SWINE EMBRYO DEVELOPMENT IN VITRO

by

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CHAPTER I

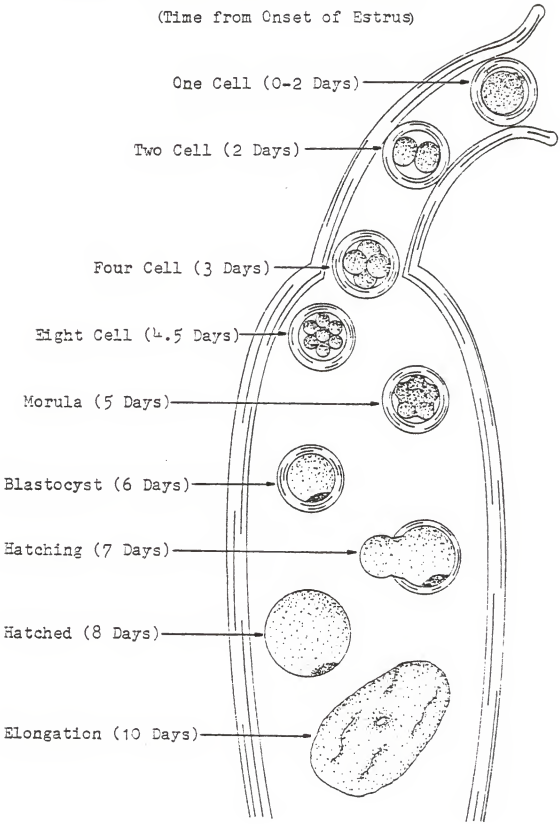
INTRODUCTION

At fertilization mammalian eggs enter a period of cleavage, which, in the pig, lasts about 6 days (Hunter, 1974). At 3 to 4 days of gestation, compaction of the 8 to 16 cell embryo occurs giving the embryo a smooth, rounded appearance typical of the morula stage. Approximately 2 days later fluid accumulates between the blastomeres and eventually forms a central cavity (Heuser and Streeter, 1929). The embryo is now a blastocyst and continues to increase in size by cell division and fluid accumulation. The zona pellucide is shed at 6 to 7 days of gestation. The blastocyst continues its free existance in the uterus until it begins attaching to the uterine endometrium at 10 to 12 days (these stages are depicted in Figure 1.). Prior to attachment, nutrients needed for growth and development are acquired either from uterine secretions present in the lumen, or from stored reserves present in the egg at ovulation. The blastocyst grows rapidly and the trophoblast elongates and begins differentiating into the placenta. Differentiation of the inner cell mass begins at 11 days and by 15 days most of the organ systems have formed. The embryo is recognizable as a pig by about 35 days of gestation marking the beginning of the fetal period (Marrable, 1971).

By this stage of pregnancy many of the eggs originally ovulated have been lost. Hanly (1961) found a reduction in embryo number of 30 to 40% by 25 days of pregnancy. Perry and Rowlands (1962) found a 34.8% decrease in the number of potential embryos by 25 to 40 days of gestation.

FIGURE 1. DEVELOPMENT OF PIG EMBRYOS

(Time from Onset of Estrus)



Other reports (Lerner et. al., 1957; Gossett and Sorenson, 1959) show losses near 30% by 25 days. Perry and Rowlands (1962), by examining earlier stages of pregnancy, found the greatest portion of this loss (24.4%) had occured by 13 to 18 days of gestation and only 4.5% of the missing eggs could be attributed to fertilization failure. Since the pig is an economically important species, and embryonic losses decrease overall reproductive efficiency, any improvement in embryonic survival would be useful. Investigations of factors affecting the survival of embryos during this period have been inconclusive, partly due to a lack of available information on the requirements for normal growth and development of swine embryos. A necessary prerequisite to investigating these requirements is a system which allows separation of the embryo from the uterine environment. Culture, in vitro, provides such a system.

Most of the information available on factors required for development of the early mammalian embryo has been obtained from embryos of laboratory animals including the mouse, rabbit, and rat. Possible requirements for the economically important farm animals, such as the pig, can be extrapolated from this information. Indeed, morphological development through these stages is very similar for most mammalian species although the time-table for development varies (Brinster, 1974). For example, the mouse embryo forms a blastocyst at 3 to 4 days of pregnancy and implants a day later. In contrast, the rabbit embryo forms a blastocyst at 3 to 4 days of pregnancy but does not attach for another 3 to 4 days. Furthermore, at implantation the mouse embryo weighs no more than the egg at ovulation or about 25mug whereas the rabbit embryo

weighs approximately 100mμg at ovulation and approximately 6000mμg at implantation (Brinster, 1972). Thus, the larger and later implanting rabbit blastocyst must be more active biosynthetically before implantation than the mouse and consequently has more complex requirements during the preimplantation period. Realizing that differences exist between species, apparent similarities may be evaluated. Comparisons between laboratory animals, which have been extensively studied, and farm animals may provide insight into the requirements of the latter species, however, the final answer must come from experiments with the species in question.

CHAPTER II

LITERATURE REVIEW

Culture Media

Physiological salines used for in vitro experiments with mammalian organs are based on the components of blood serum (Table 1.). Locke (1901) first attempted to replicate the salt components of mammalian serum in his experiments with the heart. He devised a balanced salt solution consisting of NaCl, KCl, $CaCl_2$, and $NaHCO_3$ and found the addition of 0.1% glucose also increased survival time of the heart in vitro. Tyrode (1910) modified Lock's solution by adding phosphate and increasing the bicarbonate content to improve its buffering capacity. Krebs and Henseleit (1932) further increased the bicarbonate content of this medium and equilibrated it with a 5% CO_2 gas atmosphere to maintain pH at 7.4. They also added $MgSO_4$ to the medium and this addition proved to be advantageous for organs other than heart. Physiological salines are often referred to as Ringer solutions after Sydney Ringer who first investigated inorganic salt requirements for in vitro studies of the frog heart (Ringer, 1882) and consequently the solution used by Krebs and Henseleit (1932) is more commonly known as "Krebs-Ringer solution" or "Krebs-Ringer bicarbonate" (KRB).

Physiological salines are only effective for maintaining organs and tissues a short time in vitro. Interest in investigating the long term behavior of cells separated from the animal gave rise to techniques

Table 1. CATION CONCENTRATIONS IN mM OF SOME PHYSIOLOGICAL SOLUTIONS[a]

	mM			
Solution	Na	K	Ca	Mg
Locke (1901)	156.4	5.6	2.1	0
Tyrode (1910)	149.3	2.7	1.8	1.1
Krebs and Henseleit (1932)	141.8	5.9	2.5	1.2
Pig Serum	153	5.3	2.8	1.2
Sheep Serum	143	6.5	2.9	1.0
Cow Serum	143	3.9	2.2	0.9

[a]Burton, 1975.

which allow not only survival, but growth, for extended periods of time *in vitro*. Early culture methods utilized lymph (Harrison, 1908), blood plasma or serum (Burrows, 1910) and embryo extracts (Carrel, 1912) as supporting media. Early workers recognized the importance of controlling and defining the environment in which cells were grown *in vitro* (Lewis and Lewis, 1911; 1912). Their experiments attempted to substitute physiological salt solutions for more complex components. More information soon became available on factors in serum, such as vitamins, amino acids, lipids, trace metals, proteins, and others. With these developments defined culture media became more complex as workers attempted to meet the requirements of cells in culture. One of the earliest of these media was developed by Morgan *et. al.*, (1950). Referred to as "Tissue Culture Medium 199" (TCM 199), it contains a large variety of the above factors along with a physiological salt solution. Eagle (1959) and Ham (1963), and many others have also developed complex chemically defined media which attempt to replace the serum component. However, today only a few established cell lines can be grown *in vitro* in the absence of an undefined biological component.

Progess in the development of culture systems which allow the development of mammalian embryos *in vitro* has closely paralleled advancements in tissue culture techniques. Early studies on the development of rabbit (Lewis and Gregory, 1929) and mouse (Lewis and Wright, 1935) embryos *in vitro* used media consisting of blood plasma and embryo extract. As culture techniques developed and information on the metabolism of the embryo accumulated, it was discovered that at least certain stages of

embryos of some species could be cultivated in completely defined media based on the components of media developed for organ and tissue culture (Whitten and Biggers, 1968). Subsequently, considerable information was obtained by testing the performance of embryos in tissue culture media of specified composition.

Culture Methods

Microdrop. The microdrop method was first described by Brinster (1963). In this system 25 to 100µl of culture medium is placed beneath a layer of paraffin or mineral oil in a petri dish. The oil is equilibrated with culture medium, gassed and sterilized prior to culture. The oil provides no barrier to CO_2, however, and when using a bicarbonate buffered medium the culture vessel must be kept in a controlled atmosphere (Biggers et. al., 1971). Variation in the ability of different batches of oil to maintain stability of the drops of medium is a disadvantage of this system. Also, some medium components are soluble in oil which might result in their dilution.

Dish. In one variation of the dish culture system a watch glass containing culture medium is placed on a sponge or glass wool which is moistened to keep the atmosphere inside the dish saturated with water. The dish is then placed in a gassed culture chamber. Alternatively, a layer of mineral or paraffin oil may be placed over the medium (Biggers et. al., 1971). It is possible to grow many embryos in one culture dish (Biggers et. al., 1971) and also to grow later stages of embryos in a similar system (New, 1971).

Petri dishes with or without a collagen coat have been used to grow embryos over the implantation period (McLaren and Hensleigh, 1975). The dishes contain approximately 2ml of medium and are incubated in a controlled atmosphere saturated with water. This system has been used by Hsu (1974) to culture mouse embryos to the beating heart stage.

Test tube. Test tubes may be gassed and stoppered after the embryos and medium are added. Each tube then maintains its own gas atmosphere. Alternatively the tube may be left unstoppered and in a gassed culture chamber. By filling the tubes completely with gassed medium the gassing process may be avoided, although filling the tubes half full gives a flat fluid surface when the tube is tipped horizontally making observations during culture possible under a steriomicroscope (Davis, 1977).

Whitten (1956) and Hammond (1949) have successfully cultured early cleavage stage mouse embryos in test tube cultures. New et. al., (1973) have used culture tubes to grow post-implantation rat fetuses in vitro. The tube may be rotated to provide a system with flowing medium although Brinster (1969) found no advantage to rotating the tubes when culturing cleavage stage mouse embryos.

Circulator. The circulator was designed by New (1967) for the culture of post-implantation rat embryos. Daniel (1970) modified the circulator's culture chamber to make it suitable for growing post-implantation rabbit embryos. This system uses a constant flow of gas to continually circulate culture medium over the embryo. The constant flow of gas through the medium ensures proper equilibration and embryos may be

observed continuously during culture.

Embryo Development in vitro

Mouse. Whitten and Biggers (1968) have shown that mouse embryos will develop from one-cell to the blastocyst stage in a semi-defined medium based on a KRB solution. Whittingham (1971) also reported that development of the one-cell mouse embryo to the blastocyst stage could be achieved in a medium based on Tyrode's solution. It appears that cleavage stage mouse embryos are able to tolerate a fairly broad range of ionic concentrations. Information reported by Wales (1970) on the concentrations of ions supporting development, confirms this observation. Potassium and calcium were the only ions necessary for development of two-cell embryos and optimum development occurs within a range that includes concentrations of these ions present in most physiological salines for mammals. Only in the absence of magnesium and phosphate was development retarded and sulphate omission had no effect.

Optimum osmolarity for development of early mouse embryos in vitro is approximately 0.275 osmoles (Brinster, 1965a; Whitten, 1971) but, as with ion concentrations, development occurs over a wide range of osmolarities.

Bicarbonate ion is necessary for development of early mouse embryos (Whittingham, 1971) possibly because all cleavage stages fix CO_2 (Wales et. al., 1969; Graves and Biggers, 1979). Development, again, occurs over a wide range of concentrations (Brinster, 1965a). The concentration of bicarbonate in the medium and the concentration of CO_2 in the atmosphere control the pH of the medium. In a KRB of a 5% CO_2 atmosphere

and 24.8mM $NaHCO_3$ result in a pH of 7.4 (Krebs and Henseleit, 1932). Eight-cell mouse embryos have been shown to develop in a pH range of 6.9 to 7.7 (Whitten, 1956) and two-cell embryos between 5.87 and 7.78 (Brinster, 1965a).

Energy requirements for the early mouse embryo have been shown to be very specific. Pyruvate or oxaloacetate must be present in the medium for development of the one-cell ovum (Biggers et. al., 1967). The two-cell egg will develop when lactate, oxaloacetate, pyruvate, or phosphoenol pyruvate are present. After the eight-cell stage the embryos' requirements are much less specific and cleavage will occur in the presence of glucose or several other energy substrates. Development of the two-cell ovum is enhanced when both lactate and pyruvate are present (Brinster, 1965b) and for this reason most media used for growing early stages contain both of these energy sources and glucose.

Cholewa and Whitten (1970) have shown that two-cell mouse eggs will develop to the blastocyst stage in the absence of a fixed nitrogen source. However, bovine serum albumin (BSA) is commonly added to culture media and possibly aids development by stabilizing the cell membranes, chelating toxic metal ions, or in other undefined ways (Whittingham, 1971).

It appears that a physiological saline, such as KRB, modified by the additions of pyruvate, lactate, and BSA, adequately supports development of one-cell mouse eggs to the blastocyst stage. However, after blastocysts hatch from the zona pellucida they cease development in a defined medium, although possibly remaining viable but dormant for

several days (Gwatkin, 1966a, b). For continued development the addition of serum is necessary (Cole and Paul, 1965). Using Eagle's minimum essential medium (MEM) supplemented with serum Hsu et. al., (1974) has attained complete development of mouse embryos from the two-cell to the 10-somite stage. Some embryos with beating hearts and differentiated red blood cells were observed. The embryos were cultured from the two-cell to blastocyst stage in a KRB medium with pyruvate, lactate, and BSA. Blastocysts were then transfered to MEM plus pyruvate and 10% heat inactivated calf serum where they underwent hatching. After the blastocysts attached to a collagen coated dish the medium was changed to MEM plus 20% heat inactivated human cord serum. Although only 1 to 3% of the embryos were able to progress this far in culture, development was apparently normal as determined microscopically.

Spindle and Pederson (1973) looked more specifically into the fixed nitrogen requirements for in vitro hatching, attachment, and outgrowth. Using Eagle's Basal Medium (BME) with 1% dialyzed fetal calf serum they observed a decrease in the incidence of hatching when histidine, methionine, threonine, tryptophane, tyrosine, or valine were omitted from BME. Along with these, cystine and lysine were shown to be required for attachment, and all essential amino acids except isoleucine were necessary for trophoblast outgrowth. Earlier work by Gwatkin (1966a, b) indicated less stringent requirements for leucine, methionine, tyrosine, valine and glutamine, but more stringent requirements for arginine, leucine, and histidine. Part of this variation may be attributed to the different stages at which the embryos were placed

in culture (Spindle and Pederson, 1973). These results indicate that mouse blastocysts, growing in vitro, gradually become dependent on a specific exogenous fixed nitrogen source and other factors present in serum.

Rabbit. The development of the rabbit embryo from the one-cell to hatched blastocyst stage has also been achieved in vitro in a chemically defined medium (Kane, 1972), although specific requirements for development during this period are poorly defined. Requirements for specific ionic concentrations and osmolarity have not been determined, although concentrations in oviducal fluid have been found to be similar to those present in KRB (Brinster, 1969). Similarly, hydrogen ion requirements have not been determined for the rabbit embryo, but are probably similar to that present in most standard media used for mammalian embryo culture (Brinster, 1969). Kane (1975) showed that one-cell rabbit eggs form morulae when grown in a medium with a non-bicarbonate buffer. Blastocyst formation, however, requires at least 0.31mM HCO_3 in the medium.

Requirements for energy substrates have been investigated by Kane (1972) who cultured one-cell rabbit ova to the hatched blastocyst stage in Ham's F10 medium plus 1.5% BSA. The basal medium alone supported an 81% hatching rate and supplementation with pyruvate, glucose, or pyruvate and glucose did not improve results. He concluded that rabbit ova may be able to utilize the amino acids or BSA present in the medium as an erengy source or they may rely on endogenous energy sources. Daniel (1967a), also used Ham's F10 medium without BSA and obtained no development of two-cell embryos unless pyruvate, lactate, or

phosphenol pyruvate were provided. Defatted BSA does not support development of one-cell embryos (Kane, 1976). However, partial development can be restored by adding pyruvate or certain fatty acids to medium containing the defatted BSA.

Attempts to define a fixed nitrogen requirement have been complicated by the apparent ability of rabbit embryos to utilize fixed nitrogen supplements as energy sources. Brinster (1970) found that two-cell embryos would develop to morulae with supplemental glutathione and that pyruvate was beneficial if the level of glutathione was reduced. At the blastocyst stage the rabbit embryo undergoes a large increase in tissue mass, and therefore it likely also requires exogenous amino acids during this period (Brinster, 1972). Daniel and Krishnan (1967) have suggested that a specific group of amino acids are necessary for blastocyst expansion. Due to inconclusive information on this subject, however, most media employed for culture of rabbit embryos contain a large variety of amino acids. The rabbit blastocyst may also have a requirement for exogenous vitamins. Daniel (1967b) found that the development of five-day blastocysts was improved by the addition of thiamine, riboflavin, niacin, pyridoxine, folic acid, inositol, and hypozanthene.

Culture of blastocysts across the implantation period has been achieved in the rabbit. Cole and Paul (1965) cultured six-day rabbit blastocysts in Waymouth's medium plus 5% calf serum and 2% human serum. Since blastocysts cultured in this medium do not hatch, the zona was removed with pronase after 1½ days in culture. The embryos developed beating hearts and the amniotic folds closed. Daniel (1970) recovered

hatched blastocysts on day 7, which is ½ day before implantation begins, and cultured them in Ham's F10 medium supplemented with 20 to 25% rabbit serum, or rabbit and fetal calf serum. Embryos of 18 to 20 somites developed.

It appears that the rabbit embryo, like the mouse embryo, can develop to the blastocyst stage in a relatively simple, semi-defined medium. At the blastocyst stage, however, requirements become more complex and the addition of an undefined biological component is necessary for subsequent development.

Rat. The culture of preimplantation rat embryos has not been as successful as with the mouse and rabbit. Mayer and Fritz (1974) recovered embryos from the one-cell to blastocyst stages and cultured them in a modified KRB, substituting 25% fetal calf serum for bovine serum albumin. One-, two-, and four-cell embryos progressed to the four-cell stage but developed no further. Embryos recovered at the eight-cell stage or later developed into blastocysts. Only blastocysts recovered on day 5 hatched completely from the zona. Folstad et. al., (1969) have reported culturing eight-cell embryos to the blastocyst stage in a chemically defined medium. Little information, however, is available on the specific requirements for the rat embryo in vitro. Whittingham (1975) has made an attempt to define energy sources that support the first cleavage division. Results from his experiments show that a large number of energy sources did allow one division in the absence of BSA; BSA alone supported one cleavage division and, when BSA was replaced by Ficoll, 50% of the embryos divided but degenerated by 24 hours.

Only limited information is available on the culture of rat embryos over the implantation period. Bitton-Casimiri and Psychoyos (1968) have reported that rat blastocysts will attach and produce outgrowths in vitro. In contrast, post-implantation development has received a great deal of attention. New (1973) described methods for growing post-implantation embryos from the egg cylinder stage at 8 days of gestation to the 63 somite stage at 14½ days of gestation. Development of eight-day embryos directly through to 14½ days of gestation has not been achieved, but rather periods of development from 3 days for the early embryos to 1 day for the later embryos. Rat serum was used by New (1973) and dilution of the serum with Tyrode's solution or TCM 199 gave inferior results. Cockroft (1973), in contrast, found that diluting rat serum with Tyrode's solution gave superior growth of 12½ and 13½-day fetuses.

Though specific requirements for the complete in vitro development of the early rat embryo have not been determined, it is tempting to assume that it also will fit closely into the pattern on metabolic development being established by the mouse and rabbit.

Pig. Several attempts were made by early investigators using various culture media to develop a culture system that would allow cleavage of pig embryos. Hafez et. al., (1966) cultured four-cell embryos in various media including pig serum, MEM, KRB, and two dilutions of pig serum. One to two cleavage divisions were obtained in 50% pig serum plus 50% MEM. No other treatments supported development. Rundell and Vincent (1968), using various defined culture media, sera and combinations of the two, reported cleavage for 65% of the embryos cultured

in Brinster's medium for mouse ovum culture (BMOC-2; Brinster, 1965c), which again is a modified KRB solution, and BMOC-2 plus fetal calf serum. Embryos were recovered at various cleavage stages. A cleavage rate of 42% was reported in another experiment by Rundell and Vincent (1969) in which embryos from the one- to four-cell stages were cultured in the same two media. Pope and Day (1970) have reported 89% of one-cell embryos and 96% of two-cell embryos cleaved in 24 hours after culture in BMOC-2.

Not until recently, however, has development beyond one or two cleavage divisions been reported. Schneider et. al., (1975) cultured four- to eight-cell pig embryos in Tervit's synthetic oviduct fluid (Tervit et. al., 1972). Synthetic oviduct fluid is based on the biochemical analysis of sheep oviduct fluid and differs slightly from KRB in ion concentrations. Pyruvate, lactate and glucose are provided as energy sources, and BSA concentration is increased to 3.2%. Schneider et. al., (1975) was able to culture 60% of the four- to eight-cell embryos to the blastocyst stage. Earlier stages did not progress beyond one or two cleavage divisions. Wright (1977) has investigated the ability of Whitten's medium and a modified Ham's F10 medium to support blastocyst formation in pig eggs. Whitten's medium with 15mg/ml BSA supported more cleavage divisions than the more complex culture medium. In his study 9% of the five- and seven-cell embryos, 21% of the eight- to twelve-cell embryos and 95% of the morula stage embryos formed blastocysts. Earlier stages failed to develop past the morula stage. Pope and Day (1977), using BMOC-2, reported that 23% of the embryos

cultured in one experiment formed blastocysts. Most of the embryos were at the two-cell stage. In another experiment, however, none of 97 ova formed blastocysts. Lindner and Wright (1978) have recently reported a small number of embryos hatching in vitro when cultured in Whitten's medium plus 1.5% BSA from the four-cell stage.

The development of techniques for culturing early stages of pig embryos has allowed for more detailed studies on specific requirements. Davis and Day (1978) investigated the effects of glucose, lactate, and pyruvate as energy sources. Results from their studies indicate that lactate and pyruvate were not necessary for four-cell embryos to form blastocysts and, in fact, inhibited development. The presence of glucose aided blastocyst formation but was not necessary. Embryos cultured in the salt solution of a KRB medium plus BSA were able to form blastocysts. When BSA was replaced by Ficoll or polyvinylpyrrolidine, which are large nonmetabolizable polymers used to replace some of the physical properties of BSA, no blastocysts formed. The reason for BSA's beneficial effect is not known but seems to be more than colloid osmotic pressure.

Very little work has been done on the in vitro requirements for the post-hatching pig blastocyst. Shaffer and Wright (1978) compared Ham's F10 medium and MEM each supplemented with either 0, 10, or 40% pig serum for their ability to support attachment, trophoblastic outgrowth, and differentiation of the inner cell mass on either collagen or plastic substrates. Results indicated that 10 or 40% serum with either medium and either substrate supported development equally well.

However, the authors pointed out that development in vitro did not appear to closely parallel in vivo development. Blastocysts were recovered on days 6 to 9 and initiated attachment within 48 hours without regard to day of recovery. While the morphology of embryos developing in vitro did not resemble in utero embryonic development, there was a distinctly beneficial effect of serum.

By comparing the information available on the requirements for in vitro development by three common laboratory species, it becomes apparent that the cleavage stages of development may proceed in a relatively simple culture medium with BSA as the only macromolecular component required. Development from the blastocyst stage, however, is possible only when serum is included. The pig seems to follow the same pattern. Early pig embryos can develop in a simple medium supplemented with BSA, whereas post-hatching stages appear to have a requirement for serum.

Serum

Serum components. As stated previously, one reason for developing a culture medium for a particular type of tissue, organ, or embryo is to determine the specific requirements needed for normal function. As long as the addition of a complex biological component, such as serum, which contains a great variety of small and large molecular weight fractions, is necessary, it will be difficult to gain a full understanding of the function and regulation of the cell type being studied. As a result, a large amount of work has been done to determine which factors in serum are important. Most of the studies

have utilized tissues from differentiated organs. Identification of the low molecular weight compounds that are important, and required concentrations for specific cell lines has resulted in the development of over 100 different types of culture media (Waymouth, 1972). Factors in serum, such as amino acids, vitamins, lipids, and trace metals, may account for part of the benefit of adding serum to embryo culture media, but other components are probably also important. This contention is supported by the observation that defined media are unable to support development of blastocysts and later stages unless serum is included. It has been suggested that serum contains some specific factors which are responsible for control of cell multiplication and differentiation (Temin et. al., 1972). Spindle and Pederson (1973) reported that the post-blastocyst mouse embryo gradually becomes dependent on a non-dialyzable component from serum. Gwatkin (1966b) found that 10% fetal calf serum satisfied both an amino acid requirement and a macromolecular requirement for outgrowth and differentiation of mouse trophblast cells. The macromolecular requirement was satisfied by the α-globulin fetuin fraction of fetal calf serum and neither BSA nor PVP could replace it. Fetuin, the principle protein component of fetal calf serum, was first described by Pederson (1944). Since then considerable work has resulted in the further purification and characterization of its effects on various tissue culture cell lines. Temin et. al., (1972) summarized this information and concluded that fetuin may be a large carrier protein for a smaller protein which has the property of promoting attachment and stretching in several cell types.

A great variety of other protein fractions, isolated from serum or tissue, have been shown to have biological activity in cultured cells, however, no information is available on the effects of these specific proteins on embryo development in vitro.

Steroid hormones, are present in serum and may play a role in in vitro embryonic development. Warner and Tolefson (1977) were able to find no stimulation of RNA synthesis when mouse blastocysts were incubated with estradiol. However, it may be necessary for steroid hormones to bind to carrier proteins to be transported inside the embryo before they can exert an embryotrophic effect (El-Bana and Daniel, 1972). Working with earlier stages of mouse embryos, Roblero and Izquierdo (1976) found that progesterone increased cleavage rate only in the presence of the macromolecular fraction of serum. Salomen and Sherman (1975), however, found that removal of all detectable steroids did not affect the ability of fetal calf serum to support attachment and outgrowth of mouse blastocysts. Furthermore, New (1966) found sex and pregnancy had no effect on rat serum's ability to support post-implantation rat embryos in vitro. It is possible that levels of hormones present in serum, after dilution for culture, or even undiluted, may not be high enough to give a noticeable change in developmental rate or differentiation. Roblero and Izquierdo (1976) used microgram quantities whereas serum contains progesterone at nanogram levels.

Serum preparation. The presence of an ovicidal factor in serum was demonstrated by Chang (1949). In testing sera from several species

of animals for their ability to support development of rabbit ova, Chang (1949) found that human, sheep, cattle, goat, and chick serum contained an ovicidal factor, however rabbit, horse, dog, guinea pig and rat serum did not, and pig serum contained only a small amount of this factor. When rabbit ova were exposed for a short time to serum from species in which the ovicidal factor was present, and then cultured in rabbit serum for 24 hours, none of the ova cleaved. The factor was found to be thermolabile and non-dialyzable. Further, storage of serum for 17 to 30 days at 3°C was found to destroy the factor. In tissue culture heterologous sera are often harmful if the serum is not previously heated to 55°C (New, 1966). The damage appears to be that of an immune reaction which requires complement, and it is complement that is destroyed by heat treatment (New, 1966). Chang (1949), however, demonstrated that those sera which were ovicidal did not necessarily cause hemolysis of rabbit red blood cells, which requires the participation of complement. New (1966) found that rabbit serum is rapidly lethal to rat embryos, which demonstrates that a specific type of sera may be ovicidal to one species, but not another.

Normal fusion of heart primordia in rat embryos that have grown in vitro from egg cylinder stages is dependent on the proper processing of serum (Steele, 1972). Serum that is centrifuged immediately supports fusion whereas serum left standing overnight and then centrifuged does not support fusion. The harmful factor appeared rapidly in contact with a normal bloodclot in which the cells were trapped in the fibrin coagulum, but did not develop after 18 hours contact with separated

blood cells and fibrin clot (Steele and New, 1974). New (1967) had previously found that serum, with hepatocytes added, gives poor results, possibly as a result of hemolysis. Variability in different batches of serum has been a commonly reported phenomenon (McLaren and Hensleigh, 1975; Hsu, 1972). McLaren and Hensleigh, (1975) tested different batches of fetal calf serum for protein patterns on polyacrylamide gel electrophoresis and for amino acid concentrations but failed to find differences associated with the ability to support embryos in vitro. In many cases dilution of serum improves results and it has been suggested that this might result from dilution of inhibitory factors (Cockcroft, 1973). McLaren and Hensleigh (1975) found that 20% fetal calf serum gave the best response, when comparing levels from 0 to 50% for the culture of mouse blastocysts. Serum diluted with 50 to 75% Tyrode's solution resulted in better growth of 12½ and 13½ day rat fetuses (Cockcroft, 1973). It may be that optimum growth is achieved as a result of a balance between the dilution of inhibitory factors and essential nutrients, growth regulators and other factors present in serum.

CHAPTER III

SWINE EMBRYO DEVELOPMENT IN VITRO

Introduction

In vitro development of cleavage stage pig embryos to blastocysts has been demonstrated by several investigators. Schneider et. al. (1975) cultured 24 four- to eight-cell pig embryos in Tervit's synthetic oviduct fluid (Tervit et. al., 1972) and 14 formed blastocysts. Wright (1977) found that Whitten's medium with 15mg/ml BSA supported blastocyst formation in 9% of the five- to seven-cell embryos cultured. Later stages more readily formed blastocysts with 95% of the morulae cultured forming blastocysts. Lindner and Wright (1978) reported a limited number of the embryos cultured in Whitten's medium hatching in vitro. Davis and Day (1978) reported that a modified Krebs-Ringer bicarbonate medium, which included glucose and BSA, would support blastocyst formation by four-cell pig eggs, but only a few of these blastocysts initiated hatching in vitro. Less information, however, is available on the requirements for the in vitro development of post-hatching pig blastocysts. Shaffer and Wright reported that pig blastocysts would attach to either a collagen or plastic substrate and undergo trophoblast outgrowth in vitro. Ham's F10 and MEM, with and without pig serum, were compared for their ability to support attachment and development of 6- to 9-day blastocysts. Either medium, with pig serum, supported similar development. However, BSA could not replace serum. Development was defined as trophoblastic outgrowth, inner cell mass growth and differentiation.

The objective of the research reported here was to develop an *in vitro* system for the culture of swine blastocysts. The data collected was also summarized to describe *in vivo* changes in embryo nuclei number and cell size during the first 10 days of gestation.

Experimental Procedure

Preparation of Culture Equipment. All materials which came in contact with the embryos or culture medium were thoroughly hand washed. The washing included soaking the culture equipment in a tissue culture detergent[a] for at least one day. Individual pieces were then brushed and rinsed with at least 15 exchanges of tap water, 2 of distilled water, and 1 of deionized distilled water. The equipment was then dried in an aluminum foil covered tray in an oven. Equipment was stored in autoclave bags[b] and autoclaved within 10 days of use.

Preparation of medium. Dry ingredients were individually weighed and added to a 500 or 1,000 ml volumetric flask approximately half filled with deionized distilled water. Weigh boats and papers were rinsed by flushing a stream of deionized distilled water over them. A magnetic stir bar was used to mix the solution and, after all ingredients had been added, water was added to bring the flask to volume and the mixture stirred for an additional 15 to 30 minutes. The flask was immediately placed in the refrigerator for cooling.

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[a] 7X Tissue Culture Detergent, Limbro Chemical Co., Inc., New Haven, Conn.

[b] Will Ross, Inc., Milwaukee, Wis.

Sterilization was accomplished by filtration (0.22um pore size)[a] with positive pressure supplied by a bottled gas mixture which was first equilibrated with water by bubbling through two 500ml Erlenmeyer flasks half filled with sterile deionized distilled water and sterilized by filtration (0.45um pore size)[a]. The medium was forced directly from the filtration apparatus into 100 ml sterile bottles with a 1cc syringe and needle. The bottle was vented by another needle. The bottles were later gassed for approximately 3 minutes with the same mixture.

Pig and sheep sera were obtained by vena puncture from young females. After collection, the blood was immediately placed on ice and allowed to clot. Then, within 2 to 3 hours after collection, the serum was separated by centrifugation and the supernatant removed. The serum was centrifuged a second time to remove remaining red blood cells. Any serum with visually apparent hemolysis was discarded. The serum was then either sterilized by filtration and used fresh, or frozen immediately and sterilized after thawing. Fresh serum was stored at $4^{o}C$ and frozen at $-10^{o}C$. Fetal calf serum was purchased from Grand Island Biological Co.[b] as a lyophilized powder. It was stored at $4^{o}C$ in this form and rehydrated with sterile deionized distilled water just prior to culture. All serum was heated at $63^{o}C$ for 30 minutes in a water bath within 3 days of use for embryo culture.

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[a]Millipore Corp., Bedford, Mass. 01730.

[b]Grand Island, N.Y. 14072.

Medium. The basic semi-defined medium (Table 2) used in these experiments was based on a Krebs-Ringer bicarbonate (KRB). The addition of 4g/liter of BSA and 1g/l glucose to the KRB solution was made based on the results of preliminary experiments by Davis (1977) with cleavage stage swine embryos. All sera were added directly to this medium at the specified concentrations. The osmolarities of mKRB, mKRB plus 10% sheep serum and sheep serum were .284, .287 and .270 osmols, respectively, as measured by freezing point depression.

Animals. Embryos for these experiments were recovered from 27 Yorkshire and Yorkshire cross gilts. Eight of the gilts were bred on an estrus synchronized with a synthetic progestogen (allyl tremolone; 17 α-allyl-estratriene-4-9-11, 17 beta-ol-3-one), five were prepuberal and induced to ovulate with a mixture of 400 IU of pregnant mare serum gonadotropin and 200 IU of human chorionic gonadotropin and bred on the induced estrus. Fourteen were bred on a naturally occuring estrus. Gilts were checked twice daily for estrus and bred either artificially with 100 ml of 1:1 diluted semen, or hand mated. Inseminations were made at either 0, 12 and 24 hours or 12 and 24 hours after the onset of estrus. Onset of estrus was considered to be the first estrus check that gilts exhibited the immobile reflex and embryo age was calculated to the nearest half day.

Recovery Procedure. Embryos were recovered surgically by flushing the uterus with mKRB. An approximate 22 cm length of silastic tubing[a]

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[a]Dow Corning, Midland, Mich. 48650.

Table 2. MODIFIED KREBS-RINGER BICARBONATE

Ingredient	grams/liter	mM
NaCl	7.021	120.13
KCl	.356	4.78
$CaCl_2$	.189	1.71
KH_2PO_4	.162	1.19
$MgSO_4 \cdot 7H_2O$	.294	1.19
$NaHCO_3$	2.106	25.00
Glucose	1.000	5.56
Bovine serum albumin[a]	4.000	
Penicillin G	.063	
Streptomycin sulfate	.050	

[a]Fraction V.

(I. D. .66mm) was inserted through the oviduct wall approximately 4.4cm into the lumen near the tubouterine junction. With a syringe and blunt tip needle, 20cc of mKRB culture medium was injected into the lumen near the body of the uterus. The medium was stripped through the horn, out the tubing and into a petri dish. For recovering 10 and 12-day embryos this procedure was modified slightly. A glass cannula (I. D. 7mm), which was inserted through the uterine wall near the tubouterine junction, replaced the silastic tubing. In addition, the body of the uterus was flushed for recovery of 10 and 12-day embryos. After the flushings were collected, the petri dish was examined under a stereomicroscope to locate and examine the embryos. Embryos were collected in a finely drawn Pasteur pipet and immediately placed in the well of a culture plate[a] containing fresh, warmed medium. When embryos from both horns had been collected and placed in the culture plate, they were washed to remove excess cellular debris by rinsing through four wells of clean medium. After washing the embryos were examined for differences in appearance and were measured at 50X magnification using an ocular micrometer located in the eye piece of a stereomicroscope. Size and appearance were used to allot similar embryos from each pig to all treatments.

Culture procedure. Embryos were cultured in either 12 x 75mm polystyrene tubes with polyethylene caps[a] or in 16 x 125mm glass tubes[b]. The smaller tubes contained 2 ml of medium and the larger tubes 3 ml. After

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[a]Falcon, Oxnard, C.A. 93030.

[b]Bellco Glass, Inc., Vineland, N.J. 08360.

addition of the embryos, the tubes were gassed with a 5% O_2, 5% CO_2, 90% N_2 humified gas as described earlier, for 30 seconds. The larger tubes were then sealed with white rubber stoppers[a] and placed in a roller drum inside an incubator. The small plastic tubes were placed either inside another large glass tube (30 x 200mm) or a small descicator and the larger container was also gassed. This was necessary since the polystyrene tubes are permeable to gases. All cultures were conducted in a water jacketed incubator at 37.5°C. Observations and measurements were made every 24 hours. The glass culture tubes and small plastic tubes sealed in the larger glass tubes could be examined directly under the stereomicroscope. The plastic tubes in the desiccator, however, had to be removed for observation and the desiccator was regassed after each observation.

At the end of culture embryos were stained for examination under phase contrast microscopy. This was done by placing the embryo, along with a small quantity of medium, on a glass slide between two small beads of paravaseline (paraffin, vaseline; 1:1). The beads were placed along either edge of the slide, parallel to the long axis, with a syringe and a short, blunt needle. The syringe, filled with paravaseline, was kept in the incubator which kept the paravaseline warmed and at a lower viscosity. A small amount of rubber cement was brushed on either side of the slide, next to the paravaseline, to hold the coverslip in place. The coverslip was placed on the paravaseline beads on the slide and

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[a]Bellco Glass, Inc., Vineland, N.J. 08360.

gently pressed until the embryo flattened slightly. Two beads of liquid paravaseline were then placed along the edges of the coverslip, parallel to the long axis of the slide, with an eyedropper. The slide was then placed in a Coplan jar which contained a clearing solution consisting of 30ml of acetic acid and 10ml of absolute ethanol. The slide was left to clear at least 24 hours. Degenerating embryos were too fragile for this procedure and were cleared by introducing the above solution under the coverslip. The coverslip was sealed completely with paravaseline. After clearing a minimum of 24 hours the paravaseline seal was removed with a scalpel and a staining solution of 1% orcein in 45% acetic acid was flushed under the coverslip. Again the mount was sealed with paravaseline. Usually excess stain was removed after 24 hours by introducing 45% acetic acid under the coverslip. If clearing was delayed more than a couple of days the embryo absorbed excess stain making it difficult to distinguish nuclei. Even with a second clearing observations had to be made within a few days since the slides eventually deteriorated as the paravaseline dried.

Measurements. Post-hatching embryos were measured at the start of culture and 24 and 48 hours later. Measurements were made for the long axis and the widest perpendicular to the long axis. From these measurements volume and surface areas were calculated assuming the blastocyst is a prolate spheroid (Schacht and Foote, 1978). The formulas used are as follows:

$$\text{Volume} = 4/3 \; \pi \; a \; b^2$$

$$\text{Surface Area} = \frac{\text{Sin}^{-1} E}{57.2958 \; a \; b \; 2\pi} + 2\pi \; b^2$$

where: $E = \frac{\sqrt{a^2 - b^2}}{a}$

and, a = largest radii
b = smallest radii

Growth for each 24 hour period was calculated using differences in volume measurements.

The number of nuclei were determined by examining stained embryos under the phase contrast microscope. A grid, placed in the eyepiece, was used to delineate a section of the embryo and number of nuclei within the grid counted. The total number of nuclei for the embryo was determined by averaging number of nuclei for a minimum of four grids, determining the number of grids required to cover the entire embryo and multiplying the number of nuclei per grid by the number of grids per embryo. Areas such as the inner cell mass with differing nuclei densities were counted directly and added to the total nuclei number determined for the rest of the embryo. The number of chromosomes in metaphase or anaphase was determined either by this method or by direct count.

Embryos were observed at 24-hour intervals for morphological appearance which was denoted by short descriptive comments or drawings. This information was used to categorize the embryos into either surviving or nonsurviving groups. Those embryos which remained expanded and had a relatively smooth appearance were designated surviving. In a few

cases embryos would begin to collapse in one particular place but the majority of the trophoblast would look normal and stay expanded. These embryos were considered surviving. Nonsurviving embryos were usually dark and rough in appearance and had collapsed although a small cavity was sometimes present. Some of these blastocysts disintegrated during culture.

In vitro development of morulae was evaluated by determining stage of development attained during culture, rather than size, since they were surrounded by the zona pellucida and could increase only slightly in size until after hatching as blatocysts. At the first visable sign of cavity formation the embryos were designated as blastocysts. When the trophoblast was expanded enough to eliminate the periviteline space the embryo was classified an expanded blastocyst. A partially hatched blastocyst was one for which the trophoblast was protruding through the zona and at complete shedding of the zona the blastocyst was designated hatched. The number of nuclei were also determined. This was done by a direct count of the nuclei in each embryo.

Statistical analysis. In experiments I and II embryos were cultured in groups of 2 or 3 per tube. This made following the development of individual embryos impossible. Therefore, measurements of embryos in each tube were averaged for each observation period to give one size for the group which was used to calculate growth. Growth in vitro was calculated by averaging the size of embryos that were larger than the smallest embryo at the previous observation. If an embryo had collapsed

and was smaller, the average value for the previous observation was recorded. This calculation eliminated negative growth as a factor. This seemed appropriate since negative growth is really an indication of embryo death and is better described in terms of survival. In all subsequent experiments only one embryo was cultured in each tube and least square means were calculated from observations on the individual embryos.

In experiments II and III means for all parameters except survival rates were adjusted for donor and beginning volume by least square analysis (Snedecor and Cochran, 1967). These were then subjected to a modification of Duncan's multiple range test to compare means with unequal numbers of replications (Kramer, 1956). Survival rates were analyzed by chi-square as was developmental stage achieved by morulae. Nuclei number for embryos cultured from morulae were analyzed by t-test (Snedecor and Cochran, 1967). T-tests were also used to compare culture media for 10-day blastocysts.

Growth rate for pig embryos in vivo. During these studies 232 embryos were evaluated immediately after recovery. Data on these embryos was used to characterize growth of swine embryos in vivo. Number of nuclei for embryos up to the eight-cell stage was assumed to equal the number of blastomeres as determined by stereomicroscopic examination at 50X magnification. Nuclei number for later stages were determined by staining as described earlier. Day of gestation was calculated to the nearest half day considering the first detection of estrus as time 0. Estrus was checked twice daily so a range of 18 to 36 hours from first detected estrus to ovulation could be expected (Davis and Day, 1978).

These values were then used to calculate a growth curve by linear regression.

Cell size of pig blastocysts in vivo. Cell size was determined by dividing the surface area of the blastocyst by the number of nuclei. This was based on the assumption that one nucleus was present per cell. This may not be absolutely correct. Some cells, as observed with phase contrast microscopy, had two and sometimes three nuclei. This was not an uncommon sight but was of low frequency and should not greatly distort cell size calculations. A regression of cell size on nuclei number was also calculated.

Table 3. EFFECT OF SHEEP SERUM ON SWINE BLASTOCYST DEVELOPMENT IN VITRO

Treatment	Tubes/ Treatment[c]	Nuclei/ Blastocyst mean ± S.E.[d]	Maximum Volume mean ± S.E.[d] ($\times 10^{-3}$ mm^3)
mKRB	9	502 ± 195[a]	26.1 ± 4.8[a]
mKRB + 10% sheep serum	9	1749 ± 182[b]	105.8 ± 4.6[b]

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

[c] Two or three blastocysts per tube.

[d] Standard error.

Results and Discussion

Experiment I. Preliminary investigations indicated that mKRB plus 10% heat treated sheep serum would support growth of day-8 pig blastocysts in vitro. Both number of nuclei per blastocysts and maximum volume attained were greater for embryos cultured in serum supplemented mKRB (Table 3). The growth promoting ability was also compared for serum stored at $-10^{o}C$ or at $4^{o}C$. No difference was observed and frozen serum was used in most of the subsequent experiments.

Experiment II. In Experiment II several levels of sheep serum supplementation were compared. Along with these culture treatments some blastocysts from each pig were stained immediately after recovery. Results from this experiment indicated that mKRB plus either 10 or 20% sheep serum supports growth of day-8 pig blastocysts. Number of nuclei per blastocyst (Table 5) and size (Table 4) attained during culture for these two levels of serum were superior to other treatments ($P < .01$). By comparing the numbers of nuclei for blastocysts stained at recovery it is apparent that the cultured embryos increased their nuclei number approximately three to four times in the 48-hour culture period. This corresponds to a growth period of approximately 24-hours in vivo, assuming a doubling time of 14.3 hours (Figure 2). Interestingly, a large increase in volume also occurred during the first 24 hours in vitro. However, it is not possible from these data to determine when the increase in nuclei number occurred. It is possible nuclear divisions occured at a slower than expected rate throughout the culture period. Alternatively, nuclear division may have occurred at the expected in utero rate for a time and then slowed. The later possibility, however, is difficult to reconcile with the observed mitotic indices (percent of nuclei in metaphase or anaphase) which, at the end of culture, were

Table 4. GROWTH OF BLASTOCYSTS CULTURED IN MKRB WITH DIFFERENT LEVELS OF SHEEP SERUM

Treatment	Blastocysts/ Treatment	Maximum Volume mean ± S.E.[d] ($mm^3 \times 10^{-3}$)	Maximum Surface Area mean ± S.E.[d] ($mm^2 \times 10^{-3}$)	Increase in Volume: 0-24 hrs. mean ± S.E.[d] ($mm^3 \times 10^{-3}$)	24-48 hrs. mean ± S.E.[d] ($mm^3 \times 10^{-3}$)	0-48 hrs. mean ± S.E.[d] ($mm^3 \times 10^{-3}$)
Stained at Recovery	13	24 ± 2[a]	378 ± 20[a]	--	--	--
Cultured 48 hrs. in:						
MKRB	14	32 ± 31[a]	458 ± 217[a]	3 ± 16[a]	5 ± 20	8 ± 27[a]
MKRB + 10% Sheep Serum	15	218 ± 28[b]	2003 ± 200[c]	158 ± 15[b]	32 ± 19	190 ± 26[b]
MKRB + 20% Sheep Serum	14	211 ± 27[b]	1428 ± 193[b]	135 ± 15[b]	52 ± 19	187 ± 26[b]
MKRB + 50% Sheet Serum	14	37 ± 28[a]	478 ± 193[a]	11 ± 15[a]	0 ± 19	11 ± 26[a]

[a,b,c]Means in columns with different superscripts are different ($P<0.01$).

[d]Standard Error.

Table 5. EFFECT OF SHEEP SERUM CONCENTRATION ON IN VITRO SURVIVAL AND NUCLEI CHARACTERISTICS OF SWINE BLASTOCYSTS

Treatment	Blastocysts/ Treatment	Nuclei/ Blastocyst mean ± S.E.[e]	Nuclei/mm^2 mean ± S.E.[e]	% Metaphase & Anaphase mean ± S.E.[e]	Blastocyst Survival Rate[d] %
Stained at Recovery	13	667 ± 270[a]	2050 ± 375	1.11 ± 0.37	--
Cultured 48 hrs. in:					
MKRB	9	435 ± 258[a]	1974 ± 512	0.36 ± 0.40[f]	21.4[ab]
MKRB + 10% SS[e]	14	2444 ± 240[b]	1388 ± 378	1.60 ± 0.33	73.3[c]
MKRB + 20% SS	13	2366 ± 235[b]	1478 ± 355	1.36 ± 0.33	50.0[bc]
MKRB + 50% SS	13	829 ± 236[a]	2116 ± 378	1.12 ± 0.33	0.0[a]

[a,b,c]Means in columns with different superscripts are different ($P < .05$).

[d]Surviving embryos were designated as those remaining expanded and relatively smooth in appearance.

[e]Standard error.

[f]Different from other treatments ($P < .10$).

similar to indices for blastocysts stained at recovery. Doubling time, however, is also affected by mitotic duration which may have lengthened during culture. Interestingly, degenerating embryos seemed to have similar mitotic indices to nondegenerating embryos. The expansion resulting in a large increase in volume and surface area during the first 24 hours of culture is also unexplained. Expansion may not always reflect tissue growth. For example, differences in osmolarity between uterine fluids and the culture medium could cause an increase in fluid accumulation. If this were the case, however, it would be reasonable to expect an increase in volume for the embryos cultured in the mKRB alone comparable to those cultured in media with 10 and 20% serum dilutions since measured osmolarities of these media were similar (mKRB, .284 osmol.; mKRB plus 10% sheep serum, .287 osmol.). However, the mKRB did not produce such an expansion. Evidence for active transport and selective permeability of the trophoblast wall (Brinster, 1974) as the mechanisms for controlling blastocyst expansion suggests that something other than osmolarity may have played a role in the expansion of the embryos cultured in medium with 10 and 20% serum. No attempt was made in these experiments to determine the cause for early expansion. It also must be noted that no difference was observed in number of nuclei per square millimeter of surface area between treatments (Table 5). However, observations on nuclear density changes in vivo indicate that an embryo with a nuclei similar to the embryos at the end of culture in the mKRB with 10% sheep serum would have a theoretical nuclear density of approximately 2800 nuclei per square millimeter. Figures 2 and 3 illustrate blastocysts at recovery and after culture in 10% sheep serum.

A trend towards a lower number of nuclei in metaphase or anaphase was observed ($P < .10$) for the embryos grown in mKRB alone (Table 5). This corresponds to a small number of nuclei at the cessation of

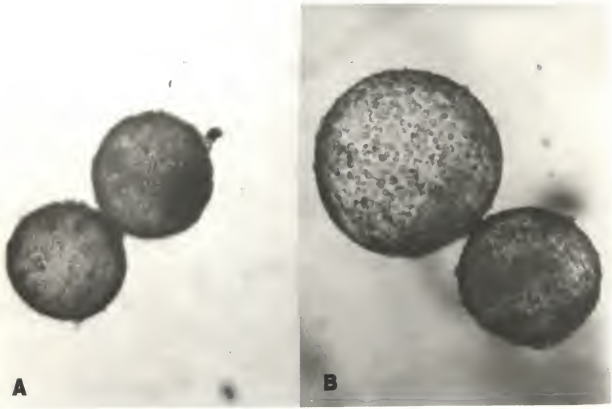

Figure 2. Pig blastocysts (A) at recovery on day 8 and (B) pig blastocysts recovered on day 8 and cultured 48 hours in mKRB plus 10% heated sheep serum (60x approx.).

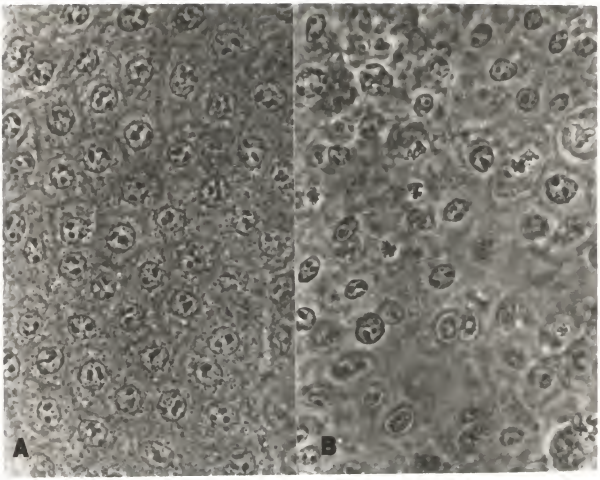

Figure 3. Stained pig blastocyst nuclei from (A) a day 8 blastocyst stained when recovered and (B) a blastocyst recovered on day 8 and cultured 48 hours in mKRB plus 10% heated sheep serum (650x approx.).

culture and a low survival rate for this treatment. The blastocysts cultured in 50% sheep serum also had low survival rates and nuclei numbers but the percent nuclei in metaphase or anaphase was similar to the other serum dilutions, and the embryos stained at recovery. This contradiction can be resolved if it is assumed that when cells die they become, after a period in cluture, more difficult to distinguish and consequently more easily overlooked during counting. In other words, the cells may have been dividing but also dying. This would decrease the total number of countable nuclei and tend to increase the mitotic index. It is also possible that the mitotic duration had increased during culture resulting in a high mitotic index and low division rate.

Experiment III. In Experiment III 10% pig serum and 10% fetal calf serum were compared to 10% sheep serum and the undiluted mKRB for their capacity to support development of day-8 blastocysts. It became apparent, after a few blastocysts had been cultured, that the basic medium supplemented with pig serum would not support growth, and in most cases survival, of the embryos (Table 6). Of nine embryos, obtained from five pigs, only one survived for the full 48 hours in mKRB plus 10% pig serum, and it had partially collapsed. Most of the embryos were degenerating by 24 hours in vitro and were too fragile to mount for nuclei counts.

Addition of 10% sheep serum again improved in vitro development as indicated by blastocyst volume and surface area ($P < .05$; Table 7) and nuclei per blastocyst ($P < .06$; Table 6).

Table 6. SURVIVAL AND NUCLEI CHARACTERISTICS OF BLASTOCYSTS GROWN IN MKRB SUPPLEMENTED WITH DIFFERENT TYPES OF SERUM

Treatment	Blastocysts/ Treatment	Nuclei/ Blastocyst mean ± S.E.[d]	Nuclei/mm^2 mean ± S.E.[d]	% Metaphase & Anaphase mean ± S.E.[d]	Blastocyst Survival Rate[e] %
MKRB	14	180 ± 540	709 ± 204	0.53 ± 0.20	50.0
MKRB + 10% Sheep Serum	17	1226 ± 509[a]	665 ± 193	1.07 ± 0.19	64.7[c]
MKRB + 10% Fetal Calf Serum	19	1680 ± 598[b]	855 ± 185	0.94 ± 0.18	68.4[c]
MKRB + 10% Pig Serum	9	--	--	--	11.1

[a]Greater than MKRB ($P < .06$).

[b]Greater than MKRB ($P < .05$).

[c]Greater than MKRB + 10% Pig Serum ($P < .05$).

[d]Standard error.

[e]Surviving embryos were designated as those remaining expanded and relatively smooth in appearance.

Table 7. GROWTH OF SWINE BLASTOCYSTS IN MKRB WITH DIFFERENT TYPES OF SERUM

Treatment	Blastocysts/ Treatment	Maximum Volume mean ± S.E.[c] ($mm^3 \times 10^{-3}$)	Maximum Surface Area mean ± S.E.[c] ($mm^2 \times 10^{-3}$)	Increase in Volume: 0-24 hrs. mean ± S.E.[c] ($mm^3 \times 10^{-3}$)	24-48 hrs. mean ± S.E.[c] ($mm^3 \times 10^{-3}$)	0-48 hrs. mean ± S.E.[c] ($mm^3 \times 10^{-3}$)
MKRB	14	45 ± 48[a]	644 ± 235[a]	4 ± 37[a]	10 ± 24	13 ± 47[a]
MKRB + 10% Sheep Serum	17	230 ± 37[b]	1700 ± 182[b]	132 ± 29[b]	32 ± 21	164 ± 37[b]
MKRB + 10% Fetal Calf Serum	19	160 ± 44[b]	1316 ± 216[b]	83 ± 32[b]	20 ± 21	104 ± 41[b]

[a,b] Means in the same column with different superscripts are different ($P < 0.05$).

[c] Standard error.

Results from this experiment indicate that 10% fetal calf serum also supports an increase in size (Table 7) and nuclei number for day-8 pig blastocysts comparable to mKRB supplemented with 10% sheep serum. Survival rates were also similar for these two media.

Experiment IV. This experiment compared the growth of several different stages of swine morulae cultured in either mKRB or mKRB supplemented with 10% sheep serum. The results are summarized in Table 8. Development followed a similar trend to that observed for hatched blastocysts. More embryos initiated hatching, and hatched when grown in the mKRB medium with 10% sheep serum. The hatching rate for embryos cultured in 10% sheep serum was 18.2%, and 54.4% initiated the hatching process. Lindner and Wright (1978) have shown a hatching rate of 2% with 10% initiating hatching when cultured in Whitten's medium with 1.5% BSA. Davis and Day (1978) also reported a limited number of embryos initiating hatching when cultured in mKRB. It is probably unfair, however, to compare these results with data presented here since embryos in the studies cited were grown in vitro from early cleavage stages. In the mouse, rabbit, and cow evidence has been presented to suggest that amino acid, or serum fractions, may be necessary or at least beneficial for the hatching process (Spindle and Pederson, 1973; Daniel and Krishnan, 1967; Wright et. al., 1976). It appears that this is also true for the pig. The mechanism by which serum increases the hatching response is not known. A simple increase in cell number does not seem to be the explanation since no difference

in nuclei number was detected between treatments producing the different hatching rates ($P > .20$).

Experiment V. Blastocysts recovered on days 10 and 12 were also cultured in mKRB and mKRB plus 10% sheep serum. None of the embryos recovered on day 12 survived to 24 hours. Most had begun to disintegrate in culture and, a few of the embryos which were mounted on slides, were degenerate in appearance and all nuclei were pyknotic. At recovery these blastocysts had elongated and displayed the characteristic wrinkled appearance described by Heuser and Streeter (1929). The trophoblast was very delicate and easily torn, which made handling of these embryos difficult.

Of the embryos recovered on day 10, those from one pig appeared similar, although smaller, to those described above. They were wrinkled and some had begun to elongate. None of these embryos survived to 24 hours when cultured in either medium. Embryos from the other day-10 pig were similar in appearance to earlier blastocysts recovered on day 8 and from nuclei counts of the embryos stained at recovery it was apparent that these embryos had not progressed as far in development. Results (Table 9) from the culture of these blastocysts indicate that mKRB with 10% sheep serum supported a doubling of nuclei number *in vitro*. Several explanations can be postulated for these observations. It is possible that the medium was lacking in some essential nutrient, or nutrients, needed by the later stages. Most *in vitro* systems for growing later stages of mammalian embryos use a more complex medium

Table 8. HATCHING OF SWINE MORULAE CULTURED 72 HRS. IN VITRO

Treatment	Embryos/ Treatment	Nuclei/ Embryo	% Blastocysts	% Expanded Blastocysts	% Initiating Hatching	% Completing Hatching
MKRB	22	80	95.5	81.8	4.5^{a}	0.0^{a}
MKRB + 10% Sheep Serum	22	96	100.0	81.8	54.5^{b}	18.2^{b}

[a,b] Means in columns with different superscripts are different ($P < 0.05$).

Table 9. DEVELOPMENT OF DAY 10 SWINE BLASTOCYSTS RECOVERED FROM ONE GILT

Treatment	Blastocysts/ Treatment	Nuclei/ Blastocyst	Volume ($mm^3 \times 10^{-3}$)	Surface Area ($mm^2 \times 10^{-3}$)	Growth 0-48 hrs. ($mm^3 \times 10^{-3}$)	% Surviving
Stained at Recovery	4	12,934	697[a]	3643[a]	--	--
Culture 48 hrs. in:						
MKRB	4	--	1178[a]	5157[a]	0.00	0.0
MKRB + 10% Sheep Serum	4	27,765	4532[b]	13005[b]	4807	75.0

[a,b]Means in columns with different superscripts are different ($P < 0.025$).

such as MEM or Ham's F10 rather than mKRB. Serum probably provides some concentration of the factors present in these media, but may not provide enough. New (1971) found it essential to increase the concentration of O_2 in the gas atmosphere when growing day-8 rat embryos. Daniel (1971) bubbled a 10% O_2 gas through his culture medium when growing day-7 rabbit blastocysts and Hsu (1974) used a 5% CO_2 in air gas phase when culturing mouse embryos to later stages. It is possible that the 5% O_2 gas used in these experiments was not adequate for the larger embryos. A build up of metabolites is not likely to have been a problem since rotating the culture tubes did not improve results. It also seems unreasonable that a toxic concentration could have been reached in the short time it took for the embryos to collapse and disintegrate.

It can be concluded that cleavage of at least some blastocysts recovered on day 10 of gestation is possible in a mKRB with 10% sheep serum although growth of later stages is questionable in this culture system.

Growth rate for pig embryos in vivo. Nuclei number for in vivo developing blastocysts by day of gestation, is shown in Figure 4. The equation for the regression line is as follows:

$$\log (\text{nuclei number/embryo}) = -3.068 + 1.176 \text{ (day of gestation)}$$

Using this equation, the calculated time required to double nuclei number, for the first 13 divisions, is 14.3 hours. This corres-

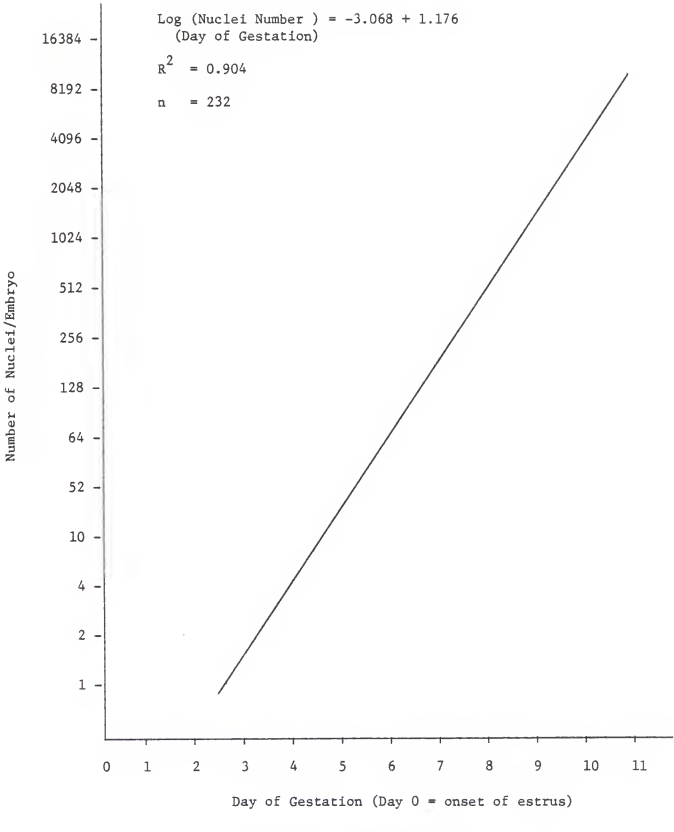

FIGURE 4. NUCLEI NUMBER FOR PIG EMBRYOS DEVELOPING IN VIVO

ponds closely to data reported by Heuser and Streeter (1929) who found that it took approximately 5½ days for the one-cell embryo to undergo nine complete divisions which corresponds to a doubling time of approximately 14.6 hours.

Cell size of pig blastocysts in vivo. Changes in cell size for pig embryos developing in vivo are depicted in Figure 5. The regression equation is as follows:

$$\log (\text{cell size}) = 3.272 - 0.256 \log (\text{nuclei number/blastocyst})$$

This line indicates a rapid decline in cell size from approximately 6.7×10^{-4} mm^2 for blastocysts with about 200 nuclei to approximately 2.6×10^{-4} mm^2 at 8,000 nuclei, then a leveling off to a cell size of 1.7×10^{-4} mm^2 at 45,000 nuclei. Daniel (1964) has shown in the rabbit, that trophoblast cell size stays relatively constant after blastocyst formation at about 3.1×10^{-4} mm^2.

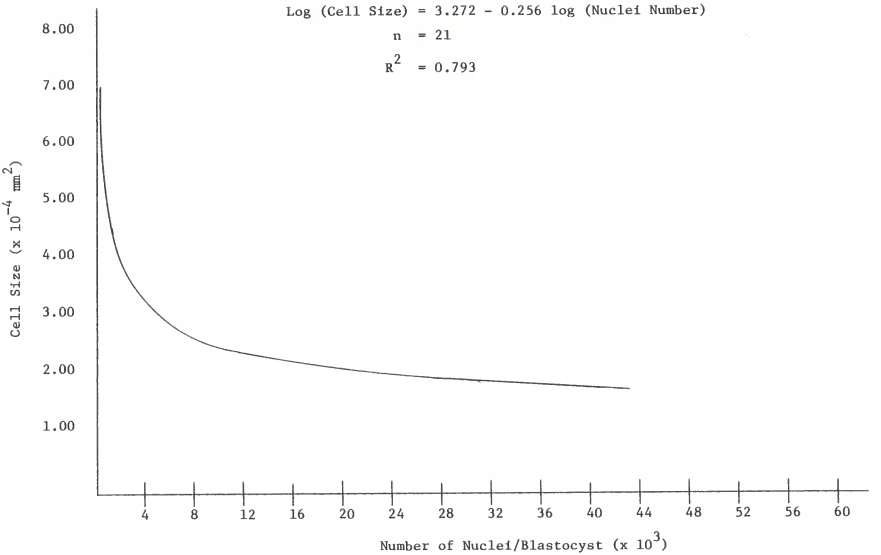

FIGURE 5. CELL SIZE FOR BLASTOCYSTS DEVELOPING IN VIVO

Conclusions. These results indicate that the development of post-hatching swine blastocysts in vitro requires supplementation with serum. Not all types of serum support development, and the serum must be present at the proper level for maximum response. Embryos grown from the morula stage in vitro exhibit a response to serum supplementation as exhibited by the proportion completing hatching.

The development of day-8 pig blastocysts in vitro should prove a useful tool for the study of early pregnancy in the pig. Culture in the experiments reported here covers the period when embryo losses in utero become prominent (Perry and Rowlands, 1962). With future improvements it should be possible to expand this culture system to include later stages of pregnancy.

CHAPTER IV

SUMMARY

The effects of serum on *in vitro* swine embryo development was investigated in five experiments. Experiment I compared a modified Krebs-Ringer bicarbonate medium, containing glucose and bovine serum albumin (mKRB), to the same medium plus 10% heat treated sheep serum. The addition of sheep serum improved *in vitro* growth of day-8 swine blastocysts as indicated by a larger number of nuclei and volume after 48 hours *in vitro* ($P < .05$).

Experiment II compared 10, 20 and 50% sheep serum to the unsupplemented mKRB. In addition, some blastocysts from each pig were stained at recovery (day-8). Blastocysts cultured in medium containing 10 or 20% sheep serum had more nuclei at the end of culture ($P < .01$) and reached larger surface areas and volumes ($P < .01$) than those cultured in medium with 0 or 50% sheep serum, and those stained at recovery.

In Experiment III mKRB was compared with a mKRB supplemented with either 10% sheep serum, 10% fetal calf serum, or 10% pig serum for the development of day-8 blastocysts. The addition of 10% pig serum resulted in very low blastocyst survival rates and no growth in size of the embryos. MKRB plus 10% fetal calf serum supported growth, as indicated by nuclei number and volume, similar to mKRB plus 10% sheep serum. Unsupplemented mKRB did not support growth.

Experiment IV investigated the effect of sheep serum on *in vitro* hatching. Morulae were cultured in mKRB or mKRB plus 10% sheep serum. The addition of 10% sheep serum improved the hatching rate (18.2% vs. 0%; $P < .05$) compared to culture in mKRB medium alone.

In Experiment V neither mKRB nor mKRB plus 10% sheep serum supported *in vitro* survival of blastocysts recovered on day 12. Only blastocysts from one gilt, recovered on day 10, survived and increased in size and nuclei number when cultured in a mKRB medium supplemented with 10% sheep serum. All blastocysts cultured in unsupplemented mKRB failed to survive. These results indicate that requirements for *in vitro* development of pig embryos from morula to 10-day blastocysts are more complex than can be provided by mKRB and that development is enhanced by the addition of serum.

In vivo growth rate and cell size (surface area/nuclei) were also investigated. A nuclei doubling time of 14.3 hours was calculated for embryos growing from one to 8,000 nuclei. Cell sizes decreased rapidly to about 2.6×10^{-4} mm^2 at 8,000 nuclei, and then leveled off.

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APPENDIX

Appendix EFFECT OF EPIDERMAL GROWTH FACTOR, INSULIN AND VITAMIN B^{12} ON SWINE EMBRYOS IN VITRO
Table 1.

Treatment	Day 7 Embryos[a]			Day 8 Blastocysts		
	Embryos/ Treatment	% Survival	Nuclei/ Embryo	Embryos/ Treatment	% Survival	Nuclei/ Embryo
MKRB[b]	11	100	119	15	93	600
MKRB + Vitamin B^{12} (500ng/ml), Insulin (100ng/ml), EGF[c] (1ng/ml)	10	60	126	13	54	655
MKRB + Vitamin B^{12} (500ng/ml), Insulin (100ng/ml), EGF (10ng/ml)	11	82	82	13	38	275
MKRB + Vitamin B^{12} (500ng/ml), Insulin (100ng/ml), EGF (100ng/ml)	12	92	116	15	73	643
Stained at Recovery	7	--	83	10	--	578

[a] Morulae or expanded blastocysts; culture for 72 hrs.

[b] Krebs-Ringers bicarbonate modified with bovine serum albumin (4g/l) and glucose (1g/l).

[c] Epidermal growth factor.

Appendix
Table 2. EFFECT OF EPIDERMAL GROWTH FACTOR ON SWINE EMBRYOS

Treatment	Recovery Day	Blastocysts/ Treatment	Volume at Start ($mm^3 \times 10^{-3}$)	Growth 0-48 hrs. ($mm^3 \times 10^{-3}$)	Nuclei/ Blastocyst	% Survival
Experiment I						
MKRB[a]	9	6	45	28	--	66.7
MKRB + Vitamin B^{12} (500ng/ml) Insulin (100ng/ml) EGF[b] (100ng/ml)	9	6	72	203	4795	42.9
Stained at Recovery	9	3	39	--	2625	--
Experiment II						
MKRB	8	3	13	0	400	0.0
MKRB + 167ng/ml EGF	8	3	21	9	543	100
MKRB + 1.67ng/ml EGF	8	3	14	3	419	100
Stained at Recovery	8	2	18	--	242	--

[a]Krebs-Ringer bicarbonate modified with bovine serum albumin (4g/l) and glucose (1g/l).

[b]Epidermal Growth Factor.

Appendix
Table 3. EFFECT OF LACTATE AND PYRUVATE ON EARLY CLEAVAGE STAGE PIG EMBRYOS

Treatment	Stage	Recovery Day	Embryo/ Treatment	% Blastocysts	Nuclei/ Embryo
Experiment I					
MKRB[a]	4-8 cell	4	14	100.0	73
MKRB + 0.25mM Pyruvate	4-8 cell	4	17	100.0	75
MKRB + 0.125mM Pyruvate	4-8 cell	4	9	88.9	74
MKRB + 0.25mM Pyruvate	4-8 cell	4	14	92.9	66
Experiment II					
MKRB	3-4 cell	3 & 4	18	33.3	52
MKRB + 0.25mM Pyruvate 25mM Lactate	3-4 cell	3 & 4	19	10.5	23
MKRB + 0.25mM Pyruvate	3-4 cell	3 & 4	21	33.3	28

[a]Krebs-Ringer bicarbonate modified with bovine serum albumin (4g/l) and glucose (1g/l).

Appendix
Table 4. COMPARISON OF TWO DEFINED MEDIA FOR SWINE EMBRYOS IN VITRO

Treatment	Stage at Recovery	Embryos/ Treatment	Culture Period	Volume at Start ($mm^3 \times 10^{-3}$)	Growth ($mm^3 \times 10^{-3}$)	% Survival	Recovery Day
Experiment I							
MKRB[a]	Expanded Blastocysts	6	72 hrs.	2.6	4.2	66.7	6
MEM[b]	Expanded Blastocysts	6	72 hrs.	2.6	0	0.0[d]	6
Experiment II							
MKRB	Morula	2	72 hrs.	--	Formed Blastocyst	100.0	5
MEM	Blastocysts	3	72 hrs.	--	0	0.0[d]	5
Experiment III							
MKRB	Expanded and Hatched Blastocysts	4	48 hrs.	3.8	12.4	100.0	7
MKRB with BME[c] amino acids + glutamine	Expanded and Hatched Blastocysts	4	48 hrs.	3.6	0	0.0[d]	7

[a]Krebs-Ringer bicarbonate modified with bovine serum albumin (4g/l) and glucose (1g/l).

[b]Eagle's minimum essential medium.

[c]Eagle's basal medium.

[d]All degenerate by 24 hours in culture.

Appendix
Table 5. EFFECT OF UTERINE FLUSHINGS ON SWINE EMBRYOS IN VITRO

Treatment	Recovery	Recovery Day	Embryos/ Treatment	Culture Period	Volume at Start ($mm^3 \times 10^{-3}$)	Growth ($mm^3 \times 10^{-3}$)	Nuclei/ Embryo	% Survival
Experiment I								
MKRB[a]	Morula & Blastocyst	5	3	48 hrs.	1.4	5.0	38	100.0
MKRB containing uterine flushings[b]	Morula & Blastocyst	5	3	48 hrs.	1.5	3.0	45	100.0
Experiment II								
MKRB	Morula	6	6	72 hrs.	1.8	3.0	--	100.0
MKRB containing uterine flushings	Morula	6	6	72 hrs.	1.8	3.8	--	100.0

[a]Krebs-Ringer bicarbonate modified with bovine serum albumin (4g/l) and glucose (1g/l).

[b]MKRB was flushed through one uterine horn of day 8 gilt.

Appendix
Table 6. EFFECT OF UTERINE SECRETIONS ON BLASTOCYST DEVELOPMENT IN VITRO

Treatment	Recovery Day[a]	Embryos/ Treatment	Volume at Start ($mm^3 \times 10^{-3}$)	Growth ($mm^3 \times 10^{-3}$)	Nuclei/ Embryo	% Survival
MKRB[c]	6.5 & 7.0	7	4.4	149.2	624	71.4
MKRB + 1 mg/ml Uterine Secretions[b]	6.5 & 7.0	7	5.6	21.2	483	42.9
MKRB + 4 mg/ml Uterine Secretions	6.5 & 7.0	8	12.3	3.4	380	12.5

[a]Recovered as blastocysts, expanded blastocysts, and hatched blastocysts.

[b]Uterine secretions recovered by flushing the uterus on day 10 with deionized distilled water and lyophilizing.

[c]Krebs-Ringer bicarbonate modified with bovine serum albumin (4g/l) and glucose (1g/l).

SWINE EMBRYO DEVELOPMENT IN VITRO

by

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B. S., Kansas State University, 1977

AN ABSTRACT OF A MASTER'S THESIS

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The effects of supplementing a modified Krebs-Ringer bicarbonate medium with serum, on *in vitro* swine embryo development, was investigated in five experiments. Embryos were recovered surgically and cultured in either stationary plastic (12 x 75mm) or rotating glass (16 x 125mm) tubes, containing 2 and 3 ml of medium, respectively. Neither culture vessel nor rotation affected *in vitro* development. The culture medium was equilibrated with a 5% O_2, 5% CO_2, 90% N_2 gas atmosphere. Morulae were cultured 72 hours and blastocysts 48 hours. The culture temperature was 37.5°C. The effects of sheep, pig and fetal calf serum were investigated. Sheep and pig serum was obtained fresh by bleeding female animals, letting the clot form while the sample was on ice, and isolating the serum by centrifugation within 2 to 3 hours. Frozen storage of the serum produced no detectable effects on swine embryo growth. Fetal calf serum was purchased as a lyophilized powder. All serum was heated to 63°C for 30 minutes within 3 days prior to culture. The basic medium used was a modified Krebs-Ringer bicarbonate solution (mKRB) containing glucose (1g/l) and BSA (4g/l). Osmolarities of the mKRB, mKRB plus 10% sheep serum and sheep serum alone were .284, .287 and .270 osmols, respectively, as measured by freezing point depression.

Experiment I compared mKRB to mKRB plus 10% sheep serum. The addition of sheep serum improved growth of day-8 blastocysts as indicated by a larger number of nuclei and volume attained *in vitro* ($P < .05$).

Experiment II compared mKRB plus 10, 20 and 50% sheep serum to the unsupplemented mKRB. In addition some blastocysts from each donor gilt were stained at recovery (day-8). Blastocysts cultured in medium containing 10 or 20% sheep serum had more nuclei at the end of culture and reached larger surface areas and volumes ($P < .01$) than those cultured in medium with 0 or 50% sheep serum, or those stained at recovery.

In Experiment III mKRB was compared with a mKRB supplemented with either 10% sheep serum, 10% fetal calf serum, or 10% pig serum for the development of day-8 blastocysts. The addition of 10% pig serum resulted in very low blastocyst survival rates and no increase in volume. Medium supplemented with 10% fetal calf serum supported growth, as indicated by nuclei number and volume, similar to mKRB plus 10% sheep serum. Unsupplemented mKRB did not support growth.

Experiment IV investigated the effect of sheep serum on in vitro hatching. Morulae were cultured in mKRB or mKRB plus 10% sheep serum. The addition of 10% sheep serum improved hatching rate (18.2% vs. 0%; $P < .05$) compared to culture in mKRB medium alone.

In Experiment V neither mKRB nor mKRB plus 10% sheep serum supported in vitro survival of blastocysts recovered on day 12. Only blastocysts from one pig recovered on day 10 survived and increased in size and nuclei number when cultured in a mKRB medium supplemented with 10% sheep serum. All blastocysts cultured in unsupplemented mKRB failed to survive. These results indicate that requirements for

in vitro development of pig embryos from morulae to 10-day blastocysts are more complex than could be met by mKRB and that development is enhanced by the addition of serum.

In vivo growth rate and cell size (surface area/nuclei) were also investigated. A nuclei doubling time of 14.3 hours was calculated for embryos growing from one to 8,000 nuclei. Cell sizes decreased rapidly to about 2.6×10^{-4} mm^2 at 8,000 nuclei, and then leveled off.

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