Original Articles

Effect of Protein Supplement Source on Porcine Oocyte Maturation and Subsequent Embryonic Development after Parthenogenetic Activation

(pig / oocyte / maturation / embryo / development)

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Abstract. The aim of this study was to compare the effect of purified GPBoS and commonly used FCS on porcine oocyte maturation and subsequent embryonic development after their parthenogenetic activation. COCs were obtained from dissected follicles and cultured for 18, 24, 30, 36, 42 and 48 h in M-199 medium either with GPBoS or FCS. After 24 h with GPBoS, 91% of oocytes reached MI stage while in the medium supplemented with FCS, only 29% of oocytes reached the same stage ($P < 0.05$). The majority of oocytes from the FCS group (61%) reached MI stage approximately 6 h later. In the time periods between 36 to 48 h both groups of oocytes reached the same stage of maturation. After 48 h of culture the oocytes with extruded polar bodies were activated by a single electric pulse and then cultured with 4 mM 6-DMAP. Activated oocytes were cultured in PZM-3 medium supplemented with 3 mg/ml of BSA. After 7 days, the development and the quality of embryos were evaluated. The results showed that the maturation of oocytes in the presence of GPBoS significantly increased their subsequent developmental ability when compared with FCS supplementation (27% vs. 19% of blastocysts, $P < 0.05$). However, differential staining revealed that once blastocysts were formed in either group, they had the same total cell number (40 vs. 41) and also the ICM/total cell ratio (0.27 vs. 0.29).

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Abbreviations: 6-DMAP – 6-dimethylaminopurine, AI-TI – anaphase-telophase I, BSA – bovine serum albumin, COCs – cumulus-oocyte complexes, FCS – foetal calf serum, FSH – follicle-stimulating hormone, GPAG – growth-promoting alpha globulin, GPBoS – growth proteins of bovine serum, GV – germinal vesicle, GVBD – germinal vesicle breakdown, ICM – inner cell mass, IVF – *in vitro* fertilization, LH – luteinizing hormone, MI (II) – metaphase I (II), NT – nuclear transfer, PBS – phosphate-buffered saline, PVA – polyvinyl alcohol, PZM-3 – porcine zygote medium, TE – trophectoderm.

Pig is a very promising model organism for many biomedical studies, for example for the establishment of embryonic stem cell lines and their subsequent differentiation, xenotransplantation, etc. (Prather et al., 2003). These experiments, however, require a large number of high-quality oocytes and embryos. Thus, the very essential step is the improvement of current culture systems for porcine oocytes collected from antral follicles that are then matured *in vitro* and used for *in vitro* fertilization or nuclear transfer (NT). Despite intensive research, the potential of *in vitro* cultured oocytes to develop into viable offspring after fertilization is not satisfactory and this is a limitation for other techniques of pig embryo biotechnologies such as NT, transgenesis, etc.

After isolation from antral follicles, porcine oocytes are arrested in prometaphase of the first meiotic division, where the nucleus, called germinal vesicle (GV), contains a clearly visible nuclear envelope and ring-shaped heterochromatin around the nucleolus. These oocytes can be successfully cultured *in vitro* up to the second metaphase (MII) with the first polar body extruded. Only these oocytes can be successfully fertilized and develop up to the blastocyst stage. This means that as is in many other mammalian species, meiotic maturation occurs spontaneously after the oocytes are removed from the follicular microenvironment (Pincus and Enzmann, 1935). In contrast to rodent oocytes, the porcine culture system must be supplemented with gonadotropins.

In many previous studies, it has been demonstrated that protein supplementation also belongs among components of *in vitro* culture medium that significantly affect meiotic maturation of porcine oocytes (Motlik,

1972; Racowsky and McGaughey, 1982; Zheng and Sirard, 1992; Yoshida et al., 1992; Abeydeera and Day, 1997).

Growth proteins of bovine serum (GPBoS) belong among protein sources that can be used as components of *in vitro* culture medium and indeed, their convenience for porcine oocyte maturation has been described by Motlik (1972). GPBoS is a protein complex isolated by Michl (1961) from the calf serum. It has been determined that the active component is the growth-promoting alpha-globulin (GPAG). It has been demonstrated that GPAG can influence the mitotic activity in mammalian cells *in vitro*. The positive effect was further determined in primary cultures and in cell lines (Michl, 1962, Macek and Michl, 1964).

In our study we compared the effects of GPBoS and foetal calf serum (FCS) on *in vitro* maturation of porcine oocytes. We focused on the timing of oocyte maturation and on embryonic development after the oocyte parthenogenetic activation. The number of developed blastocysts, their total cell number and inner cell mass (ICM)/total cell number ratio were compared for both groups.

Material and Methods

Unless stated otherwise all chemicals were purchased from Sigma (Prague, Czech Republic).

Collection of oocytes

Porcine ovaries obtained at a local slaughterhouse were transported in PBS with 0.01% PVA (phosphate-buffered saline with 0.01% polyvinyl alcohol, PBS/PVA) at 30–35°C. After washing in PBS/PVA, antral follicles between 3–6 mm in diameter were dissected from ovaries with two scalpels. Only bright clear follicles were selected and opened with two forceps and their content had been released into manipulating medium M2. Cumulus-oocyte complexes (COCs) were aspirated by a narrow glass pipette and transferred into 500 µl of maturation medium M-199 supplemented with 4 mg/ml of GPBoS (Sevapharma, Prague, Czech Republic) or 10% FCS (Gibco Invitrogen, Prague, Czech Republic), 5 µg/ml follicle-stimulating hormone (FSH) (from porcine pituitary), 5 µg/ml luteinizing hormone (LH) (from ovine pituitary), 50 µg/ml sodium pyruvate, 70 µg/ml cysteine and 50 µg/ml gentamicin (Gibco) in 4-well plates (Nunc, Roskilde, Denmark) and cultured at 38.5°C under 5% CO_2 in air.

Evaluation of the nuclear status of cultured oocytes

After the time period chosen COCs were treated with 0.1% hyaluronidase and cumulus cells were removed by pipetting. Denuded oocytes were then fixed with acetic acid-alcohol (1 : 3 v/v) for 48 h and stained with 1% aceto-orcein.

Parthenogenetic activation

After 48 h of culture, COCs with expanded cumuli were treated with 0.1% hyaluridonase and cumulus cells were removed as above. Only those oocytes containing the first polar body were washed three times in M2 medium, then three times in the activation medium (0.3 M manitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.01% PVA, Sigma) and subjected to a single DC pulse (1.5 kV/cm, for 100 µs, CF 150, BLS, Budapest, Hungary). The oocytes were then washed nine times in M2 medium, transferred into 500 µl of M199 with 4 mM 6-dimethylaminopurine (6-DMAP, Sigma) in 4-well plates and cultured for 4 h at 38.5°C under 5% CO_2 in air. Then, after nine washings in M2 medium the activated oocytes were transferred into 500 µl of PZM-3 medium (Yoshioka et al., 2002) in 4-well plates and cultured for 7 days at 38.5°C under 5% CO_2 in air.

Differential staining of blastocysts

Differential staining was performed according to a method described by Thouas et al. (2001). Briefly, blastocysts were treated with 1% Triton X-100, 0.01 % PVA and 100 µg/ml propidium iodide in PBS for 15 s. Immediately after they were transferred into 96% ethanol with 25 µg/ml of Hoechst-33342 and incubated for 10 min at 4°C. After several washings in 0.01% PVA in PBS, blastocysts were mounted in a small drop of Permafluor (Immunotech, Prague, Czech Republic). The evaluation of differential staining was done under a fluorescence inverted microscope equipped with appropriate filters (Leica). The ICM was stained blue and the trophoectodermal cells (TE) were stained pink.

Statistical analyses

Total number of cells and ICM/total number of cells ratio were analysed by Student's t-test. All other data were analysed by χ^2 test.

Results

Maturation of porcine oocytes

First, we have examined the nuclear status of oocytes after 18, 24, 30, 36, 42 and 48 h of maturation in the medium supplemented either with GPBoS or FCS. After fixation and staining a significant acceleration of oocyte maturation was observed in GPBoS medium. In this medium 91% of oocytes reached MI stage after 24 h. In contrast, supplementation with FCS resulted only in 29% of oocytes in MI stage ($P < 0.05$). Prolonged incubation for up to 30 h clearly demonstrated that whilst the majority of oocytes cultured in GPBoS medium were at AI-TI stage (61%), the oocytes from FCS medium were mainly in MI stage (61%) ($P < 0.05$). However, after prolonged culture (36 to 48 h) both groups revealed a similar number of oocytes that completed the process of maturation and reached the MII stage (Table 1, Fig. 1).

Table 1. Effect of GPBoS or FCS on meiotic maturation of porcine oocytes

Culture time (h)	Protein supplementation	No. of oocytes examined	No. (%) of oocytes					
			GV	GVBD	MI	AI-TI	MII	
18	GPBoS	38	32 (84)	6 (16)				
	FCS	35	35 (100)	0 (0)				
24	GPBoS	33	0 (0)	3 (9)	30 (91)[a]			
	FCS	35	15 (43)	10 (29)	10 (29)[b]			
30	GPBoS	36	0 (0)		7 (19)[a]	22 (61)[a]	7	(19)
	FCS	33	2 (6)		20 (61)[b]	9 (27)[b]	2	(6)
36	GPBoS	41			3 (7)	1 (2)	36	(88)
	FCS	39			3 (8)	4(10)	32	(82)
42	GPBoS	29			1 (3)		27 (93)	
	FCS	33			1 (3)		32 (97)	
48	GPBoS	32			0 (0)		32 (100)	
	FCS	43			0 (0)		42 (98)	

[a,b]Values with different superscripts within a column differ significantly ($P < 0.05$).
Empty columns mean that both groups contain no oocytes at the respective stage 0 (0).

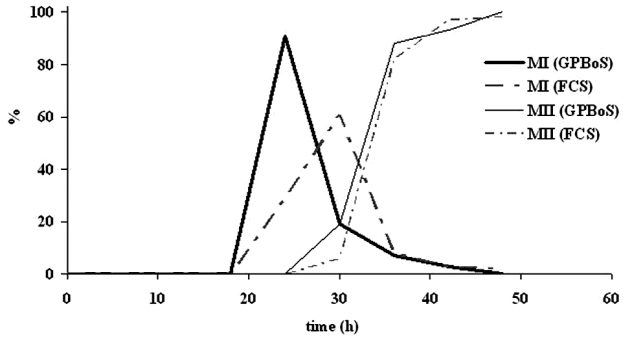

Fig. 1. Time course of maturation of porcine oocytes in media supplemented either with GPBoS or FCS

Embryonic development after electroactivation

In this part we have tested the developmental competence of matured porcine oocytes cultured in GPBoS- as well as in FCS-supplemented media. Based on our previous experiments, the electroactivation was followed by a 4-h treatment with 4 mM 6-DMAP. After a 7-day culture in PZM-3 medium, the number of blastocysts, total cell number per blastocyst and ICM/total cell ratio were evaluated.

As shown in Table 2 and Fig. 2, 27% of porcine oocytes matured in medium with GPBoS developed up to the blastocyst stage. The supplementation with FSC resulted in significantly lower developmental competence of matured oocytes (19% of blastocysts, $P < 0.05$), Table 2.

Differential staining was performed in order to evaluate the overall blastocyst quality, i.e. the total cell number per blastocyst and ICM/total cell ratio. As shown in Table 3, differences between both groups were not statistically significant (40 vs. 41 for total cell number per blastocyst and 0.27 vs. 0.29 for ICM/total cell ratio).

Discussion

The first studies analysing the active components of GPBoS - GPAG describe its ability to bind and incorporate exogenous DNA into cell nuclei and its responsibility for mitotic activity in primary cultures and within the cell lines (Macek and Michl, 1964; Marec et al., 1978; Keprtova et al., 1978; Keprtova et al., 1982). GPBoS also stimulated the entry of radiolabelled triphosphate into cells and enhanced DNA and RNA synthesis of these cells (Michl and Spurna, 1974). However, there is only one comparative study on its effects on meiosis (Motlik, 1972). The author found that porcine oocytes cultured in medium supplemented with GPBoS (15 mg/ml) reached the MII stage in 80%. Comparing to results of other studies it was concluded that GPBoS support meiotic maturation of porcine oocytes more effectively than inactivated homologous serum (Harms et al., 1970; McGaughey and Polge, 1971) or BSA (Biggers et al., 1967).

In the present study we have compared the effects of GPBoS with widely used FCS on meiotic maturation of porcine oocytes in relation with parthenogenetically activated oocyte embryonic development. We found that both protein sources support meiotic maturation;

Table 2. Developmental competence of activated porcine oocytes matured in medium supplemented with GPBoS or FCS

Protein supplementation	No. of embryos examined	No. (%) of blastocysts
GPBoS	138	38 (27)[a]
FCS	164	31 (19)[b]

The oocytes at MII stage were artificially activated after 48 h of maturational culture. The number of blastocysts was evaluated after 7 days of culture in PZM-3 medium.
[a,b]Values with different superscripts within a column differ significantly ($P < 0.05$).

Table 3. The quality of blastocysts in dependence on the oocyte maturation system (GPBoS vs. FCS)

Protein supplementation	No. of blastocysts examined	Total No. of cells	ICM / Total No. of cells
GPBoS	14	40	0.27
FCS	8	41	0.29

The oocytes at MII stage were artificially activated after 48 h of culture (GPBoS or FCS). Blastocysts were evaluated after 7 days of culture in PZM-3 medium.

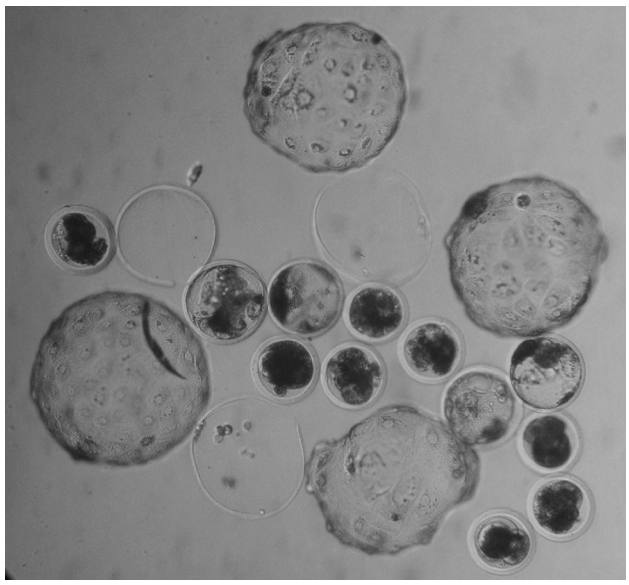

Fig. 2. Blastocysts derived from activated oocytes after *in vitro* maturation in GPBoS medium.

nevertheless, the analysis of maturation timing revealed that in medium supplemented with FCS, oocytes achieved the MI stage about six hours later than in the medium with GPBoS. This delay of meiotic maturation could be caused by some inhibitory factors contained in FCS that suppress the positive response of cumulus cells to FSH (Downs et al., 1991). FSH and LH effects on cumulus cells are important for their progesterone production, which is responsible for the acceleration of germinal vesicle breakdown (GVBD) and the resumption of meiosis (Shimada and Terada, 2002; Yamashita et al., 2003).

Interestingly, after 48 h of culture in both media systems (GPBoS and FCS) certain differences in the cytoplasmic consistency may be detected. Supplementation with GPBoS resulted in oocytes with clearly distinguished first polar bodies. Detection of these structures in oocytes cultured in FCS is very difficult. In our laboratory, certain further manipulations such as intracytoplasmic sperm injection (ICSI) or nuclear transfer work better with oocytes matured in GPBoS-supplemented medium (unpublished results). Additionally, our study showed that GPBoS also stimulate the embryonic development up to the blastocyst stage. Embryos originated from oocytes cultured in medium supplemented with GPBoS develop into a significantly higher number of blastocysts after artificial activation when compared with FCS. Surprisingly, the culture of oocytes in medium supplemented with FCS does not affect the final quality of developed blastocysts as the total cell number and the ICM/total cell number ratio was similar in both groups.

We conclude that GPBoS can be effectively used as a protein source for the *in vitro* culture system of porcine oocytes as it enhances the quality of maturation of oocytes and this results in a significantly higher number of blastocysts after parthenogenetic activation in comparison with FCS.

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