Comparison of development of pig zygotes and embryos in simple and complex culture media

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The development of pig zygotes and embryos in three culture media was investigated. Zygotes and morulae, recovered from superovulated prepubertal gilts after slaughter, were randomly allocated to culture in one of three simple media: (i) Chatot, Ziomek, Bavister medium (CZB) in 5% CO₂:95% air; (ii) Hepes-buffered CZB medium (H-CZB) in 100% air; or in a complex solution enriched with vitamins and amino acids; or (iii) modified Eagle's minimal essential medium (Pig-MEM) in 5% CO₂:95% air. The relative effectiveness of the various media and gas combinations was assessed by determining the percentage of zygotes and morulae that developed into blastocysts and hatched from their zonae, as well as by the mean number of cells per embryo. Of zygotes cultured either in CZB in CO₂:air, or in H-CZB in air alone, 65% and 57% developed into blastocysts, respectively; no zygotes cultured in Pig-MEM in CO₂:air developed beyond the four-cell stage. After 115 h of culture, embryos resulting from zygotes cultured in either CZB or H-CZB contained approximately 50 cells per embryo. Morulae differed from zygotes in their developmental potential in the three media in that virtually all morulae cultured in CZB, H-CZB or Pig-MEM developed to the expanded blastocyst stage *in vitro*. However, of blastocysts resulting from morulae cultured in Pig-MEM, 97% hatched from their zonae, whereas only 8% and 5%, respectively, of those resulting from morulae cultured in CZB or H-CZB did so. Furthermore, those blastocysts that developed from morulae in Pig-MEM contained significantly more cells than did embryos cultured in either CZB or H-CZB. More than 60% of 80 zygotes cultured in simple media without glucose developed into expanded blastocysts, but < 30% hatched. In contrast, ≥ 70% of 40 zygotes first cultured in CZB or H-CZB to the morula stage and then placed into Pig-MEM hatched from their zonae. After being held briefly in H-CZB, 42% of 91 embryos transferred into four of five recipients that became pregnant developed into fetuses or live young.

Introduction

Substantial progress has been made in devising *in vitro* culture conditions for pig zygotes and cleavage stage embryos. As described in two recent reviews (Reed *et al.*, 1992; Petters and Wells, 1993), three types of culture condition have been studied: (i) organ culture, in which the embryos are grown within oviducts either *in vivo* or *in vitro*; (ii) co-culture with somatic cells, with oviductal cells, or in media supplemented with oviductal fluid; (iii) culture in defined media. Early pig embryos develop into morulae or blastocysts with varying degrees of success in all three systems. Only organ culture or co-culture systems appear to support the development into blastocysts of zygotes and embryos earlier than the four-cell stage (Archibong *et al.*, 1989; Krisher *et al.*, 1989a; White *et al.*, 1989; Prather *et al.*, 1991). Nevertheless, because simple, defined culture media are easiest to replicate, and because the

influence of the addition or elimination of individual components can be studied easily in simple solutions, considerable attention has been paid to devising such media.

The relationship between various energy sources and metabolism of pig embryos has been examined in some detail (reviewed in Petters and Wells, 1993). The utilization of glucose by pig embryos exhibits a very significant increase at the compacted morula stage (Flood and Wiebold, 1988), suggesting that their energy requirements change at that stage. Pig embryos at various initial stages have been successfully cultured *in vitro* to the blastocyst stage in both simple and complex salt solutions, including Eagle's minimal essential medium (MEM) and Kreb's–Ringer bicarbonate medium (KRB), a derivative of a medium of Brinster, both supplemented with different sources of energy (Stone *et al.*, 1984; Rosenkrans *et al.*, 1989; Meyen *et al.*, 1989; Petters *et al.*, 1990).

Pig zygotes can also develop at high rates into blastocysts in media without glucose (Hagen *et al.*, 1991; Misener *et al.*, 1991). Using CZB medium devised by Chatot *et al.* (1989) specifically for the culture of mouse zygotes, Misener *et al.*

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(1991) briefly reported that the same high percentage of > 90% of pig zygotes developed into morulae or blastocysts in the presence or absence of glucose. Comparing several modifications of Tyrode's media and TCM199, Hagen *et al.* (1991) found that the same percentages of 50–60% of one- and two-cell embryos developed into morulae or blastocysts in modified Tyrode's medium with either glucose or glutamine or with neither energy source. Their modifications of Tyrode's solution were the marked reduction of the bicarbonate concentration, the addition of Hepes as a buffer, and the culture of the embryos in air with no increase in CO₂ content of the gaseous atmosphere. The influence of glucose in the media on the development of pig zygotes was shown to depend also on the NaCl concentration and osmolality of either modified Whitten's medium or mKRB, both of which contain glucose, pyruvate and lactate (Beckmann and Day, 1993). The addition of hyaluronic acid to Whitten's medium containing BSA also improves the development of one- and two-cell embryos into blastocysts (Miyano *et al.*, 1994).

Development of pig blastocysts ceases *in vitro* after blastocyst expansion if embryos are maintained in simple medium similar to those used for the successful culture of pig zygotes (Rosenkrans *et al.*, 1989). Continued development and hatching of cultured pig blastocysts appear to require the presence of serum and amino acid supplements in the culture media (Robl and Davis, 1981; Niemann *et al.*, 1983; Stone *et al.*, 1984; Meyen *et al.*, 1989; Rosenkrans *et al.*, 1989). Adding growth factors or oviductal fluid alone to a basal culture medium (NCSU 23) is reported to be of limited value for *in vitro* development of pig embryos (Eberhardt *et al.*, 1994), but addition of fatty acid-free BSA does enhance development.

The objective of the present study was to compare the efficacy of three culture media in supporting development of pig zygotes into expanded and hatched blastocysts. The aim was to identify media that would permit embryos to be handled in air alone (no increase in CO₂) without damage, and could also be used for *in vitro* culture to assess embryo viability. The media tested were CZB, as first described by Chatot *et al.* (1989), and Eagle's MEM, as modified by Malayer *et al.* (1988), both of which require high CO₂ to maintain a neutral pH. In addition, a Hepes-buffered modification of CZB medium (H-CZB) was tested, because it would eliminate the necessity of an increase in CO₂ content in the atmosphere for the routine recovery, handling, culture and transfer of pig embryos. CZB medium was found to be effective for culturing mouse zygotes, particularly from strains early embryos of which exhibited the so-called 'two-cell block' (Chatot *et al.*, 1990), bovine embryos (Ellington *et al.*, 1990), and pig embryos, in a preliminary study (Misener *et al.*, 1991). Modified Eagle's MEM was selected because of its common use for maintaining hatched pig blastocysts (Basha *et al.*, 1980). In addition, a 'mixed media' experiment was conducted, in which zygotes cultured in CZB or H-CZB to the morula stage were also cultured in MEM for an additional 72 h.

Materials and Methods

Chemicals were obtained from Sigma Chemical Company (St Louis, MO) and media from GIBCO (Grand Island Biologi-

Table 1. Media compositions

Component	Media (mmol l ⁻¹)	
	CZB*	H-CZB
NaCl	81.62	81.62
KCl	4.83	4.83
CaCl ₂ · 2H ₂ O	1.70	1.70
KH ₂ PO ₄	1.18	1.18
MgSO ₄ · 7H ₂ O	1.18	1.18
NaHCO ₃	25.12	4.00
Hepes	—	20.00
Sodium pyruvate	0.27	0.27
Sodium lactate	31.30	31.30
Glutamine	1.00	1.00
EDTA	0.11	0.11
BSA (Fr V; g l ⁻¹)	5.00	5.00
Sodium penicillin G (U ml ⁻¹)	100.00	100.00
Streptomycin (mg ml ⁻¹)	0.70	0.70
Phenol red (mg ml ⁻¹)	—	0.01
pH	7.4	7.4
Osmolality (mosmol)	275	270

*As described by Chatot *et al.* (1989).

cal Company, Grand Island, NY), unless otherwise stated. Embryo recovery and manipulation procedures were conducted at a temperature of 35°–38°C using warmed media and temperature-controlled warming plates, pads and water baths. Embryo age was defined in terms of time after donor gilts were injected with human chorionic gonadotrophin (hCG; Ayerst, Quebec) designated as time = 0.

Embryo culture media

The CZB medium was prepared as described by Chatot *et al.* (1989) (Table 1). The modification, called H-CZB, consisted of adding Hepes to CZB medium, decreasing the concentration of NaHCO₃, and adding phenol red. Where indicated, CZB and H-CZB were supplemented with 20% (v/v) heat-treated fetal calf serum (FCS; Hyclone Inc., Logan, UT). Modified Eagle's minimal essential medium (Pig-MEM) was prepared as described by Malayer *et al.* (1988) and contained 4 g glucose l⁻¹, 2 × non-essential MEM-amino acids, 2 × MEM-vitamins, 8.2 mg insulin l⁻¹ and 20% (v/v) FCS. All media were prepared with water from a Millipore Reverse Osmosis System (Model Milli-RO 15) and a Millipore Filtration System (Model Milli-Q ZD20 94). Media were adjusted to pH 7.4, their osmolalities measured and adjusted to about 270 mOsm when necessary, and filter-sterilized through 0.22 µm Millipore filters. The first 10 ml of media passing through each filter was discarded. Media found to differ by more than 3% from the desired osmolality were discarded.

Embryo recovery and handling

All animals were handled and experiments were conducted according to guidelines of the Canadian Council of Animal Care. Follicular maturation and ovulation were induced in

prepubertal Large White gilts by i.m. injection of 1000 iu equine chorionic gonadotrophin (eCG; Ayerst, Quebec) and 500 iu hCG was injected 72 h later. Donor gilts, which were 5–6 months old, of mass 105–115 kg, were artificially inseminated 36 h after hCG injection with semen collected from Large White boars immediately before insemination. Zygotes and morulae were recovered from donor gilts that were killed at 52 and 120 h after hCG injection, respectively. At slaughter, the animals were used by several groups of investigators for different purposes. For those animals whose ovaries were available for detailed counting, the mean ovulation rate was 24.5; an average of 20.9 embryos per gilt were collected. All embryos were recovered and handled at 35°–38°C in H-CZB. Only embryos with spermatozoa visible on their zona pellucida and of excellent morphology, as described by Krisher *et al.* (1989b), Hagen *et al.* (1991) and Petters *et al.* (1990), were used for culture. Care was taken to maintain reproductive tracts, uterine flushings and recovered embryos above 35°C from the time of slaughter of the donors to embryo culture.

Embryo culture

Within 30 min of their collection, zygotes or morulae recovered from individual donor gilts were randomly allocated to culture in the three culture media. Thus, depending on the number of embryos collected from each gilt, embryos from one gilt were cultured in two or three media, and embryos from two or more gilts were cultured in the same media. Groups of four to five embryos were cultured in 100 µl droplets of media held under 1 ml of silicone oil (Medical Fluid 200; 50 cSt viscosity; Dow Corning Corp., Midland, MI) in four-well tissue culture plates (Nunc, GIBCO-BRL, NY). After culture plates containing embryos in droplets of either CZB or Pig-MEM were placed into a modular culture chamber (Billips-Rotherberg, Del Mar, CA), the chamber itself was purged with pre-warmed and humidified 5% CO₂ in air. Other culture plates containing embryos in H-CZB droplets were transferred into a similar culture chamber filled only with air. Both culture chambers were sealed and placed into a 39°C water-jacketed incubator. High humidity within individual chambers was achieved by placing an open Petri dish of 150 mm diameter filled with Milli-Q water in the bottom of each chamber. Every 48 h, droplets of culture media were replaced with fresh media. The development of the embryos was assessed at intervals of 24 h, with embryos classified as either 1-, 2-, 4-, 6- to 8-, > 8-cell, compact morulae, early blastocyst, blastocyst, expanded blastocyst, hatching blastocyst or hatched blastocyst. The number of cells comprising individual embryos in the various treatment groups was determined at about 72, 96, 120, 144 and 168 h for cultured zygotes, and at 120, 144 and 168 h for cultured morulae. Groups of ten embryos were randomly selected from each culture treatment at the indicated times, and were fixed in methanol:acetic acid (3:1), spread on glass slides, and stained for counting (King *et al.*, 1979). To estimate the rate of the cell division of the embryos, the logs of the mean cell numbers of each group were plotted as a function of time, as described by Dawes (1969).

Experimental design

In the first experiment, the development of zygotes in CZB, H-CZB or Pig-MEM was determined. In the second experiment, the development of morulae in the same media was measured. In the third experiment, zygotes first cultured in CZB or H-CZB until they developed into morulae were then cultured in CZB alone, CZB + FCS, H-CZB alone, H-CZB + FCS, or Pig-MEM. As a preliminary test of the effects of handling embryos in H-CZB, some two-cell and four-cell embryos that had been held for > 4 h in H-CZB in air alone at 38°C were surgically transferred into the uteri of synchronized recipients.

Statistical analyses

Regression analysis or analysis of variance was performed on continuous (logarithm of cell numbers) and categorical responses (embryos developing to the blastocyst and hatched blastocyst stage) by the least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute Inc., 1988a). Categorical data were transformed by the empirical logit ($Y = \log[(\text{Observed} + 0.5)/(\text{Total number} - \text{Observed} + 0.5)]$; Cox and Snell, 1987). Homogeneity of variance was tested on residuals using the Univariate procedure (SAS, 1988b). Particular attention was placed on the degree of kurtosis (shape of the curve), skewness (tailing of the curve) and the probability associated with distribution testing of normality (W normality test) of the residuals (Zar, 1974).

Results

When zygotes were cultured in the three test media, approximately two-thirds of groups of 50 zygotes each in CZB and H-CZB developed into blastocysts; none of the 46 zygotes in Pig-MEM developed. Very few of the blastocysts actually hatched from their zonae (Table 2). The log of the number of cells of embryos cultured in CZB and H-CZB increased with time up to about 168 h after hCG treatment (Fig. 1). The mean number of cells of a total of ten embryos per medium cultured in the two media reached about 50 cells per embryo after about 120 h in culture. Those zygotes cultured in Pig-MEM did not develop beyond an average of 3–4 cells per embryo. Analysis of both the categorical (Table 2) and the continuous responses (Fig. 1) demonstrated that both the percentage of blastocysts, and the number of cells per embryo of those that developed in CZB or in H-CZB were statistically greater than those that developed in Pig-MEM.

In contrast, when embryos recovered from gilts as morulae were placed into the three media, virtually all of about 40 embryos per medium developed into blastocysts, and there was no statistical difference between the three media (Table 3). However, although only a few of the blastocysts that developed in CZB or H-CZB hatched from their zonae, 97% of 41 blastocysts that developed in Pig-MEM did so; the hatching

Table 2. Development of pig zygotes in CZB, H-CZB and Pig-MEM media

Culture medium	Number of zygotes cultured	Number of replicates	Number of blastocysts (168 h) (%)	Number of hatched blastocysts (192 h) (%)
CZB	50	5	38 (65) ^a	2 (4) ^a
H-CZB	50	5	33 (57) ^a	1 (2) ^a
Pig-MEM	46	5	0 (0) ^b	0 (0) ^a

Source	Degree of significance (blastocysts)
Treatment (orthogonal contrasts)	
CZB versus H-CZB	0.158
CZB and H-CZB versus Pig-MEM	2×10^{-9}

Values with different superscript letters in the same column are significantly different ($P < 0.05$) based on Ryan–Einot–Gabriel–Welsch multiple range test.

Table 3. Development of pig embryos whose culture was begun at the morula-stage in CZB, H-CZB or Pig-MEM media

Culture medium	Number of morulae cultured	Number of replicates	Number of blastocysts after 168 h (%)	Number of hatched blastocysts after 192 h (%)
CZB	38	6	37 (97) ^a	3 (8) ^a
H-CZB	40	6	40 (100) ^a	2 (2) ^a
Pig-MEM	41	6	41 (100) ^a	40 (97) ^b

Source	Degree of significance (Blastocysts*)	Degree of significance (Hatched blastocysts)
Treatment (orthogonal contrasts)		
CZB versus H-CZB	0.290	0.499
CZB and H-CZB versus Pig-MEM	0.501	5×10^{-8}

Values with different superscript letters in the same column are significantly different ($P < 0.05$) based on Ryan–Einot–Gabriel–Welsch multiple range test.
*Transformed by the empirical logit $\{y = \log[(\text{Observed} + 1)/(\text{Total number} - \text{Observed} + 1)]\}$.

Fig. 1. Mean number of cells of pig embryos ($n = 10$ per time per treatment) cultured from the zygote stage in CZB (■), H-CZB (●) or Pig-MEM (▲) medium.

percentages were statistically different (Table 3). Furthermore, the logs of the mean numbers of cells of embryos in the three media increased with time, although the rates of cell division in CZB and H-CZB were considerably lower than in Pig-MEM (Fig. 2). The numbers of cells per embryo that developed in

CZB and H-CZB were significantly lower than in embryos that developed in Pig-MEM (Table 4).

To determine whether morulae that were derived by *in vitro* culture of zygotes were developmentally equivalent to those morulae collected at that stage, a ‘mixed media’ experiment was conducted. Two sets of controls consisted of zygotes cultured in CZB or in H-CZB continuously. As shown in Table 5, of those cultured in CZB, 80% and 62% developed into morulae and blastocysts, respectively; of those in H-CZB, the corresponding percentages were 75% and 55%. The addition of FCS to CZB medium did not alter the percentage of zygotes that developed into blastocysts, but it did significantly increase the percentage of those blastocysts that hatched. When morulae that developed from zygotes in CZB were transferred into Pig-MEM medium, not only did a significantly greater percentage develop into blastocysts, but more importantly 75% of the blastocysts hatched from their zonae. Analogous results were obtained for zygotes cultured first in H-CZB and then transferred into either H-CZB + FCS or into Pig-MEM: that is, the hatching rate of blastocysts was enhanced by the addition of FCS to the culture medium. In addition, a significantly greater percentage of blastocysts hatched when morulae that developed from zygotes in H-CZB were transferred into Pig-MEM for the final 72 h of culture.

As a preliminary test of H-CZB as a holding medium, 113 embryos collected at the two- or four-cell stage and incubated in air alone for > 4 h in H-CZB at 38°C were transferred into five gilts, four of which became pregnant. When two of the pregnant recipients, into which 21 and 25 embryos had been transferred, were slaughtered on day 35 of gestation, they contained 9 and 12 normally developed fetuses, respectively. The other two pregnant recipients, into which 25 and 20 embryos had been transferred, were allowed to farrow, resulting in the birth of four and 13 live piglets. In total, 33.6% of 113 transferred embryos developed into normal fetuses and

Fig. 2. Mean number of cells of pig embryos cultured from the compact morula stage in CZB (■), H-CZB (●) or Pig-MEM (▲) medium.

live young, representing 41.8% of the embryos transferred into the four recipients that became pregnant.

Discussion

The *in vitro* development of pig embryos has been shown to depend upon the stage of the embryo at the initiation of culture and the composition of the culture medium (for reviews see Reed *et al.*, 1992; Petters and Wells 1993). In the study reported here, zygotes developed into blastocysts in either CZB or H-CZB medium, but did not develop in Pig-MEM. These results confirm the findings of other investigators who have demonstrated that simple culture media can support the development of pig zygotes to the blastocyst stage (Wright, 1977; Menino and Wright, 1982; Petters *et al.*, 1990; Hagen *et al.*, 1991; Beckmann and Day, 1993), but that complex media cause development of zygotes to become arrested at or before the four-cell stage (Hagen *et al.*, 1991). The development of blastocysts in H-CZB also confirms the earlier report by Hagen *et al.* (1991), who found that the zwitterion, Hepes, is a suitable replacement for the $\text{NaHCO}_3\text{--CO}_2$ buffer system. However, the equivalent efficacy of CZB and H-CZB to support zygote development to the blastocyst stage contrasts with the results of Hagen *et al.* (1991), who found that the $\text{NaHCO}_3\text{--CO}_2$ -buffered Tyrode's medium did not support development.

Table 4. Analysis of variance of cell numbers at 120, 144 and 168 h of embryos placed into culture as morulae in CZB, H-CZB or Pig-MEM media

Source	Degree of significance (number of cells)
120 h	
Treatment	0.565
Contrast	
CZB versus H-CZB	0.778
CZB and H-CZB versus Pig-MEM	0.307
144 h	
Treatment	0.002
Contrast	
CZB versus H-CZB	0.509
CZB and H-CZB versus Pig-MEM	0.0005
168 h	
Treatment	1×10^{-8}
Contrast	
CZB versus H-CZB	0.671
CZB and H-CZB versus Pig-MEM	2×10^{-9}

The rates of development of zygotes into blastocysts in either CZB or H-CZB medium are comparable to those obtained in modified Krebs–Ringer bicarbonate medium (Petters *et al.*, 1990), modified Whitten's medium (Beckmann and Day, 1993), or Hepes-buffered Tyrode's medium (Hagen *et al.*, 1991).

The actual development rates of zygotes, derived from the plot of $\log [\text{cell numbers per embryo}]$ versus time, in CZB and H-CZB were virtually identical. The respective totals of 48 and 47 cells in embryos derived from zygotes cultured in CZB or H-CZB are comparable to those obtained in studies of porcine embryos cultured in a bicarbonate medium (58 cells: Krisher *et al.*, 1989a; 29 cells: Petters *et al.*, 1990), in Whitten's medium (52 cells: Youngs *et al.*, 1993), and are similar to those of embryos maintained in organ culture (59–72 cells: Krisher *et al.*, 1989a; 48 cells: Krisher *et al.*, 1989b). The hatching percentage of embryos cultured continuously from the zygote to the blastocyst stage in CZB or H-CZB was very low. A comparison of the rates of development of embryos, collected as morulae, as a function of time in CZB, H-CZB or Pig-MEM was also made. The mean number of cells in embryos developing from morulae was greater than that in embryos developing from zygotes; nevertheless, the development rates as a function of time were similar. Further evidence that these media support normal development was provided by comparison with observations of Papaioannou and Ebert (1988). In their study, although the total numbers of cells of embryos from 31 gilts vary substantially, the logs of the mean number of cells recovered on days 5–8 after the onset of oestrus increased with time. These data provide an excellent estimate of the rate of development of pig embryos *in vivo*. Comparison of the data from the present study for *in vitro* development of pig embryos with those of Papaioannou and Ebert for *in vivo* development suggests that the time-dependent rates are similar (Fig. 3).

Table 5. Development of embryos collected as zygotes and cultured in one medium for 84 h, and then transferred into a second medium at the morula stage for an additional 72 h

Culture Media Zygote→Morulae		Number of zygotes cultured	Number of morulae (120 h) (%)	Number of blastocysts (168 h) (%)	Number of hatched blastocysts (192 h) (%)
1st medium	2nd medium				
CZB→	CZB	20	18 (80)	13 (62) ^{abc}	1 (5) ^{bc}
	CZB + FCS	20	16 (80)	14 (69) ^{abc}	6 (30) ^b
	Pig-MEM	20	18 (88)	18 (88) ^a	15 (75) ^a
H-CZB→	H-CZB	20	15 (75)	11 (55) ^c	0 (0) ^c
	H-CZB + FCS	20	16 (80)	12 (60) ^{bc}	5 (25) ^b
	Pig-MEM	20	17 (85)	17 (85) ^{ab}	14 (70) ^a
Source		Degree of significance (Blastocysts) (Hatched blastocysts [†])			
Treatment (orthogonal contrasts)					
CZB→CZB versus CZB→CZB + FCS			0.883	0.021	
CZB→CZB + FCS versus CZB→Pig-MEM			0.019	4 × 10 ⁻⁵	
H-CZB→H-CZB versus H-CZB→H-CZB + FCS			0.719	0.008	
H-CZB→H-CZB + FCS versus H-CZB→Pig-MEM			0.002	2 × 10 ⁻⁵	
CZB (1st medium) versus H-CZB (1st medium)			0.343	0.508	

Times refer to hours after hCG injection of donors.

Values with different letters in the same column are significantly different ($P < 0.05$) based on Ryan–Einot–Gabriel–Welsch multiple range test.

[†]Transformed by the empirical logit $\{y = \log[(\text{Observed} + 1)/(\text{Total number} - \text{Observed} + 1)]\}$.

The development of morulae (placed into culture immediately after collection from donors) differed significantly from those derived from the culture of zygotes. A high percentage of *in vivo* morulae developed to the expanded blastocyst stage in all of the tested media, whereas only about two-thirds of *in vitro* morulae developed in CZB and H-CZB, and none developed in Pig-MEM medium. The number of cells comprising blastocysts that developed in the different media also differed. Those morulae cultured in the complex medium Pig-MEM had a significantly higher mean number of cells than did those developing in either of the simpler media. Although high percentages of morulae developed into blastocysts in CZB or in H-CZB, very few hatched. In contrast, almost all of the blastocysts derived from morulae cultured in Pig-MEM hatched from their zonae. However, addition of fetal calf serum to both CZB and H-CZB did significantly increase the number of blastocysts that hatched *in vitro*, compared with that observed in serum-free medium. This beneficial effect of a biological macromolecule for the culture of pig embryos is similar to that found for pig oviductal fluid (Archibong *et al.*, 1989), or for addition of serum to the medium (Robl and Davis, 1981; Stone *et al.*, 1984; Rosenkrans *et al.*, 1989). The high rates of blastocyst development and zona hatching observed in the present study appear to differ from the observations of Meyen *et al.* (1989), who suggest that embryos of different genotypes may respond differently in culture. In the study reported here, 99% of a total of 119 morulae obtained from the mating of Large White gilts to Large White boars developed into

blastocysts in the three media, and 97% of 41 morulae cultured in Pig-MEM hatched from their zonae.

Although many of the cultured embryos formed blastocysts and even hatched from their zonae, the percentage of the zygotes that developed completely *in vitro* was significantly less than that reported for development *in vivo* (Pope and Day, 1972; Hunter, 1974; Davis, 1985). Such a reduction in development rate has been observed during culture *in vitro* (Davis, 1985; Papaioannou and Ebert, 1988; Rosenkrans *et al.*, 1989). The point at which embryonic development ceased in the study reported here depended upon the media and the embryo stage at the start of culture. None of the embryos, even those that hatched *in vitro*, elongated, and all slowly degenerated at about 240 h after hCG.

Identification of media suitable for handling and culture of pig zygotes and compact morulae either in air or in a CO₂-supplemented environment was achieved in the present study. The identification of methods for handling embryos without impairing their developmental capability is important for deriving and testing new techniques for domestic livestock species. This is particularly true for procedures such as cryopreservation for which rapid bioassays of cell viability after various treatments are essential.

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Fig. 3. Comparison of the log of the mean number of cells of pig embryos that developed *in vitro* in either H-CZB (---) or Pig-MEM (—●—) with the log of the mean number of embryos collected directly from pigs (●—●); data from Papaioannou and Ebert, 1988).

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