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Reactive Oxygen Species Production and Redox state in Parthenogenetic and Sperm mediated bovine oocyte activation

Short title: ROS and redox in bovine oocyte activation

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ABSTRACT

The knowledge concerning redox and reactive oxygen species (ROS) mediated regulation of early embryo development is scarce and remains controversial. The aim of this work was to determine ROS production and redox state during early in vitro embryo development in sperm mediated and parthenogenetic activation of bovine oocytes. Sperm mediated oocyte activation was carried out in IVF-mSOF with frozen-thawed semen. Parthenogenetic activation was performed in TALP plus ionomycin and then in IVF-mSOF with 6-dimethylaminopurine plus cytochalasin B. Embryos were cultured in IVF-mSOF. ROS and redox state were determined at each 2-h interval (7–24h from activation) by 2’,7’-dichlorodihydrofluorescein diacetate and RedoxSensor Red CC-1 fluorochromes, respectively. ROS levels and redox state differed between activated and non-activated oocytes (p<0.05). In sperm activated oocytes an increase was observed between 15 and 19h (p<0.05). Conversely, in parthenogenetically activated oocytes, we observed a decrease at 9h (p<0.05). In sperm activated oocytes, ROS fluctuated throughout the 24h, presenting peaks around 7, 19 and 24h (p<0.05), while in parthenogenetic activation, peaks were detected at 7, 11 and 17h (p<0.05). In the present work, we found clear distinctive metabolic patterns between normal and parthenogenetic zygotes. Oxidative activity and ROS production are an integral part of bovine zygote behavior, and defining a temporal pattern of change may be linked with developmental competence.

INTRODUCTION

Oxidative stress has been widely reported in biological sciences to describe an enhanced state of oxidants in cells, a situation in which the concentration of reactive oxygen species (ROS) increases above its biologically normal levels (Sikka et al., 2001). Oxidative stress, mediated by ROS results in an imbalance of the intracellular redox potential towards an oxidized potential (Balaban et al., 2005). The role of ROS in biological processes is still controversial. It was found that the oxidative modification of cell components due to the action of ROS is one of the most potentially damaging processes for normal cell function, leading to inactivation of proteins, lipid membrane peroxidation and DNA alterations (Yang et al., 1998). However, it has been observed that at physiological concentrations, ROS participate in normal cell processes as major factors in growth and development regulation (Hancock et al., 2001).

The procedure for producing embryos in vitro in cattle is still unsatisfactory, with results ranging from 35 to 50% blastocyst rate at day 7/8 of development (Lim et al., 2007, Shirazi et al., 2009). Chemical activation presented significantly improved success rates and...
blastocyst formation compared with IVF (Ruggeri et al., 2012), demonstrating that the process of oocyte activation is a major factor for successful production of reconstructed embryos by somatic cell nuclear transfer (Wells et al., 1999). ROS generation has been implicated as a major cause of poor development of bovine embryo in vitro. ROS has been suggested to participate in meiotic arrest in oocytes (Nakamura et al., 2002), and embryonic block and cell death (Hashimoto et al., 2000). It has been advanced that high levels of ROS may cause oocyte meiotic arrest (Downs and Mastropolo, 1994). Within the oocyte, a critical intracellular concentration of ascorbic acid is necessary for normal cytoplasmic maturation and embryo developmental competence (Tatemoto et al., 2001). It has been observed that an excessive amount of glucose in the maturation medium produces high ROS concentrations and exerts a negative effect on subsequent bovine embryo development to the blastocyst stage (Hashimoto et al., 2000). The importance of regulating ROS levels is revealed by the observation that cumulus–oocyte complexes (COCs) have developed significant antioxidant strategies to control ROS production (Cetica et al., 2001, Tatemoto et al., 2001, Dalvit et al., 2005a).

On the other hand, some evidence exists demonstrating that ROS are important to spermatozoa in regulating every aspect of sperm function examined, including their movement characteristics, capacitation, sperm–zona interaction, acrosome reaction and sperm–oocyte fusion (Baker and Aitken, 2004, Rivlin et al., 2004). Some studies have also documented that the addition of natural antioxidants to oocyte maturation medium failed to modify the percentage of bovine embryos produced in vitro (Blondin et al., 1997) or even diminished the rate of embryo production (Dalvit et al., 2005b). Other cell-permeable antioxidants inhibited the precocious resumption of meiosis in rat oocytes, suggesting a regulatory function of ROS in the maturation process (Takami et al., 1999).

Several transcription factors involved in diverse developmental processes are now known to be regulated by the intracellular redox potential (Dickinson and Forman, 2002; Funato et al., 2006; Imai et al., 2000; Liu et al., 2005; Rahman et al., 2004; Zhang et al., 2002). The recent discovery that these factors can be sensitive to oxidation by ROS or S-glutathionylation, or require NAD(P)H (the reduced form) or NAD(P)+ (the oxidised form) is opening new insights in the regulation of embryonic development (Dumollard et al., 2007). It has been observed that redox state and ROS levels are negatively associated within the cell. A high cellular oxidative activity (eg. increased mitochondrial oxygen consumption rate) is usually associated with lower ROS production and vice verse (Boveris and Cadenas, 1982). In the early mouse embryo, the fundamental importance of redox state and ROS regulation of early embryo development has also been demonstrated (Dumollard et al., 2007).

Previous studies from our group have documented variations in ROS production attributable to oocyte and early embryo metabolic activities during bovine in vitro maturation (IVM) and embryo development (Dalvit et al., 2005a; Morado et al., 2009). In addition,
temporal changes in oxygen consumption were detected in bovine oocytes undergoing the transition from oocyte to zygote (Lopes et al., 2010). Accordingly, the aim of this work was to determine the production of ROS and redox state during early in vitro embryo development in sperm mediated and parthenogenetic activation of bovine oocytes.

MATERIALS AND METHODS

The materials used in these experiments were obtained from Sigma-Aldrich (St. Louis, Missouri), unless otherwise indicated.

Recovery and classification of cumulus-oocyte complexes

Bovine ovaries were obtained from an abattoir within 30 min after slaughter and kept warm (30-33°C) until they were brought to the laboratory. Ovaries were washed in physiological saline containing 100 000 IU/l penicillin and 100 mg/l streptomycin. COCs were recovered by aspiration of antral follicles (2–5 mm in diameter) and classified according to cumulus morphology under a stereomicroscope. Only oocytes completely surrounded by compact and multiple layers of cumulus cells were employed.

Oocyte in vitro maturation

Groups of 50 COCs were cultured in 500 µl of medium 199 (GIBCO, Grand Island, NY, USA) supplemented with 0.2 mg L⁻¹ porcine follicle-stimulating hormone (FSH; Folltropin-V; Bioniche, Belleville, Ontario, Canada), 2 mg L⁻¹ porcine luteinizing hormone (LH; Lutropin-V; Bioniche), 5% (v/v) fetal bovine serum (FBS, Internegocios, Mercedes, Buenos Aires, Argentina) and 50 mg/l gentamycin sulphate under mineral oil (Squibb & Sons Inc., Princeton, NJ, USA) at 39°C for 22 h in an atmosphere of 5% CO₂ in humidified air.

Sperm mediated activation of matured oocytes

In vitro fertilization (IVF) was carried out using frozen–thawed Holstein bull semen from a male of proven fertility. Semen was thawed at 37°C in modified synthetic oviductal fluid (mSOF) (Takahashi and First, 1992) with 10 mmol/l theophylline, centrifuged at 500 x g twice for 5 min and then resuspended in fertilization medium to a final concentration of 2 x10⁶ motile spermatozoa/ml. Co-incubation of COCs and spermatozoa was performed in IVF–mSOF medium, consisting of mSOF supplemented with 10 IU/ml heparin and 5 mg/ml BSA, under...
mineral oil at 39°C, 5% CO₂ in humidified air during 24 h. Different nuclear early embryo development stages were evaluated within 24 h of culture by the fluorescent stain Hoescht 33342 as described below.

**Parthenogenetic activation of matured oocytes**

Oocytes matured in vitro were denuded in phosphate buffer saline (PBS) supplemented with 3 mg/ml bovine serum albumin (BSA) by gentle pipetting with a Pasteur pipette. Oocytes were considered mature when the first polar body was present.

Only mature oocytes were incubated in TALP supplemented with 3 mg/ml BSA with 5 µM ionomycin for 5 min and then in mSOF added with 2 mM 6-dimethylaminopurine (6-DMAP) + 7.5 µg/ml cytochalasin B for 3 h as described by Grupen *et al.* (2002). They were then washed and placed in IVF-mSOF under mineral oil at 90% N₂: 5% CO₂: 5% O₂ and 100% humidity for 21 h.

**Determination of redox state and nuclear stage**

To determine redox state and nuclear stage, matured oocytes, putative zygotes and parthenotes were collected from culture media at 2-h interval from 7 to 24 h post-activation/insemination. They were then denuded and incubated in PBS supplemented with 3 mg/ml BSA in the presence of 1 nM RedoxSensor Red CC-1 (Molecular Probes, Eugene, OR, USA) plus 1 µM of Hoechst 33342 for 10 min in the dark at 39.5°C.

All oocytes were then washed in PBS supplemented with 3 mg/ml BSA and mounted on glass slides. Fluorescence was measured by means of digital microphotographs using a Jenamed II epifluorescence microscope with an x12 objective using 450-490 nm (excitation) and 570 nm (emission) filters for RedoxSensor Red CC-1. Pixel intensity within microphotographs of each oocyte/zygote/parthenote was determined using Image J 1.240 software (National Institutes of Health, Federal Government of the United States). To normalise measurements between different replicates, the fluorescence of matured oocytes was set at a consistent level. Nuclear stage was evaluated at x400 using 330-380 nm (excitation) and 420 nm (emission) filters for Hoechst 33342.

Redox state measurements were expressed as Arbitrary Units/oocyte or zygote/parthenote.

**Determination of ROS production and nuclear stage**
To measure ROS production and nuclear stage, matured oocytes, putative zygotes and parthenotes were collected from culture media at 2-h interval from 7 to 24 h post activation/insemination, denuded and incubated in PBS supplemented with 3 mg/ml BSA for 30 min in the presence of 5 µM 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) (Le Bel et al., 1992) and 1 µM of Hoechst 33342. To measure esterase activity, 25% of the cells of each sample were incubated in the dark at 39.5°C in PBS supplemented with 3 mg/ml BSA for 15 min in the presence of 0.12 µM fluorescein diacetate (FDA).

After exposure to DCHFDA plus Hoechst 33342 or FDA, all oocytes were washed in PBS supplemented with 3 mg/ml BSA and mounted on glass slides. Fluorescence was measured as described above using 450-490 nM (excitation) and 520 nM (emission) filters for DCHFDA and FDA.

Both DCHFDA and FDA fluorescence are dependent on the endogenous esterase activity, therefore, a pixel intensity ratio between DCHFDA fluorescence and the mean FDA fluorescence (for the subset measured) at each time point for each oocyte was determined as justified by Lane et al. (2002). ROS levels were expressed as Arbitrary ROS Units/oocyte or zygote/parthenote.

**Experimental design and statistical analysis**

Data were expressed as mean ± SEM. Values at different time points were compared using ANOVA. A p-value < 0.05 was considered significant.

**RESULTS**

**Sperm mediated oocyte activation**

In sperm mediated activation, following insemination, pronuclei formation began by 9 h and peaked at 13-15 h (around 80% of the zygotes), then slightly decreased until 24 h. Syngamy began around 13 h and reached a plateau at 17-24 h (around 40% of the zygotes). First cleavage of embryos began at 21 h (Figure 1).

**Parthenogenetic oocyte activation**

In parthenogenetically activated oocytes, by the first observation at 7 h, all oocytes were at pronuclear stage and maintained up to 17 h from the initiation of activation, when they
abruptly decreased. Chromosomal fusion and cleavage began at 17 h, with most parthenotes (around 76%) appearing about 19-21 h (Figure 2).

**Redox state in non-activated, sperm and parthenogenetically activated oocytes**

In sperm activated oocytes, oxidative activity presented an increase between 15 and 19 h (p<0.05) (Figure 1; Figure 3, a - d). On the other hand, in parthenogenetically activated oocytes, we observed a decrease at 9 h (p<0.05) which did not alter until 24 h (Figure 2, Figure 3, e – h).

To determine whether the oxidative activity detected during early oocyte activation depended on activation or simply reflected the length of time since maturation, in vitro matured non-activated oocytes were cultured in vitro for 24 h, observing a significant decrease between 11 and 17 h (p<0.05) (Figure 4).

**Reactive oxygen species production in non-activated and sperm and parthenogenetically activated oocytes**

ROS production was compared between sperm-activated oocytes and parthenogenetically activated oocytes. In sperm-activated oocytes, ROS levels fluctuated throughout the 24 h of development, presenting clearly discernable peaks around 7, 19 and 24 h (p<0.05) (Figure 1; Figure 5, a - d), while in parthenogenetically activated oocytes, peaks were detected at 7, 11 and 17 h (p<0.05) (Figure 2, Figure 5, e - h).

To determine whether the rise in ROS levels depended on oocyte activation or reflected ROS production in the aging matured oocyte, non-activated oocytes were cultured in vitro for 24 h, in which we observed a significant decrease after 0 h (p<0.05) (Figure 4).

**DISCUSSION**

To our knowledge, this is the first time significant shifts in both ROS production and redox state have been observed in association with temporal developmental events in bovine oocyte sperm mediated and parthenogenetic activation.

Different temporal patterns of nuclear events were observed between both types of activation. Within putative zygotes stemming from sperm mediated activation, there was a temporal spread in the major developmental events after fertilization. In contrast, and not surprisingly, parthenogenetically activated oocytes behaved in a highly synchronized manner throughout development to the first cleavage division. The temporal sequence of developmental
events observed with both types of activation are similar to those previously reported for bovine zygotes (Gordon, 1994). The difference in the patterns observed is attributed to the asynchrony of sperm penetration, which lasts about 4 h in bovine (Jiang, 1991). Nevertheless, the synchronous nuclear progression observed in parthenogenetic activation does not necessarily equate to improved embryo development in vitro (Monaghan, 1993).

RedoxSensor Red CC-1 is a fluorescent dye that has been used as an indicator of oxidative activity in living cells (Chen and Gee, 2000). The increase in oxidative activity observed in sperm-activated oocytes corresponds with the initiation of pronuclear formation and first mitotic division in putative zygotes, suggesting increased demands of energy for these events. It has been observed that one and two cell bovine embryos are dependent on mitochondrial oxidative phosphorylation for energy supply, consuming oxidative substrates to produce ATP (Kim et al., 1993, Thompson et al., 1996). Coincidently, a higher oxygen consumption rate was detected prior to cleavage in bovine zygotes (Lopes et al., 2010).

In contrast, parthenotes initially have a high oxidative activity, which then declines from 7 h following activation and remains low thereafter during the developmental process with some small, non-significant, oscillations despite the events of chromosomal fusion and first cleavage. To our knowledge, there is no published data concerning the metabolism of bovine parthenotes, but an increase in the metabolic activity is expected in any type of embryo which undergoes cell division. In coincidence with our findings, in mouse, parthenogenetic 1- to 2-cell embryos present a lower glucose metabolism, glycogen content, ATP content and adenylate kinase activity than fertilized embryos (Han et al., 2008). This difference in metabolic behavior between both groups of activated oocytes could in part be responsible for the markedly lower developmental competence of the parthenogenetically activated oocytes.

It has been shown that the DCFHDA probe is oxidized by hydrogen peroxide, its derived oxidants, other peroxides and indirectly by the superoxide anion when generating hydrogen peroxide, thus providing a useful test to evaluate ROS production (LeBel et al., 1992). In sperm activated oocytes, ROS peaks appear before and/or during structural events associated with early embryo cleavage. The first peak occurs during preparative stages prior to pronuclear formation (7 h), such as sperm penetration and sperm head decondensation and the second and third peaks with association of pronuclei (19 h) and first mitotic division (24 h), respectively. On the contrary, in non-activated oocytes ROS levels dropped after 7 h of culture and remained low until 24 h. These results are in agreement with those reported for murine zygotes, in which only fertilized oocytes showed a rise in ROS production, while unfertilized oocytes presented declining levels over the same period (Nasr-Esfahani and Johnson, 1991). It has been suggested that certain levels of ROS are needed for the interaction between the spermatozoa and oocytes during bovine IVF, indicating that they may play different roles depending on the moment and the quantity in which they are present (Blondin et al., 1997). Very recently, a new class of
Dioxigenases have been identified, the Ten-eleven translocation proteins (Tet 1-3) that are key to the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine, thereby initiating the first steps towards DNA de-methylation (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). As DNA demethylation is a key process within early embryo development immediately following fertilization and pro-nuclear formation, perhaps the rise we have observed here in ROS production and elsewhere in oxygen consumption (Lopes et al., 2010) reflects Tet protein activity. In support of this, bovine pro-nuclear zygotes have significant levels of hydroxymethylcytosine in the male pronucleus, but not the female pronucleus, which appears largely due to the activity of Tet 3 (Wossidlo et al., 2011).

In parthenotes, high levels of ROS were observed, coinciding with a high rate of oocytes at the pro-nuclear stage (7 and 11 h) and prior to cleavage (17 h). Once again, ROS production seems to be associated with structural events related to early embryo development. Coincidently, an increase in ROS production in parthenogenetically activated murine oocytes was also observed (Nasr-Esfahani and Johnson, 1991). Of interest was the lack of synchronicity between the peaks in ROS levels and those of oxidative activity in both types of activation, indeed their behavior were contrasting during development. It is known in somatic eukaryotic cells that mitochondria in a resting respiration state (state 4) produce higher levels of ROS than those with active oxygen consumption (state 3); some of the electrons passing through the mitochondrial electron transport chain are transferred to molecular oxygen to form superoxide anion, which can then derive hydrogen peroxide (Boveris and Cadenas, 1975 and 1982).

During fertilization, the stimulation of mitochondrial respiration by sperm-triggered Ca\(^{2+}\) oscillations has been observed (Campbell and Swann, 2006; Dumollard et al., 2003, 2004; Schomer and Epel, 1998). Thus, the lack of oxidative burst observed in parthenotes could also be related to the single Ca\(^{2+}\) peak induced by parthenogenetic activation, which would not be efficient to stimulate mitochondria consistently.

There is scarce information about metabolic changes which occur in early bovine zygotes, especially in parthenogenetically activated oocytes. In the present work, we found clear and distinctive metabolic patterns between non-activated oocytes, in vitro fertilized and parthenogenetically activated oocytes. Characteristic behaviors in redox activity and fluctuations of ROS production during early development could be integrated in our understanding of measurements of oocyte and early embryo competence. The differences observed in parthenogenetic zygotes with respect to these oxidative patterns could in part explain their impaired developmental competence. Further studies into the metabolic control of parthenogenetic activation could contribute to improve the performance of these embryos for different biotechnological applications, such as somatic cell nuclear transfer for genetic improvement through cloning and transgenesis.
DECLARATION OF INTEREST, FUNDING AND ACKNOWLEDGEMENTS

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LEGEND OF FIGURES

Figure 1:
A) Nuclear stage of putative zygotes in sperm mediated oocyte activation, n= 1612 zygotes.

B) Redox state in sperm mediated oocyte activation. Values are expressed as mean arbitrary units/oocyte or zygote ± SEM. n = 134 putative zygotes. a,b Values with different superscripts are significantly different (p<0.05).

C) Reactive oxygen species production/total esterase activity in sperm mediated oocyte activation. Values are the ratio between DCHFDA and FDA assays; they are expressed as mean Arbitrary ROS Units/oocyte or zygote ± SEM. n = 1478 putative zygotes. a,b,c,d,e Values with different superscripts are significantly different (p<0.05).

Figure 2:
A) Nuclear stages observed in parthenogenetic oocyte activation, n= 630 parthenotes.

B) Redox state in parthenogenetically activated oocytes. Values are expressed as mean arbitrary units/oocyte or parthenote ± SEM. n = 172 parthenotes. a,b,c Values with different superscripts are significantly different (p<0.05).

C) Reactive oxygen species production/total esterase activity in parthenogenetically activated oocytes. Values are the ratio between DCHFDA and FDA assays; they are expressed as mean Arbitrary ROS Units/oocyte or parthenote ± SEM. n = 458 parthenotes. a,b,c Values with different superscripts are significantly different (p<0.05).

Figure 3: Representative activated oocytes or putative zygotes stained with RedoxSensor Red CC1 (x120). (a) to (d) Sperm activated oocytes at 0, 7, 11 and 19 h from activation and (e) to (h) Parthenogenetically activated oocytes at 0, 7, 11 and 19 h from activation. Bar = 50 µm.
Figure 4:

A) Redox state in non-activated oocytes. Values are expressed as mean arbitrary units/oocyte ± SEM. n = 180 oocytes. a,b Values with different superscripts are significantly different (p<0.05).

B) Reactive oxygen species production/total esterase activity in non-activated oocytes. Values are the ratio between DCHFDA and FDA assays; they are expressed as mean Arbitrary ROS Units/oocyte ± SEM. n = 200 oocytes. a,b,c Values with different superscripts are significantly different (p<0.05).

Figure 5: Representative activated oocytes or putative zygotes stained with 2,7 – dichlorodihydrofluorescein diacetate (x120). (a) to (d) Sperm activated oocytes at 0, 7, 11 and 19 h from activation and (e) to (h) Parthenogenetically activated oocytes at 0, 7, 11 and 19 h from activation. Bar = 50 µm.
Figure 2
Figure 3
Figure 5