Synergistic Effects of Glutathione and β-Mercaptoethanol Treatment During In Vitro Maturation of Porcine Oocytes on Early Embryonic Development in a Culture System Supplemented with L-cysteine

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Abstract. Various methods have been used to remove reactive oxygen species (ROS) generated from in vitro culture (IVC) conditions that can cause cell injury or death, including the application of low oxygen (O2) tension and the addition of antioxidants. The beneficial effects of antioxidants and O2 tension on IVC of porcine embryos, however, are controversial among researchers. In this study, we sought to determine the effects and optimal concentrations of antioxidants for the development of porcine embryos in an IVC system. Specifically, we examined the synergistic effects of antioxidants on development to the blastocyst stage in a culture system supplemented with L-cysteine during IVM. Of the antioxidants tested (melatonin, glutathione (GSH), β-mercaptoethanol (β-ME), N-acetylcysteine (NAC) and dithiothreitol (DTT)), addition of GSH (1 mM) or β-ME (25 μM) significantly increased development to the blastocyst stage compared with the controls without antioxidant treatment (22.2 ± 4.2% for 1 mM GSH, 25.9 ± 2.2% for 25 μM β-ME and 12–13% for the control, P<0.05). In addition, the mean cell number per blastocyst was increased by approximately 1.7-fold in the presence of GSH or β-ME. These GSH- and β-ME-induced increases in development to the blastocyst stage and total cell number, however, were not mimicked by melatonin, NAC or DTT, all of which are ROS scavengers. The combination of GSH or β-ME with L-cysteine significantly reduced high O2 tension-induced ROS production (P<0.05). These results suggest that a combination of 1 mM GSH or 25 μM β-ME with 1 mM L-cysteine could be used for production of high quality porcine blastocysts in IVC systems.

Key words: Antioxidant system, Embryo, Oxidative stress, Pig

T he academic and industrial value of porcine embryonic technology is very high, as this technology allows for production of transgenic porcine embryos with disease-resistance genes, for xenogenic organ transplantation and for preservation of superior genotypes that are at risk of extinction. Obtaining many high-quality embryos, however, is a prerequisite for improvement of embryo technology. A large number of immature oocytes can be collected from the ovaries of slaughtered pigs, and these oocytes can then be matured, fertilized and cultured in vitro. At present, the quality of in vitro produced (IVP) porcine embryos is considerably lower than that of in vivo-derived embryos, although many investigators have attempted to improve in vitro culture systems to help produce higher quality porcine embryos.

Earlier studies have shown that in vitro cultured (IVC) embryos cannot develop normally, as the oxygen (O2) tension in vitro is higher than that in the oviduct. This high O2 tension induces excessive production of reactive oxygen species (ROS), which leads to oxidative stress and impedes oocyte maturation and embryonic development [1, 2]. To remove the ROS generated from IVC conditions, various methods have been used such as the application of low O2 tension and the addition of antioxidants [3, 4]. The effects of antioxidants and O2 tension on the in vitro maturation (IVM), in vitro fertilization (IVF) and IVC of porcine embryos, however, remain controversial among researchers. Some studies have reported positive effects of low O2 tension and antioxidants on embryonic development, but others have not. Ock et al. reported that the in vitro development of porcine embryos is not affected by O2 tension [5]. The use of 20% O2 tension for IVM of porcine oocytes promoted blastocyst formation in vitro [6]. In contrast, 5% O2 tension during IVM or IVC of porcine oocytes promoted in vitro development [1, 7]. The antioxidant requirements are also varied during IVM, IVF and IVC, although antioxidants are beneficial additives to synthetic culture media as ROS scavengers. Supplementation of the medium with cysteine during IVM and IVC improves the rate of bovine embryonic development, but not during IVF [8]. Various antioxidants have shown a positive or negative effect on mammalian embryonic development depending on their concentration or culture time.

Numerous studies have reported the effects of treatment with a single or a few antioxidants on embryonic development throughout IVM, IVF and IVC [3, 9]. In this study, however, we added various antioxidants supplemented with 1 mM L-cysteine (cysteine) only...
during the IVM period to rule out other factors that affect embryonic development during the in vitro handling of oocytes or embryos such as changes in temperature or oxygen tension. We examined whether the combination of cysteine with various antioxidants during IVM had a synergistic effect on porcine embryonic development and determined the optimal concentrations of antioxidants during IVM to promote embryonic development. Antioxidants such as glutathione (GSH), β-mercaptoethanol (β-ME), N-acetyl-5-methoxytryptamine (melatonin), N-acetylcysteine (NAC) and dithiothreitol (DTT) were used as scavengers. Cysteine, GSH, β-ME and melatonin are widely used as antioxidants to obtain high quality embryos, and the use of IVM medium supplemented with cysteine is thought to be the most simple and effective method to harvest competent porcine oocytes [10]. GSH is one of the most important antioxidants and is present in cells such as sperm and oocytes [11]. An increase in GSH levels has been shown to play an important role in sperm function, maturation, fertilization and embryonic development. In particular, an important event that must occur during porcine oocyte maturation is the synthesis of intracellular glutathione, which functions in DNA and protein synthesis and amino acid transport inside mammalian cells [12] and has beneficial effects on subsequent embryonic development [9]. β-ME is a thiol compound that is involved in the reduction of substances and prevents the oxidation of cysteine to cystine, thus increasing cysteine activity [13]. β-ME acts as an antioxidant by itself and indirectly by increasing intracellular GSH and cysteine activity. Previous studies have shown that melatonin, an antioxidant that can easily cross cell membranes and the blood-brain barrier [14], accelerates oocyte maturation, fertilization, cell division and subsequent embryonic development in mice, sheep, cows, and pigs [15–18]. NAC and DTT, both thiol reducing agents, are often used as antioxidants in a variety of mammalian cells to study gene expression and signaling pathways [19]. NAC consists of the amino acid cysteine with an acetyl group attached to the amino group. DTT is often used interchangeably with β-ME, and both are frequently used to reduce the disulfide bonds of proteins. These antioxidants have critical roles in the survival of cells, including mammalian oocytes and embryos; however, they have different effects that depend on the cell type and culture conditions. In this study, we compared the effects of antioxidants on porcine embryonic development.

Materials and Methods

Chemicals

All of the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. Stock solutions of the antioxidants, cysteine (100 mM), GSH (100 mM), β-ME (14.3 M), NAC (500 mM), and DTT (100 mM, Invitrogen, Carlsbad, CA, USA), were prepared in distilled water (DW), while melatonin (10 μM) was prepared in ethanol. The antioxidants were then diluted in NCSU-23 medium to a working concentration. When ethanol was used as a solvent, a solution containing an equivalent concentration of ethanol was used as a control. The final ethanol concentration was 0.1% for melatonin.

Oocyte recovery

Porcine ovaries were collected from a local slaughterhouse, transported to the laboratory within 2–3 h after harvest and maintained in PBS containing penicillin G (100 units/ml) and streptomycin (100 μg/ml) at 35 C. Oocytes were aspirated from the antral follicles using an 18-gauge needle fitted to a 10 ml syringe and isolated from the follicular fluid under a stereomicroscope (>40). Only oocytes with more than four layers of compact cumulus cells and uniform cytoplasm were used for the present study. The isolated oocytes were washed three times with Tyrode’s albumin-lactate-pyruvate (TALP)-HEPES medium [20] supplemented with 0.1% polyvinyl alcohol (PVA).

In vitro maturation

Groups of 50 oocytes were matured in a four-well dish (500 μl/well) containing North Carolina State University (NCSU)-23 medium [21] supplemented with 10% porcine follicular fluid (PFF), 1.0 mM cysteine [9, 22], 0.5 μg/ml porcine follicle stimulating hormone (FSH), 5.0 μg/ml equine luteinizing hormone (LH), 1.0 μg/ml 17β-estradiol (E2) and 10 ng/ml epidermal growth factor (EGF) at 39 C in a humidified incubator with 20% O2 or 5% O2. IVM was performed in NCSU-23 medium supplemented with hormones (FSH, LH and E2) for 22 h, and this was followed by culture in NCSU-23 medium without hormones for 22 h.

In order to evaluate the effect of antioxidant treatment during IVM on porcine embryonic development, melatonin, GSH, β-ME, NAC or DTT was added to the NCSU-23 medium supplemented with cysteine at various concentrations as indicated. Following 44 h of maturation, the oocytes were transferred to antioxidant-free medium, fertilized and then cultured for 7 days.

In vitro fertilization

Sperm isolated from the caudal epididymis were prepared using the swim-up procedure. Briefly, sperm were washed three times by centrifugation at 1,500 rpm for 5 min in Dulbecco’s phosphate buffered saline (D-PBS). Modified tris-buffered medium (mTBM) [23] supplemented with 0.1% bovine serum albumin (BSA) and 2.5 mM caffeine and preincubated for 48 h was used for IVF. Some of the expanded cumulus cells were removed by treatment with 0.1% hyaluronidase. The oocytes were washed twice in D-PBS containing 0.1% BSA and then washed twice in mTBM. With the tubes tilted at a 45-degree angle, incubation at 39 C in a humidified incubator with 20% O2 for 20 min allowed the motile sperm to swim up. Groups of 30 oocytes were then inseminated with sperm that had been prepared by the swim-up procedure at a final concentration of 1.5 × 10⁵ sperm/ml in 100-μl drops of the mTBM medium. The gametes were co-incubated for 6 h at 39 C in a humidified incubator with 20% O2.

In vitro culture

After 6 h of co-incubation with the sperm, groups of 30 oocytes were removed from the fertilization drops. The oocytes were pipetted multiple times through a small-bore pipette to remove the cumulus cells, and predicted zygotes were cultured in a four-well dish (Nunc, Denmark) containing 500 μl of NCSU-23 medium supplemented with minimum essential medium (MEM) non-essential
amino acids, basal medium Eagle (BME) amino acids, and 0.4% BSA. The oocytes were incubated at 37°C in a gas mixture of 95% air and 5% CO2. Cleavage and blastocyst formation were assessed on days 2 and 7 of culture, respectively. The cell number per blastocyst was counted by propidium iodide staining.

Measurement of intracellular GSH content

The intracellular glutathione content of the porcine oocytes was measured using a total glutathione detection kit (Assay Designs, Ann Arbor, MI, USA) using a dithionitrobenzoic acid-glutathione disulfide (DTNB-GSSG) reductase recycling assay. For each replicate, we placed groups of 30 oocytes incubated with or without cysteine during IVM in D-PBS containing PVA. The oocytes were suspended with 100 μl of cold 5% (w/v) metaphosphoric acid to remove proteins that interfere with the assay and mixed thoroughly by repeated pipetting. The cell suspension was homogenized and stored on ice for 5 min, then transferred to a 1.5-ml tube and centrifuged at 13,000 × g for 5 min at 4°C. The 1× assay buffer was added to each well of the microtiter plate, and the oocyte supernatants were added to each well with 1× assay buffer. A freshly prepared reaction mixture with glutathione reductase was then added to each well. The absorbance was measured ten times at 1-min intervals at a wavelength of 405 nm using a plate reader (Infinite® F200, Tecan, Männedorf, Switzerland). A glutathione standard and sample blank lacking glutathione were also assayed. The total glutathione content per sample was determined from a standard curve of glutathione. The glutathione concentration per oocyte was calculated by dividing the total glutathione concentration per sample by the number of oocytes present in the sample. The oxidized glutathione assay, 1 μl of 2 M 4-vinylpyridine per 50 μl of sample and 4 μM of oxidized glutathione (GSSG) were added, and the samples were incubated for 1 h at room temperature. The 4-vinylpyridine-treated GSSG standard and samples were diluted as described above for the total glutathione assay. The reduced glutathione (GSH) concentration was obtained by subtraction of the oxidized glutathione concentration from the total glutathione concentration (reduced GSH = total glutathione − oxidized GSSG).

Measurement of reactive oxygen species

Oocytes were cultured with or without various antioxidants during IVM under 20% oxygen tension. On day 2 of culture (48 h after IVC), embryos were loaded with 5 μM H2DCFDA (DCF-DA, Calbiochem, San Diego, CA, USA) for 30 min in the dark. After incubation, the embryos were washed three times with PBS and immediately analyzed for fluorescence intensity using a confocal laser scanning microscope equipped with a fluorescence system (IX70 Fluoview, Olympus, Tokyo, Japan). For detection of green fluorescence (H2DCFDA), embryos were illuminated with 488- and 530-nm laser lines, and fluorescence was collected with a 550 nm band pass filter for green fluorescence detection. Fluorescent images were saved as TIFF files and analyzed using the Fluoview software program (version 2.0, Olympus).

Statistical analysis

Differences among groups were analyzed using one-way analysis of variance (ANOVA) by SPSS and the Student’s t-test. The data are shown as the mean ± SD. An asterisk (*) indicates a significant difference from the corresponding control. A value of P<0.05 was considered to be significant.

Results

Effects of antioxidants on porcine embryonic development

We first examined the effects of O2 tension (5% and 20%) during IVM on the development of porcine embryos. As shown in Fig. 1A, blastocyst formation under 5% O2 tension was greater than that under 20% O2 tension, but the difference was not statistically significant. As shown in Fig. 1B, the GSH content of the IVM oocytes exposed to 0.5 (13.5 ± 0.8 pmol/oocyte) and 1 mM cysteine (14.7 ± 1.0 pmol/oocyte) was significantly increased compared with the control in the absence of cysteine (10.3 ± 0.8 pmol/oocyte; P<0.05). Cleavage and blastocyst formation of the IVM oocytes exposed to 1 mM cysteine, however, did not increase compared with the control (Fig. 2A and 2B). Although there was no significant effect of cysteine on embryonic development, 1 mM cysteine was added to the IVM medium in all subsequent experiments as cysteine increased the GSH content of the oocytes compared with the control (Fig. 1B). We then examined the effects of adding antioxidants on porcine embryonic development in the presence of cysteine. The addition of 0, 1, 5 or 10 nM melatonin to oocytes cultured with 1 mM cysteine failed to increase oocyte cleavage and blastocyst formation (Fig. 2C and 2D). At all concentrations of melatonin tested, blastocyst formation was 10−13% (Fig. 2D), and there was no significant difference among the examined groups.

As shown in Table 1, the addition of GSH increased blastocyst formation, with development rates of 14.5 ± 5.9, 22.2 ± 4.2, 18.9 ± 3.9 and 15.8 ± 5.6% at GSH concentrations of 0.5, 1, 5 and 10 mM,
respectively. A GSH concentration of 1 mM significantly increased development to the blastocyst stage compared with the control cultured without GSH (0 mM, 12.1 ± 4.0%). GSH concentrations higher than 5 mM, however, did not further increase development to the blastocyst stage (P>0.05). The addition of GSH had no effect on oocyte cleavage.

Oocyte cleavage and blastocyst formation of porcine embryos exposed to various concentrations of β-ME are summarized in Table 2. When β-ME was added to the IVM medium at concentrations of 0, 10, 25, 50 and 100 μM, the percentages of development to the blastocyst stage were 13.0 ± 2.1, 19.2 ± 3.3, 25.9 ± 2.2, 18.4 ± 2.9, and 17.5 ± 2.2%, respectively. At all β-ME concentrations, blastocyst formation was significantly greater than that seen in the absence of β-ME (P<0.05). The addition of 25 μM β-ME showed the highest percentages of oocyte cleavage and blastocyst formation. Fig. 3A summarizes the effects of antioxidants on porcine embryonic development. Treatment with antioxidants (5 nM melatonin, 1 mM GSH, 25 μM β-ME, 1.5 mM NAC or 100 μM DTT) in the absence of cysteine had no significant effect on blastocyst formation; however, the combination of GSH (1 mM) or β-ME (25 μM) with cysteine significantly increased porcine embryonic development to the blastocyst stage (P<0.05). NAC and DTT failed to increase blastocyst formation. As shown in Fig. 3B and C, GSH (0.5, 1 and 5 mM) and β-ME (25 and 50 μM) significantly increased the mean number of cells per blastocyst (P<0.05). In particular, this number was the highest with 1 mM GSH and 25 μM β-ME (40.3 ± 7.0 and 46.3 ± 5.0 in the presence vs. 27.8 ± 2.2 and 27.0 ± 4.2 in the absence of GSH and β-ME, respectively). Representative photomicrographs of blastocysts (day 7) produced from oocytes cultured with GSH or β-ME are shown in Fig. 3B and C.

Table 1. Effects of glutathione treatment during IVM on the development of porcine embryos in vitro

<table>
<thead>
<tr>
<th>Concentration of GSH (mM)</th>
<th>No. of oocytes</th>
<th>cleaved (%)</th>
<th>blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>352</td>
<td>164 (46.6 ± 4.7)a</td>
<td>20 (12.1 ± 4.0)a</td>
</tr>
<tr>
<td>0.5</td>
<td>352</td>
<td>165 (46.9 ± 4.0)a</td>
<td>24 (14.5 ± 5.9)ab</td>
</tr>
<tr>
<td>1</td>
<td>344</td>
<td>162 (47.1 ± 3.7)a</td>
<td>36 (22.2 ± 4.2)</td>
</tr>
<tr>
<td>5</td>
<td>351</td>
<td>155 (43.6 ± 5.7)a</td>
<td>29 (18.9 ± 3.9)ab</td>
</tr>
<tr>
<td>10</td>
<td>389</td>
<td>177 (45.5 ± 5.8)a</td>
<td>28 (15.8 ± 5.6)</td>
</tr>
</tbody>
</table>

a,b Values with different superscripts within each column are significantly different (P<0.05). The in vitro maturation was performed in culture medium supplemented with cysteine. Percentages are means ± SD of ten replicates.

Table 2. Effects of β-mercaptoethanol (β-ME) treatment during IVM on development of porcine embryos in vitro

<table>
<thead>
<tr>
<th>Concentration of β-ME (μM)</th>
<th>No. of oocytes</th>
<th>cleaved (%)</th>
<th>blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>607</td>
<td>285 (47.0 ± 8.3)a</td>
<td>37 (13.0 ± 2.1)a</td>
</tr>
<tr>
<td>10</td>
<td>635</td>
<td>302 (47.6 ± 10.1)a</td>
<td>58 (19.2 ± 3.3)</td>
</tr>
<tr>
<td>25</td>
<td>663</td>
<td>367 (55.4 ± 11.3)a</td>
<td>95 (25.9 ± 2.2)</td>
</tr>
<tr>
<td>50</td>
<td>696</td>
<td>331 (47.6 ± 12.3)a</td>
<td>61 (18.4 ± 2.9)</td>
</tr>
<tr>
<td>100</td>
<td>690</td>
<td>326 (47.2 ± 14.9)a</td>
<td>51 (17.5 ± 2.2)</td>
</tr>
</tbody>
</table>

a,b Values with different superscripts within each column are significantly different (P<0.05). The in vitro maturation was performed in culture medium supplemented with cysteine. Percentages are means ± SD of ten replicates.
Effects of antioxidants on ROS generation in porcine embryos

As shown in Fig. 4, treatment with 1 mM cysteine showed a similar level of ROS generation compared with the control cultured without antioxidants under 20% O2 tension. Treatment with 1 mM GSH and 25 μM β-ME, however, significantly reduced the ROS levels compared with the control. The combination of cysteine with GSH or β-ME also reduced the ROS levels compared with the control. In particular, the combination of cysteine with β-ME showed the highest reduction in ROS levels for embryos cultured in vitro. The microphotographs show in vitro cultures of blastocysts derived from porcine in vitro matured oocytes in culture medium supplemented with 1 mM GSH (B) or 25 μM β-ME (C). The blastocysts were stained with PI. The numbers inside the bars represent the numbers of oocytes used. Each bar shows the mean ± SD. The asterisks (*) indicate significant differences from the corresponding control.

Discussion

This study shows that the addition of 1 mM GSH and 25 μM β-ME to media supplemented with cysteine has a beneficial effect on both development to the blastocyst stage and the mean number of cells per blastocyst. These antioxidants were also found to reduce the ROS levels produced by in vitro culture under 20% O2 tension. Treatment with only cysteine, however, failed to reduce the ROS levels in embryos, although cysteine increased the GSH concentration in oocytes. Treatment with GSH and β-ME during the IVM of porcine oocytes in a culture system supplemented with cysteine showed synergistic effects on early embryonic development. A culture system supplemented with cysteine is thought to be the simplest and most effective method to harvest competent porcine oocytes [10], and many researchers have used media supplemented with various concentrations of cysteine (0 to 3.3 mM) [9, 10, 22, 24]. In this study, we used 1 mM cysteine, which highly increased the GSH concentrations, while cysteine concentrations less than 1 mM had no effects on embryonic development (data not shown, our preliminary data).

Effects of antioxidants on mammalian embryonic development

O2 tension has been shown to be a major regulatory factor for in vitro embryonic development [3]. Generally, the O2 tension in air (20%, 150 mmHg) is higher than that in the oviduct (approximately 10%, 40–60 mmHg) [25], and this elevated O2 tension increases the intracellular O2 tension by diffusion, thus inducing excessive ROS production in cells. Unexpectedly, our results showed that low (5%) O2 tension does not significantly increase blastocyst formation. Insignificant differences in O2 tension can result from temperature and O2 tension changes during in vitro handling in IVM, IVF and IVC. If all of the procedures for IVM, IVF and IVC are carried out in an environment with 5% O2 tension, better embryonic development will likely result. We exposed porcine embryos to 5% O2 tension only during IVM. Kikuchi et al. (2002) [26] reported that blastocyst quality is significantly higher with 5% O2 tension compared with 20% O2 tension, although the rate of blastocyst formation does not differ between these O2 tension values. We measured blastocyst quality by counting the number of cells per blastocyst and found a trend indicating that 5% O2 tension could lead to improved blastocyst formation compared with 20%
O₂ tension. We further studied the effects of antioxidants on porcine embryonic development in vitro. Antioxidants decrease the production of potentially harmful hydrogen peroxide in cultured embryos.

Cysteine, the precursor of glutathione, has been shown to have beneficial effects as an antioxidant by protecting mammalian cells from oxidative damage [27]. The addition of cysteine to culture media (IVM or IVC) has a beneficial effect on bovine and porcine blastocyst formation [8, 28, 29]; however, researchers disagree about the beneficial effects of cysteine with respect to cell number, glutathione level and embryonic development [8, 28, 29]. Under our culture conditions, the addition of cysteine failed to significantly increase embryonic development to the blastocyst stage but increased the GSH concentration, indicating that cysteine may have an indirect effect on embryonic development by increasing the concentration of GSH.

Supplementing the oocyte maturation medium with 1.5 mM NAC or NACA N-acetyl-cysteine-amide has been shown to increase the percentage of development to the blastocyst stage [30]. However, the addition of NAC (0.6 mM) to the maturation medium did not improve the percentage of oocytes undergoing morula and blastocyst development. Moreover, the addition of NAC during the IVF period has been shown to significantly reduce the subsequent rate of bovine embryonic development to the morula and blastocyst stages [8]. Under our culture conditions, NAC showed no effect on porcine embryonic development. Motos et al. found that cysteine is metabolized by oocytes during IVM despite the presence of cumulus cells that protect oocytes from the extracellular medium, but that NAC did not [31]. The fact that a high concentration of NAC had no effect on embryonic development under our culture conditions could suggest that certain ROS levels are required for embryonic development [8]. Furthermore, the addition of DTT had no effect on porcine embryonic development. At this time, however, we do not understand the precise mechanistic reasons for the observed difference between β-ME and DTT. Further studies will be necessary to identify the difference in function that causes β-ME, but not DTT, to increase embryonic development.

The effects of melatonin are different depending on the concentration, cell stage and length of treatment. Although melatonin at a concentration of 10⁻⁸ M has a positive effect on embryo cleavage rates and cell number per blastocyst, it has no effect on blastocyst formation [32]. Treatment with 1 µM or 100 µM melatonin 4 h after insemination resulted in a significant increase in the rate of embryos developing to the two-cell stage 24 h later, but further development to the blastocyst stage was not increased. The addition of 10 nM melatonin, however, increased the development rates from the four-cell stage to the blastocyst stage, but did not increase the rate of embryos developing to the two-cell stage. This suggests that melatonin may function differently according to the cell stage and its concentration [15]. Other researchers have reported that melatonin treatment does not affect mouse embryonic development after fertilization [33, 34]. Our results showed a minimal effect of melatonin on porcine embryonic development. Thus, further studies are necessary to determine the effects of melatonin treatment on porcine embryonic development as a function of antioxidant concentration and length of treatment.

Additional effects of glutathione and β-mercaptoethanol treatment on porcine embryonic development

When oocytes were treated with GSH or β-ME during IVM without cysteine under our culture conditions, there was no effect on porcine embryonic development. The combination of GSH or β-ME with cysteine, however, has been shown to increase development to the blastocyst stage. Maximal GSH concentrations have been achieved by addition of cysteine to the maturation medium [35], similar to our results. Supplementation with L-α-aminobutyrate, which is linked to the synthesis and metabolism of GSH, or β-ME has been shown to lead to high success rates in IVM, IVF and IVC [36].

Earlier studies have reported that addition of high concentrations of GSH to the medium for IVF decreased the rate of blastocyst formation compared with treatment with low concentrations of GSH [24, 37], suggesting that the concentration of this antioxidant is a contributing factor to the generation of high quality embryos. In our experiments, the blastocyst development rates and mean cell numbers per blastocyst were highest in the group treated with 1 mM GSH relative to the other concentrations tested (0.5, 5 and 10 mM), which is consistent with the results of previous studies. The addition of GSH to BSA-containing media has been shown to increase blastocyst formation and to have a synergistic effect on porcine embryonic development [38]. In the presence of a thiol compound, supplementation of the IVM medium with cysteine can increase the GSH level and improve the developmental competence of porcine oocytes following fertilization [22]. Based on these findings, it could be speculated that GSH induces in vitro maturation of oocytes and that a high GSH concentration ultimately has a direct effect on embryonic development.

The positive effects of β-ME on bovine embryonic development may be mediated by increasing the availability of cysteine [39]. The addition of β-ME has been shown to significantly promote embryonic development when embryos are cultured under high oxygen tension by promoting cystine uptake at each stage of bovine embryonic development [13]. In addition, supplementation with β-ME during IVF has been shown to have a beneficial effect on the function of gametes, the incidence of normal fertilization and, consequently, the quality of IVF embryos [40]. Others have shown that the addition of β-ME and cysteine improves sperm decondensation, fertilization rates and blastocyst development to day 7, but has no effect on the blastocyst rate at day 9 [41]. In our experiments, addition of 25 µM β-ME also resulted in the highest rate of blastocyst development; however, the optimal concentration of β-ME for in vitro embryonic development differs among investigators. For example, Abeydeera et al. [22] reported that 25 µM β-ME was optimal for porcine embryonic development, while Takahashi et al. [42] reported that 50 µM was optimal. We also found that 25 µM β-ME increased the concentration of GSH (data not shown). In our study, addition of GSH or β-ME had an additional beneficial effect on cysteine-induced embryonic development, suggesting that GSH levels could be a key determinant of maturation and subsequent porcine embryonic development.

High O₂ tension (20%) induced ROS generation in porcine embryos, but the detrimental effects of ROS were overcome by the combination of GSH or β-ME with cysteine as in previous studies.
SYNERGISTIC EFFECTS OF ANTIOXIDANTS ON THE DEVELOPMENT OF PORCINE EMBRYOS

In conclusion, this study provides evidence that optimal embryonic development in vitro is partially dependent on the presence of precursor amino acids for intracellular GSH synthesis and that addition of GSH or β-ME has a synergistic effect with cysteine on subsequent embryonic development. Our results also suggest that the combination of 1 mM GSH or 25 μM β-ME with 1 mM cysteine could be used for production of high quality porcine blastocystcs in vitro culture systems by preventing oxidative stress. The results obtained with antioxidant treatment, however, are not consistent among investigators and are dependent on the culture system, including factors such as the culture medium, treatment time, concentration and cell stage. In particular, hormones and growth factors contained in culture media can impact the effects of antioxidants. Further studies will be necessary to evaluate the exact role and mechanism of GSH and β-ME treatment on porcine embryonic development in vitro system supplemented with cysteine.

Acknowledgments

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