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# The copper transporter (SLC31A1/CTR1) is expressed in bovine spermatozoa and oocytes: Copper in IVF medium improves sperm quality

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ABSTRACT

Adequate dietary intake of copper (Cu) is required for normal reproductive performance in cattle. The objective of this study was to investigate the pregnancy rates from cattle with deficient, marginal and adequate Cu plasma concentration at the beginning of artificial insemination protocol. Moreover, we determined Cu concentrations present in bovine oviductal fluid (OF), and the effects of Cu on fertilizing ability of bovine spermatozoa. Also, the presence of Cu transporter, SLC31A1 (also known as CTR1), in spermatozoa and *in vitro* matured oocyte were investigated. We found no differences in pregnancy rates among animals with adequate, marginal, and deficient Cu concentrations measured in plasma at the beginning of fixed-time artificial insemination (FTAI) protocol. Copper concentrations in OF were 38.3 ± 2.17 μg/dL (mean ± SEM) regardless of cupremia levels. The addition of 40 μg/dL Cu to IVF medium enhanced total and progressive motility, sperm viability, functional sperm membrane integrity (HOST), sperm–zona binding, and pronuclear formation. On the other hand, the presence of Cu in IVF medium did not modify acrosome integrity and cleavage rates after IVF, but impaired blastocyst rates. Cu transporter SLC31A1 was detected in bovine spermatozoa in the apical segment of acrosome, and in the oocyte matured *in vitro*. In conclusion, the results obtained in the present study determined that cupremia levels at the beginning of FTAI protocol did not influence the pregnancy rates at 60 d after insemination. The presence of CTR1 in bovine mature oocyte and spermatozoa, as well as the beneficial effect of Cu on sperm quality would suggest an important role of this mineral during the fertilization process.



## 1. Introduction

Reproductive efficiency has a dramatic economic impact on both beef and dairy cattle production. Reproductive success within the cow herd is five times more crucial in commercial operations than growth rate or milk production [1]. For this reason in recent years, there has been an increasing interest in studying the pathogenesis of different reproductive harms. Subfertility in cattle still remains a substantial problem in Argentina and many areas of the world and a considerable proportion of those cattle respond to appropriate copper (Cu) supplementation [2–4].

Hypocuprosis is the predominant deficiency that globally affects grazing cattle [5]. A survey conducted by the National Animal Health Monitoring Service classified 40.6% of US beef cattle as copper deficient [6]. Similar or higher values were reported by Ramirez et al. [5] in Salado River Basin (Argentina), an area of 55,793 km$^2$ with 6.5 × 10$^6$ beef cattle [7]. Copper status in cattle is

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defined as deficient, marginal, and adequate for plasma concentrations of <30, 31–60, and >60 μg/dL Cu, respectively [8]. Cu is involved in numerous biological processes, especially as an integral part of enzymes that function in a number of important processes including energy production, oxidant defense, extracellular matrix protein crosslinking, immune function, and iron mobilization and trafficking [9,10]. Copper enters cells via the Cu transporter, Solute Carrier Family 31 Member 1 (SLC31A1, also known as CTR1), considered the main Cu uptake transporter [11].

Copper deficiency has been associated to reproductive disorders including, low fertility, long post-partum return to oestrus period, and an increase number of services per conception [11,12]. The aims of this study were investigate: a) effect of cupremia levels at the beginning of a Fixed Time Artificial Insemination (FTAI) protocol on pregnancy rate; b) Copper concentrations in bovine oviductal fluid (OF); c) influence of different copper concentrations on fertilizing ability of bovine spermatozoa; and, d) presence of Cu transporter (SLC31A1) in spermatozoa and matured oocyte.

## 2. Materials and methods

### 2.1. Reagents and media

All reagents for media preparation 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 17b-estradiol, and 50 mg/mL kanamycin. The maturation medium used in all experiments was bicarbonate-buffered TCM-199 supplemented with 10% FCS. The fertilization medium consisted of TALP supplemented with 6 mg/mL BSA-fatty acid free, 20 μM penicillamine, 10 μM hypotaurine, and 10 mg/mL heparin sulfate. The composition of TALP medium was described previously by Parrish et al. [13]. The culture medium for embryo development consisted of modified synthetic oviduct fluid (SOFm), which was composed of SOF [14] supplemented with 1 mM glutamine, 2% (v/v) BME-essential amino acids, 1% (v/v) MEM-nonessential amino acids, and 4 mg/mL fatty acid free BSA (274–276 mOsm/kg) [15]. Primary anti-CTR1 (FL-190) and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### 2.2. Animals

The experimental procedures were approved by the Animal Care and Use Committee, (Faculty of Veterinary Sciences, National University of La Plata, Buenos Aires, Argentina). The trial was performed on an experimental cowherd in Buenos Aires, Argentina during late spring (October–November). The farm was located in Salado River Basin (35° 44′ 44.76″ S, 58° 3′ 22.29″ W), where hypocuprosis is endemic. Three hundred sixty-eight Angus cows weighing 410.1 ± 4.5 kg (mean ± SEM; range, 330–598), with a body condition score (BCS) of 3.47 ± 0.02 (mean ± SEM; range, 2.5–4) as assessed on a scale from 1 to 5 [16], and more than 55 days of post-partum at the start of the experiment were used in this experiment. Cows were managed together under an extensive grazing system based on natural pastures. Cows, at random stage of the estrous cycle (Day 0), received an intramuscular (IM) injection of 2 mg of EB (Syntex, Argentina) and an intravaginal P4-device was inserted containing 0.5 g of progesterone (DIB, Syntex, Argentina). On Day 7, the DIB was removed and cows were administered 500 μg Cloprostenol (Ciclase DL, Syntex) and 0.5 mg estradiol cypionate (Cipiosyn, Syntex) by IM injection. Artificial insemination was performed between 52 and 56 h after DIB removal by 3 experienced technicians. Semen from the same bull and batch was used in all cows. Jugular blood samples (10 mL) were collected on d0 and d9 for determination of plasma Cu concentrations. Pregnancy rate was determined by trans-rectal ultrasonography (Aloka 500V equipped with a 5.0-MHz linear-array transducer, Aloka, Wallingford, CT) at 60 d after FTAI. For data analysis (pregnancy rate), cows were divided into three groups according to Cu status at the beginning of artificial insemination protocol (d0). For this purpose, groups were established according to the criteria of Kincaid [17], and Underwood and Sutlle [8] classification for plasma Cu status in cattle: deficient (≤30 μg/dL Cu); marginal (31–59 μg/dL Cu) and adequate: (≥60 μg/dL Cu). Also, cupremia at the beginning (d0) and at the end of the FTAI protocol (d9) were compared.

### 2.3. Copper concentrations in plasma, oviductal fluid and IVF medium

Samples of jugular blood (10 mL) from heifers (immediately before slaughter) were collected 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, oviducts and mesenteries from these heifers were collected and handled independently. Oviducts and mesenteries were wrapped with plastic film, and taken to the laboratory in an icebox within 2 h after slaughter. Oviductal fluid was obtained as described Apichela et al. [18]. Briefly, a sterile pipette was carefully introduced into the ampulla and air was insufflated several times. Then liquid was collected at the utero-tubal junction level by slight pressure on the oviductal walls. Samples containing blood were rejected. Then, flushings were grouped in five different pools according to cupremia: 40–59; 60–79; 80–99; 100–119; and 120–140 μg/dL of Cu in plasma. Each pool was considered one sample and centrifuged at 5000 g × 10 min, to pellet any cellular debris. The OF thus obtained was stored at −20 °C until further analysis. Samples of OF, IVF medium, and plasma from slaughtered and pre-insemination females were centrifuged, and the supernatants were treated with 10% (v/v) trichloroacetic acid. Copper concentration was measured by double beam flame atomic absorption spectrophotometer (GBC 902) through an internal quality control [19]. For this purpose, OF and plasma from 76 heifers were used in three replicates (23–28 heifers for each day). A total of three OF samples per cupremia group (n = 5) were evaluated.

### 2.4. In vitro maturation, fertilization and culture

*In vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture of embryos (IVC) were conducted as described previously [20]. Briefly, 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 oestrous cycle stage of the donor. Cumulus–oocyte complexes (COC) were aspirated from 3 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 (20–30X) stereomicroscope, for IVM. COC were washed twice in TCM- 199 buffered with 15 mM HEPES and twice in IVM medium. Groups of 10 COC were transferred into 50 μl of IVM medium under mineral oil (Squibb, Princeton, NJ, USA) pre-equilibrated in a CO2 incubator. The incubations were performed at 39 °C in an atmosphere of 5% CO2 in air with saturated humidity for 24 h. In order to IVF, oocytes 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 and batch was used. Two 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% $CO_2$ in air with saturated humidity for 24 h. 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. The medium was changed every 48 h, and the embryos were incubated for 8 d (Day 0 = day of fertilization). Cleavage rates were recorded 48 h after insemination. At the end of incubations, the embryos were evaluated for the morphological stages of development with an inverted microscope (Diaphot, Nikon, Tokyo, Japan). For this purpose, 449 COC were matured in four replicates.

### 2.5. Analysis of structural (viability) and functional sperm plasma membrane integrity and sperm motility

In all experiments, frozen semen from the same bull and batch was used. The semen was prepared as described above (described in Section 2.3), and incubated in IVF medium supplemented with 0, 20, 40, and 60 μg/dL Cu (concentrations established according to the criteria of Kincaid [17], and Underwood and Sutlle [8] classification for Cu status in cattle). At different time points (0, 3 and 6 h), one aliquot per test group was evaluated for viability, functional sperm plasma membrane integrity and sperm motility. Structural membrane integrity was assessed using eosin–nigrosin staining, which is considered a reliable and feasible technique for this purpose [21]. A total of 200 spermatozoa were counted in at least five different microscopic fields. The hypo-osmotic swelling test (HOST) was used to evaluate the functional sperm membrane integrity [22]. The test was performed by incubation of 25-μl semen with 200 μl of HOST solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate) at room temperature (RT). A wet mount was made using a 10 μl drop of homogenized mixture and placed directly on microscopic slide and covered by a cover slip. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of spermatozoa with swollen and curved tails were recorded. In addition, total (TM) and progressive (PM) motility (200 spermatozoa in at least four different fields) were assessed by means of computer-assisted sperm analysis (Hamilton Thorne, Beverly, MA, USA). For each test, twelve straws were used in four replicates (three straws for each day). In each replicate, semen was pooled and then separated into the four treatments.

### 2.6. Acrosomal status (FITC-labeled Pisum sativum agglutinin staining)

The acrosomal status (AS) was assessed using *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (PSA-FITC, Sigma Chemical Company, St Louise, MO, USA), as described earlier [23]. Briefly, sperm smears were fixed in methanol for 30 s after air-drying and then stained using 50 μg/mL PSA-FITC in PBS for 30 min in a humidified chamber (HC) at RT. The slides were washed with distilled water and mounted. A total of 200 spermatozoa per sample were counted with an Olympus BX40 epifluorescent microscope (Olympus, Tokyo, Japan) using excitation wavelengths of 450–490 nm and a magnification of 1000×. The acrosomal region of acrosome-intact spermatozoa was PSA-FITC positive and labeled green, while acrosome-reacted spermatozoa retained only an equatorial labeled band with little or no labeling of the anterior head region. The semen was prepared and incubated with various Cu concentrations as described above (described in Section 2.4). For this purpose, twelve straws were used in four replicates (three straws for each day). In each replicate, semen was pooled and then separated into the four treatments.

### 2.7. Spermatozoa–ZP binding test

*In vitro* matured COC were incubated in 0.1% (w/v) hyaluronidase in phosphate buffer salts medium for 5 min at 37 °C and then denuded by gentle pipetting. The denuded oocytes 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 supplemented with 0, 20, 40, and 60 μg/dL Cu under mineral oil. The drops containing oocytes (10 oocytes/drop) and sperm were incubated for 2 h at 37 °C in 5% $CO_2$ in air. The semen was prepared as described above (described in Section 2.3), two straws per replicate were used. Then, the oocytes were then picked up and washed three times with the medium to remove loosely attached sperm using a wide-mouth Pasteur pipette. The oocytes were stained with Hoechst 33,342, and mounted on slides and covered with a cover slip. The number of spermatozoa bound to each egg was determined by observation under an epifluorescent microscope Olympus BX40 (with a 365 nm excitation filter and a 400 nm emission filter) at 400× magnification. For this purpose, 146 COC were used in three replicates (separate batch of ovaries for each day).

### 2.8. Pronuclear formation

COC obtained from slaughterhouse ovaries were matured and fertilized *in vitro* as described above (described in Section 2.3). The IVF medium was supplemented with 0, 20, 40, and 60 μg/dL Cu. At 18 h post-insemination, presumptive zygotes were incubated in 0.1% (w/v) hyaluronidase in the phosphate buffer salts medium for 5 min at 37 °C and then oocytes were denuded by gentle pipetting. The presumptive zygotes were incubated in 5 mg/L Hoechst 33,342 in phosphate buffer salts medium for 30 min at 37 °C. Thereafter, presumptive zygotes were examined under a fluorescent microscope Olympus BX40 (with a 365 nm excitation filter and a 400 nm emission filter) at 200× and 400× magnifications to reveal the presence of pronuclei. For this purpose, 492 COC were matured in five replicates.

### 2.9. Immunocytochemistry

#### 2.9.1. Semen

Two straws of 40 × $10^6$ bovine spermatozoa were thawed in a 37 °C water bath. Spermatozoa were washed once in 2 mL HEPES-TALP medium and centrifuged at 300*g* for 10 min. HEPES-TALP medium was removed, and 10 μ (400,000 spermatozoa) of the sample were smeared on to a slide and fixed in 3:1 methanol-acetic acid (Carnoy's fixative) for 24 h. After washing in PBS (1 min), sperm were permeabilized for 5 min with PBS/0.03% (v/v) Tritón X-100, washed, and blocked in PBS (pH 7.4) containing 5% non-fat powdered milk (Blotto) for 30 min. Slides were subsequently incubated with primary antibody for 3 h at 37 °C in a HC, washed

with PBS/0.02% (v/v) Tween 20 four times, and further incubated with goat anti-rabbit IgG-FITC for 30 min at RT in a HC. Primary antibody was anti-CTR1 (FL-190; rabbit, polyclonal; 1:20 dilution in Blotto/0.02% (v/v) Tween 20). Slides were washed three times in PBS/0.02% (v/v) Tween 20, assembled with mounting medium (PBS/50% (v/v) glycerol), and observed under a Leica TCS-SP5 Confocal Microscope (Leica Microsystems, Wetzlar, Germany) at 630× magnification.

#### 2.9.2. Mature oocyte

*In vitro* matured Oocytes were fixed in 0.01% formaldehyde in PBS without calcium and magnesium for 10 min at RT, and then incubated in PBS/3.0% (w/v) BSA at 37 °C for 1 h. Subsequently, the oocytes were incubated overnight at 4 °C with the anti-CTR1 antibody (FL-190; rabbit, polyclonal; 1:50 dilution in PBS/1% (w/v) BSA). After washing three times in PBS/1% (w/v) BSA, the oocytes were incubated at 37 °C for 1 h with goat anti-rabbit IgG-FITC, mounted onto slides, and examined using a Leica TCS-SP5 Confocal Microscope (Leica Microsystems, Wetzlar, Germany) at 630× magnification. Negative controls were obtained by substituting the incubation with primary antibody for incubation with Blotto for semen, and PBS/1% (w/v) BSA for oocytes.

### 2.10. Statistical analysis

A completely randomized block designs were used. Statistical model included the random effects of block (n = 3–5 depending on experiment) and the fixed effect of treatment (40–59 vs 60–79 vs 80–99 vs 100–119 vs 120–140 μg/dL of Cu in plasma (Experiment 1); 0 vs 20 vs 40 vs 60 μg/dL Cu (Experiments 2–5); 0 vs 40 μg/dL Cu (Experiment 6); and Deficient vs Marginal vs Adequate (Experiment 7). In Experiment 7, statistical model also included technician as fixed effect. Variables such as Cu concentrations in OF and sperm–zona binding were analyzed with linear models using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA). Viability, HOST, TM, PM, AS, pronuclear formation, cleavage rate, and pregnancy rate were analyzed by logistic regression using GENMOD procedure (SAS Institute). Results are expressed as mean ± SEM. Statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Effect of serum copper levels on FTAI outcomes

In Experiment 1, there were no differences in pregnancy rate at 60 d after insemination among adequate (52.2%), marginal (59.5%), and deficient groups (55.5%; $P > 0.05$; Fig. 1). Artificial insemination technician did not have an effect on pregnancy rate. On the other hand, a high correlation ($r = 0.93$; $R^2 = 0.87$) was found between plasma Cu concentration on d0 and d9, but cupremia was higher on d9 (68.8 ± 1.4/dL Cu) than d0 (63.4 ± 1.4 μg μg/dL Cu; $P < 0.01$).

### 3.2. Copper determination in oviduct fluid, and IVF medium

In Experiment 2, there were no differences in Cu concentrations in OF at any Cu concentration in plasma (37.5 ± 7, 41.2 ± 9, 42.9 ± 9, 36 ± 4 and 30 ± 7 μg/dL Cu in OF for 40–59, 60–79; 80–99; 100–119 and 120–140 μg/dL Cu in plasma, respectively) (Fig. 2). Moreover, there were no correlation in Cu concentrations between plasma and OF ($r = 0.44$; $R^2 = 0.20$); oviduct Cu concentrations was about 40 μg/dL regardless of cupremia levels (range, 41–139 μg/dL Cu). Copper concentration in IVF medium was 0.5 ± 0.21 μg/dL.

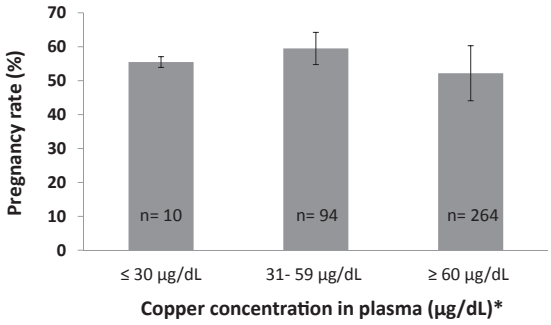


**Fig. 1.** Effect of cupremia at the beginning of the fixed-time artificial insemination protocol on pregnancy rate. *Copper plasma concentrations at the beginning of artificial insemination protocol (d0). Data are expressed as percentage ± standard error (SE). There were no differences in pregnancy rate at 60 d after insemination among deficient (≤30 μg/dL), marginal (31–59 μg/dL), and adequate groups (≥60 μg/dL; $P > 0.05$) (368 Angus cows inseminated: deficient group n = 10, marginal group n = 94 and adequate group n = 264).

### 3.3. Effect of different copper concentration in IVF medium on structural and functional sperm membrane integrity and sperm motility

In Experiment 3, spermatozoa incubated with Cu showed a significant increase in the percentage of live after the 3-h incubation period at any concentrations ($P < 0.05$), but the difference was higher when 0.4 and 0.6 μg/mL Cu was added to IVF medium ($P < 0.05$). However, after 6 h of incubation viability of spermatozoa was only higher with the addition of 0.4 μg/mL Cu ($P < 0.05$); there was no difference in viability when spermatozoa were incubated with 0, 0.2, or 0.6 μg/mL Cu ($P > 0.05$; Table 1).

Percentage of HOST positive sperm was significant decreased in Control as compared to Cu at any concentrations after 3 or 6 h of incubation ($P < 0.05$). There was no difference among different concentrations of Cu at the different time points (0, 3 and 6 h; $P > 0.05$). Besides, functional membrane integrity did not decrease after 6 h of incubation in sperm treated with Cu at any concentrations ($P > 0.05$; Table 1).

Percentage of TM at 0 h was greater when 0.4 μg/mL Cu was added to IVF medium ($P < 0.05$). Spermatozoa incubated with Cu showed a significant increase in the TM after 3-h and 6-h incubation periods at any concentrations ($P < 0.05$). After 3 h of incubation, PM did not differ with 0, 0.2, and 0.6 μg/mL Cu, but was higher with 0.4 μg/mL Cu ($P < 0.05$). There were no differences between 0 and 0.2 μg/mL Cu after 6 h of incubation, but PM was lower with 0.4 and 0.6 μg/mL Cu ($P > 0.05$; Table 1).

### 3.4. Effect of different copper concentration in IVF medium on acrosomal status

In Experiment 4, acrosome integrity did not show a significant difference in sperm treated at any Cu concentrations after 3 or 6 h of incubation.

### 3.5. Effect of different copper concentration in IVF medium on sperm–zona binding

In Experiment 5, considerably more sperm bound to ZP when sperm in the presence of 40 μg/dL Cu ($P < 0.01$). However, there were no significant differences in the number of sperm bound to ZP when 0, 20 or 60 μg/dL Cu were added to IVF medium (Fig. 3).

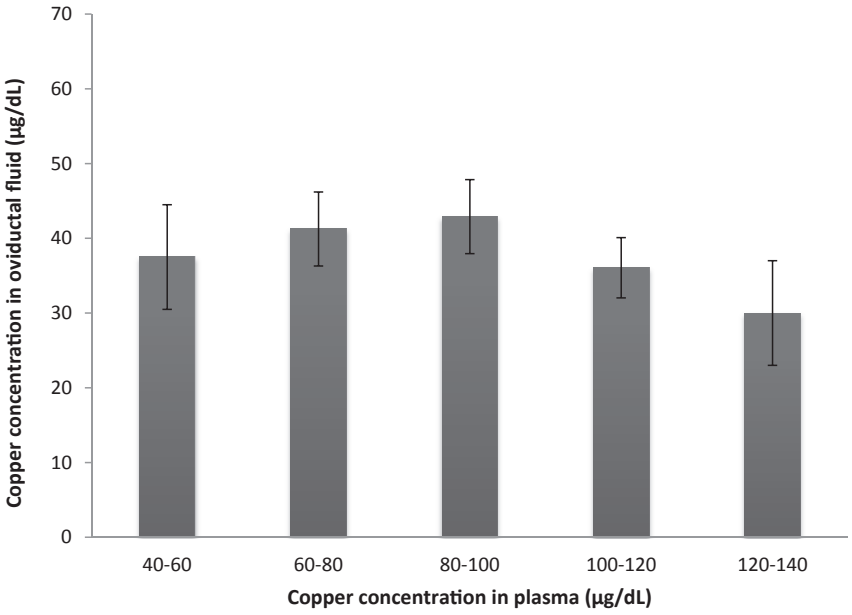


**Fig. 2.** Copper concentration in oviductal fluid according to cupremia. Data are expressed as least square means ± standard error of the mean (LSM ± SEM). There were no differences in copper concentration in oviductal fluid (OF) at any Cu concentration in plasma ($P > 0.05$); OF and plasma from 76 heifers were used in three replicates (23–28 heifers for each day). A total of three OF samples per cupremia group (n = 5) were evaluated.

**Table 1**
Effect of different copper concentration added to IVF medium on structural and functional sperm membrane integrity, and sperm motility.

| Cu µg/dL | Viability (%) | | | Total Motility (%) | | | Progressive Motility (%) | | | HOST positive (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 3 h | 6 h | 0 h | 3 h | 6 h | 0 h | 3 h | 6 h | 0 h | 3 h | 6 h |
| 0 | 80.75$^{a}$ | 53.25$^{a}$ | 55.2$^{a}$ | 91$^{a}$ | 75$^{a}$ | 55$^{a}$ | 56.5$^{a}$ | 27.5$^{a}$ | 18.5$^{a}$ | 38$^{a}$ | 16.7$^{a}$ | 22.2$^{a}$ |
| 20 | 80.25$^{a}$ | 61$^{b}$ | 59$^{a}$ | 94$^{a}$ | 86$^{bc}$ | 65.5$^{b}$ | 57$^{a}$ | 30$^{a}$ | 15$^{a}$ | 38.5$^{a}$ | 35.5$^{b}$ | 38$^{b}$ |
| 40 | 80.25$^{a}$ | 70.5$^{c}$ | 67$^{b}$ | 98$^{b}$ | 91.5$^{c}$ | 59$^{ab}$ | 60.5$^{a}$ | 36.5$^{b}$ | 14$^{a}$ | 38$^{a}$ | 33$^{b}$ | 34.2$^{b}$ |
| 60 | 79.5$^{a}$ | 70$^{c}$ | 53.2$^{a}$ | 93.5$^{a}$ | 83.3$^{b}$ | 68$^{b}$ | 58$^{a}$ | 32.5$^{a}$ | 12$^{b}$ | 37.7$^{a}$ | 36.5$^{b}$ | 40$^{b}$ |

**HOST**, hypo-osmotic swelling test (functional sperm membrane integrity).
Viability, HOST, Total Motility and Progressive Motility are expressed as percentages (four replicates on different days). Viability, HOST, Total Motility and Progressive Motility of sperm cultured in IVF medium supplemented with 0, 20, 40, and 60 µg/dL Cu were evaluated after 0, 3 and 6 h of incubation.
(a,b,c) values with different superscript within a column differ ($P < 0.05$).

### 3.6. Effect of different copper concentration in IVF medium on pronuclear formation

In Experiment 6, the incidence of polyspermy (>2 pronuclei) and the percentage of matured oocytes penetrated by spermatozoa did not differ among the treatments. However, the formation of two pronuclei (normal fertilization) was greater when 0.4 µg/mL Cu was added to IVF medium ($P < 0.05$; Table 2).

### 3.7. Effect of different copper concentration in IVF medium on subsequent embryo development

The Cu concentration that yielded clear effects on sperm viability, sperm–zona binding and pronuclear formation (40 µg/dL) was chosen for this experiment. In Experiment 7, there was no difference in cleavage rate when 0 or 40 µg/dL Cu were added to IVF medium ($P > 0.05$). The presence of Cu in IVF medium impaired the blastocyst rate ($P < 0.05$), but did not modify the hatch rate ($P > 0.05$; Table 3).

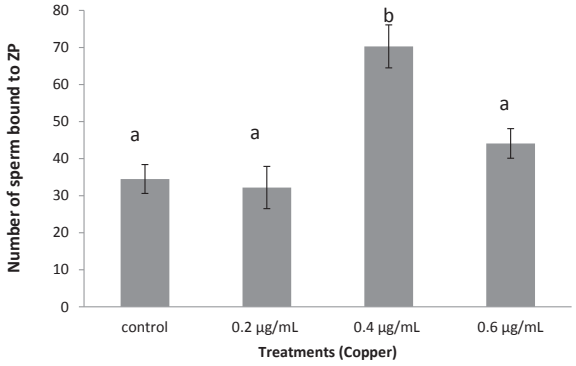


**Fig. 3.** Effect of different copper concentration in IVF medium on sperm–zona pellucid binding. $^{a,b}$Bars with different letters differ statistically ($P < 0.01$). Data are expressed as least square means ± standard error of the mean (LSM ± SEM) (146 denuded COC in three replicates). Oocytes and sperm were incubated for 2 h and then stained with Hoechst 33,342. The number of spermatozoa bound to each egg was determined by observation under an epifluorescent microscope at 400× magnification.

### 3.8. Localization of SLC31A1 in spermatozoa and in vitro matured oocyte

In Experiment 8, using a polyclonal antibody, SLC31A1 staining was detected in the apical segment of the bovine acrosome (Fig. 4A), and in the oolemma of *in vitro* matured oocyte (Fig. 4C and D). No staining was detected in the negative controls for semen and mature oocyte (Fig. 4B and E, respectively).

**Table 2**
Fertilization status of putative zygotes produced *in vitro* with various copper concentrations in IVF medium.

| Cu μg/dL | No. Oocytes | No. **(%)** 1 PN | No. **(%)** 2 PN | No. **(%)** >2 PN | No. **(%)** penetrated |
|---|---|---|---|---|---|
| 0 | 146 | 52[a] **(36)** | 80[a] **(55)** | 4[a] **(3)** | 136[a] **(93)** |
| 20 | 120 | 39[a] **(33)** | 72[a] **(60)** | 1[a] **(1)** | 112[a] **(93)** |
| 40 | 127 | 25[b] **(20)** | 87[b] **(69)** | 5[a] **(4)** | 117[a] **(92)** |
| 60 | 99 | 29[a] **(29)** | 61[a] **(62)** | 2[a] **(2)** | 92[a] **(93)** |

**PN** = Pronucleus.
Pronuclear rate was recorded 18 h after insemination (492 COCs matured and fertilized in five replicates). The presumptive zygotes were incubated in Hoechst 33,342 and then examined under fluorescent microscope at 200× and 400× magnification.
[a,b] Values with different superscript within each column differ ($P < 0.05$).

**Table 3**
Effect of different copper concentration in IVF medium on subsequent embryo development.

| Cu μg/dL | Oocytes (n) | Cleaved | Blastocyst/oocytes | Blastocyst/cleaved | Hatched |
|---|---|---|---|---|---|
| 0 | 182 | 76.3 | 34.0[a] | 44.6 | 51.1 |
| 40 | 162 | 68.5 | 23.4 [b] | 34.2 | 50.0 |

The IVF medium was supplemented with 0 (Control) and 40 μg/dL Cu. 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, development and hatch rates are expressed as percentage (344 COC in four replicates).
(a-b) Values with different superscript within each row differ ($p < 0.05$).

## 4. Discussion

Copper deficiency is reported to be responsible for reproductive disorders such as low fertility [11,12,24,25]. In this study, pregnancy rates in animals with adequate, marginal and deficient Cu concentrations in plasma at the beginning of the FTAI protocol were similar. We suggest that there are at least two possible explanations for these results: (a) the cupremia might not reflect animal Cu status. Blood Cu concentration is widely and routinely used to assess Cu status, but was shown to be influenced by fluctuating concentration of ceruloplasmin (Cp), the major Cu-carrying protein in the blood [26]. About 95% of plasma Cu is bound to Cp [26]. Ceruloplasmin is an acute-phase protein whose concentrations increase after infection/inflammation, disease and stress states and even during normal pregnancy [27–33]. Uriu-Adams et al. [34] pointed out that the use of cupremia as a marker of Cu status in these conditions would mistakenly equate high plasma Cu with "good Cu status" and underestimate the true prevalence of a Cu deficiency. In this study, we found that cupremia on d9 was higher than on d0; this difference not only confirms the variability in cupremia values, but also reflect a stressful effect of FTAI protocol on the animals (cattle must be handled three times to be inseminated). (b) On the other hand, Cu *per se* may not be a main factor in determining the fertility of cattle. This last explanation led us to study the role of this mineral during fertilization.

In mammals, fertilization occurs in the oviduct and during this period, the female and male gametes are dependent on the nutrients provided by the OF [35,36]. It has been shown, that ionic composition of OF is important for oocyte and spermatozoa maturation, and fertilization [36,37]. Even though, the concentration of macrominerals in OF has been studied extensively [37–39], the oviduct concentration of trace minerals such as Cu remain largely unknown. In a previous study, we demonstrated that Cu concentrations in plasma and follicular fluid of heifers are similar [40]. In contrast, in the present study we found no correlation in Cu concentrations between plasma and OF. Oviduct Cu concentration was about 40 μg/dL regardless of cupremia level, suggesting that there might be some kind of regulatory mechanisms for Cu concentration by bovine oviduct epithelium.

SLC31A1 is considered the main Cu uptake transporter in mammalian cells [41]. Current information suggests that the regulation of SLC31A1 occurs mainly by the transporter localization and its expression level [42,43]. SLC31A1 is present in the plasma membrane at times of cellular Cu demand and in intracellular vesicles membrane when there is a high exogenous concentration of this mineral [43–45]. Although, mammalian SLC31A1 mRNA was expressed in all tissues examined, including testes, ovary and embryos [46–48], there are very few studies that reveal the presence of this transporter on cell surface. In the present study, SLC31A1 presence was detected by immunostaining in the oolemma of *in vitro* matured oocyte and in the apical segment of the bovine acrosome. To our knowledge, this is the first study revealing the presence of this transporter in the plasma membrane of mammalian oocytes.

The relationship between Cu and mammalian spermatozoa has been substantially studied. Although, adverse effects of Cu in male reproductive capacity have been shown in a number of studies [49–52], high Cu concentrations were used in all of them. Excess of Cu can oxidize proteins and lipids, increase free radicals production and diminish oxidative processes and glucose consumption, which reduces or abolishes sperm motility [53]. Miska-Schramm et al. [52] have demonstrated that bank voles, when exposed to 600 mg/kg Cu for 12 weeks compromised spermatozoa tail membrane integrity, viability and motility. In rats, spermatozoa became less mobile and the counts of damaged and dead sperm increased after intraperitoneal injection of high concentrations of Cu [49]. Conversely, in the present study we found that addition of 40 μg/dL Cu to IVF medium enhanced total and progressive motility, sperm viability and functional sperm membrane integrity. These results are consistent with that observed by Knazicka et al. [54] who found that supplementation of culture medium with 7.8 μM (49 μg/dL) of Cu stimulates progressive motility of bull spermatozoa after 1 h of incubation. In buffalo, addition of 3.2 μg/dL Cu sulphate ($CuSO_4$) to semen extender enhanced spermatozoa motility and viability after semen dilution and cryopreservation [55]. Moreover, a positive correlation between Cu concentrations in semen and sperm motility has been shown in human [56], rooster [57], and buffalo [58]. In bovine, Machal et al. [59] reported a positive correlation between the Cu concentration in blood plasma and sperm progressive motility.

A limited generation of reactive oxygen species (ROS) by the spermatozoa is implicated in the control of normal sperm function, but an excessive ROS production induces DNA damage and impairs sperm motility, mitochondria homeostasis and membrane permeability, which render the sperm cell unable to fertilize [60–63].

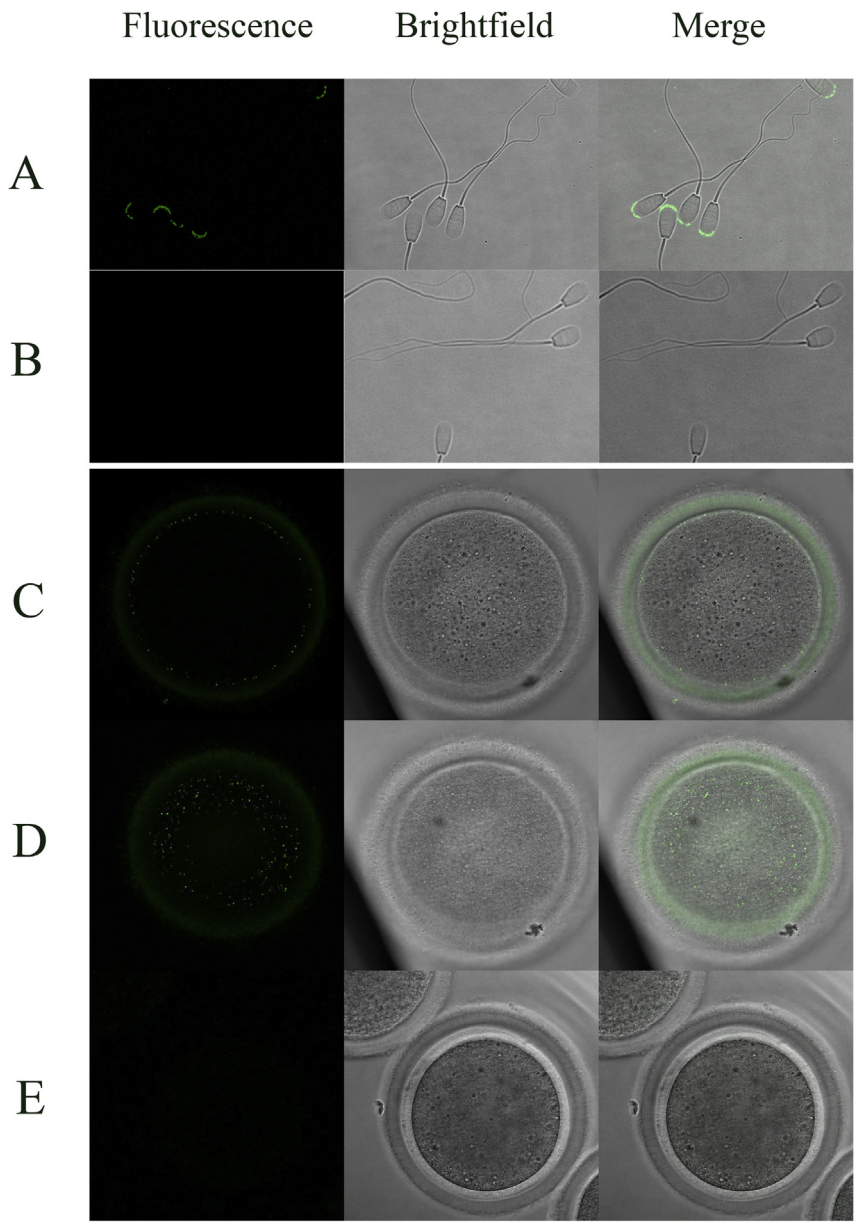


**Fig. 4.** Immunofluorescence staining of Cu transporter SLC31A1 in bovine spermatozoa and *in vitro* matured oocyte. Sperm and oocytes were stained with rabbit polyclonal anti-CTR1 antibody and observed under a *confocal microscope* at 630× magnification. SLC31A1 staining was detected in the apical segment of the bovine acrosome **(A)**, and in the oolemma of *in vitro* matured oocyte (**C** and **D**; two different focal planes of the same oocyte). No staining was detected in the negative control for semen **(B)** and mature oocyte **(E).** Negative controls were obtained by substituting the incubation with primary antibody for incubation with Blotto for semen, and PBS/1% (w/v) BSA for oocytes.

Therefore, the antioxidant role of Cu might be an important mechanism to improve spermatozoa quality. Copper is a metal cofactor for numerous enzymes including diamine oxidase, tyrosinase, cytochrome *c* oxidase (COX) and the antioxidant copper-zinc superoxide dismutase (Cu/Zn-SOD; [64]). In Cu/Zn SOD, Cu is needed to maintain full catalytic activity and cannot be substituted with other metal [65]. Superoxide dismutase plays the major role in protecting spermatozoa against oxygen toxicity and lipid peroxidation, and is produced by the testis, epididymis, accessory reproductive organs and even spermatozoa [66–69]. In mammals, SOD activity in seminal plasma was positively associated with progressive motility, viability and spermatozoa concentration [58,69,70]. Recently, Kobayashi et al. [71] concluded that SOD produced in the oviduct may be able to improve sperm quality or male fertility via protection from oxidative stress. Although we did not examine the SOD activity of spermatozoa in this study, this could be increased by the Cu supplementation of IVF medium. On the other hand, sperm motility is dependent on ATP produced by aerobic metabolism. Mitochondrial COX is the final electron acceptor and the apparent rate-limiting step of the mitochondrial respiratory chain [72]. It has been established that this copper-dependent metalloenzyme is required for aerobic ATP production and requires Cu for its biogenesis, assembly, stability and catalytic function [73].

Other effects of adequate ROS levels on sperm function are related to spermatozoa ZP binding and the activation of acrosome reaction [74,75]. It has been demonstrated that lipid peroxidation, resulting from low concentration of ROS, promotes sperm binding to the ZP [76]. Tsunoda et al. [77] demonstrated that more Cu/Zn SOD deficient (Sod1-KO) mice sperm were found to bind the ZP after insemination compared to wild-type mice sperm. In the present study, the addition of 40 μg/dL Cu to IVF medium increased

the number of sperm bound to ZP. In addition, Cu supplementation did not vary the acrosome status. These results are consistent with that observed by Roblero et al. [78], who found that the AR was not affected when human sperm were incubated in medium containing concentrations of Cu ranging from 1 μg/dL to 1 mg/dL.

Recently, it has been shown that Cu/Zn SOD deficient mice sperm have a reduced capability to penetrate the zona pellucida during IVF [77]. In the present study, although, the presence of Cu in IVF medium did not affect the rate at which oocytes were penetrated, 40 μg/dL Cu increased the formation of two pronuclei. Pronuclei formation is considered the first major cellular event following gamete union [79]. Mammalian oocytes matured *in vitro* have low competence for male pronuclear formation [80–82]. This deficiency is improved by increasing the concentration of glutathione (GSH) in oocytes [83]. GSH is an important cytoplasmic factor for decondensing spermatozoan nuclei after fusion of the spermatozoa with the egg and to form the male pronucleus [84,85]. The relationship between GSH and Cu has been substantially studied; Cu is a biological ligand forming binary complexes with GSH [86]. In a previous study, we demonstrated that supplementation of IVM medium with 40 μg/dL of Cu increased intracellular GSH concentration in bovine oocyte [40]. Moreover, de Matos and Furnus [87] concluded that stimulation of GSH synthesis during IVM increased the percentage of cleavage rate by the improved protection against oxidative stress during IVF. In the present study, the cleavage rate did not vary with Cu supplementation of IVF medium.

Interestingly, the Cu concentration that produced best results in the different sperm parameters evaluated was similar to the concentration found in OF. Collectively, the data show that the addition of 40 μg/dL of Cu to IVF medium increased bull sperm quality *in vitro*. In spite of Cu concentration found in OF (38.3 ± 2.17 μg/dL Cu) we found a negative effect on blastocyst rate when 40 μg/dL Cu was present during IVF. This may be because 95% of Cu *in vivo* is bound to ceruloplasmin [26], whereas *in vitro* the supplementation was performed with copper sulphate adding to IVF medium Cu but in the free form. Although Cu is essential for many biological processes, because it is a transition metal, excess of Cu can cause oxidative damage when it is in the free ion form [88].

The presence of the SLC31A1 transporter in both the mature oocyte and spermatozoa, as well as the beneficial effect of Cu on sperm quality would suggest an important role of this mineral during the fertilization process. Although, it has been shown that hypocupraemia does not affect fertility in cattle [89–91], perhaps reproductive dysfunction appears at very low levels of cupremia. Probably, both the gamete and each stage of embryonic development might have a specific Cu requirement. According to our studies, the adequate concentration of Cu for oocyte maturation might be 60 μg/dL [40]; however, for fertilization and intraoviductal embryo development could be 40 μg/dL, concentration that was maintained in OF even in the animals considered as marginal deficient [17]. More studies are needed to define more precisely the value of cupremia affecting reproductive performance.

## 5. Conclusions

The results from the present study showed that cupremia levels at the beginning of FTAI protocol did not influence pregnancy rate at 60 d after insemination. Cu concentration in bovine OF was about 40 μg/dL regardless of cupremia. Moreover, the presence of Cu in IVF medium, at similar concentrations to those found in OF, improved sperm quality parameters including, total and progressive motility, sperm viability and functional sperm membrane integrity. In addition, number of sperm bound to the ZP and pronucleus formation was increased by Cu. Nevertheless, blastocyst rate was impaired when 40 μg/dL Cu was added to IVF medium. Cu transporter SLC31A1 was detected for the first time in bovine spermatozoa and *in vitro* matured oocyte. The mechanism by which Cu improves sperm quality *in vitro* is under current investigation. We inferred that Cu could play an important role in the fertilization.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

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