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The Control of Reactive Oxygen Species Influences Porcine Oocyte *In Vitro* Maturation

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Contents
The aim of this study was to examine the effect of varying intracellular reactive oxygen species (ROS) levels during oocyte *in vitro* maturation with enzymatic ROS production systems (xanthine + xanthine oxidase or xanthine + xanthine oxidase + catalase), scavenger systems (catalase or superoxide dismutase + catalase) or cysteine on porcine oocyte maturation. Oocyte ROS levels showed an increase when H_2O_2 or $O_2^{\cdot -}$ production systems were added to the culture medium ($p < 0.05$). On the other hand, the presence of ROS scavengers in the maturation medium did not modify oocyte ROS levels compared with the control after 48 h of maturation, but the addition of cysteine induced a decrease in oocyte ROS levels ($p < 0.05$). The ROS production systems used in this work did not modified the percentage of oocyte nuclear maturation, but increased the decondensation of sperm head ($p < 0.05$) and decreased the pronuclear formation ($p < 0.05$). In turn, the addition of $O_2^{\cdot -}$ and H_2O_2 scavenging systems during *in vitro* maturation did not modify the percentage of oocytes reaching metaphase II nor the oocytes with decondensed sperm head or pronuclei after fertilization. However, both parameters increased in the presence of cysteine ($p < 0.05$). The exogenous generation of $O_2^{\cdot -}$ and H_2O_2 during oocyte *in vitro* maturation would not affect nuclear maturation or later sperm penetration, but most of the spermatozoa cannot progress to form the pronuclei after fusion with the oocyte. The decrease in endogenous ROS levels by the addition of cysteine would improve pronuclear formation after sperm penetration.

Introduction

The detrimental effect of O_2 concentration on *in vitro* cell culture systems has been correlated with oxidative stress generated by the production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (reviewed by Takahashi 2012). ROS have been related to several mechanisms of cell damage that impair oocyte maturation. The alteration of the meiotic spindle induced by oxidative stress was observed in mouse oocytes during *in vitro* maturation; therefore, ROS participation in the oocyte meiotic arrest was proposed (Choi et al. 2007; Downs and Mastropolo 1994; Zhang et al. 2006b). Oxidative stress also affected bovine oocytes impairing the developmental competence after fertilization (Fatehi et al. 2005). Furthermore, the alteration of redox status during *in vitro* culture of human oocytes was associated with an increased incidence of apoptosis in the gamete (Zhang et al. 2006a). In turn, oxidative stress during porcine oocyte *in vitro* maturation reduced the percentage of oocytes at metaphase II nuclear stage after culture and affected cytoplasm maturation and developmental competence. The porcine gamete also underwent DNA damage and cell death by the apoptosis pathway during *in vitro* maturation (*Tatemoto* et al. 2000, 2001, 2004).

One of the several ROS-generating systems is xanthine oxidase (XOD). This enzyme and its mechanism of action have been thoroughly studied (Olson et al. 1974a, b; Hille and Massey 1981; Hille et al. 1981; Porras et al. 1981). XOD acting aerobically upon xanthine (X) generates $O_2^{\cdot -}$ and H_2O_2 and is used frequently in model systems to investigate cytotoxic effects initiated by these compounds (Link and Riley 1988). Thus, a ROS-generating system which combines X and XOD would result in the production of $O_2^{\cdot -}$ and H_2O_2 (de Lamirande and Gagnon 1993; Blondin et al. 1997), while the X + XOD system in the presence of catalase (CAT) would mainly generate $O_2^{\cdot -}$ (O'Flaherty et al. 1999) due to the transformation of H_2O_2 into H_2O and O_2 mediated by CAT (Cory 2002).

Living organisms possess ROS scavengers to counteract the negative effects of ROS. Superoxide dismutase (SOD) eliminates $O_2^{\cdot -}$ through a reaction that converts it into H_2O_2 and O_2. In turn, CAT will transform H_2O_2 into H_2O and O_2, thereby eliminating ROS (Meister 1983; Cory 2002). In the follicular fluid of developing oocytes, enzymatic antioxidants, such as SOD and CAT, work in concert with non-enzymatic antioxidants, such as vitamin C and taurine, presumably to counteract the potentially harmful effects of ROS (Shiotani et al. 1991; Sabatini et al. 1999; Carbone et al. 2003). It has been demonstrated that glutathione (GSH) is a non-enzymatic antioxidant that increases its concentration within porcine oocyte after *in vitro* maturation in the presence of cysteine (Abeydeera et al. 1999).

Until present, the majority of studies were focused on the effects of exogenous ROS on cell function during oocyte *in vitro* maturation. Mostly, indirect evidence of ROS impact was evaluated instead of measuring oocyte endogenous ROS levels. We hypothesize that *in vitro* oocyte maturation could be influenced by the oocyte endogenous ROS levels, which in turn might be modified by the presence of the same enzymatic ROS scavenger systems present in the follicular fluid.

The aim of this study was to examine the effect of varying endogenous porcine oocyte ROS levels during *in vitro* maturation with different enzymatic ROS scavengers (CAT or SOD + CAT) or cysteine, and ROS production systems (X + XOD or X + XOD + CAT) on oocyte maturation.

Materials and Methods

Materials

Unless otherwise specified, all chemicals used were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Recovery and classification of cumulus–oocyte complexes

Ovaries from slaughtered gilts were transported in a warm environment (28–33°C) for the 2- to 3-h journey to the laboratory. Ovaries were washed in 0.9% (w/v) NaCl containing 100 000 IU/l penicillin and 100 mg/l streptomycin. COCs were aspirated from 3–8 mm antral follicles using a 10-ml syringe and an 18-gauge needle, and oocytes surrounded by a dense cumulus were selected for *in vitro* culture.

Oocyte *in vitro* maturation

COCs were cultured in medium 199 (Earle's salts, L-glutamine, 2.2 mg/l sodium bicarbonate; GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) foetal bovine serum (GIBCO), 50 mg/l gentamicin sulphate and 0.5 mg/l porcine follicle-stimulating hormone (Folltropin-V, Bioniche, Belleville, Ontario, Canada) plus 0.5 mg/l porcine luteinizing hormone (Lutropin-V, Bioniche) (control medium) under mineral oil at 39°C for 48 h in a 5% CO_2 atmosphere (Abeydeera et al. 2001).

Different compounds were added to the control medium: 2 mM X+1 IU/ml XOD (enzymatic production system of H_2O_2), 2 mM X+1 IU/ml XOD + 50 IU/ml CAT (enzymatic production system of $O_2^{\cdot-}$), 50 IU/ml CAT (enzymatic scavenger of H_2O_2), 100 IU/ml SOD + 50 IU/ml CAT (enzymatic scavenger of $O_2^{\cdot-}$) (Blondin et al. 1997) and 0.57 mM cysteine (GSH synthesis precursor).

In vitro fertilization

In vitro fertilization was performed using fresh semen from a Yorkshire boar of proven fertility. Sperm-rich fractions were collected by the gloved-hand method. Sperm samples were washed twice in phosphate-buffered saline (PBS – consisting of 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.7 mM KH_2PO_4 [pH 7.35–7.65] plus 3 g/l bovine serum albumin [BSA]) by centrifugation at 400 × ***g*** for 5 min. Pellets were resuspended in fertilization-modified Tris-buffered medium, consisting of 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 4 g/l bovine serum albumin, 2.5 mM caffeine and 50 mg/l gentamicin sulphate (Abeydeera and Day 1997). Samples were filtered through a 20-mg glass wool column (10 mm height, 4 mm diameter), previously washed with a modified Tris-buffered medium, to obtain the motile fraction (Pereira et al. 2000). Matured COCs were denuded by pipetting and inseminated to a final concentration of 5×10^8/l spermatozoa. The co-incubation of gametes was performed in 500 µl droplets of fertilization-modified Tris-buffered medium under mineral oil at 39°C for 18 h in a 5% CO_2 atmosphere.

Oocyte denudation

Immature oocytes were denuded of somatic cells by vortex agitation for 1 min at 37°C in 3 g/l BSA in PBS. They were then separated from the remaining cumulus cells with a Pasteur pipette.

In vitro matured COCs were incubated in 1 g/l hyaluronidase in PBS medium for 5 min at 37°C and the oocytes denuded by gentle pipetting.

Evaluation of endogenous oocyte ROS production

To evaluate ROS generation, 15 COCs were matured in 150 µl droplets of culture medium and ROS production was determined after 48 h of incubation.

To measure ROS level, COCs were collected from the maturation medium and denuded oocytes were incubated at 37°C in PBS supplemented with 0.3% (w/v) BSA for 30 min in the presence of 5 µM 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) (Le Bel et al. 1992; Morado et al. 2009). Fifteen immature COCs were also included in each experiment to measure the initial oocyte ROS production.

To measure esterase activity, 15 COCs were collected from the maturation medium and denuded oocytes were incubated at 37°C in PBS supplemented with 0.3% (w/v) BSA for 15 min in the presence of 0.12 µM fluorescein diacetate (FDA). Fifteen immature COCs were also included in each experiment to measure the initial oocyte esterase activity.

After exposure to their respective fluorochromes, both oocyte samples were washed in PBS supplemented with 0.3% (w/v) BSA and mounted on glass slides. Fluorescence was measured using digital microphotographs obtained with an epifluorescence microscope using 450- to 490-nm (excitation) and 520-nm (emission) filters. All microphotographs were analysed using IMAGEJ 1240 software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA), measuring the brightness of each oocyte.

The fluorescence detected by DCHF-DA is dependent on esterase activity. The ratio between the brightness of each oocyte measured by DCHF-DA and the mean brightness detected by FDA in each treatment was therefore considered a better indicator of oocyte ROS levels (Lane et al. 2002; Morado et al. 2009). ROS levels were expressed in arbitrary units per oocyte per min.

Evaluation of oocyte maturation

To evaluate oocyte *in vitro* maturation, 50 COCs were matured in 500 µl of culture medium. Metaphase II chromosome configurations were determined after 48 h of incubation. Denuded oocytes were placed in a hypotonic medium of 10 g/l sodium citrate at 37°C for 15 min, fixed on a slide with Carnoy's fixing solution (3 : 1 ethanol : acetic acid) and stained with 5% (v/v) Giemsa (Merck, Darmstadt, Germany) for 15 min. They were then observed under a light microscope at 100× and 400× magnification. Only oocytes with condensed and well-defined metaphase II chromosome configurations were considered meiotically mature (Alvarez et al. 2009).

Cytoplasmic maturation was assessed after co-incubating gametes for 18 h. Presumptively fertilized oocytes were released from attached spermatozoa by repetitive pipetting, fixed on a slide with Carnoy's fixing solution for at least 24 h, incubated in an aqueous solution of 10 mg/l Hoechst 33342 fluorochrome for 15 min at room temperature and observed under an epifluorescence microscope using 330- to 380-nm (excitation) and 420-nm (emission) filters at 250× and 400× magnification. Oocytes were considered cytoplasmically mature when at least one decondensed sperm head and/or a fully formed pronucleus could be identified (Alvarez et al. 2009).

Statistical analysis

Non-parametric values were recorded as percentages and analysed using a chi-squared test. Parametric values were reported as means ± SEM, and comparisons were made by ANOVA, using Bonferroni post hoc test. Significance was set at $p < 0.05$.

Results

Oocyte endogenous ROS levels decreased from immature oocytes to *in vitro* matured ones ($p < 0.05$). Oocyte endogenous ROS levels showed an increase when exogenous ROS production systems (X + XOD or X + XOD + CAT) were present in the culture medium during *in vitro* maturation ($p < 0.05$). The presence of exogenous ROS scavenger systems in the maturation medium (CAT or SOD + CAT) did not modify oocyte ROS levels compared with the control after 48 h of maturation. On the other hand, the addition of cysteine (intracellular GSH synthesis inductor) provoked a decrease in oocyte ROS levels ($p < 0.05$, Fig. 1).

There was no difference in the percentage of oocytes reaching metaphase II nuclear stage in the presence of exogenous ROS production systems, exogenous scavenger systems or cysteine (Fig. 2).

ROS production systems used in this work (X + XOD or X + XOD + CAT) increased the decondensation of sperm head respect to control ($p < 0.05$), but decreased the pronuclear formation respect to control ($p < 0.05$).

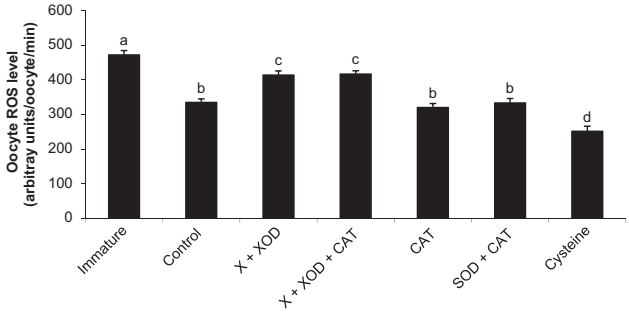

Fig. 1. Reactive oxygen species level per oocyte per minute before (immature) and after maturation with different treatments: X + XOD (xanthine and xanthine oxidase), X + XOD + CAT (xanthine, xanthine oxidase and catalase), CAT (catalase), SOD + CAT (superoxide dismutase and catalase) and cysteine. [a, b, c, d] Different superscripts over bars indicate significant differences ($p < 0.05$). n = 42–45 oocytes for each bar. Experiments include three replicates. Data are presented as mean ± SEM

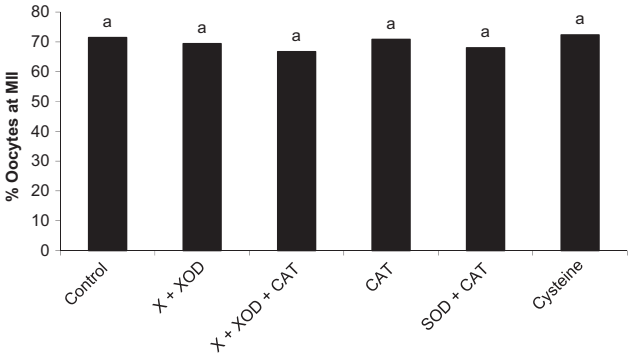

Fig. 2. Percentage of oocytes reaching metaphase II (M II) after maturation with different treatments: X + XOD (xanthine and xanthine oxidase), X + XOD + CAT (xanthine, xanthine oxidase and catalase), CAT (catalase), SOD + CAT (superoxide dismutase and catalase) and cysteine. [a] The same superscript over bars indicates no significant difference between treatments. n = 141–150 oocytes for each bar. Experiments include three replicates

The exogenous ROS scavenger systems used in this work (CAT or SOD + CAT) did not modify oocyte cytoplasmic maturation rates, evaluated by the ability of oocytes to decondense the sperm head and/or to form pronuclei. Yet, both parameters increased in the presence of cysteine ($p < 0.05$, Fig. 3).

Esterase activity showed no significant differences between immature and mature oocytes or with the different treatments used. Therefore, the differences observed using the DCHF-DA assay can be attributed to ROS production.

Discussion

In the porcine species, the high oxygen tension used during *in vitro* embryo culture produces ROS accumulation inside the cells and provokes damage in their DNA impairing embryo development (Kitagawa et al. 2004). Several protein and non-protein antioxidants

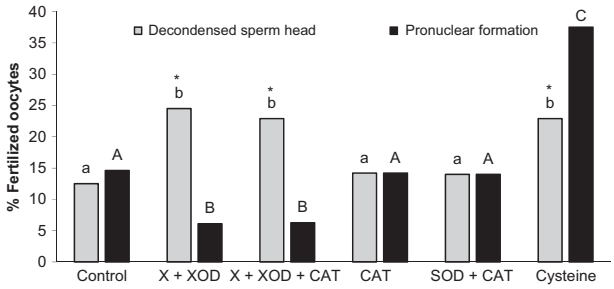

Fig. 3. Percentage of oocytes with mature cytoplasm after culture with different treatments: X + XOD (xanthine and xanthine oxidase), X + XOD + CAT (xanthine, xanthine oxidase and catalase), CAT (catalase), SOD + CAT (superoxide dismutase and catalase), cysteine, as evaluated by sperm head decondensation or pronucleus formation. a, b and A, B, C Different superscripts over bars indicate significant differences between treatments as regards the same evaluation parameter ($p < 0.05$). *Significant difference between evaluation parameters in the same treatment ($p < 0.05$). n = 144–150 oocytes for each treatment. Experiments include three replicates

have been reported to improve *in vitro* embryo development. The addition of free radical scavenger enzymes such as SOD, catalase or thioredoxin is effective for embryo development by scavenging ROS and providing embryos of low oxidative stress conditions in mouse, pig and cow (Reviewed by Takahashi 2012). The H_2O_2 and $O_2^{\cdot-}$ scavenger systems used in the present study (CAT or SOD + CAT) during porcine oocyte *in vitro* maturation did not modify the endogenous ROS levels in oocytes neither influence the percentages of nuclear and cytoplasmic maturation. The oocyte may have an appropriate capacity to regulate the endogenous ROS levels, as demonstrated by the decrease in ROS production in the matured oocytes respect to immature ones. We demonstrated for the first time the lack of effectiveness of the addition of exogenous enzymatic ROS scavenger systems to the culture medium to either decrease the ROS level inside the porcine oocyte or improve oocyte maturation. It has been proposed that cumulus cells seem to effectively protect the porcine oocyte against ROS *(Tatemoto* et al. 2000), making the addition of extracellular ROS scavenger systems to the culture medium unnecessary.

It has been shown that the DCFHDA probe is oxidized by H_2O_2, its derived oxidants, other peroxides and indirectly by the $O_2^{\cdot-}$ when generating H_2O_2, thus providing a useful test to evaluate ROS production (Le Bel et al. 1992).

The addition of cysteamine and β-mercaptoethanol during *in vitro* maturation of ovine oocytes induces GSH synthesis and produces a reduction of ROS levels within the gametes (de Matos et al. 2002). This protective effect of increased GSH content against oxidative stress was also suggested in the porcine oocyte (Tatemoto et al. 2000). It has been observed that supplementation of culture medium with cysteine and β-mercaptoethanol induces an increase in the GSH content of the porcine oocyte, improving the cytoplasmic maturation of this gamete (Sawai et al. 1997; Abeydeera et al. 1999; Tatemoto et al. 2001; Viet Linh et al. 2009).

We observed a decrease in the oocyte endogenous ROS level when *in vitro* maturation medium was supplemented with cysteine. This compound seems to improve the capacity of the oocyte to reduce ROS production during this process. The addition of cysteine to the culture medium did not modify meiotic progress, but the cytoplasm maturation rate was higher than in control medium. The contribution of cysteine to cytoplasm maturation of porcine oocytes and its lack of effect on nuclear maturation was previously reported. Some studies have explained this effect suggesting that GSH is necessary for the pronuclear formation and embryo development after fertilization (Sawai et al. 1997; Abeydeera et al. 1999; Tatemoto et al. 2001; Viet Linh et al. 2009).

Furthermore, our results suggest an additional function of cysteine during *in vitro* maturation. We demonstrated for the first time the contribution of this compound to decrease the endogenous ROS levels within the porcine oocyte during *in vitro* maturation. The decrease observed demonstrates that the GSH produced acts as a non-enzymatic ROS scavenger in the oocyte. Therefore, low levels of ROS could be determinant for porcine oocyte developmental competence.

Other authors demonstrated that cumulus cells exert a protective effect on porcine oocyte damage mediated by ROS added to culture medium (Tatemoto et al. 2000; Somfai et al. 2004), but it remains unknown whether oocyte intracellular ROS are modified during *in vitro* maturation or not. In the present work, the exogenous H_2O_2 and $O_2^{\cdot-}$ production systems added to the culture medium (X + XOD or X + XOD + CAT) were effective to increase ROS levels inside the oocytes during *in vitro* maturation. $O_2^{\cdot-}$ alone or in combination with H_2O_2 could overload the mechanism of the COC to control oocyte endogenous ROS levels. Despite the lack of effect of these ROS on oocyte nuclear maturation, both $O_2^{\cdot-}$ alone and in combination with H_2O_2 diminished pronuclear formation in the oocytes after insemination. However, sperm penetration in the oocyte was not affected by any of the two, and on the contrary, sperm head decondensation increased with respect to the control. There are many studies demonstrating the effects of ROS on the spermatozoa. Although the excessive production of ROS is detrimental to the spermatozoa, there is a growing body of evidence which suggests that low and controlled concentrations of ROS play an important role in sperm physiology (Reviewed by Griveau and Le Lannou 1997; de Lamirande et al. 1997). H_2O_2 and $O_2^{\cdot-}$ inhibit sperm–egg fusion via oxidation of the SH-proteins in the sperm membrane (Mammoto et al. 1996), increase the concentration of lipid hydroperoxides and decrease the capacity for acrosome reaction and sperm motility (Griveau et al. 1995). On the other hand, it has been demonstrated that $O_2^{\cdot-}$ participates in the capacitation of human and bull spermatozoa (de Lamirande and Gagnon 1993;

O'Flaherty et al. 1999, 2003) and that H_2O_2 participates as an inductor of the acrosome reaction in bovine spermatozoa (O'Flaherty et al. 1999). The effects mentioned above seem to be exerted mainly on the male gamete membrane. Yet, in the present study, the increase in oocyte ROS levels during *in vitro* maturation did not affect sperm–egg fusion, but considerably diminished pronuclear formation. Apparently, the oocyte membrane maintains its ability to fuse with the spermatozoa after exposure to H_2O_2 and $O_2^{\cdot -}$. In mouse oocytes, exposure to H_2O_2 and $O_2^{\cdot -}$ induced the hardening of the zona pellucida and the loss of cortical granules (Goud et al. 2008), but the sperm fusion was not evaluated in that study. It has been observed that excess of ROS during *in vitro* embryo culture causes developmental arrest, physical DNA damage, apoptosis induction and lipid peroxidation in embryos (Takahashi 2012). The sequential mechanism of H_2O_2 and $O_2^{\cdot -}$ generation, mitochondrial damage, caspase activation and apoptosis has been proposed to explain the developmental arrest of bovine embryos (Velez-Pardo et al. 2007). Particularly in oocytes, exposure to H_2O_2 and $O_2^{\cdot -}$ altered gamete microtubules (Goud et al. 2008) and H_2O_2 provoked the acetylation of histones (Cui et al. 2009). Some of the mechanisms mentioned above may be involved in the diminished pronuclear formation observed in the present study.

In conclusion, the addition of exogenous $O_2^{\cdot -}$ and H_2O_2 scavenging systems during porcine oocyte *in vitro* maturation did not modify the percentage of oocytes reaching metaphase II nor the oocytes with decondensed sperm head or pronuclei after fertilization. However, the exposure of oocytes to these ROS strongly inhibited their ability to form pronuclei. The generation of $O_2^{\cdot -}$ and H_2O_2 during oocyte maturation would not affect the later sperm penetration, but most of the spermatozoa cannot progress to form the pronuclei after fusion with the oocyte. Furthermore, the decrease in oocyte endogenous ROS level by the addition of cysteine would improve pronuclear formation.

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Conflict of interest

The authors declare they have no conflict of interests that might impede their impartiality with respect to the work performed.

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Author contributions

Mariela Soto, Gabriel Alvarez and Sergio Morado did most of the laboratory work for the experiments. Gabriel and Sergio also wrote the manuscript and did the statistical analysis. Pablo Cetica and Gabriel Dalvit directed the work, developed the experimental design and corrected the manuscript.

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