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Consequences of copper and zinc co-supplementation on DNA integrity and apoptosis of bovine cumulus cells during oocyte *in vitro* maturation*

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Minerals such as copper (Cu) and zinc (Zn) are essential trace elements with specific roles in the antioxidant defense system. They are also vital for the health and growth of dairy cattle. We hypothesised that simultaneously increased Cu and Zn concentrations would reduce DNA damage and apoptosis in cumulus cells (CC) during oocyte *in vitro* maturation (IVM). Cumulus oocyte complexes (COC) were aspirated from follicles and only intact ones were selected for IVM. Groups of 10 COC were transferred into 50 µL of IVM medium and cultured in 5% CO_2 at 39°C for 24 h. The final concentrations of Cu and Zn were 0.2 and 0.7 µg/mL, 0.5 and 1.0 µg/mL, and 0.8 and 1.5 µg/mL, respectively. DNA integrity was analysed with the alkaline comet assay. Early apoptosis induction was studied by annexin-V binding. The results obtained showed that whereas joint Cu/Zn supplementation at a ratio not greater than 0.3 could be beneficial for DNA integrity and prevent the onset of apoptosis in CC during IVM, a Cu/Zn ratio greater than 0.5 induced DNA damage and cell death.

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Minerals such as copper (Cu) and zinc (Zn) are essential trace elements with specific roles in the antioxidant defense system. Several central enzymes, e.g. cytochrome C oxidase, tyrosinase, p-hydroxyphenyl pyruvate hydrolase, dopamine beta hydroxylase, lysyl oxidase and Cu-Zn superoxidase dismutase (CuZn-SOD), include Cu and/or Zn as cofactors, which are necessary for the catalytic and structural requirements of those enzymes [Gaetke and Chow 2003, Skalny 2011].

Both Cu and Zn are also vital for the health and growth of dairy cattle. Some authors [Spears and Weiss 2008, Suttle 2010] reported that these minerals are involved in some aspects of oxidative metabolism and immune function and could therefore modulate health and reproduction in livestock Copper deficiency generates a array of symptoms in grazing cattle. It is the second most important mineral deficiency in this species [Fry *et al*. 2014, Overton and Yasui 2014], which may generate subfertility by negatively impacting follicular growth and development. It may also affect normal embryonic progress due to the increased Cu demand by the bovine fetus [Kendall *et al*. 2003, Fry *et al*. 2014]. Zinc deficiency may induce DNA damage, immune deregulation and chronic inflammation, among other symptoms, while it may also be related to reproductive disorders [Suttle 2010, Ebisch *et al*. 2007, Song *et al.* 2009ab]. It has been observed that adequate Zn supplementation improves oocyte maturation, cumulus expansion and embryo development in both cattle and pigs [Tian and Diaz 2012, Lisle *et al*. 2013, Jeon *et al.* 2014]. Cumulus cells (CC) play a preponderant role in the oocyte ability to mature and eventually be fertilised and develop into an embryo [Lonergan and Fair 2008].

Despite the importance of each of these micronutrients individually, there is a fundamental issue related to Cu/Zn balance. In fact, plasma Cu/Zn concentration is one of the parameters associated with the reduction in both maintenance and recovery of homeostasis after a destabilising event [Osredkar and Sustar 2011, Malavolta *et al*. 2015]. For this reason, the objective of this study was to explore the concomitant genotoxic effect of Cu and Zn co-supplementation on bovine CC cultured *in vitro.* We tested the hypothesis that simultaneously increased Cu and Zn concentrations would reduce DNA damage and apoptosis in CC during bovine oocyte *in vitro* maturation (IVM).

Material and methods

Reagents and cells

Ovaries were obtained from a slaughterhouse. They were collected from the viscera tray under aseptic conditions and transported to the laboratory in a sterile NaCl solution (9 g/L) (Sigma Aldrich, Cat # S9888) plus penicillin and streptomycin (Sigma Aldrich, Cat # P4333) at 39°C within 3 h after slaughter.

The IVM medium was prepared according to Furnus and co-workers [1998]. Ovary treatment to obtain intact cumulus oocyte complexes (COC) and IVM was developed according to the methodology described by Picco and co-workers [2010]. Briefly, COC were aspirated from 2 to 8 mm follicles and only intact COC with an evenly granulated cytoplasm were selected for IVM. Groups of 10 COC were transferred into 50 µL of IVM medium and cultured for 24 h at 39°C in 5% CO_2 in air with saturated humidity.

Although the protocol does not include laboratory animals, it was ethically reviewed and approved by the IGEVET (UNLP-CONICET) ethics committee.

Experimental Design

The IVM culture medium was supplemented with Cu sulphate (Sigma Aldrich, Cat # 451657) and Zn sulphate (Sigma Aldrich, Cat # Z0251) in order to obtain final concentrations of 0.2 µg/mL Cu and 0.7 µg/mL Zn (CuZn 1), 0.5 µg/mL Cu and 1.0 µg/mL Zn (CuZn 2), and 0.8 µg/mL Cu and 1.5 µg/mL Zn (CuZn 3). The control culture medium was not supplemented with minerals (0.05 µg/mL Cu and 0.3 µg/mL Zn basal concentrations). Four replicates of the experiments were carried out on different days with a separate COC batch for each day; each experiment was performed for 24 h. The Cu and Zn concentrations in the IVM medium were measured by double beam flame atomic absorption spectrometry (GBC 902) through an internal quality control [Piper and Higgins 1967]. The comet and the Annexin-V-FLUOS staining assays were performed to evaluate DNA damage and the apoptotic effect on CC after the addition of Cu and Zn sulphate to the IVM medium.

Alkaline Comet Assay

Cells were obtained as reported by Picco and co-workers [2010]. The alkaline version of single cell gel electrophoresis described by Singh *et al.* [1988]] was performed with minor modifications [Tice and Strauss 1995. Slides were stained with a 1/1000 SYBR Green I (Molecular Probes, Cat # S7585) solution [Olive *et al.* 1999]. Scoring was made at the 400 x magnification using a fluorescent microscope (Olympus BX40 equipped with a 515–560 nm excitation filter) connected to a Sony 3 CCD-IRIS Color Video Camera, and saved using the Image Pro Plus software. Two hundred nuclei from each treatment were selected at random and classified as exhibiting (comets with tails) or being free from DNA damage (comets without tails), and further assessed for Olive tail moment (OTM) in arbitrary units and DNA percentage in the tail (TDNA %) [Olive 1999].

Apoptosis Detection by Annexin-V Staining

Early apoptosis (EA) frequency was evaluated with the Annexin-V-FLUOS Staining Kit (Roche, Cat # 11-858-777-001) for phosphatidylserine membrane redistribution. The assay involves simultaneous staining with both Annexin-V-FLUOS (green) and the DNA stain propidium iodide (PI, red). Briefly, cells (1 x

10^6) 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 labeling solution (Annexin V + fluorescein, HEPES buffer and PI) and incubated in the dark for 10-15 min at 15-25 ºC. A total of 500 cells were analysed per treatment under a fluorescence microscope and the results are expressed as percentages. Normal cells excluded PI and Annexin-V-FLUOS.

Statistical analysis

Results of OTM and TDNA were analysed applying Student´s *t*-test. Parametric Pearson correlation and linear regression analysis were used to determine the relationship of Cu and Zn concentrations with OTM and TDNA. The chi-square test with the Yates correction was applied to analyse the frequency of cells without DNA damage and EA. The results are expressed as mean ± SD. These computations were performed using the SSPS® Version 11 (IBM Corp. 2011).

Results and discussion

DNA damage parameters of CC treated with Cu and Zn during IVM are presented in Figures 1-3. They decreased significantly only when exposed to the lowest Cu and Zn combination (CuZn1) (p<0.01). Conversely, values of such parameters increased significantly with the other doses tested. While OTM was significantly lower in CuZn1 than in the negative control (p<0.01), it was significantly higher in CuZn2 and CuZn3 as compared with the controls (p<0.001) (Fig. 1). TDNA exhibited a similar behavior to OTM, with a significant TDNA decrease in CuZn1 (p<0.001) and a significant increase in CuZn2 and CuZn3 (p < 0.001). The percentage of DNA-damaged cells

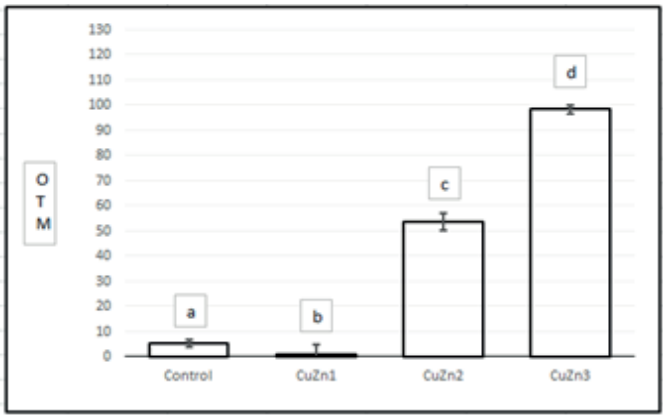

Fig. 1. Olive tail moment (OTM) in bovine cumulus cells cultured in vitro with different Cu and Zn sulphate concentrations. Values are expressed as means ± SD. Letters indicate homogeneous groups. OTM was significantly lower in CuZn1 and significantly higher in CuZn2 and CuZn3 as compared with the control ($p < 0.01$ and $p < 0.001$, respectively).

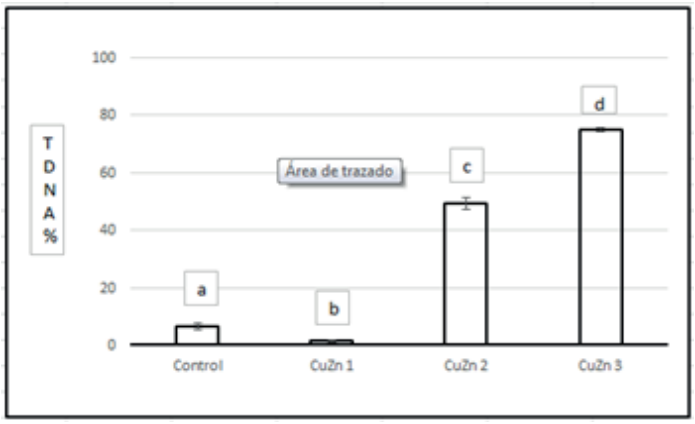

Fig. 2. DNA percentage in the tail (TDNA) in bovine cumulus cells cultured in vitro with different Cu and Zn sulphate concentrations. Values are expressed as means ± SD. Letters indicate homogeneous groups. TDNA was significantly decreased in CuZn1 and significantly increased in CuZn2 and CuZn3 as compared with the control ($p<0.001$).

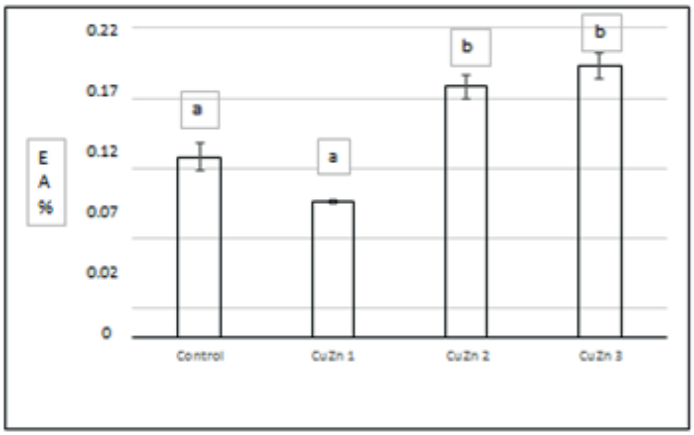

Fig. 3. Early apoptosis (EA) in bovine cumulus cells cultured in vitro with different Cu and Zn sulphate concentrations. Values are expressed as means ± SD. Letters indicate homogeneous groups. EA was lower in CuZn1 and significantly higher in CuZn2 and CuZn3 as compared with controls ($p<0.05$).

in the controls and CuZn1 was similar; however, all cells presented DNA damage in CuZn2 and CuZn3 ($p<0.01$) (Fig. 2).

Assessment of EA resulted in a higher, although non-significant, frequency of apoptotic cells in the control group as compared with cells cultured with the lowest Cu and Zn doses ($p=0.249$). In CuZn2 and CuZn3, such a frequency was significantly higher. Also, significant differences were found when comparing CuZn1 with CuZn2 and CuZn3 ($p<0.05$) (Fig. 3).

Images of the comet and EA assays are provided in Figures 4-6.

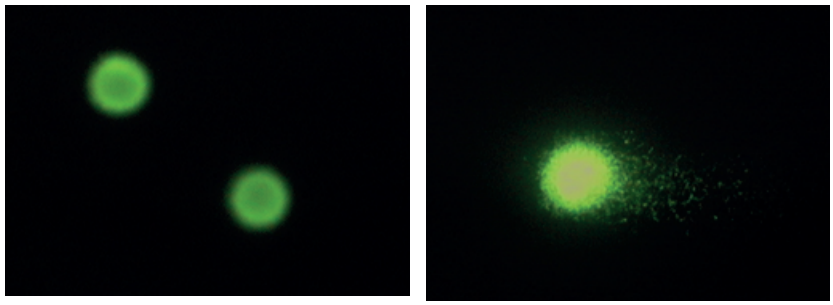

Fig. 4. Comet assay. Left: Undamaged cells. Right: Damaged cell.

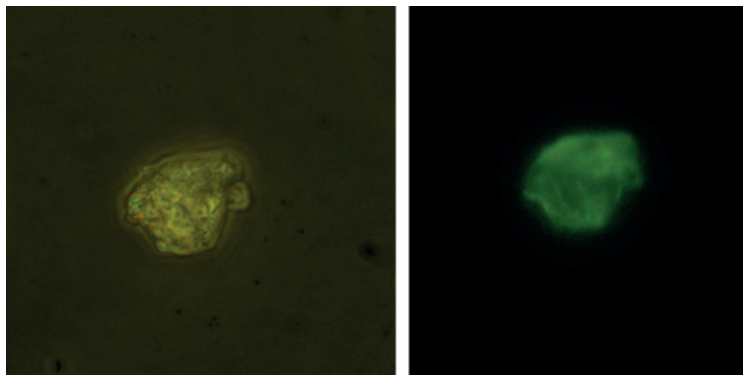

Fig. 5. Early apoptosis. Left: EA cell (phase image). Right: EA cell (fluorescence image).

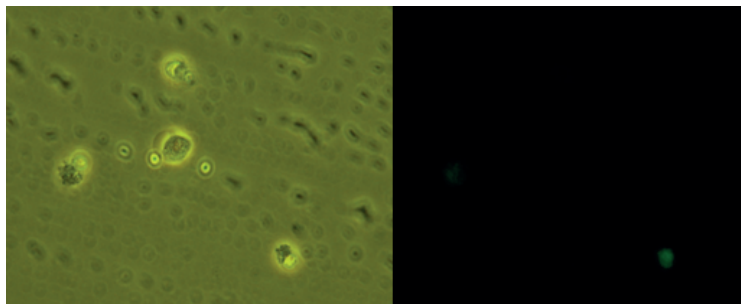

Fig. 6. Early apoptosis. Left: EA and normal cells (phase image). Right: EA cells (fluorescence image).

The independent supplementation of IVM media with Cu and Zn has previously been reported by our group [Anchordoquy *et al*. 2011, Anchordoquy *et al*. 2014, Picco *et al.* 2010, Picco *et al*. 2012]. In this study we tested the joint action of Cu and Zn at the same applied doses. The present results showed that the combined lowest Cu and Zn dose (CuZn1) induced similar effects to those exerted by the individual doses, whereas the combined higher doses (CuZn2 and CuZn3) elicited greater genotoxic

effects and EA frequency. These results were clearly different from those obtained with the individual treatments.

One explanation for our results could be provided by the antagonism between Cu and Zn. This phenomenon has been previously associated with excess or deficit of these minerals [Walsh *et al*. 1994]. Vidal and Hidalgo [1993] reported a strong embryotoxicity on preimplantation mouse embryo development *in vitro* when both metals were present, whereas Obiakor and co-workers [2010] observed increased frequencies of micronuclei in *Synodontis Clarias* and *Tilapias* under the same conditions. Moreover, the simultaneous presence of Cu and Zn may generate a disturbance in the intracellular ability to bind metals. Although evidence for the genotoxic effects of Zn excess in normal cells is contradictory [Walsh *et al*. 1994, Sliwinski *et al*. 2009, Padula *et al*. 2017], it is known that excess or free Cu is genotoxic.

According to Malavolta and co-workers [Malavolta *et al.* 2015], plasma Cu/Zn concentration ratios are associated with several age-related chronic diseases. Although both are strictly controlled under normal physiological conditions, Zn concentration has a tendency to decrease as Cu increases in the presence of inflammatory conditions [Malavolta *et al*. 2010, Guo *et al.* 2011]. Despite the two metals being important in metabolism, their relationship rather than their independent concentrations would be a better indicator. Therefore, the increased Cu/Zn ratio may be associated with a reduction in the capacity to maintain homeostasis. In this sense, our results showed that joint Cu and Zn supplementation at a ratio not greater than 0.3 could be beneficial for DNA integrity and prevent the onset of apoptosis in CC during IVM. On the other hand, a Cu/Zn ratio greater than 0.5 caused DNA damage and cell death in this cell system. These observations let us hypothesise that the higher relative amount of Cu could be responsible for the observed damage.

Efforts have been made in recent years to determine the effects of trace elements on mammalian reproduction, but only a few trials have been carried out in cattle. It is known that Cu and Zn independently improve oocyte maturation and fertilisation, but the combined effect of both micronutrients has not yet been adequately studied. Despite its limitations, our study is one of the few reporting the combined effect of these minerals.

The present study showed that the effects elicited by the combined lowest Cu and Zn dose (CuZn1) were similar to those exerted by the individual doses. On the other hand, the combined higher doses (CuZn2 and CuZn3) elicited greater genotoxic effects and EA frequency. In addition to the importance of each of these micronutrients individually, Cu/Zn balance is a fundamental issue. In fact, plasma Cu/Zn concentration is one of the parameters associated with the reduction in both maintenance and recovery of homeostasis after a destabilising event.

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