatching rate of 65% after 96 h in culture compared to an 18% hatching rate for embryos cultured in BMOC-3. The time of *in vitro* hatching was equal that of hatching *in vivo*, which is approximately day 10 in the bovine. In 1978, Boone et al. cultured bovine morulae in BMOC-2, (a variation of BMOC-3), supplemented with various carbohydrate sources, including

pyruvate, lactate, oxaloacetate, and glucose. Twenty-one of 22 embryos developed to blastocysts with a few partially hatching in an atmosphere of 5% CO_2- in air. Eight- to 32-cell embryos cultured in BMOC-3 in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 yielded 38 of 46 embryos developing to blastocysts (Kanagawa, 1979). In this experiment embryos were grouped into three categories: normal, young, and degenerate. Only normal embryos developed well, while young and degenerate ova developed poorly. The effects of exposure to microscopic light, short-term storage, and travel also were examined. These parameters were not found to be detrimental to embryo survival. These results are important because these conditions are often found in the embryo transfer industry today.

In 1982, Allen et al. tested three sera, normal steer serum (NSS), newborn calf serum (NBCS), and FCS, for their ability to promote early bovine embryo development. The experiment was further expanded by including two different percentages of sera (5% and 10%). The 10% NSS treatment was significantly better than the other treatments with 10 of 15 embryos reaching the hatched blastocyst stage. The 5% NSS treatment had a 40% hatching rate while 33% and 15% hatched in the 10% and 5% NBCS, respectively. The FCS treatment had the lowest results with only one out of 15 embryos hatching

in the 5% concentration and no hatching occurred in the 10% FCS. This study had interesting results because until this report FCS was considered to be the best sera for use in bovine embryo culture and was the leading sera used as supplementation to flushing and holding media used in the bovine embryo transfer industry. Although the authors suggested that NSS may be the best sera tested so far for use in bovine embryo culture, they cautioned that there are known variabilities in commercial lots of FCS and there may also be variations in different collections of NSS as well. Also, they could not assess the reasons that NSS was superior to FCS in its ability to promote early embryo development; only that NSS may contain more of certain growth promoting factors than FCS.

Work in our laboratory has indicated that NSS provides optimal early embryo growth and development, but its effects were not significantly different from a 10% purified BSA treatment (Canfield, 1983). Eight of 20 embryos cultured in NSS reached the hatched blastocyst stage while seven of 20 hatched in media supplemented with BSA. Since the BSA utilized in this experiment was highly purified and not contaminated with other proteins or substances, development of embryos in the BSA supplement was attributed to the albumin which constituted it. Since NSS showed no beneficial growth

promoting effects over BSA, he concluded that albumin present in NSS may be the component which was responsible for embryo development in NSS.

NUTRIENT REQUIREMENTS OF IN VITRO EMBRYOS

The nutrient requirements for development of preimplantation mammalian embryos have been studied for years. Most of the early research was conducted with laboratory species, in particular the mouse. Since there are few studies which have been conducted in the bovine with regard to an embryo's nutritive requirements, most of the recommended nutrients for use in current bovine embryo culture research resulted from their successful use in mouse embryo culture.

Although fertilization of cow ova can take place *in vitro* in a simple defined medium, more complex media are needed for cleavage to occur (Brackett, 1980). Media components studied in laboratory species and which are thought to be important for successful bovine culture include: inorganic ions, osmolarity, pH, gaseous atmosphere, energy substrates, and amino acids.

Today, research with bovine embryo culture generally utilizes Ham's F-10 media, a basic salt solution supplemented with vitamins, amino acids, and trace elements. Ham's F-10 has been reported to be superior to most other media in

promotion of early bovine embryo development (Wright et al., 1976a).

ROLE OF INORGANIC IONS: EFFECTS OF OSMOLARITY

Although little is known about the inorganic ion requirements of the bovine embryo, research with mice and rabbits has established that Na^+, Cl^-, K^+, Ca^{++}, Mg^{++}, phosphate, and bicarbonate ions all play important roles in the normal early development of mammalian embryos (Whitten, 1956; Brinster, 1963; Daniel, 1965). It still has to be determined whether the embryo actually requires these ions or if they are only necessary in the medium to prevent leaching of the same components within the embryo (Whitten, 1970). Hamner (1970), working with rat, rabbit, and dog uterine secretions, reported that uterine concentrations of phosphates were lower than their respective blood serum concentrations and that K^+ uterine concentrations were higher than their respective blood serum concentrations. Wales (1970) found that for successful culture of mouse ova optimal levels of K^+, Ca^{++}, Mg^{++}, and phosphates were close to the normal serum levels. Working with mouse embryos, Whitten (1956) reported that embryos cultured without Ca^{++}, Mg^{++}, or K^+ exhibited no development and that development was delayed when phosphates were absent.

The main role of Na^+ is its function in regulating osmolarity together with Cl^- (Brinster, 1972). Mammalian embryos can develop in a wide range of osmolarities, but optimum development occurs at different osmolarities which seem to vary among species. Two-cell mouse ova have developed to blastocysts in media ranging from 200 to 354 mOsm, but Sodium is also thought to be involved in fluid accumulation optimum results were obtained at 276 mOsm (Brinster, 1965a). Naglee et al. (1969) working with rabbit ova reported that normal early embryo development occurred between osmolarities ranging from 230 to 339 mOsm. However, generally 270 mOsm is considered to be the optimum osmolarity for the culture of early rabbit embryos (Brackett, 1969). In one of the few osmolarity studies conducted with bovine embryos, Bowen et al. (1975) reported superior development occurred in all media tested at 270 mOsm compared to culture at 300 mOsm. However, this was the only study in the bovine in which embryonic development was said to have been dependent on such a narrow range of osmolarities. In contrast to Bowen's study, Wright and Bondioli (1981) reported that the effect of osmolarities of the media on early bovine embryo development is minimal. Their study reported a wide range of osmolarities among commonly used media and supplements (270 mOsm for modified Ham's F-10 to 316 mOsm for BMOC-3).

Sodium is thought to be involved in fluid accumulation in blastocyst formation which has been suggested to depend entirely on sodium-potassium ATPase (DiZio and Tasca, 1977). Although Wales (1970) reported that in the absence of K^+, ovum cleavage was inhibited, a wide range of K^+ concentrations varying from .6 to 48 mM was able to support mouse embryo development. It is known that the level of K^+ in the female reproductive tract of the cow is considerably higher than in the plasma (Lamothe and Guay, 1970; Schultz et al., 1971).

The absence of Ca^{++} ions from media inhibited cleavage of mouse ova (Wales, 1970). Calcium has been suggested to be involved in membrane stability, permeability, and cell-cell interactions, especially at the time of compaction to form the morula (Whitten, 1970; Ducibella and Anderson, 1975). However, as with K^+, normal development occurs over a wide range of Ca^{++} concentrations (.4 to 10mM). Wales (1970) reported that the absence of Mg^{++} and phrrrrates significantly depressed the normal development of 2-cell mouse ova to blastocysts but did not completely inhibit development. Thus, these ions are found today in most commercial media that are utilized in mammalian embryo culture experiments.

Early embryo culture work has shown that the main role of the bicarbonate ion was its effect on pH. However, Brinster (1972) reported that the bicarbonate ion is essential for normal development in mammalian embryos for other reasons than its effect on pH. When other buffers (Tris, phosphate, HEPES, or TES) were substituted for the bicarbonate buffer and pH was kept constant, little or no embryo development occurred. This requirement for HCO_3^- *in vitro* may result from its relatively high concentrations *in utero*, which are especially high in uterine secretions from the rabbit (Hamner, 1970) and sheep (Restall and Wales, 1966). Cross et al. (1973) suggested that HCO_3^- was needed for blastocyst expansion in rabbit ova, probably acting through a role in fluid accumulation within the blastoceole.

Other inorganic ions and trace elements are for the most part considered to be non-essential for addition to media used in mouse or rabbit embryo culture (Kane and Foote, 1970). This does not mean that other ions and trace elements are not necessary for successful mammalian embryo culture, since embryos may contain enough endogenous reserves of these substances to allow for short-term culture (Kane, 1978). In contrast to these reports, Daniel and Millward (1969) reported that the ferrous ion was required for cleavage of day 5 rabbit ova. It was suggested that the role of

the ferrous ion was in the electron transport system where it was needed to transport electrons from the cytochromes. Also, it was hypothesized that the ferrous ion may be involved in early mitotic activity in the embryo.

The functions of the ferrous ion and the other major inorganic ions in early bovine embryo development are not well understood. More research needs to be conducted in these areas so that an embryo's ion requirements can be better explained.

ROLE OF PH

Brinster (1965a) reported that mouse embryos survived in a wide range of pH (5.9 to 7.8). Rabbit ova are able to develop in a wide range of pH (6.6 to 7.9; Kane, 1974). Thus, it appears that pH effects on the developing mammalian embryo are minimal and are difficult to recommend. Whitten (1956) reported that the availability of certain substrates (pyruvate and lactate) effect the H^+ concentration of the medium. Futhermore, he suggested that the lower the pH, the lower the concentration of substrates needed to obtain optimum development. The uptake of these compounds was thought to be related to the amount of the compound in the acid from. If this is true, it suggests that the membrane of the developing embryo is able to show selective permeability to

substrates necessary for its development, and that in some cases the selective permeability is dependent on the H^+ concentration in the medium (Brinster, 1972). Although pH effects on the bovine embryo have not been studied, the effects appear to be minimal as long as the pH is kept within a range of 6.0 to 7.8.

ROLE OF GASEOUS ATMOSPHERES

Two gaseous atmospheres have been routinely utilized for culture of mammalian embryos: 5% CO_2-in air and 5% CO_2, 5% O_2, and 90% N_2. The nitrogen component of the later mixture is considered to be inert and it is the concentrations of CO_2 and O_2 that appear to play a critical role in early embryo development.

Although most investigators agree that there is an O_2 requirement by the mammalian embryo, they differ on the percentage required. Early studies concluded that mouse ova had a low but definite O_2 requirement. However, too much O_2 was found to be detrimental to early embryo development. This was suggested to be a carry over effect from the low oxygen tension observed in the Fallopian tubes to which a mouse ova is exposed during the early cleavage stages (Bishop, 1956; Whitten, 1970). Brinster (1965c) suggested that O_2 may be involved in altering the oxidation-reduction po-

tential in the ovum and early embryo, thus, it has been shown to modify development. The most marked effect of O_2 is thought to be in the earliest stages of development when the embryo may be very dependent on the balance between NAD, NADH, pyruvate, and lactate.

The main role of CO_2 is thought to be its use as a carbon source by preimplantation embryos during development (Wales et al., 1969). Carbon dioxide use begins at the 8-cell stage and extends to the blastocyst stage, when the embryo's general metabolism is highest. Brinster (1972) reported that normal mouse embryo development occurred within a range of CO_2 concentrations of one to 10%. In a later study Brinster and Troike, (1979) blocked carbon fixation in de novo RNA synthesis by use of a selective inhibitor of aspartate carbamyl transferase (phosphonacetyl-L aspartate). The results showed that development of mouse embryos was arrested at the morula stage and confirmed the active role of CO_2 in early embryo development. Another role of CO_2 in a gaseous atmosphere is that it is needed by several bicarbonate media for maintenance of pH. Without the CO_2 the pH increases with time and may become detrimental to normal embryo development.

There has been some evidence in the bovine that an atmosphere of 5% O_2 is superior to 20% O_2 for promoting early

embryo development (Tervit et al., 1972; Wright et al., 1976a). However, other investigators have continued to use 5% CO_2-in air (20% O_2) and have obtained excellent results (Boone, 1978; Allen et al., 1982). Thus, while a gaseous atmosphere of 5% CO_2, 5% O_2, and 90% N_2 is the most common atmosphere utilized in bovine embryo culture today, whether or not the atmosphere is comprised of 5% O_2 or 20% O_2 appears to have little effect on normal embryo development.

ROLES OF ENERGY SUBSTRATES

Little is known about the energy requirements and metabolism of the early bovine embryo, but much work has been conducted with embryos obtained from different laboratory species. It is well documented that embryos require certain energy substrates at certain developmental stages. Whitten (1957) found that the use of lactate in two-cell mouse embryo culture allowed development of some embryos to the four- and eight-cell stages. Brinster (1967a) measured CO_2 production from radio-isotopically labeled substrates that had been supplemented into mouse embryo culture media. His results supported his previous work which concluded that in the beginning the oocyte needs pyruvate or oxaloacetate (which can be converted by the oocyte to pyruvate). After the two-cell stage, the embryo can use pyruvate, oxaloace-

tate, lactate, or phosphoenolpyruvate. By the eight-cell stage the mouse embryo can use glucose, malate, alpha-ketogluterate, citrate, and acetate, as well as the aforementioned substrates. By the time the embryo reaches the blastocyst stage, its energy metabolism and energy requirements are similar to most adult cells (Brinster, 1965b).

During the first two days a mouse embryo derives from 90 to 100% of its oxygen consumption from the oxidation of pyruvate (Brinster, 1967a). Follicular cells produce pyruvate as a metabolite of glucose and this results in its availability as a substrate for the early cleavage stages while the embryo is in the oviduct (Brinster, 1972). Whitten (1957) reported that concentrations of lactate and pyruvate are high in the rabbit oviduct, while glucose concentrations are low. In contrast to pyruvate's beneficial effects on mouse and rabbit ova, Davis and Day (1977) reported that four-cell porcine ova were inhibited in media supplemented with pyruvate. This could be explained by their addition at too high a concentration of pyruvate, as high concentrations can inhibit cleavage of mouse ova (Brinster, 1967a). Also, Graves et al. (1977) successfully cultured early porcine embryos in media supplemented with pyruvate.

Between the two to eight-cell stage in the mouse embryo, there is a shift in utilization of the metabolic pathways with the rate of oxidation of glucose increasing via glycolysis and the TCA cycle as the embryo increases in age (Pike, 1981). This is thought to result from decreasing ATP and increasing ADP levels which are believed to remove the inhibition of the glycolytic enzyme phosphofructokinase (PFK; Quinn and Wales, 1973). Also, in rabbit ova, after the morula stage, there is a shift from the pentose phosphate shunt to utilization of the glycolytic pathway and the TCA cycle at around the blastocyst stage. This is thought to occur through increases in PFK and pyruvate kinase, two of the rate-limiting enzymes of glycolysis along with increases in malate and lactate dehydrogenases, which are enzymes used in the TCA cycle (Paria et al., 1984). Thus, as a mammalian embryo develops it begins using more and more glucose as its energy source and pyruvate utilization begins to level off, and even decreases in mouse embryos (Brinster, 1967b).

The opportunity for the mammalian embryo to utilize glucose is demonstrated by its presence in uterine secretions. Measurable quantities of glucose as well as fructose, lactate, and citrate have been found in uterine secretions collected from the rabbit, ewe, and cow (Menezo,

1981). Renard et al. (1980) using radio-isotopically labeled glucose, reported that glucose can be taken up by the bovine embryo, degraded, and incorporated into fats, proteins, and nucleic acids. Also, the quantity of glucose in the uterus of the gilt and pony mare has been shown to increase through out the cycle and early pregnancy (Zavy et al., 1982). Furthermore, Wordinger and Brinster (1976) reported that blastocyst stage mouse embryos rely on glucose at least in vitro for hatching, attachment, and trophoblastic outgrowth. Renard (1980) reported that glucose was an essential ingredient in bovine embryo culture in order for hatching to occur and that zona pellucida loss was completely inhibited when glucose was replaced by pyruvate and lactate. Therefore, media utilized for early bovine embryo culture should include pyruvate and glucose, both of which are found in most commercial media available today.

The role of glycogen as an energy substrate has been studied in mouse ova. Thomson and Brinster (1966) reported that early mouse embryos contain large stores of glycogen which are rapidly used up during blastulation. It was suggested that this glycogen reserve was not available at the earlier stages due to lack of the enzyme glycogen phosphorylase.

Some research with rabbit ova concluded that one-cell rabbit embryos are capable of developing in media supplemented with several long-chain fatty acids or with acetic and proprionic acids (Kane, 1976). Short-chain fatty acids may be capable of acting as energy sources for bovine embryos since acetate and proprionate form a major part of the energy substrates in bovine blood. Many bovine embryo culture experimentors have utilized BSA as a protein supplement. It has been suggested that many BSA preparations are contaminated with both long- and short-chain fatty acids carried by the albumin (Kane and Headon, 1980; Kane, 1983).

ROLE OF AMINO ACIDS

Early cleavage stages of two-cell mouse ova were found to develop in simple salt solutions without free supplementation of free amino acids, but containing polyvinylpyrrolidine (PVP) or BSA (Cholewa and Whitten, 1970; Kuzan et al., 1982). Addition of amino acids to rabbit ova culture may be more important than in the mouse. Kane and Foote (1970) reported 0% rabbit embryo development in media containing no free amino acids compared to 72% developing to blastocysts in media containing a full complement of the 20 major amino acids. Mintz (1964) reported the incorporation of radio-isotopically labeled H^3-leucine into embryonic protein in

preimplantation mouse ova. The incorporation of radio-isotopically labeled amino acids into embryonic proteins in the mouse and rabbit was shown to be low in the early cleavage stages, but gradually increased between the eight- to 16-cell stages to very high levels in the morula and blastocyst (Manes and Daniel, 1969). The incorporation of amino acids in the bovine embryo has been reported to occur as early as day 3 (Epstein, 1975).

Brinster (1965c) reported that no one amino acid is essential for mouse embryo development and that the addition of PVP provides some of the physical properties of protein. Previous studies suggest that mammalian embryos can rely to a substantial degree on endogenous amino acid supplies, but require exogenous amino nitrogen to supplement their endogenous stores (Brinster, 1972). In contrast to these results, Gwatkin (1967) found that after blastulation of mouse ova five amino acids were essential and five more were beneficial for further development. Daniel and Olson (1968) reported that the first cleavage of rabbit ova can take place without exogenous amino acids in the supporting medium, but that subsequent cleavage requires cysteine, tryptophan, phenylalanine, lysine, arginine, and valine. As the embryo continues to divide, methionine, threonine, and glutamine also become essential.

Many mammalian embryo culture media contain BSA as a protein supplement. Bovine serum albumin functions include: stabilization of cellular membranes and supplementation of 21 amino acids (Kane and Headon, 1980); a reduction in the leakage of endogenous amino acids from the embryo (Brinster, 1965c); removal of toxic metal ions in the culture media such as copper and zinc ions (Cholewa and Whitten, 1970; Wright et al., 1978); and supplementation of unidentified nutrients (Kane and Headon, 1980).

Ham's F-10, the media of choice for most research involving bovine embryo culture, contains a full complement of the 20 major amino acids. Little or no research has been conducted testing bovine embryos in amino acid free media.

ROLES OF MISCELLANEOUS NUTRIENTS

There are a number of miscellaneous nutrients which may affect the development of mammalian embryos *in vitro*. Many nutrients such as trace elements, have not been studied to a large extent, but may be important.

Kane and Foote (1970) showed that the omission of a group of 11 water-soluble vitamins from complex culture media decreased the percentage of rabbit embryos forming blastocysts. Also, an increase in development was reported in day 5 rabbit blastocysts when thiamine, riboflavin, niacin,

pyridoxine, folic acid, inositol, or hypoxanthine were added separately to the culture media (Daniel, 1967). However, vitamins do not seem to be necessary for blastocyst formation in the mouse (Brinster, 1963; Kane, 1978), bovine (Tervit et al., 1972; Bowen et al., 1975; Kanagawa et al., 1975), ovine (Tervit et al., 1972), and porcine species (Davis and Day, 1977).

Alkaline and acid phosphatases have been suggested to be involved in mammalian embryo development. Schultz et al. (1971) reported high levels of both acid and alkaline phosphatases in collected bovine uterine secretions. These membrane bound enzymes were reported to increase upon blastulation and may be involved in embryonic transport processes (Fernley, 1971).

Although successful results are obtained in culture systems available today, the search for the ideal energy source continues. If further progress is made, one may be able to actually speed up in vitro development, either by elimination of deterrents or by the supplementation of specific promoting factors, with the result of more vigorous embryos with a higher degree of viability (Brackett, 1980).

THE ROLE OF THE UTERINE ENVIRONMENT: RELATIONSHIP TO THE EMBRYO

The uterine luminal fluid is the medium in which a number of important events in the reproductive processes of the bovine take place. In the cow, the embryo lies free floating in the uterine environment for 30 days of more, during which time extensive embryonic differentiation takes place before the conceptus becomes firmly attached to the uterine wall (Wislocki and Padykula, 1961). During this time the uterine fluid provides the medium in which the early embryo develops (Roberts and Parker, 1974a). Thus, any transfer of nutrients or exchange of information between the mother and the embryo must occur through the uterine fluid and consequently any component to this fluid is potentially important (Casida, 1961; Kirby, 1962). The idea that a proper uterine environment is needed for normal embryo development in the bovine has been investigated for a number of years (Hawk et al., 1955; Casida, 1961).

Previous research has shown that there is a high percentage of fertilization following insemination (Boyd et al., 1969; Diskin and Sreenan, 1980). Although fertilization rates are generally over 90% following insemination with high quality semen (Boyd et al., 1969), calving rates to a single insemination are closer to 50% (Ayalon, 1978). Most research suggests that the major factor causing this

loss in reproductive efficiency is the condition termed early embryonic death (Hawk et al., 1955; Ayalon, 1978; Diskin and Sreenan, 1980).

Most researchers agree that there are some components which are contained in the uterine fluids, whether in excesses or in deficiencies, which are responsible for the high incidence of early embryonic death in dairy cattle today. It is well documented in many mammalian species that the components of the early maternal uterine environment are constantly changing (Schultz et al., 1971; Ayalon, 1978). And it is known that the early embryonic stages possess stringent synchrony requirements which must be met by the uterine secretions in order for normal embryo development to occur (Rowson et al., 1969; Rowson et al., 1972; Newcomb and Rowson, 1975). By using embryo transfer, these investigators concluded that any deviation of plus or minus 2 days between donors and recipients can be tolerated but that deviations of as little as plus or minus 1/2 day caused decreases in pregnancy rates.

Repeat breeder cattle are subfertile cattle that usually have been inseminated more than three times without a full term pregnancy (Bulman and Lamming, 1978). Many repeat breeder cattle may result from such factors as: poor estrus detection, improper nutrition, genetic defects, uterine in-

fections, and other miscellaneous factors. But controlled research studies have concluded that the highest percentage of cattle classified as repeat breeders have a high incidence of early embryonic death. Linares et al. (1980) collected ova from repeat breeder heifers and from virgin heifers. Of 60 embyros collected from the repeat breeders, only 27, of which 20 were 4 days or younger, were classified as normal. This is in contrast with the embryos collected from the virgin heifers, where 27 out of 33 were in the expected stage of development.

Many investigators suggest that there is some critical stage in early bovine embryo development which is correlated with the greatest percentage of embryonic deaths. Hawk et al. (1955) reported that embryonic death rates in subfertile cows approached 42% in the first 16 days of gestation and 52% in the second 16 days. Renard and Heyman (1979) concluded that the transition from morula to blastocyst corresponded to a critical stage of development in vitro after superovulation in the cow. They reported at day 7 after breeding, that the percentage of normal embryos was significantly lower than at day 6 (59 of 100 compared to 70 of 100). Also, Ayalon (1973) reported that day 7 rather than day 6 was the critical day on which embryonic death becomes evident. Other investigators suggest that the major portion

of embryo loss occurs between days 8 and 16 of gestation (Diskin and Sreenan, 1980).

Repeat breeder cattle have been shown to have deficiencies or excesses of certain components contained in uterine secretions. They have been reported to have lower concentrations of sodium, phosphorus, glucose, and total protein on days 5 and 11. Concentrations of calcium, potassium, and magnesium were higher in the repeat breeders and showed cyclic variations (Lamothe and Guay, 1970). Ayalon (1978) reported that cows with abnormal ova had significantly higher concentrations of potassium, zinc, phosphorus, and calcium. Especially impressive was the differences in calcium ions which rose on day 7 in flushings from cows with abnormal embryos to more than 12 times the concentrations of the ion in uterine flushings from cows with normal embryos. Attempts to influence the electrolyte composition of uterine fluids by glucocorticoid therapy and by a varied calcium/phosphorous ratio in the diet were without significant effects (Lamothe et al., 1976). Jordan et al. (1983) reported that cows fed high crude protein diets had significantly higher urea levels in uterine secretions. They found that this was correlated with the concentration of ammonia in the blood and suggested that high ammonia concentrations could have detrimental effects on fertility as it is toxic to mammalian cells.

The integrative action of ovarian steroids causes cyclic changes in numerous uterine responses including: blood flow (Ford et al., 1979), endometrial histology and cytology (Priedkalns, 1976), and uterine fluid profiles (Roberts and Parker, 1976). Such changes occur to establish an intrauterine environment capable of sustaining the bovine embryo. Most of the data collected comparing repeat breeder cattle with normal cattle suggest a hormonal difference between the two groups. Guise (1978) reported that serum progesterone concentrations were lower in repeat breeder cattle than in normal cattle. Although many investigators have suggested that a progesterone deficiency may be a cause of early embryonic death, there are numerous other factors involved, and as of yet there has been no significant increases in pregnancy rates resulting from the use of supplemental progesterone administration.

The role of uterine specific proteins (USP) in early embryo development has been studied for years, but their function in the bovine is unclear. Suggested roles include: nutritional (amino acids), osmotic regulation, inhibitor removal, membrane stabilization, hormonal, and enzymatic. Krishnan and Daniel (1967) reported that blastokinin, a USP discovered in rabbit uterine secretions, was necessary for blastocyst formation and thus for further growth of rabbit

embryos. Since blastokinin was stored frozen and embryo development occurred in media supplemented with uterine secretions, it appeared that freezing was not detrimental to the USP contained in these secretions. A USP, uteroferrin, has been found in porcine uterine secretions. This specific progesterone-induced protein is believed to be involved in iron transport to the fetus (Bazer, 1975).

Very few bovine USP studies have been undertaken, but Mills et al. (1973) have reported the presence of a USP (MW=100,000) on days 15 and 16 of the estrous cycle following polyacrylamide disc gel electrophoresis (PAGE). In a later study, Mills (1975) reported the presence of a protein between days 13 and 20 of the estrous cycle in 62% of the uterine secretion samples which was absent from concomitant plasma samples. This same protein was observed in all samples collected from days 15 and 16 of the estrous cycle and there was a significant correlation between its presence and the concentration of plasma progesterone. Guise (1978) also identified this protein in the electrophoretic gels of cycling cattle. Bartol et al. (1979) utilizing sodium deodecyl sulfate-PAGE (SDS-PAGE) reported a total of 32 proteins of which six were present only from days 8 to 16 of the estrous cycle. These six USP had molecular weights of 18,700, 21,600, 32,400, 38,000, 45,500, and 162,500.

Early work by Laster (1974a) with uterine secretions collected from pregnant cattle revealed no difference in the total amount of protein between samples collected from open and pregnant heifers. Although total protein did not differ, a few specific proteins were present in lesser amounts and one particular protein was absent in a large number of the open heifers. In 1977, Laster described a protein with a molecular weight between 50 to 60,000 that can be detected in the uterus of cows 15 days after mating only when blastocysts are present. At day 15, this protein represents about 1% of the total protein in endometrium and uterine flushings.

Mainly serum proteins were found in bovine uterine secretions collected at various days throughout early pregnancy, but two USP were observed in day 14 uterine samples which disappeared by day 20 (Roberts and Parker, 1974b). Roberts and Parker (1976) studied uterine secretions collected from cattle that were between 14 and 75 days pregnant. Following protein fractionation by Sephadex G-100 chromatography, fractions were analyzed by SDS electrophoresis. The presence of USP in cows pregnant more than 17 days was greater than in cows pregnant less than 17 days. Molecular weights of the USP ranged between 38 to 48,000. Two of these USP were also observed in uterine fluid samples collected from early pregnant (day 7 to 12) cattle.

Bartol et al. (1981a) reported that the array of proteins present within the bovine uterus changed quantitatively and qualitatively during the estrous cycle following SDS-PAGE analysis of luminal proteins from pregnant and open cattle. Maximal stimulation occurred during the luteal phase. They concluded from their results that maximum stimulation of uterine protein synthesis or uptake occurs in response to progestational stimulation. They also reported that the concept of protein production by the bovine conceptus was supported by their results obtained from the analyses of the uterine secretions collected on day 19 of pregnancy. The quantitative and qualitative array of proteins found in utero on day 19 of pregnancy differed significantly from that found on day 19 of the estrous cycle. Furthermore, four proteins were indentified in the uterine flushings collected from day 19 pregnant cows which were absent from the uterine flushings of cyclic cattle.

There is much evidence that the production of USP may be regulated by the actions of progesterone and estrogens; especially in the pig (Knight et al., 1973; Bazer, 1975). Laster (1974b) working with ovariectomized heifers reported no significant differences between total protein or electrophoretic patterns among groups treated with progesterone, estrogen, or both for 4 days. However, these results may be

due to the shortness of the administration period. Dixon and Gibbons (1979) reported that prolonged progesterone treatment for a 2 to 3 month period resulted in nine non-serum USP in analyzed uterine secretions from treated cows. One USP was found to be identical to bovine milk lactoferrin (MW=81,000). Bartol et al. (1981b) reported that estradiol-17β (3 mg, i.v.), adminstered to cyclic cattle on day 14 or day 15 postestrus, caused increases in total protein as measured in uterine flushings obtained postmortem. Miller and Moore (1983) suggested that estrogens and progesterone regulate the secretion of different uterine proteins in the ewe, some which stimulate embryo development and some which inhibit it. They also found that progesterone given before estradiol inhibited the secretion of uterine proteins at 4 to 7 days after estrus which impaired embryo development in ovariectomized ewes not receiving the progesterone.

Very few embryo culture experiments have been conducted using uterine secretions as a media supplement. If the production of uterine proteins is necessary for embryo development *in vivo* it follows that the addition of uterine proteins to *in vitro* culture systems will maintain or enhance embryo development. This has been observed in the rabbit where the addition of unfractionated uterine proteins from day 5 pregnant rabbits was found to stimulate the growth of

early embryos (eight-to 16-cell), morulae, and day 4 blastocysts in culture (Maurer and Beier, 1976). Also, El-Bana and Daniel (1972) reported increased uptake of amino acids with addition of uterine flushings to rabbit embryo culture. Similar work in the bovine by Laster et al. (1978) using uterine secretions from day 12 uterine flushings as a supplement to day 10 embryos in culture found increased survival rates and greater increases in diameter than embryos cultured in BSA control media. However, this report is somewhat misleading since the uterine secretions were combined with more than 13.5 mg of BSA.

Work in our laboratory by Anderson (1982) reported the presence of USP in uterine flushings obtained from ovariectomized cows that had been administered progesterone and estrogen alone, or in combination. Acidic proteins unique to collected uterine flushings were associated with treatments including progesterone except one cow which received only an estrogen treatment. A basic protein was observed in 11 of 18 cattle receiving treatments which included progesterone compared with none from cattle receiving only estrogen treatments. He suggested that this basic protein may be a histotrophe component instrumental in early embryonic growth and development. Further research was conducted in our laboratory to test these uterine proteins for their ability to

promote *in vitro* development of bovine embryos. Canfield (1983) tested uterine secretions which had been obtained from cows that had been treated with 1:4000 estrogen:progesterone implants as a supplement to Ham's F-10 medium for their ability in promoting the development of bovine morulae. Other supplements tested were steer serum and BSA. Although it was hypothesized that the addition of uterine proteins to embryo culture media would allow normal development, the results were that only two of 35 morulae cultured in media supplemented with uterine proteins developed to blastocysts compared to 34 out of 35 in the steer serum and 18 out of 20 in the BSA.

Chapter III

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

This study was designed to test the effects of uterine proteins as supplements to Ham's F-10 media on early bovine embryo development. The study consisted of six experiments. Steer serum was used as a control in all experiments. Experiment I tested estrogen and progesterone induced uterine proteins obtained from previously ovariectomized cows (10% v/v) and Ham's F-10 medium with no protein supplement added. Experiments II through V tested uterine proteins from day 6, 10, or 15 from previously superovulated cows in different concentrations. Experiment VI tested 1% uterine proteins from day 6, 10, or 15 combined with 10% steer serum. Evaluation of fair and poor embryos was conducted by recording their development in steer serum. Also, the effects of varying superovulation regimes on subsequent superovulatory responses was noted.

The experiments and the protein supplements tested were as follows:

Experiment I:

1. Concentrated uterine proteins (UP; 10%) from ovariectomized estrogen treated cows.

2. Concentrated UP (10%) from ovariectomized progesterone treated cows.
3. Ham's F-10 medium (100%) with no protein supplement added.
4. Steer serum (10%).

Experiment II:

1. Day 6 UP (10%) collected from previously superovulated cows.
2. Day 10 UP (10%) collected from previously superovulated cows.
3. Day 15 UP (10%) collected from previously superovulated cows.
4. Steer serum (10%)

Experiment III:

1. Day 6 UP (1%) collected from previously superovulated cows.
2. Day 10 UP (1%) collected from previously superovulated cows.
3. Day 15 UP (1%) collected from previously superovulated cows.
4. Steer serum (10%).

Experiment IV:

1. Day 6 UP (.1%) collected from previously superovulated cows.

2. Day 10 UP (.1%) collected from previously superovulated cows.
3. Day 15 UP (.1%) collected from previously superovulated cows.
4. Steer serum (10%).

Experiment V:

1. Day 6 UP (.01%) collected from previously superovulated cows.
2. Day 10 UP (.01%) collected from previously superovulated cows.
3. Day 15 UP (.01%) collected from previously superovulated cows.
4. Steer serum (10%).

Experiment VI:

1. Day 6 UP (1%) collected from previously superovulated cows combined with 10% steer serum.
2. Day 10 UP (1%) collected from previously superovulated cows combined with 10% steer serum.
3. Day 15 UP (1%) collected from previously superovulated cows combined with 10% steer serum.
4. Steer serum (10%).

Evaluation of fair and poor embryos:

1. Culture results in 10% steer serum.

Superovulation and estrus synchronization:

1. Doses of FSH-P administered.
2. Synchronization protocol.

COWS

A total of 98 superovulations were conducted on 49 cows (14 used 2X; 6 used 3X; 5 used 4X; 2 used 5X). Thirty-six were dry cows maintained together as a group in an open lot without housing, while 13 were lactating cows (11 early lactation; 1 mid lactation; 1 late lactation). Dry cows were group fed hay and/or corn stalks ad libitum plus either 11 kg corn silage/cow/day or 7 to 9 kg total mixed ration. Dry cows on a superovulation regime were housed in a group pen and fed 5 kg corn silage and all the grass hay they could eat. Cows remained in the pen until flushed for embryos. The lactating cows were housed in a conventional free stall barn. They were fed ad libitum a complete mixed ration (15% crude protein, 20% acid detergent fiber) consisting of corn or alfalfa-grass silage, high-moisture corn, and high-protein soybean pellets.

SUPEROVULATION

Before cows were started on a superovulation regime (Appendix, fig. 1) they were palpated by a veterinarian to eliminate potential problem cows for reasons such as cystic ovaries, anatomical abnormalities, uterine infections, and other miscellaneous reasons. Such conditions generally interfere with successful superovulation and anatomical abnormalities may interfere with the non-surgical flushing procedures. Cows also were palpated for reasons such as failure to detect estrus and abnormal discharges. Cows passing this initial screening test were then observed until two consecutive estrous cycles of normal duration had been completed. Estrous cycle lengths were determined via twice daily estrus detection (0900 and 1900h) with additional observations occurring during farm visits to perform other tasks. To aid in the detection of estrus, K-mar heat mount detectors and orange livestock crayons (Nasco) were utilized.

Cows having two consecutive estrous cycles of a normal duration were than palpated by a veterinarian (days 8 to 13) to verify the presence of a normal mid-cycle corpus luteum. All cows with normal corpora lutea were then superovulated with follicle-stimulating hormone (FSH-P; Burns Biotech) using various dose regimes containing 28 to 50 mg (Appendix, table 1) between days 9 to 13 of the estrous cycle. A total

of six to eight intramuscular (im) injections were administered twice daily at 12 h intervals. Prostaglandin-F2-Alpha (PG; Lutalyse, Upjohn Co.) was administered to induce luteal regression following follicular stimulation. A total of 25 mg of PG was administered (im) in 1 or 2 injections at various times after the initial FSH-P injection (Appendix, table 2).

Additionally, age (mos) of the cow at the time of superovulation, number of times a cow was superovulated, stage of lactation, and maximum temperature the day of breeding and the day of flush were recorded each time the cow was superovulated.

BREEDING

Cows were artificially inseminated with 2 to 3 doses of frozen semen from a high fertility bull (bull #64; VPI & SU). Cows observed in standing estrus were inseminated either 3 times (at first observation of estrus and 12 and 24 h later) or 2 times (+12, +24 h). Cows not observed in standing estrus were inseminated at 72 and 84 h post initial PG injection. Semen straws (1/2 cc) were thawed in 37 C water for 45 sec. Approximately 1/3 of each straw was deposited into each uterine horn with the remaining 1/3 being deposited in the uterine body just anterior to the cervix.

PREPARATION OF FLUSHING MATERIALS

Dulbecco's phosphate-buffered saline (DPBS) was used as the flushing media. The DPBS was purchased pre-made (Gibco) in a dehydrated form in 1 liter quantities. Twice-distilled deionized water was heated to boiling to raise its pH above the levels it reaches in storage due to equilibrium with carbon dioxide in air. Also, the warmer water aids in dissolving the powdered DPBS. After the water reached boiling, it was cooled rapidly in an ice water bath until the temperature dropped below 30 C. The powdered DPBS was then added to 950 ml of the distilled water and allowed to dissolve under constant stirring. Next, the media was sterilized by membrane filtration (.45 u; Millipore) directly into 500 cc sterilized bottles. An antibiotic, Antibiotic-Antimycotic solution (Gibco), was then added to the media (.2cc/15ml) and the media was stored at 5 C. All materials possibly coming in contact with the embryos were washed, dryed, and sterilized in an autoclave before their use.

NON-SURGICAL FLUSHING PROCEDURES

The non-surgical flushing procedures were modifications of Seidel et al. (1978). Cows were flushed 6 days after the first breeding. Initially, the rectum was cleansed of feces and the ovaries were palpated to estimate the number of cor-

pora lutea. If this was the cows first flush, the cervix was also palpated to estimate its size so that the the appropiate sized Foley catheter could be used. Next, the tail head was clipped and scrubbed with Povidine surgical scrub (Henry Schein), rinsed with water, and sprayed with 70% ethanol in water. A local injection of Lidocaine HCl (5 ml; Butler) was administered between the first to third coccygeal vertebrae to deaden nerve sensations in control of rectal contractions.

The tail was then tied to the neck chain, allowing free access to anal and vulvular regions. The vulva and surrounding area were cleansed as was the tailhead. At this point, the vulva was spread slightly and a 40 cm 2-way Foley catheter (18 to 24 gauge) was placed through the cervix with the aid of a stylet from a standard A.I. gun and held in place by inflating a small cuff at the end of the Foley with approximately 3.5 to 4.5 ml PBS (Appendix, fig. 2). The fluid canal was then clamped with a hemostat to prevent leakage and the stylet was withdrawn. Next, an extension (made from old Foley catheters) was attached to the end of the catheter to facilitate the introduction and collection of the flush media.

Meanwhile, the DPBS was allowed to reach room temperture (25 C). Two 50 ml syringes were then filled with DPBS

and the fluid was introduced into the uterus and the catheter was clamped off with a hemostat. Care was taken to insure that the fluid reached each horn in approximately equal amounts. The fluid was then gently massaged to the tip of each uterine horn to dislodge the embryos in the upper ends of the horns. The hemostat was then removed and the massage was reversed forcing the fluid to flow out the catheter and into a graduated collecting bottle (500 cc). This procedure was repeated three to four times resulting in a total flushing volume between 400 and 500 ml.

Between collections, the collection bottle was capped and kept in an environment near 25 C. After recovering as much fluid as one could easily obtain, 100 ml of air was inserted. The air helped fill the uterine horns, displacing fluid back toward the catheter tip allowing for more fluid return. This procedure was usually conducted two to three times or more depending on the amount of fluid recovered. The cuff at the end of the catheter was then deflated and the catheter was removed and allowed to drain into the collecting bottle.

POSTFLUSH TREATMENTS

After flushing was completed, one of two post flush treatments were administered. Either PG was administered on day 10 (4 days post flush) or an iodine solution (Lugols solution; 5% iodine, 10% potassium iodide in water) was infused into the uterus. Both treatments insured return to estrus and the iodine solution also helped guard against future infection. The iodine treatment was prepared by mixing 4 ml of Lugol's iodine solution in 20 ml of sterile distilled water. Twenty ml of the solution was then administered using a uterine infusion rod which was passed through the cervix into the uterus.

EMBRYO SEARCHING

The collected flushings were transported to the laboratory and allowed to settle for at least 20 min. After the top 150 to 250 ml was slowly siphoned off, the remaining fluid was carefully poured off into 100 ml beakers. The fluid in the beakers was allowed to settle for a few minutes and then the top 60 to 80 ml was poured off leaving approximately 20 to 40 ml to be searched for embryos. Aliquots (10 to 15 ml) of the remaining fluid were placed in a 635 mm diameter sterile watch glass and swirled gently to move sediment including embryos towards the center of the glass.

The aliquots were then examined under a stereomicroscope with a magnification of 10X. After searching the bottoms of each beaker, the fluid was again allowed to settle and the fluid was searched again.

As embryos were found, they were transferred to fresh DPBS via a sterile drawn pipette and held at room temperature until morphlogically evaluated using a magnification of 60X.

MORPHOLOGICAL EVALUATION

Embryos were morphologically evaluated using a system provided by Renard and Heyman (1979). Embryos were placed into one of five categories: excellent, good, fair, poor, or unfertilized ova (UFO; fig. 1). Only excellent and good embryos were utilized in the experimental treatments. However, all fair and poor embryos were cultured in steer serum and development was recorded. Since all cows were flushed on day 6, morula stage embryos were expected and no later stage embryos were found.

Embryos rated excellent were symmetrical in appearance with blastomeres which were polygonal in shape and merging to form a tight inner cellular mass (ICM). These embryos had no signs of lysis, swelling, vacuoalization or degeneration of the single blastomeres and the zona pellucida was

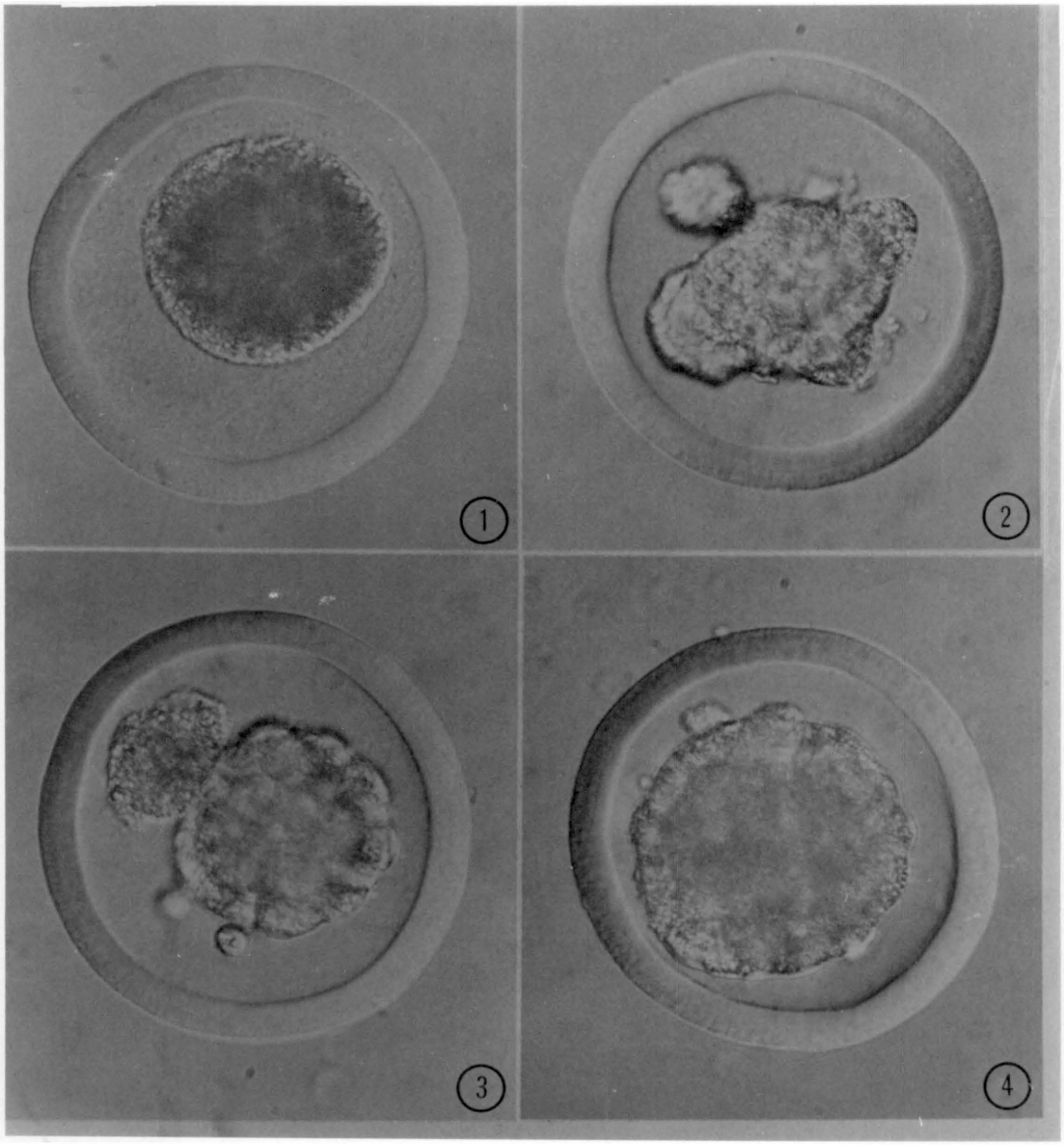
FIGURE 1. DIFFERENTIAL INTERFERENCE CONTRAST MICROGRAPHS OF BOVINE EMBRYOS: QUALITY CATEGORIES (Figures 1.1 - 1.4; X770)

Fig. 1.1 Unfertilized bovine ovum. There is no evidence of cleavage and the center mass is relatively dark and compact. Unfertilized ovum often are also unsymmetrical and when viewed from the side, appear flat or one dimensional.

Fig. 1.2 A fair morula (rated 3). This embryo would be considered transferable but not a good candidate for culturing. The center cell mass is intact and compact but there is a lack of symmetry to the cell mass shape and lack of uniformity of blastomere size. Also present are cell fragments and extruding blastomeres.

Fig. 1.3 This is a good morula (rated 2). The main cell mass is intact, compact, and both the cell mass and zona pellucida are symmetrical in shape. The blastomeres are of uniform size and density throughout. There are some extruded blastomeres and extraneous material between the cell mass and zona pellucida but not enough to interfere with development.

Fig. 1.4 This is an example of an excellent morula (rated 1). The cell mass is intact and symmetrical in shape, with and even, compact cell mass containing blastomeres of uniform size and density. The zona pellucida is also symmetrical in shape.


1
2
3
4

even and neither wrinkled or collasped. Good embryos were similar to excellent embryos, but less perfect. They usually contained a few spherical cells in the ICM and/or the extrusion of a few blastomeres outside the ICM. Fair embryos were embryos with larger amounts of either spherical cells in the ICM or detached and degenerative cells outside the ICM. Many of the fair embryos also showed some degree of developmental retardation. Embryos with an ICM which would normally be rated excellent or good, but which possessed wrinkled or abnormal zona pellucida were also rated as fair. Poor embryos exhibited more severe developmental retardation or consisted of a majority of detached and degenerative cells in the ICM.

BOVINE EMBRYO DEVELOPMENT

Bovine embryos were evaluated as they progressed from morula through the hatched blastocyst stage. The following description defines the various stages of development.

As embryo development reaches 16 to 32 cells, the embryo is referred to as a loose morula. At this point, the cells are still spherical and have yet to coalesce together to form a compact mass. However, they are too numerous to be counted. The loose morula then undergoes compaction. The cells become polygonal in nature and merge to form a

tight compact intercellular mass (ICM). This stage is termed the tight morula. All embryos used in this study were obtained from cows six days post insemination and thus were at the morula stage of development. In culture cells continue to divide and the embryo begins to form a fluid filled cavity (blastoceole) which has the appearance of a signet ring. This stage is termed the early blastocyst stage. As the blastocoele expands it loses the signet ring appearance and is called a blastocyst. Until this time the blastocoele has not expanded to completely fill the zona pellucida.

The formation of the blastocoele forces the cells of the ICM to one side of the ova and the support cells or trophoblastic cells to the outside against the zona pellucida. Prior to the blastocyst stage, the diameter of the bovine embryo remains rather constant at approximately 100 u. As development continues, the zona enlarges as the blastoceole cavity continues to increase in size. At this point, the embryo is termed an expanded blastocyst and generally reaches a diameter of over 200 u. The thickness of the zona also begins to decrease at this time from around 20 u to less than 5 u. Development continues until eventually the zona pellucida ruptures at one site and the cells of the embryo begin to work their way out. This is called the hatching

blastocyst stage and continues until the embryo is totally free of the zona at which time it is called a hatched blastocyst (fig. 2).

BASIC CULTURE PROCEDURES

The basic culture procedures were similar to those used by Wright et al. (1976a). Ham's F-10 adjusted to pH 7.2 to 7.6 was the medium utilized. This medium was purchased from Gibco in 100 ml sealed bottles. Antibiotics were then added as previously descibed and a rubber stopper was placed on the bottle. When not using the Ham's F-10, the air was suctioned out so that the pH of the medium would not rise (due to limited air contact), and the medium was stored at 5 C.

Following morphological evaluation, the embryos were placed in a watch glass containing Ham's F-10 and then placed in culture. First 10 ul of a protein supplement was added to a culture dish via an Eppendorf pipette and a sterile tip. Culture dishes (Lux) were 5-well microscope slides holding a maximum volume of 150 ul. Next 10 ul of Ham's F-10 containing a randomly selected embryo was added to the culture well and mixed with the protein supplement. Finally, 80 ul of Ham's F-10 was added and stirred to make up a total culture volume of 100 ul/embryo. Therefore, for each 100 ul of culture, there would be 90 ul of Ham's F-10 and 10

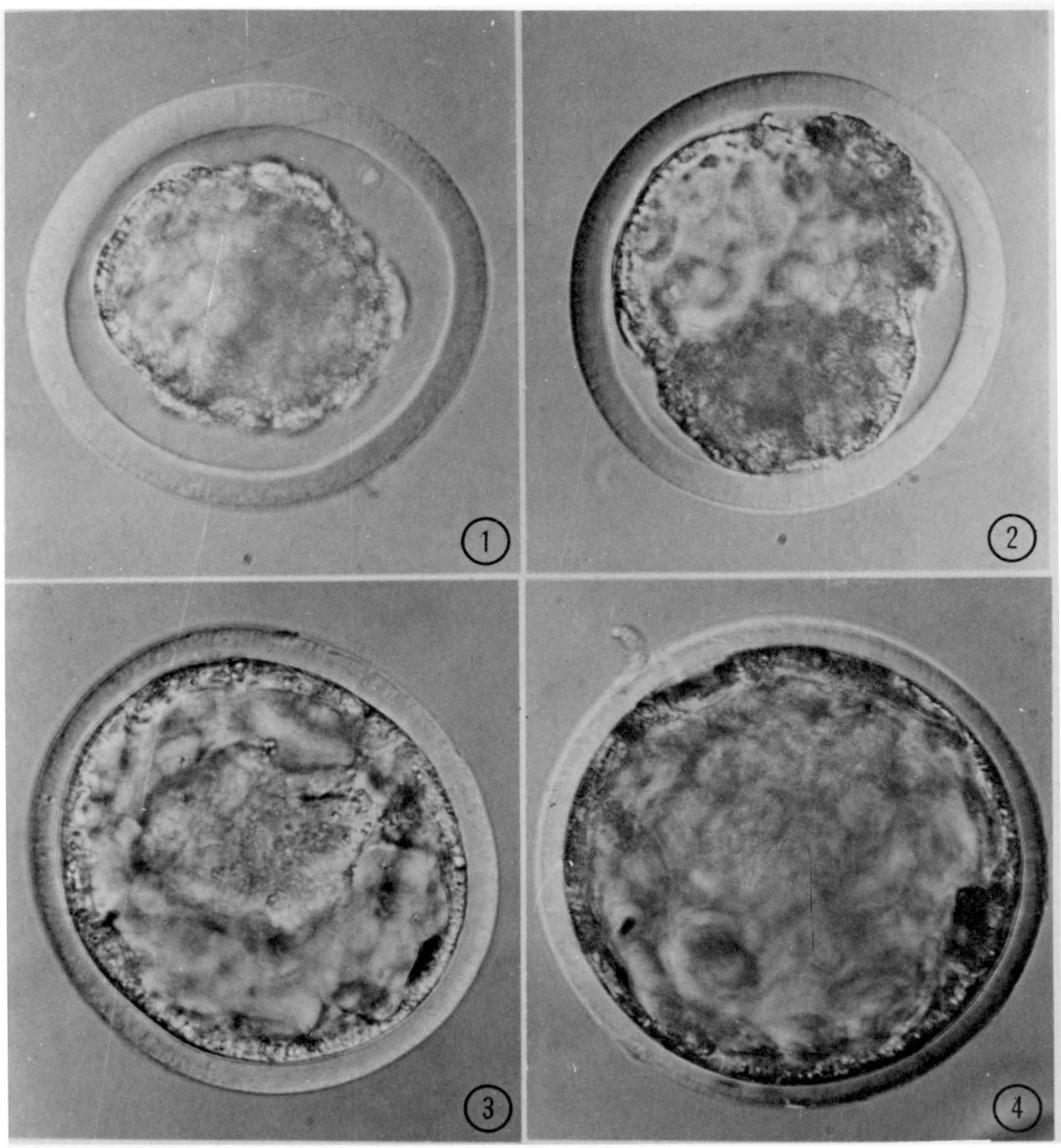
FIGURE 2. DIFFERENTIAL INTERFERENCE CONTRAST MICROGRAPHS OF BOVINE EMBRYOS: STAGES OF DEVELOPMENT IN VITRO (Figures 2.1 - 2.4; X770)

Fig. 2.1 An early blastocyst. Blastomeres are compact and beginning to migrate to one side to form an inner cell mass. The trophoblast cells are being pushed outward toward the zona pellucida by the expanding, fluid-filled blastocoele cavity.

Fig. 2.2 A good blastocyst. The inner cell mass is compressed to one side by the expanding blastocoele cavity which is extending outward toward the zona pellucida. There is some extraneous material which has been pushed outside the expanding ring of trophoblast cells but is not significant enough to interfere with development.

Fig. 2.3 An expanding blastocyst. Trophoblast cells line the inside of the zona pellucida surrounding the fluid-filled blastocoele cavity. The inner cell mass has been flattened to the top of this embryo and there has been a definite size increase resulting in the zona being stretched thin.

Fig. 2.4 Extremely expanded blastocyst just prior to hatching. Size increase is very obvious with the zona pellucida stretched very thin and beginning to tear. Trophoblast cells are flattened against the inside of the zona by the turgid, fluid-filled blastocoele cavity.


1
2
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4

ul of a particular protein supplement. A drop of previously equilibrated paraffin oil (5% CO_2, 5% O_2, 90% N_2) was added to minimize evaporation. Paraffin oil has been reported to present no significant barrier to the exchange of gases between the medium and atmosphere in the incubator (Brinster, 1963).

Embryos were cultured in an incubator at 37 C with an atmosphere of 5% O_2, 5% CO_2, and 90% N_2, bubbled through distilled water. Cultured embryos were examined every 12 h and development was recorded.

PROTEIN PREPARATION

Steer Serum

The steer serum was obtained by pooling blood from five Holstein steers. The blood was pooled into a beaker and allowed to stand for several hours. The ensuing clot was then loosened and the beaker placed in a refrigerator overnight (5 C). The next morning the clot dropped to the bottom allowing the serum to be poured off. Next, it was centrifuged at 7833 X g for 20 min to remove any remaining cells. The serum was heat-treated (30 min at 56 C) to destroy complement and frozen in 50 ml vials at -70 C (Canfield, 1983). At the start of the first experiment (and later as needed) a 50 ml vial was thawed at room temperature and small aliquots

(55 ul) were frozen in snap cap vials. The small aliquots could then be thawed as needed for their use in the different culture experiments. The protein concentration was 64 mg/ml (Lowry et al., 1951) and the osmolarity was 283 mOsm.

Estrogen and Progesterone Uterine Secretions

The estrogen and progesterone uterine secretions (US) were obtained from cattle ovariectomized on day 4 after an observed estrus following a normal estrous cycle. The cattle were implanted with either two implants, each containing 1.25 mg estradiol-17β, or four 2 g progesterone implants. Uteri of the experimental animals were flushed non-surgically to obtain US at 18, 36, and 54 days after implantation (Anderson, 1982).

At the start of this experiment, samples from each progesterone or estrogen treatment were thawed and pooled. From the initial calculations, the estrogen and progestrone induced US were too dilute to use as they were. Thus, pools of US were concentrated using a 10 ml Amicon stir cell (Amicon Corporation, model 52) equipped with a UM-2 membrane having a molecular weight exclusion of 1,000 daltons. During the concentration procedure, samples were washed with 10X the original volume with deionized water, which effectively desalted and concentrated the protein components.

The stir cell was run in a 4 C cold room under nitrogen pressure not exceeding 2069 mm Hg with constant stirring supplied by a magnetic stirrer. Secretions and washings were concentrated to approximately 3 ml. The remaining concentrated fluid was then sterilized by membrane filtration (.45 u) and the protein concentration was determined.

The US pools were over-concentrated and then diluted to 64 mg/ml to equal the protein concentration of steer serum. The osmolarity of the estrogen induced US was 294 mOsm while the progesterone induced US had an osmolarity of 314 mOsm. The estrogen and progesterone induced US were next heat-treated by placing the samples in a hot water bath (30 min at 56 C), and then placed in 35 ul aliquots and frozen (-70 C) prior to their use.

Day 6, 10 and 15 Uterine Secretions

Day 6 US were obtained from fluid recovered from non-surgical embryo flushes (Canfield, 1983). All cows were previously superovulated with 50 mg of FSH-P. Minimum requirements for use were that the flush had no visible trace of blood contamination (no pink or red color) and that the flush contained at least 4 normal embryos. Flushings from seven different cows were then pooled and concentrated using a 50 ml Amicon stir cell as described previously. After

concentration of the flushings, the protein concentration was adjusted with distilled water to 64 mg/ml. The osmolarity was determined to be 305 mOsm. The day 6 US were then heat-treated (30 min at 56 C), sterilized (via a .45u millipore filter), placed in 35 ul aliquots and frozen (-70 C) prior to their use.

Day 10 US were obtained by non-surgically flushing previously superovulated cows 4 days after they had been flushed for embryos. A 40 cm Foley catheter (18 to 24 ga.) was inserted through the cervix into one uterine horn with the aid of a stylet from a standard A.I. gun. The cuff of the catheter was then inflated with 10 to 15 ml of air to insure that the catheter would stay in place (at least 3 cm beyond the palpable uterine bifurcation). The stylet was withdrawn and rubber tubing was inserted. Next, 50 ml of a 1.5% (.33 M) sterile sodium chloride solution followed by 100 ml of atmospheric air was introduced into the uterine horn. The fluid was recovered by a gentle massaging motion into a sterile 50 ml Erlenmeyer flask which was corked, and placed on ice. The cuff of the catheter was then deflated and the catheter was postioned in the opposite uterine horn. The procedure was then repeated and samples were transported to the laboratory for analysis.

The iced US samples were then decanted into a 50 ml graduated cylinder from which a recovery estimate was calculated based on the initial flushing volume. To assess red blood cell contamination of the flushings, a subjective color classification was assigned to each collection (clean, pink I, pink II, or red) and later objectively confirmed by hemacytometer counts. A 10 ul Eppendorf pipette was used to fill the counting chamber of the hemacytometer with the sample of the flushings from one horn. The chamber was counted under 100X magnification using a standard light microscope. Erythrocytes were counted in 16 outer large blocks and their average was multiplied by 160,000 to give a concentration on a per ml basis. Samples with red blood cell concentrations of more than 3.0 X 10^5/ml were discarded and remaining samples were centrifuged at 12,602 X g to remove cellular and mucal debris.

The samples were sterlized by filtering through a millipore filter (.45 u) and then placed into 50 ml polypropylene storage bottles and frozen at -70 C until concentrated. Samples from 14 different cows were then pooled and concentrated via a 50 ml Amicon stir cell as descibed previously. The protein concentration was then determined and the sample was diluted with distilled water to a concentration of 64 mg/ml. Small aliquots (35 ul) were heat-treated (30 min at

56 C) and frozen at -70 C until their use. Osmolarity was 297 mOsm.

Day 15 US were obtained by non-surgically flushing previously superovulated cows 9 days after they had been flushed for embryos. The flushing procedures and laboratory preparations were the same as described for day 10 US. Samples from 8 different cows were pooled, concentrated, and the protein concentration was adjusted to 64 mg/ml. Aliquots of 35 ul were heat-treated (30 min at 56 C) and frozen at -70 C. Osmolarity was 292 mOsm. Erythrocyte concentrations for the uterine flushings were 1.37 ± .98 X 10^5/ml (X ± SD). These samples were later centrifuged and filtered before their use.

ANALYSIS OF DATA

Treatment differences were evaluated using the system of Wright et al. (1976a). A numerical value of 0 to 5 was assigned to each embryo representing the most advanced stage of development reached in culture (table 1). A score of 0 corresponds to an embryo showing no development or one which remained as a morula before degenerating; a score of 5 represents development to the hatched blastocyst stage.

Mean developmental scores for each treatment were calculated and the data analyzed by analysis of variance. The

TABLE 1. CODING OF OBSERVATIONS FOR STATISTICAL ANALYSIS

Assigned Code	Stage of Development
0	Morula (no development)
1	Early Blastocyst
2	Blastocyst
3	Expanded Blastocyst
4	Hatching Blastocyst
5	Hatched Blastocyst

(Wright et al., 1976a)

dependent variable was final developmental score and the independent variables in the model were treatment, initial quality, and cowflush, which was the combination of the particular cow and her flush date effect.

Mean developmental times to each stage were determined for all treatments. The dependent variables used were times to the early blastocyst, blastocyst, expanded blastocyst, hatching blastocyst, and hatched blastocyst stages, and the time to embryo degeneration. Independent variables were treatment, initial embryo quality, and cowflush.

The effects of the type of superovulation regime used were calculated using a model consisting of the dependent variables: embryo final score, number of embryos obtained per flush, number of culturable embryos obtained per flush, number of unfertilized ova obtained per flush, hours to heat following PG injection, and the response to PG injection (yes or no). Independent variables were maximum temperatures the day of breeding and flushing, day on which the superovulation scheme was initiated (relative to estrus), dose of FSH-P used, the type of PG injection relative to FSH-P initiation, and interactions: FSH-P schedule by PG schedule, FSH-P schedule by day of superovulation initiation, day of superovulation initiation by PG schedule, and FSH-P schedule by PG schedule by day of superovulation initiation.

Chapter IV

RESULTS

Overall analysis of variance for final developmental scores for embryos cultured in the different protein treatments is presented in table 2. Both protein supplement and cowflush significantly effected final developmental score ($P<.01$). The initial quality of the embryo (excellent or good) was found to have no significant effect. Variation in developmental scores of embryos cultured *in vitro* was explained adequately, in the statistical model ($r^2=.76$). Results of the individual experiments are presented as follows.

EXPERIMENT I.

Final mean developmental scores, comparing the effects of 10% estrogen (E) and 10% progesterone (P) induced uterine proteins (UP), Ham's F-10 alone, and 10% steer serum (SS) on early bovine embryo development, are presented in table 3. Treatment initial embryo quality, and cowflush effects (Appendix, table 3) were all significant ($P<.01$). Only SS promoted early embryo development with a mean developmental score of 3.62. These results were significantly higher than the poor development achieved with E and P induced UP and

TABLE 2. OVERALL ANALYSIS OF VARIANCE FOR FINAL DEVELOPMENTAL SCORES FOR EMBRYOS CULTURED IN DIFFERENT PROTEIN SUPPLEMENTS

Source	df	Mean Square
Treatment[a]	18	34.4**
Quality	1	1.9
Cowflush	52	2.3**
Error	251	.9

**P<.01

[a]Treatment was tested by cowflush.

with Ham's F-10 media alone. Only one embryo in the P induced UP treatment advanced past the morula stage while no embryos cultured in the Ham's F-10 alone or the E induced UP exhibited any further development.

The final stages of development reached by all embryos in the experiment and the percentages of embryos reaching each developmental stage are presented in Appendix, table 4. The data are presented in this manner to give an overall view of the number of embryos which progressed to each developmental stage. Least squares means for development time to each stage were also determined (Appendix, table 5). Twelve of 23 morulae cultured in SS reached the hatched blastocyst stage, which required an average of 96.0 h in culture.

EXPERIMENT II.

Final mean developmental scores for embryos cultured in 10% SS or 10% UP from day 6, 10, or 15 of the estrous cycle are presented in table 4. Analysis of variance of final developmental score showed that only treatment had a significant effect on final score (Appendix, table 6). Only SS exhibited an ability to promote early embryo development with a least squares mean score of 4.16. The number of embryos in each protein treatment reaching higher developmental

TABLE 3. FINAL MEAN DEVELOPMENTAL SCORES FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS), HAM'S F-10 ALONE, 10% PROGESTERONE (P), OR 10% ESTROGEN (E) INDUCED UTERINE PROTEINS

Treatment	n	Least Squares Mean Score	±	S.E.
SS	23	3.62**		.19
Ham's F-10	12	.19		.28
P	20	.07		.22
E	20	-.12		.21

*P<.01

stages are presented in Appendix, table 7. Embryos cultured in 10% UP from day 6, 10, or 15 showed little development as only one embryo in day 10 UP developed to an early blastocyst and no development was recorded in day 6 or day 15 UP. Least squares means for development time to each stage are presented in Appendix, table 8. Time to hatched blastocyst stage for embryos cultured in SS was 108.0 h.

EXPERIMENT III.

Since, the 10% UP supplements from day 6, 10, or 15 exhibited little or no embryo development and since the concentrations of UP which embryos *in vitro* are exposed to are between .1 and 1%, we decided next to test lower concentrations of UP. Experiment III utilized 10% SS and 1% UP from day 6, 10, or 15 to determine if lower concentrations of UP would promote early embryo development. Final mean developmental scores are presented in table 5. Analysis of variance showed a significant treatment effect ($P<.05$; Appendix, table 9). Embryos cultured in 1% UP from day 6, 10, or 15 still showed little further development as only one embryo in each of the day 10 and day 15 UP treatments advanced to the early blastocyst stage while no further development was recorded for embryos cultured in day 6 UP. The number of embryos reaching each developmental stage in each treatment

TABLE 4. FINAL MEAN DEVELOPMENTAL SCORES FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS) OR 10% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	n	Least Squares Mean Score	±	S.E.
SS	10	4.16**		0.18
Day 6	10	-0.18		0.20
Day 10	10	-0.10		0.18
Day 15	10	-0.20		0.19

**P<.01

is presented in Appendix, table 10. Embryos cultured in SS exhibited greater development than other treatments and had a least squares mean score of 4.06. Least squares means for development time to each stage are presented in Appendix, table 11. Embryos cultured in SS exhibited a mean time of 108.0 h to become hatched blastocysts.

EXPERIMENT IV.

Final mean developmental scores for embryos cultured in 10% SS or .1% UP from either day 6, 10, or 15 were determined (table 6). Analysis of variance showed a significant treatment effect ($P<.01$; Appendix, table 12). Embryos cultured in UP showed little further development while SS promoted early embryo development with a least squares mean score of 4.37. Six of 10 embryos hatched in the SS while only one embryo in each of the UP treatments from day 6, 10, or 15 reached the early blastocyst stage (Appendix, table 13). Time to complete hatching for embryos cultured in SS was 108.0 h. Least squares means for development time to each stage were determined for all treatments (Appendix, table 14).

TABLE 5. FINAL MEAN DEVELOPMENTAL SCORES FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS) or 1% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	n	Least Squares Mean Score	±	S.E.
SS	10	4.06**		0.25
Day 6	10	0.32		0.29
Day 10	10	0.52		0.29
Day 15	10	0.46		0.28

**P<.01

TABLE 6. FINAL MEAN DEVELOPMENTAL SCORES FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS) or 0.1% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	n	Least Squares Mean Score	±	S.E.
SS	10	4.37**		0.22
Day 6	10	0.25		0.21
Day 10	10	0.20		0.19
Day 15	10	0.23		0.22

**P<.01

EXPERIMENT V.

Final mean developmental scores for embryos cultured in 10% SS or .01% UP from day 6, 10, or 15 are presented in table 7. Analysis of variance showed a significant treatment and cowflush effect (Appendix, table 15). Steer serum was the only protein supplement which allowed early embryo development and embryos had a mean final developmental score of 4.26. No development past the early blastocyst stage occurred in either of the UP treatments. Final developmental stages for all embryos in Exp. V are presented in Appendix, table 16. Least squares means for development time to each stage were also determined (Appendix, table 17). Embryos reaching the hatched blastocyst stage had a least squares mean time to complete hatching of 96.0 h.

EXPERIMENT VI.

Our final experiment utilized a combination of 10% SS and 1% UP from either day 6, 10, or 15. This experiment was conducted to determine whether these UP contained inhibitory substances or if components in the uterine secretions might act synergistically with SS to promote early embryo development. Steer serum (10%) was again used as the control. Final mean developmental scores are presented in table 8. Analysis of variance showed both a treatment and cowflush

TABLE 7. FINAL MEAN DEVELOPMENTAL SCORES FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS) or .01% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	n	Least Squares Mean Score	±	S.E.
SS	10	4.26**		0.18
Day 6	10	-0.09		0.18
Day 10	10	-0.21		0.19
Day 15	10	-0.23		0.19

**P<.01

effect on final score (Appendix, table 18). Steer serum had a significantly higher final mean developmental score ($P<.05$; 4.19) than the three other UP treatments. Developmental scores for embryos cultured in day 6, 10, or 15 UP were 2.57, 3.28, and 3.34, respectively. Final developmental stages for all embryos in Exp. VI are presented in table 9. Twenty-three of 25 embryos cultured in SS developed to the hatched blastocyst stage which was better ($P<.01$) than the results obtained in 1% day 6, 10, or 15 UP combined with 10% SS.

Mean times to the early blastocyst stage were 28.6 h for SS, 28.7 h for day 10, and 30.0 h for day 15 UP combined with 10% SS (table 10). Mean time to the early blastocyst stage for embryos cultured in day 6 UP plus 10% SS was higher (35.6h) and nearly significantly different ($P<.058$) from the other treatments. Mean times to the blastocyst stage were 42.9, 44.3, and 44.7 h for SS and day 10 or day 15 UP plus 10% SS, respectively. Mean time to the blastocyst stage for embryos cultured in day 6 UP plus 10% SS was also higher (50.0h) and nearly significantly different ($P<.059$) from the other treatments. Mean times to the expanded blastocyst stage were similar among treatments with times of 59.4, 58.8, 63.0, and 57.3 h for embryos cultured in 10% SS, and 1% UP from day 6, 10, or 15 plus 10% SS, respectively.

TABLE 8. FINAL MEAN DEVELOPMENTAL SCORES FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS) OR 1% DAY 6, 10, AND 15 UTERINE PROTEINS COMBINED WITH 10% SS

Treatment	n	Least Squares Mean Score	±	S.E.
SS	25	4.19[a]		0.30
Day 6 + SS	21	2.57[b]		0.34
Day 10 + SS	22	3.28[b]		0.33
Day 15 + SS	20	3.34[b]		0.34

[a,b]Numbers with different superscripts differ at $P<.05$.

TABLE 9. STAGE OF EMBRYO DEVELOPMENT AFTER CULTURE IN 10% STEER SERUM (SS), OR 1% DAY 6, 10, AND 15 UTERINE PROTEINS COMBINED WITH 10% SS

Treatment	SS		Day 6 + SS		Day 10 + SS		Day 15 + SS	
Stage of Development	n	(%)	n	(%)	n	(%)	n	(%)
Morula	25	(100)	21	(100)	22	(100)	20	(100)
Early blastocyst	24	(96)	19	(90)	20	(91)	17	(85)
Blastocyst	24	(96)	17	(81)	20	(91)	17	(85)
Expanded blastocyst	24	(96)[a]	12	(57)[b]	15	(68)[b]	15	(75)[a]
Hatching blastocyst	23	(92)[a]	9	(43)[b]	12	(55)[b]	10	(50)[b]
Hatched blastocyst	23	(92)[a]	7	(33)[b]	10	(45)[b]	10	(50)[b]

[a,b]Numbers with different superscripts differ at $P<.01$.

TABLE 10. LEAST SQUARES MEANS (±SE) FOR DEVELOPMENTAL TIME TO EACH STAGE FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS) OR 1% DAY 6, 10, AND 15 UTERINE PROTEINS COMBINED WITH 10% SS

Treatment	SS	Day 6 + SS	Day 10 + SS	Day 15 + SS
Stage of Development	Time (hours)			
Morula	- (25)[a]	- (21)	- (22)	- (20)
Early blastocyst	28.6 ± 2.5(24)	35.6 ± 3.0(19)	28.7 ± 3.0(20)	30.0 ± 3.2(17)
Blastocyst	42.9 ± 2.5(24)	50.0 ± 3.1(17)	44.3 ± 3.0(20)	44.7 ± 3.2(17)
Expanded blastocyst	59.4 ± 2.5(24)	58.8 ± 3.7(12)	63.0 ± 3.4(15)	57.3 ± 3.4(15)
Hatching blastocyst	77.6[b] ± 3.3(23)	72.3 ± 5.8(9)	82.2[b] ± 5.2(12)	61.7[c] ± 5.6(10)
Hatched blastocyst	96.5[b] ± 3.8(23)	90.7[b] ± 7.4(7)	96.1[b] ± 6.3(10)	72.8[c] ± 6.5(10)

[a]number at each stage

[b,c]Numbers with different superscripts differ at $P<.05$.

Embryos cultured in day 15 UP plus 10% SS had a mean time to the hatching blastocyst stage of 61.7 h which was significantly less ($P<.05$) than embryos cultured in 10% SS or 1% day 10 UP combined with 10% SS which had mean times to hatching of 77.6 and 82.2 h, respectively. Also, mean time to the hatched blastocyst stage for embryos cultured in 1% day 15 UP combined with 10% SS was significantly reduced (72.8h) compared to 96.5, 90.7, and 96.1 h for embryos cultured in SS, and day 6, or day 10 UP plus 10% SS, respectively.

EVALUATION OF FAIR AND POOR EMBRYOS

A total of 67 fair embryos and 56 poor embryos were cultured in 10% SS and development was recorded. Only 12% of the fair embryos reached the hatched blastocyst stage (8 of 67) and only 4% of the embryos rated as poor hatched from the zona pellucida (2 of 56; Appendix, table 19).

SUPEROVULATION AND NON-SURGICAL FLUSHING RESULTS

The superovulation and non-surgical flushing data for all experiments are summarized in table 11. During the course of this study 776 embryos were obtained by superovulating 98 dairy cows. The average fluid recovery per flush was 94.0% of the total flushing volume. Mean number of cor-

pora lutea (CL) per cow superovulated was 8.0 with an average of 8.6 embryos collected per flush. Of this number, 4.5 embryos were graded excellent or good and thus usable in culture because of the quality acceptance standards. Although 98 cows were superovulated, there were only 77 which responded to the FSH-P with more than 1 CL. These cows averaged 10.0 CL with a mean of 10.1 embryos per flush and an average of 5.3 usable embryos.

Cow response to PG, as measured by the observation of standing heat, was significantly affected ($P<.01$) by PG schedule (Appendix, table 20). A 96.55% response rate was obtained when two injections (25 mg total) were given compared to a 60% response rate after one 25 mg injection (table 12). A PG schedule of one 25 mg injection 84 h after initial FSH-P injection resulted in a significantly lower response rate ($P<.05$) compared to all other PG schedules. (Appendix, table 2). A PG schedule of two injections (25 mg total) administered 60 and 72 h after the initial FSH-P injection was found to have a higher response rate ($P<.01$) than one 25 mg PG injection administered 72 h after the first FSH-P injection.

Hours to heat following PG injection was not significantly affected ($P>.05$) by any of the independent variables evaluated (Appendix, table 20). Whether one or two injec-

TABLE 11. SUMMARY OF FLUSHING RECOVERY, OVARIAN RESPONSE, AND EMBRYO RECOVERY

	Cow Flushes	% Fluid Recovered	Mean # CL	Mean # Embryos Rec.	Mean # Embryos Cult.
All cow flushes	91[a,b]	94.0 ± 7.2	8.0 ± 4.9	8.6 ± 7.6	4.5 ± 4.8
Cow flushes > 1 Cl	77	94.0 ± 7.6	10.0 ± 3.4	10.1 ± 7.3	5.3 ± 4.8

[a]mean ± S.D.

[b]ninety-eight total superovulations

TABLE 12. SUMMARY OF TIME TO HEAT FOLLOWING PG INJECTION(S) AND PG RESPONSE

PG Schedule		Hours to Heat	Response (%)
1 injection[a]	(40)[c]	58.7 ± 5.5[b]	60.0
2 injections[a]	(58)	55.0 ± 3.8	96.55**

[a] 25 mg total

[b] mean hr ± S.D.

[c] number per treatment

**P<.01

tions of PG were administered did not have an effect on the subsequent time to estrus (table 12). Cows receiving one injection of PG had a least squares mean time to heat of 58.7 h compared to 55.0 h for cows administered two injections (25 mg total).

The number of embryos obtained per flush was not influenced by environmental temperature, superovulation scheme used, age, or by individual cows ($P>.05$).

Maximum temperature on flush day, which ranged from -5.0 C to 34.4 C, had a significant effect ($P<.055$) on the number of culturable embryos obtained per flush (Appendix, fig. 3; Appendix, table 20). There was a highly significant interaction ($P<.01$) between dose of FSH-P and PG schedule. Generally, the total number of culturable embryos obtained was greater when a low dose or a decreasing dose regime of FSH-P was used in conjunction with a split PG injection scheme (Appendix, table 21). Greatest number of culturable embryos occurred with a 32 mg FSH-P and a single PG injection. Lowest number of culturable embryos was obtained with 30 mg FSH-P/6 injections and a single PG injection.

The mean number of unfertilized ova (UFO) per flush was 2.09. This number was influenced ($P<.05$) by individual cows which averaged a high percentage of UFO each flush. One individual had 32 UFO on one particular flush date. Day of

the cycle which FSH-P was started also influenced ($P<.05$) the number of UFO obtained per flush. Cows starting the superovulation scheme on day 13 had significantly higher numbers of UFO than cows started on days 9 to 12 (Appendix, table 20; Appendix, table 22). Age had a significant ($P<.01$) negative effect on the number of UFO ($b=-.5814$). The effect of maximum temperature at breeding on the number of UFO obtained per flush is shown in Appendix, fig. 4.

Final score was significantly affected ($P<.01$) by the particular superovulation regime utilized (Appendix, table 20). Embryos cultured after administering a total dose regime of 28 mg/8 injections in decreasing amounts (table 1) exhibited higher resulting final scores ($P<.05$) than embryos cultured after superovulation with 40 mg/8 injections, 50 mg/8 injections, or 30 mg/6 injections (Appendix, table 23). Embryos cultured after utilizing a superovulation regime of 32 mg/8 injections in decreasing amounts or a regime consisting of 35 mg/7 injections had significantly higher resulting final scores than embryos obtained after utilizing higher dose regimes of 40 mg/8 injections, 50 mg/8 injections, or 30 mg/6 injections (Appendix, table 20).

Cows superovulated more than once averaged 121.9 d between flushes. Time to next heat after embryo flushing averaged 23.5 d for all postflush treatments. The time to the

next heat after treatment with Lugols solution averaged 22.5 d (n=48) compared to an average of 24.4 d obtained after a postflush PG injection on day 10 following insemination (n=47).

Chapter V

DISCUSSION

EMBRYO DEVELOPMENT

In this study, different uterine proteins (UP) were tested for their ability to promote early bovine embryo development. Steer serum (SS) was chosen as the control since studies by Allen et al. (1982) and Canfield (1983) reported successful bovine embryo development in culture media supplemented with SS. The reasons for the success of SS in promoting early embryo development are not well understood. Brinster (1967b) suggested that sera in general may provide an exogenous nitrogen source which is required by the embryo to support the marked increase in protein synthesis which occurs between the morula and early blastocyst stages. Since serum contains a high percentage of albumin (3.63 g/100ml; Swenson, 1970), the uterus may have a need for albumin to support early embryo development. This is supported by the fact that uterine fluids often have higher concentrations of albumin than concomitant blood plasma samples (Anderson, 1982) and that albumin is able to pass from the blood stream into the uterus in limited amounts (Swenson, 1970). Additionally, Kane and Foote (1971) have suggested that rabbit embryos require albumin to blastulate.

Many researchers believe that the albumin component of sera may also be responsible for supplying short- and long-chain fatty acids which are often carried by albumin molecules (Kane and Headon, 1980; Kane, 1983). Canfield (1983) reported that purified BSA (99.5%) also promoted optimal early embryo development. Cholewa and Whitten (1970) showed that sera may provide a nutritive function by chelating heavy metal ions.

In addition to an albumin requirement, Saito et al. (1984b) suggested that mouse embryos also require some protein component in the large molecular weight fraction of serum for hatching and attachment. Saito et al. (1984a) reported that a medium containing at least 10% serum was necessary for culturing mouse embryos. Sera concentration requirements for the culture of bovine embryos have not been well established, but successful results have been obtained with sera concentrations ranging from 5 to 50% (Allen et al., 1982; Wright and Bondioli, 1981).

Our results support the concept that SS provides optimal early bovine embryo development. Sixty of 88 embryos cultured in SS developed to the hatched blastocyst stage which corresponds to a hatching rate of 68.2%. These results are higher than those reported by Canfield (1983) who found that bovine morulae had a 31.4% hatching rate in SS

(n=35), but agree with results obtained by Allen et al. (1982) who reported a 67.0% hatching rate for embryos cultured in 10% SS. Time to hatching for all embryos cultured in SS averaged between 96 to 108 h which is similar to the time of hatching in vivo and occurs at approximately day 10 in the bovine (Chang, 1952).

No development was recorded for embryos cultured in Ham's F-10 media alone. This agrees with Saito et al. (1984a) who found that the nitrogen source provided in Ham's F-10 media was not sufficient for mouse embryos to develop and that embryos at the compaction stages to the later blastocyst stages required a serum supplement for further development.

The results of our study also indicate that the UP utilized in the different experiments were incapable of promoting early bovine embryo development without additional supplementation with SS. Embryos cultured in 10% estrogen (E) or progesterone (P) induced UP demonstrated times to degeneration which were significantly shorter ($P<.05$) then embryos cultured in Ham's F-10 alone. Least squares mean times to degeneration were 58.5 ± 4.2, 68.2 ± 4.8, and 75.3 ± 6.1 h for E and P induced UP and for Ham's F-10 alone, respectively. A possible reason for the poor development in the E and P induced UP is that the sources of these secretions

were ovariectomized cows. It seems likely that these secretions may differ from secretions obtained from normal intact animals.

Furthermore, many studies have been conducted that suggest that supplemental hormone therapy may be detrimental to early mammalian embryo development. When pregnant rats were subjected to supplemental progesterone treatments many of the embryos were arrested at the morula stage (Dickmann, 1970). Daniel and Levy (1964) reported that the addition of progesterone to rabbit ova culture resulted in the inhibition of further development. They concluded that progesterone blocked cleavage by limiting the supply of protein or amino acids and thus inhibiting protein synthesis within the ovum. This inhibition was overcome by increasing the concentrations of amino acids or increasing the serum component in the medium. Supplementing the progesterone media with estradiol did not reverse the inhibition. Also, Maurer and Beier (1976) reported that the administration of physiological levels of progesterone appeared to retard embryonic development in the rabbit.

Estrogen and progesterone treatment of normal pregnant rabbits resulted in arrested development of blastocysts (Beier et al., 1970). The protein fractionations differed in uterine secretions collected from hormone treated rabbits

and those found in pregnant rabbits without hormone therapy. They also reported that estrogen treatment alone, strongly arrested blastocyst development. Additionally, it has been suggested that an inhibitory protein can be produced in rabbit uterine secretions by certain hormonal ratios (Stone et al., 1977). The possibility exists that a hormone induced inhibitory substance may have been produced by the progesterone and estrogen treatments which may be magnified upon concentration.

Uterine proteins collected from previously superovulated cows on day 6, 10, or 15 after insemination were also unable to support early embryo development when added to Ham's F-10 medium as the only supplemental protein source. There is a possibility that the UP secretions may also contain inhibitory substances. This is supported by the fact that as UP concentrations were diluted from 10% to .1%, times to degeneration increased from 45.3 ± 3.6, 49.8 ± 3.4, and 51.9 ± 3.5 h for 10% day 6, 10, and 15 UP, respectively to 66.1 ± 5.8, 63.9 ± 5.6, 60.5 ± 6.0 h for embryos cultured in diluted UP concentrations (.1%).

Development occurred in all treatments where 1% UP were combined with 10% SS. However, embryos cultured in 1% day 6 UP combined with 10% SS demonstrated delayed times to the early blastocyst and blastocyst stages which approached sig-

nificance. This suggests that day 6 uterine secretions (US) may contain some substance(s) which account for this retardation of development in the early stages. Embryos cultured in 1% day 6 UP plus 10% SS which survived to the blastocyst stage then exhibited times to the further developmental stages which were normal or below normal. These results suggest that the embryo may be more susceptible to certain inhibitory substances in the period from morula to blastocyst. Embryos which reach the blastocyst stage seem to overcome their susceptibility and development proceeded normally and even accelerated to compensate for the early retardation experienced.

Embryos cultured in 1% day 15 UP combined with 10% SS exhibited developmental stage times up to the expanded blastocyst stage which were similar to controls. However, the time to hatching was significantly reduced for embryos in 1% day 15 UP plus 10% SS with a least squares mean time of 72.8 h compared to 96.5 h for control embryos. This suggests that day 15 UP may contain a factor or factors which may shorten the time of embryo hatching in vitro. However, only 10 of 20 embryos reached the hatched blastocyst stage in the 1% day 15 UP plus 10% SS treatment compared with 23 of 25 hatching in SS alone. These results support the concept that if the early bovine embryo can survive in the US

through the first stages of development then the later developmental stages may not be as susceptible to inhibitory substances which may be present in the concentrated US. The embryo may then compensate for any early retardation effects by decreasing the time of development to the later stages. Times to the developmental stages for embryos cultured in 1% day 10 UP combined with 10% SS were similar to the times observed in SS alone.

A possible factor contributing to the poor embryo development in UP collected from day 6, 10, and 15 is the source of these UP. Results reported by Newcomb et al. (1976) suggest that the high percentage of abnormal embryos recovered from superovulated donors may result from an unfavorable uterine environment due to grossly distorted levels of circulating steroids in these animals. It is well known that estrogens are abnormally high at estrus in superovulated cows and it is noteworthy that estrogens are also known to be high after ovulation in a large number of sterile cows (Greve et al., 1979). Greve (1984) suggested that the process of superovulation has a profound effect on a dairy cow's normal endocrine balance with resultant abnormal embryo development. He suggested that abnormal embryo development may be a result of the unusually high progesterone concentrations which are prevalent in many superovulated

cows. In his study a high proportion of superovulated animals (15 of 38) developed abnormal progesterone profiles with concurrent abnormal ova and embryo development.

Estrogens and progesterone are known to influence the production and concentrations of certain US (Bartol et al., 1981b). Anderson (1982) reported that acidic proteins were unique to collected uterine flushings associated with progesterone treatments. Also, a basic protein was observed in 11 of 18 cattle receiving treatments including progesterone compared to none from cattle receiving only an estrogen treatment. Since superovulated cows have abnormal levels of estrogens and progesterone, they may have US which differ from normal cows and thus may be detrimental to early embryo development. Also, the exact composition of these US is unknown and the composition of the UP was not elucidated.

Martin (1984) reported that many so called luminal fluid proteins obtained by flushing mouse uteri may originate from luminal and stromal cells, intercellular fluid, and blood. It was concluded that the composition and concentration of these proteins may reflect alterations in the extent and type of damage produced by the flushing procedures. Possibly this could also pertain to flushing cow uteri as high red blood cell concentrations were observed in collected flushings. Even after centrifugation and filtra-

tion, there is a strong possibility that the hemoglobin component of the red blood cell may account for a significant percentage of the total protein recovered from these flushings.

Another possibility contributing to the poor development in UP is that the synchrony requirements between the embryo and the US may not have been met. It is well known that the composition of the uterine environment in early pregnancy is constantly changing (Lamothe and Guay, 1970; Schultz et al., 1971; Bartol et al., 1981a). Furthermore, embryo transfer experiments have found the need for stringent synchrony requirements between the donor and recipients. Deviations of more than two days caused dramatic decreases in pregnancy rates and generally best results were obtained when exact synchrony occurred (Newcomb and Rowson, 1975; Rowson et al., 1972; Nelson et al., 1982). Culturing day 6 morulae in UP from day 10 or day 15 flushings may mean that we were placing the embryos in an environment for which they are not prepared. It was hypothesized that day 6 UP would achieve the best results of the three different days in which collections were made since we were culturing day 6 morulae in day 6 UP.

Another possibility for the poor development observed in the UP treatments is that certain inhibitory substances

as well as the proteins may have been concentrated. Schumacher (1980) reported the presence of large numbers of immunoglobulins and lysozymes in human US. Upon concentration, these substances may become detrimental to early embryo development. He reported that IgG and IgA antibodies were capable of inhibiting in vivo and in vitro fertilization in the rabbit. Also, IgA type antibodies crossreacted with rabbit blastocyst cells and caused subsequent degeneration of the blastocyst.

The presence of immunoglobulins in US obtained from previously superovulated cows has been documented in day 10 and day 18 flushings (Gimenez et al., 1984). Thus, it is possible that the procedures employed to concentrate the UP may have concentrated immunoglobulins which became toxic to the early bovine embryo. Preliminary data from our laboratory suggests that the addition of IgM antibodies to bovine embryo culture media may interfere with the normal developmental process.

Only a small percentage of the fair and poor embryos cultured in SS developed to the hatched blastocyst stage (12% and 4%, respectively). This is in contrast to the 68.2% hatching rate achieved by excellent and good embryos cultured in SS. These results suggest that the initial quality scores assigned to the embryos were accurate for the

most part. However, even though initial quality scores have significant effects on resulting pregnancy rates (Scanlon et al., 1968; Sreenan, 1978; Greve et al., 1979), there are still some embryos rated excellent or good that do not continue to develop and some fair and poor embryos which result in pregnancies upon their transfer (Greve et al., 1979; Renard and Heyman, 1979).

SUPEROVULATION AND EMBRYO RECOVERY

It is generally agreed that the success of superovulation is effected by various factors such as the individual response, the physiological state of the animal at the time of treatment, age, breed, season, number of consecutive stimulations, and the type of gonadotrophin used (Lauria et al., 1982). Superovulation responses in this study varied greatly from no ovulations to multiple ovulations too large to count accurately. Underestimating the number of CL in cows with multiple ovulations explains the reason for obtaining an average of 8.6 embryos per flush which was higher than the mean number of CL estimated (8.0). Many multiple ovulations which were too numerous to accurately count were recorded as 14 or more for the purpose of data analysis. Thus, a cowflush may have resulted in more than 14 embryos but CL number would have been estimated at 14. Twenty-five

cows had CL numbers that were underestimated. An underestimation of recoverable embryos per CL counted was expected as previous studies indicate embryo recovery rates resulting from non-surgical methods were between 50 and 85% of the number of palpable CL (Elsden et al., 1976; Brand and Drost, 1977; Monnieux et al., 1983). This underestimation may be explained in part by technician error in the flushing technique and also by research which suggests that embryo transport takes longer in the superovulated cow with 6% or more of the embryos still in the oviduct 13 days after ovulation (Betteridge et al., 1980).

Total number of embryos recovered was lower than the 10.1 mean number of embryos obtained per flush reported by Donaldson (1984) but nearly equaled the number of good embryos (4.6/flush). These results are higher than the average of only 3 to 6 embryos recovered in some studies (Greve et al., 1980; Chupin and Procureur, 1982; Darrow et al., 1982; Canfield, 1983). Of the 98 cows superovulated, there were 77 which had two or more palpable CL. Thus, there were 21 cows which did not respond to the superovulation regime. These results are comparable to other research which suggest a range in unresponsiveness of 14% for healthy animals to 51% in infertile animals (Hasler et al., 1983). Eight cows utilized in this study failed to produce recoverable embryos

the first time they were superovulated. Five of these animals were superovulated again and three produced recoverable embryos. Nineteen of 27 cows (70.4%) did produce recoverable embryos on their initial superovulation. Cows flushed with two or more CL averaged 10.0 embryos per flush with 5.3 classified as excellent or good. The percentage of excellent and good embryos in this study averaged 53% which is comparable to that of other research (Seidel, 1981; Donaldson, 1984).

Dry cows (n=36) averaged 8.1 embryos per flush with an average of 3.8 classified as culturable. Total ova and embryos per collection from lactating cows (n=13) averaged 11.3; the number of culturable embryos averaged 9.0. These results agree with Darrow et al. (1982), who found that although superovulatory responses were similar in dry and lactating cows, only about one-half as many transferable embryos were recovered from dry cows.

A total of 168 unfertilized ova (UFO) were collected of which 116 resulted from seven individual cows. The incidence of UFO in this study was 22.0% which is comparable to the rates published by previous investigators who suggest that fertilization rates between 70 to 85% for superovulated ova are expected (Elsden et al., 1976; Sreenan and Diskin, 1982). Also, a trend toward an increase in the number of

UFO obtained per flush was observed as cows were superovulated more than four times. This agrees with Hasler et al. (1983) who reported a significant decrease in fertilization rates after five superovulations. In our study, day of the cycle in which FSH-P was started influenced the number of UFO obtained per flush. Cows starting the superovulation scheme on day 13 had significantly higher numbers of UFO than cows started on day 9 to 12. These results may be explained in part by individual cows having large numbers of UFO on day 13. It may also be plausible that since day 13 is an extreme of the recommended period to start a superovulation regimen, that many ova introduced to exogenous hormone therapy may not be able to develop in a natural way to allow normal fertilization. In contrast to these results, Hasler et al. (1983) reported significantly lower fertilization rates on days 11 and 12 when compared to days 8, 9, 10, and 13.

Maximum temperature on the flush day had a significant effect on the number of culturable embryos obtained per flush. Also, as maximum temperature the day of breeding increased, a significant positive relationship was observed between increasing temperature and the total number of UFO obtained per flush. Although temperature has been shown to affect reproduction in cattle (Tucker, 1982), little infor-

mation is available reqarding the influence of ambient temperature, specifically on superovulation and embryo quality. Some studies suggest that temperature affects are only minimal (Betteridge, 1977), while others suggest that best results are obtained in winter and spring (Hasler et al., 1983). A possible explanation for the significant effect of maximum temperature at flushing on the number of culturable embryos obtained may be that higher environmental temperatures are more conducive for short-term holding of embryos during embryo flushing in an uncontrolled temperature environment. Embryo flushings conducted on cold days may have been detrimental to final scores and embryo quality if the embryos were exposed to these low temperatures for too long a time.

The number of culturable embryos obtained was also influenced by the interaction between the dose of FSH-P used and the type of PG injection scheme utilized. Although numbers of culturable embryos were generally higher when a decreasing dose regime of FSH-P was combined with a two injection scheme of PG, the highest number was obtained when a decreasing dose of 32 mg of FSH-P was combined with one PG injection administered at 72 h after the initial FSH-P injection. However, only one animal received this combination of superovulation schemes. A trend for higher numbers of

culturable embryos was clearly established when a two injection PG schedule was used instead of a single PG injection. This may be a result from the increase in PG response by cows receiving two PG injections compared to those receiving only one PG injection, as synchrony between follicular stimulation and time to estrus is needed to obtain optimal superovulation responses (Elsden et al., 1976). There may be a correlation between high doses of FSH-P and decreases in the quality of embryos obtained due to resulting abnormal hormone levels.

FSH-P treatments using 7.5 and/or 5 mg injections given at 12 h intervals for 8, 6, or 7 injections, were similar to FSH regimes used in earlier superovulation studies (Seidel et al., 1978; Sreenan, 1978). Since recent research suggests that best superovulatory responses are obtained when 28 to 32 mg of FSH-P are administered in a decreasing dose regime (Chupin and Procureur, 1982; Garcia et al., 1982), we tested treatments of 28 and 32 mg given in decreasing dosages. Results showed that embryo final score was significantly affected by the particular superovulation regime utilized. Embryos obtained after superovulation with a decreasing dose regime of 28 mg/8 injections were found to have higher final scores ($P<.05$) than embryos obtained utilizing higher dosages of FSH-P. Also, administration of

lower dose regimes (35 mg/7 injections; 32 mg/8 injections) resulted in embryos with higher final scores when compared to embryos obtained after superovulation with 40 or 50 mg of FSH-P. These results suggest that high doses of FSH may have a negative effect on subsequent embryo development. Elsden et al. (1976) reported higher percentages of abnormal embryos were obtained when high dose regimes of FSH were administered during a superovulation regime. They suggested that the high doses of FSH may cause hormonal imbalances which may affect normal embryo development. Although excellent and good embryos obtained from the high dose regimes appeared normal at the start of culture, it is possible that they may have been adversely affected in a way which was not distinguishable by our subjective assignment of initial quality scores but in a way which affected subsequent development in culture.

Hasler et al. (1981) reported that significantly more ova were obtained from animals that were from 3 to 10 years old than from younger animals less than 3 years of age. In this study, cow age had an effect on the number of unfertilized ova obtained per flush. As cow age increased there was a significant decrease ($P<.05$) in the number of unfertilized embryos recovered. In contrast to these results Hasler et al. (1983) reported that age had no effect on supero-

vulatory response. Little research has been conducted on donor age and its effects on bovine embryo development in culture. Embryos from certain cows on certain days were observed to develop differently. Groups of embryos from a particular cow may have all developed well in culture while another group of embryos from a different cow may have developed poorly although initial quality scores were similar. These results agree with previous reports which noted significant differences in the behavior of the embryos between donor animals and large variations in the viability of eggs of the same stage, put into the same conditions, but coming from different cows (Trounson et al., 1976; Renard and Heyman, 1979).

Various PG schemes were used in an effort to maximize PG response and concomitant superovulation response. A two injection schedule of 25 mg PG resulted in significantly higher response rates (96.55%) compared to a 60% response rate after one 25 mg injection (table 12). These results agree with those obtained by Donaldson (1983) who reported that the number of times the donor was treated with PG was more important than the dose rate. Research in sheep has shown that during luteolysis, PGF2-alpha is released from the uterus as a series of pulses each lasting about 1 h at a frequency of approximately 1 pulse every 6 h. This pulsa-

tile release of PGF2-alpha is also observed in the cow. Such an episodic release of PGF2-alpha from the uterus appears to play a crucial role for the induction of luteolysis. Furthermore, under physiological conditions, a relatively short pulse frequency of PGF2-alpha (every six h) over a period of 25 to 30 h has been found to be a minimal requirement for CL regression in sheep (McCracken et al., 1984). Thus, it appears that increasing the number of injections may better mimic the natural sequences of events which occur during luteolysis with the result of more cows responding to PG treatment. Also, since PG is rapidly metabolized in the lungs, increasing the number of injections may also increase the time in which it has to act on the CL before it is degraded.

Cows exhibiting signs of standing estrus were bred at +12 and +24 h and some cows were bred at estrus. Inseminations were conducted in this manner as previous research with superovulated cattle suggests that two breedings at +12 and +24 h after the onset of estrus results in optimal fertilization rates (Schiewe et al., 1983; West et al., 1984).

Time to heat following the initial PG injection in our study averaged 51.3 h which is somewhat higher than the range of 42 to 48 h reported by previous investigators (Greve, 1980; Donaldson, 1983).

Lugols iodine was a modification of the postflush treatment used by Brand and Drost (1977). They utilized Lugols solution to counter infection or pregnancy from residual embryos. In our study, there was a 1.9 day difference between Lugols solution and PG treatment for the time interval to estrus following the administration of the particular postflush treatment.

From this study, we concluded that SS promoted embryo development in all experiments, while UP alone were unable to promote in vitro development. Secondly, embryos cultured in UP from day 6, 10, or 15 had significantly longer times to the early developmental stages then developmental times recorded for embryos cultured in SS alone. This suggests that the uterine secretions utilized in this study may have also contained substances which may retard early bovine embryo development in vitro. Lastly, uterine secretions obtained from cows on day 15 post insemination may contain a factor or factors which shorten the time of hatching in vitro.

The results clearly show that the UP supplements were unable to support the in vitro development of the early bovine embryo. However, this does not mean that UP do not play important roles in early embryo development in vivo. The exact composition and nature of the uterine secretions

and the UP need to be determined before we can more fully explain why they were unable to promote early embryonic development. Current work in our laboratory suggests that uterine secretions contain immunoglobulins which may become toxic to the early embryo after uterine secretion concentration. Possibly, if immunoglobulins were removed from the uterine secretions, the UP could then be able to support embryonic development.

Also, if superovulated cows contain abnormal uterine secretions, it may be beneficial to collect uterine flushings from normal early pregnant cows. Then, by culturing embryos in concentrated UP from these flushings which are in exact synchrony with the developmental stage of the embryo, one can remove the influences of abnormal uterine secretions and maintain synchrony requirements so that the effects of UP on early embryo development may be better evaluated.

Chapter VI

SUMMARY

A total of 98 superovulations were conducted on 49 dairy cows using various injection schemes of follicle stimulating hormone (FSH; 28 to 50 mg). Estrus was synchronized using 25 mg of Prostaglandin F2-alpha in either one or two injections at various times following the initiation of superovulation. Cows were then bred and flushed non-surgically six days post insemination. Embryos were morphologically evaluated and those meeting quality specifications were cultured. Ham's F-10 was used as the basic media and various uterine protein (UP) supplements were added and tested for their ability to promote early bovine embryo development. Embryo growth was observed every 12 h and development was recorded. A coding system (0 to 5) was used to assess final embryo development achieved *in vitro*.

This study consisted of six separate experiments. Steer serum was used as a protein supplement control in all experiments. Experiment I tested estrogen (E) and progesterone (P) induced UP from previously ovariectomized cows and Ham's F-10 media with no protein supplement added. Experiments II through V tested UP from day 6, 10, or 15 from previously superovulated cows in different concentrations (10%,

1%, .1%, .01%). Experiment VI tested 1% UP from day 6, 10, or 15 combined with 10% SS.

In experiment I only SS exhibited the ability to promote early embryo development with 12 of 23 morulae reaching the hatched blastocyst stage while no development past the early blastocyst stage was observed in the E and P induced UP supplements or in Ham's F-10 media alone. The results were similar in Exp. II through V as 10%, 1%, .1%, and .01% UP concentrations were unable to support development while embryos cultured in 10% SS developed best with mean final scores of 4.16, 4.06, 4.37, and 4.26 for Exp. II to V, respectively.

Results from Exp. VI showed that a mean final score of 4.19 for SS cultured embryos was not significantly different than mean scores of 2.57, 3.28, and 3.34 for 1% day 6, 10, or 15 UP combined with 10% SS, respectively. Embryos cultured in 1% day 6 UP plus 10% SS had longer times to the early stages of development than in the other treatments. This suggests that day 6 UP may contain some substance(s) which may retard early embryo development <u>in vitro</u>. Embryos cultured in 1% day 15 UP plus 10% SS had stage of development times similar to controls up to the expanded blastocyst stage, but the time to the hatching and hatched blastocyst stages were significantly reduced ($P<.05$) compared to times

of development observed for control embryos. This suggests that day 15 UP may contain a factor(s) which may shorten the time of embryo hatching in vitro.

Initially, it was hypothesized that supplementation of culture media with UP would enhance embryo development, but this did not occur. There are many possible factors which may have contributed to the poor embryo development in UP. Firstly, the sources of UP were cows which may have hormonal imbalances. The UP utilized in Exp. I were collected from ovariectomized, hormonally treated cows while the UP utilized in Exp. II to VI were obtained by flushing previously superovulated cows which have elavated levels of estrogens and progesterone during the period of superovulation and in the luteal phase of the estrous cycle which follows. Since the exact nature and composition of these proteins were not determined, they may vary drastically from the UP contained in the normal early pregnant uterus.

Secondly, inhibitory substances as well as UP may have been concentrated. Upon concentration, substances such as lysozymes and immunoglobulins may become detrimental to early embryo development. Lastly, the synchrony requirements between the embryo and the uterine secretions may have not been met. Previous research suggests that uterine secretion components during the period of early pregnancy are cons-

tantly changing and that the embryo requires a stringent synchrony between its developmental stage and the uterine environment in which it is placed. Since UP used in this study were collected at different days, they may contain substances which could be harmful to early embryo development or which may be lacking in some essential nutrients.

Cow response to PG injection was significantly affected ($P<.01$) by PG schedule. A 96.55% response rate was obtained when two injections totaling 25 mg were given compared to a 60% response rate after one 25 mg injection. Maximum temperature at breeding was positively related to the total number of unfertilized ova recovered per flush, while maximum temperature on the flush day had a significant effect ($P<.055$) on the number of culturable embryos obtained per flush. Furthermore, day of the cycle which FSH was started was found to influence the number of unfertilized ova (UFO) obtained per flush. Cows initiating a superovulation regime on day 13 had significantly higher ($P<.05$) numbers of UFO than cows started superovulation on days 9 to 12. Final developmental scores which embryos obtained in culture were significantly affected ($P<.01$) by the particular superovulation regime utilized.

From this study we conclude that SS promoted early embryo development in all experiments, while UP alone were

unable to promote *in vitro* development. Secondly, although it was found that embryos cultured in 1% UP from day 6, 10, or 15 combined with 10% SS had significantly ($P<.01$) greater development than embryos cultured in UP alone, it appeared that the UP supplements used may also contain certain substances which are detrimental to early embryo development. Lastly, it was concluded that the 1% day 15 UP plus 10% SS protein supplement may shorten the time of embryo hatching *in vitro*.

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APPENDIX

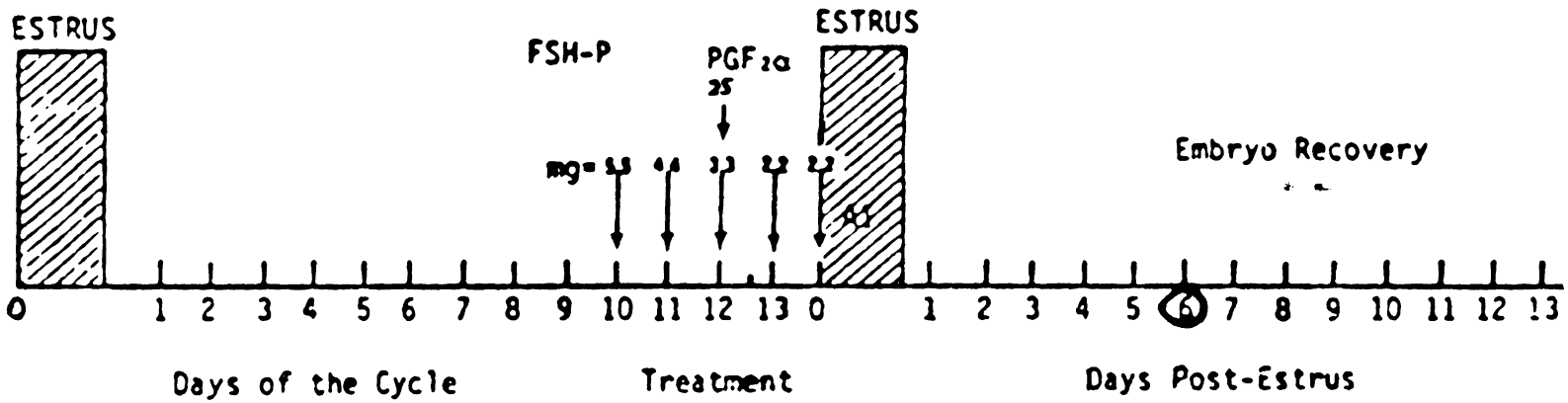

FIGURE 1. DIAGRAMIC ILLUSTRATION OF A SUPEROVULATION REGIME AND EMBRYO RECOVERY.

TABLE 1. FSH-P INJECTION SCHEMES

Treatment #	(n)	Total Dose	Day of Superovulation 1	2	3	4
1	19	40[a,b]	5:5	5:5	5:5	5:5
2	4	50	7.5:7.5	5:5	5:5	5:5
3	8	30	5:5	5:5	5:5	-
4	44	35	5:5	5:5	5:5	5:0
5	4	28	5:5	4:4	3:3	2:2
6	19	32	6:6	5:5	3:3	2:2

[a]mg FSH-P

[b]FSH-P started either AM or PM.

TABLE 2. PROSTAGLANDIN F-2 ALPHA INJECTION SCHEMES

Treatment #	(n)	Total Dose	# Injections	# Hours After FSH-P[a]	# Response
1	2	25[b]	1	60	100.0[c]
2	34	25	1	72	61.8[c,e]
3	4	25	1	84	0.0[d]
4	2	25	2	48:60	100.0[c]
5	49	25	2	60:72	95.9[c,f]
6	7	25	2	72:84	100.0[c]

[a]Time of administration after initial FSH-P injection.

[b]PG started AM or PM.

[c,d;c,f]Numbers with different superscripts differ at $P<.01$.

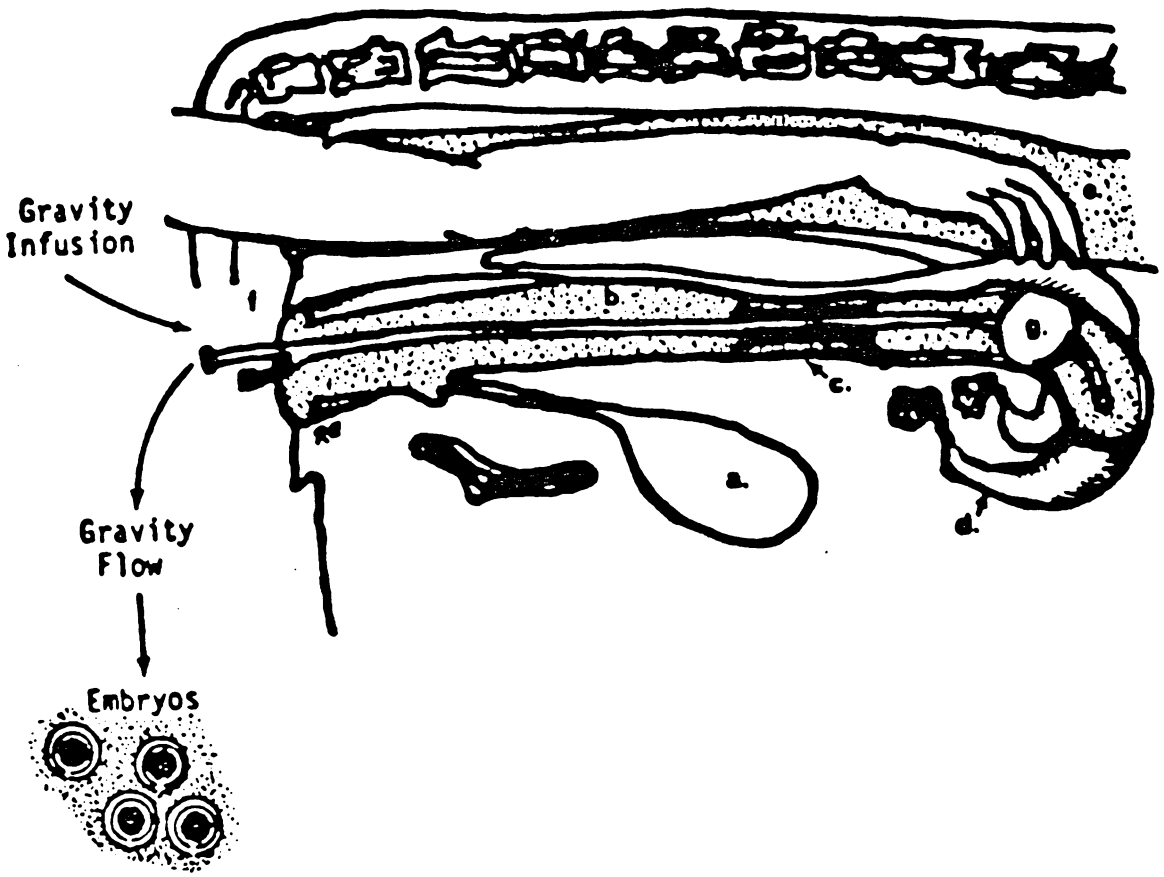

Figure 2. A gravity flow non-surgical embryo collection system for cattle: (a) bladder, (b) vagina, (c) cervix, (d) uterine horn, (e) rectum, (f) two-way Foley catheter, and (g) inflated cuff.

TABLE 3. ANALYSIS OF VARIANCE OF FINAL DEVELOPMENTAL SCORE FOR EMBRYOS CULTURED IN 10% ESTROGEN AND 10% PROGESTERONE INDUCED UTERINE PROTEINS, HAM'S F-10 ALONE, AND 10% STEER SERUM

Source	df	Mean Square
Treatment[a]	3	64.2**
Quality	1	3.0**
Cowflush	11	1.5**
Error	59	0.7

**P<.01

[a]Treatment was tested by cowflush.

TABLE 4. STAGE OF EMBRYO DEVELOPMENT AFTER CULTURE IN 10% STEER SERUM (SS), HAM'S F-10 ALONE, 10% PROGESTERONE (P), OR 10% ESTROGEN (E) INDUCED UTERINE PROTEINS

Treatment	SS		P		E		Ham's F-10	
Stage of Development	n	(%)	n	(%)	n	(%)	n	(%)
Morula	23	(100)	20	(100)	20	(100)	12	(100)
Early blastocyst	22	(96)[a]	1	(05)[b]	--		--	
Blastocyst	20	(87)	--		--		--	
Expanded blastocyst	15	(65)	--		--		--	
Hatching blastocyst	13	(57)	--		--		--	
Hatched blastocyst	11	(48)	--		--		--	

[a,b]Numbers with different superscripts differ at $P<.01$.

TABLE 5. LEAST SQUARES MEANS (± SE) FOR DEVELOPMENTAL TIME TO EACH STAGE FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS), HAM'S F-10 ALONE, 10% PROGESTERONE (P), OR 10% ESTROGEN (E) INDUCED UTERINE PROTEINS

Treatment	SS	Ham's F-10	P	E
Stage of Development	Time (hours)			
Morula	- (23)[a]	- (12)	- (20)	- (20)
Early blastocyst	38.7 ± 3.1(22)	-	38.7 ± 19.4(1)	-
Blastocyst	58.3 ± 3.9(20)	-	-	-
Expanded blastocyst	67.2 ± 3.4(15)	-	-	-
Hatching blastocyst	77.1 ± 4.1(13)	-	-	-
Hatched blastocyst	96.0 ± 2.1(11)	-	-	-

[a]number at each stage

TABLE 6. ANALYSIS OF VARIANCE OF FINAL DEVELOPMENTAL SCORE FOR EMBRYOS CULTURED IN 10% DAY 6, 10, AND 15 UTERINE PROTEINS AND 10% STEER SERUM

Source	df	Mean Square
Treatment[a]	3	40.2513**
Quality	1	0.0004
Cowflush	7	0.3912
Error	28	0.2772

**P<.01

[a]Treatment was tested by cowflush.

TABLE 7. STAGE OF EMBRYO DEVELOPMENT AFTER CULTURE IN 10% STEER SERUM (SS), OR 10% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS		Day 6		Day 10		Day 15	
Stage of Development	n	(%)	n	(%)	n	(%)	n	(%)
Morula	10	(100)	10	(100)	10	(100)	10	(100)
Early blastocyst	10	(100)[a]	--		1	(10)[b]	--	
Blastocyst	10	(100)	--		--		--	
Expanded blastocyst	9	(90)	--		--		--	
Hatching blastocyst	8	(80)	--		--		--	
Hatched blastocyst	6	(60)	--		--		--	

[a,b]Numbers with different superscripts differ at $P<.01$.

TABLE 8. LEAST SQUARES MEANS (± SE) FOR DEVELOPMENTAL TIME TO EACH STAGE FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS), OR 10% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS	Day 6 + SS	Day 10 + SS	Day 15 + SS
Stage of Development	Time (hours)			
Morula	- (10)[a]	- (10)	- (10)	- (10)
Early blastocyst	33.7 ± 3.9(10)	-	25.7 ± 13.2(1)	-
Blastocyst	46.6 ± 5.4(10)	-	-	-
Expanded blastocyst	62.9 ± 7.0(9)	-	-	-
Hatching blastocyst	80.7 ± 5.3(8)	-	-	-
Hatched blastocyst	108.0 ± 7.2(6)	-	-	-

[a]number at each stage

TABLE 9. ANALYSIS OF VARIANCE OF FINAL DEVELOPMENTAL SCORE FOR EMBRYOS CULTURED IN 1% DAY 6, 10, AND 15 UTERINE PROTEINS AND 10% STEER SERUM

Source	df	Mean Square
Treatment[a]	3	19.21**
Quality	1	.75
Cowflush	10	.85
Error	25	.46

**P<.01

[a]Treatment was tested by cowflush.

TABLE 10. STAGE OF EMBRYO DEVELOPMENT AFTER CULTURE IN 10% STEER SERUM (SS), OR 1% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS		Day 6		Day 10		Day 15	
Stage of Development	n	(%)	n	(%)	n	(%)	n	(%)
Morula	10	(100)	10	(100)	10	(100)	10	(100)
Early blastocyst	10	(100)[a]	--		1	(10)[b]	1	(10)[b]
Blastocyst	9	(90)	--		--		--	
Expanded blastocyst	8	(80)	--		--		--	
Hatching blastocyst	8	(80)	--		--		--	
Hatched blastocyst	8	(80)	--		--		--	

[a,b]Numbers with different superscripts differ at $P<.01$.

TABLE 11. LEAST SQUARES MEANS (± SE) FOR DEVELOPMENTAL TIME TO EACH STAGE FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS), OR 1% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS	Day 6 + SS	Day 10 + SS	Day 15 + SS
Stage of Development	Time (hours)			
Morula	- (10)[a]	- (10)	- (10)	- (10)
Early blastocyst	27.7 ± 4.1(10)	-	39.7 ± 17.5(1)	27.7 ± 17.5(1)
Blastocyst	41.3 ± 2.9(9)	-	-	-
Expanded blastocyst	60.0 ± 0(8)	-	-	-
Hatching blastocyst	83.6 ± 3.1(8)	-	-	-
Hatched blastocyst	108.0 ± 6.2(8)	-	-	-

[a]number at each stage.

TABLE 12. ANALYSIS OF VARIANCE OF FINAL DEVELOPMENTAL SCORE FOR EMBRYOS CULTURED IN .1% DAY 6, 10, AND 15 UTERINE PROTEINS AND 10% STEER SERUM

Source	df	Mean Square
Treatment[a]	3	32.27**
Quality	1	.02
Cowflush	10	.28
Error	25	.07

**P<.01

[a]Treatment was tested by cowflush.

TABLE 13. STAGE OF EMBRYO DEVELOPMENT AFTER CULTURE IN 10% STEER SERUM (SS), OR .1% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS		Day 6		Day 10		Day 15	
Stage of Development	n	(%)	n	(%)	n	(%)	n	(%)
Morula	10	(100)	10	(100)	10	(100)	10	(100)
Early blastocyst	10	(100)[a]	1	(10)[b]	1	(10)[b]	1	(10)[b]
Blastocyst	10	(100)		--		--		--
Expanded blastocyst	10	(100)		--		--		--
Hatching blastocyst	7	(70)		--		--		--
Hatched blastocyst	6	(60)		--		--		--

[a,b]Numbers with different superscripts differ at $P<.01$.

TABLE 14. LEAST SQUARES MEANS (± SE) FOR DEVELOPMENTAL TIME TO EACH STAGE FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS), OR .1% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS	Day 6 + SS	Day 10 + SS	Day 15 + SS
Stage of Development	Time (hours)			
Morula	- (10)[a]	- (10)	- (10)	- (10)
Early blastocyst	20.4[b] ± 3.6(10)	52.3[c] ± 14.5(1)	52.3[c] ± 1.45(1)	27.7 ± 17.5(1)
Blastocyst	38.3 ± 4.4(10)	-	-	-
Expanded blastocyst	63.0 ± 5.6(10)	-	-	-
Hatching blastocyst	89.0 ± 9.9(7)	-	-	-
Hatched blastocyst	108.0 ± 6.2(6)	-	-	-

[a]number at each stage

[b,c]Numbers with different superscripts differ at $P<.01$.

TABLE 15. ANALYSIS OF VARIANCE OF FINAL DEVELOPMENTAL SCORE FOR EMBRYOS CULTURED IN .01% DAY 6, 10, AND 15 UTERINE PROTEINS AND 10% STEER SERUM

Source	df	Mean Square
Treatment[a]	3	34.17**
Quality	1	.01
Cowflush	10	1.41**
Error	25	.24

**P<.01

[a]Treatment was tested by cowflush.

TABLE 16. STAGE OF EMBRYO DEVELOPMENT AFTER CULTURE IN 10% STEER SERUM (SS), OR .01% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS		Day 6		Day 10		Day 15	
Stage of Development	n	(%)	n	(%)	n	(%)	n	(%)
Morula	10	(100)	10	(100)	10	(100)	10	(100)
Early blastocyst	10	(100)[a]	4	(40)[b]	3	(30)[b]	3	(30)[b]
Blastocyst	10	(100)	--		--		--	
Expanded blastocyst	8	(80)	--		--		--	
Hatching blastocyst	8	(80)	--		--		--	
Hatched blastocyst	6	(60)	--		--		--	

[a,b]Number with superscripts differ at $P<.01$.

TABLE 17. LEAST SQUARES MEANS (± SE) FOR DEVELOPMENTAL TIME TO EACH STAGE FOR EMBRYOS CULTURED IN 10% STEER SERUM (SS), OR .01% DAY 6, 10, AND 15 UTERINE PROTEINS

Treatment	SS	Day 6 + SS	Day 10 + SS	Day 15 + SS
Stage of Development	Time (hours)			
Morula	- (10)[a]	- (10)	- (10)	- (10)
Early blastocyst	20.3 ± 2.5(10)[b]	40.7 ± 4.1(4)[c]	44.8 ± 5.2 (3)[c]	37.2 ± 4.9(3)[c]
Blastocyst	36.5 ± 2.3(10)	-	-	-
Expanded blastocyst	48.7 ± 2.7(8)	-	-	-
Hatching blastocyst	76.0 ± 0(8)	-	-	-
Hatched blastocyst	96.0 ± 6.2(6)	-	-	-

[a]number at each stage

[b,c]Numbers with different superscripts differ at P<.01.

TABLE 18. ANALYSIS OF VARIANCE OF FINAL DEVELOPMENTAL SCORE FOR EMBRYOS CULTURED IN 10% STEER SERUM OR 1% DAY 6, 10, AND 15 UTERINE PROTEINS COMBINED WITH 10% STEER SERUM

Source	df	Mean Square
Treatment[a]	3	9.3
Quality	1	.2
Cowflush	12	7.2**
Error	71	1.8

**P<.01

[a]Treatment was tested by cowflush.

TABLE 19. STAGE OF EMBRYO DEVELOPMENT FOR FAIR AND POOR QUALITY EMBRYOS CULTURED IN STEER SERUM

Stage of Development	Fair Embryos		Poor Embryos	
	n	%	n	%
Morula	67	(100)	56	(100)
Early blastocysts	37	(55)	4	(7)
Blastocysts	30	(45)	4	(7)
Expanded blastocysts	22	(33)	3	(5)
Hatching blastocysts	14	(21)	2	(4)
Hatched blastocysts	8	(12)	2	(4)

TABLE 20. MEAN SQUARES FOR FACTORS AFFECTING EMBRYO QUALITY AND RESPONSE TO PG

Source	PG Response	df	Hours to Heat	df	# Embryos Per Flush	df	# Culturable Embryos	df	#UFO[a]	df	Final Score	df
Max T^{O}/flush day	.26	1	.02	1	43	1	73.06[b]	1	9	1	7.7	1
Max T^{O}/breeding	.05	1	98.24	1	14	1	.03	1	100*	1	.3	1
Day FSH started	.04	4	375.69	4	54	4	4.50	4	73*	4	5.5	4
FSH scheme	.20	5	313.90	5	57	5	26.57	5	37	5	9.7**	5
PG scheme	.53**	5	293.44	4	34	5	29.64	5	18	5	2.2	5
Age	.08	1	297.62	1	62	1	3.81	1	95*	1	6.2	1
Cow	.07	1	260.77	1	73	1	3.75	1	104*	1	6.4	1
FSH scheme*PG Scheme[c]	—	[d]	—	[d]	—	[d]	93.99**	3	—	[d]	—	[d]
Residual	.10	78	162.76	61	57	72	19.18	69	22	69	2.7	36

**P<.01
*P<.05

[c]interaction between FSH scheme and PG scheme

[d]interaction not significant (P>.05)

TABLE 21. LEAST SQUARES MEANS (+ SE) FOR NUMBER OF CULTURABLE EMBRYOS OBTAINED FOLLOWING DIFFERENT COMBINATIONS OF FSH-P AND PG SCHEDULES

PG Schedule[b,c]	FSH-P Schedule[a]					
	1	2	3	4	5	6
1	-	-	2.43 ± 3.37[g]	-	-	-
2	2.86 ± 2.08[g]	4.62 ± 2.59[d,g]	1.64 ± 4.81[g]	1.34 ± 1.82[g]	-	19.09 ± 4.62[f]
3	-	-	-1.75 ± 2.64[e,g]	-	-	-
4	-	-	4.20 ± 4.53[g]	9.13 ± 4.59[d]	-	-
5	-	-	-	4.79 ± 1.32[g]	-	6.39 ± 1.82[d,g]
6	-	-	-	5.55 ± 3.25[g]	4.48 ± 2.42[g]	-

[a]FSH-P schedule 1 = 40 mg, 8 injections; 2 = 50 mg, 8 injections; 3 = 30 mg, 6 injections; 4 = 35 mg, 7 injections; 5 = 28 mg, 8 injections (5:5, 4:4, 3:3, 2:2); 6 = 32 mg, 8 injections (6:6, 5:5, 3:3, 2:2).

[b]25 mg total

[c]PG schedule 1 = 2 injection 60 h after 1st FSH-P injection; 2 = 1 injection 72 h after 1st FSH-P injection; 3 = 2 injection 84 h after 1st FSH-P injection; 4 = 7 injections, 48 and 60 h after 1st FSH-P injection; 5 = 2 injections, 60 and 72 h after 1st FSH-P injection; 6 = 2 injections 72 and 84 h after 1st FSH-P injection.

[d,e;f,g]Numbers with different superscripts differ at P<.05.

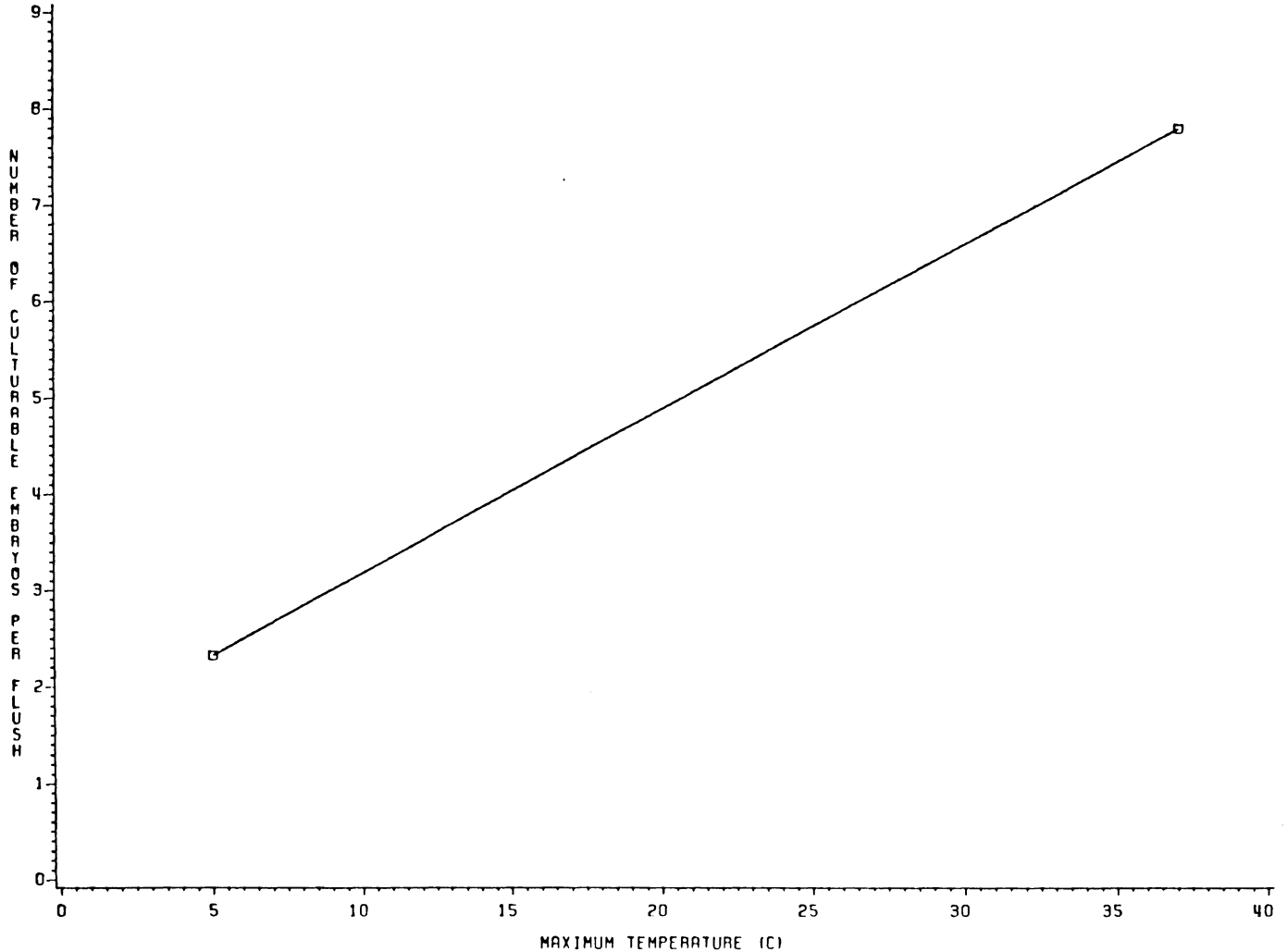

FIGURE 3. EFFECT OF MAXIMUM TEMPERATURE AT FLUSHING ON TOTAL NUMBER OF CULTURABLE EMBRYOS RECOVERED.

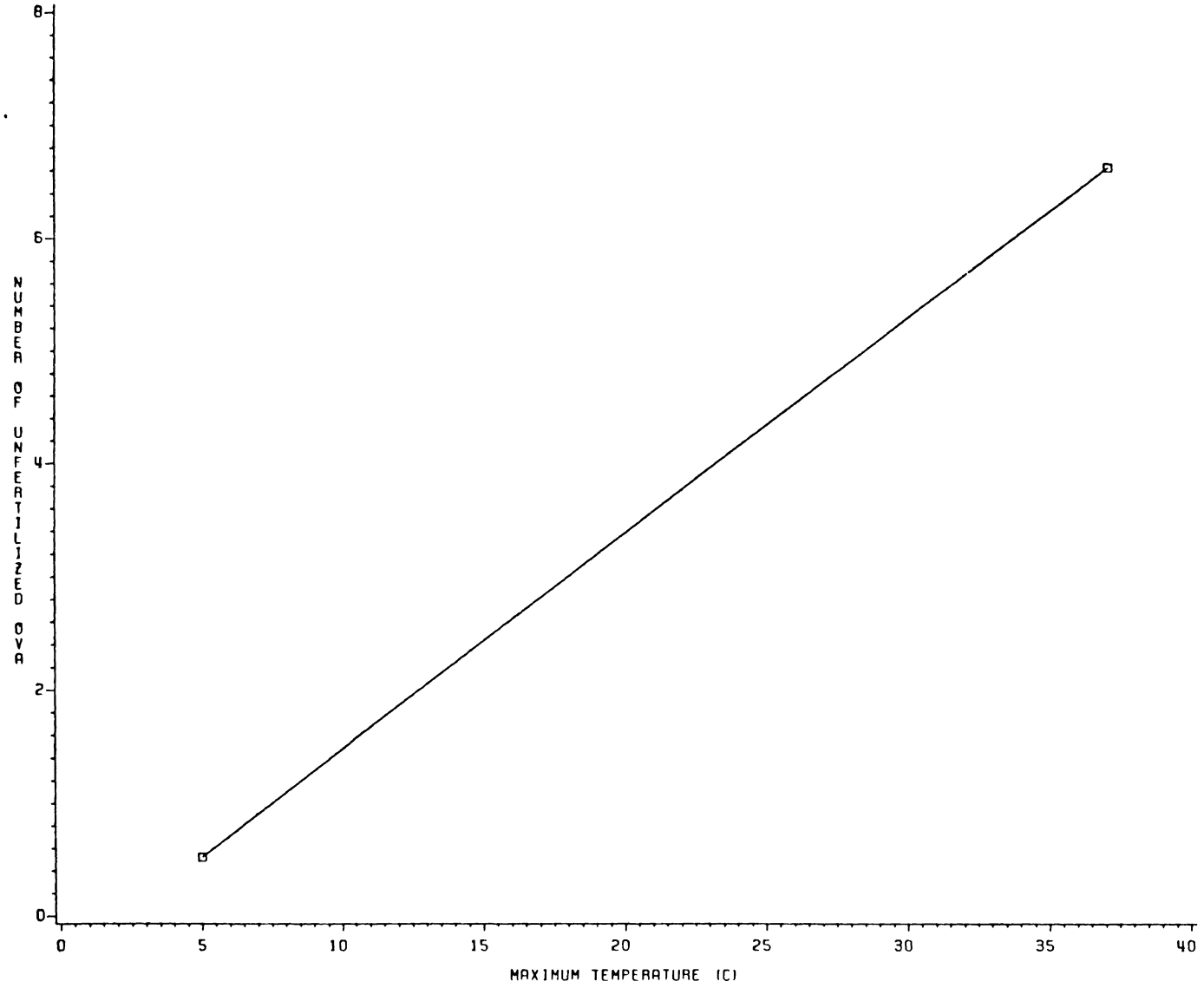

FIGURE 4. EFFECT OF MAXIMUM TEMPERATURE AT BREEDING ON TOTAL NUMBER OF UNFERTILIZED OVA RECOVERED.

TABLE 22. LEAST SQUARES MEANS (±SE) FOR NUMBER OF UNFERTILIZED OVA OBTAINED PER FLUSH PER DAY OF FSH INITIATION

Day FSH Initiated	n	Number of Unfertilized Ova Per Flush
9	23	.97 ± 1.73[a]
10	25	-1.38 ± 1.57[a]
11	20	-1.43 ± 1.99[a]
12	16	-2.04 ± 2.28[a]
13	4	7.14 ± 2.77[b]

[a,b]Numbers with different superscripts differ at $P<.05$.

TABLE 23. LEAST SQUARES MEANS (±SE) FOR FINAL SCORES FOLLOWING UTILIZATION OF VARIOUS FSH-P SCHEDULES

FSH Schedule	n	Final Score
40[a]	11	-1.7 ± 1.3[b]
50	3	-2.4 ± 1.5[b]
30	4	1.0 ± 1.1[b,d]
35	27	1.7 ± 1.1[c,f]
28	4	6.2 ± 1.7[c,e,g]
32	6	2.6 ± 1.4[c]

[a]total dose (mg)

[b,c;d,e;f,g]Numbers with different superscripts differ at $P<.05$.

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