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Culture of pig embryos

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Pig embryos can be cultured using a number of different strategies including complex approaches like culture *in vivo* in a surrogate oviduct (rabbit, sheep, mouse), culture in mouse oviducts in organ culture, and co-culture of embryos with cells in addition to simple approaches like culture in defined media or salt solutions. Addition of serum to medium has been of particular importance where blastocyst development and hatching are required. Pig conceptuses (day 10–15), embryonic discs or cell lines derived from conceptuses can be cultured in complex media like Eagle's minimal essential medium or Dulbecco's modified Eagle's medium with serum, although embryonic discs can be cultured in the absence of serum. In contrast, early stage pig embryos (one-cell to blastocyst) are best cultured in simpler media such as those used for mouse embryos. The media that have been used are all relatively similar in composition. They contain salts and one or more energy sources such as glucose, lactate, or pyruvate with BSA as a macromolecular component. Early attempts to culture pig embryos were not very successful, but some embryos did develop to the blastocyst stage. More recent reports indicate a much higher rate of development with relatively little or no change in media composition. Some workers have reported improved development in medium lacking glucose, which is consistent with findings with laboratory animals such as hamsters. Glutamine can serve as the sole exogenous energy source in medium lacking glucose, lactate and pyruvate. Addition of taurine and hypotaurine to culture medium enhances development of pig embryos *in vitro*. We suggest, where possible, adoption of a standard medium that could be used by different laboratories and, perhaps, with different species. Use of one medium for different species would simplify experimental protocols, enhance studies of comparative embryonic physiology and metabolism, and expedite transfer of information obtained in different species.

Introduction

Considerable progress has been made in the culture of pig embryos since the comprehensive review of Davis (1985). It is now reasonable to expect that pig embryos can be cultured from the zygote to the blastocyst stage *in vitro* (Reed *et al.*, 1992). At the same time, requirements for a particular experiment may require consideration of alternative methods for the culture of pig embryos. These may include *in vivo* culture in surrogate oviducts or *in vitro* culture in systems ranging from complex to simple or completely defined (Petters, 1992). Furthermore, the culture requirements of pig embryos at different stages may be distinctive and require special solutions. Applications in basic research and in biotechnology will benefit from the increased embryo viability associated with improved culture methods. This review documents current methods for pig embryo culture and indicates areas where further research is warranted. Other aspects are covered in Reed *et al.* (1992a).

In Vivo Culture

The optimal culture environment for pig embryos is the pig reproductive tract itself. Early stage embryos, once removed and manipulated in some way, can be returned to the reproductive tract for short-term culture or for development to term (Day, 1979). The choice of recipients (donor female as recipient, synchronous versus asynchronous) is important and depends upon the experimental goals. If the embryos are to be recovered within a few days, recovery rate can be of some concern, but a high rate of embryo recovery should be expected.

Pig embryos have been cultured in the reproductive tracts of other species. Early stage pig embryos can develop to the morula and blastocyst stages following culture in the ligated oviducts of anoestrous sheep (Prather *et al.*, 1991). The recovery rate from the sheep oviducts was high (83%) and development of one- and two-cell embryos to morula or blastocyst stage was good (72%). Transfer of embryos to recipient females (12 h asynchronous) resulted in offspring. In contrast, the ligated rabbit oviduct seems to be less suited for the culture of pig embryos (Herrmann and Holtz, 1985). Two-cell embryos developed well (95% normal development) in rabbit oviducts, whereas three- to four-cell embryos did not (24–45%). With 48 h of culture, 146 of 170 two-cell embryos (86%) developed beyond the four-cell stage. However, results following embryo transfer indicated a decline in viability if culture was for longer than 24 h in the rabbit oviduct. These results are in contrast to those for bovine or ovine embryos in rabbit oviducts where storage can be for a longer period (Hermann and Holtz, 1985).

The immature mouse oviduct can also be used for the culture of pig embryos. Morula and blastocyst stage embryos developed better in the mouse oviducts than in a complex culture medium (Ham's F-12, 10% fetal calf serum (FCS)) as judged by morphology and number of cells (Papaioannou and Ebert, 1988). However, the immature mouse oviduct could not support the development of pig zygotes beyond the four-cell stage (Ebert and Papaioannou, 1989).

The results cited above can be considered from a different viewpoint. What causes the different responses from pig embryos to different oviduct systems? The sheep oviduct appears to be permissive for pig embryo development, whereas the rabbit and mouse oviduct are not for certain cell stages. The pig oviduct itself only provides a suitable environment for development to the morula stage (Murray *et al.*, 1971). Comparative studies of the oviduct environment could provide much information about early embryonic development which may lead to further improvement of pig embryo culture.

Culture in Mouse Oviducts in Organ Culture and Co-culture with Cells

The explanted mouse oviduct is a suitable culture environment for early embryos of a number of species (Biggers *et al.*, 1962; Whittingham, 1968a, b, c; Minami *et al.*, 1988). Pig embryos can develop from the one-cell to the blastocyst stage in the mouse oviduct in organ culture (Krisher *et al.*, 1989b). Development of the pig embryos does not depend upon the presence of mouse embryos, which are included in the explant as a positive control (Krisher *et al.*, 1989a). Approximately 78% of the embryos ($n = 63$) reached the morula or blastocyst stage in the mouse oviducts compared with 17–36% in modified Krebs' Ringer bicarbonate (mKRB) medium alone (Krisher *et al.*, 1989a, b). These results have been confirmed by Williams *et al.* (1992); however, the overall rate of development was much lower in this study. Bovine embryos can also develop in mouse oviducts, but only when CZB medium (Chatot *et al.*, 1989) is used to culture the explants (Sharif *et al.*, 1991a, b, 1992). Attempts are being made to identify components in the mouse oviduct environment that are beneficial for embryo development (Minami *et al.*, 1992).

Co-culture of pig embryos with cell monolayers including oviductal epithelial cells has been shown to be beneficial (Allen and Wright, 1984; White *et al.*, 1989). However, this technique is not used extensively because of the relative success of defined media.

Addition of Complex Biological Fluids to Culture Media

Addition of oviductal fluid to culture medium enhances the *in vitro* development of one- and two-cell pig embryos (Archibong *et al.*, 1989). Such fluid is not readily available for routine embryo culture

experiments so it has not been used extensively. Pig oviductal fluid has been shown to contain insulin-like growth factor I (IGF-I) and IGF-II (Wiseman *et al.*, 1992). Dilution of culture medium with oviductal fluid results in a predictable dilution of certain culture medium ingredients that are present at lower concentrations in oviductal fluid (Nichol *et al.*, 1992).

Serum supplementation of pig embryo culture media is best discussed by referring to results with different stage embryos. Early stage embryos (one to eight cells) are most often cultured in media using BSA as a protein supplement. However, some studies have used serum supplementation during these stages. Niemann *et al.* (1983) reported the use of 10% heat-inactivated lamb serum with Krebs Ringer bicarbonate medium (KRB), although development in the absence of lamb serum was not assessed. This medium worked well for the culture of single blastomeres from eight-cell embryos when combined with an extracellular matrix of fibronectin (Saito and Niemann, 1991). Offspring were produced from single blastomeres that were cultured *in vitro* for 24 h before embryo transfer. Wollenberg *et al.* (1990) reported that addition of 20% heat-inactivated bull serum to phosphate-buffered saline (PBS) supported the development of two- to eight-cell pig embryos to the hatched blastocyst stage. In this report, it is not possible to determine whether the development of two-cell embryos was supported by this medium or whether only four-cell stage or greater developed. It is not clear whether previous reports from the same group (Herrmann and Holtz, 1981; Herrmann *et al.*, 1981) used PBS also. Furthermore, the earlier reports indicated much lower rates of development with the same medium. For a historical account of repeatability problems, see Davis (1985).

The beneficial effect of serum (fetal calf, FCS or lamb, LS) on pig morulae or blastocysts can be traced to Robl and Davis (1981). Morulae cultured to blastocyst in modified KRB (mKRB) medium hatched only in the presence of serum (Robl and Davis, 1981; Rosenkrans *et al.*, 1989). In another study, hatching did occur in the absence of serum, but at a reduced rate (Meyen *et al.*, 1989). Dialysis of FCS apparently removed a factor that is important for cell division, but factors important for blastocyst expansion were not affected. Serum was also shown to support the development of later stage blastocysts (Robl and Davis, 1981). Day 8 blastocysts grew well over a 48 h period when KRB medium was supplemented with sheep serum or fetal calf serum. In this study, no attachment of the blastocysts to the dish occurred. Day 10 blastocysts grew only if they appeared developmentally similar to the day 8 embryos. Day 12 embryos, which were elongated and wrinkled in appearance, did not survive even for 24 h.

Pig conceptuses (days 10–15) have been cultured *in vitro* for up to 30 h for certain biochemical analyses (Harney *et al.*, 1990). The method used was that of Godkin *et al.* (1982) which uses Eagle's minimal essential medium (MEM) without protein supplementation. Documentation of cell viability was based on a linear release of radioactively labelled proteins from certain day 16 conceptuses over the period of 4–24 h of culture. Primary cell cultures established from day 14 and day 16 pig conceptuses (MEM plus 15% FCS) resulted in large, free-floating spheres as well as confluent cell cultures (Godkin *et al.*, 1985). These cultures did not produce the same proteins as are found in the short-term cultures. It was suggested that the trophoblast cells did not survive well in culture. The formation of vesicles was also noted by Allen and Wright (1985) using MEM plus 10% FCS. Vesicles were formed only when cells were enzymatically dispersed from day 12–15 blastocysts, not from day 10 and 11 blastocysts. The hypothesis is that these vesicles are formed from embryonic disc material rather than from trophoblast cells.

Silcox and Johnson (1988) reported the culture of day 13 porcine embryonic discs in DMEM (Dulbecco's modified Eagle's) plus 10% newborn bovine serum. Vesicles formed during the first 24 h of culture. These explants could be cultured for up to 10 days with a number developing pulsating tissue implying mesoderm differentiation. Estrada *et al.* (1991) cultured embryonic discs in DMEM without serum and demonstrated that IGF-I stimulated protein synthesis *in vitro*, suggesting a physiological role for the IGF found in oviductal fluid.

Culture in Defined Media

Research with laboratory animals, such as mice and hamsters, has been very important for pig embryo culture. The formulations used for pig embryo culture and base media used first with mouse embryos are

shown (Table 1). In preparing this table, we noted a number of apparent mistakes in medium formulation tables. Medium formulations should therefore be evaluated from the original reference.

Research with laboratory animals such as hamsters has influenced pig embryo culture. Hamster embryos are very difficult to culture and *in vitro* developmental 'blocks' occur at the two-cell and eight-cell stages. Bavister and co-workers have developed a culture medium for hamster embryos. Among the concepts that came from research with hamster embryos was that glucose and phosphate are inhibitory and cause the *in vitro* developmental blocks (Schini and Bavister, 1988b; Seshagiri and Bavister, 1989a, b; Seshagiri and Bavister, 1991). This concept has been found to be important for the *in vitro* culture of embryos from mice (Chatot *et al.*, 1989), rats (Reed *et al.*, 1992b), cattle (Ellington *et al.*, 1990; Moore and Bondioli, 1993) and pigs (Petters *et al.*, 1990; Youngs and McGinnis, 1990; Misener *et al.*, 1991).

In our own laboratory, experiments were performed that resulted in a number of modifications to a culture medium for pig embryos. Pig embryos can develop from the one-cell stage using glutamine or glucose as their sole exogenous energy source (Petters *et al.*, 1990). Glucose alone was not inhibitory. However, the interaction of glucose and phosphate was statistically significant. Other workers have reported favourable results either in the presence (Petters and Reed, 1991; Beckmann and Day, 1993; French *et al.*, 1993) or absence (Misener *et al.*, 1991; Youngs and McGinnis, 1990; Youngs *et al.*, 1993) of glucose. Recent reports of the use of Whitten's medium (Beckmann and Day, 1993; French *et al.*, 1993) have used the same modification of Whitten's formulation (Whitten and Biggers, 1968) as reported by Wright and co-workers (Wright, 1977; Linder and Wright, 1978; Menino and Wright, 1982). However, the more recent reports indicate a much higher rate of development with this medium.

High levels of embryonic development have been obtained with a medium designated NCSU-23, containing taurine and hypotaurine (Fig. 1 and Petters and Reed, 1991; Reed *et al.*, 1992). Recently, workers in other laboratories have successfully used NCSU-23 for the *in vitro* culture of pig embryos (Illera *et al.*, 1992; Torres and Rath, 1992). Three media have been tested in our laboratory: a modified Whitten's medium with glucose (5.56 mmol l^{-1}) and high BSA (1.5%); a modified Whitten's medium lacking glucose and with 0.4% BSA; and NCSU-23 medium (see Table 1 for formulations). These findings (Table 2), although limited, are interesting. First, these results are consistent with those of Youngs and McGinnis (1990) who found better development of pig embryos in the absence of glucose when using a Whitten's medium. NCSU-23 contains glucose and has a higher NaCl concentration than Whitten's medium, yet the proportion of embryos developing to the blastocyst stage was similar to that in Whitten's medium without glucose.

In vitro culture of early stage pig embryos is quite feasible as has been demonstrated by Petters *et al.* (1990); Petters and Reed (1991); Reed *et al.* (1992); Youngs and McGinnis (1990); Youngs *et al.* (1993); Misener *et al.* (1991); Hagen *et al.* (1991) and Beckmann and Day (1993). Further considerations of *in vitro* culture of pig embryos can be best considered by a discussion of embryo metabolism.

Embryo Metabolism and Culture *In Vitro*

Energy sources

Oxaloacetate, phosphoenolpyruvate, pyruvate or lactate can be used as the exclusive energy source by early cleavage stage mouse embryos (Brinster, 1965b). Lactate and pyruvate are generally included in media formulations and lactate is thought to be required for optimal development (Biggers, 1987). Lactate inhibits porcine embryo development (Davis and Day, 1978; Davis, 1985; Stone *et al.*, 1984) and neither lactate nor pyruvate is required for pig embryo development (Petters *et al.*, 1990; Petters and Reed, 1991). All experiments that have been interpreted to demonstrate an inhibitory effect of lactate on pig embryos have been performed in the presence of glucose. Since lactate and pyruvate are included in all mouse embryo culture media, the inhibitory effect of glucose has been described only in the presence of lactate (Chatot *et al.*, 1989). Glucose has been added or deleted in the presence of lactate in mice and interpreted to be inhibitory, whereas lactate was deleted or included in the presence of glucose in pigs and interpreted to be inhibitory. The inhibitory treatment is the same for both species – glucose plus lactate. Recently, glucose has been interpreted to be inhibitory in pig (Youngs and McGinnis, 1990; Misener *et al.*, 1991), but again the inhibitory treatment contained lactate and glucose.

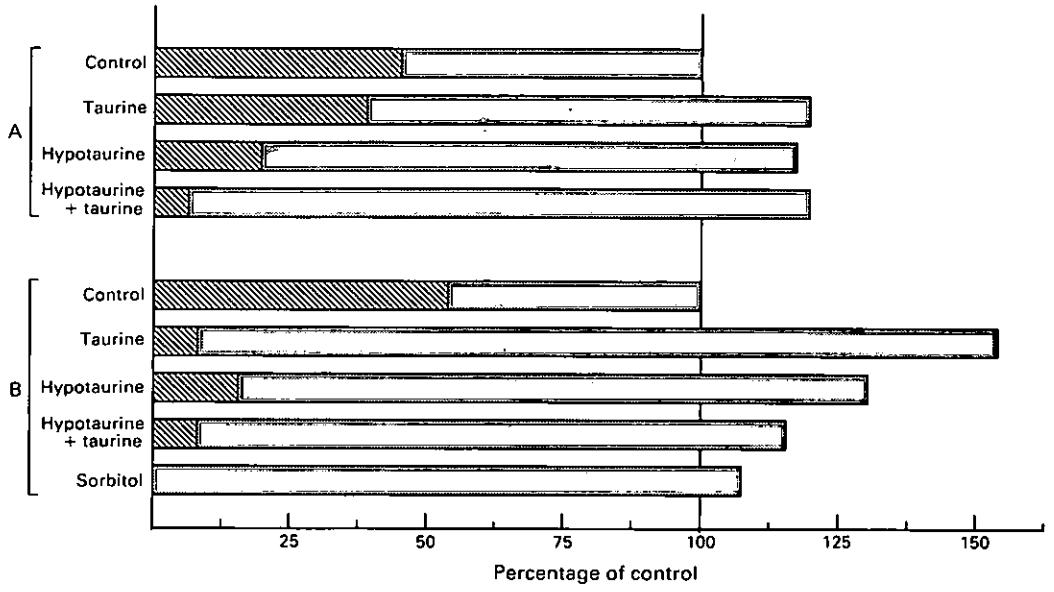

Fig. 1. Development of one- or two-cell pig embryos in media with or without taurine and hypotaurine: (▨) morula; (■) blastocyst. Data from two experiments (A and B) are displayed in this figure. Treatment with hypotaurine and taurine is medium NCSU-23 (see Table 1 for composition). Treatment with sorbitol is as in formulation of medium NCSU-37 (Table 1). Data are shown as percentage of control. For experiment A, 38–44 embryos were cultured in each treatment; for experiment B, 22 embryos were cultured in each treatment (Petters, 1992).

The Crabtree effect (Crabtree, 1929) has been proposed as the mechanism by which glucose exhibits its negative effect on preimplantation embryo development in hamsters (Seshagiri and Bavister, 1991) and it has been suggested that this occurs in pigs (Beckmann and Day, 1993). As reviewed by Koobs (1972), the Crabtree effect involves the depletion of phosphate (and perhaps adenosine diphosphate, ADP) in the presence of hexoses. The effect is caused by an overabundance of glycolytic enzymes (primarily hexokinase) and can be alleviated by the addition of phosphate. Since phosphate enhances the inhibitory effect of glucose in pig embryos (Petters *et al.*, 1990), this effect is probably not a Crabtree effect, as the addition of phosphate would be expected to alleviate the effect, not enhance it. However, similar data have been interpreted as the Crabtree effect in hamsters (Schini and Bavister, 1988b). In the hamster the phosphate–glucose interaction inhibits respiration as is the case for glucose alone in the Crabtree effect (Seshagiri and Bavister, 1991).

The low oxygen environment preferred by embryos and some cancer cells demonstrates a similarity that may be related to metabolism. The Pasteur effect describes the differential rates of glycolysis under aerobic and anaerobic conditions. For example, anaerobic glycolysis can be four times faster than aerobic glycolysis (Wu and Racker, 1959). Koobs (1972) suggests that the Pasteur effect (anaerobic glycolysis running faster than aerobic) and the Crabtree effect (depression of respiration via overactive hexokinase and abundant glucose) may be caused by the same mechanism – insufficient phosphate to simultaneously satisfy glycolysis and oxidative phosphorylation. In the Pasteur effect, mitochondria out-compete glycolysis for phosphate in the presence of oxygen, whereas in the Crabtree effect there is an overabundance of glycolytic enzymes. Nevertheless, in both cases, addition of phosphate to the media is the cure – rather than the cause. Pigs and hamsters may provide a unique opportunity to study the control of glycolysis and respiration. The Crabtee effect, the Pasteur effect, and the phosphate–glucose effect observed by Bavister in hamsters (Schini and Bavister, 1988b; which appears to be present in the pig; Petters *et al.*, 1990; Youngs and McGinnis, 1990) are closely related and reflect the metabolic interconnections between glycolysis and respiration.

Table 1. Formulations of media used for pig embryo culture

Ingredient (mmol l^{-1})	Whitten's medium[a]	Whitten's medium[b]	Whitten's medium[c]	Whitten's medium[d]	Whitten's medium[e]	BMOC-2[f]	mKRB[g]	NCSU 6[h]	NCSU 23[i]	NCSU 37[i]	CZB[k]	TLH[l]
NaCl	68.49	68.49	68.49	68.49	68.49	94.88	94.88	118.70	108.73	108.73	81.62	114.00
KCl	4.78	4.78	4.78	4.78	4.78	4.78	4.78	4.78	4.78	4.78	4.83	3.20
$CaCl_2$	—	—	—	—	—	1.71	1.71	1.70	1.70	1.70	1.70	2.00
KH_2PO_4	1.19	1.19	1.19	1.19	1.19	1.19	1.19	—	1.19	1.19	1.18	—
$MgSO_4 \cdot 7H_2O$	1.19	1.19	1.19	1.19	1.19	1.19	1.19	1.19	1.19	1.19	1.18	—
$NaHCO_3$	25.07	25.07	25.07	25.07	25.07	25.07	25.07	25.07	25.07	25.07	25.12	2.00
Glucose	5.56	5.56	—	—	—	—	5.56	—	5.55	5.55	—	—
Glutamine	—	—	—	—	—	—	—	1.00	1.00	1.00	1.00	—
Taurine	—	—	—	—	—	—	—	—	7.00	—	—	—
Hypotaurine	—	—	—	—	—	—	—	—	5.00	—	—	—
Sorbitol	—	—	—	—	—	—	—	—	—	12.00	—	—
Sodium lactate	25.20	25.20	25.40	12.93	21.55	25.00	29.20	—	—	—	31.30	10.00
Sodium pyruvate	0.33	0.33	0.33	0.33	0.33	0.25	0.25	—	—	—	0.27	0.25
Calcium (lactate)$_2$	1.71	1.71	1.71	1.61	0.85	—	—	—	—	—	—	—
EDTA	—	—	—	—	—	—	—	—	—	—	0.11	—
$NaH_2PO_4 \cdot H_2O$	—	—	—	—	—	—	—	—	—	—	—	0.40
$MgCl \cdot 6H_2O$	—	—	—	—	—	—	—	—	—	—	—	0.50
HEPES	—	—	—	—	—	—	—	—	—	—	—	10.00
BSA (mg ml^{-1})	4.00	15.00	4.00	4.00	4.00	1.00	4.00	4.00	4.00	4.00	5.00	6.00
Selected component Summary												
Total Na^+	119.09	119.09	119.29	106.82	115.44	145.13	149.33	143.77	133.80	133.80	138.53	136.65
Total Cl^-	73.27	73.27	73.27	73.27	73.27	103.08	103.08	126.88	116.91	116.91	89.85	122.20
L(+)lactate$^-$	16.02	16.02	16.12	9.89	12.48	12.50	14.60	—	—	—	15.65	10.00
Lactate/pyruvate	49	49	49	30	39	50	58	—	—	—	58	40

[a]Whitten and Biggers (1968).
[b]Wright, 1977; Beckmann and Day (1993); French *et al.* (1993).
[c]Youngs and McGinnis (1990).
[d]Youngs *et al.* (1993).
[e]Wells and Petters (unpublished; see Table 2).
[f]Brinster (1969).
[g]Davis and Day (1978).
[h]Petters *et al.* (1990).
[i]Petters and Reed (1991); Petters (1992).
[j]Petters (1992).
[k]Chatot *et al.* (1989); Misener *et al* (1991).
[l]Hagen *et al.* (1991).

Table 2. Development of one- and two-cell pig embryos in three media

Medium	*n*	Degenerate	Cleavage	Morula	Blastocyst (%)
mWM* + Glucose + 1.5% BSA	28	5	8	3	12 (43)[a]
mWM* Glucose + 0.4% BSA	28	1	3	2	22 (75)[b]
NCSU 23†	28	0	5	3	20 (71)[b]

Values with different superscripts are statistically different ($P < 0.05$).
mWM: modified Whitten's medium.
*Wells and Petters (unpublished; see footnote e, Table 1).
†Petters and Reed (1991); Petters (1992).

In mice, it has been suggested that the glucose effect depends on NaCl (Lawitts and Biggers, 1991a). The Crabtree effect would not lead to a prediction of NaCl dependency. Glucose inhibition of development of pig embryos has been demonstrated in modified Whitten's media with relatively low NaCl (Youngs and McGinnis, 1990) and in CZB which also has relatively low NaCl (Misener *et al.*, 1991). In some types of cell, glucose transport into the cell is Na^+-dependent which could connect the Crabtree effect with Na^+ dependency. However, this is probably not the case in preimplantation embryos since Na^+-dependent glucose transport could not be detected (Powers and Tupper, 1977; Gardner and Leese, 1988). The nature of glucose metabolism in pig embryos has recently been described (Flood and Wiebold, 1988).

Amino acids – energy, nitrogen and toxicity

As in mice (Chatot *et al.*, 1990; Lawitts and Biggers, 1992), glutamine has been demonstrated to enhance *in vitro* embryo development in pigs (Petters *et al.*, 1990). Glucose or glutamine was shown to support embryo development as the sole energy source from the one-cell stage to the blastocyst stage.

Mice and pigs can develop to the blastocyst stage in media that contain no free amino acids. It has been proposed (Gardner and Lane, 1993) that amino acids could serve as osmoregulators/osmoprotectants, pH regulators, and energy sources in preimplantation embryos. The amino acids found to be beneficial for embryo development in hamsters (Schini and Bavister, 1988a) and mice (Gardner and Lane, 1993) parallel those that have been found to be abundant in mouse oocytes (Schultz *et al.*, 1981) and rabbit oviductal fluid (Miller and Schultz, 1987). Only recently has the use of amino acids in murine embryo culture media shown promise (Gardner and Lane, 1993). Mouse embryos do possess specific transport mechanisms for amino acids throughout the preimplantation period (Van Winkle, 1988). Likewise, amino acid transporters are beginning to be described for pigs (Prather *et al.*, 1993).

Gardner and Lane (1993) suggest that any detrimental effect of amino acids could be attributed to ammonia production from the spontaneous degradation of amino acids during embryo culture as well as metabolism of endogenous nitrogen sources. Murine embryos used in these experiments appear to have an extreme sensitivity to ammonia which could account for observations that the culture volume and the presence or absence of an oil overlay can affect embryo development (Menino and Wright, 1982). The apparent difference in amino acid requirements between the hamster model and the mouse and pig may be explained by the inability of the hamster embryo to maintain endogenous amino acid pools (Schini and Bavister, 1988a) and could explain the benefit of culturing in volumes of less than one microlitre when no amino acids are present. Again, different responses to manipulation of culture conditions are not necessarily indicative of metabolic differences. Under permissive conditions, amino acids other than glutamine may therefore prove to be beneficial in pigs though not required. Such benefits have been described for blastocyst expansion (Rosenkrans *et al.*, 1989; Gardner and Lane, 1993).

Osmolarity

The mouse model was used to demonstrate that a reduction in media osmolarity by reducing NaCl enhances embryo development (Lawitts and Biggers, 1991a, b). Likewise, addition of components known to have osmoprotectant effects against high NaCl in other systems also enhances development (Biggers *et al.*, 1993). Modified Whitten's medium, (mWM) which has been used to support development of pig embryos to the blastocyst stage (Wright, 1977; Menino and Wright, 1982; Menino *et al.*, 1989; Beckmann and Day, 1993), has a much lower NaCl concentration than does modified Krebs Ringer bicarbonate (mKRB; Davis and Day, 1978). However, culture media for pig embryos with relatively high NaCl can support development (Petters *et al.*, 1990). It must also be noted that upon addition of taurine, a potential osmoprotectant, embryo development was improved (Fig. 1; Petters and Reed, 1991). Lawitts and Biggers (1991b) suggested that high Cl^- may be the detrimental ion in the mouse system. The media used by Petters *et al.* (1990) had slightly higher Na^+ than did mWM but much higher Cl^- (see Table 1) which would lend credence to the hypothesis that Na^+ is the more detrimental ion in NaCl.

Recently, the effect of osmolarity and ionic conditions was addressed in pigs (Beckmann and Day, 1993). A series of experiments was performed by adding (mKRB) or deleting (mWM) water in the media preparations. The osmolarity of the diluted mKRB was also adjusted back to undiluted values by the addition of sorbitol as an osmotic balance and similarly sorbitol was used to adjust the osmolarity of undiluted mWM to the level of undiluted mKRB. The results were interpreted to demonstrate that Na^+ concentration and not necessarily osmolarity had a negative effect on development *in vitro*. This conclusion could be supported by Galvin *et al.* (1993) and by research with mouse embryos (Lawitts and Biggers, 1991a, b). However, the results are difficult to interpret because when modifying osmolarity with the volume of solvent, all media component concentrations are altered – including those that have been considered inhibitory. We used media based on the NCSU-23 formulation with reduced NaCl (68 mmol l^{-1}; concentration used in Beckmann and Day, 1993). In those treatments with reduced NaCl, pig embryo development was extremely low ($n = 32$; 3% morula or blastocyst), whereas embryos developed well in media with higher NaCl concentration (109 mmol l^{-1}; $n = 31$; 84% morula or blastocyst). These data may reflect a lower limit of permissible Na^+ concentration or osmolarity.

The dilution of mKRB and readjustment of osmolarity with sorbitol produced a media that supported development of 100% of the one- and two-cell embryos to the morula or blastocyst stage (Beckmann and Day, 1993). The addition of sorbitol to murine embryo culture media can enhance development and accelerate compaction, blastocyst expansion and hatching (Wells *et al.*, 1992). This information further complicates the interpretation of these experiments (Beckmann and Day, 1993), as a basic assumption was that sorbitol is an inert osmolyte. Whether sorbitol can cross the cell membrane or can be metabolized at all by pig embryos is unknown. However, it is known that intracellular concentrations of sorbitol increase in glucose sensitive cell types in response to glucose in the media (Hod *et al.*, 1986; Eriksson *et al.*, 1989; DelMonte *et al.*, 1991; Nakamura *et al.*, 1992) and in other cell types in response to osmotic stress of high NaCl (Bagnasco *et al.*, 1987). Data are therefore available that provide precedence for a role of sorbitol in both glucose sensitivity and osmotic stress or high Na^+ which brings the assumption that sorbitol is inert into question.

Universal Basal (UB) Medium

There are many advantages to having a medium that can be easily compounded and used for a number of species. Use of such a medium will help standardize work between laboratories and allow studies of comparative embryo physiology. The medium, universal basal (UB) medium, is designed to provide a common starting point for embryo culture experiments in different species (Table 3). The formulation of this medium was based on recent research on oviductal fluid, many of the findings reviewed above on pig embryo culture and media used in other systems. The rationale for the inclusion and amount of each ingredient of the medium is discussed below.

The osmolarity chosen for UB was based on osmolarities of media currently in use and the optimum determined by Brinster (1965a) for mice. The actual NaCl concentration (nearly that of CZB) is intermediate and was modified to balance Na^+ to that of Whitten's medium (Whitten and Biggers, 1968). KCl

Table 3. Universal basal (UB) medium formulation

UB – first formulation	(mmol l^{-1})
Base Medium	
NaCl	80.42
KCl	5.36
$MgSO_4 \cdot 7H_2O$	1.06
$NaHCO_3$	25.00
Sodium DL lactate	12.08
Sodium pyruvate	0.18
L(+)Ca (lactate)$_2$	2.00
Glutamine	1.03
Additives	
Taurine	7.03
Hypotaurine	5.04
Sorbitol	7.14
Sodium acetate	0.49
Sodium aspartate	1.93
Glycine	7.06
Glutamate HCl	0.98
BSA	0.4%
Selected component summary	
Na^+	118.17
Cl^-	86.76
L(+)lactate	10.50
L(+)lactate/pyruvate	55

was also increased in response to the finding of Biggers *et al.* (1993) that the K^+:Na^+ ratio can be altered intracellularly under stress conditions, but total K^+ was not significantly altered since potassium phosphate was deleted. Phosphate was deleted as suggested by Schini and Bavister (1988a) for hamsters and Lawitts and Biggers (1991b) for mice and supported by data of Petters *et al.* (1990) which indicated that phosphate was not necessary for pig embryo development. The calcium concentration was increased based on data from oviduct secretions (Hamner and Fox, 1969) and the concentration used in hamster medium (Schini and Bavister, 1988a). The concentration for L(+)lactate was decreased based on measurements of pig (Nichol *et al.*, 1992) and rabbit (Holmdahl and Mastroianni, 1965) oviductal fluid and data from Youngs *et al.* (1993) with pig embryo development at lower lactate concentrations. It is still near the optimal value of lactate for mouse embryo development (Brinster, 1965b). The pyruvate concentration was based on measurements in pig (Nichol *et al.*, 1992) and rabbit (Hamner and Fox, 1969) oviduct and is near the optimum concentration found by Brinster (1965b). Glutamine was included on the basis of data in mice (Chatot *et al.*, 1990), hamsters (Schini and Bavister, 1988a), and pigs (Petters *et al.*, 1990). Other components to be considered as additives would include: glucose, fructose, acetate, citrate and malate, since they can be used as the exclusive energy source by eight-cell mouse embryos (Brinster, 1969); hypotaurine and taurine owing to their benefit in hamsters (Barnett and Bavister, 1992), mice (Eppig *et al.*, 1990), pigs (Petters and Reed, 1991) and rabbits (Li *et al.*, 1993); amino acids for reasons indicated above; sorbitol for its benefit in mice (Wells *et al.*, 1992) and possibly pigs (Beckmann and Day, 1993); betaine for its osmotic protection (Biggers *et al.*, 1993); and EDTA for its benefits in mice (Fissore *et al.*, 1989) and it may allow the replacement of BSA with polyvinylalcohol (PVA; Youngs *et al.*, 1991).

We have recently tested the formulation in Table 3 (UB-first formulation) on one-cell (n = 11, 91% blastocyst) and four-cell pig embryos (n = 31, 94% blastocyst). Experiments testing this formulation in

other species are in progress. The additional amino acids chosen for this first formulation were based on those included in HECM-1 (Schini and Bavister, 1988a) and the experiments of Gardner and Lane (1993). The amount chosen was the lower concentration of a comparison of HECM and oviductal fluid. The other additives included are based on the above justification and the concentration used was based on previous experiments – taurine (Schini and Bavister, 1988a; Eppig *et al.*, 1990; Petters and Reed, 1991; Li *et al.*, 1993), hypotaurine (Petters and Reed, 1991; Barnett and Bavister, 1992), sorbitol (Wells *et al.*, 1992) and acetate (Carney and Bavister, 1987; Moore and Bondioli, 1993). It must be reiterated that UB media is suggested as a starting point and the optimum additives and their concentration would be determined for each species. It is also suggested that adjustment of NaCl be considered when additives in the form of a sodium salt are used.

Conclusion

The culture of preimplantation pig embryos is now sufficiently efficient for most laboratory and agricultural applications with current embryo culture media. The above discussion illustrates, however, that information on the nature of embryo culture requirements is limited. Why is embryo development *in vitro* so poor in media with Na^+ levels found in oviduct secretions? What are the ionic conditions most suited for embryo development? What are the optimum energy substrates? Are these questions even relevant *in vitro* and are there optimum concentrations for media components? To understand the intricacies of early embryo development and the role of the oviductal environment, an *in vitro* model must first be developed. Although embryo survival is high *in vitro*, the culture conditions do not model the conditions *in vivo*. Some oviductal fluid components may be found to be incidental simply owing to the nature of their origin (plasma and interstitial fluid). Other components may be found that allow the embryo to develop in the presence of components which would otherwise be detrimental, whereas other factors will be identified, the presence of which is specifically required for metabolic use by the embryo. More information is needed on oviductal secretions and the metabolism of preimplantation embryos to understand early development. We have suggested the advantages in attempting to develop a universal basal medium so that information gained in each species may be more easily evaluated in other species. Such a system would aid the study of reproduction and will become essential for the use of culture research in less studied species – especially those that are endangered.

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