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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