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RESEARCH ARTICLE

Influence of manganese on apoptosis and glutathione content of cumulus cells during in vitro maturation in bovine oocytes

Juan Patricio Anchordoquy[1,2], Juan Mateo Anchordoquy[1,2], Sebastián J. Picco[1,2], Matías A. Sirini[1,2], Ana Lía Errecalde[3] and Cecilia C. Furnus[1,3]*

1 Instituto de Genética Veterinaria Prof. Fernando N. Dulout, (UNLP-CONICET), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina
2 Cátedra de Fisiología, Laboratorio de Nutrición Mineral, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina
3 Cátedra de Citología, Histología y Embriología 'A', Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Argentina

Abstract

We have investigated the effect of different Mn concentrations on (1) DNA integrity of cumulus cells by olive tail moment (OTM); (2) cumulus cells apoptosis by Annexin V staining assay; (3) intracellular total glutathione (GSH-GSSG) content; and (4) oocyte nuclear maturation and embryo cleavage after in vitro fertilisation (IVF). For this purpose, 0 (control), 2 (Mn1), 5 (Mn2) and 6 ng/mL (Mn3) Mn concentrations were added to IVM medium. Comet assay analysed by OTM was significantly higher in cumulus cells arising from COCs matured without Mn (control, $P < 0.01$) respect to cumulus cells obtained from COCs matured with Mn (control: 5.18 ± 2.3; Mn1: 2.93 ± 2.2; Mn2: 2.63 ± 2.4; Mn3: 2.92 ± 2.4). The frequency of apoptotic cells was higher in the control group (control: 6.63 ± 0.59; Mn1: 5.05 ± 0.5; Mn2: 4.61 ± 0.49; Mn3: 3.33 ± 0.42). Intracellular concentration of GSH-GSSG increased in oocytes and cumulus cells matured in the presence of Mn ($P < 0.01$). There were no differences in percentages of nuclear maturation when Mn was added to IVM medium at any concentration, but at 6 ng/mL Mn a higher cleavage rate was observed respect to the control group ($P < 0.05$). In conclusion, deficiency in Mn concentration during in vitro maturation increased the damage in the DNA molecule and the frequency of apoptotic cumulus cells. However, the addition of an adequate Mn concentration (6 ng/mL Mn) to IVM medium improved the health of cumulus-oocyte complexes and produced more cleaved embryos 48 h after IVF.

Keywords: apoptosis; follicular fluid; glutathione; in vitro fertilisation; manganese; oocyte maturation

Introduction

Manganese (Mn) is a trace element present in all mammalian tissues. The redox-active metal Mn plays a key role in cellular adaptation to oxidative stress (Aguirre and Culotta, 2012). This element is involved in several functions including glucose metabolism and several enzymes activation (Baly and Schneiderman, 1990). Besides, Mn has an important role preventing oxidative damage by regulation superoxide dismutase activity via mitochondrial protein influx (Candas and Li, 2013). Furthermore, the periodic fluctuation in MnSOD activity during the cell cycle inversely correlates with cellular superoxide anion levels as well as glucose and oxygen consumption (Sarsour et al., 2013).

Manganese plasma concentration ranges from 5 to 10 ng/mL (Gibbons et al., 1976). Kincaid (1999) defined bovine Mn status taken into account plasma concentrations as: deficient (<5 ng/mL Mn); marginal (5–6 ng/mL Mn) and adequate (6–70 ng/mL Mn). Manganese deficiency in cattle produces a delay in the first estrous (Bentley and Phillips, 1951; Rojas et al., 1965), and in small ruminants not only impairs estrous cycle, but also reduces birth weight (Underwood and Suttle, 1999). LH enhances the viability of lutheal cells by stimulating the intralutheal expression of Mn-SOD, Cu–Zn-SOD and catalase, and by stimulating SOD activity to maintain lutheal function in cows (Kawaguchi et al., 2013).

Apoptosis essentially occurs when cellular damage, including damage to genetic material, has exceeded the capacity for repair (Rana, 2008). It is a genetically programmed and physiological mode of cell death involved in normal tissue remodelling, and is one of the processes that keeps cell proliferation and cell loss in balance (van Engeland

*Corresponding author: e-mail: cfurnus@fcv.unlp.edu.ar

et al., 1997; Zeuner et al., 2003). High rates of apoptotic bovine follicular cells affect subsequent oocyte development (Zadák et al., 2009). Besides, low zinc concentrations induce apoptosis and damage in the integrity of DNA of cumulus cells (Anchordoquy et al., 2010) with detrimental effects on development of preimplantation embryos (Picco et al., 2010). Adequate zinc concentrations added to IVM medium also improve cleavage rates after IVF of oocytes matured in vitro compared with those matured in zinc-deficient medium (Picco et al., 2010). Similarly, deficiency of copper concentrations during in vitro maturation of bovine oocytes affects DNA integrity of cumulus cells, and the competence (cytoplasmic maturation) of these oocytes to develop until the blastocyst stage (Picco et al., 2012).

Glutathione (GSH) synthesis during in vitro maturation (IVM) has an important role in embryo development. The increase in GSH concentrations during IVM of cattle oocytes improved subsequent embryo development to blastocyst stage (de Matos et al., 1997; Furnus et al., 2008). Glutathione is the major non-protein sulphydryl compound in mammalian cells and protects cells from oxidative damage (Pastore et al., 2003). Multiple actions have been described for this compound, including an effect on amino acid transport, DNA and protein synthesis, and reduction of disulfides (Lafleur et al., 1994). Glutathione has an important role in cellular defense against hazardous agents of endogenous and exogenous origin (Meister and Anderson, 1983). The increase in GSH content provides oocytes with large stores of GSH available for protection during subsequent embryo development (de Matos et al., 1995, 1996).

The present study is an investigation of the effect of the availability of Mn during in vitro maturation of bovine oocytes. Consequently, experiments were designed to evaluate the effect of different Mn concentrations added to IVM medium on: (1) DNA integrity and apoptosis in cumulus cells; (2) intracellular total glutathione (GSH-GSSG) content in both oocytes and cumulus cells; and (3) the oocyte capacity to mature and cleave after IVF.

Materials and methods

Reagents and media

All reagents for media preparation, comet assay, and GSH determinations were purchased from Sigma Chemical Co. (St. Louis, MO, USA), whereas FSH was purchased from Bioniche (Belleville, Ontario, Canada). The maturation medium used in all experiments was bicarbonate-buffered TCM-199 with Earle's salts supplemented with 10% FCS. The fertilisation medium consisted of TALP supplemented with 6 mg/mL fatty acid free BSA, 20 mM penicillamine, 10 mM hypotaurine, and 10 μg/mL heparin sulphate (Parrish et al., 1986). The culture medium for embryo development consisted of modified synthetic oviductal fluid (mSOF), which was composed of SOF (Tervit et al., 1972) supplemented with 1 mM glutamine, 2% (v/v) BME-essential amino acids, 1% (v/v) MEM-nonessential amino acids, and 4 mg/mL fatty acid free BSA (274–276 mOsm/kg) (Gardner et al., 1994). Annexin-V-FLUOS was purchased from Roche (Penzberg, Germany) and FSH from Serono, Inc. (Rockland, MA, USA).

Manganese concentrations in plasma, follicular fluid, and IVM medium

Samples of jugular blood (10 mL) were collected from 40 healthy heifers immediately before slaughter. The plasma was separated and the ovaries from these heifers were collected and handled independently to give 40 values for Mn in each follicle class. The follicle diameter was measured with a Vernier caliper and classified in two groups: small follicles (<10 mm) and large follicles (>10 mm). The follicular fluid (FF) from each group was aspirated with disposable sterilised insulin syringes. Samples of IVM medium with 10% FCS were also collected ($n = 6$). Manganese concentration was measured by atomic absorption spectrophotometer (GBC 902) with graphite furnace through an internal quality control (Picco et al., 2012) and classified according to Kincaid (1999). In bovine, Kincaid (1999) defined status of Mn according to the values determined in plasma. For this purpose, proposed three groups: deficient (<5 ng/mL Mn); marginal deficient (5–6 ng/mL Mn) and adequate (6–7 ng/mL Mn).

Oocyte recovery and classification

Bovine ovaries were obtained from an abattoir (Frigorífico Gorina S.A., Buenos Aires, Argentina) and transported to the laboratory in sterile NaCl solution (9 g/L) with antibiotics streptomycin (100 mg/L) and penicillin (59 mg/L) at 37°C within 3 h after slaughter. Ovaries were pooled, regardless of stage of the estrus cycle of the donor. The COCs (cumulus oocyte-complexes) were aspirated from 3 to 8 mm follicles, using an 18-G needle connected to a sterile test tube and to a vacuum line (50 mmHg). Only cumulus-intact complexes with evenly granulated cytoplasm were selected, using a low-power (20–30×) stereomicroscope, for IVM. Replicates of experiments (4–6) were performed on different days, with a separate batch of COCs for each day.

In vitro maturation (IVM)

COCs were washed twice in TCM-199 buffered with 15 mM HEPES containing 5% (v/v) FCS, and twice in IVM medium. Groups of 10 COCs were transferred into 50 μL of IVM medium under mineral oil (Squibb, Princeton, NJ, USA) pre-equilibrated in a CO_2 incubator. The COCs were cultured in

IVM medium at 39°C in 5% CO_2 in air with saturated humidity for 24 h. Oocyte maturation was assessed by mounting and staining the oocytes with the fluorescent DNA-specific dye Hoechst 33342 (2.5 μg/50 μL IVM medium), under an epifluorescence microscope (Nikon, Optiphot). We verified the presence of MII + PB (metaphase II + polar body) after 24 h of IVM. The manganese sulphate used for in vitro maturation was purchased by Merck (Japan, Cat. 25824-2B) and was diluted in Tissue Culture Water (Sigma–Aldrich Co, Germany) to provide 2 ng/mL Mn (deficient); 5 ng/mL Mn (marginal) and 6 ng/mL (adequate) concentrations (Kincaid, 1999) to IVM medium. At the end of incubation, the COCs were assessed for cumulus expansion. Only oocytes with an expanded cumulus (generally 90–95%) were used for in vitro fertilisation (IVF).

Effect of manganese on DNA integrity of cumulus cells evaluated by comet assay

At the end of IVM, all oocytes from each treatment were stripped of surrounding cumulus cells by repeated pipetting with a narrow-bore glass pipette in TCM 199 buffered with HEPES, and the cumulus cells were washed three times in calcium- and magnesium-free PBS containing 1 mg/mL PVP. Complete cell disruption was achieved by repeated aspiration using a narrow bore pipette. Samples were mixed with low melting point agarose. Single cell gel electrophoresis was performed using the alkaline version described by Singh et al. (1988) with modifications (Piper and Higgins, 1967; Tice and Strauss, 1995). Briefly, slides were covered with a layer of 180 μL of 0.5% normal agarose (Carlsbad, CA, USA). Then, 75 μL of 0.5% low melting point agarose (Carlsbad) was mixed with cells and layered onto the slides, which were immediately covered with cover slips. After agarose solidification at 4°C for 10 min, cover slips were removed and slides were immersed overnight at 4°C in fresh lysis solution. The slides were equilibrated in alkaline solution for 20 min. Electrophoresis was done for 30 min at 25 V and 300 mA (1.25 V/cm). Thereafter, slides were neutralised by washing (5 min each) three times with TRIS buffer (pH 7.5), and then with distilled water. Slides were stained with 1/1,000 SYBR Green I (Molecular Probes, Eugene, OR, USA) solution (Olive et al., 1999). Scoring was made at 400× magnification using a fluorescent microscope (Olympus BX40 equipped with a 515–560 nm excitation filter) connected through a Sony 3 CCD-IRIS Colour Video Camera, and saved using Image Pro Plus software. Based on the extent of strand breakage, cells were classified according to their tail length into five categories, ranging from Grade 0 (no visible tail), Grade 1 (comets with tiny tail), Grade 2 (comets with a dim tail), Grade 3 (comets with a clear tail), and Grade 4 (comets with a clear decrease in the diameter of the head and a clear tail). Arbitrary units of DNA damage value was established according to Collins (2004). This method was used in order to quantify DNA damage from the comets. The index damage (ID) was obtained as follows: if 100 comets are scored, and each comet assigned a value of 0–4 according to its class, the total score for the sample gel will be 0–400 'arbitrary units'. Visual scoring (arbitrary units) is rapid as well as simple, and there is a very close agreement between this method and computer image analysis (percentage DNA in tail) (Collins, 2004). In Experiment 1, the effect of Mn on DNA damage of cumulus cells following the addition of 0, 2, 5, or 6 ng/mL Mn to IVM medium was measured by a comet assay. COCs were matured for 24 h (as described above), and thereafter, DNA damage was measured. For this purpose, 800 COCs during four replicates from different days (200 COCs per replicate, 50 COCs per treatment) were matured in vitro with various Mn concentrations. Each batch of 50 COCs was processed for preparing slides to analyse at least 250 single cells per treatment for comet assay.

Effect of manganese on cumulus cells apoptosis by Annexin V staining assay

Annexin V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine (PS) (Glander and Schaller 1999; Paasch et al. 2004). Early apoptosis was evaluated by membrane redistribution of PS with Annexin-V-Fluos Staining Kit (Roche, Cat # 11-858-777-001). The assay involves simultaneous staining with both Annexin-V-FLUOS (green) and the DNA stain propidium iodide (PI, red). Normal cells exclude PI and Annexin-V-FLUOS. The apoptotic cells are visible in green and can be differentiated from necrotic cells by the PI iodide staining. Necrotic cells take up PI and stain orange/green, while apoptotic cells stain green only. Briefly, cells (1 × 106) were washed twice with PBS and centrifuged at 200*g* for 5 min. The pellet was resuspended in 100 μL of Annexin-V-Fluos labelling solution (Annexin V + fluorescein, HEPES buffer and propidium iodide), and incubated in the dark 10–15 min at 15–25°C. Cells suspension (50 μL) was layered onto the slides, which were immediately covered with cover slips. A total of 200 cells were analysed under a fluorescence microscope per treatment. The effect of adding 0, 2, 5, and 6 ng/mL Mn to IVM medium, on early apoptosis of cumulus cells, were evaluated (Experiment 2). The COCs were matured for 24 h and the frequencies of apoptotic cumulus cells were analysed by Annexin–FLUOS V staining as described above.

Effect of manganese on intracellular total GSH-GSSG content

After completion of IVM, all oocytes from each treatment in a batch were combined and stripped of surrounding cumulus cells. The GSH-GSSG assay was carried out as described

previously using a double-beam spectrophotometer (Model 35, Beckman, Irvine, CA, USA) (Furnus et al., 2008). Blanks consisted of 10 μL of PBS or 10 μL aliquots of wash medium. Total GSH content in oocytes and cumulus cells were calculated from a standard curve of GSH (Furnus et al., 1998). Under these conditions, the minimum detectable concentration of assay was 25 pmol of GSH-GSSG. The effect of adding 0, 2, 5, and 6 ng/mL Mn to IVM medium on intracellular GSH-GSSG content in oocytes and cumulus cells, were evaluated (Experiment 3). The COCs were matured for 24 h (as described above), and total GSH-GSSG concentration were evaluated. For this purpose, 800 COCs were matured on different days (four replicates) where 200 COCs were treated each day (batch of ovaries) and distributed in groups of 50 COCs per treatment.

Determination of cumulus cell number in COC

COC, either compact or expanded, were dispersed by pipetting the cells up and down several times under a stereomicroscope. The cell suspensions were transferred to Eppendorf tubes, and the number of cells in each suspension was estimated by counting in a hemocytometer chamber.

Effect of manganese concentrations during oocyte maturation and after in vitro fertilisation

In vitro fertilisation (IVF) and in vitro culture of embryos (ICV) were conducted as described previously (Furnus et al., 2003). Briefly, expanded COCs were placed into 50 μL drops of IVF medium under mineral oil. In all experiments, frozen semen from the same bull was used, and motile spermatozoa were selected by a discontinuous Percoll gradient. The final sperm concentration in IVF medium was 2×10^6 spermatozoa/mL. Incubation was at 39°C in 5% CO_2 in air with a saturated humidity for 24 h. After IVF, presumptive zygotes were stripped of cumulus cells by passing through a drawn pipette, washed twice in Hepes-TALP and cultured in mSOF. On Day 2 of IVC (48 h post IVF) the embryos were assessed for the morphological stages of development with an inverted microscope (Diaphot, Nikon, Tokyo, Japan). In Experiment 4, the effect of adding 0, 2, 5, and 6 ng/mL Mn during IVM on nuclear maturation was investigated. Also, the rate of cleavage up two-cell stage embryo was determined 48 h after IVF as described above. For this purpose, 400 COCs were matured on 4 days (100 COCs/day).

Processing and classification of matured oocytes and two-cell-embryos

Cumulus cells surrounding the mature oocytes were removed by gently pipetting these up and down with a plastic pipette in 400 μL of Hepes-TALP 24 h after IVM. Similarly, two-cell-embryos were assessed 48 h after IVF. The oocytes or embryos were individually transferred to individual wells of a four-well dish (Nunc, Roskilde, Denmark) containing 500 μL Hepes-Talp and stained with Hoechst 33342 (Molecular Probes; 2.5 μg/50 μL mSOF). The oocytes or 2-cell-embryos were subsequently washed in Dulbecco's PBS and individually loaded into a 10 μL drop of 4-morpholinepropanesulfonic acid (MOPS) – buffered medium (G-MOPS without phenol-red; Lane and Gardner, 2004) on a clean microscope slide. A cover slip was fixed to the slide using Vaseline as a spacer and the oocytes or embryos were imaged immediately using fluorescence microscopy, to determine the nuclear stage. Oocytes were classified as in vitro matured when a metaphase II chromosome configuration was present and two-cell-embryos by the presence of one nucleus per blastomera.

Statistical analysis

DNA damage was analysed by olive tail moment (OTM) and the relationship between Mn and OTM was studied using a parametric correlation and linear regression analysis. The frequency of cells without DNA damage and early apoptosis were analysed using Chi-square test with the Yates correction. Percentages of maturation and cleavage were also analysed by ANOVA and Student-Newman–Keuls Multiple Comparison post-test after angular transformation of data (CSS: Statistica, module C, Statsoft, Tulsa, OK, USA). Results are expressed as mean ± SEM.

Results

Manganese determination in plasma, follicular fluid, and IVM medium

Manganese concentration was 7.1 ng/mL ± 0.2 in 40 plasma samples; 7.0 ng/mL ± 0.7 in FF from large follicles; 7.1 ng/mL ± 0.4 in FF from small follicles; and 0.3 ng/mL ± 0.02 in IVM medium. There were no significant differences in Mn concentrations between plasma and FF, but both were significantly higher compared to IVM medium ($P < 0.01$).

Effect of manganese on DNA integrity of cumulus cells

In Experiment 1, cumulus cells from oocytes cultured with supplemental Mn during IVM had a significant decrease in the OTM (control: 5.18 ± 2.3; Mn1: 2.93 ± 2.2; Mn2: 2.63 ± 2.4; Mn3: 2.92 ± 2.4) (Figure 1). The percentage of DNA in the head (%DNAH) was 90.3 ± 4.09; 94.1 ± 4.11; 94.7 ± 4.12 and 93.6 ± 4.10 for control, Mn1, Mn2 and Mn3, respectively. All treatments were statistically different from the control group ($P = 0.006$ for Mn1; $P = 0.002$ for Mn2 and $P = 0.016$ for Mn3).

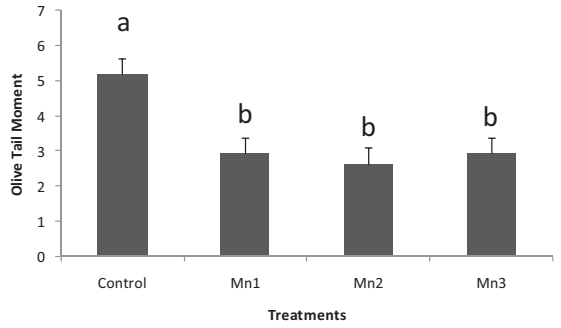

Figure 1 DNA damage in cumulus cells of COC matured with different Mn concentrations (0 ng/mL Mn: control; 2 ng/mL: Mn1; 5 ng/mL: Mn2 and 6 ng/mL: Mn3). Olive tail moment was significantly higher in control ($P < 0.01$) respect to cells matured with Mn1, Mn2 and Mn3.

Effect of manganese on cumulus cells apoptosis by Annexin V staining assay

In Experiment 2, cumulus cells matured with Mn were measured for early apoptosis by Annexin V-Fluos (Figure 2). The frequency of apoptotic cells decreased in cumulus cells arising from COCs matured with higher Mn concentrations (Mn2: 4.61 ± 0.49; Mn3: 3.33 ± 0.42), with respect to the groups matured in the presence of deficient Mn concentrations (control: 6.63 ± 0.59; Mn1: 5.05 ± 0.5). Statistical differences were found between control and Mn2 ($P < 0.05$), control and Mn3 ($P < 0.01$), Mn1 and Mn3 ($P < 0.01$) and Mn2 and Mn3 ($P < 0.05$). No differences were found between Mn1 and Mn2 ($P = 0.65$).

Effect of manganese on total intracellular glutathione concentration

Intracellular concentration of GSH-GSSG increased in oocytes and cumulus cells matured in the presence of Mn ($P < 0.01$; Table 1). However, the use of 5 and 6 ng/mL Mn concentrations ($P < 0.01$) increased GSH content in oocytes and cumulus cells compared to 2 ng/mL Mn. There were no differences in cumulus cell number per COC either before ($n = 4$) or after IVM ($n = 4$ per treatment) at any Mn concentration (before IVM: $12{,}900 \pm 1{,}100$; after IVM: $13{,}032 \pm 1{,}110$ (0 ng/mL Mn), $13{,}180 \pm 1{,}107$ (2 ng/mL Mn), $13{,}544 \pm 1{,}320$ (5 ng/mL Mn), and $14{,}010 \pm 1{,}200$ (6 ng/mL Mn) cumulus cells/COC. In all experiments, the cell number per COC did not vary significantly among Mn concentrations.

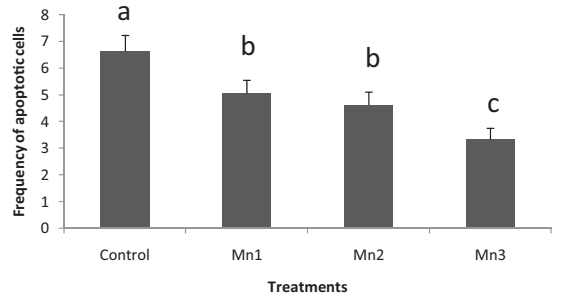

Figure 2 Frequencies of early apoptosis in cumulus cells of COC matured with different Mn concentrations. Cumulus cells incubated with Mn (0 ng/mL Mn: control; 2 ng/mL: Mn1; 5 ng/mL: Mn2 and 6 ng/mL: Mn3) were evaluated for early apoptosis by Annexin V-Fluos. Statistical differences were found between control and Mn1 ($P = 0.049$), control and Mn2 ($P = 0.02$), control and Mn3 ($P = 0.0001$), Mn1 and Mn3 ($P = 0.01$) and Mn2 and Mn3 ($P = 0.049$). No differences were found between Mn1 and Mn2.

Effect of manganese concentrations during oocyte maturation and on subsequent embryo development up to two cell-stage embryo

In Experiment 4, 800 COCs in four replicates were matured, fertilised and developed in vitro 48 h post-IVF. There were significant increases in maturation (M II + PB) and cleavage rates when Mn was added to IVM medium at 6 ng/mL (Mn3) ($P < 0.05$; Table 2). No differences were found between control, Mn2 and Mn3.

Discussion

The addition of adequate manganese concentration (6 ng/mL) to maturation medium reduced DNA damage and apoptosis in cumulus cells, and increased GSH-GSSG content in oocytes and cumulus cells, improving the competence of oocyte to be fertilised. Cumulus cells surround the oocyte during the maturation process within the follicle, and protect the developing oocyte providing nutrients through gap junctions (Mori et al., 2000; Tatemoto et al., 2000; Fatehi et al., 2002). Addition of Mn during oocyte in vitro maturation diminished the DNA damage and the frequency of apoptotic cumulus cells. The antioxidant role of Mn may be an important mechanism in maintaining DNA integrity in the COC by preventing oxidative DNA damage in cumulus cells. IVM medium without Mn (control) had a detrimental effect on the DNA integrity of cumulus cells after in vitro maturation. In contrast, different Mn concentrations (2, 5, 6 ng/mL Mn) added to the IVM medium reduced the DNA damage in the cumulus cells after in vitro maturation. Van Remmen et al. (2003) using heterozygous MnSOD knock-out mice found that a reduction of MnSOD activity leads to increase DNA oxidative damage.

Reactive oxygen species are implicated as mediators of apoptosis (Hampton and Orrenius, 1997). The mechanism by which Mn prevents apoptosis is unclear, but several reports established a correlation between MnSOD activity and cell injury resistance (Keller et al., 1998; Epperly et al., 2002; Holley et al., 2011). We found that apoptosis in cumulus cells was diminished in COCs matured in the

Table 1 Total intracellular glutathione concentration in bovine oocytes and cumulus cells matured with various Mn concentrations.

	Mn supplementation (ng/mL)			
	0	2	5	6
Oocyte GSH-GSSG (pmol/oocyte)	3.22 ± 0.08^{a}	4.02 ± 0.28^{b}	5.15 ± 0.24^{c}	5.0 ± 0.26^{c}
Cumulus GSH-GSSG (nmol/10^6 cells)	0.31 ± 0.02^{a}	0.39 ± 0.02^{b}	0.5 ± 0.02^{c}	0.5 ± 0.04^{c}

All values for oocytes (pmol GSH/GSSG/oocyte) and cumulus cells (nmol GSH/GSSG/10^6 cumulus cells) are expressed as mean ± SEM (800 COCs in four replicates, 200 COCs per replicate, 50 COCs per treatment for GSH-GSSG).
[a,b,c] Values with different superscripts within each row differ ($P < 0.01$).

presence of Mn. The degree of apoptosis is correlated with developmental competence of the enclosed bovine oocytes (Ikeda et al., 2003). van Montfoort et al. (2008) conducted a microarray experiment comparing gene expression in cumulus cells from oocytes resulting in early cleavage embryos and oocytes resulting in non-early cleavage embryos. The results showed that a total of 611 genes were differentially expressed; these genes were involved in a number of biological processes including cell cycle, angiogenesis, and apoptosis.

A large store of GSH during oocyte maturation is important for cumulus expansion in vitro and embryo protection up to the blastocyst stage (de Matos et al., 1995). Glutathione content was increased in oocytes and cumulus cells when Mn was present during IVM; however, the best condition was provided when manganese concentration in IVM medium was 6 ng/mL. Manganese increases tissues GSH levels in vivo on developing rat brain (Weber et al., 2002), and exerts a protective effect on hepatic oxidative damage and trace elements level in mice (Eybl and Kotyzová, 2010). The relationship between Mn and GSH has been studied; the antioxidant nature of Mn increases intracellular GSH/GSSG content by reducing GSH usage (Bansal and Kaur, 2009). Mn also stimulates the enzymes of the GSH cycle and affects GSH, GSSG and GSH-GSSG content (Bansal and Kaur, 2009). We observed that the percentages of in vitro matured oocytes and embryo cleavage were significantly higher when an adequate concentration of Mn (6 ng/mL) was present in IVM medium. The presence of 5 or 6 ng/mL Mn during IVM was the best condition to achieve an improvement in GSH/GSSG content in both oocytes and cumulus cells.

Thus: (1) Mn concentration is similar in plasma and follicular fluid; (2) DNA damage decreases in cumulus cells from oocytes matured with the addition of Mn during in vitro maturation; (3) the frequency of apoptotic cells decreases in cumulus cells arising from COCs matured with higher Mn concentrations (Mn2 and Mn3); (4) intracellular content of GSH-GSSG increases in oocytes and cumulus cells matured in the presence of Mn; and (5) Mn added to IVM medium increases the percentage of matured oocytes and cleavage rates.

In conclusion, the presence of adequate Mn concentration during in vitro maturation of bovine oocytes provides oocytes with favourable intracellular conditions to support fertilisation and early embryonic development in vitro. It should be considered that, although the global process from oocyte collection to early embryo development was analysed, Mn was added only to the maturation medium, and the effect of this compound could be beneficial also in subsequent steps.

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Table 2 Percentages of in vitro maturation and two-cell-embryos with various concentrations of Mn.

	Manganese supplementation (ng/mL Mn)			
	0	2	5	6
No. oocytes	200	200	200	200
MII + PB (%)	85.08 ± 4.31^{a}	85.38 ± 3.61^{a}	90.11 ± 2.14^{a}	93.67 ± 3.65^{b}
Cleaved (%)	72.18 ± 3.41^{a}	75.48 ± 2.61^{a}	78.11 ± 1.16^{a}	80.57 ± 0.65^{b}

Percentages of two-cell-embryos were recorded 48 h after insemination. Metaphase + polar body (MII + PB) and cleavage rates are expressed as mean ± SEM (800 COCs in four replicates on different days).
[a–b]Values with different superscripts within each row differ ($P < 0.05$).

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