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

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Short Title: Apoptosis in cumulus cells cultured with zinc

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Abstract

The purpose of this study was to investigate the effect of zinc (Zn) concentration on cumulus cells (CC) during in vitro maturation. For this purpose, DNA integrity of CC 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 culture medium was evaluated by comet assay. In addition, early apoptosis was analyzed by annexin staining assay. Cumulus cells treated with Zn showed a significant decrease in the DNA damage in a dose dependent manner. Comet assay analyzed for Tail Moment was significantly higher in cells cultured without Zn (Control, $p < 0.01$) 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 ($p=0.014$ for Zn1; $p< 0.01$ 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 CC cultured in vitro. However, adequate Zn concentrations "protect" the integrity of DNA molecule and diminish the percentage of apoptotic CC.

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1. Introduction

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., 2009 a,b).

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 (Zadak et al., 2009). Apoptosis is a genetically programmed and physiological mode of cell death, which plays an important role in normal tissue remodeling, 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 investigate the effect of Zn concentration on CC cultured in vitro. We tested the hypothesis that low Zn concentrations in culture medium for oocyte in vitro maturation, induces damage on DNA integrity and apoptosis of CC.

2. Material and Methods

2.1. Reagents and media

All reagents for media preparation were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The culture medium used in all experiments for CC culture was bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FCS, 0.2 mM sodium pyruvate, 1 mM glutamine, 10 mg/mL LH (NIH-oLH-S1), 1 mg/mL FSH and 50 mg/mL kanamycin (Furnus et al, 1998) Annexin-V-FLUOS was purchased from Roche (Penzberg, Germany) and FSH from Serono, Inc, Rockland, MA.

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 37ºC 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 (X20 to X30) stereomicroscope for culture in vitro. Replicates of experiments (4–6) 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, Princeto, NJ, USA) pre-equilibrated in a CO_2 incubator. The COCs were cultured in culture medium at 39 ºC in 5 % CO_2 in air with saturated humidity for 24 h.

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2.4. Zinc determination in plasma, 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 (ethylene diamine tetra acetic acid). The samples were centrifuged at 3000 rpm for 10 min and the plasma was separated and stored at 4°C. 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 (< 10mm) and large follicles (> 10 mm). The follicular fluid (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 CC by repeated pipetting with a narrow-bore glass pipette in TCM 199 buffered with HEPES and washed 3 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. Cumulus cells samples (1 x 10^6 cells) were processed for single cell gel electrophoresis using the alkaline version described by Singh and colleagues (1988) with modifications (Tice and Strauss, 1995). Samples were stained with 1/1000 SYBR Green I (Molecular Probes, Eugene, Oregon, USA) solution (Olive et al., 1999). Images were captured with a Sony CCD 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 analyzed for Tail Moment (TM) and percentage of DNA in the head (%DNAH) (Olive et al. 1990; Bocker et al. 1997).

2.6. Apoptosis by Annexin V staining assay

Anexin 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 x 10^6) are washed twice with PBS and centrifuge at 200 x g for 5 min. Then the pellet was resuspended in 100 µL of Annexin-V-Fluos labeling solution (Annexin V + fluorescein, HEPES buffer and propidium iodide), and incubated in the dark 10 to 15 min at 15 -25 ºC. A total of 200 cells were analyzed under a fluorescence microscope per treatment.

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2.7. Data Analysis

DNA damage was analyzed by Tail Moment (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 analyzed using Chi-square test with the Yates correction. Results are expressed as mean ± SD.

2.8 Experimental Design

In **Experiment 1a** Zn concentrations were determinate 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 0µg/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 hr as described above and after this period DNA damage was evaluated as described in 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 CC was analysed. The cells were cultured during 24 hr and apoptosis was evaluated as described in the section 2.6.

4. 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**).

Cumulus cells 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.

Cumulus cells treated with Zn during in vitro culture showed a significant decrease in the DNA damage parameters in a dose dependent manner (**Experiment 1b**). Tail moment (TM) was significantly higher in CC cultured without Zn added to culture medium (Control, $p < 0.01$) 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). The percentage of DNA in the head (%DNAH) was 93.46 ±11.82; 96.65 ±7.44; 99.04 ±1.71 and 99.80 ± 0.50 for Control group, Zn1, Zn2 and Zn3 respectively. All treatments were statistically different from the Control group ($p=0.014$ for Zn1; $p< 0.01$ for Zn2 and Zn3) (**Figure 1**). The relationship between comet parameters and Zn concentrations were: CC= 0.874; r2=0.647; p=0.02 for TM and CC= 0.968; r2= 0.907; p=0.034 for %DNAH.

In **Experiment 2** CC treated with Zn were evaluated for early apoptosis by Annexin V-Fluos (**Figure 2**). The frequency of apoptotic cells was higher in the Control group than in cells cultured with 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=

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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.

5. 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 CC. Moreover, low Zn concentration induced apoptosis and damage in the integrity of DNA.

Antioxidant role of Zn may be an important mechanism in maintaining DNA integrity in cumulus oocyte-complex by preventing oxidative DNA damage. In our study, low Zn concentrations had a deleterious effect on DNA integrity. Indeed, DNA damage in CC 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 alter the antioxidant capacity of cells (Ho E, 2002, Picco et al., in press). 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., in press). 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, Tatemoto 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 CC.

6. Acknowledgments

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7. References

Bocker W, Bauch T, Muller WU, Streffer C. Technical Report: Image analysis of comet assay measurements. Int J Radiat Biol 1997;72:449-60.

Chimienti F, Jourdan E, Favier A, Seve M. Zinc resistance impairs sensitivity to oxidative stress in Hela cells: Protection through metallothioneins expression. Free Radic Biol Med 2001;31:1179-90.

Cousins RJ, Liuzzi JP, Lichten LA. Mammalian zinc transport, trafficking, and signals. J Biol Chem 2006;281:24085-89.

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Emonet-Piccardi N, Richard MJ, Ravanat JL, Signorini N, Cadet J, Beani JC. Protective effects of antioxidants against UVA-induced DNA damage in human skin fibroblasts in culture. Free Radic Res 1998;29:307-13.

Fatehi AN, Zeinstra EC, Kooij RV, Colenbrander B, Bevers MM. Effect of cumulus cell removal of in vitro matured bovine oocytes prior to in vitro fertilization on subsequent cleavage rate. Theriogenology 2002; 57:1347-55.

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

Ho E, Ames BN. Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappaB and AP1 binding and affects DNA repair in a rat glioma cell line. Proc Natl Acad Sci USA 2002;99:16770-5.

Ho E. Zinc deficiency, DNA damage and cancer risk. J Nutr Bioch 2004;15:572-8.

Ikeda S, Imai H, Yamada M. Apoptosis in cumulus cells during in vitro maturation of bovine cumulus-enclosed oocytes. Reproduction 2003;125:369-76.

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

Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW. The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 1996;272:1013-6.

Mori T, Amano T, Shimizu H. Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. Biol Reprod 2000; 62:913-9.

Olive PL, Banáth JP, Durand RE. Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells. J Natl Cancer Inst 1990; 2:779-83.

Olive PL, Durand RE, Jackson SM, Le Riche JC, Luo C, Ma R, McLaren DB, Aquino-Parsons C, Thomson TA, Trotter T. The comet assay in clinical practice. Acta Oncol 1999;8:839-44.

Picco JS, Anchordoquy JM, de Matos DG, Anchordoquy JP, Seoane A, Mattioli GA, Errecalde AL, Furnus CC. Effect of increasing zinc sulphate concentration during in vitro maturation of bovine oocytes. Theriogenology (In Press).

Piper HG, Higgins G. Estimation of trace metals in biological material by atomic absorption spectrophotometry. Proc Assoc Clin Biochem 1967;7:190-5.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184-91.

Song Y, Chung C, Bruno R, Traber M, Brown K, King J, Ho E. Dietary zinc restriction and repletion affects DNA integrity in healthy men. Am J Clin Nutr 2009a;90 321-328.

Song Y, Leonard SW, Bruno RS, Traber MG, Ho E. Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses, and DNA repair in rats. J Nutr 2009b;139:1626-31.

Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: role of cumulus cells. Biol Reprod 2000; 63:805-10.

Tice RR, Strauss GH. The single cell gel electrophoresis/comet assay: a potential tool for 469 detecting radiation-induced DNA damage in humans. Stem Cells 1995;1:207-14.

Underwood E, Suttle N. The mineral nutrition of livestock. 3rd edition. CABI Publishing. 1999; pp214-235.

van Engeland M, Kuijpers H, Ramaekers F, Reutelingsperger C, Schutte B. Plasma membrane alterations and cytoskeletal changes in apoptosis. Exp Cell Res 1997;235:421-430.

Zadák Z, Hyspler R, Tichá A, Hronek M, Fikrová P, Rathouská J, Hrnciariková D, Stetina R. Antioxidants and vitamins in clinical conditions. Physiol Res 2009;58:S13-7.

Zeuner A, Müller K, Reguszynski K, Jewgenow K. Apoptosis within bovine follicular cells and its effect on oocyte development during in vitro maturation. Theriogenology 2003; 59:1421-33.

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Figure 1. DNA damage in CC cultured with different Zn concentrations (0µg/mL Zn: Control; 0.7 µg/mL: Zn1; 1.1 µg/mL: Zn2 and 1.5 µg/mL: Zn3). Tail moment was significantly higher in Control ($p < 0.01$) respect to cells treated with Zn1, Zn2 and Zn3. The percentages of DNAH were statistically different in all treatments 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 (0µg/mL Zn: Control; 0.7 µg/mL: Zn1; 1.1 µg/mL: Zn2 and 1.5 µg/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 ($p< 0.0001$), Zn1 and Zn3 ($p< 0.001$) and Zn2 and Zn3 ($p= 0.0004$). No differences were found between Zn1 and Zn2.

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Figure 1

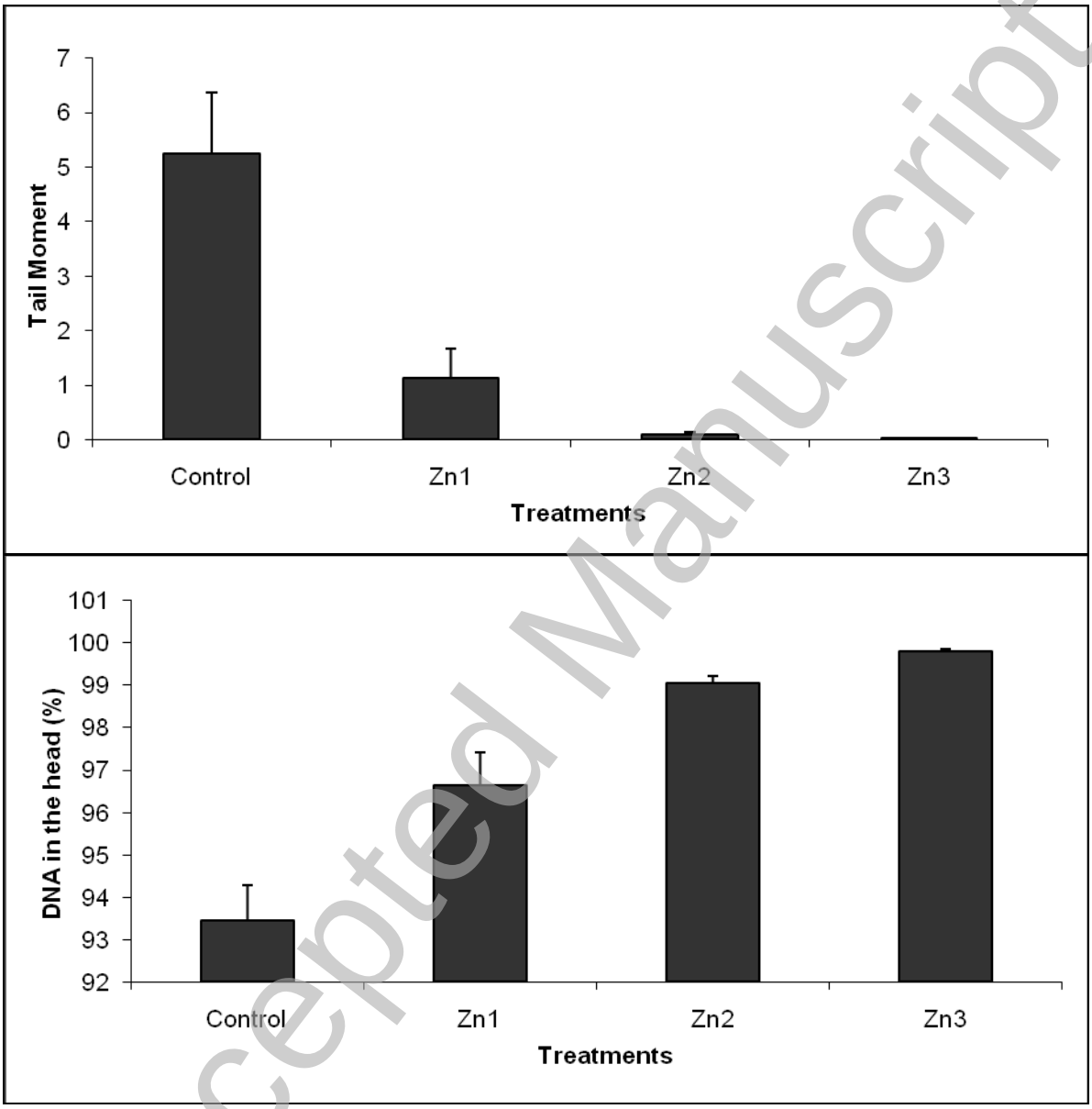

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Figure 2