Reproduction in Domestic Animals

Reprod Dom Anim **49**, 865–874 (2014); doi: 10.1111/rda.12385
ISSN 0936–6768

The Importance of Having Zinc During *In Vitro* Maturation of Cattle Cumulus–Oocyte Complex: Role of Cumulus Cells

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Contents
The aim of this study was to investigate the influence of zinc (Zn) on the health of cumulus–oocyte complex (COC) during *in vitro* maturation (IVM). Experiments were designed to evaluate the effect of Zn added to IVM medium on: DNA integrity, apoptosis, cumulus expansion and superoxide dismutase (SOD) activity of cumulus cells (CC). Also, role of CC on Zn transport during IVM was evaluated on oocyte developmental capacity. DNA damage and early apoptosis were higher in CC matured with 0 μg/ml Zn compared with 0.7, 1.1 and 1.5 μg/ml Zn ($p < 0.05$). Cumulus expansion did not show differences in COC matured with or without Zn supplementation ($p > 0.05$). Superoxide dismutase activity was higher in COC matured with 1.5 μg/ml Zn than with 0 μg/ml Zn ($p < 0.05$). Cleavage and blastocyst rates were recorded after IVM in three maturation systems: intact COCs, denuded oocytes with cumulus cells monolayer (DO + CC) and denuded oocytes (DO). Cleavage rates were similar when COC, DO + CC or DO were matured with 1.5 μg/ml Zn compared with control group ($p > 0.05$). Blastocyst rates were significantly higher in COC than in DO + CC and DO with the addition of 1.5 μg/ml Zn during IVM ($p < 0.01$). Blastocyst quality was enhanced in COC and DO + CC compared with DO when Zn was added to IVM medium ($p < 0.001$). The results of this study indicate that Zn supplementation to IVM medium (i) decreased DNA damage and apoptosis in CC; (ii) increased SOD activity in CC; (iii) did not modify cumulus expansion and cleavage rates after *in vitro* fertilization; (iv) improved subsequent embryo development up to blastocyst stage; and (v) enhanced blastocyst quality when CC were present either in intact COC or in coculture during IVM.

Introduction

Zinc (Zn) is a relevant trace element in the body (Mills 1989). The function of Zn involves a wide range of biological processes including cell proliferation, immune function, and defence against free radicals (Prasad 1998; Prasad 2003 Powell 2000). Its role in cell division and differentiation, gene transcription and many enzymatic activities has led to considerate Zn as a leading element that ensures correct functioning of body homoeostatic mechanisms (Prasad 2003; Mocchegiani et al. 2006).

During oocyte maturation, the mammalian oocytes are surrounded by cumulus cells that are coupled through gap junctions to the oocyte, and also, these gap junctions connect one cumulus cell with another one around the oocyte. These communications are evident in the transporting of low molecular weight compounds, ions and amino acids to the oocyte and implicate a metabolic support for the oocyte (Larsen 1998; Albertini et al. 2001; Sutton et al. 2003; Gilchrist et al. 2008). Moreover, Kim et al. (1996) demonstrated that although the presence of cumulus cells coupled to bovine oocytes was not necessary for nuclear maturation, they play an important role on subsequent oocyte capacity for developing to blastocyst stage (Lonergan and Fair 2008).

The cumulus–oocyte complex (COC) is the structural and functional unit present in mammalian antral follicles (Camaioni et al. 1993). The space between cumulus cells is enlarged by extracellular matrix production as a consequence of an increase of endogenous gonadotrophins during the pre-ovulatory phase. This process, called cumulus expansion, facilitates COC removal from the follicle wall, its extrusion during ovulation and its capture by the oviductal fimbriae. Furthermore, optimum expansion of cumulus mass is essential for subsequent fertilization of the oocyte (Camaioni et al. 1993; Borg and Holland 2008; Nagyova et al. 2012). It has been demonstrated that the degree of cumulus expansion is directly related to the amount of hyaluronic acid (HA) synthesized in the extracellular matrix (Chen et al. 1990) and that Zn participates as a cofactor for pSmad 2 and 3 transcription factors that are related to the synthesis and organization of HA within the extracellular matrix (Tian and Diaz 2012).

The enzyme superoxide dismutase (SOD) protects cell from damage caused by free radicals, and catalyse the dismutation of superoxide to hydrogen peroxide (Chihuailaf et al. 2002). In mammals, there are three SOD isoforms: two isoforms are bound copper and Zn (Cu/Zn-SOD), one is located in the cytosol (Cu/Zn-SOD 1) and the other one in the extracellular medium (Cu/Zn-SOD 3), and the third isoform is bound manganese (Mn-SOD) and is present in the mitochondrial matrix (Miao and St Clair 2009). The Cu/Zn-SOD1 is mainly located in the cytosol but also in the nucleus, lysosomes, peroxisomes and between the membranes of the mitochondria (Weisiger and Fridovich 1973; Chang et al. 1988; Field et al. 2003). Studies in knock-out mice for Cu/Zn-SOD indicate that deprivation of this enzyme is associated with liver cell damage (Polavarapu et al. 1998; Kessova et al. 2003), female infertility (Matzuk et al. 1998), axonal injury (Reaume et al. 1996) and shortening of life (Elchuri et al. 2005). The binding of SOD protein to the metal is essential for the suitable function of this enzyme. Zinc loss disrupts the tertiary structure of enzyme-active site which generates adverse oxidative reactions (Sahawneh et al. 2010).

There is a correlation between apoptosis and Zn deficiency in different cells types such as liver cells, vascular endothelial cells and rat testes tissues (Oteiza et al. 1995, 1996, 1999, 2001; Sharif et al. 2012). Zinc deficiency either *in vivo* or *in vitro* increases the percentages of apoptotic cells (Fraker et al. 1977; Fernandes et al. 1979; Martin et al. 1991; Anchordoquy et al. 2011). Moreover, *in vitro* studies with Zn supplementation reduces DNA fragmentation (Cohen and Duke 1984; Sellins and Cohen 1987; Migliorati et al. 1993; Anchordoquy et al. 2011). In response to low Zn concentration, rat glioma C6 cells decrease proliferation, increase oxidative stress and single-strand breaks (Provinciali et al. 1995).

The aim of this study was to investigate the influence of Zn on the health of COC during *in vitro* maturation (IVM). For this purpose, experiments were designed to evaluate the effect of different Zn concentrations added to the IVM medium on: apoptosis, DNA integrity and SOD activity of cumulus cells. Also, the role of cumulus cells in the transport of Zn during IVM was evaluated on oocyte developmental capacity.

Materials and Methods

Reagents and media

All reagents for media preparation and Comet Assay were purchased from Sigma Chemical Co. (St. Louis, MO, USA), whereas FSH was purchased from Bioniche (Belleville, Ontario, Canada). The maturation medium was bicarbonate-buffered TCM-199 supplemented with 5% (v/v) FCS, 0.2 mM sodium pyruvate, 1 mM glutamine, 10 mg/ml LH (NIHoLH-S1), 1 mg/ml FSH, 1 mg/ml 17β-estradiol and 50 mg/ml kanamycin (Furnus et al. 1998). Standard zinc sulphate water solution was purchased from Merk (Tokyo, Japan). The fertilization medium consisted of TALP supplemented with 6 mg/ml BSA-fatty acid free, 20 mM penicillamine, 10 mM hypotaurine and 10 mg/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 oviduct fluid (SOFm), 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).

Oocytes

Bovine ovaries were obtained from an abattoir and transported to the laboratory in sterile NaCl solution (9 g/l) with antibiotics (streptomycin and penicillin) at 37°C within 3 h after slaughter. Ovaries were pooled, regardless of stage of the oestrous cycle of the donor. Cumulus–oocyte complexes were aspirated from 3 to 8 mm follicles, using an 18-G needle connected to a sterile syringe. Only cumulus–intact complexes with evenly granulated cytoplasm were selected, using a low-power (20–30×) stereomicroscope, for IVM. Replicates (3–5) were performed on different days, with a separate batch of COC for each day.

In vitro maturation

Cumulus–oocyte complexes were washed twice in TCM-199 buffered with 15 mM HEPES and twice in IVM medium. Groups of 10 COC were transferred into 50 µl of IVM medium under mineral oil (Squibb, Princeton, NJ, USA) pre-equilibrated in a CO_2 incubator. The incubations were performed at 39°C in an atmosphere of 5% CO_2 in air with saturated humidity for 24 h. In a preliminary experiment, the presence of PB + MII plate was evaluated in sampled oocytes from treatments and IVM medium alone with Hoechst 33342 after 24 h of IVM.

Culture of cumulus cells

Cumulus cells monolayers (CC) were prepared by pipetting COC with a narrow-bore pipette. The oocytes were discarded, and cumulus mass were vigorously pipetted to allow separation. Cumulus cells were counted in a hemocytometer chamber, and aliquots of the cell suspension (0.5 ml, 1×10^6 cells/ml in IVM medium) were placed in four-well plate under mineral oil, at 39°C in an atmosphere of 5% CO_2 in air with saturated humidity. The medium was changed every 48 h until 70–80% confluence, which was normally attained within 4–5 days. The percentage of live cells, evaluated by vital stain with trypan blue, was over 80% at the beginning of the culture.

Comet assay

At the end of IVM, oocytes were stripped of surrounding cumulus cells by repeated pipetting with a narrow-bore glass pipette in TCM-199 buffered with HEPES. Then, cumulus cells were washed three times in calcium- and magnesium-free PBS containing 1 mg/ml polyvinylpyrrolidone (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 previously described (Singh et al., 1988) with modifications (Tice and Strauss 1995). Briefly, slides were covered with 180 µl of 0.5% normal agarose (Carlsbad, 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 lyses solution. 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 solution (Molecular Probes, Eugene, OR, USA) (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. Two hundred randomly selected nuclei from each treatment were

classified into two groups: with (comets with tail) or without DNA damage (comets without tail) and then analysed for Olive Tail Moment (OTM) and percentage of DNA in the head (%DNAH) (Olive et al. 1990; Bocker et al. 1997).

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 (Cat # 11-858-777-001; Roche Diagnostics GmbH, Roche Applied Science, Penzberg, Germany). The assay involves simultaneous staining with both Annexin-V-FLUOS (green) and the DNA stain propidium iodide (PI, red). Intact 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, at the end of IVM, oocytes were stripped of surrounding cumulus cells as described above, washed twice with PBS and centrifuged at 200 × ***g*** for 5 min. Then, the pellet was resuspended in 100 µl of Annexin-V-FLUOS labelling solution (Annexin V + fluorescein, HEPES buffer and PI) and incubated in the dark 10–15 min at 15–25°C. A total of 200 cells were analysed under a fluorescence microscope per treatment.

Cumulus expansion

After IVM, cumulus expansion was measured in each COC using a computerized image-digitizing system with Image ProPlus® 3.1 which allows measurement of irregular areas. The system units were transformed to μm^2 by calibration with a Maklert chamber. For comparison, each COC area was measured before IVM.

SOD activity

Cumulus–oocyte complexes ($n = 400$) were freezing and thawing twice in distilled water and then centrifuged at 10 000 × ***g*** for 20 min at 4°C. Supernatants were used to determine SOD activity with the RANSOD kit (RANDOX, Antrim, UK). Superoxide radical produced in the incubation medium from xanthine oxidase reacts with INT [2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride] producing red formazan. This coloured compound is measured by an spectrophotometer at 505 nm. One SOD unit causes 50% inhibition of INT reduction. The chromogen inhibition is proportional to SOD activity present in the sample.

In vitro fertilization

Oocytes were washed twice in HEPES-TALP supplemented with 3 mg/ml bovine serum albumin-fatty acid free (BSA-FAF) and placed into 50 µl drops of *in vitro* fertilization (IVF) medium under mineral oil. In all experiments, frozen semen from the same bull was used. Three straws, each containing 40×10^6 spermatozoa, were thawed in a 37°C water bath. Spermatozoa were washed in a discontinuous Percoll gradient prepared by depositing 2 ml of 90% Percoll under 2 ml of 45% Percoll in a 15-ml centrifuge tube. Semen samples were deposited on the top of the Percoll gradient and centrifuged for 20 min at 500 × ***g***. The pellet was removed and resuspended in 300 µl of HEPES-TALP solution and centrifuged at 300 × ***g*** for 10 min. After removal of the supernatant, spermatozoa were resuspended in IVF medium, counted in a hemocytometer chamber and further diluted. The final sperm concentration in IVF was 2×10^6 sperm/ml. Incubations were conducted at 39°C in 5% CO_2 in air with saturated humidity for 24 h.

In vitro culture (IVC)

After IVF, presumptive zygotes were washed twice in HEPES-SOF and then cultured in SOFm. 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 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. The medium was changed every 48 h, and 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).

Differential staining of blastocysts

Embryo quality (Fukui et al. 1991; O'Hara et al. 2014) was evaluated by Total, Inner Cells Mass (ICM) and Trophoectoderm (TE) cell numbers. For this purpose, total, ICM and TE cell numbers were determined in Day 8 blastocysts as described by Fields et al. (2011). Briefly, before beginning the fixation and staining steps, embryos were exposed to 1 µg/ml RNAse for 1 h at 38.5°C. After incubation, blastocysts were washed in 0.01 M PBS containing 1 mg/ml PVP, stained with 100 µg/ml PI in PBS-PVP with 0.2% (v:v) Triton for 30 s, repeatedly washed in PBS-PVP, and then fixed in PBS-PVP containing 4% (wt/vol) paraformaldehyde and 1 µg/ml Hoechst 33342 for 15 min. After repeated washing in PBS-PVP, blastocysts were placed on microscope slides with cover slips containing a small drop of glycerol. ICM and TE cell numbers were visualized by a Nikon Optiphot epifluorescent microscope with a 40× fluor objective (Nikon) equipped with a 330–490 nm excitation filter and a 420–520 nm emission filter to distinguish nuclei of TE cells (red) and ICM (blue).

Experimental Design

Zinc and DNA integrity of cumulus cells

Zinc concentrations (0.7; 1.1; and 1.5 µg/ml Zn) to be added to IVM medium were established in a previous

study (Picco et al. 2010). In *Experiment 1*, the effect of Zn (0; 0.7; 1.1; or 1.5 μg/ml Zn) during IVM on DNA damage of cumulus cells from COC matured *in vitro* was evaluated by Comet Assay. The COC were matured for 24 h (as described above), and thereafter, DNA damage was evaluated as described (section 'Comet assay'). For this purpose, 800 COC in four replicates from different days (200 COC per replicate, 50 COC per treatment) were matured *in vitro* with various Zn concentrations. Each batch of 50 COC was processed for preparing slides to analyse at least 250 single cells per treatment for the Comet Assay.

Zinc on apoptosis of cumulus cells

In *Experiment 2*, the addition of 0, 0.7, 1.1 or 1.5 μg/ml of Zn to IVM medium was evaluated on cumulus cells apoptosis (section 'Apoptosis by Annexin V staining assay') after 24 h of IVM as described above. For this purpose, 800 COC were matured in four replicates (separate batch of ovaries for each day), with 200 COC distributed in groups of 50 COC per treatment.

Zinc and cumulus expansion

In *Experiment 3*, the influence of 0, 0.7, 1.1 or 1.5 μg/ml Zn added to IVM medium was evaluated on cumulus expansion. The COC were matured individually for 24 h as described above, and cumulus expansion was measured either before or after IVM (described in section 'Cumulus expansion'). For this purpose, 300 COC were matured in five replicates (separate batch of ovaries for each day), with 60 COC distributed in groups of 15 COC per treatment.

Zinc and SOD activity

In *Experiment 4*, the addition of 0 or 1.5 μg/ml of Zn to IVM medium was evaluated on SOD activity (section 'SOD activity') after 24 h of IVM as described above. For this purpose, 400 COC were matured in four replicates (separate batch of ovaries for each day), with 100 COC distributed in groups of 50 COC per treatment.

Role of cumulus cells in the transport of zinc during IVM

In *Experiment 5*, oocytes were matured *in vitro* during 24 h with or without 1.5 μg/ml Zn in three maturation systems: (i) intact COC; (ii) denuded oocytes with cumulus cell monolayer (DO + CC); and (iii) denuded oocytes (DO). Denuded oocytes were obtained by pipetting COC with a narrow-bore pipette when was required for the experimental design. Cleavage rates were recorded 48 h after insemination. Data reported for development to the blastocyst stage included embryos that progressed to the expanded or hatched blastocyst stages (COC, $n = 400$; DO+CC, $n = 400$; and DO, $n = 400$ in four replicates on different days). Total, ICM and TE cell numbers of blastocysts were evaluated on 72 embryos, 12 embryos per treatment (three replicates) in *Experiment 6*.

Statistical analysis

A completely randomized block designs were used and statistical models included the random effects of block ($n = 3$–5 depending on experiment) and the fixed effect of treatment (0 vs 0.7 vs 1.1 vs 1.5 μg/ml Zn in Experiments 1–3; and 0 vs 1.5 μg/ml Zn in Experiments 4, 5 and 6). Continuous response variables such as area of cumulus, % DNAH, OTM and SOD activity were analysed with linear models using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA). Cumulus area before IVM (T0) was used as covariate in the analysis of cumulus expansion. Apoptosis (%) was analysed by logistic regression using GENMOD procedure (SAS Institute). Percentages of cleavage, embryo development and cell number per blastocyst (ICM, TE and Total cell number) were 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. Statistical significance was set at $p < 0.05$.

Results

Effect of zinc on DNA integrity of cumulus cells

In Experiment 1, OTM was significantly higher in CC from COC matured without Zn (0 μg/ml Zn, $p < 0.01$) respect to cells treated with Zn (OTM: 2.30 ± 0.36; 0.82 ± 0.36; 0.22 ± 0.36; 0.04 ± 0.31 for COC exposed to 0, 0.7, 1.1 and 1.5 μg/ml Zn, respectively). The percentages of DNA in the head (%DNAH) were 93.15 ± 0.7; 96.81 ± 0.7; 98.94 ± 0.7; and 99.82 ± 0.7 for 0, 0.7, 1.1 and 1.5 μg/ml Zn, respectively. The percentages of DNAH were statistically different in all treatments respect to 0 μg/ml Zn ($p < 0.001$) (Fig. 1).

Effect of zinc on apoptosis of cumulus cells

In Experiment 2, cumulus cells from COC matured *in vitro* with Zn were evaluated for early apoptosis by Annexin-V-FLUOS (Fig. 2). The percentage of apoptotic cells after IVM was higher in cumulus cells matured without Zn than in cumulus cells matured with different Zn concentrations (Early apoptosis: 18%; 8%; 9% and 6% for COC exposed to 0, 0.7, 1.1 and 1.5 μg/ml Zn, respectively). Statistical differences were found between 0 and 0.7 μg/ml Zn ($p < 0.05$), 0 and 1.1 μg/ml Zn ($p < 0.05$), and 0 and 1.5 μg/ml Zn ($p < 0.01$). No differences were found among Zn concentrations.

Effect of zinc on cumulus expansion

In Experiment 3, cumulus expansion did not show significant differences in COC treated with 0, 0.7, 1.1 or 1.5 μg/ml Zn concentrations during IVM (Table 1, $p \geq 0.05$). No differences were found in cumulus cells

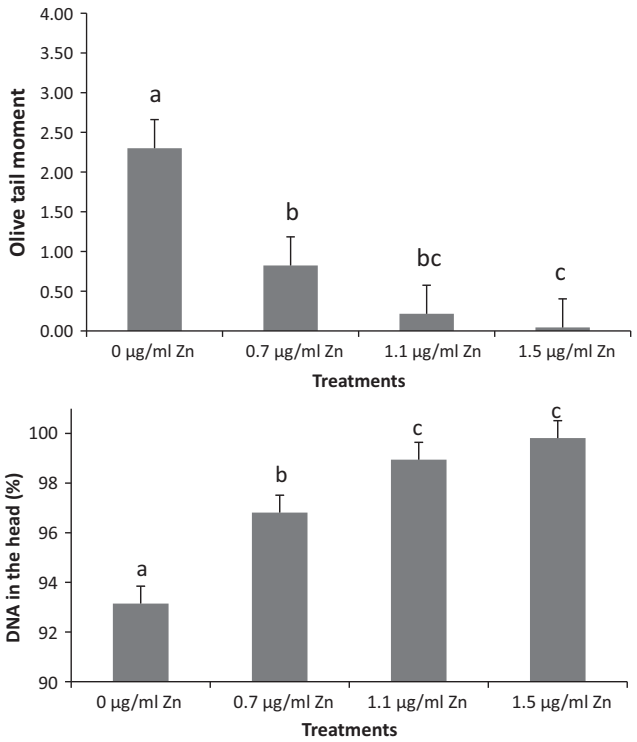

Fig. 1. DNA damage in cumulus cells from cumulus–oocyte complex (COC) matured with different Zn concentrations. Bars with different letters differ ($p < 0.05$). Bovine COCs were incubated in *in vitro* maturation medium alone (0 µg/ml Zn); 0.7 µg/ml Zn; 1.1 µg/ml Zn; and 1.5 µg/ml Zn

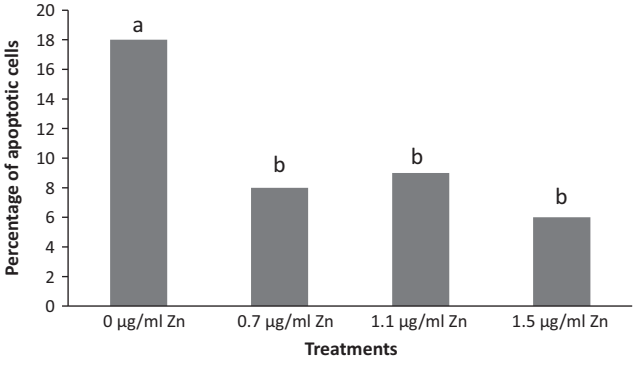

Fig. 2. Percentages of early apoptosis in cumulus cells from cumulus–oocyte complex (COC) matured with different Zn concentrations. Bars with different letters statistically differ ($p < 0.05$). Bovine COCs were incubated in *in vitro* maturation medium alone (0 µg/ml Zn) or supplemented with 0.7 µg/ml; 1.1 µg/ml and 1.5 µg/ml Zn

number per COC either before or after IVM at any Zn concentration (Before IVM: 14800 ± 1250; after IVM: 14950 ± 1190, 14870 ± 1205, 15130 ± 1280 and 15205 ± 1310 cumulus cells/COC for 0, 0.7, 1.1 and 1.5 µg/ml Zn, respectively). In all experiments performed, the cell number per COC did not vary significantly with any Zn concentration as well as the percentage of nuclear maturation (89–96%) evaluated by Hoechst 33342.

Table 1. Cumulus expansion with different zinc concentrations in *in vitro* maturation (IVM) medium

IVM	Treatments	Area of cumulus (µm²)
Before		116.529 ± 4.527[a]
After	0 µg/ml Zn	647.616 ± 35.700[b]
	0.7 µg/ml Zn	596.716 ± 35.879[b]
	1.1 µg/ml Zn	541.038 ± 37.060[b]
	1.5 µg/ml Zn	601.447 ± 36.869[b]

Data are expressed as LSM ± SEM.
Values with different superscripts within each column differ ($p < 0.05$).

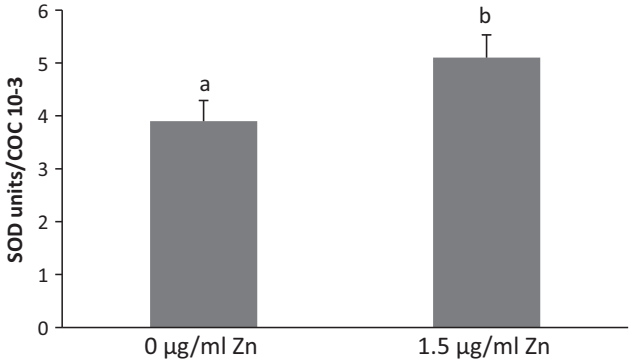

Fig 3. Superoxide dismutase (SOD) activity in cumulus-oocyte complexes matured with or without Zn supplementation. Bars with different letters statistically differ ($p < 0.05$). Superoxide dismutase activity (units/COC) are expressed as LSM ± SEM (400 COC in four replicates). Cumulus cells-oocytes complex were matured in *in vitro* maturation medium alone (0 µg/ml Zn) or with 1.5 µg/ml Zn. COC, cumulus–oocyte complex

Effect of zinc on SOD activity

In Experiment 4, SOD activity was significantly higher in COC matured with 1.5 µg/ml Zn (5.1 ± 0.43 × 10^{-3} units/COC) than with 0 µg/ml Zn (3.9 ± 0.39 × 10^{-3} units/COC) ($p < 0.05$; Fig. 3).

Role of cumulus cells in the transport of zinc during IVM

Oocytes were *in vitro* matured with or without Zn supplementation to IVM medium. Cleavage and blastocyst rates were recorded after IVM in three maturation systems: intact COCs, DO + CC and DO. Cleavage rates were significantly higher in COC matured with or without Zn (0 µg/ml Zn: 76.35 ± 1.1 and 1.5 µg/ml Zn: 80.4 ± 3.25) than in DO + CC (0 µg/ml Zn: 70.85 ± 1.3 and 1.5 µg/ml Zn: 72.65 ± 0.95; $p < 0.05$). The lowest cleavage rates were obtained in DO (0 µg/ml Zn: 59.3 ± 3.8 and DO 1.5 µg/ml Zn: 55.05 ± 1.8 ($p < 0.05$; Fig. 4A). In addition, blastocyst rates were significantly higher in COC (0 µg/ml Zn: 24.7 ± 1.1 and 1.5 µg/ml Zn: 40.46 ± 2.8) than in DO + CC (0 µg/ml Zn: 22.11 ± 0.9 and 1.5 µg/ml Zn: 26.85 ± 0.7; $p < 0.01$) and DO (0 µg/ml Zn: 8.35 ± 1.2 and 1.5 µg/ml Zn: 12.47 ± 1.5; $p < 0.01$) (Fig. 4B). Independently of the presence of cumulus cells (COC, DO + CC or DO), the blastocyst rates were higher when 1.5 µg/ml Zn was added to IVM medium compared with medium alone ($p < 0.01$) (Fig. 4B).

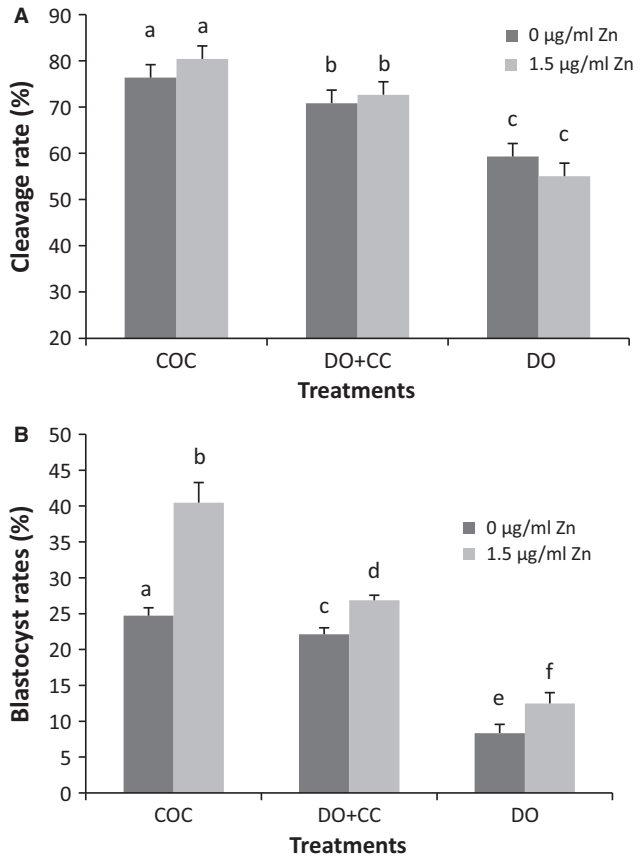

Fig. 4. Role of cumulus cells during *in vitro* maturation on the developmental capacity of oocytes matured with or without zinc. COC, cumulus-oocyte complex; DO+CC, denuded oocytes cultured with cumulus cell monolayer; DO, denuded oocytes. (A) (a–c) indicates significant differences ($p < 0.05$). (B) (a–f) indicates significant differences ($p < 0.01$). 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 (COC, $n = 400$; DO+CC, $n = 400$; and DO, $n = 400$ in four replicates on different days)

Total cells number per blastocyst (Experiment 6) was higher in COC and DO + CC compared with DO when Zn was added to IVM medium ($p < 0.01$) (Table 2). The number of cells in the ICM and TE was significantly higher when COC were matured with Zn addition to IVM medium ($p < 0.01$). However, the number of cells in ICM and TE diminished in blastocysts derived from DO either with or without Zn addition during IVM ($p < 0.01$). No differences were found in ICM:/TE cell ratio, but only in DO without Zn supplementation during IVM ($p < 0.01$, Table 2).

Discussion

The results of the present study indicate that Zn supplementation to IVM medium (i) decreased DNA damage and apoptosis in cumulus cells; (ii) increased SOD activity in cumulus cells; (iii) did not modify cumulus expansion and cleavage rates after IVF; (iv) improved subsequent embryo development up to blastocyst stage; and (v) enhanced blastocyst quality, evaluated by mean cell number per blastocyst, when cumulus cells were present in the intact COC or in coculture during IVM.

Zinc added during oocyte maturation decreased both apoptosis and DNA damage of cumulus cells. The role of Zn as antioxidant may be an important mechanism in maintaining DNA integrity in COC by preventing oxidative DNA damage. In this study, DNA damage of cumulus cells was reduced when 1.1 and 1.5 µg/ml Zn were added during IVM. It has been demonstrated that changes in intracellular Zn concentrations alter the antioxidant capacity of *in vitro* cultured cells (Ho and Ames 2002). Moreover, Ho (2004) exhibited that Zn added to culture medium *in vitro* prevents cellular death. *In vivo* studies showed an increase in protein oxidation and DNA damage in rat leucocytes, neumocytes and monkey hepatocytes with Zn deficiency (Taylor et al. 1988; Olin et al. 1993; Oteiza et al. 2000; Song et al. 2009). In addition, long time Zn supplementation in human and rat ensures protection from cellular dead and DNA damage (Emonet-Piccardi et al. 1998; Chimienti et al. 2001). The present study indicates that adequate Zn concentrations 'protect' cumulus cells from DNA damage during IVM. Zinc is an essential component of Cu/Zn-SOD that is one of the most important mechanisms for cellular defence from ROS (Ho 2004). On the other hand, Zn prevents the oxidation of sulfhydryl groups in proteins and exerts an antagonist role on transition metals such as iron and cooper to compete for membrane binding sites (Kelly et al. 1986;

Table 2. Effect of presence or absence of cumulus cells during *in vitro* maturation (IVM) with or without zinc added to IVM medium on cells number per blastocyst

Treatments	Blastocysts No	Total Cells No	ICM Cells No	TE Cells No	ICM/TE Cell Ratio
COC 0 µg/ml Zn	12	108.5 ± 2.0^{a}	27.1 ± 2.1^{a}	81.0 ± 6.7^{a}	0.33 ± 0.012^{a}
COC 1.5 µg/ml Zn	12	132.2 ± 3.1^{b}	34.3 ± 2.3^{b}	98.0 ± 6.3^{b}	0.36 ± 0.014^{a}
DO+CC 0 µg/ml Zn	12	106.0 ± 5.8^{a}	25.7 ± 2.3^{a}	79.8 ± 5.2^{a}	0.32 ± 0.015^{a}
DO+CC 1.5 µg/ml Zn	12	120.0 ± 4.0^{c}	30.7 ± 3.0^{ab}	89.7 ± 4.8^{ab}	0.34 ± 0.012^{a}
DO 0 µg/ml Zn	12	85.1 ± 6.6^{d}	18.9 ± 1.9^{c}	66.1 ± 5.0^{c}	0.28 ± 0.014^{b}
DO 1.5 µg/ml Zn	12	87.0 ± 4.5^{d}	22.1 ± 1.5^{c}	65.3 ± 6.3^{c}	0.34 ± 0.013^{a}

COC, cumulus–oocyte complex; DO + CC, denuded oocytes cultured with cumulus cell monolayer; DO, denuded oocytes; Total, Trophoectoderm (TE) and inner cell mass (ICM) cells of Day 8 blastocysts developed from oocytes matured with or without zinc.
Values with different letters within each column differ ($p < 0.01$).
Data are expressed as Mean ± SEM.

Conte et al. 1996; Powell 2000; Ho 2004). Also, Zn is involved in the metabolism of metallothionein. This low molecular weight protein acts as a potent hydroxyl radical scavenger. Zinc induces metallothionein expression raising its cytoplasmic concentrations through the activation of MTF-1 (metal transcription factor 1) (Ho 2004).

In the present study, SOD activity was significantly higher when COC were matured in IVM medium supplemented with Zn. Enzyme SOD scavenges the harmful superoxide radicals and protects cells from the related damaging effects. In mammals, two types of cellular SOD have been identified: cytoplasmic copper-zinc SOD (Cu/Zn-SOD) and mitochondrial manganese SOD (Mn-SOD) (Chihuailaf et al. 2002). Metal binding to SOD protein is essential for its biological function. Zinc deficiency disorganizes the tertiary structure of the active site of Cu/Zn-SOD, leaving the copper available to catalyse adverse oxidative reactions (Sahawneh et al. 2010). It has been described that Zn supplementation in neurons culture medium restores SOD activity (Estévez et al. 1999). The loss of Cu/Zn-SOD activity increases apoptosis and reduces cellular survival (Estévez et al. 1999; Roberts et al. 2007; Srivastava et al. 2007). Most likely, the highest SOD activity observed in Zn-supplemented COC is due to an increase in the availability of Zn.

The mechanism by which Zn acts as an antioxidant is partially unknown. It has been demonstrated that changes in Zn status *in vitro* alter the antioxidant capacity of cells (Ho and Ames 2002; Picco et al. 2010). Our study provides evidence that adequate Zn concentration 'protects' cumulus cells against DNA damage increasing SOD activity. High number of apoptotic cumulus cells is associated with a lessened ability of oocyte developmental capacity (Høst et al. 2002; Ikeda et al. 2003). Also, high rates of apoptotic cumulus cells have been traduced in lower fertilization (Høst et al. 2002) and blastocysts percentages (Corn et al. 2005). In addition, Seino et al. (2002) demonstrated that increasing DNA damage to human cumulus cells during IVM decreases fertilization and blastocysts rates. Indeed, when 1.5 μg/ml Zn was added to IVM medium, the competence of bovine oocytes was improved regardless of the presence of cumulus cells during IVM manifested by an increase in blastocyst rates.

In our study, the degree of cumulus expansion did not vary in presence of Zn. The cumulus cells are a specific subset of granulosa cells that surround the developing mammalian oocyte. These cells and their metabolically coupled communication pathway create a microenvironment conducive for oocyte growth and development that is necessary for ovulation and fertilization (Heller et al. 1981; Brower and Schultz 1982; Herlands and Schultz 1984). Extensive gap junction network links the cumulus cells to one another and to the developing oocyte (Anderson and Albertini 1976; Gilula et al. 1978; Larsen et al. 1986). These gap junctions may allow the oocyte to be exposed to cellular factors (i.e. cAMP, steroid hormones and prostaglandins) (Hess et al. 1999). After IVM, COC undergo dramatic changes. Cumulus cells dissociate from each other and then synthesize and secrete large amounts of a HA-enriched extracellular matrix (Chen et al. 1992). The matrix is then deposited into extracellular spaces leading to the process of expansion (Eppig 1979). The presence of an intact and successfully expanded cumulus mass aids in oocyte maturation, optimal ovulation and fertilization (Chen et al. 1993), oocyte transport to the oviduct (Mahi-Brown and Yanagimachi 1983), zona reaction (Fowler et al. 1986), sperm motility (Tesarik et al. 1990), sperm attraction (Hunter 1988), sperm selection (Bedford and Kim 1993) and acrosome reaction (Stock et al. 1989). Recently, Zn has been postulated as an essential cofactor for the transcription factors pSMAD2/3 that in the presence of epidermal growth factor (EGF) stimulates cumulus expansion in mouse COC (Tian and Diaz 2012). The degree of cumulus expansion did not vary in presence of Zn; however, this might be explained by the absence of EGF in the IVM medium.

Blastocyst rates were increased when oocytes was maturated in the presence of cumulus cells and was significantly higher in COC. Removal of cumulus cells before IVM is detrimental to oocyte maturation in various species (pigs, rats and cattle). Nevertheless, DO co-cultured with cumulus cells partially 'restored' their developmental capacity (Chian et al. 1994; Zhang et al. 1995; de Matos et al. 1997; Wongsrikeao et al. 2005). In our study, cleavage rates were higher in the presence of attached cumulus cells (COC) indicating that quality of cytoplasmic maturation was superior. The beneficial effects of cumulus cells on oocyte maturation and early development are well known in mouse (Cross and Brinster 1970); rat (Vanderhyden and Armstrong 1989); human (Kennedy and Donahue 1969); ovine (Staigmiller and Moor 1984); and bovine (Sirard et al. 1988; Fukui 1990). The cumulus cells are intimately connected with the oocyte through long microvilli that traverse through the zona to contact the oolemma to form gap junctions and desmosomes (Motta et al. 1994). The gap junctions help to mediate the transport of certain molecules that are necessary for oocyte metabolism (Brower and Schultz 1982; Haghinat and Van Winkle 1990). In addition to these effects, the granulosa cells also appear to exert some of their effects on the oocyte via paracrine signals which may explain the beneficial effect of co-culture of granulosa cells (Dandekar et al. 1991; Schramm and Bavister 1996). Diffusible factors secreted by cumulus cells play a key role in developmental competence acquisition in bovine and mouse DO (Luciano et al. 2005; Ge et al. 2008). Oocytes clearly depend on the presence of follicle cells to generate specific cellular signals that coordinate their growth and maturation (Assidi et al. 2008). Ménézo and colleagues (Ménézo et al. 2011) have been demonstrated that ZIP family transporters are expressed in human COC. Blastocyst quality was enhanced in COC and DO cocultured with cumulus cells when Zn was present during IVM. The number of cells in the ICM and TE was higher when COC were matured with Zn; however, ICM:/TE cell ratio was similar.

In conclusion, Zn increased the blastocyst rates regardless of the presence of cumulus cells during IVM, highlighting the importance of this mineral in oocyte cytoplasmic maturation. In addition, Zn improved embryo quality when oocytes were matured with cumulus cells (COC) or in the presence of cumulus cells (CC + DO). Our study suggest that gap junctions (intact COC) but also paracrine factors might be involved in Zn transport into the oocyte.

Acknowledgements

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) for make available bovine frozen semen; and the staff of Frigorífico Gorina S.A. for providing the bovine ovaries.

Conflict of interest

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

Author contributions

Juan Mateo Anchordoquy and Juan Patricio Anchordoquy Experimental Design and Laboratory work, Matías Sirini Laboratory work, Sebastián Picco DNA Damage, Pilar Peral Garcia Statistical Analysis, Cecilia Furnus coordination and manuscript writing, revision and editing.

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Submitted: 19 Mar 2014; Accepted: 22 Jun 2014

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