**CSIRO** PUBLISHING

*Reproduction, Fertility and Development*, 2009, **21**, 608–614 www.publish.csiro.au/journals/rfd

# Reactive oxygen species in bovine oocyte maturation *in vitro*

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**Abstract.** The role of reactive oxygen species (ROS) in the *in vitro* maturation (IVM) of oocytes remains controversial. The aim of the present study was to determine possible fluctuations in ROS production during bovine oocyte IVM in the presence of different modulators of ROS generation. Cumulus–oocyte complexes were cultured in medium 199 (control) in the absence or presence of 0.6 mM cysteine, 1 mM 1-choro-2,4-dinitro benzene (CDNB), 2 μM diphenyliodonium, 0.5 mM *N*-nitro-L-arginine methyl ester or 10 μM sodium nitroprusside (SNP) at 39°C, in 5% $CO_2$ in humidified air for 22 h. In addition, the respiratory chain effectors potassium cyanide (KCN; 1 mM) and carbonyl cyanide m-chlorophenylhydrazone (0.42 μM) were used. Meiotic maturation was determined by the presence of MII. ROS production was evaluated in denuded oocytes at different time points as the ratio of 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) to fluorescein diacetate (FDA). ROS levels, expressed as DCHF-DA : FDA, fluctuated throughout the 22 h of maturation depending on the treatment applied. At 12 h incubation in the presence of KCN and SNP, ROS levels were increased, whereas ROS levels after 12 h in the presence of cysteine were reduced ($P < 0.05$). Both CDNB and SNP impaired meiotic progression. The higher metabolic activity demand during bovine oocyte maturation coincides with a concomitant reduction in ROS generation. These results suggest that 12 h would be a critical point for bovine oocyte IVM because it is closely related to the production of ROS at this time.

## Introduction

The production of reactive oxygen species (ROS), such as superoxide anion ($O_2^-$), hydroxyl radical ($OH^-$), hydrogen peroxide ($H_2O_2$) and organic peroxides, is a normal process that occurs in the cell when electrons are diverted during their transportation along the mitochondrial respiratory chain and other electron transfer systems. Although the oxidative modification of cell components as a result of the action of ROS is one of the most potentially harmful processes for proper cell function, causing DNA fragmentation, protein oxidation and lipid peroxidation (Yang *et al.* 1998), biological systems have developed several anti-oxidant mechanisms, both enzymatic (superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic (α-tocopherol, ascorbic acid, β-carotene and glutathione; Johnson and Nasr-Esfahani 1994). Therefore, damage to biological systems depends on the balance between ROS production and their removal by the anti-oxidant machinery of the cell (Ho *et al.* 1996). An enzymatic anti-oxidant system has been detected in bovine cumulus–oocyte complexes (COCs; Cetica *et al.* 2001).

The role of ROS in the *in vitro* maturation (IVM) of oocytes and embryonic development remains controversial. It has been observed that high ROS concentrations due to excessive glucose in the maturation medium have a negative effect on bovine embryos until the blastocyst stage (Hashimoto *et al.* 2000). However, accumulating evidence indicates that ROS can regulate cell function by both controlling the production and activation of substances that have biological activity and by activating key downstream signalling pathways (Schreck *et al.* 1991; Meyer *et al.* 1993; Li and Karin 1999; Buhimschi *et al.* 2000; Marshall *et al.* 2000). Moreover, cellular adaptations have been observed that not only protect against the harmful effects of ROS, but to use the reactive nature of ROS for beneficial purposes (Riley and Behrman 1991).

Anti-oxidants that can cross the plasma membrane inhibit meiotic resumption in mouse oocytes, suggesting that ROS have a regulatory function in the maturation process (Takami *et al.* 1999). In bovine oocytes, the addition of α-tocopherol and ascorbic acid to the maturation medium does not modify meiotic maturation and fertilisation percentages; however, a decrease in the percentage of blastocysts produced *in vitro* has been observed (Dalvit *et al.* 2005). Natural anti-oxidants, when present in the fertilisation medium, reduce the percentage of cleaved embryos, which suggests that certain ROS could be implicated in some events of oocyte maturation and fertilisation (Dalvit *et al.* 1998).

A critical intracellular anti-oxidant of the cell is glutathione, the major non-protein sulfydryl compound in mammalian cells (de Matos and Furnus 2000). Glutathione has the ability to cycle readily between reduced (GSH) and oxidised (glutathione disulfide; GSSG) forms, thus participating in many biological processes such as DNA and protein synthesis, as well as



10.1071/RD08198 1031-3613/09/040608

chemical metabolism (Zuelke *et al.* 1997). In both male and female gametes, GSH is involved in the protection of these cells against oxidative damage and in maintaining the meiotic spindle morphology of the oocyte (Luberda 2005). Some studies have demonstrated that the addition of cysteine to the maturation medium induces GSH synthesis, increasing oocytes developmental competence (de Matos *et al.* 1997; de Matos and Furnus 2000). Conversely, the effect on reproductive processes of reagents such as 1-chloro-2,4-dinitrobenzene (CDNB), which removes GSH non-oxidatively (Muzyamba *et al.* 2000), has not been studied until now.

Nitric oxide (NO) is a ubiquitous free radical (Ignarro 1999) that plays a role in early development, such as oocyte activation, oocyte maturation, embryonic development and implantation (Gouge *et al.* 1998; Jablonka-Shariff and Olson 1998; Kuo *et al.* 2000; Sengoku *et al.* 2001; Petr *et al.* 2005). At sub-micromolar concentrations, NO has two main effects on the mitochondrial respiratory chain: (*1*) competitive inhibition of cytochrome oxidase (Antunes *et al.* 2004); and (*2*) stimulation of $O_2^-$ production by inhibition of electron transfer at complex III (Poderoso *et al.* 1996). The metabolism of NO and $O_2^-$ in the mitochondrial matrix is linked by the very fast reaction between NO and $O_2^-$ to produce peroxynitrite ($ONOO^-$; Kissner *et al.* 1997). It is now well documented that NO and its toxic metabolite $ONOO^-$ can inhibit components of the mitochondrial respiratory chain, leading to cellular energy deficiency and, eventually, cell death (Calabrese *et al.* 2004). Some studies have been performed in the mouse investigating the effects of NO donors and NO synthase (NOS) inhibitors on oocyte maturation. The NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) has been shown to inhibit the formation of the first polar body (Hattori and Tabata 2006), whereas the NO donor, sodium nitroprusside (SNP) delays germinal vesicle breakdown (GVBD) and further suppresses the formation of the first polar body (Bu *et al.* 2004).

Diphenyleneiodonium (DPI) and diphenyliodonium (IDP) are well known inhibitors of flavoprotein oxidoreductases that have been reported to form phenol radicals that attack a wide range of targets, such as NADPH oxidase, NOS, xanthine oxidase, cytochrome P450, NADPH reductase and mitochondrial respiratory chain complex I NADH reductase (Li *et al.* 2003).

Recent studies have shown that mitochondrial proximal electron transport chain (ETC) inhibitors, such as rotenone, inhibit ROS generation, whereas the distal ETC inhibitor cyanide (CN) increases ROS production. Another respiratory chain effector is the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), which disrupts mitochondrial transmembrane potential and depletes mitochondrial $Ca^{2+}$ (Wang *et al.* 2003).

Although the effects of anti-oxidants on the IVM of mammalian oocytes has been documented, no studies have described variations in ROS in these cells during this process. Determining variations in ROS during the IVM of mammalian oocytes would increase our knowledge of the function of ROS in reproductive biotechnology *in vitro*, particularly during female gamete maturation. Thus, the aim of the present work was to determine possible fluctuation in ROS production during the IVM of bovine oocytes in the presence of different modulators of ROS generation and the relationship between ROS levels and the main events of the maturation process.

## Materials and methods

### *Recovery and classification of immature COCs*

Bovine ovaries were collected in an abattoir from slaughtered cows and kept warm (30–33°C) during the 2 h transit to the laboratory. Ovaries were washed in physiological saline containing 100 000 IU $L^{-1}$ penicillin and 100 mg $L^{-1}$ streptomycin. The COCs were recovered by aspiration of antral follicles (2–5 mm) and selected using a stereomicroscope. Only good-quality oocytes, completely surrounded by a compact and thick cumulus, were used (Class A; Cetica *et al.* 1999).

### *Oocyte IVM*

The COCs were matured in medium 199 supplemented with 5% fetal calf serum and 50 μg $mL^{-1}$ gentamicin sulfate under mineral oil at 39°C in a humidified atmosphere of 5% $CO_2$ for 22 h.

### *Oocyte denudation and evaluation of nuclear status*

Oocytes were completely denuded by gentle pipetting in phosphate-buffered saline (PBS) supplemented with 0.3 mg $mL^{-1}$ bovine serum albumin (BSA).

After 22 h maturation, COCs were collected from the maturation media, denuded, incubated in 10 mg $mL^{-1}$ sodium citrate hypotonic medium for 15 min, fixed on a glass slide with acid–ethanol (Tarkowski 1966), stained with 5% Giemsa for 15 min and then observed at ×100 and ×1000 magnification under a light microscope to determine nuclear status. The meiotic maturation rate was determined by the number of oocytes that presented the MII chromosome configuration at 22 h.

### *Experiment 1: determination of ROS production*

To measure ROS production COCs were collected from the maturation medium at 2-h intervals over the 22 h of incubation, denuded and incubated in PBS supplemented with BSA for 30 min in the presence of 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA; Le Bel *et al.* 1992).

To measure esterase activity, COCs were collected from the maturation medium at 2-h intervals over the 22 h of incubation, denuded and incubated in PBS supplemented with BSA for 15 min in the presence of 0.12 μM fluorescein diacetate (FDA; $n = 88$ oocytes).

After exposure to their respective fluorochrome, both COC samples were washed in PBS supplemented with BSA and mounted on glass slides. Fluorescence was measured using digital microphotographs in a Jenamed II epifluorescence microscope (Carl Zeiss Jena, Buenos Aires, Argentina) with 450–490 nm (excitation) and 520 nm (emission) filters. The photographs were analysed using ImageJ 1.240 software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA), measuring brightness for each oocyte.

Fluorescence levels detected by DCHF-DA are dependent on esterase activity; therefore, a ratio between the brightness of each oocyte measured by DCHF-DA and the mean brightness detected by FDA at each time point was considered a better indicator of

the level of ROS in each oocyte (Lane *et al.* 2002). ROS levels are expressed in arbitrary units per oocyte per min.

### *Experiment 2: ROS production in the presence of cysteine or CDNB*

In order to examine the role of glutathione in bovine oocyte meiotic maturation and ROS production, COCs were matured in control medium as described for Experiment 1, but with the addition of 0.6 mM cysteine (de Matos *et al.* 1997), which induces GSH synthesis, or 1 mM CDNB (Muzyamba *et al.* 2000), which removes GSH.

The COCs were collected from the maturation medium at 0, 6, 12, 18 and 22 h, times at which protein synthesis is required for oocyte developmental competence (Sirard *et al.* 1989), to measure ROS production using the DCHF-DA : FDA ratio, as described above.

### *Experiment 3: ROS production in the presence of L-NAME or SNP*

The COCs were matured as described for the control group but in the presence of 0.5 mM L-NAME (Jablonka-Shariff and Olson 2000), an endothelial NOS (eNOS) inhibitor, or 10 μM SNP (Bu *et al.* 2004), an NO donor, in order to determine the effect of NO on ROS production and oocyte meiotic maturation.

The COCs were collected from the maturation medium at the same time points analysed in Experiment 2 to measure ROS generation using the DCHF-DA : FDA ratio.

### *Experiment 4: ROS production in the presence of IDP*

To investigate the role of NADPH oxidase in ROS production and oocyte maturation, COCs were matured as described for the control group but with the addition of 2 μM IDP (O'Flaherty *et al*. 2005), an inhibitor of NADPH oxidase.

The COCs were collected from the maturation media at the same time points analysed in Experiment 2 to measure ROS generation using the DCHF-DA : FDA ratio.

### *Experiment 5: ROS production in oocytes exposed to KCN or CCCP*

In this experiment, COCs were matured in control medium and then exposed to 1 mM KCN (Wang *et al.* 2003), a cytochrome *c* oxidase inhibitor, or 0.42 μM CCCP (Beconi *et al.* 1993), an uncoupler of oxidative phosphorylation, during the 30-min incubation in DCHF-DA solution at 0, 6, 12, 18 and 22 h to elucidate the participation of the mitochondrial respiratory chain in ROS production at these time points during the maturation process.

### *Statistical analysis*

Data are expressed as the mean ± s.e.m. In Experiment 1 ROS levels and FDA values at different time points were compared using Kruskal–Wallis analysis. In Experiment 4, ROS levels were compared by a 5 × 2 factorial design, whereas in the other experiments they were compared using a 5 × 3 factorial design. Meiotic maturation rates between treatments were compared using Chi-squared analysis. In all tests, significance was set at $P < 0.05$.

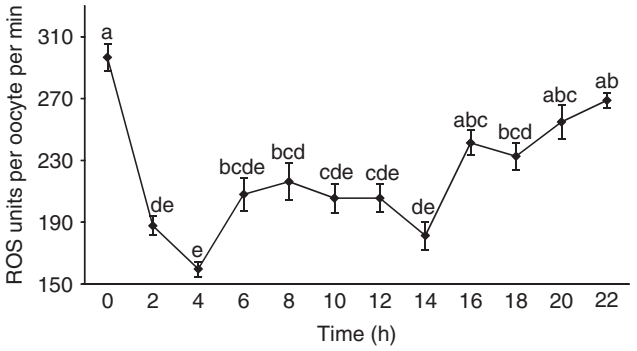


**Fig. 1.** Reactive oxygen species (ROS) production : esterase activity during *in vitro* maturation. Values are the ratio of 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) to fluorescein diacetate (FDA) and are expressed as the mean ± s.e.m. (in arbitrary ROS units per oocyte per min) for 282 oocytes. Approximately 24 oocytes were used at each time point to determine ROS production. Values with different superscript letters differ significantly ($P < 0.05$).

## Results

### *Experiment 1*

The production of ROS, expressed as a ratio between brightness registered by both fluorescent assays (DCHF-DA : FDA), was found to fluctuate during bovine oocyte *in vitro* culture. A significant decrease in ROS production was detected at 4 h of IVM compared with 0 h ($P < 0.05$). Beyond this time-point, ROS levels increased until 14 h, when another significant decrease was detected ($P < 0.05$). Then, ROS levels increased to reach similar values to those seen at 0 h (Fig. 1).

Esterase activity, determined by FDA, did not differ significantly at the different times evaluated during IVM or within the different treatment groups (data not shown).

At 18 h, 80% of oocytes exhibited protrusion of the first polar body ($n = 24$ oocytes) and 87% had reached MII by 22h (26/30 oocytes).

### *Experiment 2*

Compared with the control group, ROS levels were lower in oocytes matured in the presence of cysteine throughout the entire IVM process; significant differences were detected at 12 and 18 h ($P < 0.05$). In contrast, no significant differences were observed between oocytes treated with CDNB and the control group (Fig. 2).

In Experiment 2, there was no significant difference in the meiotic maturation percentage at 22 h in the presence of cysteine (54 matured oocytes/67 oocytes; 81%) compared with the control group (78/100 oocytes; 78%). However, in the presence of CDNB, oocytes were arrested at the germinal vesicle stage (0 oocytes matured of the 61 oocytes in this group).

### *Experiment 3*

There were no significant differences in ROS levels in oocytes matured in the presence of L-NAME compared with control. Conversely, a significant increase in ROS compared with control

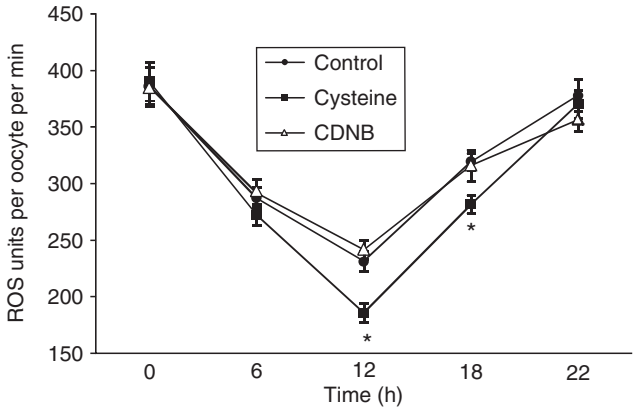


**Fig. 2.** Reactive oxygen species (ROS) production : esterase activity during bovine oocyte *in vitro* maturation in control medium and in the presence of cysteine or 1-choro-2,4-dinitro benzene (CDNB). Values are the ratio of 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) to fluorescein diacetate (FDA) and are expressed as the mean ± s.e.m. (in arbitrary ROS units per oocyte per min) for 575 oocytes. Between 32 and 40 oocytes were used for each treatment at each time point to determine ROS production. *$P < 0.05$ compared with control and CDNB treatment at the same time point.

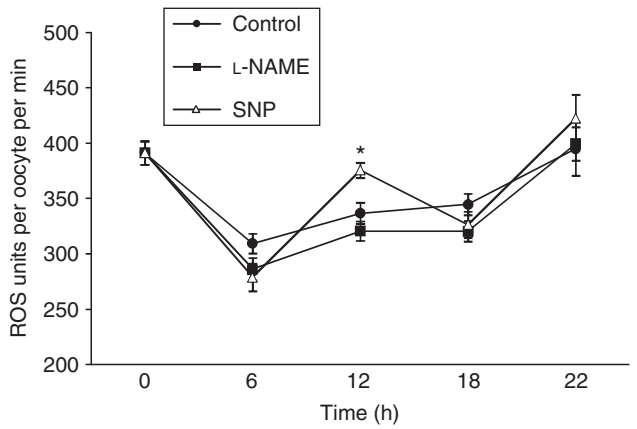


**Fig. 3.** Reactive oxygen species (ROS) production : esterase activity during bovine oocyte *in vitro* maturation in control medium and in the presence of *N*-nitro-L-arginine methyl ester (L-NAME) or sodium nitroprusside (SNP). Values are the ratio of 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) to fluorescein diacetate (FDA) and are expressed as the mean ± s.e.m. (in arbitrary ROS units per oocyte per min) for 565 oocytes. Between 29 and 42 oocytes were used for each treatment at each time point to determine ROS production. *$P < 0.05$ compared with control and L-NAME treatment.

was observed at 12 h in oocytes cultured in the presence of SNP ($P < 0.05$; Fig. 3).

The proportion of control oocytes that reached MII at 22 h was 16/20 oocytes (80%). Of oocytes matured in the presence of L-NAME, 24 of 29 reached MII (83%), which did not differ significantly compared with control. However, in the presence of SNP, only seven of 33 oocytes reached metaphase II (21%; $P < 0.05$), with the remaining oocytes stopped at the germinal vesicle–MI stage.

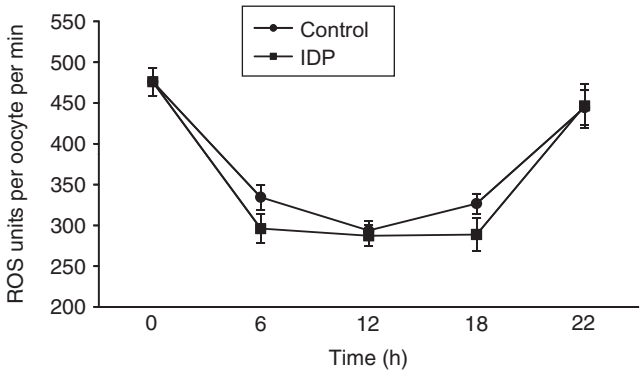


**Fig. 4.** Reactive oxygen species (ROS) production : esterase activity during bovine oocyte *in vitro* maturation in control medium and in the presence of diphenyliodonium (IDP). Values are the ratio of 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) to fluorescein diacetate (FDA) and are expressed as the mean ± s.e.m. (in arbitrary ROS units per oocyte per min) for 312 oocytes. Between 29 and 39 oocytes were used for each treatment at each time point to determine ROS production. No significant differences were observed between treatments at the same time points.

### *Experiment 4*

There were no significant differences in ROS levels in oocytes matured in the presence of IDP compared with control (Fig. 4). In addition, there was no significant difference in the meiotic maturation rate at 22 h between oocytes matured in the presence of IDP and control (68/91 oocytes (75%) *v.* 72/90 oocytes (80%), respectively).

### *Experiment 5*

There was a significant increase in ROS production at 12 h in oocytes treated with KCN compared with control ($P < 0.05$). However, ROS production in oocytes exposed to CCCP did not differ significantly from control (Fig. 5).

## Discussion

In the present study, ROS production was determined during IVM of a mammalian female gamete for the first time. Previously, it had been demonstrated that the oxygen consumption of COCs increases through IVM (Sutton *et al.* 2003), with mitochondrial oxidative phosphorylation being the main source of ATP synthesis in the non-fertilised oocyte (Dumollard *et al.* 2004). The significant fluctuation observed in ROS levels over the 22 h of culture in the present study (Fig. 1) could be attributed to variations in the metabolic activity of the oocyte at different phases of maturation, with active participation of the respiratory chain.

The mitochondrial ETC has been recognised as one of the major cellular generators of ROS, including $O_2^-$, $H_2O_2$ and $OH^-$ (Chance *et al.* 1979). Some of the electrons passing through the mitochondrial ETC leak out to molecular oxygen ($O_2$) to form $O_2^-$, which is quickly dismutated to $H_2O_2$ by mitochondrial superoxide dismutase (Boveris and Cadenas 1975). The production of $H_2O_2$ in 'controlled or resting respiration' (State 4) is

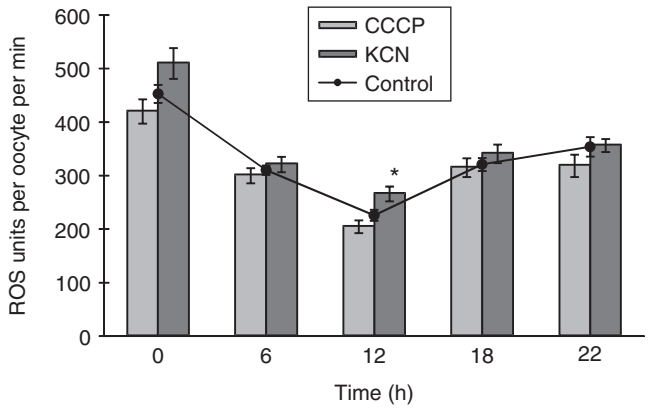


**Fig. 5.** Reactive oxygen species (ROS) production : esterase activity during bovine oocyte *in vitro* maturation in control medium and exposed to carbonyl cyanide m-chlorophenylhydrazone (CCCP) or potassium cyanide (KCN). Values are the ratio of 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) to fluorescein diacetate (FDA) and are expressed as the mean ± s.e.m. (in arbitrary ROS units per oocyte per min) for 296 oocytes. Between 15 and 28 oocytes were used for each treatment at each time point to determine ROS production. $*P < 0.05$ compared with control.

approximately four- to fivefold higher than in 'active respiration' (State 3), a fact that indicates that the $H_2O_2$ generator is a component of the respiratory chain, markedly changing during the transition from State 4 to State 3 (Boveris and Cadenas 1982). In bovine oocyte maturation, protein synthesis is needed at four different steps: (*1*) for GVBD (6 h); (*2*) to proceed towards MI (6–9 h); (*3*) during a short period of time during MI (12 h); and (*4*) to maintain the MII configuration (18 h; protrusion of the first polar body; Sirard *et al.* 1989). Therefore, we consider that these are the main events for the acquisition of developmental competence by the oocyte. The significant decrease in ROS generation between 2 and 18 h of maturation (Fig. 1) could correspond to a period that demands high energy production for the synthesis of proteins required during the preparatory stages for the main events of IVM. The low ROS concentrations observed during this time could also trigger cell signalling events implicated in meiotic maturation, as has been demonstrated in other cell models (Irani *et al.* 1997; Finkel 1998; Forman and Torres 2002).

Cysteine added to the maturation medium induces GSH synthesis, which may prevent oxidative damage (de Matos and Furnus 2000) and maintain the meiotic spindle morphology of the oocyte (Luberda 2005). The decrease observed in ROS production during IVM in the presence of cysteine compared with control (Fig. 2) demonstrates that the GSH produced acts as a non-enzymatic ROS scavenger in the oocyte. Low levels of ROS, particularly at 12 h of IVM, could determine the developmental competence of bovine oocytes.

The presence of an enzymatic anti-oxidant system in the bovine oocyte may regulate, in part, ROS levels during IVM (Cetica *et al.* 2001). The removal of GSH following the formation of a complex with CDNB during maturation had no effect on ROS levels with regard to control. Other components of the anti-oxidant system may be compensating the lack of GSH. However, changes in the total glutathione pool size may lead to oxidative damage of key mitochondrial proteins. Inhibition of GSH synthesis or its removal causes the inactivation of complexes I (Hsu *et al.* 2005), II/III and IV (Heales *et al.* 1995; Seyfried *et al.* 1999). Another target for thiol oxidation is the adenine nucleotide translocator (Majima *et al.* 1995), disruption of which restricts mitochondrial ATP export to the cytoplasm (Vesce *et al.* 2005). This reduction in ATP in the cytoplasm could impair the synthesis of proteins required for the progression of meiotic maturation.

The inhibition of eNOS by L-NAME in the present study had no effect on either ROS generation or meiotic maturation. Therefore, it is unlikely that eNOS actively participates in bovine oocyte IVM. The addition of exogenous NO by SNP increased ROS levels at 12 h (Fig. 3). This increase may be attributed to the competitive inhibition of cytochrome oxidase by NO (Antunes *et al.* 2004) and the stimulation of $O_2^-$ production by inhibition of electron transfer at complex III (Poderoso *et al.* 1996). The lower activity of the respiratory chain leads to a reduction in ATP production, diminishing the meiotic maturation rate. Conversely, under the present culture conditions, the $O_2^-$ that normally dismutates to $H_2O_2$ could combine with NO to form $ONOO^-$, which could produce oxidation, nitration and/or nitrosation of proteins, thus impairing meiotic progression. The fluorescent assay used in the present study detects different peroxides, so the increase observed at 12 h may also correspond to $ONOO^-$.

At low concentrations, IDP only inhibits membrane NADPH oxidase, flavoprotein that generates $O_2^-$, as has been reported for bovine spermatozoa (O'Flaherty *et al.* 2005). During IVM in the present study, no changes were observed in ROS generation in meiotic maturation following the addition of a low concentration of IDP (Fig. 4). Unlike what happens in sperm capacitation, it appears that there is no active participation of a membrane NADPH oxidase in bovine IVM.

The treatment of oocytes with KCN at the time of the main events of maturation increased ROS generation at 12 h (Fig. 5). Although cytochrome oxidase is not a source of ROS, its inhibition may facilitate ROS production by complexes I or III (Chen *et al.* 2003), which are the main sites of mitochondrial superoxide production (Muller *et al.* 2004). The inhibition of respiration with KCN prevents the oxidation of complexes I and III of the respiratory chain. These reduced complexes transfer electrons to molecular $O_2$, generating $O_2^-$. The dismutation of $O_2^-$ could be responsible for the increase observed in ROS production.

The uncoupler of the oxidative phosphorylation CCCP increases $O_2$ consumption without the production of ATP, leading to a decrease in $O_2^-$ generation. Even though no significant differences were detected between control and CCCP-treated COCs, there was a tendency for lower generation of ROS after treatment of oocytes with CCCP. This tendency would confirm the participation of ETC in ROS generation by the bovine oocyte during IVM.

## Conclusion

In conclusion, the higher metabolic activity demand that takes place during bovine oocyte maturation coincides with a concomitant reduction in ROS generation. Of note, an increase in ROS levels was detected with pro-oxidant modulators (SNP and

KCN) at 12 h of IVM, indicating that this time coincides with an important meiotic event that is sensitive to an increase in ROS. In addition, supplementation of the IVM medium with cysteine reduced ROS production at 12 h. These results suggest that 12 h is a critical point during bovine oocyte IVM because it is closely related to the production of ROS at this time. Further studies to identify pertinent ROS and their biochemical mechanisms of action in oocyte maturation should be conducted.

## Acknowledgements

This work was supported by a grant from the University of Buenos Aires. The authors thank the Japanese International Cooperation Agency (JICA) for technology transfer and equipment, Astra Laboratories for ultrapure water, Deltacar abbatoir for providing ovaries and Fernando Delicia for recovering the ovaries.

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Manuscript received 10 September 2008, accepted 26 January 2009