The effect of horse serum on \textit{in vitro} development of porcine parthenogenetic embryos

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SUMMARY

The objective of this study was to examine the effect of different sera and serum-like substances on the preimplantation development of porcine parthenogenetic embryos. Chemically activated (calcium ionophore A23187+cytochalasin B) pig oocytes were pre-cultured for five days. On day 5, the parthenogenetic embryos were treated with porcine follicular fluid (PFF), fetal bovine serum (FBS), horse serum (HS) or porcine serum albumin (PSA), and were cultured two more days. Horse serum was found to be the most effective protein source in enhancing parthenote development judging by blastocyst formation and hatching. Next, three different concentrations of HS (10, 20 and 30\%) were used to determine the optimal
HS concentration needed to improve the development of porcine parthenogenetic embryos. All HS concentrations increased the blastocyst cell number and decreased the incidence of blastocyst apoptotic cells with 20% being the most effective. In conclusion, horse serum enhanced parthenogenetic embryo development and the quality of porcine parthenogenetic embryos. 


**Key words:** pig, parthenotes, preimplantation development, blastocyst hatching, blastocyst apoptosis

## INTRODUCTION

Embryological technology can be widely applied in agricultural or biomedical research. The pig has been extensively used as an animal model of human diseases, a genetically defined model for xenotransplantation and a source of human therapeutical proteins. Molecular genetic manipulation of the porcine genome holds promise for producing high quality products and disease-resistant animals with improved reproductive capacity. Somatic cell nuclear transfer (SCNT) is widely used to produce genetically identical animals. However, only 1-2% of the embryos reconstructed by SCNT develop to term [15]. To enhance the efficiency of SCNT production, almost all steps of the procedure such as *in vitro* culture, oocyte activation, micromanipulation, embryo transfer need to be improved. Artificial activation of reconstituted eggs and optimal *in vitro* culture conditions for the developing egg are essential for producing viable embryos capable of full-term development after transfer into surrogates [20].

Different media have been developed for culturing pig embryos including Whitten’s medium [26]; North Carolina State University (NCSU)-23 medium [21]; Beltsville embryo culture medium (BECM)-3 [5]; porcine zygote media PZM-3, PZM-4, and PZM-5 [28]. NCSU-23 medium has been widely used because of its effective support of porcine embryo culture after *in vitro* fertilization or SCNT. The PZM supports post-fertilization blastocyst development [28] and parthenogenesis [9] to the same extent as NCSU-23 [4].

Many experiments have been conducted to optimize *in vitro* culture system leading to the production of high quality embryos for successful
animal production after SCNT. It was found that increased developmental competence and quality of embryos depends upon using specific factors (e.g. hormones) or complex supplements (e.g. serum). Melatonin, for example, increases the cleavage rate of porcine preimplantation embryos in vitro [24] and leptin enhances the cleavage rate and blastocyst formation rate in porcine preimplantation embryos [3]. Moreover, adiponectin, relaxin, ghrelin, insulin-like growth factor-1, luteinizing hormone (LH), follicle stimulating hormone (FSH) and progesterone may positively affect embryonic developmental competence [2, 7, 11, 27, 29].

Specific metallic elements such as iron/copper, selenium may also be beneficial for embryo quality [6, 25]. Selenium prevents damage by reactive oxygen species and inhibits lipid peroxidation. Serum, in turn, is a common supplement used in embryo and cell culture, and is well known to contain growth factors that chelate heavy metal cations serve as osmolytes and surfactants. Serum affects the later stages of preimplantation embryo development by improving blastocyst quality, increasing cell number and blastocyst hatching rate, and decreasing apoptosis in defined regions. The addition of serum after 20-24 h of culture increased day-6 blastocyst yields and day-8 blastocyst cell numbers [1, 14]. Additionally, serum has a biphasic effect on embryo development, inhibiting the first cleavage division but stimulating blastocyst development [22]. Additional studies aimed to define the specific serum component(s) responsible for these effects are required. The aim of the study was to compare the effects of four sera and serum-like substances on in vitro embryo culture system, and to specifically investigate the effect of horse serum (HS) as a medium supplement used for the development of porcine parthenogenetic eggs.

**MATERIALS AND METHODS**

**Oocyte maturation**

All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless stated otherwise. Ovaries were collected from
prepubertal gilts at a local abattoir and transported in saline to the laboratory (2 h, 30°C). Ovarian follicular fluids and cumulus-oocyte complexes (COCs) were aspirated from antral follicles using a 10 ml disposable syringe with an 18-gauge needle. After sedimentation, COCs were recovered using a stereomicroscope (Olympus, Tokyo, Japan). Oocytes surrounded by several compact layers of cumulus cells were selected for in vitro maturation. Approximately 50 COCs were transferred into 500 μl of TCM-199 medium (Gibco-BRL, Grand Island, NY, USA), that had been covered with mineral oil, in a four-well culture dish (Nunc, Roskilde, Denmark). TCM-199 medium was supplemented with 0.1% polyvinyl alcohol (PVA, w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μl/ml LH, 0.5 μl/ml FSH, 75 μl/ml penicillin G, and 50 μl/ml streptomycin. Porcine follicular fluid (PFF; 10%) was also added to the maturation medium. Oocytes were matured for 42 to 44 h (38.5°C; 5% CO₂, humidified conditions).

Parthenogenetic activation and in vitro embryo culture

After in vitro maturation of oocytes, expanded COCs were vortexed in 0.1% hyaluronidase (HY) solution for 40 s. Cumulus cells were removed, the first polar body-extruded, and oocytes were collected for further experiments. Metaphase II stage oocytes were activated with calcium ionophore (5 μM) in NCSU-23 medium (5 min, room temperature-RT) and then immediately transferred and cultured in NCSU-23 medium supplemented with 7.5 μg/ml cytochalasin B (CB) for 4 h. Then, parthenotes were cultured in 50 μl of PZM-5 droplets (IFP, Yamagata, Japan) for five days (38.5°C; 5% CO₂, humidified conditions).

Experimental design

To evaluate the effects of HS on parthenogenetic pig embryo development in vitro, four different sera or serum like substances were used. In experiment 1, we examined the effects of fetal bovine serum (FBS), porcine serum albumin (PSA), HS, and PFF on the formation of blastocyst and hatched blastocyst from porcine parthenogenetic embryos. The activated embryos were
randomly distributed in five different dishes (four treatment groups and one untreated control group), and cultured in the PZM-5 for five days (38.5°C; 5% CO₂ in air). On day 5, the embryos were treated with the sera, and cultured in the PZM-5 for next two days (38.5°C; 5% CO₂ in air). The rates of blastocysts and hatched blastocysts formation were evaluated at the end of culture on day 7. Five replications of the experiment were carried out.

In experiment 2, the effects of three different concentrations (10%, 20% and 30%) of HS were investigated. The activated embryos were randomly distributed into four different dishes (three treatment groups and one untreated control group), and cultured as above. On day 5, the embryos were treated with HS and cultured in supplemented PZM-5 for the subsequent two days. The rate of blastocyst formation was evaluated on days 5, 6 and 7, and the rate of hatched blastocysts was determined on day 7. At the end of day 7, embryo quality was also assessed by nuclei staining of fully expanded and hatched blastocysts. Eight replications of the experiment were carried out.

In experiment 3, the effects of three different concentrations (10%, 20% and 30%) of HS on the apoptosis rate of blastocysts were determined. The activated embryos were randomly separated into four groups and cultured for five days, and then HS was directly added into culture droplets. At the end of day 7, the apoptosis rate was assessed by TUNEL assay of fully expanded and hatched blastocysts. Five replications of the experiment were carried out.

**Cell staining and counting**

HEPES-buffered TCM-199 medium supplemented with 0.1% BSA was used as a basic medium (BM). Nuclei of whole blastocysts were stained with 5 mM SYTO® 13 green fluorescent nucleic acid stains (Molecular Probes, Eugene, OR, USA) for 20 min within micro drops. After being washed three times in BM, SYTO® 13 stained blastocysts were preserved for 30-40 min in BM with mineral oil in an incubator under 5% CO₂ to reduce nonspecific cytosolic staining. Next, saponin (100 μg/ml) and propidium iodide (PI; 10 μg/ml) were used to stain trophectoderm (TE) cells. The cells were observed by fluorescence microscopy at 1-3 min intervals.
Illumination times were kept to 1-2 s to prevent dye bleaching due to light emitted by the mercury lamp. The dye permeations were confirmed by the appearance of red (TE cells) and green (inner cell mass, ICM, cells) color caused by PI and SYTO® 13, respectively. Then, embryos were rinsed three times in BM and carefully mounted on a glass slide under cover slips with an anti-fading gel mount (Molecular Probes, Eugene, OR, USA). The numbers of cells in ICM and TE were counted directly under fluorescence microscope (Nikon, Tokyo, Japan).

**TUNEL assay**

After culture, the parthenogenetically-derived blastocysts were washed three times in phosphate buffered saline (PBS) supplemented with 0.1% polyvinylpyrrolidone and fixed in 4% paraformaldehyde solution for 24 h at 4°C. Membranes were permeabilized with 0.5% of Triton X-100 for 30 min at RT. The TUNEL assay was used for assessing the presence of apoptotic regions using the *In Situ* cell death detection kit (TMR Red, Roche Diagnostics, Mannheim, Germany) for one hour at 38.5°C in the dark. Broken DNA ends were labeled with terminal deoxyribonucleotidyl transferase (TDT) and fluorescein-dUTP. Next, the embryos were washed and stained with 10 μg/ml of Hoechst-33342 (blue color) for 30 min at RT in the dark. The embryos were washed at least three times and mounted on slides with a ProLong Antifade kit (Molecular Probes, Eugene, OR, USA). The slides were stored at −20°C, and the numbers of apoptotic regions were counted using an epifluorescence microscope (Olympus, Tokyo, JAPAN).

**Statistical analysis**

Statistical analysis was performed using the SAS (Statistical Analysis System, Inc., Cary, NC, USA) program. Comparisons of blastocyst and hatched blastocyst formation, number of apoptotic cells and number of nuclei in blastocysts were based on “least squares” procedures performed by means of the General Linear Models Procedure (PROC GLM). Data are presented as mean±SD. The value of p<0.05 was regarded as statistically significant.
RESULTS

Experiment 1

The effect of four sera and serum-like substances on the formation of blastocysts and hatched blastocysts was investigated during the pig parthenote development. Horse serum significantly (p<0.05) increased blastocyst formation rate (tab. 1). Both HS and FBS (10%) enhanced (p<0.05) the formation of hatched blastocysts compared to that of controls (without serum). In addition, the blastocyst hatching rate was significantly higher in cultures treated with HS than in those treated with FBS (tab. 1).

Experiment 2

The effect of different HS concentrations on the development of pig parthenotes was examined in the experiment. The addition of 20% of HS resulted

Table 1. Effects of sera and serum like substances on development of pig parthenotes

<table>
<thead>
<tr>
<th>Supplements</th>
<th>No. of oocytes</th>
<th>No. of cleaved eggs (%)</th>
<th>No. of developed eggs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Blastos (%)</td>
<td>Hatched blastocysts (%)</td>
</tr>
<tr>
<td>Control group</td>
<td>323</td>
<td>231 (71.5±0.9)a</td>
<td>91 (39.4±9.9)b</td>
<td>4 (1.7±1.6)a</td>
</tr>
<tr>
<td>PSA-treated group</td>
<td>305</td>
<td>221 (72.5±2.4)a</td>
<td>27 (12.2±1.4)a</td>
<td>0 (0.0±0.0)a</td>
</tr>
<tr>
<td>PFF-treated group</td>
<td>267</td>
<td>188 (70.4±1.8)a</td>
<td>70 (37.2±3.4)b</td>
<td>0 (0.0±0.0)a</td>
</tr>
<tr>
<td>FBS-treated group</td>
<td>319</td>
<td>226 (70.8±0.7)a</td>
<td>84 (37.2±1.6)b</td>
<td>13 (5.8±1.3)b</td>
</tr>
<tr>
<td>HS-treated group</td>
<td>370</td>
<td>275 (74.3±2.3)a</td>
<td>128 (46.5±2.3)c</td>
<td>47 (17.1±7.0)c</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. All supplements (10%, v/v) were added into culture medium on day 5 of culture. The rates of blastocyst and hatched blastocysts formation were calculated from oocyte number and cleaved rates, respectively. The experiment was replicated five times. PSA: porcine serum albumin; PFF: porcine follicular fluid; FBS: fetal bovine serum; HS: horse serum. Different superscripts show significant differences within a column (p<0.05).
Table 2. Effects of horse serum doses on blastocyst formation and hatching ability of pig blastocyst produced by parthenogenetic activation

<table>
<thead>
<tr>
<th>Horse serum</th>
<th>No. of oocytes</th>
<th>No. of cleaved eggs (%)</th>
<th>No. of eggs developed to blastocysts (%)</th>
<th>No. of eggs developed to hatched blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 5</td>
<td>Day 6</td>
</tr>
<tr>
<td>0%</td>
<td>351</td>
<td>290 (82.6±3.2)</td>
<td>44 (15.2±6.2)</td>
<td>76 (26.2±6.0)</td>
</tr>
<tr>
<td>10%</td>
<td>335</td>
<td>287 (85.7±4.0)</td>
<td>58 (20.2±8.9)</td>
<td>112 (39.0±5.4)</td>
</tr>
<tr>
<td>20%</td>
<td>323</td>
<td>278 (86.1±3.8)</td>
<td>59 (21.2±9.1)</td>
<td>117 (42.1±6.0)</td>
</tr>
<tr>
<td>30%</td>
<td>323</td>
<td>276 (85.4±3.1)</td>
<td>45 (16.3±7.0)</td>
<td>99 (35.9±7.6)</td>
</tr>
</tbody>
</table>

Data are presented as means±SD. All porcine embryos derived from parthenogenetic activation were randomly distributed and cultured for two days in PZM-5 with or without horse serum (HS); HS was added to culture media on day 5 of culture. The formation rates of blastocyst and hatched blastocyst were calculated from oocyte numbers and cleaved rates, respectively. Different superscripts depict significant differences within a column (p< 0.05)
in a slightly higher blastocyst and hatched blastocyst formation compared to other HS concentrations (tab. 2; fig. 1). Moreover, all HS supplemented groups had a significantly (p<0.05) higher numbers of ICM and TE cells than the control group. Especially, the total number of blastocysts was significantly (p<0.05) increased after HS supplementation, with 20% HS being the most effective (fig. 2; tab. 3).

Experiment 3

The effect of different concentrations of HS on cell apoptosis in blastocysts was examined in the experiment. The number of apoptotic cells in blas-
Figure 2. Differential staining of porcine parthenogenetic blastocysts. Horse serum was added to culture medium at following concentrations: 0% (A-A1), 10% (B-B1), 20% (C-C1), and 30% (D-D1). Blastocysts were treated with SYTO 13® and propidium iodide on day 7 of culture. Green and red color indicate inner cell mass and trophectoderm cells, respectively; bar=100 μm.

Table 3. Effects of horse serum on blastocyst cell number of pig parthenotes

<table>
<thead>
<tr>
<th>Horse serum concentration</th>
<th>No. of blastocysts</th>
<th>No. of nuclei (mean±SD)</th>
<th>Percentage of ICM/total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TE</td>
<td>ICM</td>
</tr>
<tr>
<td>0%</td>
<td>43</td>
<td>41.9±11.6a</td>
<td>3.8±1.5a</td>
</tr>
<tr>
<td>10%</td>
<td>51</td>
<td>74.2±9.8c</td>
<td>8.6±2.3bc</td>
</tr>
<tr>
<td>20%</td>
<td>59</td>
<td>79.7±8.7d</td>
<td>9.4±2.5c</td>
</tr>
<tr>
<td>30%</td>
<td>50</td>
<td>68.7±7.8b</td>
<td>8.4±2.9b</td>
</tr>
</tbody>
</table>

Data are presented as means±SD. All porcine embryos derived from parthenogenetic activation were randomly distributed and cultured for seven days in PZM-5 and treated with horse serum for last two days of culture. Analysis of differential staining and cell counting was performed on expanded or hatched blastocysts and was replicated 10 times. ICM: inner cell mass; TE: trophectoderm cells. Different superscripts depict significant differences within a column (p< 0.05).
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tocysts was significantly lower in the presence of HS. In comparison to the other HS concentrations, the 20% HS was slightly the most effective in lowering the number of apoptotic cells (fig. 3; tab. 4).

Table 4. Effects of horse serum on cell apoptosis in porcine parthenogenetic blastocysts

<table>
<thead>
<tr>
<th>Horse serum concentration</th>
<th>No. of blastocysts</th>
<th>No. of total cells (mean±SD)</th>
<th>No. of apoptotic cells (mean±SD)</th>
<th>Percentage of apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>20</td>
<td>43.3±10.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.7±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10%</td>
<td>20</td>
<td>79.0±8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20%</td>
<td>20</td>
<td>82.7±7.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30%</td>
<td>20</td>
<td>78.4±6.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0±1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.5±1.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as means±SD. Apoptosis and cell counting analysis was performed on fully expanded or hatched blastocysts and replicated five times. Different superscripts depict significant differences within a column (p< 0.05)
DISCUSSION

In this study, the supplementation of culture medium with bovine or horse serum enhanced *in vitro* preimplantation development of porcine parthenogenetic embryos. The effect of HS was significantly more pronounced than that of FBS. It is possible that the rapid increase in cell number of the expanding blastocysts cultured in medium supplemented with serum resulted in a weak protease secretion by the zona pellucida [17]. The increase in embryonic cells resulted in a sufficient pressure to the zona pellucida, and thus, to the formation of hatched blastocyst [19].

According to manufacturer’s data, the FBS used in our study contained 10 times more hemoglobin (Hb) than HS. It was reported that the addition of 1 μg/ml Hb enhances bovine blastocyst development [16]; we used 1 μg/ml (10% HS) and 11 μg/ml (FBS) of Hb. In contrast, we found that porcine serum, containing 29 μg/ml Hb, did not support embryonic development (unpublished data). These findings suggest that the addition of serum at later preimplantation embryo stages support embryo development to hatched blastocysts. Moreover, although a high dose of Hb had a deleterious effect on embryo development, 1-3 μg/ml of Hb improved the development of pig parthenotes.

Medium supplementation with HS increased the embryonic cell number. This is in agreement with previous reports on embryonic development [5, 13, 18, 23]. Cell number of *in vivo*-derived porcine blastocysts are known to be significantly higher than those of IVF-derived or SCNT-derived blastocyst [12]. In this context our results are very promising, especially in view that SCNT-derived embryos present a problem related to implantation and progression of pregnancy. The TE cell component of SCNT-derived blastocyst is significantly lower than that of *in vivo- and vitro*-fertilized porcine blastocysts [12]. We found that TE cell number was significantly increased by HS supplementation. Since TE cells become part of the placenta, this could enhance the implantation rate thereby improving the success rate of embryo transfer. The increased cell number suggests that HS treatment from day 5 to day 7 of culture improves embryo quality. This culture system can be applied for somatic cell nuclear transferred embryos to improve successful implantation and pregnancy.
In this study, significant differences in apoptotic cell numbers were found in embryos cultured in media supplemented with different HS concentrations. Generally, the frequency of apoptotic nuclei incidence in blastocysts is around 7% [8]. In the present study, the low proportion of apoptotic cells may be attributable to the HS supplementation which also increased the mean number of total cells per blastocyst. Since Hb reduces \( \text{H}_2\text{O}_2 \) accumulation and decreases the frequency of apoptosis [10], the horse serum-derived Hb in our experiment might positively affect the development of preimplantation embryos in pigs.

In conclusion, medium supplementation with HS increased the quality of porcine parthenogenetic embryos. The mechanisms of action which are responsible for the effects of HS on parthenogenetic embryo development are being actively investigated.

Acknowledgments

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