THE EFFECT OF MEDIUM AND SERUM ON DEVELOPMENT OF SWINE EMBRYOS *IN VITRO*

by

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iv
LIST OF TABLES	v
CHAPTER	
I. INTRODUCTION	1
II. LITERATURE REVIEW	4
Culture Media Characteristics	4
Osmolarity	4
Hydrogen ion and inorganic ion concentration	5
Bicarbonate ion concentration	6
Substrates In Culture Media	6
Amino acid requirements	6
Amino acid composition of the uterine milieu and embryo	8
Amino acid metabolism	9
Serum requirements for in vitro development	11
Energy requirements	15
III. THE EFFECT OF MEDIUM AND SERUM ON DEVELOPMENT OF SWINE EMBRYOS IN VITRO	17
Introduction	17
Experimental Procedures	18
Animals	18
Recovery of embryos	18
Preparation of culture equipment	19
Medium	19

TABLE OF CONTENTS (Continued)

CHAPTER		Page
	Preparation of culture medium	20
	Preparation of serum	28
	Embryo culture	28
	Measurements	29
	Preparation of slides	29
	Statistical analysis	30
	Results	31
	Experiment I	31
	Experiment II	33
	Experiment III	33
	Experiment IV	36
	Discussion	36
IV.	SUMMARY	41
LITERATURE CITED		44
APPENDIX TABLES		50
1.	CONTENT OF TOTAL FREE AMINO ACIDS IN RABBIT REPRODUCTIVE TRACT FLUID (μM/ml) . . .	51
2.	CONTENT OF TOTAL FREE AMINO ACIDS IN SOW OVIDUCT FLUID (μM/ml)	53
3.	CONTENT OF TOTAL FREE AMINO ACIDS IN SOW UTERINE FLUID (μM/ml)	53

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LIST OF TABLES

TABLE		PAGE
1.	MODIFIED KREBS-RINGER BICARBONATE	21
2.	FORMULATIONS OF MEDIA USED	22
3.	MEM SUPPLEMENTS	26
4.	MEASURED OSMOLARITY AND pH OF VARIOUS MEDIA . . .	27
5.	DEVELOPMENT OF EMBRYOS IN EXP. I	32
6.	DEVELOPMENT OF EMBRYOS IN EXP. II	34
7.	DEVELOPMENT OF EMBRYOS IN EXP. III	35
8.	DEVELOPMENT OF EMBRYOS IN EXP. IV	37

CHAPTER I

INTRODUCTION

In mammals, the multicellular body of the offspring is derived from a single cell, the fertilized egg. The initial cell divisions, called cleavage, are mitotic divisions with no net growth. Cleavage continues until a cavity (blastocoele) appears (Balinsky, 1970).

During the early period of gestation the embryo floats free in the oviduct and uterus. During the pre-attachment period the embryo relies on nutrients in its own cytoplasmic stores and uterine secretions. The latter assume a dominant role after the late morula to early blastocyst stage is attained (Brinster, 1971; Epstein and Smith, 1973).

The fertilized egg of the pig reaches the uterus at about 46 hours after ovulation when it consists of two to eight cells (Hunter, 1974). Cleavage continues with compaction obvious at the 16 to 32 cell stage resulting in a round clump of cells, the morula, approximately 3 to 4 days after ovulation (Hunter, 1974). Morulae cavitate forming blastocysts with two types of cells, the inner cell mass and the trophoblast (Brinster, 1974) 5 to 6 days after ovulation (Hunter, 1974). The blastocyst has markedly

different metabolic patterns as compared to earlier stages. These include increased oxygen consumption, incorporation of precursors into nucleic acids and protein synthesis (Brinster, 1974). The accumulation of fluid in the blastocoele functions to transport substances and as a means of selective permeability. At 6 to 8 days after ovulation the blastocyst escapes from its zona pellucida, a phenomenon termed hatching. During the next few days the embryo grows, elongates, and attachment begins. The connection between the embryo and uterus is noninvasive and superficial (Perry, 1981). By the 18th day of pregnancy the surface microvilli have disappeared and the first adherence of trophoblast to uterine epithelium occurs at isolated points (Perry, 1981).

Most embryo culture studies have used laboratory animals such as the mouse, rabbit, and rat. The morphological development is chronologically faster for these species. The mouse and rate experience their first cleavage by 24 hours after copulation with subsequent cleavages at intervals of 12 hours (Snell, 1941). After the first few cleavages the rat lags about 2 days behind the mouse. On day 4 to 5 post coitum the mouse blastocyst emerges from the zona pellucida and implants into the uterine wall (Snell, 1941), whereas the rat begins implantation at day 6.5 and is implanted by 7.5 to 8 days after copulation

(Witschi, 1956). The rabbit blastocoel and inner cell mass appear at 70 hours post copulation, entering the uterus on day 4, and implant on day 7 (Lewis and Gregory, 1929).

The first 10 days of pregnancy is critical in swine pregnancies and embryo losses can be recognized as early as day 8 (Dawydiak, 1981). Some 25 to 40% of embryos are lost during the first 30 days of gestation. Therefore, it is economically important to study early embryonic development. Many approaches to this unanswered question have been taken but as yet the requirements of the swine embryo are not resolved. To determine requirements one must first remove the embryo from its uterine environment for study under controlled conditions. Embryo culture provides a controlled environment and is the subject of this thesis.

CHAPTER II

LITERATURE REVIEW

Culture Media Characteristics

Effective culture systems must provide an environment that supports cell or tissue growth. The culture medium has a key position in the culture system and should be as close to the *in vivo* environment of the embryo as practical. Three basic characteristics of the culture medium are osmolarity, hydrogen ion and inorganic ion concentration, and the bicarbonate ion concentration. These characteristics are determined by the inorganic salts in the medium.

Osmolarity. Whitten (1971) showed that mouse embryos could develop in a wide range of osmolarities (240 to 310 mosm). Brinster (1965a) extended Whitten's observations and found a hypoosmotic optimum of 256 mosm as compared to the physiological range of 308 to 315 mosm. Blood sera of most species is approximately 308 mosm (Brinster and Troike, 1979). Naglee et al. (1969) showed that two-cell rabbit embryos would develop to blastocys in media ranging from 230 to 339 mosm. Osmolarity effects have not been studied for the embryos of farm animals. For the study of physiological processes an

osmolarity in the physiological range would appear best on theoretical grounds.

Hydrogen ion and inorganic ion concentrations. Whitten (1956a) showed that a modified Krebs-Ringer bicarbonate (Krebs and Henseleit, 1932) containing the cations; Na^{+}, K^{+}, Ca^{++}, Mg^{++}, and the anions; CI^{-}, $PO_4^{=}$ and HCO_3^{-} would support growth from eight-cell to blastocyst by mouse embryos (Whitten, 1971). Ducibela and Andersen (1975), stated that Ca^{++} was necessary for membrane stability, permeability and cell to cell interaction, especially at the time of compaction to form morulae. Ducibella and Andersen (1975) demonstrated a large majority of embryos had completed compaction in control medium whereas in Ca^{++} depleted medium, compaction was completely inhibited. Compaction at the eight-cell stage of the mouse embryo occurs *in vitro*, is accompanied by the formation of tight and gap junctions and can be reversibly inhibited *in vitro* by lowering the Ca^{++} concentration of the medium. The percent of partially and completely compacted embryos in 0.17 and 0.017 mM Ca^{++} was 85 and 0%, respectively (Ducibella and Andersen, 1975).

Hydrogen ion concentration (pH) is similar to osmolarity in that the embryo can tolerate a wide range. Whitten (1956) reported an optimum pH of 6.9 to 7.7 for eight-cell mouse embryos and Brinster (1965) found that two-cell mouse embryos developed in a range of 5.87 to 7.78. Whitten

(1971) suggested that a culture medium should have a pH of about 7.2. Brinster (1974) considers pH important since it might play a role in membrane permeability and thus interact with substrate concentration. The pH of the sow uterine lumen is reported to be 7.02 and the oviduct 6.98 (Mather and Day, 1972). In bicarbonate buffered media pH is maintained by equilibrating the medium and environment with an enriched CO_2 gas atmosphere (5% CO_2 for a 25 mM bicarbonate medium).

Bicarbonate ion concentration. Bicarbonate ions serve as a buffer in culture media and are also involved in the formation of Krebs cycle metabolites and carbon metabolism (Kane, 1975). Development of one-cell rabbit embryos will occur in a Hepes buffered medium without HCO_3^- but blastocyst formation requires a low level of bicarbonate (Kane, 1975). Whittingham (1971) showed that bicarbonate was a necessary ingredient in the culture medium of early mouse embryos for development to occur. Quinn and Wales (1973) showed that limited growth of mouse ova in HCO_3^- free phosphate buffered solution was possible.

Substrates in Culture Media

Another role for culture media is the provision of substrates for energy metabolism, macromolecular synthesis and growth.

Amino acid requirements. Eagle (1959) reported that in cell culture, whether animal or human, 13 amino acids

were required for survival and growth. These were isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine for nitrogen balance plus arginine, cystine, glutamine, histidine, and tyrosine. Daniel and Krishnan (1967) showed that rabbit blastocysts *in vitro* required 10 amino acids: arginine, histidine, lysine, tryptophan, methionine, phenylalanine, leucine, valine, threonine and serine. Arginine and lysine were required in relatively high concentrations, 10^{-2}M and 10^{-3}M, respectively, for optimum growth. Even though none of the nonessential amino acids were shown to be required individually by Daniel and Krishnan (1967), their complete omission markedly reduced blastocyst growth and they probably serve as an amino nitrogen source. Spindle and Pederson (1973) found that hatching, attachment, and outgrowth of mouse blastocysts *in vitro* is dependent upon the presence of specific free amino acids in the medium. Eagle's Basal Medium (BME) plus 1% dialyzed fetal calf serum in their study resulted in 67% of the embryos hatching. When histidine, methionine, threonine, tryptophan, tyrosine and valine were omitted from the medium the number of embryos hatching were significantly reduced (w/o tyrosine 18% of the blastocysts hatched vs 70% hatching rate with tyrosine, omission of methionine, threonine, or tryptophan decreased the control level by 40% with the absence of valine or histidine the hatching

rate was 45 to 50% of the control level). Cystine and lysine were also required for attachment to take place. Every essential amino acid was shown to be required for outgrowth except isoleucine. They also investigated the addition of 10^{-5} to 10^{-2}M concentrations of non-essential amino acids but these did not support hatching, attachment, or outgrowth. In some cases the non-essential amino acids showed inhibitory effects. Glutamic acid had the greatest inhibitory effect; at a concentration of 10^{-3} to 10^{-2}M, hatching was significantly inhibited and the extent of outgrowth was reduced to less than 50% of controls.

Brinster (1965b) suggested that two-cell embryos required several amino acids in approximately the proportion in which they occur in serum albumin.

Whitten (1957) stated mouse embryos will develop from the eight-cell stage to the expanded blastocyst with glycine as the only source of fixed nitrogen. Cholea and Whitten (1970) demonstrated that two-cell mouse embryos do not require any external source of fixed nitrogen (96% of 2-cell formed blastocysts).

Amino acid composition of the uterine milieu and embryo. Iritani et al. (1971) identified 19 amino acids in both oviduct and uterine fluids of the rabbit. Glycine was the predominant amino acid followed by alanine, serine and glutamic acid. In appendix table 1 the amino acids present in the uterine and oviducal milieus are presented.

Schultz et al. (1981) measured endogenous pool sizes of 17 amino acids of mouse eggs, eight-cell embryos and blastocysts by estimation of the fluorescent product of the reaction of o-phthalaaldehyde and primary amines. Taurine, glycine, alanine, glutamate and aspartate were detected at high levels.

Iritani (1974) identified 17 amino acids in oviduct and uterine fluid of the sow. Glycine, as in the rabbit (Iritani et al. 1971) was the predominant followed by alanine, valine and lysine. The individual amino acids and concentrations are given in appendix table 2 as found in the oviduct or uterus during estrus and diestrus.

Amino acid metabolism. It has been shown that a significant increase in protein synthesis occurs between the morula and early blastocyst stages. This is also the stage of increased growth and differentiation following cleavage (Brinster, 1971, and Epstein and Smith, 1973). This follows a period of endogenous protein use by the mouse embryo during the first three days after ovulation and fertilization. Brinster (1967) showed a 26% decrease in protein content of mouse embryos developing from one-cell to blastocysts. Brinster (1971) pointed out that there is a relatively low level and rate of amino acid incorporation into new protein before the eight-cell stage. This

occurs even though the number of cells were double every 12 hours, suggesting that the new nuclei are not yet functioning. Similar observations for early rabbit embryos were reported by Manes and Daniel (1969).

Most studies have employed radioactive amino acid precursors in the culture medium and measured the amount of incorporation (acid insoluble fraction) of amino acids between fertilization and implantation (Monesi and Salfi, 1967; Manes and Daniel, 1969). However, when measurements are made of the total uptake (incorporation plus acid soluble uptake), it is found that soluble uptake increases at a rate similar but not identical to incorporation (Tasca and Hillman, 1970; Brinster, 1971c).

Schultz et al. (1981) found a two to three fold increase in amino acid pools during blastocyst formation, however, taurine and glycine pools decreased to about 50 and 10%, respectively of the egg value. This change in amino acid pool size is relevant to the increase in protein metabolism which has been observed by many investigators to accompany the morula to blastocyst transition (Monesi and Sulfi, 1967; Brinster 1971; Epstein and Smith, 1973; Brinster et al. 1976; Abreu and Brinster, 1978; Schultz et al. 1981).

Smith and Smith (1971) showed that estradiol 17 B can stimulate uptake and incorporation of amino acids in

preimplantation embryos by acting directly on the blastocyst, and they suggest that the mouse embryo may develop an estrogen sensitive mechanism just prior to implantation.

Serum requirement for in vitro development. As a source of macromolecules, homologous or heterologous inactivated blood serum or its albumin fraction is an essential component of most culture media (Kane, 1978). Eagle (1959) observed that most mammalian cell cultures required a serum protein and serum protein plays a role in the adhesion of the cells to glass in culture as well as acting as a carrier of unidentified growth factors which are bound to the protein and which are slowly released into the medium.

Several studies have investigated the role of serum on growth of mouse embryos. Spindle and Pedersen (1973) determined that during post-blastocyst development in vitro the mouse embryo becomes dependent on nitrogen sources other than its endogenous amono acid pool, one being a non-dialyzable component of serum. Hsu (1973) has found that, in mouse embryos, fetal calf serum helps in inducing the egg cylinder stage and human cord serum is required for formation of the embryo proper and the somites. Hsu's system used 10% fetal calf serum added to medium for early culture. Later a mixture of 10% fetal calf serum and 10% human cord serum (on days 3 and 4 of culture) then

on day 7 of culture and after 20% human cord serum plus Minimum Essential Medium (MEM).

Kane and Foote (1970) found that the addition of between 6.25 and 25% serum to a modified Hams F10 medium was optimal for blastocoel formation by rabbit embryos. They also found that the components necessary for blastocyst formation were present in the dialyzable portion and that the nondialyzable serum fraction did not promote blastocyst formation. Kane (1979) stated that one-cell rabbit ova may grow to morphological morulae in a simple salt solution containing defatted Bovine Serum Albumin (BSA), however these are not capable of later development to the blastocyst stage unless the defatted BSA has been supplemented with either pyruvate or certain fatty acids.

Nilausen (1978) demonstrated that dialyzed serum albumin had considerable growth-promoting effects on cultivated hamster cells. With the fatty acids removed, the effect was virtually lost and it was completely restored by recombination of the fatty acid-free albumin with the isolated and purified fatty acids. Nilausen (1978) also considers that BSA may be a protectant against the toxic effects of fatty acids in free solution. When fatty acids were added in the absence of albumin growth inhibition occurred, except in extremely dilute solutions. Beta-lactoglobulin, a protein also possessing the ability to bind and release fatty acids, replaced albumin in the

presence of fatty acids with a similar growth-promoting effect. As far as individual fatty acids and their effect, Nilausen (1978) showed that all unsaturated fatty acids tested were growth-promoting, whereas the saturated acids were growth inhibiting, with the exception of stearic acid in low concentrations. This protecting effect, by albumin, shown by Nilausen (1978) was demonstrated to be effective against toxic effects of cuprous and cupric chloride by Holland and Pike (1978). With the absence of BSA, cuprous and cupric chloride completely inhibited the development of mouse morulae and early blastocysts. Adding BSA to the medium partly protected the embryos against the toxic effect of both copper ions (Holland and Pike, 1978).

Bovine Serum Albumin is a necessary component of most mammalian embryo culture systems using otherwise chemically defined media. In swine embryo culture, Davis and Day (1978) showed growth with buffered salt solutions supplemented with BSA. Graves (1978) suggested that lower osmolarity (276 mosm) and higher BSA concentration (4g/l) were beneficial for blastocyst formation by eight-cell porcine embryos. Hermann, Schafer and Holtz (1981) reported a failure of two-cell swine embryos to cleave unless heat inacted bovine serum was added to the Dulbecco's medium used. Robl and Davis (1981) stated that requirements for swine embryo development <u>in vitro</u>

change at about the time of blastocoel formation, since modified Krebs-Ringer Bicarbonate (mKRB) will not support development of day 8 blastocysts unless supplemented with serum, and that hatching percentage is improved by including serum in the culture medium for morulae. They used 10% sheep serum, 10% fetal calf serum, or 10% pig serum, but pig serum did not support growth or survival. When mKRB plus 10% sheep serum was used 54.4% initiated hatching with 18.2% escaping from the zona pellucida, while culture in mKRB alone resulted in 4.5% initiate hatching with none completely escaping from the zona pellucida. Shaffer and Wright (1978) supplemented Ham's F-10 and Minimum Essential Medium (MEM) with 0, 10, or 40% porcine serum over substrates of plastic or collagen. Blastocysts in serum-free media showed some attachment, however only 31% of those blastocysts which attached showed subsequent development as compared to 93% of those blastocysts which attached in serum added medium showing trophoblastic outgrowth. There were no significant differences between treatments with collagen or plastic substrates. They also concluded that using MEM+10% porcine serum would be the optimum medium since MEM contains fewer ingredients than Ham's F-10 and no differences between 10% and 40% porcine serum were observed.

Serum contains an ovicidal factor as demonstrated by Chang (1949). This factor is present in sera of humans,

sheep, cattle, goats, and chickens, however the rabbit, horse, dog, guinea pig and rat serum lack the factor. Pig serum has a small fraction of ovicidal factor. Ovicidal factor, probably part of the compliment system, is destroyed by heat treatment of 30 min. at 55 C (New, 1966).

Energy requirements. Wordinger and Brinster demonstrated that, in mouse embryos, glucose is a necessary factor for in vitro hatching. Presumably, glucose is used as an energy source for blastocyst expansion and contraction during hatching. They also showed that hatching can be delayed by culturing blastocysts in media without glucose. Thompson and Brinster (1966) found that energy was primarily stored as glycogen. Brinster (1969) examined the incorporation of pyruvate and glucose into glycogen and found an incorporation rate of about 2% of that which is required to account for the increase in glycogen content. The rest must come from other sources, possibly endogenous protein. Ozias and Stern (1973) found that external glucose has significant positive effect on blycogen accumulation in the embryo.

In sheep (Boone, 1978), pyruvate and/or lactate can be used by the early embryo. Brinster's Medium for Ovum Culture (BMOC-2) salts plus glucose failed to support growth of eight-cell ovine, or 16-cell or morula bovine embryos. However the balanced salt solution plus

α-ketoglutarate was highly effective for culturing 16-cell bovine embryos to the "hatching" stage, with 95% (21/22) of the 16-cell and early morulae embryos developing to expanded or partially hatched blastocysts.

In the pig, an inhibitory effect was seen by Davis and Day (1978) when pyruvate and lactate were included in the media, and an increase in percentage of four-cell eggs reaching the blastocyst stage when they were removed (13% of 4-cell eggs reaching blastocyst in the presence of pyruvate and lactate and 42-66% of four-cell eggs forming blastocysts with pyruvate and lactate omitted).

Another point to consider is the interaction of the energy substrate and hydrogen ion concentration of the medium. Brinster (1965c) demonstrated that the optimum concentration of the substrate (pyruvate, lactate, oxaloacetate and phosphoenol pyruvate) was influenced by the pH of the medium. This was a positive relationship with an increase in pH necessitating an increase in substrate concentration for optimum growth.

CHAPTER III

THE EFFECT OF MEDIUM AND SERUM ON DEVELOPMENT OF SWINE EMBRYOS IN VITRO

Introduction

Several investigators have shown that pig embryos will develop in vitro. Hafex et al. (1966) reported one to two cleavage divisions in a medium with equal parts Minimum Essential Medium (MEM) and pig serum. Pope and Day (1970) cultured one and two cell pig embryos in Brinster's Medium for Ovum Culture (BMOC-2) with 89 and 96% cleaving in 24 hours, respectively. Linder and Wright (1978) reported hatching of swine embryos cultured from the four-cell stage in Whitten's medium containing 1.5% BSA, and Davis and Day (1978) found that a balanced salt solution containing 4% BSA would support blastocyst formation by four-cell pig embryos.

Embryo transfer in farm animals, especially cattle, has drawn considerable interest in recent years. The use of embryo transfer provides a means of increasing the offspring from genetically superior individuals, introducing new genes in a closed herd, and improving the breeding performance of economically important livestock. Embryo transfer necessitates the handling of embryos in an artificial environment. Therefore embryo culture

has applications in this animal breeding technique.

In order to study the requirements of preattachment pig embryos, we have utilized embryo culture of blastocyst and cleavage stage embryos recovered on days 4 to 6 of pregnancy.

Experimental Procedures

Animals. Embryos for this research were collected from Yorkshire and Yorkshire x Duroc gilts and sows which were checked for estrus twice daily with an intact boar. Breeding was either by artificial insemenation or hand mating at 12 and 24 hours and 24 and 36 hours after first detected estrus for gilts and sows, respectively.

Recovery of embryos. Embryos were recovered surgically under aseptic conditions on day 4 to 6 of pregnancy (onset of estrus=day 0). A midventral incision was made, approximately 13 cm in length, to expose the reproductive tract. One uterine horn was exposed at a time and kept moist with .9% NaCl. A small incision was made with the blunt end of a scalpel handle approximately two-thirds of the distance from the oviduct to the uterine bifurcation. A Foley catheter[1] was inserted through this incision into the uterine lumen. The uterine horn was flushed twice with 20 ml of medium which was injected into the ampulla with an 18 ga. animal feeding needle

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[1]Silicone rubber, (24Fr, 5cc).

inserted through the infundibulum. The uterine horn was then massaged to force the fluid through the catheter and into a petri dish. The catheter was removed and the incision closed using 00 absorbable suture. The number of corpora lutea were counted and recorded. This procedure was repeated for the other uterine horn and the abdominal incision closed.

Preparation of culture equipment. Glassware used for embryo culture was washed in 7x cleaning solution[2], rinsed 15 times with tap water, three times with distilled water, and once with deionized distilled water. Equipment was then dried in an oven and stored in autoclave bags[3] until autoclaved for use.

Glass pasteur pipettes were used for handling of embryos. These were prepared by washing as described above and forming a fine curved tip by pulling over a bunsen burner. A curved tip enables easier handling of embryos in small culture plates.

Medium. The control and flush medium used in this research was a modified Krebs-Ringer Bicarbonate (mKRB, Davis and Day, 1978). Two commercially prepared complex media were used in comparisons through portions

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[2]Flow Laboratories, Inc., McLean, Va., 22102.

[3]Central States Diversified, Inc., St. Louis, Mo., 63115.

of this research, CMRL 1066 and MEM[4]. The individual components[4] of MEM were also used separately and are presented along with the previously mentioned media formulations in tables 1-3. These media were selected from results reported by Hsu (1972, 1973, 1979) for mouse embryo culture. Osmolarity for the various combinations of media had an observed ranged from 251 to 298 mosm as measured by freezing point depression (table 7). PH was also taken and all media fell in the range of 6.9-7.3 (table 4).

Preparation of culture medium. The mKRB used for flushing and control treatments was made up in 500 ml or 1000 ml lots at time of use. The medium was sterilized by filtration, accomplished by positive pressure through a .22µm filter into 100 or 250 ml sterilized bottles. The bottles were two-thirds filled and the remaining space replaced with 5% CO_2:95% air. The 100 ml culture medium bottles were gassed 3 minutes and the 250ml bottles 5 minutes. CMRL and MEM were also equilibrated with 5% CO_2:95% air and all media were warmed to 37C prior to culture. In experiments where different combinations of ingredients were used, each ingredient was added separately at the concentration indicated.

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[4]GIBCO Laboratories, Grand Island, New York, 14072.

Table 1. MODIFIED KREBS-RINGER BICARBONATE[a]

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[b]	4.000	
Penicillin G	.063	
Streptomycin sulfate	.050	

[a]Davis and Day, 1978.

[b]Fraction V.

Table 2. FORMULATIONS OF MEDIA USED

Ingredient	MEM[a] (mg/l)	MEMα[b] (mg/l)	CMRL1066[c,d] (mg/ml)
$CaCl_2$(anhyd)	200.00	200.00	200.00
KCl	400.00	400.00	400.00
$MgSO_4 \cdot 7H_2O$	200.00	200.00	200.00
NaCl	6800.00	6800.00	6799.00
$NaHCO_3$	2200.00	2200.00	2200.00
$NaH_2PO_4 \cdot H_2O$	140.00	140.00	140.00
Deoxyadenosine	---	10.00	10.00
Deoxycytidine	---	11.00	10.00
Deoxyquanosine	---	10.00	10.00
Diphasphopyridine nuclecticle	---	---	7.00
D-Glucose	1000.00	1000.00	1000.00
Phenol red	10.00	10.00	20.00
Lipoic acid	---	.20	---
Sodium pyruvate	---	110.00	---
Deoxythmidine	---	10.00	---
Thymidine	---	---	10.00
L-alanine	---	25.00	25.00
L-arginine	---	105.00	---
L-arginine·HCl	126.00	---	70.00
L-aspartic acid	---	30.00	30.00
L-asparagine·H_2O	---	50.00	

Table 2. (Continued)

Ingredient	MEM[a] (mg/l)	MEMα[b] (mg/l)	CMRL1066[c,d] (mg/ml)
L-cystine	24.00	24.00	20.00
L-cysteine $HCl \cdot H_2O$	---	100.00	260.00
L-glutamic acid	---	75.00	75.00
L-glutamine	---	297.00	---
Glycine	---	50.00	50.00
L-histidine	42.00	---	20.00
L-isoleucine	52.00	52.50	20.00
L-leucine	52.00	52.40	60.00
L-lysine (free base)	---	58.00	
L-lysine HCl	72.50	---	70.00
L-methionine	15.00	15.00	15.00
L-phenylalanine	32.00	32.00	25.00
L-proline	---	40.00	40.00
L-serine	---	25.00	25.00
L-threonine	48.00	48.00	30.00
L-tryptophone	10.00	10.00	10.00
L-tyrosine	36.00	36.00	40.00
L-valine	46.00	---	25.00
L-ascorbic acid	---	50.00	50.00
Biotin	---	.10	.01
D-Ca pantothenate	1.00	1.00	.01
Choline Chloride	1.00	1.00	.50

Table 2. (Continued)

Ingredient	MEM[a] (mg/l)	MEMα[b] (mg/l)	CMRL1066[c,d] (mg/ml)
Folic acid	1.00	1.00	.01
i-inositol	2.00	2.00	.05
Nicotinamide	1.00	1.00	.03
Pyridoxal HCl	1.00	1.00	.03
Riboflavin	.10	.10	.01
Thiamine HCl	1.00	1.00	.01
Vitamin B_{12}	---	1.36	---
Adenosine	---	10.00	---
Cytidine	---	10.00	---
Guanosine	---	10.00	---
Uridine	---	10.00	---
Uridine triphosphate	---	---	1.00
Cholesterol	---	---	.20
Pyridoxine HCL	---	---	.03
Para-aminobenzoic acid	---	---	.05
Niacin	---	---	.03
Co carboxylase	---	---	1.00
Coenzyme A	---	---	2.50
Ethanol for solubilizing lipid components	---	---	16.00
Flavin adenine dinucleotide	---	---	1.00
Glutathione (reduced)	---	---	10.00
S-methyl-deoxycyticline	---	---	.10

Table 2. (Continued)

Ingredient	MEM[a] (mg/l)	MEMα[b] (mg/l)	CMRL1066[c,d] (mg/ml)
Sodium acetate·$3H_2O$	---	---	83.00
Na glucoronate·H_2O	---	---	4.70
Triphosphopyridine	---	---	1.00
Tween 80	---	---	5.00

[a,b,c,] GIBCO Laboratories, Grand Island, New York, 14072.

[d] CMRL + is equivalent to CMRL plus 100 mg/l of L-glutamine (only included in Exp. I, Trial 2).

Table 3. MEM SUPPLEMENTS[a]

Essential Amino Acids (mg/ml)		Non-Essential Amino Acids (mg/ml)		Vitamin (mg/ml)	
L-arinine·HCL	21.00	L-alanine	8.90	NaCL	85.00
L-cystine	12.00	L-asparagine·H_2O	15.00	Biotin	1.00
L-glutamine	---	L-aspartic acid	13.30	D-Ca pantothenate	1.00
L-histidine	8.00	L-glutamic acid	14.70	Choline chloride	1.00
L-isoleucine	26.00	Glycine	7.50	Folic acid	1.00
L-lysine HCL	36.47	L-proline	11.50	i-inositol	2.00
L-methionine	75.00	L-serine	10.50	Nicotinamide	1.00
L-phenylalanine	16.50			Pyridoxal HCL	1.00
L-threonine	24.00			Riboflavin	.10
L-tryptophane	4.00			Thiamin HCL	1.00
L-tyrosine	18.00				
L-valine	23.50				

[a]GIBCO Laboratories, Grand Island, New York, 14072.

Table 4. MEASURED OSMOLARITY AND pH OF VARIOUS MEDIA

Medium	mosm	pH
mKRB+fcs	278	7.0
mKRB without BSA	251	7.1
Fcs	285	7.0
CMRL	298	6.9
CMRL+L-glutamine	291	6.9
MEM	272	6.9
MEMα	287	7.0
MEM:10[a]	277	7.1
MEM:50[b]	276	7.1
mKRB	252	7.0
mKRB+Vitamin solution	259	7.2
mKRB+Essential Amino Acids	290	7.3
mKRB+Non-Essential Amino Acids	257	7.2

[a]Ten percent MEM, 90% mKRB.

[b]Fifty percent MEM, 50% mKRB.

Preparation of serum. Fetal Bovine Serum[5] (fcs) prior to use was heated in a water bath at 56 C for 30 min. (New, 1966). The serum was allowed to cool and added to the culture medium within two days prior to culture.

In experiment 4, dialyzed fcs was prepared by placing fcs in a dialysis "sack"[6] and suspending in a 200 ml Erlenmeyer flask filled with deionized distilled water (ratio of 400:1, water:fcs), a sterilized magnetic stirring rod was then added to the flask before covering the flask opening with alluminum foil. The flask was then placed in an ice bath contained in a plastic tub on top of a stir plate. The serum was allowed to dialyze for 8 hours with distilled water changed every 2 hours. The distilled water was again changed after 8 hours dialysis and the flask was allowed to sit in a refrigerator overnight before the serum was removed and heat treated as described above.

Embryo culture. After recovery the embryos were handled under a bench type hood made of plexiglass equipped with a UV light. The petri dish, containing a flush medium and embryos, was placed on the stage of a Zeiss stereomicroscope for observation (5-50x) of the recovered embryos. The embryos were removed with a sterilized glass

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[5]KC Biological, Inc. Lenexa, Kansas, 66215.

[6]SIGMA Chemical Co., St. Louis, Mo., 63178.

pipette and rinsed three times in fresh medium. Embryos were pooled by sow and allocated equally, by initial cell stage, to all treatments. The embryos were cultured in microtiter plates,[7] 96-wells with U-bottoms (radiation sterilized) containing approximately .35 ml of medium. The plate was then placed in a desicator and gassed with a humidified mixture of 5% CO_2:95% air and the desicator sealed. The desicator was incubated at 37 C in a water jacketed incubator for 96 hours. Embryos were transferred to fresh medium after 48 hours.

Measurements. Three indicators of growth and development were used; nuclei number, blastocyst diameter, and cell stage.

Nuclei number was determined from aceto-orcein stained slides using phase contrast microscopy. The technique of slide preparation is outlined in a later section. Nuclei were counted with the aid of an ocular grid in the eyepiece. Nuclei for each embryo were counted twice and averaged. Diameter was determined for all blastocysts using an ocular micrometer eye piece. Cell stage was recorded at beginning of culture, and after 24, 48, 72, and 96 hours.

Preparation of slides. Whole mount slides were prepared for all cultured embryos. Four drops of a paraffin-vaseline (50:50 mixture) mixture was placed on one end of

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[7]Costar, Cambridge, Ma. 02139

a microscope slide to support a 22x22 mm cover slip. A small amount of rubber cement was placed between the top two and bottom two drops. The embryo was transferred to the slide and extra fluid removed leaving a minimal amount of fluid on the slide with the embryo. The cover slip was placed over the four drops of para-vaseline and then pressure carefully applied until the cover slip came in contact with the zona pellucida. A bead of para-vaseline was placed on the top and bottom edges of the coverslip and the slide placed in 40ml acetic ethanol (1:3) to clear for 12 to 24 hours. After clearing the slide was stained by drawing acetic orcein (1% orcein in 45% acetic acid) under the cover slip. The cover slip was sealed with a bead of vaseline and allowed to stain for 6 to 10 hours after which excess stain was removed by drawing 45% acetic acid under the cover slip.

Statistical analysis. Percentage datum was analyzed by Chi-square (Snedecor and Cochran, 1980). All other datum was analyzed by least square procedures (SAS, 1979) using the following model:

$$Yij=u + TI + Sj + Ti*Sj + Eijn$$

where,

Yij= nuclei or diameter or volume of the nth embryo.
u= overall mean
Ti= effect of the ith treatment
Sj= cell stage at the beginning of culture
Ti*Sj= treatment by initial cell stage effect
Eijn= random error associated with the nth embryo

The above model was used for all experiments measuring those parameters except for the nuclei number in experiment IV where a missing cell prevented direct examination of the interaction. Least square means and standard errors were calculated using the model following:

$$Yij = u + Ti + Sj + Eijn$$

where the same values are associated with the variables. For nuclei number in experiment IV this model has been modified by removing the interaction term to enable the standard error to be calculated on treatment 15, initial cellstage 7 (early blastocyst). The remaining cells of the design were evaluated for a significant interaction but none was present.

Results

Experiment I. In experiment I two trials were conducted to compare two commercially prepared complex culture media to mKRB. The two commercially prepared media were Minimum Essential Medium (MEM) and CMRL 1066 (CMRL). All media were supplemented with 10% fcs and the initial embryo stage was morula. Percentage of morulae forming blastocysts was superior for mKRB as compared to CMRL ($P < .05$) but not different from MEM (table 5). Embryos cultured in mKRB had greater nuclei numbers than MEM or CMRL ($P < .05$) and embryos cultured in MEM had more nuclei than those cultured in CMRL ($P < .05$) (table 5).

Table 5. DEVELOPMENT OF EMBRYOS IN EXP. I

Culture medium[d]	No. forming blastocyst		Nuclei/embryo (no. observations)
	Initial cell stage		
	2-8 cell (no. obs.)	morula (no. obs.)	
Trial 1			
mKRB	1 (7)	7[a] (7)	40.00[a] (7)
CMRL	1 (6)	2[b] (9)	6.25[b] (11)
MEM	0 (7)	6[a,b] (10)	15.36[b] (12)
Trial 2			
mKRB-BSA	3 (11)	7 (8)	21.2[a] (10)
CMRL+	0 (8)	7 (10)	15.94[b] (16)
MEM alpha	0 (8)	5 (8)	8.5[c] (10)

[a,b,c]Values with different superscripts are different (P .05).

[d]All medium supplemented with 10% fetal calf serum.

in trial 2, L-glutamine was added to CMRL (100 mg/ml) and MEM alpha (which contains ribonucleosides and deoxyribonucleosides) replaced MEM. All media were supplemented with 10% fcs and BSA was omitted from mKRB. There were no differences, in percentage of morulae forming blastocysts (table 5). Nuclei numbers were greater in mKRB treatment ($P<.05$) than other treatments and morulae cultured in CMRL had more nuclei than those cultured in MEM alpha ($p < .05$) (table 5).

Experiment II. The preliminary trials in experiment I indicated a detrimental effect for the more complete media. Experiment II was designed to compare increments of MEM (added to mKRB) to mKRB. These treatments were mKRB, mKRB plus 10% MEM (MEM 10), and mKRB plus 50% MEM (MEM 50). The percentage of morulae and expanded blastocysts that hatched during culture was greater for mKRB and MEM 10 ($P < .05$) than for MEM 50. No differences in nuclei number were detected (table 6).

Experiment III. Since the more complete media were consistantly inferior to mKRB in supporting in vitro development, the effect of three groups of ingredients in the complex media were studied. These ingredient groups were vitamins, non-essential amino acids, and essential amino acids. There was no differences in nuclei number or percentage of morula stage embryos reaching the blastocyst stage for any of the treatments (table 7).

TABLE 6. DEVELOPMENT OF EMBRYOS IN EXP. II

Culture medium[a]	No. Hatched[b] (no. obs.)	% Hatched	Nuclei/embryo (no. obs.)
mKRB	7[c] (41)	17.07	53.05 (38)
MEM:10	8[c] (41)	19.51	50.51 (35)
MEM:50	0[d] (41)	.00	36.56 (34)

[a]All treatments supplemented with 10% fetal calf serum.

[b]Initial cell stage morula-expanded blastocyst.

[c,d]Values with different superscript are different ($P < .05$).

Table 7. DEVELOPMENT OF EMBRYOS IN EXP. III

Culture medium[a]	No. morula forming blastocyst (no. obs.)	Nuclei/embryo (no. obs.)
mKRB	11(12)	80.67(21)
mKRB+vitamins	13(15)	88.64(25)
mKRB+non-ess. amino acids	10(13)	80.45(20)
mKRB+ess. amino acids	7(9)	41.86(14)

[a]All treatments supplemented with 10% fetal calf serum.

However, mKRB plus essential amino acids tended to be lower than the others in nuclei number (table 7).

Experiment IV. Experiment IV was designed to examine the effect of the serum components on development. Four treatments were used in this experiment: mKRB, mKRB plus serum, mKRB plus dialyzed serum, and also mKRB plus dialyzed serum plus vitamins plus essential amino acids plus non-essential amino acids. Beginning cell stage was blastocyst. The nondialyzable portion of the serum should contain only large molecules. The percentage of blastocysts hatching were not different (table 8). The lowest percent hatching occurred in mKRB without serum treatment (32.14%) with the highest occurring in the mKRB plus dialyzed serum (63.33%). Blastocysts cultured in mKRB plus serum and mKRB plus dialyzed serum had more nuclei and reached greater volumes than blastocysts grown in mKRB or mKRB plus individual components ($P < .05$). There was no difference in nuclei number between mKRB and mKRB plus individual components (table 8). There was no difference between mKRB plus serum or mKRB plus dialyzed serum in maximum volume, nor was there a difference between treatments mKRB and mKRB plus individual components for this characteristic (table 8).

Discussion

In the preliminary trials of experiment I, mKRB tended to be superior to the two commercial media in

Table 8. DEVELOPMENT OF EMBRYOS IN EXPERIMENT IV

Culture Medium	No. Hatched (no. obs.)	% Hatched	Nuclei/embryo ± S.D.[d]		Volume (mm^3)
			Mean	S.D.	
mKRB	9(28)	32.14	40.36[c]	34.45	3680.94[b]
mKRB+serum	15(29)	51.72	231.64[a]	35.88	9674.80[a]
mKRB+dialyzed serum+	19(30)	63.33	115.37[b]	33.15[b]	8602.90[a]
mKRB+dialyzed serum+ individual components	14(32)	43.75	50.34[c]	33.82	3557.13[b]

[a,b,d]Values with different superscripts are different (P .05).

[d]Standard Error

percent forming blastocyst as well as being superior in nuclei number. The more complete media seemed to be detrimental to embryo growth. All treatments contained 10% fetal calf serum and possibly the protein content or amino acids were at two high of levels when combined with the amino acids already in the complex medium. Spindle and Pederson (1973) showed inhibitory effects, in mouse embryos, in some cases with addition of non-essential amino acids. Glutamine had the greatest inhibitory effect in their experiments. Brinster (1965) also suggested two-cell embryos required several amino acids in approximately the proportion in which they occur in serum albumin. The greater amino acid content of the complete media may be toxic.

In experiment II mKRB plus 50% MEM was detrimental by the embryos cultured in complete medium added at 10% to mKRB responded similarly to embryos cultured in mKRB. These results support the earlier conclusion that the concentration of ingredient(s) was at too high a level resulting in suppression of development. However in experiment III no individual group of nutrients displayed the inhibitory effects observed in the first two experiments. Possibly a combination of factors is required to suppress growth.

Serum is definitely growth promoting (experiment IV) as indicated by nuclei number and blastocyst volume. This

agrees with earlier results (Robl and Davis, 1981). While no significant effects were observed for percent hatching, the lowest hatching percent was observed for nonsupplemented mKRB (32.14%). mKRB plus dialyzed serum had the highest percentage hatching but blastocysts grown in medium containing dialyzed serum had fewer nuclei (P .05). Spindle and Pederson (1973) determined that for post-blastocyst development of mouse embryos, the embryo becomes dependent on nitrogen sources other than its endogenous amino acid pool as well as a non-dialyzable compent of serum. In our studies (experiment IV) addition of dialyzed serum increased the number of nuclei and maximum volume attained during culture as compared to nonsupplemented mKRB. However, dialyzed serum was not equivalent to nondialyzed serum since blastocysts cultured in the presence of the latter had more nuclei. When mKRB plus dialyzed serum was made more complete by adding essential and nonessential amino acids and vitamins development was inhibited as compared to mKRB plus dialyzed serum alone. This suggests that the negative effects observed for the more complete media in experiment I and II may be attributable to the combination of the ingredients. The negative effects of these ingredients is puzzling but must be resolved if further progress in the culture of swine blastocysts is to be attained. In addition this effect may have relevance to <u>in utero</u> embryo survival since high levels of maternal nutrient

intake, which might be expected to increase nutrient levels in the uterine lumen, is widely believed to decrease embryo survival in early pregnancy (Robertson, et al., 1951; Bazer, et al., 1968).

Using nuclei number and blastocyst volume as indicators of growth, a growth promoting effect was seen demonstrated for mKRB serum supplementation (either non-dialyzed and dialyzed; experiment IV). Nondialyzed serum was superior to dialyzed serum in nuclei number ($P < .05$) and tended to support a greater maximum volume obtained during culture. Therefore a component in non-dialyzed serum is beneficial to blastocyst growth and is not present after dialysis, however a growth promoting effect is still present in dialyzed serum. These effects of serum are similar to those observed with mouse blastocysts (Spindle and Pederson, 1973; Gwatkin, 1966). Addition of vitamins and essential and nonessential amino acids to mKRB+dialyzed serum resulted in embryonic development comparable to nonsupplemented mKRB, a medium which is inadequate for blastocyst culture (Robl and Davis, 1981). Therefore, the additions were either toxic, or growth suppressive. In experiment III none of these components, added individually, was suppressive. Therefore, two or more ingredients, present in different groups, must interact to decrease in vitro growth. Further research will be required to determine the ingredients responsible for this effect.

CHAPTER IV

SUMMARY

The effects of two commercially prepared complex culture media, and certain of their constituents, on in vitro swine embryo development was compared to a modified Krebs-Ringer bicarbonate medium (mKRB) in four experiments. The effect of dialyzed and nondialyzed serum, as a supplement to mKRB, was also investigated in the fourth experiment. The two commercially prepared media were Minimum Essential Medium (MEM) and CMRL 1066 (CMRL). Experiment I, trial 1, compared MEM and CMRL to mKRB, all treatments were supplemented with 10% fetal calf serum (fcs). Percentage of morulae forming blastocysts were superior for mKRB as compared to CMRL ($P < .05$) but not different from MEM. MKRB embryos had greater nuclei number than MEM or CMRL ($P < .05$) and embryos cultured in MEM had more nuclei than those cultured in CMRL ($p < .05$). In trial 2, experiment I CMRL plus L-glutamine (100 mg/ml) and MEM alpha (which contains ribonucliosides and deoxyribonucleosides) were compared to mKRB without BSA. All media were supplemented with 10% fcs. There was no difference in percentage of morulae forming blastocysts, however, nuclei numbers were greater in mKRB treatment ($P < .05$) with CMRL plus L-glutamine

having more nuclei than MEM alpha (P .05).

In experiment II, either 10% or 50% MEM (MEM 10 and MEM 50, respectively) was added to mKRB and compared to mKRB, all media were supplemented with 10% fcs. The percentage of morulae and expanded blastocyst that hatched during culture was greater for mKRB and MEM 10 ($p < .05$) than for MEM 50 with no differences in number of nuclei at end of culture.

Experiment III compared the addition of three individual components of MEM (essential amino acids, non-essential amino acids, and vitamins) to mKRB. There were no differences in nuclei number or percentage of morula stage embryos reaching the blastocyst for any of the treatments.

Experiment IV was designed to examine the effect of serum components on development. Modified Krebs-Ringer bicarbonate served as a control which was compared to mKRB plus either dialyzed serum, nondialyzed serum, or dialyzed serum plus the individual components of MEM used in experiment III. There were no differences among treatments for percentage of blastocysts hatching. The lowest percent hatching was observed in unsupplemented mKRB (32.14%) with the highest occurring in the mKRB plus dialyzed serum (63.33%). Modified KRB plus serum and mKRB plus dialyzed serum blastocysts had more nuclei and reached greater volumes than those cultured in mKRB or mKRB plus the MEM supplements ($P < .05$). Treatments mKRB and mKRB

plus MEM supplements did not differ in nuclei number or volume. There was also no difference between mKRB plus serum and mKRB plus dialyzed serum for volume.

The results of this research indicate that the mKRB plus 10% fcs supported the greatest development in vitro of the media examined. A growth-promoting effect was definitely observed with the addition of 10% fcs and a partial effect with dialyzed fcs added to mKRB. There appeared to be toxic effect from the complex media (MEM and CMRL) by either an interaction among substrates or too high a concentration of certain ingredients present.

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APPENDIX

APPENDIX TABLE 1. CONTENT OF TOTAL FREE AMINO ACIDS IN RABBIT REPRODUCTIVE TRACT FLUID(μM/ml)[a].

Amino acids	Oviduct fluid[b]	Uterine fluid[c]
Alanine	.469 ± .020	1.017 ± .203
Arginine	.064 ± .013	.141 ± .056
Aspartic acid	.024 ± .005	.193 ± .045
Cysteine and proline	.086 ± .002	.210 ± .070
Cystine	.015 ± .001	.035 ± .008
Glutamic acid	.192 ± .032	.840 ± .079
Glycine	2.766 ± .308	3.559 ± .181
Histidine	.067 ± .027	.143 ± .045
Isoleucine	.069 ± .001	.271 ± .065
Leucine	.129 ± .006	.545 ± .135
Lysine	.165 ± .047	.485 ± .127
Methione	.022 ± .002	.125 ± .047
Phenylalanine	.065 ± .005	.247 ± .060
Serine	.318 ± .034	.909 ± .181
Taurine	.123 ± .015	.491 ± .196
Threonine	.125 ± .006	.425 ± .092
Tryptophan	N[d]	N[d]
Tyrosine	.079 ± .006	.281 ± .039
Valine	.172 ± .010	.417 ± .088
Total	4.936 ± .266	10.364 ± 1.254

[a]Source: Iritani et al. (1971).

[b]Mean ± Standard Deviation for determinations of four pooled samples from 5 rabbits.

[c]Mean ± Standard Deviation for determinations of four pooled samples from 7 rabbits.

[d]Not detected.

APPENDIX TABLE 2. CONTENT OF TOTAL FREE AMINO ACIDS IN SOW OVIDUCT FLUID(μM/ml)[a]

Amino acids	Oviduct fluid[b]	
	Estrus	Diestrus
Alanine	.26 ± .02	.31 ± .11
Arginine	.08 ± .01	.09 ± .02
Aspartic acid	N[bc]	.02 ± .00
Cystine	.01 ± .00	.01 ± .00
Glutamic acid	N[bc]	.14 ± .02
Glycine	.38 ± .10	.57 ± .11
Histidine	.06 ± .00	.06 ± .02
Isoleucine	.07 ± .01	.12 ± .03
Leucine	.13 ± .01	.11 ± .06
Lysine	.12 ± .01	.25 ± .08
Methionine	T[d]	T[d]
Phenylalanine	.07 ± .01	.07 ± .00
Proline	T[d]	T[d]
Serine	N[b]	.05 ± .01
Threonine	N[b]	.08 ± .03
Tyrosine	.05 ± .01	.06 ± .02
Valine	.14 ± .02	.31 ± .11
Total	1.46 ± .25	2.27 ± .63

[a]Source: Iritani et al. (1971)

[b]Estimated Mean ± Standard Deviation for determinations of each three pooled samples from three estrous sows.

[c]Not detected; no peak was detected on the sheet when 5ml of whole fouid was used (sensitivety of analysis: .05 μM).

[d]Trace amounts; peak was detected but less than 101 μM/ml.

APPENDIX TABLE 3. CONTENT OF TOTAL FREE AMINO ACIDS IN SOW UTERINE FLUID (μM/ml)[a]

Amino acids	Uterine fluid[b]	
	Estrus	Diestrus
Alanine	.28 ± .01	.94 ± .31
Arginine	.06 ± .03	.07 ± .01
Aspartic acid	.03 ± .01	.04 ± .01
Cystine	.01 ± .00	.04 ± .01
Glutamic acid	.21 ± .02	.39 ± .10
Glycine	.40 ± .11	1.32 ± .30
Histidine	.07 ± .02	.29 ± .05
Isoleucine	.12 ± .02	.36 ± .08
Leucine	.25 ± .01	.68 ± .19
Lysine	.23 ± .03	1.00 ± .02
Methionine	.02 ± .00	.09 ± .03
Phenylalanine	.09 ± .00	.29 ± .04
Proline	.01 ± .00	.78 ± .21
Serine	.08 ± .00	.06 ± .00
Threonine	.06 ± .01	.06 ± .01
Tyrosine	.08 ± .00	.26 ± .06
Valine	.37 ± .03	.79 ± .05
Total	2.37 ± .50	7.46 ± 2.05

[a]Source: Iritani et al. (1971).

[b]Estimated Mean ± Standard Deviation for deterination of each three pooled samples from three diestrous sows.

THE EFFECT OF MEDIUM AND SERUM ON DEVELOPMENT OF SWINE EMBRYOS IN VITRO

by

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The effects of two commercially prepared complex media and of serum components on in vitro swine embryo development was compared to a modified Krebs-Ringer bicarbonate medium (mKRB) in four experiments. Embryos were recovered surgically on day 4 to 6 of pregnancy (onset of estrus = day 0) from Yorkshire and Yorkshire x Duroc gilts and sows which were checked for estrus twice daily with an intact boar and artificially inseminated or hand mated. The commercial media used were minimum essential medium, (MEM) MEM alpha, and CMRL 1066, plus L-glutamine. The effects of MEM essential amino acids, nonessential amino acids and vitamins were also evaluated. Embryos were cultured in microtiter plates, containing approximately .35 ml of medium (equilibrated with 5% CO_2:95% air), placed in a desicator and gassed with a humidified mixture of 5% CO_2:95% air. Embryos were evaluated every 24 hours for the 96 hours of culture.

Experiment I consisted of two trials. Trial 1 compared MEM and CMRL to mKRB (all treatments supplemented with 10% fcs). Percentage of morulae forming blastocysts was superior for mKRB as compared to CMRL ($P < .05$) but not different from MEM. MKRB embryos had greater nuclei numbers than MEM or CMRL ($P < .05$) and embryos cultured in MEM had more nuclei than those cultured in CMRL ($P < .05$). Trial 2 compared CMRL plus L-glutamine and MEM alpha to

mKRB without BSA; again all media contained 10% fcs. There was no differences in percentage of morulae forming blastocysts, however nuclei numbers were greater for embryos cultured in mKRB ($P < .05$) with embryos cultured in CMRL plus L-glutamine having more nuclei than those cultured in MEM alpha ($P < .05$).

In experiment II, increments of 10 and 50% MEM were added to mKRB and compared to mKRB. All media contained 10% fcs. The percentage of morulae and expanded blastocyst that hatched during culture was greater for mKRB and MEM 10 ($P < .05$) than for MEM 50 with no differences in number of nuclei at the end of culture.

Experiment III compared the addition of certain components of MEM to mKRB (essential amino acids, non-essential amino acids and vitamins). There were no differences in nuclei number or percentage of morulae reaching the blastocyst for any of the treatments.

Experiment IV was designed to examine the effect of the serum components on development. Modified KRB served as a control and was compared to mKRB plus either dialyzed serum, nondialyzed serum, or dialyzed serum plus the MEM supplements. There were no differences among treatments for percentage of blastocysts hatching. However, the lowest percent hatching was observed in unsupplemented mKRB (32.14%) with the highest occurring in the mKRB plus dialyzed serum (63.33%). Modified KRB plus serum and

mKRB plus dialyzed serum blastocysts had more nuclei and reached greater volumes than those cultured in mKRB or mKRB plus MEM supplements ($P < .05$). Culture treatments mKRB and mKRB plus MEM supplements did not differ in nuclei number or volume. There was also no differences between volumes attained by blastocysts cultured in mKRB plus serum or mKRB plus dialyzed serum.

The results of this research indicate that the mKRB plus 10% fcs supported the greatest development *in vitro* of the media examined. A growth-promoting effect was definitely observed with the addition of 10% fcs and a partial effect with dialyzed fcs added to mKRB. There appeared to be a toxic effect from the complex media (MEM and CMRL) by either an interaction among substrates or due to high concentrations of certain ingredients present in the medium.