Reproduction in Domestic Animals

Reprod Dom Anim **48**, 905–911 (2013); doi: 10.1111/rda.12184
ISSN 0936–6768

Effect of Different Manganese Concentrations during *in vitro* Maturation of Bovine Oocytes on DNA Integrity of Cumulus Cells and Subsequent Embryo Development

JP Anchordoquy[1,2], JM Anchordoquy[1,2], MA Sirini[1,2], G Mattioli[2], SJ Picco[1,2] and CC Furnus[1,3]

[1]*Instituto de Genética Veterinaria Prof. Fernando N. Dulout (IGEVET), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CONICET, La Plata, Argentina;* [2]*Cátedra de Fisiología, Laboratorio de Nutrición Mineral, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Argentina;* [3]*Cátedra de Citología, Histología y Embriología "A", Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina*

Contents
Manganese (Mn) is a trace element present in forages and cereals, and its concentration depends on soil status. Manganese deficiency in cattle, goats and ewes not only impairs oestrous cycle but reduces calf birth weight. The achievement of the first oestrus is delayed, and more attempts are necessary to obtain a successful conception. This study was conducted to investigate the effect of the availability of supplemental Mn during IVM on DNA damage of cumulus cells and total glutathione (GSH) content in oocytes and cumulus cells. The effect of supplementary Mn during IVM on subsequent embryo development was also studied. The results reported here indicate (i) DNA damage in cumulus cells decreased with 0, 2, 5 and 6 ng/ml Mn supplementation during IVM ($p < 0.05$). (ii) Intracellular GSH-GSSG content increased ($p < 0.01$) with different Mn concentrations in oocytes and cumulus cells. Also, cumulus cell number per cumulus oocyte-complexes (COC) did not differ either before or after IVM. (iii) Addition of Mn to maturation medium resulted in similar cleavage rates ($p > 0.05$) at 0, 2, 5 and 6 ng/ml Mn. However, subsequent embryo development to blastocyst stage was significantly higher ($p < 0.01$) in oocytes matured with 5 and 6 ng/ml Mn. (iv) There was also an increase ($p < 0.05$) in mean cell number per blastocyst obtained from oocytes matured with 5 and 6 ng/ml respect to zero Mn (IVM alone) and 2 ng/ml Mn. This study provides evidence that optimal embryo development to the blastocyst stage was partially dependent on the presence of Mn during IVM. Moreover, the availability of Mn during oocyte maturation ensures 'normal' intracellular GSH content in COCs and protects DNA integrity of cumulus cells.

Introduction

Manganese (Mn) is a trace element present in forages and cereals, and its concentration depends on soil status (Underwood and Suttle 1999). In cattle, Mn is absorbed by small intestine and bound to plasma transferrin (Davidsson et al. 1989; Forrest 1993; Keen et al. 2009). Manganese plasma concentration ranges from 5 to 10 ng/ml (Gibbons et al. 1976). Kincaid (1999) defined bovine Mn status by taking into account plasma concentrations as deficient (<5 ng/ml Mn), marginal (5–6 ng/ml Mn) and adequate (6–70 ng/ml Mn).

Misregulation of Mn2 + homeostasis impairs genome replication and cell cycle progression. Furthermore, genome instability and endomitosis can be triggered by alterations in cytosolic or Golgi Mn2 + levels (García-Rodríguez et al. 2012). Homeostasis of Mn tissue content is controlled by uptake, distribution and elimination mechanisms (Abrams et al. 1976). To decrease intracellular concentration, Mn is transported across the plasma membrane by ferroportin transporter (Yin et al. 2010), whereas ZIP family (He et al. 2006; Himeno et al. 2009), divalent metal transporter 1 (DMT1) and transferrin receptor are mechanisms for increasing intracellular Mn level (Gunshin et al. 1997; Garrick et al. 2003).

In mammalian cells, Mn plays an important role in protecting DNA damage (Zidenberg-Cherr et al. 1983; Holley et al. 2011). Manganese is a constituent of several metalloenzymes and is involved in enzymes activation such as hydrolases, decarboxylases, transferases and kinases (Forrest 1993; Keen et al. 2009). Besides, Mn prevents oxidative damage by increasing superoxide dismutase (SOD) activity, trapping hydroxyl and superoxide radicals, promoting metallothionein synthesis and competing with iron transport systems (Chihuailaf et al. 2002; Aschner and Aschner 2005; Santamaria 2008). Furthermore, MnSOD regulates a 'metabolic switch' during progression from quiescent through the proliferative cycle (Sarsour et al. 2012). It has been reported that an inadequate Mn dietary intake produces skeletal defects, generates abnormal glucose tolerance and perturbs lipid and carbohydrate metabolisms (Freeland-Graves and Llanes 1994; Keen et al. 2009).

Manganese deficiency in cattle produces reproductive disturbs. The achievement of the first oestrus is delayed, and more attempts are necessary to obtain a successful conception (Bentley and Phillips 1951; Rojas et al. 1965). In addition, Mn deficiency in cattle, goats and ewes not only impairs oestrous cycle but reduces calf birth weight (Underwood and Suttle 1999). In dairy cows, dietary organic zinc, Mn, copper and cobalt supplementation increase milk production in lactation, but has no detectable benefits on early post-calving follicular dynamics and embryo quality (Hackbart et al. 2010). Also, it has been reported that pregnancy rate, conception rate, age at conception and services to conception are not affected by supplemental Mn in growing beef heifers (Hansen et al. 2006).

The present study was carried out to investigate the effect of Mn supplementation during *in vitro* maturation of cattle oocytes. For this purpose, experiments were designed to evaluate (i) manganese concentrations in plasma, follicular fluid (FF) and IVM medium; (ii) the effect of different Mn concentrations added to the IVM medium on DNA integrity of cumulus cells; (iii) intracellular GSH-GSSG level in oocytes and cumulus cells; and (iv) subsequent embryo development up to blastocyst stage. As far as we know, this is the first report in literature of using Mn as supplement for *in vitro* maturation of mammalian oocytes.

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 fertilization 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. The composition of TALP medium was described previously by 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 non-essential amino acids and 4 mg/ml fatty-acid-free BSA (274–276 mOsm/kg) (Gardner et al. 1994).

Manganese concentrations in plasma, FF 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 then classified in two groups: small follicles (<10 mm) and large follicles (>10 mm). The FF from each group was aspirated with disposable sterilized 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 (Piper and Higgins 1967; Picco et al. 2012) and classified according to Kincaid (Kincaid 1999).

Oocyte recovery and classification

Bovine ovaries were obtained from an abattoir 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 oestrous cycle of the donor. The COCs (cumulus oocyte-complexes) were aspirated from 3 to 6 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 (20–30X) 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)

The COCs were washed twice in TCM-199 buffered with 15 mM HEPES containing 5% (v/v) FCS and then 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* fertilization (IVF).

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 then mixed with low melting point agarose. Single-cell gel electrophoresis was performed using the alkaline version described by Singh et al. (1988) with modifications (Tice and Strauss 1995). Briefly, slides were covered with a layer of 180 _L of 0.5% normal agarose (Carlsbad; Carlsbad, CA, USA). Then, 75 _L 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 performed for 30 min at 25 V and 300 mA (1.25 V/cm). Thereafter, slides were neutralized by washing (5 min each) three times with TRIS buffer (pH 7.5) and then with distilled water. Slides were stained with 1/1000 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 Color Video Camera and saved using Image Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA). Based on the extent of strand breakage, cells were classified according to their tail length into five categories (Fig. 1), 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 were established according to Collins (2004). The method of Collins (2004) was used to quantify DNA damage from the comets. The index damage (ID) was obtained as follows: if 100 comets are scored, and each

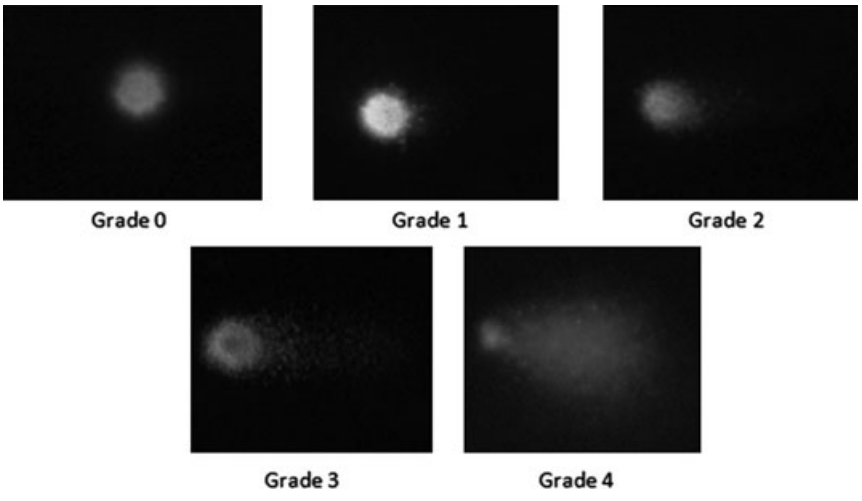

Fig. 1. DNA damage at the individual cumulus cell level: Grade 0 (no visible tail), Grade 1 (comets with tiny tail), Grade 2 (comets with a dim tail), Grade 3 (comets with a clear tail) and Grade 4 (comets with a clear decrease in the diameter of the head and a clear tail). (Collins 2004)

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).

GSH-GSSG assay

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.

Determination of cumulus cell number in COC

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

In vitro fertilization (IVF) and *In vitro* culture (IVC),

In vitro fertilization and IVC 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 was 2×10^6 spermatozoa/ml. Incubations were conducted at 39°C in 5% CO_2 in air with 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. Embryo culture was carried out in 40-μl drops of medium under mineral oil (10 presumptive zygotes per drop) at 39°C in an atmosphere composed of 7% O_2, 5% CO_2 and 88% N_2 with saturated humidity. All embryos were cultured in the absence of glucose during the first 24 h and further cultured for 7 days in the presence of 1.5 mM glucose (Furnus et al. 1997). The embryos were incubated for 8 days (Day 0 = day of fertilization). At the end of incubations, the embryos were evaluated for the morphological stages of development with an inverted microscope (Diaphot, Nikon; Tokyo, Japan).

Blastocyst staining for total cell number

Day 8 (8D) blastocysts were fixed in 4% formaldehyde after washing three times in 1% polyvinylpyrrolidone (PVP) in PBS overnight. Embryos were placed in 1% Triton X-100 overnight, stained with Hoechst 33342 (2.5 μg/50 μl mSOF) and mounted on slides and covered with a cover slip. Total cell numbers of blastocysts were visualized by a Nikon Optiphot epifluorescent microscope with a 40 × fluor objective (Nikon; Tokyo, Japan) equipped with a 365-nm excitation filter, a 400-nm barrier filter, and a 400-nm emission filter.

Experimental Design

Effect of manganese on DNA integrity of cumulus cells

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 evaluated by a Comet Assay. The COCs were matured for 24 h (as described above), and thereafter, DNA damage was evaluated as described (Comet assay). 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 intracellular total GSH level

The effect of adding 0, 2, 5 and 6 ng/ml Mn to IVM medium on intracellular GSH-GSSG concentration in

oocytes and cumulus cells was evaluated (Experiment 2). The COCs were matured for 24 h (as described above), and total GSH concentration was evaluated (described in GSH-GSSG assay). For this purpose, 800 COCs were matured on different days (4 replicates) where 200 COCs were treated each day (batch of ovaries) and distributed in groups of 50 COCs per treatment.

Effect of manganese concentrations during IVM on subsequent embryo development and embryo quality

In Experiment 3, the developmental capacity of oocytes matured in IVM medium supplemented with 0, 2, 5 and 6 ng/ml Mn was investigated. For this purpose, 1140 COCs were matured on 6 days (between 180–200 COCs/day). Cleavage rates were recorded 48 h after insemination. Percentages reported for development of the blastocyst stage included embryos that progressed to the expanded or hatched blastocyst stages. The total cell numbers of blastocysts (8D–Grade 1) were determined by counting the number of nuclei under an epifluorescent microscope.

Statistical analysis

Chi-square analysis with the Yates correction was used to compare, among groups, the proportion of DNA damage determined with comet assay. Differences among treatments for GSH concentrations in oocytes and cumulus cells were analysed by ANOVA and Student–Newman–Keuls Multiple Comparison post-test, after logarithmic transformation of data. Percentages of cleavage and embryo development 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). Results are expressed as mean ± SEM.

Results

Manganese determination in plasma, FF and IVM medium

Manganese concentration was 7.3 ng/ml ± 0.2 in 40 plasma samples; 6.9 ng/ml ± 0.7 in FF from large follicles; 7.0 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.

Effect of manganese on DNA damage of cumulus cells

In Experiment 1, cumulus cells from oocytes cultured with supplemental Mn during IVM had a significant decrease in the index damage of DNA (Index Damage was 47.14 ± 9.1, 29.17 ± 6.26, 28 ± 6.25 and 24.7 ± 7.63 in 0, 2, 5 and 6 ng/ml, respectively; $p < 0.05$). Percentages of cells without DNA damage (Degree 0) were higher with Mn supplementation ($p < 0.05$; Table 1). In addition, there were significant differences in the degree of DNA damage in cumulus cells treated with 6 ng/ml Mn compared with the other two Mn supplemented groups.

Table 1. DNA damage in cumulus cells matured *in vitro* with different manganese concentrations

Mn (ng/ml)	Damage degree (Grades)				
	0	1	2	3	4
0	73.71 (3.87)[a]	4.00 (2.83)[a]	6.00 (1.38)[a]	4.00 (1.75)[a]	2.28 (1.27)[a]
2	79.42 (5.01)[b]	14.85 (4.14)[b]	13.71 (1.34)[b]	1.14 (0.59)[b]	0.85 (0.40)[b]
5	77.14 (5.01)[b]	19.42 (4.61)[b]	2.28 (1.27)[c]	0.57 (0.37)[b]	0.70 (0.37)[b]
6	83.14 (5.11)[c]	11.71 (4.43)[c]	4.00 (2.14)[c]	0.85 (0.40)[b]	0.57 (0.37)[b]

DNA damage in bovine cumulus cells obtained from COC matured *in vitro* with various Mn concentrations (Cells analysed: 1275–1330 for each level of Mn supplementation).
Cells were classified according to their tail length in five categories: 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).
All values for damage degree in cumulus cells are expressed as Mean ± SEM, (800 COCs in four replicates for comet assay).
[a–c]Within a column means without a common superscript differed ($p < 0.05$).

Effect of manganese on total intracellular GSH concentration

Intracellular concentration of GSH-GSSG increased in oocytes and cumulus cells matured in the presence of Mn ($p < 0.01$; Table 2). However, the use of 5 and 6 ng/ml Mn concentrations ($p < 0.01$) increased GSH content in oocytes and cumulus cells compared with 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: 13900 ± 1100; after IVM: 14032 ± 1100 (0 ng/ml Mn), 14180 ± 1207 (2 ng/ml Mn), 14544 ± 1390 (5 ng/ml Mn) and 15010 ± 1237 (6 ng/ml Mn) cumulus cells/COC. In all experiments performed, neither cell number per COCs nor the percentage of nuclear maturation (90–95%; evaluated with Hoechst 33342) varied significantly among Mn concentrations.

Effect of manganese concentrations during IVM on subsequent embryo development and embryo quality

In Experiment 3, 1240 oocytes in six replicates were matured, fertilized and developed *in vitro*. There was no difference in cleavage rate when Mn was added to IVM medium at any concentration ($p \geq 0.05$). Furthermore,

Table 2. 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)	3.52 ± 0.08[a]	4.32 ± 0.28[b]	5.45 ± 0.24[c]	5.2 ± 0.26[c]
Cumulus GSH-GSSG (nmol/10^6 cells)	0.29 ± 0.02[a]	0.38 ± 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–c]Within a row means values without a common superscript differed ($p < 0.01$).

Table 3. Developmental capacity of bovine oocytes matured *in vitro* with various concentrations of Mn

	Manganese supplementation (ng/ml Mn)			
	0	2	5	6
No. oocytes	288	262	285	305
Cleaved (%)	72.38 ± 3.41[a]	75.48 ± 2.61[a]	80.55 ± 1.16[a]	75.57 ± 0.65[a]
Blastocyst/ oocytes	20.76 ± 2.25[a]	29.19 ± 1.02[b]	37.62 ± 1.31[c]	37.45 ± 1.78[c]
Blastocyst/ cleaved	28.82 ± 2.97[a]	38.73 ± 0.91[b]	46.67 ± 1.26[bc]	49.58 ± 2.43[c]
Cell number/ blastocyst	102.3 ± 8.5[a]	91.2 ± 5.5[a]	117.4 ± 6.7[b]	119.0 ± 5.0[b]

Cleavage rates were recorded 48 h after insemination. Data reported for development to the blastocyst stage included those embryos that progressed to the expanded or hatched blastocyst stages after 8 days in culture. All values for cleavage and development rates are expressed as mean ± SEM (1140 COCs in six replicates on different days).
Mean cell numbers of Day 8 blastocysts developed from oocytes matured with 0 ng/ml (n = 15), 2 ng/ml (n = 19), 5 ng/ml (n = 20) and 6 ng/ml Mn (n = 22).
[a–c]Within a row means values without a common superscript differed ($p < 0.01$).

there was an increase in the percentages of blastocyst yield ($p < 0.01$) in oocytes matured with 2, 5 and 6 ng/ml Mn concentrations. Embryo production did not differ between 5 and 6 ng/ml Mn (Table 3). Mean cell number per blastocyst increased ($p < 0.05$) when oocytes were matured with 5 or 6 ng/ml Mn added to IVM media (Table 3).

Discussion

The results of the present study indicate that (i) DNA damage in cumulus cells decreased by addition of Mn to the maturation medium. (ii) The supplementation of Mn in IVM medium increased intracellular GSH-GSSG content in oocytes and cumulus cells. (iii) The presence of Mn in IVM medium improves subsequent embryo development up to the blastocyst stage. (iv) Embryo quality in terms of the number of cells per blastocyst was enhanced when Mn was added to IVM medium at marginal or adequate concentrations.

Manganese concentrations obtained in bovine FF were similar to plasma concentrations. Previous studies demonstrated that both Zn and Cu also have similar values when plasma and FF concentrations were compared (Picco et al. 2010, 2012). According to Kincaid's classification (1999), the values of Mn obtained in this study for plasma and FF correspond to adequate status in cattle. Nevertheless, IVM medium presented values corresponding to deficient Mn status (0.3 ng/ml Mn).

The antioxidant function of Mn may be an essential system in preserving DNA integrity in cumulus–oocyte complex. In the present study, IVM medium without Mn had a detrimental effect on the DNA integrity of cumulus cells after *in vitro* maturation. Manganese-superoxide dismutase protects human cell lines *in vitro* against radiation-induced DNA damage (Pollard et al. 2009). Besides, Mn appears to be able to counteract oxidative stress and modulate apoptosis depending on the cell type and concentration used (Schrantz et al. 1999). *In vivo*, it has been reported that a reduction in MnSOD activity increases DNA oxidative damage in brain, liver, spleen and heart of heterozygous MnSOD knock-out mice (Van Remmen et al. 2003). Manganese mode of action remains partly unknown in COCs; however, the present study provides evidence that adequate concentrations of Mn 'protect' cumulus cells against DNA damage.

Glutathione content was lower in oocytes and cumulus cells when Mn was deficient during IVM. It has been reported that Mn increases tissues' GSH levels *in vivo* on developing rat brain (Weber et al. 2002). Furthermore, Mn 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). Besides, Mn stimulates the enzymes of GSH cycle and affects the GSH, GSSG and GSH-GSSG content (Bansal and Kaur 2009). A large store of GSH during oocyte maturation is important for cumulus expansion *in vitro* and embryo protection up to the blastocyst stage (Takahashi et al. 1993; de Matos et al. 1995), improving the efficiency of *in vitro* blastocyst production from immature oocytes (de Matos et al. 1995). Therefore, adequate concentrations of Mn during oocyte maturation ensured an adequate GSH store for subsequent embryo development.

In the present study, Mn (2, 5 and 6 ng/ml) added to IVM medium did not increase cleavage rates from oocytes matured in medium alone. Nonetheless, the yield of embryo development to blastocyst stage was significantly higher when oocytes were matured with both 5 and 6 ng/ml Mn. It has been reported that micronutrients deficiencies and excesses have profound and sometimes persistent effects on the developmental competence of conceptuses and neonates in all species studied to date (Ashworth and Antipatis 2001). The consequences of a single micronutrient deficiency can be more severe than the effects of more general undernutrition (Ashworth and Antipatis 2001). The presence of micronutrients such us Zn or Cu during oocyte maturation not only increased GSH content and protect DNA integrity of cumulus cells, but also improved subsequent embryo development to the blastocyst stage (Picco et al. 2010, 2012). In agreement with these previous studies, we have found a correlation between embryo production and Mn concentration in the IVM media. As the oocyte is particularity susceptible to oxidative stress, excessive generation of reactive oxygen species (ROS) in culture media has the potential to deplete antioxidant stores and compromise the embryo developmental potential through DNA damage and ROS-mediated apoptotic event (Liu et al. 2000; Guerin et al. 2001).

In conclusion, inadequate Mn supply influenced total intracellular GSH content and DNA integrity of cumulus cells during *in vitro* maturation of bovine oocytes, with detrimental effects on development of pre-implantation embryos. The incubation of COC with adequate Mn concentrations increased intracellular GSH-GSSG content in oocytes. Manganese supplementation during *in vitro* maturation of cattle oocytes

might be considered to improve oocyte developmental competence *in vitro* to develop to blastocyst stage.

Acknowledgments

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Tecnológicas (PIP 0990 - CONICET); Ministerio de Ciencia, Tecnología e Innovación Productiva de la Nación Argentina. We are grateful to Centro de Inseminación Artificial La Elisa S.A. (CIALE) to make available bovine frozen semen and the staff of Frigorifico Gorina S.A. for providing the bovine ovaries.

Conflict of interest statement

None of the authors have any conflict of interest to declare.

Author contributions

JP Anchordoquy and JM Anchordoquy designed the study, M Sirini analysed the data, SJ Picco analysed the images of comet assay, GA Mattioli determine Mn concentratiions in plasma and FF (Mineral Lab) and C. Furnus coordinate the experiemnts, wrote and revised the manuscript. All the team participate in lab job.

References

Abrams E, Lassiter JW, Miller WJ, Neathery MW, Gentry RP, Scarth RD, 1976: Absorption as a factor in manganese homeostasis. J Anim Sci **42**, 630–636.

Aschner JL, Aschner M, 2005: Nutritional aspects of manganese homeostasis. Mol Aspects Med **26**, 353–362.

Ashworth CJ, Antipatis C, 2001: Micronutrient programming of development throughout gestation. Reproduction **122**, 527–535.

Bansal AK, Kaur AR, 2009: Cooperative functions of manganese and thiol redox system against oxidative stress in human spermatozoa. J Hum Reprod Sci **2**, 76–80.

Bentley OG, Phillips PH, 1951: The effect of low manganese rations upon dairy cattle. J Dairy Sci **34**, 396–403.

Chihuailaf RH, Conteras PA, Wittwer FG, 2002: Pathogenesis of oxidative stress: consequences and evaluation in animal health. Vet. Mex **33**, 265–283.

Collins AR, 2004: The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol **26**, 249–261.

Davidsson L, Lonnerdal B, Sandstrom B, Kunz C, Keen CL, 1989: Identification of transferrin as the major plasma carrier protein for manganese introduced orally or intravenously or after *in vitro* addition in the rat. J Nutr **119**, 146–144.

Eybl V, Kotyzová D, 2010: Protective effect of manganese in cadmium-induced hepatic oxidative damage, changes in cadmium distribution and trace elements level in mice. Interdiscip Toxicol **3**, 68–72.

Forrest HN, 1993: Ultratrace minerals. In: Shils ME, Olson JA, Shike M (eds), Modern Nutrition in Health and Disease8th edn. Lea and Febiger, Philadelphia, PA, pp. 269–286.

Freeland-Graves J, Llanes C, 1994: Models to study manganese deficiency. In: Klimis-Tavantzis DJ (ed), Manganese in Health and Disease. CRC Press, Boca Raton, LA, pp. 59–86.

Furnus C, de Matos D, Martínez A, Matkovic M, 1997: Effect of glucose on embryo quality and post-thaw viability of in-vitro-produced bovine embryos. Theriogenology **47**, 481–490.

Furnus CC, de Matos DG, Moses DF, 1998: Cumulus expansion during *in vitro* maturation of bovine oocytes: relationship with Intracellular glutathione level and its role on subsequent embryo development. Mol Reprod Dev **51**, 76–83.

Furnus CC, Valcarcel A, Dulout FN, Errecalde AL, 2003: The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and early stage embryos. Theriogenology **60**, 1633–1644.

Furnus CC, de Matos DG, Picco S, García PP, Inda AM, Mattioli G, Errecalde AL, 2008: Metabolic requirements associated with GSH synthesis during *in vitro* maturation of cattle oocytes. Anim Reprod Sci **109**, 88–99.

García-Rodríguez N, Díaz de la Loza Mdel C, Andreson B, Monje-Casas F, Rothstein R, Wellinger RE, 2012: Impaired manganese metabolism causes mitotic misregulation. J Biol Chem **287**, 18717–18729.

Gardner DK, Lane M, Spitzer A, Batt PA, 1994: Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biol Reprod **50**, 390–400.

Garrick MD, Dolan KG, Horbinski C, Ghio AJ, Higgins D, Porubcin M, Moore EG, Hainsworth LN, Umbreit JN, Conrad ME, Feng L, Lis A, Roth JA, Singleton S, Garrick LM, 2003: DMT1: a mammalian transporter for multiple metals. Biometals **16**, 41–54.

Gibbons RA, Dixon SN, Hallis K, Russell AM, Sansom BF, Symonds HW, 1976: Manganese metabolism in cows and goats. Biochim Biophys Acta **444**, 1–10.

Guerin P, El Mouatassim S, Menezo Y, 2001: Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update **7**, 175–189.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA, 1997: Cloning and characterization of a mammalian proton-coupled metal–ion transporter. Nature **388**, 482–488.

Hackbart KS, Ferreira RM, Dietsche AA, Socha MT, Shaver RD, Wiltbank MC, Fricke PM, 2010: Effect of dietary organic zinc, manganese, copper, and cobalt supplementation on milk production, follicular growth, embryo quality, and tissue mineral concentrations in dairy cows. J Anim Sci **88**, 3856–3870.

Hansen SL, Spears JW, Lloyd KE, Whisnant CS, 2006: Growth, reproductive performance, and manganese status of heifers fed varying concentrations of manganese. J Anim Sci **84**, 3375–3380.

He L, Girijashanker K, Dalton TP, Reed J, Li H, Soleimani M, Nebert DW, 2006: ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of transporter properties. Mol Pharmacol **70**, 171–180.

Himeno S, Yanagiya T, Fujishiro H, 2009: The role of zinc transporters in cadmium and manganese transport in mammalian cells. Biochimie **91**, 1218–1222.

Holley AK, Bakthavatchalu V, Velez-Roman JM, St Clair DK, 2011: Manganese superoxide dismutase: guardian of the powerhouse. Int J Mol Sci **12**, 7114–7162.

Keen CL, Ensunsa JL, Lönnerdal , Zidenberg-Cherr S, 2009: Manganese. In: Caballero B (ed) Guide to Nutritional Supplements. Elsevier Ltd., Oxford, UK.

Kincaid RL, 1999: Assessment of trace mineral status of ruminants: a review. Proc Am Soc Anim Sci, 1–10.

Liu L, Trimarchi JR, Keefe DL, 2000: Involvement of mitochondria in oxidative stress-induced cell death in mouse zygotes. Biol Reprod **62**, 1745–1753.

de Matos DG, Furnus CC, Moses DF, Baldassarre H, 1995: Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*. Mol Reprod Dev **42**, 432–436.

Olive PL, Durand RE, Jackson SM, Le Riche JC, Luo C, Ma R, McLaren DB, Aquino-Parsons C, Thomson TA, Trotter T, 1999: The comet assay in clinical practice. Acta Oncol **8**, 839–844.

Parrish JJ, Susko-Parrish J, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NF, 1986: Bovine *in vitro* fertilization with frozen–thawed semen. Theriogenology **25**, 591–600.

Picco SJ, Anchordoquy JM, de Matos DG, Anchordoquy JP, Seoane A, Mattioli GA, Errecalde AL, Furnus CC, 2010: Effect of increasing zinc sulphate concentration during *in vitro* maturation of bovine oocytes. Theriogenology **74**, 1141–1148.

Picco SJ, Rosa DE, Anchordoquy JP, Anchordoquy JM, Seoane A, Mattioli GA, Furnus CC, 2012: Effects of copper sulphate concentrations during *in vitro* maturation of bovine oocytes. Theriogenology **77**, 373–381.

Piper HG, Higgins G, 1967: Estimation of trace metals in biological material by atomic absorption spectrophotometry. Proc Assoc Clin Biochem **7**, 190–195.

Pollard JM, Reboucas JS, Durazo A, Kos I, Fike F, Panni M, Gralla EB, Valentine JS, Batinic-Haberle I, Gatti RA, 2009: Radioprotective effects of manganese-containing superoxide dismutase mimics on ataxia-telangiectasia cells. Free Radic Biol Med **47**, 250–260.

Rojas MA, Dyer IA, Cassatt WA, 1965: Manganese deficiency in the bovine. J Anim Sci **24**, 664–667.

Santamaria AB, 2008: Manganese exposure, essentiality and toxicity. Indian J Med Res **128**, 484–500.

Sarsour EH, Kalen AL, Xiao Z, Veenstra TD, Chaudhuri L, Venkataraman S, Reigan P, Buettner GR, Goswami PC, 2012: Manganese superoxide dismutase regulates a metabolic switch during the Mammalian Cell Cycle. Cancer Res **72**, 3807–3816.

Schrantz N, Blanchard DA, Mitenne F, Auffredou MT, Vazquez A, Leca G, 1999: Manganese induces apoptosis of human B cells: caspase-dependent cell death blocked by Bcl-2. Cell Death Differ **6**, 445–453.

Singh NP, McCoy MT, Tice RR, Schneider EL, 1988: A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res **175**, 184–191.

Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N, Okano A, 1993: Effect of thiol compounds on *in vitro* development and intracellular glutathione content of bovine embryo. Biol Reprod **49**, 228–232.

Tervit HR, Whittingham DG, Rowson LEA, 1972: Successful culture *in vitro* of sheep and cattle ova. J Reprod Fertil **30**, 493–497.

Tice RR, Strauss GH, 1995: The single cell gel electrophoresis/comet assay: a potential tool for detecting radiation-induced DNA damage in humans. Stem Cells **1**, 207–214.

Underwood EJ, Suttle NF, 1999: The Mineral Nutrition of Livestock. CABI Publishing, London UK.

Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, Alderson NL, Baynes JW, Epstein CJ, Huang TT, Nelson J, Strong R, Richardson A, 2003: Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiol Genomics **16**, 29–37.

Weber S, Dorman DC, Lash LH, Erikson K, Vrana KE, Aschner M, 2002: Effects of manganese (Mn) on the developing rat brain: oxidative-stress related endpoints. Neurotoxicology **23**, 169–175.

Yin Z, Jiang H, Lee ES, Ni M, Erikson KM, Milatovic D, Bowman AB, Aschner M, 2010: Ferroportin is a manganese-responsive protein that decreases manganese cytotoxicity and accumulation. J Neurochem **112**, 1190–1198.

Zidenberg-Cherr S, Keen CL, Lönnerdal B, Hurley LS, 1983: Superoxide dismutase activity and lipid peroxidation in the rat: developmental correlations affected by manganese deficiency. J Nutr **113**, 2498–2504.

Submitted: 15 Feb 2013; Accepted: 23 Apr 2013

Author's address (for correspondence): CC Furnus, Instituto de Genética Veterinaria Prof. Fernando N. Dulout (IGEVET), Facultad de Ciencias Veterinarias, CONICET; Cátedra de Citología, Histología y Embriología "A", Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina. E-mail: cfurnus@fcv.unlp.edu.ar