

High copper concentrations produce genotoxicity and cytotoxicity in bovine cumulus cells

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Abstract The aim of this study was to investigate the cytotoxic and genotoxic effects of high copper (Cu) concentrations on bovine cumulus cells (CCs) cultured in vitro. We evaluated the effect of 0, 120, 240, and 360 µg/dL Cu added to in vitro maturation (IVM) medium on CC viability assessed by the trypan blue (TB)–fluorescein diacetate (FDA) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays, apoptosis, and DNA damage. Differences in cell viability assessed by TB–FDA were not significant among CC treated with 0, 120, 240, and 360 µg/dL Cu. However, mitochondrial activity assessed by MTT was lower in CC cultured with 120, 240, and 360 µg/dL Cu as compared with the control ($p < 0.01$). Percentages of apoptotic cells were higher when CCs were treated with 120, 240, and 360 µg/dL Cu ($p < 0.05$) due to higher frequencies of late apoptotic cells ($p < 0.05$). The frequency of live cells diminished in a dose-dependent manner when Cu was added to the culture medium. Whereas genetic damage index (GDI) increased significantly in CC cultured in the presence of 240 and 360 µg/dL Cu ($p < 0.05$), DNA damage increased at all Cu

concentrations tested ($p < 0.05$). These results indicate that Cu induces cytotoxic and genotoxic effects in bovine CC.

Keywords Cumulus cells · MTT · Apoptosis · Comet assay · Copper

Introduction

Environmental quality plays an important role in human health. One quarter of global diseases are associated with environmental factors such as the presence of heavy metals (Rzymiski et al. 2015). Healthy environments can decrease morbidity rate in humans (WHO 2006). Although several metals, such as chromium, manganese, zinc, and copper (Cu), are essential for living in small quantities, they are toxic at high concentrations. The sources of global contamination by heavy metals include industry, transport, waste management, and soil fertilizers (Fenga 2016). In the environment, emission of heavy metals can pollute the atmosphere by combustion; oceans, rivers and soil by direct deposition; and thereby crops and other organisms through the food chain (Fenga 2016). Heavy metals mainly bioconcentrate in tissues and body fluids (Taupeau et al. 2001; Choi et al. 2007). They have been detected in follicular fluid (FF) of women (Younglai et al. 2002; Tolunay et al. 2016), sheep (Bires et al. 1995), and bovine (Picco et al. 2012). In mammals, certain pollutants contribute to the underlying causes of fertility problems (Kamarianos et al. 2003; Campagna et al. 2009). Exposure to lead and cadmium produce follicular atresia and early luteinization in cattle, and the presence of these metals in cervical mucus is toxic to spermatozoa (Rob and Dolezalova 1986). Previous studies have demonstrated that heavy metal exposure during in vitro maturation (IVM) affects oocyte maturation and causes chromosomal aberrations in bovine (Rodriguez-Tellez et al. 2005). Moreover, Leoni et al. (2002) found a negative effect of

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heavy metals on oocyte maturation and subsequent fertilization competence in ovine.

Cu plays an important role in mammalian cells as a cofactor of metabolic enzymes involved in radical detoxification, iron metabolism, and other physiological processes (Petris et al. 2000; Steveson et al. 2003; Lutsenko et al. 2007). However, the same redox properties that make Cu an essential micronutrient also contribute to its toxicity. Since Cu is a transition metal, it can enhance the production of reactive oxygen species (ROS) through the Fenton reaction (Lovejoy and Guillemain 2014). Cu has the capacity to produce oxidative damage in cells, interfering with essential cellular processes such as protein synthesis, membrane permeability, DNA structure, enzyme activities, essential ion functions, and respiration (Yruela 2005; Grãto et al. 2005).

Numerous layers of cumulus cells (CCs) surround the mammalian oocytes (Eppig 1991; Gilchrist et al. 2008). Their role in oocyte maturation, ovulation, and fertilization has been widely studied (Heller et al. 1981; Herlands and Schultz 1984; Camaioni et al. 1993; Sutton et al. 2003; Gilchrist et al. 2008; Lonergan and Fair 2008; Nagyova et al. 2012). CCs are closely related to the oocyte by gap junctions, providing nutrients and regulatory molecules. In addition, CCs play a key role in oocyte competence acquisition for subsequent development (Luciano et al. 2005; Ge et al. 2008). CC damage produces low fertilization and blastocyst rates, even in humans (Høst et al. 2002; Seino et al. 2002; Corn et al. 2005).

Fertility problems are an important human health issue (Evers 2002; Snijder et al. 2012). Bovine is a widely held experimental model for studying mechanisms and properties related to ovarian function in humans owing to physiological and structural similarities between both species biological systems (Babaei et al. 2012; Santos et al. 2014; Ceko et al. 2015). The aim of this study was to investigate the effect of high Cu concentrations on bovine CC cultured *in vitro*. For this purpose, experiments were designed to evaluate the effect of 0, 120, 240, and 360 $\mu\text{g/dL}$ Cu added to IVM medium on CC viability, apoptosis, and DNA damage.

Materials and methods

All reagents for media preparation were purchased from Sigma Chemical Co. (Sigma-Aldrich, St. Louis, MO, USA). The maturation medium was bicarbonate-buffered TCM-199 supplemented with 10% (*v/v*) FCS, 0.2 mM sodium pyruvate, 1 mM glutamine, 1 mg/mL FSH, 1 mg/mL 17β -estradiol, and 50 mg/mL kanamycin. Standard aqueous copper sulfate solution was purchased from Merck (Tokyo, Japan). The Cu concentrations used (120, 240, and 360 $\mu\text{g/dL}$ Cu) were higher than normal plasma Cu concentrations in bovine. Cu status in cattle is defined as deficient, marginal, and adequate at plasma

Cu concentrations of <30, 31–60, and >60 $\mu\text{g/dL}$, respectively (Underwood and Suttle 1999).

COCs

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 the estrous cycle stage of the donor. Cumulus cell-oocyte complexes (COCs) were aspirated from 3 to 8 mm follicles, using an 18-G needle connected to a sterile syringe. Only intact COC with evenly granulated cytoplasm were selected for IVM, using a low power ($\times 20$ – $\times 30$) stereomicroscope (Nikon, Tokyo, Japan).

IVM and CC samples

COCs were washed twice in TCM-199 buffered with 15 mM HEPES and 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. Incubations were performed at 39 °C in an atmosphere of 5% CO_2 in air with saturated humidity for 24 h. At the end of IVM, CCs from each treatment were obtained from COCs 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.

CC viability by the TB–FDA technique

After IVM of COCs, CC viability was evaluated. For this purpose, CCs were incubated for 10 min at 37 °C in PBS medium with 2.5 $\mu\text{g/L}$ fluorescein diacetate fluorochrome and 2.5 g/L trypan blue. Then, CCs were washed in PBS and observed in a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a 330–490 nm excitation filter and 420–520 nm emission filter at $\times 100$ magnification. Live CCs were visible in green fluorescence, whereas dead ones showed a characteristic blue staining under white light (Hoppe and Bavister 1984).

MTT assay

The MTT assay evaluates the respiratory activity of the mitochondrial succinate–tetrazolium reductase system, which converts the yellow tetrazolium salt into a blue formazan dye (Robb et al. 1990). Since the conversion takes place in living cells, the amount of formazan produced is directly correlated with the number of viable cells. The MTT assay was performed following the protocol of Wu et al. (2013). Briefly, 1×10^4 CCs/well was cultured in TCM-199 on 96-well microplates for 4 days until the cells were nearly confluent. Afterwards, the culture medium was removed, and CCs were

treated with 0, 120, 240 and 360 $\mu\text{g}/\text{dL}$ Cu dissolved in 100 μL TCM-199 for further 24 h. Ethanol was used as positive control. MTT was added to each well at a final concentration of 0.25 mg/mL, and incubated at 37 °C for 3 h. Afterwards, 100 μL dimethyl sulfoxide (DMSO) was added to dissolve formazan blue crystals. Absorbance at 490 nm was measured by a microplate spectrophotometer (Biotek Instruments Inc., Bedfordshire, UK). Data were normalized to measurements from control cultures which were considered 100% cell survival.

Apoptosis detection by annexin V-affinity assay

Apoptosis was evaluated by membrane redistribution of phosphatidylserine with the Annexin V-FLUOS Staining Kit (cat no. 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. CCs were classified following the criteria reported by Pläsier et al. (1999) as live (annexin V negative/PI negative), early-apoptotic (annexin V positive/PI negative), late-apoptotic (annexin V positive/PI positive), and necrotic (annexin V negative/PI positive) cells. Briefly, at the end of IVM, CCs were washed twice with PBS and centrifuged at $200\times g$ for 5 min. Then, the pellet was resuspended in 100 μL of annexin V-FLUOS labeling solution and incubated in the dark for 10–15 min at 15–25 °C. Cells were analyzed under a fluorescence microscope (Nikon, Tokyo, Japan).

Comet assay

CC samples were processed by single cell gel electrophoresis (SCGE) 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 μL of 0.5% normal agarose (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 48 °C for 10 min, cover slips were removed and slides were immersed overnight at 48 °C in fresh lysis solution. The slides were equilibrated in alkaline solution for 20 min. Electrophoresis was done 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 $\times 400$ magnification using a fluorescence microscope (Olympus BX40) equipped with a 515- to 560-nm excitation filter. Based on the extent of strand breakage, cells were classified according to their tail length into five categories, 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), to grade 4 (comets with a clear decrease in the diameter of the head and a clear tail). DNA damage expressed as arbitrary units was established according to Collins (2004). Results are expressed as the mean number of damaged nucleoids (sum of grades II, III, and IV) and the mean comet score for each treatment. The genetic damage index (GDI) of each treatment was obtained using the formula $\text{GDI} = [(I) + 2(II) + 3(III) + 4(IV)]/N(0-IV)$, where 0–IV represent the nucleoid type, and N0–NIV represent the total number of nucleoids scored (Pitarque et al. 1999).

Statistical analyses

The SCGE data were compared by applying one-way ANOVA using Statgraphics 5.1 Plus software. Variables were tested for normality with the Kolmogorov–Smirnov test, and homogeneity of variances between groups was verified by the Levene's test. Pairwise comparisons between the different groups were made using the post hoc least significant difference test (LSD). Differences in GDI, viability, and apoptosis in treated and control cells were evaluated by χ^2 test. The two-tailed Student's *t* test was used to compare MTT data between treated and control groups. The chosen level of significance was $p < 0.05$ unless indicated otherwise.

Experimental design

Assessment of CC viability with different Cu concentrations by the TB–FDA technique

In Experiment 1, CC viability after 24 h IVM with 0, 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu was evaluated as described above (“[CC viability by the TB–FDA technique](#)” section). For this purpose, 243 COCs were matured in three replicates (a separate batch of ovaries for each day). Each batch of COCs was processed for preparing slides to analyze at least 400 single CCs per treatment.

Assessment of CC mitochondrial activity with different Cu concentrations by the MTT assay

In Experiment 2, CC mitochondrial activity after 24 h of incubation with 0, 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu was evaluated (“[MTT assay](#)” section). For this purpose, CCs were cultured in three independent replicates (a separate batch of ovaries for each day).

Assessment of CC apoptosis with different Cu concentrations

In Experiment 3, CC apoptosis after 24 h IVM with 0, 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu was determined as described above

(“Apoptosis detection by annexin V-affinity assay” section). For this purpose, 246 COCs were matured in three replicates (a separate batch of ovaries for each day). At least 300 CCs were analyzed per treatment in each replicate.

Assessment of CC DNA damage with different Cu concentrations

In Experiment 4, DNA damage of CC following the addition of 0, 120, 240, or 360 $\mu\text{g}/\text{dL}$ Cu to the IVM medium was measured by the comet assay. CCs were matured for 24 h (as described above) and, thereafter, DNA damage was measured. For this purpose, a total of 240 COCs in three replicates obtained on different days were matured in vitro. Each batch of COCs was processed for preparing slides to analyze at least 200 single CCs per treatment in each replicate.

Results

Effect of Cu concentrations on CC viability by TB–FDA

In Experiment 1, CC viability did not show significant differences among CCs treated with 0, 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu concentrations during IVM (89.8 ± 0.31 , 87.1 ± 0.34 , 88.0 ± 0.33 , and $89.2 \pm 0.31\%$, respectively; $p > 0.05$).

Effect of Cu concentrations on CC mitochondrial activity

In Experiment 2, the results demonstrated a significant depression in mitochondrial activity in ethanol-treated cultures (positive control, $52.91 \pm 6.68\%$) as compared with the control ($p < 0.001$; Fig. 1). Mitochondrial activity was significantly lower in CC cultured with Cu as compared with the control (100 ± 0 , 92.83 ± 1.09 , 91.75 ± 0.90 , $89.74 \pm 1.05\%$ for CC exposed to 0, 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu, respectively; $p < 0.01$). No differences were found in CC mitochondrial activity when comparing Cu concentrations (Fig. 1).

Effect of Cu concentrations on CC apoptosis

Data for apoptosis in CC cultured for 24 h showed significant differences when comparing the percentages of apoptotic cells of control vs. positive control ($p < 0.001$; Table 1). The frequency of live cells diminished in a dose-dependent manner when Cu was added to culture medium (Table 1). Percentages of apoptotic cells were higher when CCs were treated with 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu ($p < 0.05$) due to higher frequencies of late apoptotic cells ($p < 0.05$). Moreover, the addition of 240 and 360 $\mu\text{g}/\text{dL}$ Cu increased the frequency of necrotic cells ($p < 0.05$).

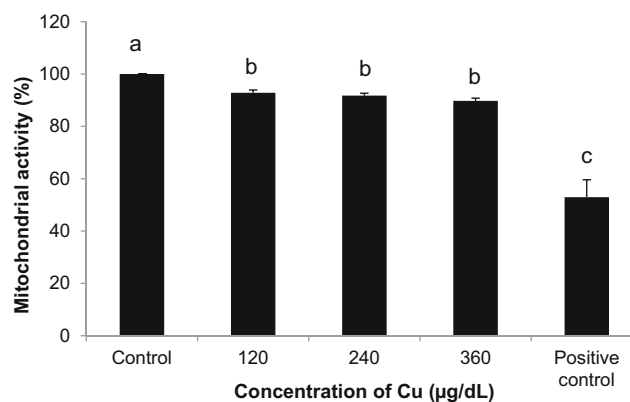


Fig. 1 Mitochondrial activity evaluated by MTT in bovine cumulus cells exposed to high copper concentrations for 24 h. Bars with different letters statistically differ ($p < 0.01$). Cultures were incubated for 3 h with MTT after 24 h of Cu treatments. Results are expressed as the mean percentage \pm SEM of cell growth inhibition from three independent experiments. Five percent ethanol-treated cells were used as positive controls

Effect of Cu concentrations on CC DNA damage

In Experiment 4, 1 $\mu\text{g}/\text{mL}$ bleomycin (positive control) induced an increase in damaged cell frequency and GDI as compared with the control ($p < 0.01$). GDI increased significantly in CC cultured in the presence of 240 and 360 $\mu\text{g}/\text{dL}$ Cu during IVM ($p < 0.05$; Table 2). DNA damage (grades II, III, and IV) increased with all Cu concentrations ($p < 0.05$; Table 2). We observed that such increase was due to the existence of higher type III and IV cells ($p < 0.05$).

Discussion

In the present study, we evaluated the effect of high Cu concentrations during bovine COC maturation. CC viability was assessed by the TB–FDA and MTT assays; apoptosis and comet assay results were used to determine whether treatment with high Cu concentrations could induce cytotoxicity and genotoxicity. Our study demonstrated that Cu supplementation to IVM medium (I) did not modify cell viability by the TB–FDA technique, (II) decreased mitochondrial activity, (III) increased apoptosis, and (IV) increased DNA damage in bovine CC. These results indicate that exposure of bovine CC to high Cu concentrations during COC IVM generates genotoxic and cytotoxic effects.

Cu is an essential trace element and an integral part of many intracellular and extracellular Cu-dependent enzymes and structural proteins (Jazvinščak Jembrek et al. 2014; Roychoudhury et al. 2014). Cu is involved in a number of biochemical and physiological functions (Jazvinščak Jembrek et al. 2014; Roychoudhury et al. 2014) such as cellular respiration, iron oxidation, pigment production, neurotransmitter biosynthesis, antioxidant defense, and collagen synthesis (Nevitt et al. 2012;

Table 1 Analysis of apoptosis in bovine cumulus cells exposed to high copper concentrations for 24 h

Copper concentration	No.	Percentage of cells (%)			
		Alive	Early apoptotic	Late apoptotic	Necrotic
Control	1022	60.86 ± 0.49a	1.56 ± 0.12a	5.38 ± 0.22a	12.62 ± 0.32a
120 µg/dL	1001	55.24 ± 0.50b	2.59 ± 0.16a	9.69 ± 0.29b	12.48 ± 0.32a
240 µg/dL	1041	46.01 ± 0.50c	2.20 ± 0.15a	10.08 ± 0.30b	22.47 ± 0.41b
360 µg/dL	1035	41.64 ± 0.50d	2.12 ± 0.14a	8.79 ± 0.27b	28.11 ± 0.45c
Positive control	1003	17.13 ± 0.40e	9.6 ± 0.18b	55.7 ± 0.61c	18.30 ± 0.47b

Values with different lowercase letters within each column differ ($p < 0.05$). Bovine COCs were incubated in IVM medium alone (control); IVM + 120, 240, and 360 µg/dL Cu; and IVM + 5% ETOH (positive control) during 24 h. COCs were denuded and CC suspensions were exposed to annexin V-FITC-propidium iodide (PI). CCs were classified as live (annexin V negative/PI negative), early-apoptotic (annexin V positive/PI negative), late-apoptotic (annexin V positive/PI positive), and necrotic (annexin V negative/PI positive). Each experiment was repeated three times and samples were performed in duplicate for each experimental point. Results are presented as mean ± SEM

Tchounwou et al. 2012; Jazvinščak Jembrek et al. 2014). Cu is necessary for living organisms in small quantities, but toxic at higher concentrations (Nevitt et al. 2012). In physiological conditions, there are mechanisms that ensure adequate supplies of Cu ions preventing toxic effects (Rae et al. 1999; Kaplan and Lutsenko 2009). However, acute and chronic Cu excess can provoke cellular damage (Jazvinščak Jembrek et al. 2014). Cu is present in natural ecosystems (Spatari et al. 2002). In addition, Cu-containing substances are extensively used in industry, agriculture, medical, and home products, representing a growing biological hazard (Wang et al. 2013). The main artificial sources of Cu are non-ferrous metal mining, metal ore smelting, galvanization, oil and gasoline combustion, waste incineration, and excessive use of certain plant protectants (Georgopoulos et al. 2001; Miska-Schramm et al. 2014; Zhang et al. 2015).

Even though the negative effects of overexposure to Cu on different organs such as the lung, spleen, liver, kidney, and intestine are well known, there exists scarce information about the effects of Cu toxicities on female genital organs and their cells

(Minervino et al. 2009; Babaei et al. 2012; Tolunay et al. 2016). Our current observations reveal that Cu was able to induce an increase in the frequency of apoptotic bovine CC after 24 h of treatment by increasing late apoptotic cell percentages. Moreover, 240 and 360 µg/dL Cu induced a strong increase in necrotic cell rates. Previous studies reported that Cu was one of the most potent metals in apoptosis cell induction (Formigari et al. 2013). It has been demonstrated that apoptosis rates are correlated with Cu excess in different cell types such as liver cells (Oe et al. 2016), mouse fibroblasts (Cao et al. 2012), osteoblasts (Cortizo et al. 2004), PC-3 human prostate cancer cells (Wang et al. 2014), and P19 neurons (Jazvinščak Jembrek et al. 2014). This effect was observed in both in vivo and in vitro systems (Handy 2003; Lu et al. 2006; Yu et al. 2008; Wang et al. 2014; Zhong et al. 2015; Pramanik et al. 2016). However, molecular and cellular mechanisms that link Cu to programmed cell death remain largely unknown (Jazvinščak Jembrek et al. 2014). Cu apoptotic potential is associated with its capacity to generate ROS through participation in the Haber–Weiss redox cycling

Table 2 Analysis of DNA damage by the comet assay in bovine cumulus cells after in vitro culture with different copper concentrations

Treatment	Proportion of damaged nucleoids (%)					DNA damage (%) (II + III + IV)	GDI
	0	I	II	III	IV		
Control	82.52 ± 0.37a	7.36 ± 0.26a	6.86 ± 0.24a	2.37 ± 0.14a	0.87 ± 0.09a	10.11 ± 0.3a	0.32 ± 0.08a
120 µg/dL Cu	78.02 ± 0.41b	5.55 ± 0.22a	8.02 ± 0.27a	4.44 ± 0.20b	3.95 ± 0.20b	16.62 ± 0.3b	0.51 ± 0.07a
240 µg/dL Cu	74.18 ± 0.44bc	5.98 ± 0.24a	9.1 ± 0.29a	6.98 ± 0.26c	3.74 ± 0.19b	19.82 ± 0.4b	0.60 ± 0.09b
360 µg/dL Cu	73.25 ± 0.44c	7.87 ± 0.27a	7.75 ± 0.27a	7.12 ± 0.26c	4.00 ± 0.20b	18.85 ± 0.3b	0.61 ± 0.08b
Positive control	48.54 ± 0.42d	23.74 ± 0.21b	18.14 ± 0.29b	5.64 ± 0.19b	3.78 ± 0.19b	27.71 ± 0.2c	0.92 ± 0.02c

Values with different lowercase letters within each column differ ($p < 0.05$). Bovine COCs were incubated in IVM medium alone (control); IVM + 120, 240 and 360 µg/dL Cu, and IVM + 1 µg/mL bleomycin (positive control) during 24 h. DNA damage in CC was evaluated by SCGE in four replicates. The extent of DNA damage was quantified by the length of DNA migration (comet), which was visually determined in 200 randomly selected and non-overlapping cells per replicate. DNA damage was classified into four classes: 0-I (undamaged), II (minimum damage), III (medium damage), and V (maximum damage). Results are expressed as mean comet score for each treatment group and mean damaged nucleoids (sum of classes II, III, and IV). Genetic damage index (GDI) of each treatment was determined with the formula $GDI = [(I) + 2(II) + 3(III) + 4(IV)]/N(0-IV)$, where 0–IV represent the nucleoid type, and N0–NIV the total number of nucleoids scored. Results are presented as mean ± SEM

as well as the inhibition of complex I of the electron transport chain, the main source of intracellular ROS (Pourahmad and O'Brien 2000; Formigari et al. 2013). Previous studies using peripheral blood lymphocytes provide evidence that Cu produces apoptosis by H_2O_2 and hydroxyl radical generation, resulting in mitochondrial depolarization, caspase-3 activation, and nuclear fragmentation (Jiménez Del Río and Vélez-Pardo 2004; Formigari et al. 2013). Intracellular ROS production activates and modulates apoptosis by regulating p53 activity in certain cell types (Narayanan et al. 2001; VanLandingham et al. 2002), but not in others (Jiménez Del Río and Vélez-Pardo 2004). Although Cu cell overexposure induces ROS formation, ROS do not appear to cause all Cu-mediated DNA damage because Cu is mutagenic (Formigari et al. 2013).

Even though, we previously demonstrated that “adequate” (60 $\mu\text{g}/\text{dL}$) Cu concentration added to IVM medium decreased DNA damage and apoptosis in bovine cumulus cells (Rosa et al. 2016), we observed an increase of necrotic cell percentages when Cu was added at high concentrations to the culture medium in concordance with other authors (Aston et al. 2000; Krumschnabel et al. 2005). Cu excess might interact in a non-specific manner with several macromolecules either by modifying their conformation or by causing site-specific damage that result in a disruption of vital cellular processes which further lead to apoptotic and necrotic cell death (Didenko et al. 2003; Krumschnabel et al. 2005; Rana 2008; Cao et al. 2012; Kuku et al. 2016; Philipp et al. 2016).

DNA damage can be an end-product of the apoptosis phenomenon or one of the factors for its progress (Wang 2001; Rana 2008). Recently, it has been reported that Cu accumulation causes apoptosis mediated by DNA damage (Roychoudhury et al. 2014). In the present study, the comet assay was used to analyze DNA damage in CCs incubated with Cu. We demonstrated that Cu had a detrimental effect on DNA integrity of CC after 24 h of treatment with 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu. These findings are in agreement with the genotoxic profile shown in other cellular systems. Addition of Cu to culture media produced DNA damage in C6 cells (Jazvinščak Jembrek et al. 2014), neurons (Lévay et al. 1997; Nzengue et al. 2012), human peripheral blood mononuclear cells (Singh et al. 2006), HepG-2 cells (Liu et al. 2016), HeLa cells (Liu et al. 2016), and WIL2-NS human B lymphoblastoid cells (Alimba et al. 2016). The affinity of Cu for specific sites on double-stranded DNA is higher than that of other metals (Formigari et al. 2013). It is suggested that Cu bound to DNA reacts with H_2O_2 to generate hydroxyl radicals, which then attack the DNA bases in a site-specific manner (Aruoma et al. 1991). Therefore, Cu might be capable of inducing DNA strand breaks and oxidation of DNA bases (Gaetke and Chow 2003).

In the present study, CCs incubated with Cu concentrations resulted in a reduction of mitochondrial activity. The cytotoxicity analyzed by the MTT method was higher when CCs were

cultured with 120, 240, and 360 $\mu\text{g}/\text{dL}$ Cu. Our results are in agreement with those presented by Cao et al. (2012). Using the MTT assay, these authors found that supplementation with Cu (10, 50, 100, 500, 1000, 2500, 4000, 5000 and 10,000 $\mu\text{g}/\text{dL}$) resulted in a significant decrease in L929 fibroblast viability. Also, the accumulation of Cu oxide nanoparticles (CuO-NPs) in C6 glioma cells produces a severe loss in cell viability (assessed by MTT reduction and cellular lactate dehydrogenase activity) and cell membrane integrity (Joshi et al. 2016). Similar results have been observed when Cu chloride was used (Nzengue et al. 2012); also, HepG2 cell exposure to 10, 200, and 500 μM Cu sulfate for 8 or 24 h reduced the viability as compared with untreated cells (Liu et al. 2014). In the present study, loss in cellular MTT reduction capacity was observed for all Cu conditions, suggesting that the MTT assay is a more sensitive indicator of Cu-induced cellular viability decrease than the TB-FDA assay.

To our knowledge, we have conducted the first genotoxic and cytotoxic evaluation of high Cu concentrations in bovine CC during COC IVM. As stated previously, our results revealed that Cu was able to induce genotoxicity and cytotoxicity in bovine female reproductive cells. Bovine ovary is a popular experimental model for studying mechanisms and properties related to ovarian function in humans (Babaei et al. 2012; Santos et al. 2014; Ceko et al. 2015). COC is the structural and functional unit present in mammalian antral follicles (Camaioni et al. 1993). COCs are embedded in FF, which is a type of blood plasma ultrafiltrate containing secretion from the ovarian follicle cells (Tolunay et al. 2016). Any change in FF may potentially affect CC. It is interesting to point out that the lowest Cu concentration used in this study (120 $\mu\text{g}/\text{dL}$) is very close to that found in FF of bovine (59.9–110.8 $\mu\text{g}/\text{dL}$) (Picco et al. 2012) and women (65.8–123.8 $\mu\text{g}/\text{dL}$, Cavallini et al. 2016; and 33–177 $\mu\text{g}/\text{dL}$, Tolunay et al. 2016). CCs surround the oocyte during the maturation process within the follicle and protect the developing oocyte providing nutrients through gap junctions (Tatemoto et al. 2000; Fatehi et al. 2002). Any alteration or damage of these cells can impair the oocyte developmental capacity (Ikeda et al. 2003). In conclusion, the present study indicates that Cu induces a genotoxic and cytotoxic effect on bovine CC. In addition, our findings support the view that CC might be a sensitive cell model for the study of Cu-induced genotoxicity and cytotoxicity.

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Compliance with ethical standards

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Conflict of interest The authors declare that there are no conflicts of interest.

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