Analysis of apoptosis and DNA damage in bovine cumulus cells after exposure in vitro to different zinc concentrations

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Abstract

The purpose of this study was to investigate the effect of Zn (zinc) concentration on CCs (cumulus cells) during in vitro maturation. For this purpose, DNA integrity of CCs by addition of different Zn concentrations [0 (control); 0.7 µg/ml (Zn1); 1.1 µg/ml (Zn2); and 1.5 µg/ml (Zn3)] to the culture medium was evaluated by comet assay. In addition, early apoptosis was analysed by annexin staining assay. CCs treated with Zn showed a significant decrease in the DNA damage in a dose-dependent manner. Comet assay analysed for TM (tail moment) was significantly higher in cells cultured without Zn (control, \( P < 0.01 \)) with respect to cells treated with Zn (control: 5.24 ± 16.05; Zn1: 1.13 ± 5.31; Zn2: 0.10 ± 0.36; Zn3: 0.017 ± 0.06). All treatments were statistically different from the control \(( P = 0.014 \text{ for Zn1}; P < 0.01 \text{ for Zn2 and Zn3})\). The frequency of apoptotic cells was higher in the control group (control: 0.142 ± 0.07; Zn1: 0.109 ± 0.0328; Zn2: 0.102 ± 0.013; Zn3: 0.0577 ± 0.019). Statistical differences were found between control and Zn1 \(( P = 0.0308)\), control and Zn2 \(( P = 0.0077)\), control and Zn3 \(( P < 0.0001)\), Zn1 and Zn3 \(( P < 0.001)\) and Zn2 and Zn3 \(( P = 0.0004)\). No differences were found between Zn1 and Zn2. In conclusion, low Zn concentrations increase DNA damage and apoptosis in CCs cultured in vitro. However, adequate Zn concentrations ‘protect’ the integrity of DNA molecule and diminish the percentage of apoptotic CC.

Keywords: annexin; apoptosis; comet assay; damage; DNA cumulus cells; zinc

1. Introduction

Zn (Zinc) is an essential trace element involved in several cell functions including signal transduction, transcription and replication (Cousins et al., 2006). Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses and DNA repair in rats, and dietary Zn restriction and repletion affects DNA integrity in healthy men (Song et al., 2009a, 2009b).

Damage of DNA and apoptosis are important parameters that are emerging as useful indicators of cellular health. It has been demonstrated that high rates of apoptotic bovine follicular cells affect subsequent oocyte development (Zadák et al., 2009). Apoptosis is a genetically programmed and physiological mode of cell death, which plays an important role in normal tissue remodelling, and is one of the factors that keeps the balance between cell proliferation and cell loss (van Engeland et al., 1997; Zeuner et al., 2003).

The aim of this study was to investigate the effect of Zn concentration on CCs (cumulus cells) cultured in vitro. We tested the hypothesis that low Zn concentrations in culture medium for oocyte in vitro maturation induce damage on DNA integrity and apoptosis of CC.

2. Materials and methods

2.1. Reagents and media

All reagents for media preparation were purchased from Sigma Chemical Co. The culture medium used in all experiments for CC culture was bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS (fetal bovine serum), 0.2 mM sodium pyruvate, 1 mM glutamine, 10 mg/ml LH (lutening hormone) (NIH-oLH-S1), 1 mg/ml FSH and 50 mg/ml kanamycin (Furnus et al., 1998). Annexin-V-FLUOS was purchased from Roche, and FSH from Serono, Inc.

2.2. Cumulus cells

Cattle 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 of slaughter. The COCs (cumulus oocyte complexes) were aspirated from 2- 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 to ×30) stereomicroscope for culture in vitro. Replicates of experiments (four to six) were performed on different days with different batches of ovaries.

2.3. Cumulus cells culture during in vitro maturation

The COCs were washed twice in TCM-199 buffered with 15 mM Hepes containing 5% (v/v) FCS and twice in culture medium. Groups of 10 COCs were transferred into 50 μl of culture medium under mineral oil (Squibb) pre-equilibrated in a CO2 incubator. The COCs were cultured in culture medium at 39ºC in 5% CO2 in air with saturated humidity for 24 h.

2.4. Zinc determination in plasma, FF (follicular fluid) and culture medium

Samples of jugular blood (10 ml) were collected from 40 healthy heifers at the time of slaughter in a test tube containing EDTA. The samples were centrifuged at 3000 rev./min for 10 min, and the plasma was separated and stored at 4 ºC. Also, ovaries from the same heifers were collected and handled independently to give 40 values for Zn in each follicle class, wrapped with film, and taken to the laboratory in an icebox within 2 h of the slaughter. 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 culture medium supplemented with 10% FCS were also collected (n=6). Samples of FF and culture medium were centrifuged, and the supernatants were treated with 10% (w/v) trichloroacetic acid. Zinc concentration was measured by double beam flame atomic absorption spectrophotometer (GBC 902) through an internal quality control (Piper and Higgins, 1967) and classified according to Kincaid (1999).

2.5. Comet assay

At the end of the culture, oocytes were stripped of surrounding CCs by repeated pipetting with a narrow-bore glass pipette in TCM-199 buffered with Hepes and washed three times in calcium- and magnesium-free PBS containing 1 mg/ml PVP (polyvinylpyrrolidone). Complete cell disruption was achieved by repeated aspiration using a narrow-bore pipette. CC samples (1 × 106 cells) were processed for single-cell gel electrophoresis using the alkaline version described by Singh et al. (1988) with modifications (Tice and Strauss, 1995). Samples were stained with 1:1000 SYBR Green I (Molecular Probes) solution (Olive et al., 1999). Images were captured with a Sony CCD (charge-coupled-device) camera and saved by 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 TM (tail moment) and %DNAH (percentage of DNA in the head) (Olive et al., 1990; Bocker et al., 1997).

2.6. Apoptosis by annexin V staining assay

Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for PS (phosphatidylserine) (Glander and Schaller, 1999; Paasch et al., 2004). Early apoptosis was evaluated by membrane redistribution of PS with Annexin-V-Fluos Staining Kit (Roche, catalogue no. 11-58-777-001). The assay involves simultaneous staining with both Annexin-V-Fluos (green) and the DNA stain PI (propidium iodide, 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 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. 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 to 15 min at 15–25 ºC. A total of 200 cells were analysed under a fluorescence microscope per treatment.

2.7. Data analysis

DNA damage was analysed by TM, and differences between treatments were compared by the Student’s t test (SSPS® Version 11). The relationship between Zn and TM 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. Results are expressed as means ± S.D.

2.8. Experimental design

In Experiment 1a, Zn concentrations were determine in plasma, FF and culture medium as described in section 2.4. In Experiment 1b, the effect of Zn on DNA integrity of CC by addition of 0 μg/ml (control), 0.7 μg/ml Zn1, 1.1 μg/ml Zn2 and 1.5 μg/ml Zn3 to culture medium was evaluated by comet assay. The cells were cultured during 24 h as described above, and after this period, DNA damage was evaluated as described in section 2.5. In Experiment 2, the effect of the addition of 0 μg/ml (control), 0.7 μg/ml, 1.1 μg/ml and 1.5 μg/ml Zn to culture medium on early apoptosis by Annexin-V-Fluos staining assay in CCs was analysed. The cells were cultured during 24 h, and apoptosis was evaluated as described in section 2.6.

3. Results

Zinc concentration was 1.32 μg/ml ± 0.22 in plasma samples, 1.49 μg/ml ± 0.37 in FF from large follicles, 1.55 μg/ml ± 0.33 in FF from small follicles and 0.3 μg/ml ± 0.02 in culture medium supplemented with serum. No significant differences were found in Zn concentrations between plasma and FF (Experiment 1a).

CCs number per COC did not show significant differences either before (n=4) or after culture (n=4 per treatment) at any Zn concentration [before culture: 15100 ± 1100; after culture: 15133 ± 1180 (control), 15187 ± 1207 (Zn1), 15554 ± 1377 (Zn2) and 16032 ± 1308 (Zn3) CC/COC]. In all experiments performed, the cell number per COC did not vary significantly with any Zn concentration.
CCs treated with Zn during in vitro culture showed a significant decrease in the DNA damage parameters in a dose-dependent manner (Experiment 1b). TM was significantly higher in CCs cultured without Zn added to culture medium (control, $P<0.01$) with respect to cells treated with Zn (Table 1). The %DNAH values were statistically different in all treatments with respect to control ($P=0.014$ for Zn1; $P<0.01$ for Zn2 and Zn3).

In Experiment 2, CCs treated with Zn were evaluated for early apoptosis by Annexin V-Fluos (Figure 2). Statistical differences were found between control and Zn1 ($P=0.0308$), control and Zn2 ($P=0.0077$), control and Zn3 ($P<0.001$), Zn1 and Zn3 ($P<0.001$) and Zn2 and Zn3 ($P<0.0004$). No differences were found between Zn1 and Zn2.

**Figure 1** DNA damage in CC cultured with different Zn concentrations (0 $\mu$g/ml Zn: control; 0.7 $\mu$g/ml: Zn1; 1.1 $\mu$g/ml: Zn2 and 1.5 $\mu$g/ml: Zn3)

Tail moment was significantly higher in control ($P<0.01$) with respect to cells treated with Zn1, Zn2 and Zn3. The %DNAH values were statistically different in all treatments with respect to control ($P=0.014$ for Zn1; $P<0.01$ for Zn2 and Zn3).

**Figure 2** Frequencies of early apoptosis in CC cultured with different Zn concentrations

CC treated with Zn (0 $\mu$g/ml Zn: control; 0.7 $\mu$g/ml: Zn1; 1.1 $\mu$g/ml: Zn2 and 1.5 $\mu$g/ml: Zn3) were evaluated for early apoptosis by Annexin V-Fluos. Statistical differences were found between control and Zn1 ($P=0.0308$), control and Zn2 ($P=0.0077$), control and Zn3 ($P<0.001$), Zn1 and Zn3 ($P<0.001$) and Zn2 and Zn3 ($P<0.0004$). No differences were found between Zn1 and Zn2.
different Zn concentrations (control: 0.142 ± 0.07; Zn1: 0.109 ± 0.0328; Zn2:0.102 ± 0.013; Zn3: 0.0577 ± 0.019). Statistical differences were found between control and Zn1 (P=0.0308), control and Zn2 (P=0.0077), control and Zn3 (P<0.0001), Zn1 and Zn3 (P<0.001) and Zn2 and Zn3 (P=0.0004). No differences were found between Zn1 and Zn2.

4. Discussion

In the present study, adequate Zn concentration added to culture medium during oocyte maturation reduced the frequency of apoptotic cells and preserve DNA integrity in CCs. Moreover, low Zn concentration induced apoptosis and damage in the integrity of DNA.

An antioxidant role of Zn may be an important mechanism in maintaining DNA integrity in COC by preventing oxidative DNA damage. In our study, low Zn concentrations had a deleterious effect on DNA integrity. Indeed, DNA damage in CCs was low when Zn was added at high concentration. It has been reported that Zn added to culture in vitro can prevent cell death (Ho, 2004), and a long-term Zn addition protects cells against death and DNA damage (Koh et al., 1996; Emonet-Piccardi et al., 1998; Chimienti et al., 2001). The mechanism by which Zn acts as an antioxidant is unclear, but compromised Zn status in vitro alters the antioxidant capacity of cells (Ho, 2002; Picco et al., 2010). However our study provides evidence that adequate Zn concentration ‘protects’ CC against DNA damage.

Adequate Zn concentration improved competence of bovine oocytes manifested by increased blastocyst rates (Picco et al., 2010). We observed that apoptosis in CC was significantly low in cells cultured with Zn. The CC surround the oocyte during the maturation process within the follicle and protect the developing oocyte providing nutrients through gap junctions (Mori et al., 2000; Tattemoto et al., 2000; Fatehi et al., 2002). The degree of apoptosis has been correlated with the developmental competence of the enclosed oocytes in the bovine (Ikeda et al., 2003).

In conclusion, deficiency of Zn increases DNA damage and apoptosis of CC; however, adequate Zn concentration in culture medium ‘protect the’ integrity of DNA and diminish the percentage of apoptosis in CC.

Author contribution

Juan Anchoordoquy was responsible for the cumulus cells culture during in vitro maturation and data analysis. Sebastián Picco and Analía Seoane were responsible for the Comet assay. María Ponzinibbio was responsible for the detection of apoptosis by the annexin V staining assay. Guillermo Mattioli and Pilar Garcia were responsible for zinc determination in plasma, follicular fluid and culture medium. Cecilia Furnus was responsible for manuscript preparation.

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