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In vitro fertilization and embryo development in pigs

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Considerable progress has been made in the *in vitro* production of pig embryos using improved methods for *in vitro* maturation (IVM) and fertilization (IVF). Despite the progress, polyspermic penetration remains a problem for *in vitro*-matured oocytes. Variation among boars, ejaculates and IVF protocols used in different laboratories appears to influence the incidence of polyspermy. Recent studies indicate that oviduct cells and their secretions play a role in reducing polyspermy. Very early attempts to culture *in vivo*-derived pig embryos met with little success and most were arrested at the four-cell stage. At present, many culture media are available that can overcome the four-cell block and support development to the blastocyst stage. In contrast, blastocyst development of *in vitro*-produced (IVP) embryos in these culture media varies significantly. Significant differences in morphology and numbers of cells have been observed in *in vitro*-produced blastocysts compared with *in vivo*-derived blastocysts. Surgical transfer of *in vitro*-produced embryos to recipient animals has resulted in acceptable pregnancy rates with moderate litter sizes. Although several systems are available for the generation of *in vitro*-produced embryos, the problems of polyspermy and poor embryo survival prevent large-scale production of embryos. Further research should be directed to improve oocyte and embryo quality, and to develop methods to minimize polyspermy through development of better IVM, IVF and embryo culture techniques.

Introduction

Owing to their physiological similarities to humans, pigs are increasingly important in biomedical research. Interest has grown in the use of transgenic pigs to produce specific proteins and as potential xenograft donors. Attempts to produce transgenic pigs by pronuclear microinjection require early embryos. Surgical collection of early embryos from donor animals is time-consuming, expensive and offers limited numbers of embryos. Therefore, it is important to produce large numbers of developmentally competent embryos by *in vitro* techniques for biomedical and basic research purposes.

Numerous reports describe maturation and fertilization of pig oocytes under *in vitro* conditions. Initially, nuclear maturation was achieved but problems with poor male pronuclear formation and high incidence of polyspermic penetration were observed routinely after *in vitro* fertilization (IVF) (Niwa, 1993; Funahashi and Day, 1997; Day *et al.*, 2000a). Oocyte maturation processes can be divided broadly into two aspects, namely nuclear and

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Table 1. Sperm penetration obtained under various IVF conditions

IVF medium and supplements	Type of spermatozoa	Sperm concentration ($\times 10^6$ ml^{-1})	Type of oocyte	Penetration rate (%)	Reference
TCM + 10% FCS + 2 mmol caffeine l^{-1}	Fresh	2	*In vivo* (follicular)	80–100[a]	Yoshida, 1987
			In vivo (ovulated)	100[a]	
BO + 0.1% BSA + 2 mmol caffeine l^{-1}	Frozen–thawed (epididymal)	24	IVM	42[a]	Nagai *et al.*, 1988
			In vivo (ovulated)	79[b]	
TCM + 10% FCS + 5 mmol caffeine l^{-1}	Frozen–thawed (ejaculated)	1	IVM	0	Wang *et al.*, 1991
		6		12	
		12		43	
		25		88	
		50		85	
TCM + 10% FCS + 5 mmol caffeine l^{-1}	Fresh	1	IVM	94[a]	Funahashi and Day, 1993
TCM + 0.4% BSA + 5 mmol caffeine l^{-1}	Fresh	1	IVM	85[a]	
BO + 0.1% PVA + 5 mmol caffeine l^{-1}	Frozen–thawed (ejaculated)	4–5	IVM	28[a]	Wang *et al.*, 1995
		7–8		45[b]	
		10–12		44[b]	
mTBM + 0.1% BSA	Frozen–thawed (ejaculated)	1	IVM	48[a]	Abeydeera and Day, 1997a
mTBM + 0.1% BSA + 1 mmol caffeine l^{-1}	Frozen–thawed (ejaculated)	1	IVM	96[b]	
TALP + 0.6% BSA	Fresh	1	IVM	71–100[a]	Cordova *et al.*, 1997
	Frozen–thawed (ejaculated)	1	IVM	56–94[a]	

TCM: tissue culture medium; FCS: fetal calf serum; BO: Brackett and Oliphant medium; PVA: polyvinylalcohol; mTBM: modified Tris-buffered medium; TALP: Tyrode's albumin lactate pyruvate medium; IVM: *in vitro*-matured.

[ab]Within a study, different superscripts indicate significant differences ($P \leq 0.05$).

cytoplasmic maturation. Although nuclear maturation appears to be normal, the degree of cytoplasmic maturation under those initial maturation conditions is unknown.

After many years of research, various modifications to the *in vitro* maturation (IVM) system have alleviated the problem of male pronuclear formation. However, polyspermy remains a major unresolved problem. It has not been established whether this abnormality is due to inadequate conditions during IVM, IVF or both. Despite the problem of polyspermy, successful production of pig embryos via *in vitro* techniques has improved markedly. Furthermore, transfer of embryos to recipient animals has resulted in acceptable pregnancy rates and litter sizes. This review will discuss recent progress in IVF and embryo culture techniques in pigs. It is envisaged that the development of new or optimization of existing *in vitro* techniques will result in further advances in embryo technology.

In vitro fertilization

Factors affecting sperm penetration

Fertilization medium and source of spermatozoa. Various types of fertilization medium, in conjunction with fresh or frozen–thawed spermatozoa at different concentrations, have been used to achieve *in vitro* penetration of pig oocytes (Table 1). Freshly ejaculated semen is still the main source of spermatozoa for routine IVF studies. Nevertheless, large variations among boars, as well as among different fractions within the same ejaculate, are observed in oocyte penetration and polyspermy (Xu *et al.*, 1996a,b). These authors found that the use of a specific sperm-rich fraction of the ejaculate can reduce the variability among different ejaculates collected from the same boar. Cryopreservation of semen from a single ejaculate would allow the optimization of IVF protocols and minimize the variability between trials. However, the same IVF protocol may not provide optimal conditions for frozen semen from other boars. It is necessary to optimize the IVF protocol for each individual batch of frozen semen to realise desirable IVF parameters because of variation in the ability of spermatozoa from different boars to withstand cryopreservation.

Bicarbonate concentration. Most IVF media contain $NaHCO_3$ concentrations that maintain a desirable pH under specific culture conditions. *In vivo*, spermatozoa undergo a process called capacitation before they are capable of penetrating an oocyte. Once ejaculated, spermatozoa are confronted with higher bicarbonate concentrations within the female tract, perhaps indicating a role for this ion (Harrison, 1996). In the presence of bicarbonate, a considerable number of sperm proteins become tyrosine-phosphorylated during *in vitro* capacitation (Flesch and Gadella, 2000). Bicarbonate concentration in IVF medium can influence the sperm penetration of pig oocytes (Table 2). Assessment of the functional status by chlortetracycline fluorescent staining indicated that HCO_3^- stimulated capacitation and acrosome reaction of spermatozoa in a concentration- and time-dependent manner (Abeydeera *et al.*, 1997). Suzuki *et al.* (1994a) showed that HCO_3^- was essential during IVF of pig oocytes under these conditions. However, successful sperm penetration in a Tris-buffered medium (Abeydeera and Day, 1997a,b) is evidence against the universal requirement for bicarbonate during pig IVF.

Calcium concentration. Extracellular Ca^{2+} is required for sperm capacitation and the ability to undergo an acrosome reaction (Flesch and Gadella, 2000). Current evidence indicates that calcium plays a critical role in modulating sperm function and is obligatory for successful fertilization in mammals (Fraser, 1995). Indeed, calcium is necessary during IVF of pig

Table 2. Sperm penetration of pig oocytes in the presence of bicarbonate

IVF condition	Bicarbonate (mmol l^{-1})	Penetration rate (%)	Reference
mBO + 0.1% PVA + 5 mmol caffeine l^{-1}	37	37[a]	Wang *et al.*, 1995
	41	51[a]	
	45	76[b]	
	50	74[b]	
TCM + 10% FCS + 5 mmol caffeine l^{-1}	26	63[a]	Abeydeera *et al.*, 1997
	36	88[b]	
	46	90[b]	
	56	88[b]	

mBO: modified Brackett and Oliphant medium; PVA: polyvinylalcohol; FCS: fetal calf serum; TCM: tissue culture medium.
[ab]Within a study, different superscripts indicate significant differences ($P < 0.05$).

Table 3. Effect of various types of macromolecule on fertilization parameters in pigs

Type of supplement	Penetration rate (%)	Polyspermy (%)
None	14[a]	5
PVA (5 mg ml^{-1})	19[ab]	15
PVP (5 mg ml^{-1})	27[b]	8
FCS (10%)	57[c]	79
BSA (5 mg ml^{-1})	77[d]	82

Data from Suzuki *et al.* (1994b).
PVA: polyvinylalcohol; PVP: polyvinylpyrrolidone; FCS: fetal calf serum.
[abcd]Different superscripts indicate significant differences among values ($P \leq 0.05$).

oocytes, as Abeydeera and Day (1997a) showed that penetration did not occur in its absence and that penetration rate increased with increasing concentrations of calcium.

Macromolecular supplements. Most pig IVF media are supplemented with fetal calf serum (FCS; Yoshida, 1987; Wang *et al.*, 1991) or BSA (Nagai *et al.*, 1988; Abeydeera and Day, 1997a) as a protein source but some studies have used polyvinylalcohol (PVA; Wang *et al.*, 1995) or polyvinylpyrrolidone (PVP; Suzuki *et al.*, 1994b) under defined conditions. Using modified Tyrode's medium containing 4.6 mmol $CaCl_2$ l^{-1} and 2 mmol caffeine l^{-1}, Suzuki *et al.* (1994b) showed that the type of macromolecule added during IVF has a significant influence on sperm penetration (Table 3). In addition, the amount of BSA or FCS added to IVF medium could also influence the fertilization rate.

Caffeine. Most pig IVF media are supplemented with caffeine, a phosphodiesterase inhibitor, which could increase cAMP within sperm cells, leading to capacitation (Flesch and Gadella, 2000). In a previous study, Wang *et al.* (1991) failed to obtain sperm penetration in the absence of caffeine and higher penetration rates were obtained at 2.5–10.0 mmol caffeine l^{-1} followed by a decrease at 20 mmol caffeine l^{-1} (Table 4). Nagai *et al.* (1994) obtained a similar pattern but failed to obtain penetration with 2 mmol caffeine l^{-1}. In contrast, recent

Table 4. Sperm penetration of pig oocytes in the presence of caffeine

IVF condition	Source of spermatozoa	Caffeine (mmol l^{-1})	Penetration rate (%)	Reference
TCM + FCS	Frozen–thawed (ejaculated)	0	0[a]	Wang *et al.*, 1991
		2.5	70[b]	
		5	89[c]	
		20	9[a]	
BO + BSA	Frozen–thawed (epididymal)	0	0[a]	Nagai *et al.*, 1994
		2	0[a]	
		5	45[b]	
		15	59[b]	
		20	32[c]	
mTBM + BSA	Frozen–thawed (ejaculated)	0	48[a]	Abeydeera and Day, 1997a
		1	96[b]	
		5	95[b]	
mBO + BSA	Frozen–thawed (ejaculated)	0	50[a]	Funahashi *et al.*, 2000
		1	98[b]	

TCM: tissue culture medium; FCS: fetal calf serum; mBO: modified Brackett and Oliphant medium; mTBM: modified Tris-buffered medium.

[abc]Within a study, different superscripts indicate significant differences ($P \leq 0.05$).

studies have shown penetration in about 50% of oocytes without caffeine (Abeydeera and Day, 1997a; Funahashi *et al.*, 2000). However, penetration rate was increased when caffeine was added during IVF. The disparity among these studies can be attributed to different IVF media or the type of spermatozoa used.

Preincubation of spermatozoa. Preincubation of spermatozoa at various concentrations (Nagai *et al.*, 1984) and the presence of pig follicular fluid during preincubation (Funahashi and Day, 1993) both influence fertilization parameters. Preincubation in the absence of pig follicular fluid stimulates sperm capacitation and results in higher penetration rate and polyspermy. In the presence of pig follicular fluid, capacitation and spontaneous acrosome reaction are stimulated in a concentration-dependent fashion. Such conditions effectively reduce the proportion of capacitated spermatozoa but maintain a high penetration rate with a low incidence of polyspermy. However, high concentrations (10%) of pig follicular fluid tend to reduce penetration by inducing a higher rate of spontaneous acrosome reaction (Funahashi and Day, 1993).

Sperm concentration and co-incubation interval. Penetration rate and polyspermy are also influenced by sperm concentration and duration of co-incubation with oocytes (Abeydeera and Day, 1997b). More spermatozoa are undergoing capacitation at higher sperm concentrations and may be responsible for the higher penetration rate. However, co-incubation of oocytes at a constant sperm concentration for a longer period allows sufficient sperm–oocyte interactions to result in a high incidence of sperm penetration. After reaching the highest penetration rate, a further increase in sperm concentration or co-incubation time tends to increase the incidence of polyspermic penetration.

Male pronuclear formation

Much research has been directed towards increasing the male pronuclear formation in pig oocytes after sperm penetration. Various modifications to IVM, such as supplementation of follicular fluid, co-culture with extroverted follicles, limited exposure to gonadotrophins or low NaCl concentration in culture medium have been used to improve the ability of oocytes to stimulate male pronuclear formation (see Day *et al.*, 2000a). A significant improvement in male pronuclear formation was obtained after supplementation of maturation media with cysteine or glutathione (Yoshida *et al.*, 1992). A later study showed that improvement in male pronuclear formation due to cysteine supplementation is correlated with higher concentrations of intracellular glutathione in matured oocytes (Yoshida *et al.*, 1993). Synthesis of glutathione during oocyte maturation is a prerequisite for sperm nuclear chromatin decondensation and successful male pronuclear formation (Perreault *et al.*, 1988). It appears that male pronuclear formation is compromised in oocytes with lower concentrations of intracellular glutathione.

Polyspermy

Despite the significant improvements in male pronuclear formation, polyspermy remains a major unresolved problem with IVF of pig oocytes. Under *in vivo* conditions, fertilization occurs within a few hours after ovulation and in most instances monospermic penetration ensues. Evidence from laboratory and farm animals indicates that the sperm : egg ratio at the time of initial penetration of the egg membranes is close to unity and this ratio increases only after the establishment of a zona block (Hunter, 1993). Under most IVF conditions, oocytes are always exposed to excessive numbers of spermatozoa, which may be a predisposing factor for multiple sperm penetrations. An ideal IVF system should result in a high penetration rate (> 80%) with a low incidence of polyspermy (< 10%). A tight correlation has been established between the absolute number of spermatozoa per oocyte at fertilization and the degree of polyspermy (Rath, 1992). Theoretically, the problem of polyspermy could be overcome by reducing the number of spermatozoa within the IVF droplets. However, in most cases, such adjustments are associated with a low oocyte penetration rate.

In mammals, sperm penetration triggers oocyte activation and subsequent cortical granule exocytosis leading to modification of the zona pellucida and block to polyspermy. Cortical granule density within the cytoplasm and exocytosis of these granules after sperm penetration *in vitro* appears to be similar between *in vitro*-matured and ovulated pig oocytes (Wang *et al.*, 1998). However, the incidence of polyspermy is higher in *in vitro*-matured oocytes. The limited perivitelline space in *in vitro*-matured oocytes may interfere with the proper dispersal of cortical granule contents and delay the establishment of the zona block. It is possible that under IVF conditions, accessory spermatozoa gain entry before establishment of a functional zona block. Pig oocytes matured in Whitten's medium containing a low NaCl concentration resulted in oocytes with a wider perivitelline space that were less polyspermic (Funahashi *et al.*, 1994a). Therefore, use of an IVM medium that results in matured oocytes with a wider perivitelline space may, at least in part, resolve the problem of polyspermy.

The *in vivo* counterparts of *in vitro*-matured oocytes are the mature oocytes from preovulatory follicles. Day *et al.* (2000b) examined the morphological, physical and fertilization parameters of *in vitro*-matured, ovulated and preovulatory oocytes (Table 5). For the properties evaluated, *in vitro*-matured oocytes are similar in quality to preovulatory oocytes. It is clear that after ovulation, major changes to the oocyte take place within the oviduct, which may be important in preventing polyspermy. Compared with controls, surgical transfer of *in vitro*-matured oocytes to an oestrous oviduct for 4 h resulted in similar

Table 5. Morphological, physical and fertilization parameters of ovulated, preovulatory and *in vitro*-matured pig oocytes

Parameter	Oocyte category		
	IVM	Preovulatory	Ovulated
Diameter (μm)	152[a]	157[a]	165[b]
Zona thickness (μm)	16[a]	16[a]	18[b]
Size of PVS (μm)	2.6[a]	2.6[a]	9.4[b]
Zona digestion time (min)	3–6[a]	3–6[a]	> 60[b]
Penetration rate (%)	85	82	93
Polyspermy (%)	64[a]	86[b]	28[c]

Data from Abeydeera *et al.* (1999).

PVS: perivitelline space.

[abc]Different superscripts within a row indicate significant differences ($P \leq 0.05$).

morphological and physical changes as observed in ovulated oocytes. Furthermore, oocytes exposed to the oviduct showed similar penetration rates (87 versus 83%) but a lower rate of polyspermy (26 versus 67%). Many *in vitro* studies have indicated the beneficial effects of oviduct cells and conditioned media in reducing polyspermy (Table 6). In these studies, oviductal cells were obtained from either prepubertal gilts or animals showing signs of oestrus. Vatzias and Hagen (1999) showed that supplementation of IVF medium with conditioned medium derived from periovulatory oviduct explant culture reduced the incidence of polyspermy compared with conditioned medium from mid-luteal phase oviduct explants and the control group. These results indicate strongly that oviductal secretions contain factors that interact with oocytes or spermatozoa to prevent or reduce entry of multiple spermatozoa.

The oviduct synthesizes and secretes multiple proteins in response to ovarian hormones, thereby creating a microenvironment capable of supporting the events of fertilization and embryo development. A variety of proteins synthesized and secreted by the pig oviduct have been identified (Buhi *et al.*, 2000). Most abundant is the oestrogen-dependent glycoprotein identified as pig oviduct-specific secretory glycoprotein (pOSP). Some other major synthesized proteins include protease inhibitors (tissue inhibitor of metalloproteinase 1 (TIMP-1), plasminogen activator inhibitor I), clusterin, growth factors and cytokines (Buhi *et al.*, 2000). The presence of pOSP in the zona pellucida and perivitelline space of oviductal oocytes and embryos indicates that it may have a physiological role during sperm–oocyte interaction (Buhi *et al.*, 1993). Exposure of oocytes to semi-purified pOSP (10 μg ml^{-1}) before and during fertilization significantly reduced the incidence of polyspermy (29 versus 61%) without compromising sperm penetration (63 versus 74%; Kouba *et al.*, 2000). There was no evidence of zona hardening but a reduction in sperm binding to zona pellucida was observed in the presence of pOSP. A different oviductal secretory product may be responsible for the zona hardening observed in ovulated oocytes. However, it is not clear whether the reduction of polyspermy by pOSP is due to its interaction with the oocyte or spermatozoa, as oocytes are exposed to the protein before and during fertilization. According to Suzuki *et al.* (2000), sperm preincubation or sperm–oocyte co-incubation in the presence of hyaluronic acid can reduce polyspermy. Hyaluronic acid has been localized in the tissues and intraluminal fluid of pig oviduct (Tienthai *et al.*, 2001), which indicates that it may be involved during *in vivo* fertilization. In addition to oviduct glycoproteins, hyaluronic acid may also play a role in modulation of sperm penetration and polyspermy.

Table 6. Effect of pig oocyte or sperm exposure to oviduct cells or conditioned media before or during IVF on sperm penetration and polyspermy

Pre-fertilization conditions	Fertilization condition	Penetration rate (%)	Polyspermy (%)	Reference
Preincubation of IVM oocytes with POEC	IVF with (+) or without (–) POEC			Romar *et al.*, *2000*
0 h	+	61	50[ab]	
0 h	–	77	57[b]	
2 h	+	59	41[a]	
4 h	+	66	24[a]	
3 h preincubation of IVM oocytes before IVF in				Bureau *et al.*, 2000
Medium only		90	45[a]	
Medium conditioned with POEC for 24 h		87	25[b]	
Medium with POEC added just before 3 h preincubation		93	37[a]	
None	Fertilization medium only (FM)	95	93[a]	Kano *et al.*, 1994
	FM + oviduct cell monolayer	85	64[b]	
	FM only	97	90[a]	
	FM + oviduct conditioned medium	90	73[b]	
Co-culture of spermatozoa with oviductal cells	Presence of oviduct cells during IVF			Nagai and Moor, 1990
0 h	–	89[a]	81[a]	
0 h	+	89[a]	85[a]	
1.0 h	+	94[a]	71[ab]	
2.5 h	+	84[a]	53[b]	
3.5 h	+	19[b]	14[c]	

IVM oocytes: *in vitro*-matured oocytes; POEC: pig oviduct epithelial cells.
[abc]Within a study, different superscripts indicate significant differences ($P \leq 0.05$).

Most pig IVF media are supplemented with caffeine, a phosphodiesterase inhibitor known to increase intracellular cAMP. A recent study, using frozen–thawed ejaculated semen, indicated that when IVF medium (modified Brackett and Oliphant medium; mBO) contained caffeine (1 mmol l^{-1}), almost all oocytes (98%) were penetrated, and 87% of penetrated oocytes were polyspermic (Funahashi *et al.*, 2000). However, when IVF medium was supplemented with fertilization-promoting peptide (FPP; 100 nmol l^{-1}) or adenosine (10 μmol l^{-1}) a high penetration rate (71–75%) was maintained with a significantly reduced frequency of polyspermy (20–25%). Furthermore, analysis of the functional state of spermatozoa exposed to these compounds revealed that FPP and adenosine stimulated

capacitation but inhibited spontaneous acrosome reaction. In contrast, caffeine stimulated both capacitation and acrosome reaction. Accordingly, it may be possible to minimize the problem of polyspermic penetration by replacing the caffeine with FPP or adenosine.

The type of IVF medium may also affect the incidence of polyspermy (A. Kidson, personal communication). This worker examined fertilization parameters using either modified Tyrode's albumin lactate pyruvate (mTALP) or modified Tris-buffered medium (mTBM) as IVF medium. At a low sperm concentration (4×10^5 spermatozoa ml^{-1}), penetration (54 versus 32%) and polyspermy (40 versus 10%) were higher in mTALP than mTBM, respectively. A ten-fold higher sperm concentration increased penetration rates (82 versus 79%) similarly for mTALP and mTBM, respectively. Incidence of polyspermy was 76% for mTALP and 26% for mTBM. Therefore, medium and sperm concentration interact to produce different rates of polyspermy.

Developmental competence of pig embryos

Early attempts to culture *in vivo*-derived one-cell pig embryos in various culture media were consistently met with a developmental arrest at the four-cell stage (Davis, 1985). However, when collected at the four-cell stage and placed in culture, embryos continued development to the blastocyst stage. Numerous approaches have been used to circumvent the *in vitro* developmental block by using oviduct organ culture, co-culture with oviductal cells and oviductal fluid supplementation (see Day *et al.*, 2000a). In more recent studies, > 70% of *in vivo*-derived embryos developed to the blastocyst stage in modified Whitten's medium (mWM; Beckmann and Day, 1993), North Carolina State University 23 medium (NCSU 23; Petters and Wells, 1993), Iowa State University medium (ISU; Youngs *et al.*, 1993) and Beltsville embryo culture medium 3 (BECM-3; Dobrinsky *et al.*, 1996).

Type of culture medium and embryo development. Although various culture media have proven to be equally competent to support development of *in vivo*-derived embryos to the blastocyst stage, various degrees of success have been observed with *in vitro*-produced embryos, thus indicating the different sensitivity of these embryos to the type of culture medium. Abeydeera *et al.* (1999) compared the developmental ability of *in vitro*-matured and fertilized embryos in four different culture media. Highest (30%) and lowest (5%) proportions of blastocyst development were observed in NCSU and mWM, respectively, with the other two media (ISU and BECM) producing intermediate rates of blastocyst formation. One difference between NCSU and mWM is the presence of a higher sodium lactate concentration (25 mmol l^{-1}) in mWM. Supplementation of NCSU with 25 mmol sodium lactate l^{-1} significantly decreased blastocyst development (14 versus 32%). A similar lactate concentration has been found to be inhibitory to development of early stage pig embryos (Davis, 1985). However, reducing the lactate concentration in mWM did not improve blastocyst development. Blastocyst development in ISU (14%) was higher than in mWM (5%). Differences between these two media are the presence of low lactate concentrations (12.9 mmol l^{-1}) and absence of glucose in ISU medium. These results indicate that the presence of a higher concentration of lactate or glucose in mWM may be detrimental to the development of *in vitro*-matured and fertilized embryos. Indeed, culture of embryos in NCSU lacking glucose but supplemented with low concentrations of lactate (4.5 mmol l^{-1}) and pyruvate (0.33 mmol l^{-1}) for the first 72 h followed by NCSU with glucose for 72 h improved blastocyst development (L. R. Abeydeera, unpublished). It is concluded that NCSU appears to be the most suitable medium available for *in vitro* production of pig blastocysts, whereas other media are effective for culture of *in vivo*-derived embryos.

Table 7. Improvement in pig blastocyst development with modifications to the oocyte maturation medium

Maturation medium	Modification	Blastocysts (%)	Reference
NCSU 23 + 10% pFF	Control	9[a]	Funahashi *et al.*, 1997a
	Preincubation in IVM medium for 12 h before addition of hormones	23[b]	
NCSU 37 + 10% pFF	Control	9[a]	Funahashi *et al.*, 1997b
	cAMP for 20 h	22[b]	
NCSU 23 + 10% pFF	Control	18[a]	Abeydeera *et al.*, 1998a
	Follicular shell pieces	36[b]	
TCM-199 + 25% pFF	Control	1[a]	Grupen *et al.*, 1995
	500 μmol cysteamine l^{-1}	12[b]	
NSCU 23 + 10% pFF	Control	26[a]	Abeydeera *et al.*, 1998b
	12.5 μmol BME l^{-1}	34[b]	
	25 μmol BME l^{-1}	41[b]	
NCSU 23 + 10% pFF	Control	21[a]	Abeydeera *et al.*, 1998c
	1 ng EGF ml^{-1}	33[b]	
	10 ng EGF ml^{-1}	42[b]	
TCM-199 + 0.1% PVA	Control	22[a]	Abeydeera *et al.*, 2000
	10 ng EGF ml^{-1}	37[b]	

NCSU: North Carolina State University; pFF: pig follicular fluid; IVM: *in vitro* maturation; TCM-199: tissue culture medium 199; BME: β-mercaptoethanol; EGF: epidermal growth factor; PVA: polyvinylalcohol.
[ab]Within a study, different superscripts indicate significant differences ($P \leq 0.05$).

Embryo development with modifications to IVM medium. Production of blastocysts through IVM–IVF techniques has resulted in variable success. Many of the failures can be attributed to poor cytoplasmic maturation or a high incidence of polyspermy or both. However, improvements in embryo development to the blastocyst stage have been achieved by introducing various modifications to the IVM system (Table 7). Many of the effective modifications increased the intracellular glutathione concentration in oocytes and can be related to their developmental competence. After sperm penetration, some of the glutathione is used for sperm nuclear decondensation and oocytes with a higher glutathione content may retain more glutathione than do those with a low glutathione contents. Day *et al.* (2000a) suggested that intracellular glutathione could eliminate the oxidative damage caused by reactive oxygen species generated during culture in a conventional 5% CO_2 in air environment, which is detrimental to embryo development. Therefore, intracellular glutathione content of pig oocytes after IVM could be a potential biochemical marker to determine the effectiveness of an IVM system and subsequent developmental competence of oocytes.

Embryo development with modifications to IVF system. A significant improvement in blastocyst development has been observed when glutathione was supplemented during IVF in pigs (Boquest *et al.*, 1999). The higher blastocyst yields did not appear to be related to an

increase in glutathione concentrations in putative zygotes. Although the mechanism is not known, it is possible that extracellular glutathione may have effects on spermatozoa or oocytes or both. It was suggested that any detrimental effects on spermatozoa and oocytes by reactive oxygen species generated during IVF might have been counteracted by the presence of glutathione to ensure normal embryo development.

In a recent study, a higher rate of penetration (80 versus 57%) and blastocyst development (30 versus 8%) was observed when spermatozoa and oocytes were co-incubated in IVF medium for 10 min followed by transfer of oocytes with zona-bound spermatozoa to a fresh IVF medium for 5 h (Grupen and Nottle, 2000). It is surprising that this IVF strategy gave a higher penetration rate than controls. Intuitively, presence of spermatozoa for the entire co-incubation period would be expected to result in a similar or a higher penetration rate than the 10 min sperm–oocyte co-incubation method. Nevertheless, such an IVF strategy could reduce the detrimental effects caused by reactive oxygen species that may otherwise occur when spermatozoa and oocytes are left together for longer co-incubation periods. It would be worthwhile to re-examine and use such IVF techniques to produce pig embryos *in vitro*.

Embryo development with hyaluronic acid. An improvement in blastocyst development (70 versus 45%) was observed when *in vivo*-derived one- to two-cell stage pig embryos were cultured in the presence of hyaluronic acid (Miyano *et al.*, 1994). This stimulatory effect was not observed when culture medium contained a higher concentration (0.4 versus 1.5% w/v) of BSA, indicating that a factor or factors in BSA may become toxic to embryos at higher concentrations. Similarly, Kano *et al.* (1998) observed the beneficial effects of hyaluronic acid on blastocyst development of *in vitro*-matured and fertilized pig embryos. Hyaluronic acid has been localized in tissues and intraluminal fluid of pig oviduct (Tienthai *et al.*, 2001), which indicates a possible involvement during embryo development. Glycosaminoglycans promote the viability of pig oocytes (Sato *et al.*, 1990). The exact mechanism of hyaluronic acid-improved embryo development is not known. However, the involvement of hyaluronic acid in sustaining embryo viability should be considered in future work.

Embryo morphology and quality

Although the success achieved in improving the production of blastocysts by *in vitro* techniques is notable, distinct morphological differences have been observed between *in vitro*- and *in vivo*-produced embryos, including blastocysts (Wang *et al.*, 1999). Well-defined blastomeres in early stage embryos and a prominent inner cell mass in blastocysts are evident in embryos recovered *in vivo*. According to Papaioannou and Ebert (1988), the number of cells in *in vitro*-produced blastocysts is lower than in *in vivo*-produced blastocysts. It is possible that inadequate cytoplasmic maturation of *in vitro*-matured oocytes or suboptimal embryo culture conditions is responsible for the poor embryo quality. Transfer of *in vitro*-matured and fertilized zygotes to the oviducts of recipient animals and then recovery by retrogate flushing 5 days later resulted in blastocysts with cell numbers several-fold higher (106–136 versus 10–21) than blastocysts developed *in vitro* (Funahashi *et al.*, 1994b). In another study, the number of blastocyst cells after *in vitro* and *in vivo* development of *in vivo*-derived one- to two-cell embryos was examined (Machaty *et al.*, 1998). Compared with blastocysts developed in culture, a two-fold increase in cell number (25 versus 55) was observed for blastocysts developed *in vivo*. Collectively, the above findings indicate that suboptimal embryo culture conditions are likely to be the reason for low numbers of cells.

Recent evidence showed that partial inhibition of oxidative phosphorylation at the morula stage can significantly increase the proportion of blastocysts and their number of cells

Table 8. Results of transfer of *in vitro*-produced pig embryos

Source of spermatozoa	Embryonic stage	Piglets/recipients	Reference
Fresh	Two- to four-cell	9/1	Mattioli *et al.*, 1989
Fresh	Two- to four-cell	19/3	Funahashi *et al.*, 1997b
Frozen–thawed	Two- to four-cell	18/5	Abeydeera *et al.*, 1998a
Frozen–thawed	Two- to four-cell	7/1	Abeydeera *et al.*, 1998c
	Eight-cell to morula	11/2	
Frozen–thawed	≥ One-cell	17/3	Kikuchi *et al.*, 1999
Frozen–thawed	Eight-cell to morula	82/12	Abeydeera *et al.*, 2000

(Machaty *et al.*, 2001). In the presence of inhibitors, it is assumed that the total ATP production by the embryo is low. A similar increase in blastocyst development and number of cells was also observed when morula stage embryos were cultured under low (5%) oxygen tension (Machaty *et al.*, 2001). In a previous study, no improvement in blastocyst development was observed when zygotes were cultured at a low oxygen tension for the entire culture period (Machaty *et al.*, 1998). The above findings indicate that ATP production in embryos after compaction may favour glycolysis over oxidative phosphorylation. In turn, it may be important to establish a sequential culture environment, 20% oxygen up to the morula stage and 5% oxygen for later stages, to reduce ATP production and generate better quality pig embryos *in vitro*.

Embryo transfer

Production of pig litters through embryo transfer techniques using *in vitro*-matured and fertilized embryos would eliminate the necessity of *in vivo*-derived embryos. Although initial embryo development can be achieved in culture, the ultimate test of their viability is to establish pregnancies and live births after transfer to recipient animals. Various degrees of success in pregnancies and live births have been achieved after transfer of *in vitro*-matured and fertilized embryos to the oviduct or uterus of recipient gilts (Table 8). A potential problem is asynchrony between the embryo and oviduct or uterus. Routine transfers with *in vitro*-produced embryos are performed to a recipient at least 24 h behind the embryonic age and accurate detection of oestrus is a critical factor. In addition, > 30 embryos are deposited in the oviduct or uterus at transfer. There is little information available about the ability of *in vitro*-produced embryos to hatch *in vivo*. Under *in vitro* conditions, < 10% of blastocysts hatch in NCSU 23 medium containing BSA (L. R. Abeydeera, unpublished). However, addition of 5–10% FCS at about days 4.5–5.0 of culture can increase hatching to 30–40%. Problems associated with *in vivo* hatching of *in vitro*-produced embryos could negatively affect the litter size. Therefore, development of embryo culture systems that stimulate hatching would probably improve pregnancy rate and litter size.

Fate of polyspermic oocytes

Polyspermic fertilization in mammals is considered pathological and usually results in early death of the zygote (Hunter, 1991). Recent studies indicate that polypronuclear oocytes produced from IVM–IVF cleave and develop to the blastocyst stage *in vitro* or *in vivo* at a rate similar to that of two-pronuclear oocytes (Han *et al.*, 1999a). However, they have fewer inner cell mass nuclei compared with blastocysts derived from oocytes with two pronuclei. It

should be noted that polypronuclear oocytes used in the study of Han *et al.* (1999a) contained one female pronucleus and two male pronuclei. In contrast to blastocysts derived from two-pronuclear oocytes, some blastocysts developed from polypronuclear oocytes showed abnormal ploidy, including haploids, triploids and tetraploids. Interestingly, transfer of polypronuclear oocytes to recipients resulted in pregnancies (Han *et al.*, 1999b). At day 40 of pregnancy, 16 fetuses were recovered from three recipients that received polyspermic oocytes. Analysis of ploidy in eight fetuses revealed one triploid, one mosaic (diploid and tetraploid) and the remainder had diploid cells. When four pregnant recipients were allowed to continue beyond day 40, one recipient showed oestrus at day 60 and one on day 97. The remaining animals farrowed five live piglets with a normal ploidy. It seems that some of the polypronuclear pig oocytes possess an as yet unknown mechanism to prevent or correct their ploidy. The location of pronuclei within the cytoplasm of polypronuclear oocytes appears to have a significant effect on determining the ploidy of the resulting embryo before the first cell division (Han *et al.*, 1999b).

Conclusion

It is now possible to produce viable pig embryos through *in vitro* techniques. Supplementation with cysteine during oocyte maturation can alleviate the problem of poor male pronuclear formation. It may also be possible to reduce polyspermy, at least in part, by changing the NaCl concentration of culture medium. Consideration should also be given to exposure of oocytes to oviductal cells and their secretory products before or during sperm–oocyte interaction to reduce polyspermy. It may be necessary to replace caffeine with adenosine to stimulate sperm capacitation and realise normal penetration. A sequential embryo culture environment may improve the quality of blastocysts. It is envisaged that combinations of these improved systems will yield a higher proportion of viable blastocysts capable of establishing successful pregnancies and eventually reduce the number of embryos necessary per transfer. *In vitro* production of pig embryos, in conjunction with improvements in non-surgical embryo transfer techniques, has tremendous potential for basic research and commercial applications.

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