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Effects of Protein Source, Vitamin E and Phenazine Ethosulfate on Developmental Competence and Quality of Porcine Embryos Cultured *in vitro**

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Effects of fetal calf serum (FCS) or bovine serum albumin (BSA), with or without vitamin E (vit. E) or phenazine ethosulfate (PES) supplementation on developmental competence and quality of cultured porcine embryos were examined. The experiment was done on zygotes and 2-cell embryos obtained from superovulated gilts. Morphologically normal zygotes were cultured *in vitro* in NCSU-23 medium supplemented with: experiment 1-0.004 g/ml BSA, 10% FCS, protein-free (control); experiment 2-0 (control), 25, 50 or 100 μM vit. E; experiment 3-0 (control), 0.025, 0.05 or 0.075 μM PES. Embryo quality criteria were developmental competence (cleavage, morula and blastocyst rates), total cell number per blastocyst and degree of apoptosis as assessed by TUNEL staining. Presence of BSA in culture medium increased significantly morula and blastocysts production as compared to FCS ($P<0.001$) and protein-free group ($P<0.05$ and $P<0.001$, respectively). The blastocysts cultured in protein-free medium had higher average number of apoptotic nuclei and DNA fragmented nucleus index as compared to the BSA ($P<0.05$ and $P<0.01$, respectively) and FCS ($P<0.5$) group. Supplementation in culture medium of 100 μM vit. E increased blastocyst production as compared to control and 50 μM vit-E ($P<0.05$). Both the number of cells per and percentage of TUNEL positive nuclei per blastocyst were slightly lower in PES treated than control groups.

Key words: Protein, vitamin E, phenazine ethosulfate, pig, embryo, *in vitro*.

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The main factor affecting porcine blastocyst yield and quality during embryo development *in vitro* are culture conditions. Since WRIGHT (1977) first reported that porcine embryos could develop from the one-cell stage into blastocyst *in vitro*, several culture media have been used for *in vitro* culture of porcine embryos (HAGEN *et al*. 1991; PETERS &WELLS 1993; DOBRINSKY *et al*. 1995; YOSHIOKA *et al*. 2002). Among these media North Carolina State University (NCSU)–23 medium is known to be one of the most successful for porcine embryo culture (RATH *et al.* 1995; GAJDA 1998; LONG *et al*. 1998; GAJDA & SMORĄG 2004). However, RUBIO POMAR *et al*. (2005) and our reports (BRYŁA *et al*. 2006) have shown that quality of porcine embryos cultured *in vitro* is still lower compared to *in vivo* derived embryos.

Basic culture media are usually supplemented with albumin or serum, which contain amino acid that play an important role as energy sources, osmoregulators, and pH stabilizers (BAVISTER 1995). Although bovine early stage embryos (one to eight cells) are most often cultured in media using albumin as a protein supplement (BAVISTER 1995), some studies have used serum supplementation during these stages (WOLLENBERG *et al*. 1990; BAVISTER 1995). On the other hand, serum was shown to be beneficial for advanced pig embryo development (ROBL & DAVIS 1981; DOBRINSKY *et al*. 1996). A few studies have reported no difference in embryo development using fetal calf serum, bovine serum albumin or no protein in culture medium.

Supplementation of culture media with vitamin E (alpha-tocopherol) increased survival rates *in vi-*

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tro of rat (STEELE *et al*. 1974), mouse (ARECHIGA *et al*. 1994) and bovine (OLSON *et al*. 2000) embryos. Vitamin E is a fat-soluble antioxidant in animal cells that suppresses peroxidation of membrane lipids (TAPPEL 1980). Peroxidation of fatty acids is known to inhibit the function of cells and might induce cell death (SPITELLER 2001). Porcine embryos contain a considerably higher concentration of fatty acids than other mammalian embryos (MCEVOY *et al.* 2000) and they should be more susceptible to lipid peroxidation.

It has recently been demonstrated that bovine blastocyst produced by culture with phenazine ethosulfate (PES) may be a promising approach to improving *in vitro* production of embryos (SEIDEL 2006). The PES treatment increased glucose metabolism, tended to increase the pentose phosphate pathway flux of glucose and clearly reduced accumulation of lipids in bovine embryos produced in chemically defined media (DE LA TORRE-SANCHEZ *et al*. 2006).

It seems interesting, then, to determine whether supplementation of protein (serum or albumin), with or without vitamin E or phenazine ethosulfate, improved developmental competence and quality of cultured porcine embryos.

Material and Methods

Embryo donors

Six-month-old gilts at about 90-110 kg body weight were used as donors. Gilts were superovulated by i.m. injection of 1500 IU of PMSG (Serogonadotropin, Biowet) followed by 1000 IU of hCG (Biogonadyl, Biomed) administered 72 hours later. At the onset of estrus (24 h after hCG administration) the gilts were artificially inseminated twice at 12-hour intervals with the standard dose of semen. The gilts were slaughtered within 24-28 hours of insemination.

Embryo recovery

Embryos were collected after flushing oviducts with PBS medium (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 20% fetal calf serum (FCS, Sigma Chemical Company, St. Louis, MO, USA) at about 30°C. The recovered embryos were examined morphologically under a stereomicroscope, in a laminar chamber at about 30°C.

Embryo culture *in vitro*

The cultured medium North Carolina State University (NCSU)–23 (PETTERS & REED 1991) was prepared in our laboratory and its components (Sigma Chemical Company, St. Louis, MO, USA) were dissolved in H_2O (Sigma Chemical Company, St. Louis, MO, USA). After dissolving the pH of media was adjusted to 7.2. Prior to use the media were filtered with the Millipore 0.22 μm filter (Millex-GS, France). The media were stored at 4°C and used for the period of 3 weeks (GAJDA & SMORĄG 2004).

Experiment 1

Morphologically normal zygotes or 2-cell embryos were cultured *in vitro* in NCSU-23 medium supplemented with: 0.004 g/ml bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO, USA) (group A1), 10% fetal calf serum (FCS; Sigma Chemical Company, St. Louis, MO, USA) (group A2), protein-free (group A3).

Experiment 2

Morphologically normal zygotes or 2-cell embryos were cultured *in vitro* in NCSU-23 medium supplemented with bovine serum albumin (BSA) and: 25 μM vitamin E (α-tocopherol) (Sigma Chemical Company, St. Louis, MO, USA) (group B1), 50 μM vitamin E (group B2), 100 μM vitamin E (group B3), 0 vitamin E (control group) (GAJDA *et al.* 2006).

Experiment 3

Morphologically normal zygotes or 2-cell embryos were cultured *in vitro* in NCSU-23 medium supplemented with bovine serum albumin and: 0.025 μM phenazine ethosulfate (PES; Sigma Chemical Company, St. Louis, MO, USA) (group C1), 0.05 μM PES (group C2), 0.075 PES (group C3), 0 PES (control group).

The embryos assigned for culture were washed in one of the experimental or control media and then transferred for culture to plastic 4-well dishes (Nunc, Denmark), which contained approximately 1.0 ml of the medium. About 10 embryos were placed in each well. The cultured embryos were placed in a CO_2 incubator (5% CO_2 in air at 39°C) for 96 to 120 hours (GAJDA & SMORĄG 2004).

Embryo evaluation

During culture embryos were evaluated for morphological stage of development 1-5 days after being transferred to culture media. Embryo quality criteria were developmental competence (cleavage, morula and blastocyst rates), total cell number per blastocyst and degree of apoptosis as assessed by TUNEL staining (Fig. 1).

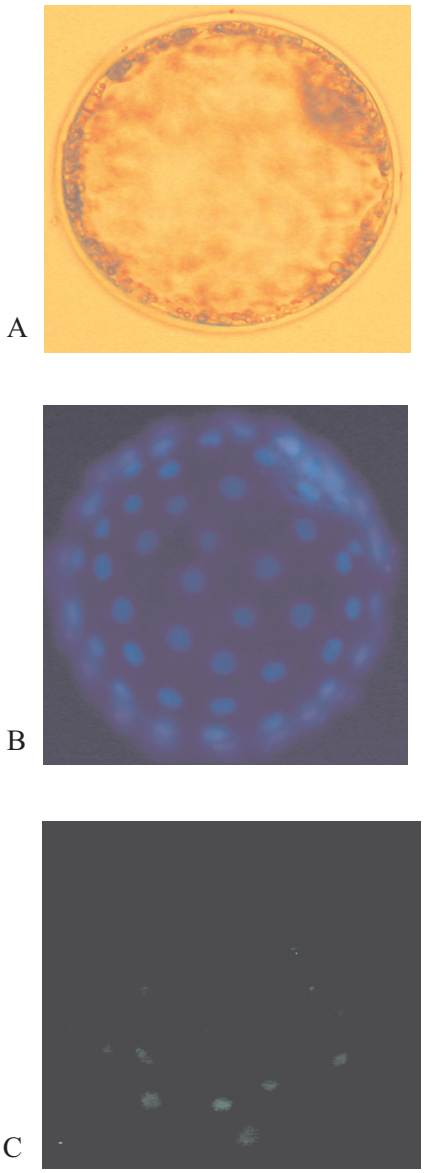

Fig. 1. *In vitro* derived pig expanded blastocyst. A – Normal light microphotograph; B – DAPI staining for total number of cells counting; C – TUNEL staining for number of cells with DNA-fragmented nucleus counting (green, arrow) ×100.

TUNEL assay

DNA fragmentation of embryos was analyzed by using a combined technique for simultaneous nuclear staining and TUNEL by a modification of the procedures used by BRISON & SCHULTZ (1997).

The embryos were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. Then the embryos were washed three times in 50 μl drops of PBS-PVP (1 μg/ml polyvinylpyrrolidone in PBS), and permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature in a humidified box and washed again in drops of PVP solution. Next the embryos were incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; In Situ Cell Detection kit, Roche Diagnostics, Germany) for 1h in an incubator at 38.5°C and 5% CO_2 in air. As positive controls, one or two embryos per TUNEL analysis were incubated in 50 U/ml DNase (Promega) for 20 min at 38,5°C. As negative controls, one or two embryos per TUNEL analysis were incubated in fluorescein-dUTP in the absence of TdT. After TUNEL, the embryos were washed three times in drops of PVP solution and transferred through a gradient of Vecta-Shield with DAPI (Vector Laboratories, Burlingame, CA) at 75 and 100% (v/v) in PBS/PVP and mounted on a glass slide. Labelled nuclei were examined under a Nikon Eclipse E600 microscope fitted with epi-fluorescent illumination (DARZYNKIEWICZ *et al*. 2001; MULLEN & CRISTER 2004). The total number of cells and number of cells with DNA-fragmented nucleus were counted, and DNA fragmented nucleus index was calculated by dividing the number of cells with DNA-fragmented by total number of cells, which included DNA-fragmented nuclei.

Statistical analysis

Results were analyzed by Chi-square and Fisher tests.

Results

The results are summarized in Tables 1-6. There was no difference in percentages of cleaved embryos between NCSU-23 medium supplemented with BSA, NCSU-23 supplemented with FCS and NCSU-23 protein free treated groups (92.8, 88.4 and 92.4%, respectively) (Table 1). Presence of BSA in culture medium increased significantly morula and blastocysts production as compared to FCS ($P<0.001$) and protein-free group ($P<0.05$ and $P<0.001$, respectively) (Table 1). The blastocysts cultured in protein-free medium had higher average number of apoptotic nuclei and DNA frag-

Table 1

Effect of protein supplementation on developmental competence of 1- and 2-cell porcine embryos

Treatment group	No. of embryos cultured/ replic.	No. of cleaved embryos (%)	No. of morulae (%)	No. of expanded blastocysts (%)
A1	73 / 9	69 (94.5)	66 (90.4)[a]	59 (80.8)[c]
A2	71 / 9	65 (91.5)	59 (83.1)	20 (28.2)[d]
A3	77 / 9	69 (89.6)	59 (76.6)[b]	17 (22.1)[e]

Group A1 – NCSU-23 + BSA
Group A2 – NCSU-23 + FCS
Group A3 – NCSU-23 protein-free
a,b – $P<0.05$ c,d; c,e – $P<0.001$

Table 2

The quality of porcine expanded blastocysts cultured in NCSU-23 medium supplemented with different protein

Treatment group	No. of blastocysts assessed/ replic.	Average number of cell/ blastocyst	Average number of apoptotic nuclei/ blastocyst	% TUNEL positive
A1	13 / 4	32.3[a]	3.31[d]	10.25[f]
A2	14 / 4	39.5[b]	6.01	15.34[g]
A3	13 / 4	30.5[c]	7.46[e]	24.46[h]

Group A1 – NCSU-23 + BSA
Group A2 – NCSU-23 + FCS
Group A3 – NCSU-23 protein-free
a,b; b,c; d,e;g,h – P<0.05 f,h – P<0.01

Table 3

Effect of vitamin E on developmental competence of 1- and 2-cell porcine embryos

Treatment group	No. of embryos cultured/ replic.	No. of cleaved embryos (%)	No. of morulae (%)	No. of expanded blastocysts (%)
B1	75 / 6	72 (96.0)	65 (86.7)	38 (80.0)
B2	76 / 6	70 (92.1)	67 (88.2)	55 (72.4)[a]
B3	74 / 6	70 (94.6)	67 (90.5)	66 (89.1)[b]
Control	49 / 6	47 (95.9)	46 (93.9)	43 (87.7)[c]

Group B1 – NCSU-23 + BSA + 25 μM vitamin E
Group B2 – NCSU-23 + BSA + 50 μM vitamin E
Group B3 – NCSU-23 + BSA + 100 μM vitamin E
Control – NCSU-23 + BSA
a,b; a,c – P<0.05

Table 4

The quality of porcine expanded blastocysts cultured in NCSU-23 medium supplemented with vitamin E

Treatment group	No. of blastocysts assessed/ replic.	Average number of cell/ blastocyst	Average number of apoptotic nuclei/ blastocyst	% TUNEL positive
B1	24 / 4	31.50[a]	1.25	4.15
B2	25 / 4	29.70[b]	1.40	4.85
B3	24 / 4	33.20	1.80	5.75
Control	15 / 4	35.60[c]	4.40	7.50

Group B1 – NCSU-23 + BSA + 25 μM vitamin E
Group B2 – NCSU-23 + BSA + 50 μM vitamin E
Group B3 – NCSU-23 + BSA + 100 μM vitamin E
Control – NCSU-23 + BSA
a,c; b,c – P<0.05

Table 5

Effect of phenazine ethosulfate (PES) on developmental competence of 1- and 2-cell porcine embryos

Treatment group	No. of embryos cultured/ replic.	No. of cleaved embryos (%)	No. of morulae (%)	No. of expanded blastocysts (%)
C1	97 / 7	96 (99.0)[a]	88 (90.7)	49 (70.0)
C2	98 / 7	95 (96.9)	86 (87.8)	74 (75.5)[c]
C3	80 / 7	78 (97.5)	67 (83.8)	48 (60.0)[d]
Control	35 / 7	32 (91.4)[b]	28 (80.0)	23 (65.7)

Group C1 – NCSU-23 + BSA + 0.025 μM PES
Group C2 – NCSU-23 + BSA + 0.05 μM PES
Group C3 – NCSU-23 + BSA + 0.075 μM PES
Control – NCSU-23 + BSA
a,b;c,d – P<0.05

Table 6

The quality of porcine expanded blastocysts cultured in NCSU-23 medium supplemented with phenazine ethosulfate (PES)

Treatment group	No. of blastocysts assessed/ replic.	Average number of cell/blastocyst	Average number of apoptotic nuclei/ blastocyst	% TUNEL positive
C1	24 / 4	30.5	1.37	4.49
C2	26 / 4	30.9	1.46	4.72
C3	26 / 4	35.8	2.08	5.81
Control	8 / 4	38.1	2.25	5.91

Group C1 – NCSU-23 + BSA + 0.025 μM PES
Group C2 – NCSU-23 + BSA + 0.05 μM PES
Group C3 – NCSU-23 + BSA + 0.075 μM PES
Control – NCSU-23 + BSA

mented nucleus index as compared to the BSA (P<0.05 and P<0.01, respectively) and FCS group (P<0.5) (Table 2).

There was no difference between control (NCSU-23+BSA) and treated B1, B2 and B3 groups (supplementation of NCSU-23+BSA with 25, 50 and 100 μM vitamin E) in percentage of cleaved embryos or embryos developed to morula. Supplementation in culture medium of 100 μM vitamin E (group B3) increased blastocyst production as compared to control (difference statistically non-significant) and B2 (50 μM vitamin E) (P<0.05) group (Table 3). Average number of apoptotic nuclei and DNA-fragmented nucleus index was lower in blastocyst cultured with 25 μM (group B1), 50 μM (group B2) and 100 μM (group B3) vitamin E compared to the control (Table 4).

There was difference between C1 (0.025 μM PES treated) and control group in percentage of cleaved embryos ($P<0.05$), C1, C2, C3 and control in percentage of morula (difference statistically non-significant), C1, C2 and control in percentage of blastocysts (difference statistically non-significant) rates (Table 5). Both the number of cells per and percentage of TUNEL positive nuclei per blastocyst were lower in PES treated than control groups (difference statistically non-significant) (Table 6).

Discussion

The results of the first experiment have shown that NCSU-23 medium supplemented with BSA significantly improved porcine embryo development and the quality of the resulting blastocysts. Staining with TUNEL revealed that apoptosis occurred in expanded blastocysts from all tested group (A1, A2 and A3), but significantly lower apoptotic cell ratio was found in embryos cultured in medium with BSA. It was reported that BSA was beneficial to all stages of embryo development: the first cleavage division of hamster embryos (MCKIERNAN & BAVISTER 1992), improved *in vitro* development of porcine embryos (YOUNGS & MCGINNIS 1990; BAVISTER 1995; KIM *et al*. 2004), the formation of blastocysts in porcine embryo (RHO & HWANG 2002) and cell number in bovine embryo (KIRCHER *et al.* 1999). In this study we tested the effect of two macromolecules, BSA or FCS on porcine zygotes and 2-cell embryo development. In some previous reports, culture of early embryos with FCS was detrimental to blastocyst development *in vitro* and significantly reduced the formation of hatched blastocysts (DOBRINSKY *et al*. 1996; BAVISTER 1995). In the present study we confirmed the detrimental effect of FCS supplementation on early pig embryo development *in vitro*. However, the quality of blastocysts, as evaluated by the total cell number, was higher after culture in medium with FCS than with BSA. In this study the increased rates of cell number of blastocysts in presence of serum confirmed the beneficial role of FCS for advanced pig embryo development (ROBL & DAVIS 1981; POLLARD *et al*. 1995) and total cell number (DOBRINSKY *et al.* 1996). Therefore, an interesting, biphasic culture system with substitution of FCS for BSA following the morula-blastocyst transition has been suggested for culture bovine embryos (WANG *et al*. 1997) and further for pig embryos (KIM *et al*. 2004).

Moreover, our results of the first experiment demonstrated that culture in protein-free medium reduced significantly morula and blastocyst rates, total cell number in blastocysts and increased DNA fragmented nuclei as compared to protein supplementation media. This fact supports the concept that protein is strictly required for culture of pig embryos.

During culture detrimental effects of oxygen-derived free radicals on embryos have been demonstrated (JOHNSON & NASR-ESFAHANI 1994). Vitamin E occurs naturally in cell membranes and protects polyunsaturated fatty acids in membranes from oxidative stress. Peroxidation of these membrane lipids can lead to structural damage, affecting function and permeability of membranes, resulting in irreversible cell injury and death (OLSON & SEIDEL 2000). In the second experiment we examined whether vitamin E improved porcine embryo development *in vitro* as determined by developmental competence, number of cells per blastocyst and the incidence of apoptosis. We showed that more pig embryos developed to expanded blastocysts when NCSU-23 medium was supplemented with 100 μM vitamin E than with control treatment. Similar observations were reported for IVM/IVF porcine (KITAGAWA *et al*. 2004) and bovine (OLSON & SEIDEL 2000) embryos. Moreover, in the second study, *in vitro* produced bovine embryos cultured 5.5 days in medium with 100 μM vitamin E were transferred nonsurgically to recipient cows and then collected 7 days later. Embryos cultured with vitamin E were approximately 63% larger in surface area than controls.

Furthermore, in our experiment a lower number of apoptotic nuclei and proportion of cells with fragmented DNA (nuclei positive for TUNEL) was recovered from all vitamin E (25, 50 and 100 μM) groups. A similar result was reported previously for 8-16 cell pig embryos (KITAGAWA *et al*. 2004), in which vitamin E (100 μM) was shown to reduce the H_2O_2 content and, as a consequence, reduced DNA fragmentation.

In this study culture medium was supplemented with vitamin E containing bovine serum albumin. REIS *et al*. (2003) showed that in the presence of serum, supplementation with vitamin E increased both total and good quality bovine blastocyst yields and lipid accumulation. Our study confirms that some benefit is achieved when vitamin E is added to porcine culture medium with serum albumin. Further research is required to determine the lipid content of porcine embryos cultured in medium with vitamin E.

It is possible to improve *in vitro* production of embryos by modification of cultured cells (DE LA TORRE-SANCHEZ *et al*. 2006). In the third experiment we studied the effect of one of the metabolic regulators – phenazine ethosulfate (PES) – on developmental competence and quality of porcine embryos. Up to now, the effects of PES supplementation of NCSU-23 medium on porcine early embryo development are not known. In this study,

we observed the slightly higher rates of cleaved embryos, morula and blastocyst in PES treated groups compared to control however, slightly more cell numbers in blastocysts were observed in control group. Results from embryo quality showed that apoptosis had a low incidence in embryos cultured with PES compared to those cultured without PES. This observation was consistent with recent reports that use of phenazine ethosulfate in bovine culture media may be a promising approach to improving *in vitro* production of bovine embryos (DE LA TORRE-SANCHEZ *et al.* 2006). Moreover, the authors demonstrated that the PES treatment increased glucose metabolism and reduced accumulation of lipids in bovine embryos. It is known that porcine embryos have a considerably higher content of lipids than other mammalian embryos. The success rates of cryopreservation of porcine embryos appear to be highly correlated with cytoplasmic lipid content. It would be interesting (especially considering cryoconservation) to determine whether and how the process of lipid accumulation progresses in porcine embryo cultured in media containing PES.

It is concluded that supplementation of culture medium with protein, vitamin E or phenazine ethosulfate changes culture conditions at the level influencing the quality of cultured embryos. Therefore, the question may be raised whether the changes in embryo quality may affect cryconservation or cloning effectiveness.

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