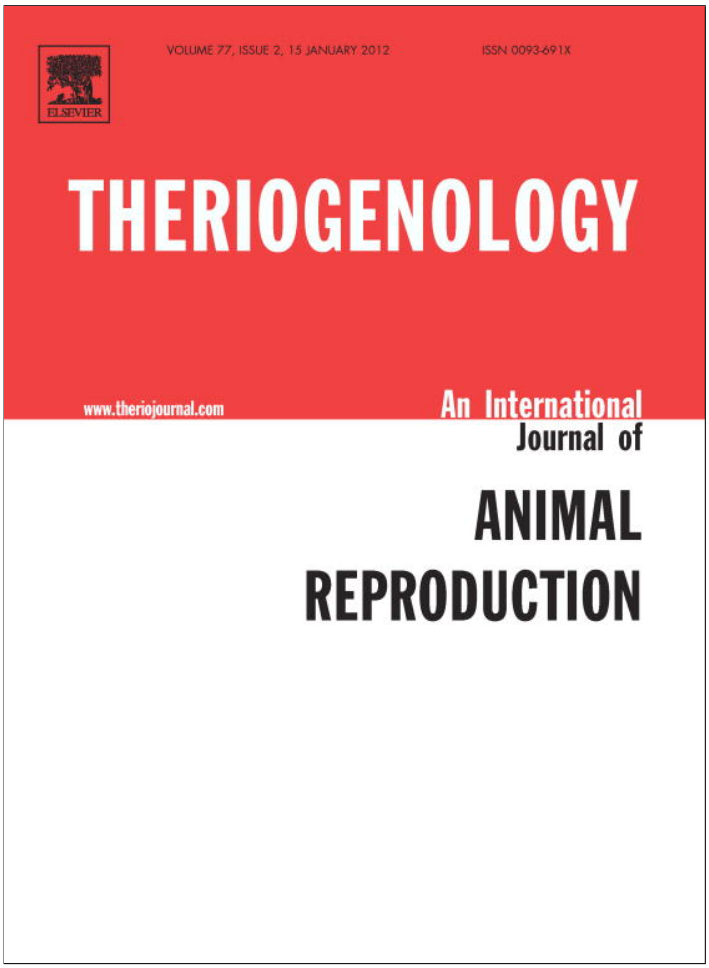

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Effects of copper sulphate concentrations during *in vitro* maturation of bovine oocytes

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Abstract

The objectives were to evaluate: 1) copper (Cu) concentrations in plasma and follicular fluid (FF) from cattle ovaries; 2) the effects of supplemental Cu during *in vitro* maturation (IVM) on DNA damage of cumulus cells and glutathione (GSH) content in oocytes and cumulus cells; and 3) supplementary Cu during IVM on subsequent embryo development. Copper concentrations in heifer plasma (116 ± 27.1 μg/dL Cu) were similar ($P > 0.05$) to concentrations in FF from large (90 ± 20.4 μg/dL Cu) and small (82 ± 22.1 μg/dL Cu) ovarian follicles in these heifers. The DNA damage in cumulus cells decreased with supplemental Cu concentrations of 4 and 6 μg/mL ($P < 0.01$) in the IVM medium (mean ± SEM index of DNA damage was: 200.0 ± 27.6, 127.6 ± 6.0, 46.4 ± 4.8, and 51.1 ± 6.0 for supplementation with 0, 2, 4, and 6 μg/mL Cu respectively). Total GSH concentrations increased following supplementation with 4 μg/mL Cu (4.7 ± 0.4 pmol in oocytes and 0.4 ± 0.04 nmol/10^6 cumulus cells) and 6 μg/mL Cu (5.0 ± 0.5 pmol in oocytes and 0.5 ± 0.05 nmol/10^6 cumulus cells, $P < 0.01$) compared with the other classes. Cleavage rates were similar ($P \geq 0.05$) when Cu was added to the IVM medium at any concentration (65.1 ± 2.0, 66.6 ± 1.6, 72.0 ± 2.1, and 70.7 ± 2.1 for Cu concentrations of 0, 2, 4, and 6 μg/mL). Percentages of matured oocytes that developed to the blastocyst stage were 18.7 ± 0.6, 26.4 ± 0.03, and 29.0 ± 1.7% for 0, 2, and 4 μg/mL Cu, and was highest (33.2 ± 1.6 %) in oocytes matured with 6 μg/mL Cu ($P > 0.01$). There was an increase ($P > 0.05$) in mean cell number per blastocyst obtained from oocytes matured with 4 and 6 μg/mL Cu relative to 0 Cu (IVM alone) and 2 μg/mL Cu. In conclusion, Cu concentrations in the FF and plasma of heifers were similar. Adding copper during oocyte maturation significantly increased both intracellular GSH content and DNA integrity of cumulus cells. Since embryo development was responsive to copper supplementation, we inferred that optimal embryo development to the blastocyst stage was partially dependent on the presence of adequate Cu concentrations during IVM.

Keywords: Oocyte; Copper; Glutathione (GSH); DNA integrity; *In vitro* maturation; Oocyte metabolism

1. Introduction

Hypocuprosis is the second most widespread mineral deficiency affecting grazing cattle [1]. A survey conducted by the National Animal Health Monitoring Service classified 40.6% of US beef cattle as copper

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deficient [2]. Similar or higher values were reported by Ramirez and colleagues [1] in the Salado River Basin (Argentina), an area of 55793 km^2 with 6.5×10^6 beef cattle [3]. Copper deficiency is linked to a variety of clinical signs, including pale coat, poor fleece quality (in sheep), anemia, spontaneous fractures, poor capillary integrity, myocardial degeneration, hypomyelinization of the spinal cord, impaired reproductive performance, decreased resistance to infectious disease, diarrhea, and generalized ill-health causing severe economic losses [4]. Inadequate copper status is associated with poor calf performance and health [5].

Copper is absorbed in the duodenum and transported bound to ceruloplasmin [6,7]. Serum copper concentrations are higher at estrus than 21 d after conception heifers, but are decreased in beef cows on the day of calving [8]. Copper status in cattle is defined as deficient, marginal, and adequate for plasma concentrations of ≤ 30, 31–60, and ≥ 60 μg/dL Cu, respectively [9]. Copper has a role in a diverse and increasing number of pathways, physiological and disease processes. Copper acts as an electron transfer intermediate in redox reactions, being an essential cofactor for oxidative and reductase enzymes [10]. The mammalian Cu-transporting P-type ATPases ATP7A and ATP7B are two key proteins that regulate Cu status; they transport Cu across cellular membranes for biosynthetic and protective functions, enabling Cu to fulfill its role as a catalytic and structural cofactor for many essential enzymes, and to prevent a toxic build-up of Cu inside cells [11].

The consequences of hypocuprosis are associated with failure of copper metalloenzymes (MT) [12,13]. Copper deficiency induced DNA damage through an increase in oxidative stress [12,14–16]. However, whether DNA damage occurred in oocytes was not assessed. The teratogenicity of Cu deficiency results from increased oxidative stress and oxidative damage [17]. In rat embryos, copper deficiency caused malformations and reduced SOD enzyme activity [18]. Also, Cu-deficient mouse embryos exhibited brain and heart anomalies, and yolk sac vasculature abnormalities with high levels of protein nitration [19]. Reduced glutathione (GSH) is an abundant compound with strong compartmentalization and various functions in cellular metabolism and defenses, including detoxication of heavy metals [20]. Copper uptake and incorporation into MT is strongly influenced by GSH levels [21]. Copper is rapidly complexed by GSH after entering the cell and is then transferred to MT [22]. Iron and copper play an important role in the success of culture of eight-cell embryos morulae and blastocysts; a long-term lack of iron or copper increased the number of apoptotic blastomeres [23].

The objective of this study was to investigate Cu concentrations in follicular fluid and the effects of Cu during *in vitro* maturation of bovine oocytes. In that regard, experiments were designed to evaluate the effect of various Cu concentrations added to the IVM medium on DNA integrity of cumulus cells by comet assay and intracellular GSH-GSSG concentrations in both oocytes and cumulus cells. In addition, developmental capacity of oocytes matured with various Cu concentrations was evaluated.

2. Materials and methods

2.1. Reagents and media

All reagents for media preparation, Comet Assay, and GSH determinations were purchased from Sigma Chemical Co. (St. Louis, MO, USA), whereas FSH was purchased from Serono Inc Rockland, MA, USA. The maturation medium was bicarbonate-buffered TCM-199 supplemented with 10% (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 [24]. Standard copper 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 sulfate. The composition of TALP medium was described previously by Parrish et al [25]. The culture medium for embryo development consisted of modified synthetic oviduct fluid (SOFm), composed of SOF [26] supplemented with 1 mM glutamine, 2% (v/v) BME-essential amino acids, 1% (v/v) MEM-non-essential amino acids, and 4 mg/mL fatty acid free BSA (274–276 mOsm/kg) [27].

2.2. 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 estrus cycle of the donor. Cumulus oocyte-complexes (COC) were aspirated from 2 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 X) stereomicroscope, for

IVM. Replicates (4 to 6) were performed on different days, with a separate batch of COCs for each day.

2.3. In vitro maturation (IVM)

The COC were washed twice in TCM-199 buffered with 15 mM Hepes containing 5% (v/v) FCS, and twice in IVM medium, and then transferred (in groups of 10 COCs) into 50 μL of IVM medium under mineral oil (Squibb, Princeton, NJ, USA) pre-equilibrated in a CO_2 incubator. The COCs were cultured in IVM medium at 39 °C in 5% CO_2 in air with saturated humidity for 24 h, and then assessed for cumulus expansion. Only oocytes with an expanded cumulus (90–95% expanded COCs) were used for IVF. 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 *in vitro* maturation.

2.4. Comet assay

At the end of IVM, all oocytes from each treatment were stripped of surrounding cumulus cells by repeated pipetting with a narrow-bore glass pipette in TCM 199 buffered with Hepes, and cumulus cells were washed three 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. Samples were then mixed with low melting point agarose. Single-cell gel electrophoresis was performed using the alkaline version previously described [28] with modifications [29]. 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 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 solution (Molecular Probes, Eugene, OR, USA) [30]. 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. Based on the extent of strand breakage, cells were classified according to their tail length into five categories (Fig. 1), 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), and

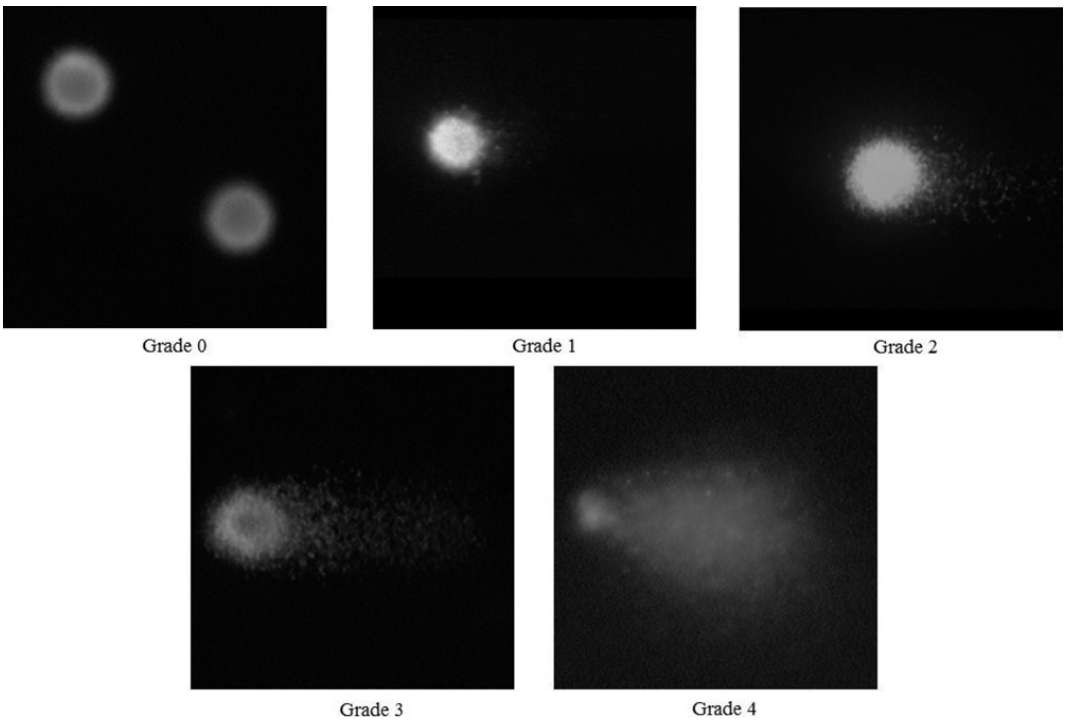

Fig. 1. DNA damage at the individual cumulus cell level: Grade 0 (no visible tail), Grade 1 (comets with tiny tail), Grade 2 (comets with a dim tail), Grade 3 (comets with a clear tail), and Grade 4 (comets with a clear decrease in the diameter of the head and a clear tail).

Grade 4 (comets with a clear decrease in the diameter of the head and a clear tail). Arbitrary units of DNA damage value was established according to Collins [30,31]. The Index Damage (ID) was obtained as follows: if 100 comets were scored, and each comet assigned a value of 0 to 4 according to its class, the total score for the sample gel was 0 to 400 "arbitrary units". Visual scoring (arbitrary units) was rapid and simple, with very close agreement between this method and computer image analysis (percentage DNA in tail) [31].

2.5. GSH-GSSG assay

After completion of IVM, all oocytes from each treatment in a batch were combined and surrounding cumulus cells were removed by repeated pipetting with a narrow-bore glass pipette in Hepes-TCM 199, and washed three times in Mg^{2+}/Ca^{2+} free PBS containing 1 mg/mL PVP. For each replicate, pools of at least 50 oocytes in 10 μL of PBS from each treatment were placed in microtubes, frozen at −20 °C, and subsequently thawed at room temperature. This procedure was repeated three times. Complete oocyte disruption was achieved by repeated aspiration using a narrow-bore pipette. Cumulus cells from at least 50 COC were placed in microtubes and washed twice by resuspension in PBS and centrifugation at 14000 × g for 10 s. The pellets were resuspended in 500 μL of PBS and counted in a hemocytometer chamber. After centrifugation at 14,000 × g for 10 s, pellets were resuspended in 40 μL of PBS, frozen at −20 °C, and thawed at room temperature. Complete cell disruption was performed by addition of 400 μL of distilled water and repeated aspiration with a 26-G needle. Distilled water was added to samples (oocytes or cumulus cells) to increase the volume to 1.2 mL, and then mixed with 1.2 mL of 0.2 M phosphate buffer containing 10 mM EDTA. After rapid addition of 100 μL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid (DTNB), 1 unit (in 50 μL) of glutathione reductase, and 50 μL of 4.3 mM NADPH, the increase in absorbance at 412 nm was measured, every 30 s up to 5 min, using a double-beam spectrophotometer (Model 35, Beckman, Irvine, CA, USA). Blanks consisted of 10 μL of PBS or 10 μL aliquots of wash medium. Total GSH content in oocytes and cumulus cells were calculated from a standard curve of GSH [24]. Under these conditions, the minimum detectable concentration of assay was 25 pmol of GSH-GSSG.

2.6. In vitro fertilization (IVF)

The expanded COC 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 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% CO2 in air with saturated humidity for 24 h.

2.7. In vitro culture (IVC)

After IVF, presumptive zygotes were stripped of cumulus cells by passing through a drawn pipette, washed twice in Hepes-TALP, 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 d in the presence of 1.5 mM glucose [26]. The medium was changed every 48 h, and the embryos were incubated for 8 d (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).

2.8. Blastocyst staining for total cell number

Day 8 blastocysts were fixed in 4% formaldehyde after washing three times in 1% polyvinylpyrrolidone (PVP) in PBS overnight. Embryos were placed in 1% Triton X-100 overnight, stained with Hoechst 33342, and mounted on slides and covered with a cover slip. The total cell numbers of blastocysts (8 d, Grade 1) from the groups of Experiment 3 were determined by counting the number of nuclei under an epifluorescent microscope. Total cell numbers of blastocysts were visualized by a Nikon Optiphot epifluorescent microscope with a 40 × fluor objective (Nikon, Tokyo, Japan) equipped with a 365 nm excitation filter, a 400 nm barrier filter, and a 400 nm emission filter.

2.9. Copper concentrations in plasma, follicular fluid, and IVM medium

Samples of jugular blood (10 mL) were collected from 40 healthy heifers (immediately before slaughter) into a test tube containing EDTA. The samples were centrifuged at 350 × g for 10 min, and the plasma was separated and stored at 4 °C. Also, ovaries from these heifers were collected and handled independently to give 40 values for Cu in each follicle class. Ovaries were wrapped with plastic film, and taken to the laboratory in an icebox within 2 h after slaughter. Follicle diameter was measured with a vernier caliper. Follicles were aspirated, measured and separated according to their diameter into two groups: (1) small and medium follicles: < 10 mm (the lowest follicle size was 2 mm, but most follicles were ~3 mm) and (2) large follicles (the largest follicles were 11–12 mm, but most of them were ~10 mm). These size ranges were selected based on established key transitions in antral folliculogenesis and accompanying changes in the acquisition of oocyte developmental competence [32,33]. Follicular fluid was collected from each follicle by aspiration with an insulin syringe. Due to the low volume of follicular fluid collected from small follicles, samples were pooled (usually between 5 and 10 follicles) within a given ovary. No sample pooling was needed for the medium and large size categories. Samples were kept on ice until centrifugation. Samples of IVM medium with 10% FCS were also collected (n = 6). Samples of FF and IVM medium were centrifuged, and the supernatants were treated with 10% (w/v) trichloroacetic acid. Copper concentration was measured by double beam flame atomic absorption spectrophotometer (GBC 902) through an internal quality control [34]. The IVM medium was supplemented with 2, 4, and 6 μg/mL copper, with concentrations established according to the criteria of Kincaid [35], and Underwood and Sutlle [9] classification for copper status in cattle.

3. Experimental design

3.1. Effect of copper on DNA integrity of cumulus cells

In Experiment 1, the effect of Cu on DNA damage of cumulus cells following the addition of 0, 2, 4, or 6 μg/mL Cu to IVM medium was evaluated by a Comet Assay. The COCs were matured for 24 h (as described above), and thereafter, DNA damage was evaluated as described (Section 2.4.). For this purpose, 800 COCs in four replicates from different days (200 COCs per replicate, 50 COCs per treatment) were matured *in vitro* with various Cu concentrations. Each batch of 50 COCs was processed for preparing slides to analyze at least 250 single cells per treatment for the Comet Assay.

3.2. Effect of copper on intracellular GSH level

In Experiment 2, the effects of adding 0, 2, 4, and 6 μg/mL Cu to maturation medium on intracellular GSH-GSSG concentrations in both oocytes and cumulus cells were evaluated. The COCs were matured for 24 h (as described above), and total GSH concentrations were evaluated (described in Section 2.5.). For this purpose, 800 COCs were matured in four replicates (separate batch of ovaries for each day), with 200 COCs distributed in groups of 50 COCs per treatment.

3.3. Effect of copper during IVM on subsequent embryo development and embryo quality

In Experiment 3, the developmental capacity of oocytes matured in IVM medium supplemented with 0, 2, 4, and 6 μg/mL Cu was investigated. For this purpose, 1265 COCs were matured in six replicates. Cleavage rates were recorded 48 h after insemination. Data reported for development to the blastocyst stage were calculated, either from oocytes or cleaved embryos, including embryos that progressed to the expanded or hatched blastocyst stages. Percentages of cleavage and embryo development rates are expressed as mean ± SEM.

3.4. Statistical analysis

Chi-square analysis with the Yates correction was used to compare, among groups, the proportion of DNA damage determined with comet assay. Differences among treatments for GSH concentrations in oocytes and cumulus cells were analyzed by ANOVA and Student-Newman-Keuls Multiple Comparison post-test, after logarithmic transformation of data. Percentages of cleavage and embryo development were also analyzed by ANOVA and Student-Newman-Keuls Multiple Comparison post-test after angular transformation of data (CSS: Statistica, module C, Statsoft, Tulsa, OK). Results were expressed as mean ± SEM.

4. Results

4.1. Copper determination in plasma, follicular fluid, and IVM medium

Copper concentrations were116 ± 27.1 μg/dL Cu in 22 plasma samples, 90 ± 20.8 μg/dL in FF from large

Table 1
Mean (± SEM) DNA damage in bovine cumulus cells matured in vitro with various concentrations of copper (800 COCs in four replicates for comet assay).

Treatment (μg/mL Cu)	Damage degrees (%)				
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
0	37.4 [a] (3.7)	17.4[a] (1.9)	12.3[a] (1.1)	11.2[a] (1.6)	11.0[a] (4.5)
2	38.9[a] (3.7)	25.0[a] (3.84)	18.0[a] (1.0)	7.9[a] (0.9)	10.1[a] (3.0)
4	72.7[b] (3.0)	13.0[b] (2.1)	10.9[b] (2.3)	1.6[b] (0.6)	1.7[b] (0.3)
6	66.2[b] (2.0)	19.5[b] (0.6)	7.2[b] (1.8)	0.9[b] (0.4)	0.6[b] (0.2)

[a,b] Within a row, means without a common superscript differed ($P < 0.01$).

follicles, 82 ± 22.1 μg/dL in FF from small follicles, and 0.05 μg/mL ± 0.01 in IVM medium. There were no differences ($P > 0.05$) in Cu concentrations between plasma and FF.

4.2. Effects of copper on DNA damage of cumulus cells

In Experiment 1, cumulus cells from oocytes cultured with supplemental Cu during IVM had a significant decrease in the Index Damage of DNA with 4 and 6 μg/mL Cu (Index Damage was 200.0 ± 27.6, 127.6 ± 6.0, 46.4 ± 4.8, and 51.1 ± 6.0, for oocytes exposed to 0, 2, 4, and 6 μg/mL Cu, respectively). Percentages of cells without DNA damage (Degree 0) increased with Cu concentration (Table 1); damage was lower ($P < 0.01$) in oocytes matured in medium with 4 or 6 μg/mL Cu compared to medium without Cu. However, there were no significant differences in the degree of DNA damage in cumulus cells treated with 0 and 2 μg/mL Cu added to IVM media.

4.3. Effects of copper on intracellular GSH concentration

In Experiment 2, 800 COCs in four replicates (200 COCs per replicate, 50 COCs per treatment) were matured *in vitro* with various Cu concentrations. Intracellular concentrations of GSH-GSSG increased with the addition of 4 and 6 μg/mL ($P < 0.01$ Table 2). However, GSH-GSSG did not differ significantly in oocytes and cumulus cells in the presence of 4 or 6 μg/mL Cu during IVM. There were no differences in cumulus cell number per COC either before ($n = 4$) or after IVM ($n = 4$ per treatment) at any Cu concentration (before IVM: 15200 ± 1200; after IVM: 15233 ± 1280 (0 μg/mL Cu), 15287 ± 1197 (2 μg/mL Cu), 15354 ± 277 (4 μg/mL), and 15599 ± 1230 (6 μg/mL Cu) cumulus cells/COC. In all experiments, neither cell number per COC, nor the percentage of nuclear maturation (PB + M II) varied significantly among Cu concentrations (Control: 83 ± 4.1%; 2 μg/mL Cu: 84 ± 4.7; 4 μg/mL Cu: 83 ± 3.2; and 6 μg/mL Cu: 85 ± 4 evaluated with Hoechst 33342).

4.4. Effects of copper concentrations during IVM on subsequent embryo development

In Experiment 3, 1265 oocytes in six replicates were matured and fertilized *in vitro*. There was no difference in cleavage rate when Cu was added to IVM medium at any concentration ($P \geq 0.05$). Copper increased blastocyst rates (blastocysts/oocytes) when oocytes were matured with 2, 4, or 6 μg/mL Cu concentrations ($P < 0.01$), but the difference was highest when 6 μg/mL Cu was added to IVM medium ($P < 0.001$; Table 3). Similarly, Cu increased the percentages of blastocyst calculated from cleaved embryos ($P < 0.01$). Mean cell number per blastocyst increased ($P < 0.05$) when oocytes were matured in the presence of 4 or 6 μg/mL Cu in IVM media (Table 4).

Table 2
Total intracellular glutathione concentrations in bovine oocytes and cumulus cells matured with various copper concentrations.

	Cu supplementation (μg/mL)			
	0	2	4	6
Oocyte GSH-GSSG (pmol/oocytes)	3.0 ± 0.9[a]	3.2 ± 0.5[a]	4.7 ± 04[b]	5.0 ± 05[b]
Cumulus GSH-GSSG (nmol/10^6 cells)	0.3 ± 0.05[a]	0.3 ± 0.02[a]	0.4 ± 0.04[b]	0.5 ± 0.04[b]

All values for oocytes (pmol GSH/GSSG/oocyte) and cumulus cells (nmol GSH/GSSG/10^6 cumulus cells) are expressed as mean ± SEM (800 COCs in four replicates, 200 COCs per replicate, 50 COCs per treatment for GSH-GSSG).
[a,b] Within a row, means without a common superscript differed ($P < 0.01$).

Table 3
Developmental capacity of cattle oocytes matured in vitro with various copper concentrations.

	Cu supplementation (μg/mL)			
	0	2	4	6
No. oocytes	313	339	309	304
Cleaved (%)	65.1 ± 2.0	66.6 ± 1.6	72.0 ± 2.1	70.7 ± 2.1
Blastocyst/oocytes	18.7 ± 0.6[a]	26.4 ± 0.03[b]	29.0 ± 1.7[b]	33.2 ± 1.6[c]
Blastocyst/cleaved	29.3 ± 1.1[a]	36.4 ± 2.2[b]	40.5 ± 2.7[b]	47.0 ± 1.5[c]

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 d in culture. All values for cleavage and development rates are expressed as mean ± SEM (1200 COCs in six replicates).
[a-c] Within a row, means without a common superscript differed ($P < 0.001$).

5. Discussion

In the present study, Cu concentrations in FF and plasma of heifers were similar. The addition of Cu during oocyte maturation decreased DNA damage of cumulus cells and increased intracellular GSH-GSSG content in oocytes and cumulus cells. Moreover, when the Cu concentration was 6 μg/mL during IVM, competence of bovine oocytes was improved, manifested by increased blastocyst rates from oocytes and cleaved embryos.

Copper status in cattle is defined as deficient, marginal, and adequate for plasma concentrations of ≤ 30, 31–60, and ≥ 60 μg/dL Cu, respectively [9]. In the present study, Cu concentrations in heifers were similar in blood plasma (116 ug/dL Cu) and FF from large and small follicles (82 to 90 μg/dL Cu). Therefore, plasma Cu status might be useful to predict Cu status in the *in vivo* follicle environment. Concentrations of copper, zinc, and selenium in serum and follicular fluid were decreased in women undergoing IVF, but were normalized by giving multivitamin/mineral supplements [36]. Furthermore, mineral supplementation had a positive impact on lipid peroxidation, reduced glutathione, and glutathione peroxidase concentrations in serum and follicular fluid, reinforcing the antioxidant defense system by decreasing oxidative stress [37].

In mammalian cells, copper operates as a free radical scavenger. The antioxidant role of Cu may be an important mechanism in maintaining DNA integrity in cumulus oocyte-complex by preventing oxidative DNA damage in cumulus cells. In the present study, IVM medium with 2 μg/mL Cu had no effect on the DNA integrity of cumulus cells after *in vitro* maturation. However, DNA damage in cumulus cells was significantly decreased when Cu was added to IVM medium at 4 and 6 μg/mL concentrations. The association between copper plasma concentrations and DNA damage in lymphocytes of hypocupremic heifers has been previously demonstrated [38]. Furthermore, this increase of DNA damage found in hypocupremic animals is explained by higher oxidative stress [38]. Hypocupremia in cattle is associated with an increase in the frequency of chromosomal aberrations, as well as in DNA migration (as assessed by the comet assay). Whereas the latter assay differentiated copper plasma level groups, chromosomal aberrations only detected differences between normal and hypocupremic animals [38].

Intracellular glutathione concentration (GSH-GSSG) was increased in 4 and 6 μg/ml Cu supplemented media, in both oocytes and cumulus cells. It has been reported that depletion of intracellular Cu caused a depletion of GSH in CHO (Chinese hamster ovary) cells cultured *in vitro* [39]. The relationship between Cu and GSH has been substantially studied; Cu is a biological ligand forming binary complexes with GSH [40]. Furthermore, formation of a copper-glutathione

Table 4
Mean cell numbers of Day-8 bovine blastocysts developed from oocytes matured with various concentrations of copper.

	Cu supplementation μg/mL			
	0	2	4	6
No. blastocysts	35	39	32	40
Cell number/blastocyst	102.3 ± 5.0[a]	107.2 ± 5.5[a]	127.4 ± 6.7[b]	129.0 ± 5.0[b]

Values are expressed as mean cell number/blastocyst ± SEM.
[a,b] Within a row, means without a common superscript differed ($P < 0.05$).

complex which is stable to the presence of some copper-chelators, lacks all thiol reactivity, but fully conserves the free-radical scavenging properties of GSH [40]. The majority of plasma Cu is transported bound to ceruloplasmin (> 95%), whereas the remainder is bound to albumin, transcuprein, and copper-amino acid complexes [41]. Copper is toxic in its unbound form, and causes redox imbalance due to its highly redox active nature [41,42]. The total thiol pool contributes the majority of antioxidant activity of the body. A major contribution to the total thiol pool is from reduced glutathione and free thiol (SH) group present on protein mainly on albumin [43,44]. A large store of GSH during oocyte maturation was important for cumulus expansion *in vitro* and embryo protection up to the blastocyst stage [24,45,46]. Moreover, high GSH concentrations during IVM improved embryo development and quality, yielding more embryos reaching the blastocyst stage, and an increased proportion suitable for cryopreservation [47,48].

In this study, independent of Cu status in the animal, we evaluated the incidence of the supplementation of various Cu concentrations during 24 h of oocyte *in vitro* maturation and the competence (cytoplasmic maturation) of these oocytes to develop until the blastocyst stage. Various concentrations of Cu (2, 4, and 6 μg/mL) added to IVM medium did not increase cleavage rates compared to oocytes matured in medium without Cu supplementation. Nevertheless, the yield of embryo development to blastocyst stage was significantly higher when oocytes were matured with Cu (2, 4, and 6 μg/mL) during IVM. Furthermore, the blastocyst rate was significantly higher when 6 μg/mL Cu was used. These results were in agreement with a previous report that copper during IVM had no effect on bovine oocyte maturation [23]. However, in our study, the improvement of blastocysts rates was a consequence of the addition of copper during oocyte maturation, whereas Gao and collegues [23] found that the supplementation of zygote medium with copper improved embryo development. Furthermore, the addition of 0.68 and 0.46 mg/L Cu to zygote culture media improved the yield of bovine embryos [23].

In conclusion, copper concentrations were similar in plasma and follicular fluid of heifers. Adequate copper concentrations improved intracellular GSH content in oocytes and cumulus cells, DNA integrity of cumulus cells during *in vitro* maturation of bovine oocytes, and development of preimplantation embryos. We inferred that there may be an association between embryo production and copper concentration during *in vitro* maturation.

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