



## ***In vitro* effect of copper treatments on mitogenic response and superoxide dismutase expression of lead exposed leukocytes from periparturient dairy cows**

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### ABSTRACT

The aim of this study was to evaluate whether copper (Cu) treatment in lead (Pb)-exposed lymphocytes can modulate their proliferation and superoxide dismutase (SOD) expression. Blood samples were collected from advanced pregnant crossbred dairy cows at –30, –15, 0, 15, and 30 days of calving and evaluated for lymphocytes proliferation and mRNA expression of SOD. A fixed number of lymphocytes ( $2 \times 10^6$ ) were cultured for 72 h with  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  molar (M) levels of Pb. Pb-exposed lymphocytes were treated with 30, 35 and 40 micro molar ( $\mu$ M) Cu. Mitogenic response of lymphocytes and mRNA expression of SOD reduced as the days of parturition advanced and were noted to be lowest at the day of calving. Reduced mitogenic response of lymphocytes and mRNA expression of SOD was reported in Pb-treated groups as compared to control. Addition of Cu in the Pb-exposed lymphocytes culture improved lymphocytes proliferation and relative mRNA SOD expression. Lymphocytes proliferation and relative mRNA SOD expression was reported highest in 40  $\mu$ M Cu-treated groups. These results indicated that Cu can ameliorate the adverse effects of Pb on lymphocytes proliferation and SOD expression in periparturient dairy cows.

**Key words:** Copper, Lead, Lymphocytes proliferation, Periparturient cows, Superoxide dismutase

The immune system of dairy cows is challenged at the peri-parturition period, resulting in an increased susceptibility to infectious diseases (Deka *et al.* 2014). Negative energy balance, increased reactive oxygen species (ROS) synthesis and elevated blood cortisol at the peri-parturition period have been postulated to be the major causes for reduced antioxidant status and immunosuppression (Burton *et al.* 1995, Gabai *et al.* 2004). Besides periparturient period, heavy metals exposure also suppresses antioxidant activity and immune status of animals (Aboud 2010, Kumar *et al.* 2013). Pb-exposure inhibits sulphhydryl dependent enzymes or antioxidant enzymes activities and/or increases susceptibility of cells to oxidative attack by altering membrane integrity and fatty acid composition (Hande and Naran 2000). Pb may also

compete with essential metallic cations for binding sites, or altering the transport of essential cations such as calcium, Cu, Zn etc. (Flora *et al.* 2008). ROS generated due to lead exposure reduces the immunity status of animals by suppressing B-cell, helper T-cell, macrophage functions or neutrophil function (Rada and Leto 2008).

Dietary supplementation of antioxidants micronutrients helps to ameliorate the adverse effect of oxidative stress results due to periparturient stress and heavy metals (Poljsak and Fink 2014). SOD requires Cu for its biological activity and loss of Cu results in complete inactivation of SOD and induces many diseases in dairy animals (Mizuno 1984, Noor *et al.* 2002). Considerable research has indicated that dietary Cu affects phagocytic as well as specific immune function (Weiss and Spears 2006, Wintergrest *et al.* 2007, Djoko *et al.* 2015). The effects of Cu supplementation on antioxidant activity and immune response of Pb-exposed periparturient animals are not fully understood. Therefore, this study was designed to determine whether supplementation of Cu in Pb-exposed periparturient lymphocytes can modulate their proliferation and SOD expression.

### MATERIALS AND METHODS

*Animals and experimental design:* Animal care procedures were approved and conducted under the established standard of the Institutional Animal Ethics Committee (IAEC).

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The effect of different levels of Pb and Cu on lymphocytes proliferation and SOD expression was studied in periparturient Karan Fries cow (Tharparker × Holstein-Friesian) maintained at Cattle Yard of the Institute. The nutrient requirements of cows were met by feeding concentrated mixture, wheat straw and available fodder (NRC 2001) and animal had free access to drinking water. The ingredients and chemical composition of basal diet fed during experimental period is depicted in Table 1.

Blood samples were collected at 07:00 h in heparinized vacutainer tubes by venipuncture of anterior vena cava at –30, –15, 0, 15 and 30 days around calving. Collected blood samples were used for determination of lymphocytes proliferation index and relative abundance of SOD mRNA expression.

**Lymphocytes proliferation index:** Lymphocytes were isolated from whole blood samples with the help of lymphocytes separation medium (*Histopaque 1077*) and  $2 \times 10^6$  cells were grown in F-bottom 96 well ELISA plates containing 10% foetal bovine serum supplemented Dulbecco's Modified Eagle's medium. Lymphocytes were grown in culture medium for 72 h with  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  M Pb. To counteract the adverse effect of Pb, 30, 35, and 40  $\mu$ M Cu was added into the culture medium.

**Lymphocytes proliferation assay:** The proliferative response of lymphocytes was estimated using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide] assay (Mosmann 1983). Cells were seeded at a final volume of 0.25 ml in 96-well flat-bottom microtiter plates in triplicate aliquots. The T-cell selective mitogen used was concanavalin A (Con A), added at 1  $\mu$ g/ml to the micro cultures. Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere for 72 h. After incubating the plate at room temperature for 15 min, the optical density was read using ELISA reader in dual wavelength measuring system, at a test wavelength of 540 nm and a reference wavelength of 630 nm.

**SOD mRNA expression:** SOD mRNA expression level was estimated from cultured lymphocytes using the real-time PCR technique with fluorogenic primers.

**Primers:** Primers for SOD were i.e. forward primer (CAC GAC GAG GCA AAG GGA GAT ACA GTC GTG) and reverse primer (TCC AAA CTG ATG ACA GTG GAA) and primers for  $\beta$ -actin (reference gene) i.e. forward primer (GTA CGA TGG GCC AGA AGG ACT CGT AC) and reverse primer (TGA CGA TGC CGT GCT CCA T) - (desalted oligonucleotides, 25 nmol, 15–45 bases, 3 ODs). The amplicon product size was 93 bp for Cu/ZnSOD gene, and 96 bp for  $\beta$ -actin reverse keeping gene.

**cDNA synthesis and quantitative real-time PCR:** Total RNA was isolated from cultured lymphocytes and cDNA was synthesized by using a cells-to-cDNA II kit. Real-time PCR was performed using the SYBR Green qPCR SuperMix which served as a double-stranded DNA-specific fluorescent dye in 25  $\mu$ l reaction to assess the SOD mRNA expression relative to housekeeping  $\beta$ -actin gene. Each cDNA sample was analyzed in triplicate for quantitative assessment of RNA amplification with PCR primers. To examine the sensitivity and linearity of the assay, a 10 fold serial dilution of a positive sample was used. The correlation between RNA concentration and the threshold (Ct) value of reverse transcription real-time PCR was determined. The initial RNA concentration was 100 ng/ $\mu$ l, after which the samples were serially diluted 10-fold for the real-time PCR assay. The PCR efficiencies were calculated according to the equation:

$$E = 10^{-1/\text{slope}}$$

**Statistical analysis:** Results of the reverse transcription real-time PCR were represented as Ct values. The  $\Delta Ct$  was computed as the difference of the Ct values derived from the target gene being assayed and the  $\beta$ -actin, considered as reference gene. The  $\Delta\Delta Ct$  was computed as the difference between the paired samples, calculated as

$$\Delta\Delta Ct = \Delta Ct_i \text{ of basal sample} - \Delta Ct_i \text{ of sampling time}$$

The  $n$ -fold differential expression in a target gene of sampling time compared to the basal counterpart was expressed as  $E^{\Delta\Delta Ct}$ . Differences with  $P < 0.05$  were considered significant.

Generated data for lymphocytes proliferation were analysed using the repeated measure analysis of the GLM model procedure of SPSS version 21.0.0. Factor terms included in the model were treatment (different levels of Pb and Cu), sampling times (–30, –15, 0, 15 and 30), and the interaction of treatments and sampling times.

Table 1. Ingredients and nutrient compositions of experimental diet

Composition	Total mixed ration
<i>Ingredients (g/kg DM)</i>	
Maize fodder	305
Wheat straw	195
Ground yellow maize	187
Groundnut cake	57
De-oiled mustard cake	95
Wheat bran	64
Rice bran	83
Dicalcium phosphate	5
Salt	3
Trace minerals and vitamins premix <sup>a</sup>	6
<i>Nutrient compositions (g/kg DM)</i>	
Dry matter	633
Organic matter	931
Crude protein	175
Ether extract	24
Ash	69
Neutral detergent fiber	526
Acid detergent fiber	290
Copper (mg/kg DM)	20
Zinc (mg/kg DM)	45

<sup>a</sup>Premix composition per kilogram: vitamin A, 500,000 IU; vitamin D<sub>3</sub>, 10,000 IU; vitamin E, 100 mg; Ca, 190,000; P, 90,000; Na, 50,000; Cu, 300 mg; Fe, 3000 mg; Mn, 2000 mg; I, 100 mg; Co, 100 mg; Se, 1 mg; Mg, 19,000 mg; BHT antioxidant, 3000 mg.

RESULTS AND DISCUSSION

**Mitogenic response of lymphocytes:** As the days of calving advanced, mitogenic response of lymphocytes decreased ( $P < 0.05$ ) and was reported lowest (Fig.1) on the day of calving (day 0). Lymphocytes cultured with Pb had further reduced ( $P < 0.05$ ) mitogenic response as compared to non Pb-exposed lymphocytes. However, within treatment, no effect of different Pb levels was observed on lymphocytes proliferation index. There was no interaction between days in relation to calving and Pb treatment on lymphocytes proliferation.

The adverse effect of Pb on lymphocytes proliferation was ameliorated by supplementation of 30, 35 and 40  $\mu\text{M}$  levels of Cu (Table 2). As the level of supplementation of Cu over Pb-exposed lymphocyte increased, lymphocytes proliferation also increased and was noted maximum ( $P > 0.05$ ) in 40  $\mu\text{M}$  Cu-treated group. Lymphocytes proliferation showed positive correlation with Cu levels. Interaction exists between lymphocyte proliferations, days

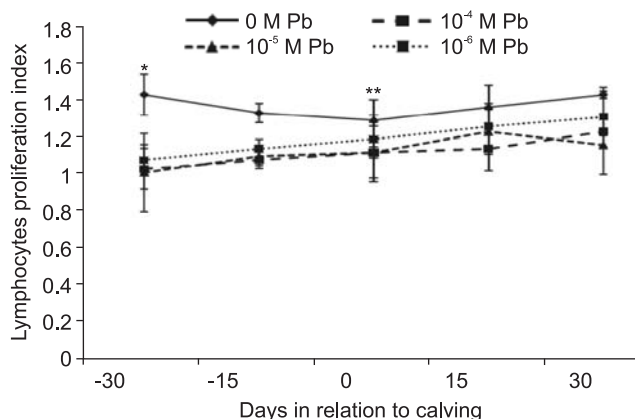


Fig. 1. Effect of different levels of Pb on mitogenic response of lymphocytes. \* and \*\* indicate significant effect of treatment and days in relation to calving, respectively. “-”, days before calving; “0”, day of calving. Differences between the treatments, days and treatment and day interaction were considered significant at  $P < 0.05$ .

in relation to calving, Pb and Cu treatment.

During transition period, dairy cows experience an abrupt change in metabolic status that can lead to an imbalance between ROS and antioxidants, which is referred to as oxidative stress (Gabai *et al.* 2004). The reason for reduced lymphocytes proliferation on the day of calving might be due to increased oxidative stress. Immune cells are particularly sensitive to oxidative stress because their membranes contain high concentrations of polyunsaturated fatty acids that are very susceptible to peroxidation, and they produce large amounts of ROS when stimulated (Spears and Weiss 2008). In present study, days in relation to calving and Pb treatment had negative effect on lymphocytes proliferation. Similar to the findings of present study, decrease in lymphocytes proliferation towards calving was also reported by Deka *et al.* (2014) in periparturient buffaloes. In another study, Panda (2006) recorded a decreased proliferation index from 2.33 to 1.40 on the day of parturition from 15 days prepartum. Rajiv (2001) also reported substantial decrease in the proliferation index of cell-mediated immunity at parturition from one week prepartum values in crossbred cows. Accordingly, Daniel *et al.* (1991) also reported a significant decrease in lymphocytes proliferation at calving. Moreover, a generalized reduction of blood leukocyte functions during the periparturient period was observed, due to the physiological demands imposed on the dairy cow by the lactating mammary gland (Nonnecke *et al.* 2003).

Besides transitional stress, bovine leukocytes are susceptible to the immunomodulatory effects of *in vitro* exposure to heavy metals such as mercury, Pb and cadmium (De Guise 1996). Aboud (2010) noted decreased percentage of phagocytosis in lead acetate, mercury chloride and cadmium chloride exposed *T. nilotica* fish indicated that heavy metals have a suppressive effect on cellular immune functions. ROS generated due to Pb exposure reduces immunity status of animals by affecting cell-mediated immunity and neutrophil function (Rada and Leto 2008). Pb exposure suppresses B-cell, helper T-cell or macrophage

Table 2. Effect of different levels of Cu and Pb on lymphocytes proliferation

Days of calving	Treatment <sup>a</sup>												P value <sup>b</sup>			
	30 $\mu\text{M}$ Cu				35 $\mu\text{M}$ Cu				40 $\mu\text{M}$ Cu				Period (P)	Pb treatment (LT)	Cu treatment (CT)	PxLT $\times$ CT
	10 <sup>-4</sup> M Pb	10 <sup>-5</sup> M Pb	10 <sup>-6</sup> M Pb	SEM	10 <sup>-4</sup> M Pb	10 <sup>-5</sup> M Pb	10 <sup>-6</sup> M Pb	SEM	10 <sup>-4</sup> M Pb	10 <sup>-5</sup> M Pb	10 <sup>-6</sup> M Pb	SEM				
-30	1.13	1.17	1.23	0.03	1.17	1.23	1.28	0.01	1.21	1.27	1.34	0.02	<0.001	0.014	0.038	0.029
-15	1.11	1.20	1.24	0.01	1.16	1.26	1.29	0.05	1.18	1.34	1.39	0.02	0.046	0.049	0.001	0.038
0	1.08	1.12	1.18	0.03	1.11	1.18	1.27	0.03	1.16	1.22	1.38	0.04	0.015	0.031	0.028	<0.001
15	1.17	1.21	1.28	0.02	1.27	1.34	1.39	0.01	1.37	1.41	1.48	0.03	0.028	0.028	0.042	0.042
30	1.32	1.32	1.38	0.02	1.38	1.37	1.41	0.02	1.46	1.46	1.53	0.05	0.019	0.033	0.016	0.011

SEM, standard error of the mean; “-”, days before calving; “0”, day of calving. <sup>a</sup>In treatment groups, effect of 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup> M concentration of Pb on mitogenic response of lymphocytes were counteracted by 30, 35, and 40  $\mu\text{M}$  Cu. <sup>b</sup> $P < 0.05$  denote significant difference from the control.

functions or any combination of these, to indirect effects of the pathogens or antigens employed, or to the alteration of aspects of innate immunity such as neutrophil function (Ewers *et al.* 1982). Kumar *et al.* (2013) reported reduced lymphocytes proliferation in Pb-exposed periparturient cows.

Some strategies aimed at reducing oxidative stress-related alterations by antioxidants and micronutrients supplementation in animals were tried (Colitis *et al.* 2002). For this study, Pb as heavy metal and Cu as protective mineral were chosen. In present study, Cu ameliorates adverse effect of Pb and transitional stress on lymphocytes proliferation. Recovered proliferation of Pb-exposed Zn-treated lymphocytes was noted by Kumar *et al.* (2013) in transitional crossbred cow. Bartoskewitz *et al.* (2007) supplemented 200 ppm Cu to deer maintained in captivity and observed improved lymphocytes proliferation. Bala *et*

*al.* (1991) showed suppressed proliferative response to T cell mitogens in Cu-deficient animals.

**SOD mRNA expression:** The relative expression of SOD in Pb and Cu-treated lymphocytes are presented in Figs. 2 and 3. mRNA expression of SOD reduced in all treatments as the day of calving advanced and maximum decrease was noted at the day of parturition. Culturing of leukocytes with different levels of Pb and Cu showed significant effect on SOD expression (P<0.05). Group with 10<sup>-4</sup> M Pb showed minimum mRNA expression of SOD. Addition of Cu over Pb-exposed periparturient lymphocytes improved relative mRNA abundance of SOD (P<0.05) and increase was reported highest in 40 µM Cu-treated lymphocytes.

The efficacy of neutralization of excess production of ROS might be due to transition stress or Pb-exposure, dependent on the genome for the enzymatic defense system, such as SOD, glutathione peroxidase(GPx), glutathione reductase, catalase, and on nutrition for the vitamins and minerals (Chen *et al.* 2010). It was reported that Pb exposure has a dose-response relationship with changes in antioxidant enzyme levels (Bechara *et al.*1993, Adonaylo and Oteiza 1999). These enzymes include SOD, catalase, and GPx, the expressions of which changed in the presence of some antioxidant molecules in animals (Hsu 1985 and McGowan and Donaldson 1986). Reverse transcriptase PCR analysis using specific primers for the phospholipid hydroperoxidase glutathione peroxidase (PHGPx), SOD, and glutathione peroxidase (GPx) cDNA sequences were performed on the total RNAs isolated from the brains of Pb-exposed animals by Kang *et al.* (2004). They showed similar SOD and GPx expression in the low- and medium-Pb groups. However, the expression levels of SOD and GPx were reduced in the high-dose Pb group. This finding suggested that Pb-exposure reduced the expression of SOD mRNA in the high-dose group because of structural tissue damage caused by lead toxicity and accumulation. In studies on animals with high exposure to Pb, a decreased SOD activity in erythrocytes was noted (Ito *et al.* 1985, Mylroie *et al.* 1986), which is in accordance to the findings of the present study. In erythrocytes, from the workers exposed occupationally to Pb, activity of SOD was remarkably lower than the non-exposed workers (Sugawara *et al.* 1991). In accordance to the finding of this study, Sandhir *et al.* (1994) also noted a decrease in the activity of SOD enzyme in Pb-exposed rat.

The findings of various studies indicated that Cu is required for biological activity of SOD and loss of Cu results in complete inactivation of Cu-Zn SOD (Noor *et al.* 2002). Cu shows interaction with Pb, therefore, higher Pb intake induced Cu deficiency resulting in decreased SOD activity (Mytroie 1986). In the findings of this study, addition of Cu over Pb-exposed lymphocytes improved relative mRNA abundance of SOD and mRNA abundance was comparable to control in 40 µM Cu-treated group. Panemangalore and Bebe (1996) observed a significant linear relationship between dietary Cu levels and SOD activity. Colitis *et al.* (2002) demonstrated that natural antioxidants counteract the adverse effect of oxidative stress in periparturient cows

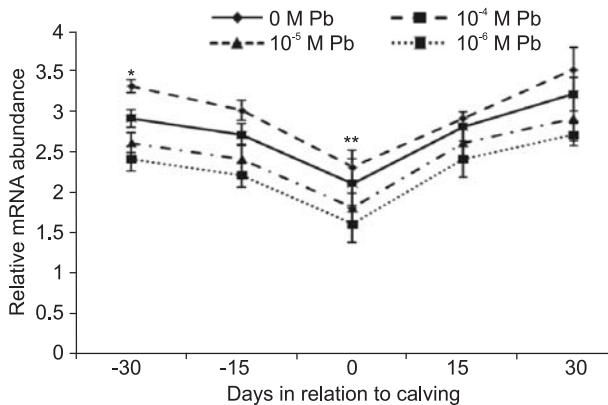


Fig. 2. Effect of different levels of Pb on *in vitro* SOD expression in cultured lymphocytes. \*, \*\* and \*\*\*, indicate significant effect of treatment, days in relation to calving and treatment and day interaction respectively. “-”, days before calving; “0”, day of calving. Differences between the treatments, days and treatment and day interaction were considered significant at P<0.05.

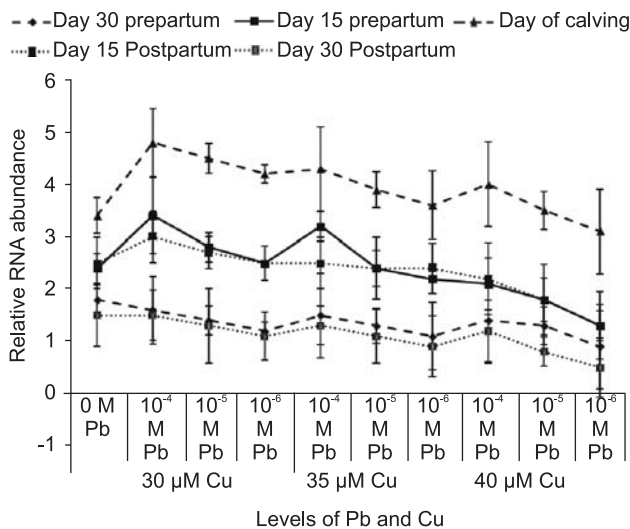


Fig. 3. Effect of different levels of Pb and Cu on *in vitro* SOD expression in cultured lymphocytes.

through modulation of SOD mRNA expression in blood leukocytes.

In conclusion, with the advancement of days of calving, proliferation of lymphocytes and mRNA expression of SOD decreased and was noted minimum on the day of calving. Exposure of lymphocytes with Pb further reduced lymphocytes proliferation and Cu/Zn SOD expression. All tested Cu levels reduced adverse effects of transitional stress but they were not able to counteract the adverse effect of Pb except at 40  $\mu\text{M}$  Cu-exposed to  $10^{-6}$  M Pb.

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