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Development of porcine embryos produced by IVM/IVF in a medium with or without protein supplementation: effects of extracellular glutathione

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Summary

This study was designed to examine the effects of extracellular reduced glutathione on development of pig embryos, produced by *in vitro* maturation (IVM) and *in vitro* fertilisation (IVF), in a chemically defined North Carolina State University (NCSU) 23 medium or in NCSU 23 medium with bovine serum albumin (BSA). Microfilament distribution, as a marker of embryo quality, was also examined by immunocytochemical staining and confocal microscopy. When the inseminated oocytes were cultured in the defined medium containing 0–0.5 mM glutathione, blastocyst formation as observed only in the media with glutathione (8.5–16.0%). Increased numbers of blastomeres were observed in the blastocysts as the concentration of glutathione was increased (18.8±7.2 to 31.0±8.6). In NCSU 23 medium with 4 mg BSA/ml, addition of glutathione at concentrations of 0.125–0.5 mM significantly increased the proportions of oocytes that developed to blastocysts (39.2–52.5%) compared with the control (29.5%). However, no difference was observed in the average cell number in the blastocysts (41.9±15.6 to 49.1±15.5). There were no significant differences in the microfilament distribution in the embryos produced in the defined medium and in the BSA-containing medium. These results indicate that pig embryos produced by IVM/IVF can develop to the blastocyst stage in a defined medium. BSA and glutathione have a synergistic effect on pig embryo development.

Keywords: Embryo development, Glutathione, Pig, Protein-free medium

Introduction

When pig oocytes matured *in vitro* were inseminated with cryopreserved ejaculated spermatozoa, about 30% of inseminated oocytes developed to the blastocyst stage in North Carolina State University (NCSU) 23 medium supplemented with bovine serum albumin (BSA) (Abeydeera *et al*., 1998*a*; Wang *et al*., 1999). However, when the blastocysts were compared with those produced *in vivo*, lower cell numbers (about one-third) were found in *in vitro* produced blastocysts (Wang *et al*., 1999, 2000*a*). Fewer microfilaments were observed in *in vitro* produced embryos (Wang *et al*., 1999) and *in vitro* blocked embryos (Wang *et al*., 2000*b*). These results suggest that microfilaments play an important role in embryo development. In order to further understand the mechanisms by which pig embryos grow *in vitro*, and to develop a more effective *in vitro* culture system, it will be necessary to clarify the effects of each component in medium on embryo development. Proteins, such as serum and BSA, are important components in culture medium in order for the embryos to develop to blastocysts. However, there are many unknown factors in serum or BSA and they may interact with other components to mask the effects of the components in the medium. If a defined medium that supports embryo development can be developed,

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it would help examine the exact role(s) of all components in the culture medium.

It has been found that glutathione is a major intracellular free thiol that regulates cell proliferation, amino acid transport, synthesis of protein and DNA and reduction of disulphides and other chemicals (Meister & Anderson, 1983). Increased normal fertilisation and subsequent blastocyst development were observed by increasing intracellular glutathione in pig oocytes during *in vitro* maturation, such as supplementation of cysteine (Yoshida *et al*., 1993; Abeydeera *et al*., 1999), cysteamine (Grupen *et al*., 1995; Yamaguchi & Nagai, 1999), β-mercaptoethanol (Abeydeera *et al*., 1998*b*), epidermal growth factor (Wang & Niwa, 1995*a*, *b*; Abeydeera *et al*., 1998*c*, 2000) or co-culture of oocytes with extra granulosa cell mass (Abeydeera *et al*., 1998*d*). Increased blastocyst formation was also reported when reduced glutathione was added in sperm washing medium (Earl *et al*., 1997), insemination medium (Van Soom *et al*., 1998; Boquest *et al*., 1999) or embryo culture medium (Brinster, 1968; Takahashi *et al*., 1993; Luvoni *et al*., 1996). However, it has not been examined whether glutathione can stimulate pig embryo development. Therefore, in the present study we first examined the effects of glutathione on pig embryo development in a chemically defined medium so that the interactions between glutathione and unknown factors in BSA can be minimised. Then we supplemented glutathione in BSA-containing medium to investigate whether glutathione and BSA have a synergistic effect on pig embryo development. Microfilament distribution in embryos produced in the defined medium and BSA-containing medium was also examined in order to compare embryo quality.

Materials and methods

In vitro maturation of oocytes

In vitro maturation (IVM) of pig oocytes was based on the procedures reported in our previous studies in a serum-free medium (Abeydeera *et al*., 1998*a*; Wang *et al*., 1999, 2000*a*). Briefly, oocytes were aspirated from antral follicles (3–6 mm in diameter) of ovaries collected from slaughtered prepuberal gilts. After washing four times with Hepes-buffered Tyrode's medium containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA), each group of 50 oocytes surrounded by compact cumulus was cultured for 44 h in Tissue Culture Medium (TCM)-199 supplemented with 0.57 mM cysteine (Sigma Chemical, St Louis, MO), 10 ng epidermal growth factor/ml (Sigma), 10 IU eCG/ml (Intervet America, Millsboro, DE), 10 IU hCG/ml (Intervet) and 0.1% PVA at 39 °C, 5% CO_2 in air in 500 µl of medium.

In vitro fertilisation of oocytes

After maturation, oocytes were separated from the enclosed cumulus by pipetting in maturation medium containing 0.02% hyaluronidase (Sigma). Oocytes without obvious degeneration were used for insemination. Cumulus-free oocytes were inseminated *in vitro* by frozen-thawed ejaculated spermatozoa (Wang *et al*., 1991) in a modified Tris-buffered medium (Abeydeera & Day, 1997; Wang *et al*., 1997). Six hours after insemination, oocytes were removed from fertilisation drops, washed three or four times and cultured in 500 µl of culture medium, NCSU 23, with or without 4 mg BSA/ml, in a four-well culture plate until examination (Wang *et al*., 1997). With this system, about 80% of oocytes can be fertilised and 50–60% of the fertilised oocytes were monospermic (Abeydeera *et al*., 2000). Oocytes were cultured in NCSU 23 medium irrespective of polyspermy or monospermy as it was difficult to evaluate the fertilisation status without fixation and staining.

Assessments of developmental ability of embryos

At 48 h after insemination, cleavage of oocytes was evaluated under a stereomicroscope and blastocyst formation was examined 144 h after insemination.

Assessments of nucleus number in blastocysts

Blastocysts were fixed with 3.7% paraformaldehyde in a calcium-free PBS for 2 h at room temperature. After fixation, samples were washed with PBS-Tween 20 (0.1%, v:v) for there times and then stained with 100 nM YO-Pro-1 iodide (Molecular Probe, Eugene, OR) for 5–10 min. Number of nuclei in each blastocyst was counted under a fluorescence microscope.

Assessments of microfilaments

Microfilaments were examined according to the methods used before (Wang *et al*., 1999; 2000*a*, *b*). In brief, embryos (2-cell and blastocyst stages) were fixed in 3.7% paraformaldehyde, treated with 1% (v:v) Triton X-100 in PBS and stained with 1 µg rhodamine-phalloidin/ml (Sigma) for microfilaments and 100 nM YO-Pro-1 (Molecular Probe) for DNA. Finally, embryos were mounted on slides with anti-fade solution and examined using a Bio-Rad MRC-600 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA).

Experimental designs

In experiment 1, at 6 h after insemination oocytes were washed three times in the defined medium (BSA-free NCSU 23 medium) with 0.1% PVA and then randomly

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cultured in the same medium containing 0, 0.125, 0.25 and 0.5 mM glutathione. The embryos were cultured for 6 days at 39 °C, 5% CO_2 in air. In experiment 2, at 6 h after insemination oocytes were washed three times in NCSU 23 medium with 4 mg BSA/ml and then randomly cultured in medium with 4 mg BSA/ml and with 0, 0.125, 0.25 and 0.5 mM glutathione (Sigma). Glutathione was prepared just before making culture dishes in NCSU 23 medium. After examination of blastocyst development at day 6 under a stereomicroscope, all blastocysts were fixed for count of nuclei or for examination of microfilaments.

Statistical analysis

All percentage data are expressed as mean ± SD and were subjected to arc sine transformation before statistical analysis. The transformed data and the average cell numbers in the embryos were compared by ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

Results

When inseminated oocytes were cultured in the chemically defined medium, cleavage rates of oocytes at 48 h after insemination were 53.6–62.0% and no differences were observed among treatments. However, as shown in Fig. 1, no blastocyst formation was observed in the medium without glutathione. When glutathione was supplemented in the medium, the rates of blastocyst formation were significantly ($p < 0.05$) higher (16.0 ± 4.2%) in the medium with 0.5 mM glutathione than in the medium with 0.125 (10.0 ± 5.6%) and 0.25 mM (8.5 ± 7.8%) glutathione. Significant higher ($p < 0.05$) cell number was also observed in the blastocysts produced in the medium with 0.5 mM glutathione (31.0 ± 8.6) than in the medium with 0.125 (18.8 ± 7.2) and 0.25 mM (23.2 ± 8.5) glutathione.

When inseminated oocytes were cultured in the medium with BSA, as shown in Fig. 2, no differences were observed in the cleavage rates (60.0–76.0%) at 48 h after insemination. Rates of blastocyst formation in the medium with 0, 0.125, 0.25 and 0.5 mM glutathione were 29.5 ± 3.0%, 40.3 ± 6.5%, 52.5% ± 10.9% and 39.2 ± 5.8%, respectively, with significantly higher ($p < 0.05$) rates in the medium with glutathione than in the medium without glutathione. Also, a higher rate of blastocyst formation was observed in the medium with 0.25 mM glutathione than in medium with 0.125 and 0.5 mM glutathione. However, no differences in the average cell number in the blastocysts, ranging from 41.9 ± 15.6 to 49.1 ± 15.5, were observed in the medium with 0–0.5 mM glutathione, respectively.

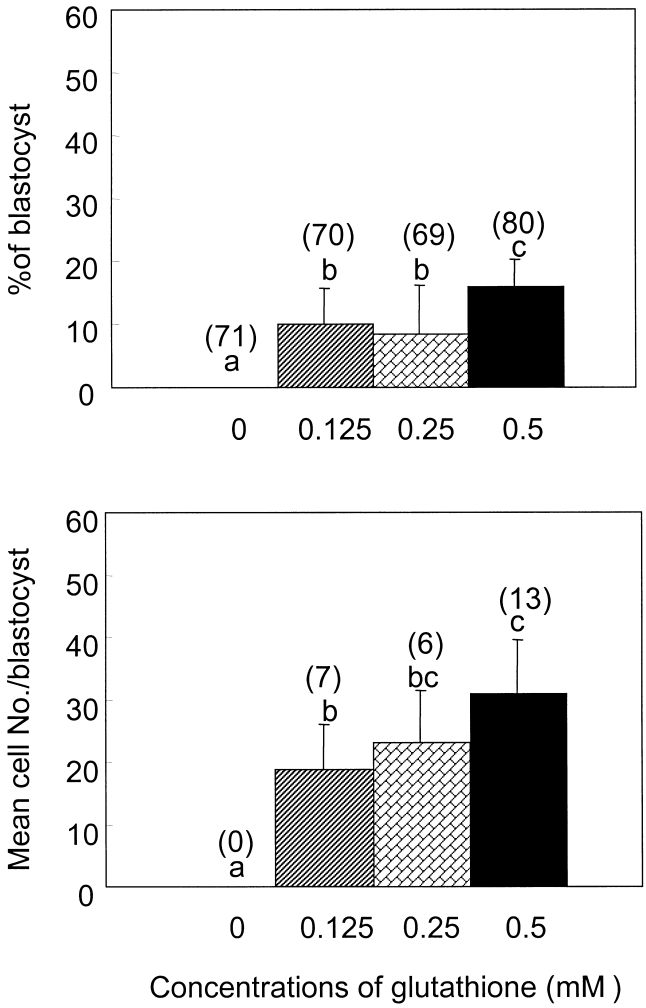

Figure 1 Blastocyst development and average number of nuclei in the blastocysts produced in a chemically defined medium containing 0, 0.125, 0.25 and 0.5 mM glutathione. The numbers of oocytes examined and the numbers of blastocysts used for count of nuclei are indicated in parentheses. ^{a,b,c} $p < 0.05$.

The morphology of blastocysts produced in the defined medium and BSA-countaining medium was the same (Fig. 3). Although we did not directly compare cell numbers in the blastocysts produced in the defined medium and BSA-containing medium, it would appear that more cells were present in the blastocysts produced in BSA-containing medium than in the defined medium. Fig. 4 shows the microfilament distribution in 2-cell and blastocyst stage embryos obtained in the defined medium and in the medium with BSA. Microfilaments were distributed in the cortex (under the membrane) of 2-cell stage embryos with more at the joint of blastomeres. Microfilaments were also observed in the cytoplasm around the nucleus in some blastomeres. There were no significant differences in the microfilament distributions between the embryos produced in the defined medium and in BSA-containing medium.

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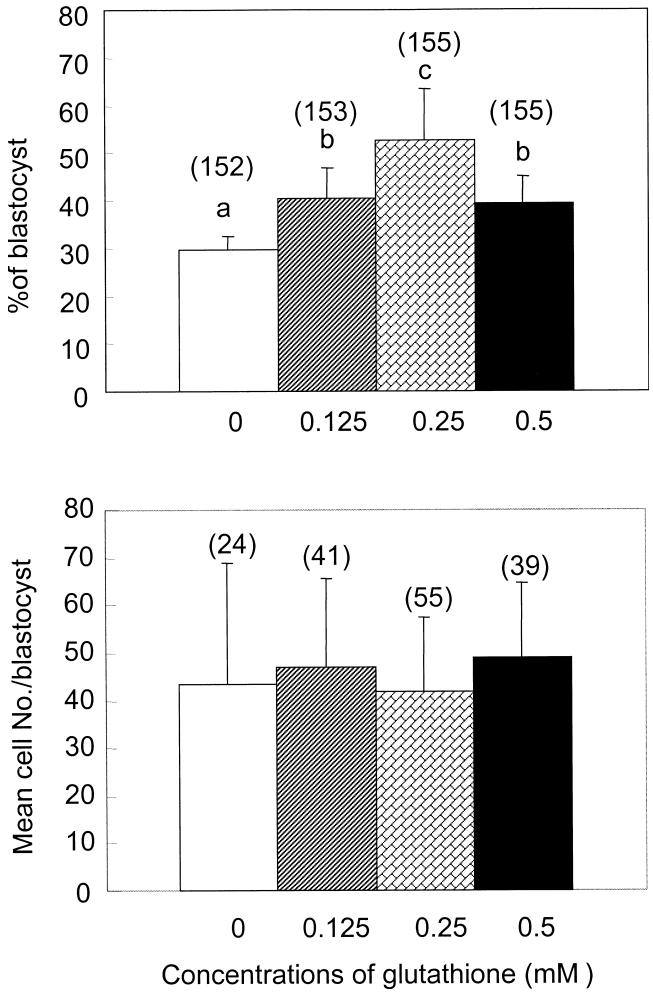

Figure 2 Blastocyst development and average number of nuclei in the blastocysts produced in medium with BSA and 0, 0.125, 0.25 and 0.5 mM glutathione. The numbers of oocytes examined and the numbers of blastocysts used for count of nuclei are indicated in parentheses. ^{a,b,c} $p < 0.05$.

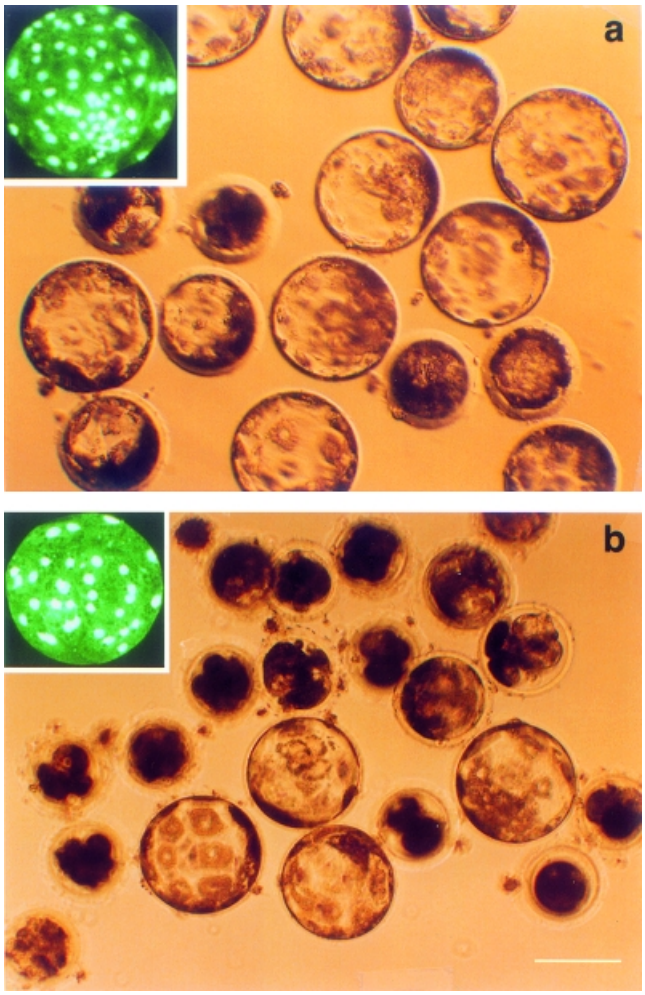

Figure 3 Micrographs of pig embryos produced in medium containing BSA and 0.25 mM glutathione (*a*) and in a chemically defined medium containing 0.5 mM glutathione (*b*). Inserts in (*a*) and (*b*) show the nuclear staining in one of the blastocysts. Scale bar represents 250 µm.

Discussion

The results obtained in the present study indicate that supplementation of glutathione in embryo culture medium increases the blastocyst formation in pig oocytes matured and inseminated *in vitro*. The results also indicate, for the first time to our knowledge, that pig oocytes matured and inseminated *in vitro* can develop to blastocyst stage in a chemically defined embryo culture medium.

A chemically defined medium is important for the study of components in the medium so that a beneficial or detrimental effect of a chemical on embryo development can be addressed. In the present study, blastocyst development was successfully obtained in a chemically defined medium containing glutathione. Although the blastocyst formation rate is low, there is an advantage in the use of this system to evaluate other components in the culture medium, and develop a more beneficial system for the production of pig embryos. On the basis of the finding that glutathione improved embryo development in the defined medium, we added glutathione in the medium with BSA and found that the rate of blastocyst formation was significantly increased compared with the control. Our results indicate that with this improved *in vitro* system, more than 50% of inseminated oocytes can develop to blastocyst stage.

Protein supplementation, such as serum and serum albumin, is important for embryo development by serving as nutrients, pH buffer, anti-oxidants and others (Gardner & Lane, 1997). Growth factors and other hormones are present in serum and unpurified serum albumin, such as BSA. Although BSA as a supplementation of protein in embryo culture media has been used for many animals, its exact effects are still unclear, as it contains many unknown factors.

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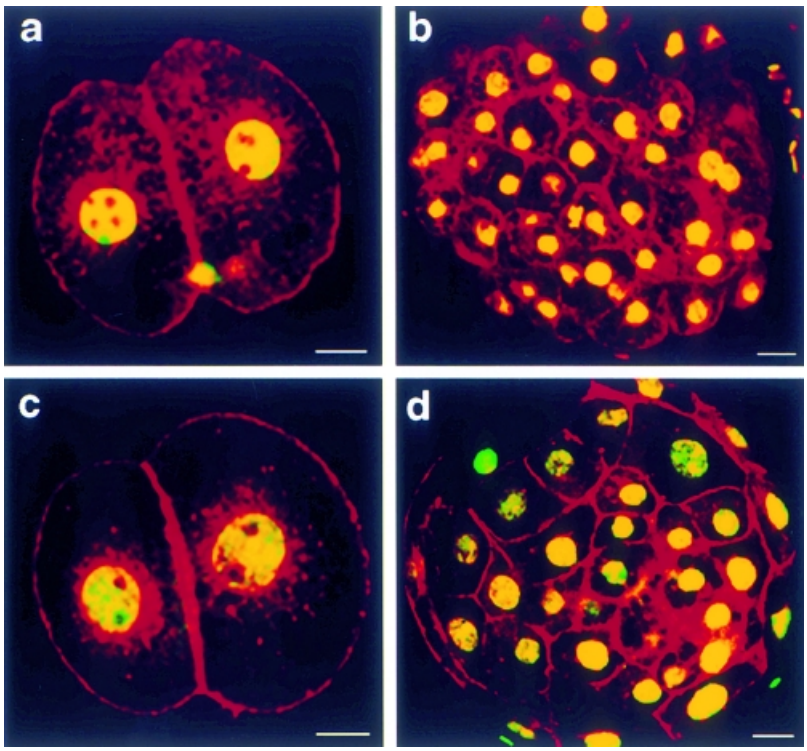

Figure 4 Micrographs of microfilaments in pig embryos at the 2-cell stage and blastocyst stage in medium containing both BSA and glutathione (*a*, *b*), and in a chemically defined medium containing 0.5 mM glutathione (*c*, *d*). Red images represent microfilaments and green images represent nuclei. Yellow images are the overlay of red and green images. Scale bars in (*a*) and (*c*) represent 20 μm and in (*b*) and (*d*) represent 60 μm.

NCSU-23 medium containing BSA has been used to culture pig embryos collected from the oviducts (Petters & Wells, 1993). This medium has also been successfully used to culture pig embryos produced by IVM/IVF (Wang *et al*., 1997, 1999; Abeydeera *et al*., 1998*a–d*, 1999). However, in the present study we found that no blastocyst formation was observed if BSA was not included, indicating that BSA or components in BSA are important for pig embryo development. Blastocyst formation was observed in the medium without BSA but with glutathione. When both BSA and glutathione were added in the medium, they had synergistic effects on blastocyst development.

Glutathione, a tripeptide thiol found in all cells, functions in metabolism as a coenzyme for a number of enzymes, in amino acid transport and in protecting cells against oxidative stress. It is also important in protein and DNA synthesis and reduction of disulphide bonds (Meister & Anderson, 1983). It has been found that increasing intracellular glutathione of oocytes in the pig during IVM increases subsequent normal fertilisation (male pronuclear formation) and blastocyst development (Yoshida *et al*., 1993; Grupen *et al*., 1995; Wang & Niwa, 1995*b*; Wang *et al*., 1997; Abeydeera *et al*., 1998*b–d*, 1999). All these findings indicate that there is a positive correlation between intracellular glutathione concentration and blastocyst formation. Increased blastocyst development was also reported when glutathione was supplemented in sperm separating medium (Earl *et al*., 1997), insemination medium (Van Soom *et al*., 1998; Boquest *et al*., 1999) or embryo culture medium (Brinster, 1968; Takahashi *et al*., 1993; Luvoni *et al*., 1996). The mechanisms by which glutathione regulates embryo development are not known. It has been found that glutathione is also present in oviductal secretions and helps protect preimplantation embryos from the adverse effects of endogenous depletion of embryonic glutathione (Gardiner & Reed, 1994; Gardiner *et al*., 1998). Intracellular glutathione concentration also decreased during embryo development from 1-cell to blastocyst (Abeydeera *et al*., unpublished data). Therefore, supplementation of glutathione in culture medium would increase intracellular glutathione content, regulate cell function by acting as an antioxidant, or perform other functions which remain to be addressed by further studies. Actin filament is one of the preferential and earliest targets in cell injury induced by oxidative stress and thiol-depleting agents (Bellomo *et al*., 1990; Huot *et al*., 1996; Palladini *et al*., 1996). Oxidative stress can induce severe fragmentation of F-actin in many types of cells (Mocali *et al*., 1995; Huot *et al*., 1996). The

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antioxidant *N*-acetyl-cysteine was able to protect cells from oxidative stress (Malorni *et al*., 1995). It is probable that oxidative stress induces delay in or failure of polymerisation of the cytoskeleton, thus preventing embryo division (Gardiner & Reed, 1994). Glutathione and BSA may be beneficial to the dynamic behaviour of the cytoskeleton, thus protecting embryos from oxidative stress (Malorni *et al*., 1995) and supporting embryo development.

In the present study we found that there were no significant differences in the morphology of embryos or in the microfilament distribution in the embryos produced in defined medium and BSA-containing medium. However, lower blastocyst rate and fewer cell numbers in the blastocysts were observed in the defined medium. It would appear that other components in BSA, such as growth factors, hormones, amino acids or vitamins, stimulated cell division. The present defined medium can be used to investigate the effects of these components on pig embryo development.

In conclusion, the present study indicates that pig embryos produced by IVM/IVF can develop to blastocyst in a chemically defined medium and that a synergistic effect was observed when both BSA and glutathione were supplemented in the embryo culture medium.

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