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Superoxide Dismutase Activity, Hydrogen Peroxide Steady-State Concentration, and Bactericidal and Phagocytic Activities Against *Moraxella bovis*, in Neutrophils Isolated from Copper-Deficient Bovines

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Abstract Copper (Cu) deficiency increases occurrence of certain infectious diseases in animals, including infectious keratoconjunctivitis in bovines, a bacterial ocular inflammation caused by *Moraxella bovis*. Neutrophil leukocytes constitute the first phagocytic cells to arrive at infection sites for bacterial neutralization. The objective of this work was to evaluate whether the functionality of neutrophils against *M. bovis* is impaired in experimentally induced Cu deficiency in bovines using high molybdenum and sulfur levels in the diet. The Cu tissue values and the periocular achromotrichia observed in +Mo animals showed that the clinic phase of Cu deficiency was reached in this group. Instead, +Cu animals have not evidenced clinical signs or biochemical parameters of hypocuprosis. On the basis of our observations, we concluded that Cu deficiency has no effect on phagocytic and bactericidal activities of neutrophils against *M. bovis*. However, superoxide dismutase activity and peroxide hydrogen generation were significantly different between groups. Therefore, additional research to explain these results is merited to fully characterize the consequences of Cu status on the risk for infections under field conditions.

Keywords Trace elements · Copper · Innate immunity · Phagocytic cells · Respiratory burst · Calves

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Introduction

Although the essential role of copper (Cu) for the optimal function of the immune system of animals and man has become increasingly apparent [1–4], our current understanding regarding the functionality of the host defense systems in ruminants with Cu deficiency remains speculative [5]. Cu deficiency increases occurrence of certain infectious diseases in animals [6–10], including infectious keratoconjunctivitis in bovines [8], a bacterial ocular inflammation caused by *Moraxella bovis* [11]. Neutrophil leukocytes constitute the first phagocytic cells to arrive at infection sites for bacterial neutralization. The release of preformed enzymes and proteins and the generation of potentially damaging reactive oxygen species (EROS) are intracellular defense mechanisms referred to as a respiratory burst, which are required to kill engulfed pathogens during microbicidal activity [12]. The alteration of Cu status can result in changes in the activity of specific cuproenzymes which can affect EROS production, such as superoxide dismutase (SOD) [5, 12, 13]. When the SOD activity is decreased, superoxide anion (O_2^-) cannot be converted into molecular oxygen and hydrogen peroxide (H_2O_2), the substrate for the generation of bactericidal hypochlorite and hydroxyl radicals [12]. This work was performed with the objective to evaluate whether the functionality of neutrophils against *M. bovis* is impaired in bovines with Cu deficiency experimentally induced with high molybdenum and sulfur levels in the diet.

Materials and Methods

Animals

All procedures received prior review and approval by the Institutional Experimental Animal Care and Use Committee of

Buenos Aires University, Faculty of Veterinary Science. Seventeen castrated male Holstein calves, approximately 2 months old and 77 ± 10.2 kg average weigh at the beginning of the experiment, were used.

Diet

Calves were fed with a basal diet containing corn grains (60 %), feather flour (15 %), wheat straw (22 %), and a mineral and vitamin mix without Cu (3 %), prepared to meet the nutritional requirements recommended by the National Research Council [14] to gain an average of 500 g per day. This diet contained 3.8 mg of Cu/kg dry matter (DM), 0.4 mg of Mo/kg DM, and 0.4 g of S/kg DM. The animals had free access to water. After 4 weeks of adaptation of the animals to the diet, calves were allocated randomly by weight and liver Cu level in two groups, into a covered facility with slatted concrete floors (0 day). One group (n = 8) received the diet supplemented with 9 mg of Cu/kg DM as copper sulfate (+Cu group), while the other group (n = 9) received the diet supplemented with molybdenum (Mo) as sodium molybdate (11 mg of Mo/kg DM), to get a Cu/Mo relation of 1/3, and sulfur (S) as sodium sulfate, to reach 3 g of S/kg DM (+Mo group). This diet was successfully used to induce Cu deficiency by our group in previous experiments [8, 15].

Plasma and Liver Samples

Body weights were measured and samples of blood and liver were taken every 28 days.

Blood samples were obtained by jugular venipuncture with disposable syringes (20 ml, Prexajet) and needles (40 × 12, Alfadoves). Fifteen milliliters (ml) of blood was collected in trace mineral-free heparinized tubes. The tubes were placed in a storage container at 4 °C and then centrifuged at 2195g for 15 min at 4 °C. Plasma was removed for Cu analysis.

Liver samples were obtained by biopsy under local anesthesia, as described by Engle and Spears [16].

Copper Determination

Cu levels were measured in the liver (expressed as μg/g DM) and plasma (expressed as μg/dl) by flame atomic absorption spectroscopy (Model 250 AA, Metrolab). Plasma Cu levels were determined after precipitation by 10 % trichloroacetic acid (Biopack). Hepatic biopsies were dried overnight at 90 °C and were digested by wet ashing procedure using nitric and perchloric acids (1:1) (Carlo Erba). The residue was resuspended in 10 % nitric and perchloric acids (1:1), for mineral estimation.

Standard curves were prepared from certified Cu reference solution (Carlo Erba), at different concentrations (0, 0.25, 0.5, 1, and 2 ppm).

Preparation of *M. bovis*

M. bovis EPP63 was kindly provided by Dr. Gustavo Zielinski (INTA, National Institute of Technology, Marcos Juárez, Córdoba, Argentina). Glycerol stock cultures were stored frozen at −20 °C. Subsequently, bacteria were streaked onto blood agar plates (Britania) and grown overnight at 37 °C in aerobic conditions. Cultures were scraped from the surface of blood agar plates after 24 h of growth. Harvested bacterial cells were suspended in 10 ml of sterile phosphate-buffered saline (PBS). Serial tenfold dilutions of bacteria were made in sterile PBS. The final work concentration was 3×10^6 colony forming unit (CFU) per milliliter (confirmed 24 h later by triplicate plate counts).

Isolation of Polymorph Nuclear Cells and Cell Viability

When calves from the +Mo group showed Cu deficiency, polymorph nuclear neutrophils were isolated from fresh whole blood under sterility conditions, according to the procedure described by Carlson and Kaneko [17] with some modifications. Fourteen milliliters of blood was collected from all the animals, by jugular venipuncture with disposable syringes (20 ml, Prexajet) and needles (40 × 12, Alfadoves), in plastic heparinized tubes. The tubes were placed in a storage container at 4 °C immediately. The blood was divided into four 10-ml aliquots and centrifuged at 1000g for 15 min at 4 °C. Plasma was harvested at 4 °C. Mononuclear cell band and the top portion of the packed red blood cells were discarded. The remaining erythrocytes were suspended in 4 ml of 0.8 % NaCl in 0.0132 M phosphate buffer (pH 6.8). Red cells were subjected to hypotonic lysis by the addition to each sample of 20-ml ice-cold deionized distilled water for 30 s before isotonicity was restored with 10 ml of 2.7 % NaCl in 0.0134 M phosphate buffer (pH 6.8). The tubes were centrifuged at 200g for 8 min, and the supernatants were aspirated and discarded. The cell pellets were pooled and gently resuspended in 5 ml PBS. The pooled pellets were washed two to four times with 35 ml PBS at 200g for 8 min. The final white cell pellets were resuspended in 1.5 ml of sterile Hank's balanced salt solution (HBSS). Cell concentration and viability were determined by hemocytometer count using 1 % aqueous trypan blue dye exclusion [18]. Cells were adjusted to the final concentration for each procedure with HBSS. An aliquot of 2 μl was diluted 1:1 with plasma to perform the differential cell count by light microscopy examination of Wright's stained smears. The neutrophils were immediately used to measure antibacterial and phagocytic activities against *M. bovis* in vitro. A fraction of neutrophils from six animals (two from the +Cu group and four from the +Mo group) was used immediately to determine H_2O_2 steady-state concentration, in a medium with *M. bovis* in vitro. Another fraction was kept frozen at −20 °C for SOD determination.

Bactericidal and Phagocytic Activities

The technique described by Xin et al. [19] and Gengelbach et al. [20] for *Staphylococcus aureus* with brief modifications was used to determine neutrophil bactericidal and phagocytic activities. Viable *M. bovis* (50 μl, 3×10^6 CFU/ml sterile PBS) were opsonized with homologous fresh undiluted sera (50 μl, pooled from each group of calves that provided the neutrophils), at 37 °C for 3 h in a shaking water bath. Following preincubation, 500 μl of neutrophil suspensions (9×10^6 cells/ml; neutrophil/bacteria ratio 1:12) were added to each tube, and the mixture was incubated at 37 °C with gentle shaking for another 30 min. Control tubes (blank) contained 500 μl of HBSS instead of neutrophil suspension. After incubation, 2 μl of the mixtures were placed in slides, stained with May-Grünwald/Giemsa, and examined microscopically to determine phagocytic activity. One hundred randomly phagocytic neutrophils were counted, and the percentage of those containing intracytoplasmatic *M. bovis* was calculated. Neutrophils were lysed by freezing and thawing (three cycles of −20 to 20 °C for 10 min each). Aliquots of 100 μl were serially diluted with PBS and plated in triplicate on dried blood agar (Britania). Following incubation at 37 °C for 24 h in aerobic conditions, the average CFU/ml was determined on both control and sample group plates, and neutrophil-mediated killing was determined by subtracting the log of CFU/ml in blank from the log of CFU/ml in samples and expressed as percentage (%) of bactericidal killing.

Superoxide Dismutase Activity Determination

SOD activity was determined as described by Jones and Suttle [21]. The assay involves the ability of SOD to inhibit the reduction of nitroblue tetrazolium violet (NBT) by O_2^-, which is generated by the reaction of xanthine and xanthine oxidase (XO) enzyme. For calibration purposes, SOD standards (Sigma Chemical Co.) prepared from a 30 UI/ml stock in 0.01 M phosphate buffer (pH 7.0) and stored at −20 °C were assayed in parallel to samples. SOD activity was determined in 1 ml of final reaction solution containing 0.100 ml of standard or sample (undiluted leukocyte lysate after three freeze-thaw cycles); and 0.850 ml of 50 mM carbonate buffer (pH 10.2) containing 0.26 g of sodium carbonate, 0.19 g of sodium bicarbonate, 0.017 ml of 10 mM xanthine in 1 M sodium hydroxide, and 0.00425 ml of 2.5 mM NBT in 11 % ethanol/l. 0.050 ml of XO (0.2 UI/ml) was added. Absorbance was read for 5 min at a wavelength of 500 nm (Model 250 AA, Metrolab) at room temperature. SOD activity was expressed as UI/9×10^6 neutrophil cells.

Hydrogen Peroxide Steady-State Concentration

Hydrogen peroxide (H_2O_2) was measured by fluorescence in a suspending medium during the respiratory burst of neutrophils against *M. bovis* in vitro, according to the procedure described by Boveris et al. [22]. It is understood that H_2O_2 is produced in all aerobic cells and is highly diffusible across biological membranes, so the concentration of H_2O_2 in the extracellular suspension reaches a diffusion equilibrium with the internal H_2O_2 steady-state concentration ($[H_2O_2]ss$) [22]. Horseradish peroxidase (HRP) reacts with H_2O_2 forming an enzyme-substrate complex that in combination with a hydrogen donor, *p*-hydroxyphenylacetic acid (*p*-HPA), yields a fluorescent tetramer.

Viable *M. bovis* (50 μl, 1.25×10^8 CFU/ml sterile PBS) were opsonized with homologous fresh undiluted sera (50 μl, pooled from each group of calves that provided the neutrophils), at 37 °C for 3 h in a shaking water bath. Following preincubation, neutrophil cells (500 μl, 3×10^6 cells/ml; neutrophil/bacteria ratio 1:8.3) of each animal were added, and the mixture was incubated at 37 °C with gentle shaking. Aliquots of 500 μl were taken from the incubation medium at 5, 15, 25, and 45 min, and analyzed for $[H_2O_2]$. A fast centrifugation at 11,000*g* for 30 s at 4 °C provided a clear supernatant for analysis. Medium aliquots were divided into two parallel samples for each animal (measurement and control), both added a volume of 1 ml of 100 mM phosphate buffer (pH 7.4) containing 0.056 μl HRP 5000 UI/ml (Sigma Chemical Co.), and 1 μl *p*-HPA 40 μM (Sigma Chemical Co.) as hydrogen donor, being the control sample pretreated with 12 μl catalase enzyme 0.1 mM (Sigma Chemical Co.) [23]. Fluorescence was measured with excitation at 317 nm and emission at 414 nm, in a Hitachi fluorometer model F-3010. The assay was calibrated with a standard 40 μM H_2O_2 solution (Merck Química) tested spectrophotometrically at 240 nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$). The differential fluorescence readings (measurement minus control) were translated into H_2O_2 concentrations by the calibration relationship. With the purpose of evaluating basal neutrophil activation by the transportation and manipulation movements, the $[H_2O_2]$ in the surrounding space was carried out using neutrophils that were none incubated with *M. bovis* in two animals from each group.

Statistical Analysis

Cu levels in plasma and liver, and body weight were analyzed by ANOVA. Differences among treatment means were separated using single degrees of freedom orthogonal contrasts. The SOD activity, bactericidal and phagocytic activities, and $[H_2O_2]$ in the suspension media were analyzed by Student's *t* test. For all the analyses, *P* values ≤0.05 were considered to be statistically significant. Statistix program was used (7.0

Table 1 Effect of treatments on parameters indicative of Cu deficiency during the experiment

Parameters	0 day		341 days	
	+Cu group	+Mo group	+Cu group	+Mo group
Body weight, kg	74.06 ± 12.2	79.12 ± 7.6	292.06 ± 74.1	269.78 ± 51.6
Plasma Cu, μg/dl	91.57 ± 14.97	91.87 ± 12.75	73.04 ± 9.51a	23.84 ± 6.74b
Hepatic Cu, μg/g DM	793.59 ± 254.3	777.84 ± 275.9	182.26 ± 52.84a	7.58 ± 1.84b

Data was expressed as mean ± standard deviation. Different letters indicate significant differences between groups ($P < 0.001$)

version, 1990, Analytical Software). Data was expressed as mean ± standard deviation.

Results

Induction of copper deficiency lasted 341 days. At this time, significant differences between groups were observed in liver and plasma (Table 1). Body weights were not affected by dietary treatment throughout the trial ($P = 0.19$).

Levels indicative of Cu deficiency for bovines [13] were detected at 224 days (23 ± 11.6 μg/g DM) for liver Cu concentration and at 198 days (55 ± 10.4 μg/dl) for plasma Cu concentration, in calves of the +Mo group.

After 33 weeks of the trial, all the animals from the +Mo group showed changes in hair pigmentation around the eyes. This sign is associated with a depressed tyrosinase enzyme activity, since Cu is an essential constituent of this enzyme, particularly involved in melanin synthesis [24].

Phagocytic activity of neutrophils was 85 ± 3.9 % for the +Cu group and 86 ± 4.1 % for the +Mo group (Fig. 1). No differences were found between groups ($P = 0.62$). Bactericidal activity of polymorph nuclear cells was 52 ± 21.6 % for the +Cu group and 64 ± 25.5 % for the +Mo group (Fig. 2). There were no differences ($P = 0.32$) between groups.

Superoxide dismutase activity (Fig. 3) in neutrophils from the +Cu group was 0.06 ± 0.01 UI/9 × 10^6 neutrophil cells and 0.03 ± 0.01 UI/9 × 10^6 neutrophil cells for the +Mo group, with significant differences between treatment and control animals ($P = 0.002$).

Hydrogen peroxide steady-state concentration ($[H_2O_2]ss$) in nonstimulated neutrophils did not differ among treatments at all evaluated times, with an average value of 0.08 nmol/10^7 cells. The stimulation of polymorph nuclear cells from the +Cu animals with *M. bovis* produced a marked increase of $[H_2O_2]ss$ in the suspension medium up to 0.8 ± 0.07 nmol/10^7 cells at 25 min of reaction. Conversely, polymorph nuclear cells from the +Mo animals stimulated with *M. bovis* have slightly modified the $[H_2O_2]ss$ in the suspension medium (0.1 ± 0.06 nmol/10^7 cells), being significant the differences between groups ($P = 0.005$) at 25 min after *M. bovis* stimulation (Fig. 4).

Discussion and Conclusion

The Cu tissue values and the periocular achromotrichia observed in the animals from the +Mo group showed that the clinic phase of Cu deficiency was reached in this group. Instead, +Cu animals have not shown clinical signs or biochemical parameters of hypocuprosis.

The phagocytic and bactericidal activities in neutrophils isolated from the +Mo group were not significantly different from those isolated from the +Cu group. The phagocytic activity seemed not to be affected in polymorph nuclear cells from bovines [8, 19, 20, 25–27], ovine [21], and male rats [28], with Cu deficiency. In contrast to the current study,

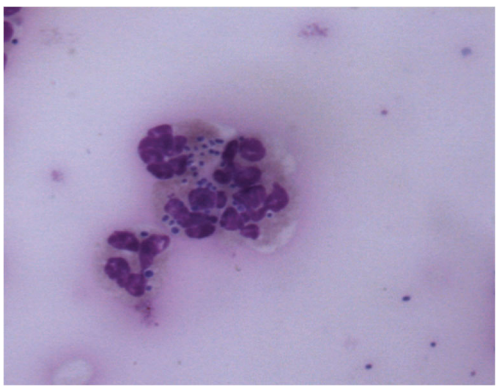

Fig. 1 Five neutrophils with intracytoplasmatic bacteria (May-Grünwald/Giemsa, ×1000)

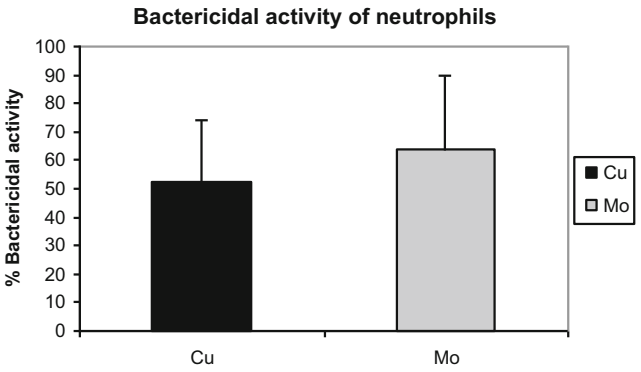

Fig. 2 Effect of treatments on bactericidal activity of neutrophils

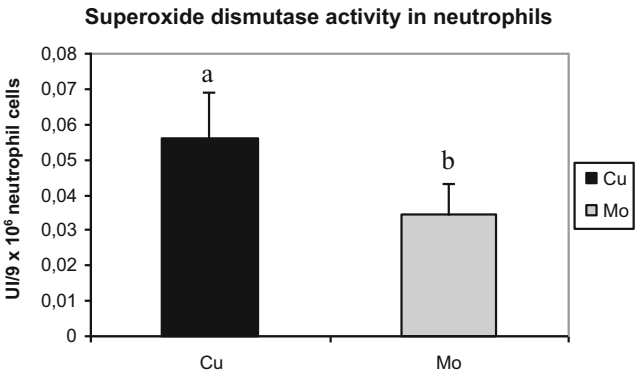

Fig. 3 Effect of treatments on SOD activity of neutrophils. *Different letters* indicate significant differences between groups

however, several investigators have found that the ability of neutrophils from Cu-deficient bovines to kill different pathogens is usually compromised [19, 21, 25, 27, 29], and only a few earlier reports are in agreement with our result [20, 26, 30]. A significant decreased activity of SOD in neutrophils isolated from Cu-deficient bovines in comparison to the cells isolated from controls has also been reported previously [8, 19–21, 29, 31, 32]. Moreover, the lower [H_2O_2] in the suspension medium with polymorph nuclear cells from the +Mo group is consistent with other studies in which a decreased production of H_2O_2 by neutrophils [26] or macrophages [33] from Cu-deficient bovines was observed. It is important to emphasize that the basal [H_2O_2] situation indicates that movement had no significant effects on prior cell activation. If neutrophils with an altered SOD activity are exposed to stimuli, a minor generation of H_2O_2 might cause a decreased supply of substrate to the myeloperoxidase-halide system to produce bactericidal killing agents into the phagosome, such as hypochlorite ions and hydroxyl radicals. Surprisingly, we have found similar values for neutrophil bactericidal capacity in both groups, so several hypotheses could be stated to explain this result.

During respiratory burst, the potent free radical O_2^- dismutates to H_2O_2 in a spontaneous or SOD-catalyzed reaction, or reacts with nitric oxide to produce the highly cytotoxic peroxynitrite ($ONOO^-$) [12], as we show in Fig. 5. It is therefore conceivable that neutrophils from the +Mo group could generate more $ONOO^-$ from O_2^-, as a potential mechanism of adaptation to kill *M. bovis* efficiently.

Another interesting point to consider is the influence of *M. bovis* extracellular leukotoxins over neutrophil functionality. Kagonyera et al. [34, 35] have evaluated the cytotoxicity of living *M. bovis* for bovine neutrophils. The cytotoxic effect seemed to be rapid, dependent on the ratio of bacteria to phagocytic cells, and was related to hemolysin production. Therefore, the liberation of these toxins into the liquid media might lead to an impaired bactericidal activity and prevented the detection of any treatments effects. In previously cited investigations, they have used yeast [21, 25, 29] or *S. aureus* bacteria [19, 27] as the target organism, whereas we used *M. bovis* in our experiments. It is possible that phagocytic cells may respond differently due to the nature and toxicity of the pathogen. *Mannheimia* (formerly *Pasteurella*) *haemolytica*, a bacterium that causes an acute fibrinous pleuropneumonia in cattle, produces a potent extracellular leukotoxin that is lethal for bovine leukocytes, similarly to *M. bovis*. Earlier studies have reported a rapidly diminished chemiluminescence response to opsonized zymosan of bovine neutrophils preincubated with *M. haemolytica* leukotoxin, which may implicate a mechanism that abbreviates the oxidative burst and compromise the ability of bovine neutrophils to produce bactericidal oxygen intermediates [36, 37].

Technical differences in the procedure may have contributed to eliminate any differences between groups. One possibility could be the result of the relationship bacteria/neutrophils used in our experiment, related to the action of leukotoxins on phagocytic cells viability, previously explained. Other reports have obtained significant differences in neutrophil microbicidal activity using a lower target/effector cell ratio or a prolonged time to put in evidence the outcome of the respiratory burst [20, 21, 25, 26, 29].

Our data indicate that neutrophil capacity to engulf and kill *M. bovis* was not altered in Cu-deficient bovines. However,

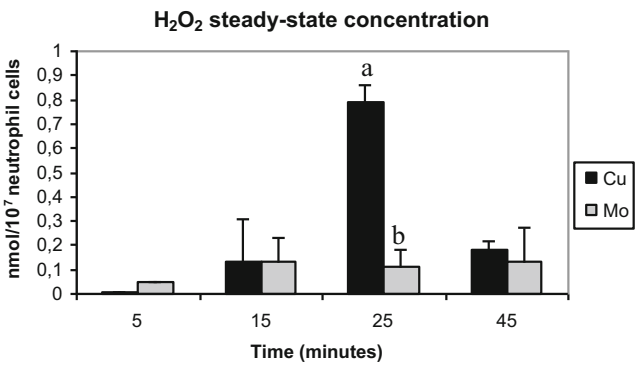

Fig. 4. Effect of treatments on [H_2O_2] in the suspension medium. *Different letters* indicate significant differences between groups

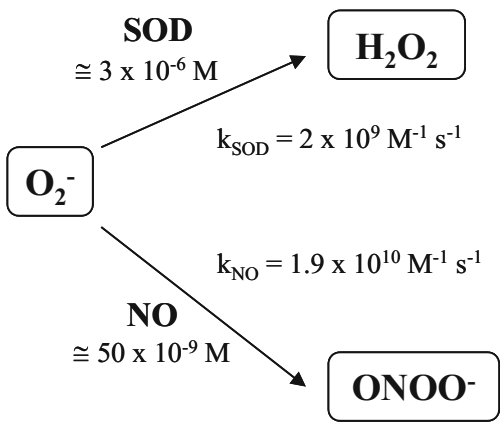

Fig. 5 H_2O_2 and $ONOO^-$ formation from O_2^-

dietary treatment differences were detected for SOD activity and [H_2O_2] in the suspending medium. The reasons for this discrepancy are not clear, and the in vivo implications of the present findings are uncertain. Therefore, additional research to explain these results is merited to fully characterize the consequences of Cu status on the risk for infections under field conditions, as was observed in ruminants with experimentally induced secondary Cu deficiency [8, 10].

Compliance with Ethical Standards

Ethics Approval All procedures received prior review and approval by the Institutional Experimental Animal Care and Use Committee of Buenos Aires University, Faculty of Veterinary Science.

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