



## Ameliorating proliferation and superoxide dismutase mRNA expression of cadmium exposed leukocytes through copper treatment in transitional dairy cows

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### ABSTRACT

The study was conducted to determine the effect of cadmium on lymphocyte proliferation and mRNA superoxide dismutase (SOD) expression and to evaluate whether copper treatment in Cd exposed lymphocytes can modulate their proliferation and SOD mRNA expression. Blood samples were collected from crossbred transitional dairy cows at -30, -15, 0, 15, and 30 days of calving and evaluated for lymphocytes proliferation and SOD expression. A fixed number of lymphocyte were cultured for 72 h with  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  M levels of Cd. Effect of different Cd levels on proliferation and mRNA SOD expression was counteracted by 30, 35 and 40  $\mu$ M Cu. Mitogenic response of lymphocyte and mRNA expression of SOD reduced as the days of parturition advanced and was noted lowest at the day of calving. Lymphocyte proliferation and mRNA SOD expression showed negative correlation with Cd levels. Addition of Cu in the Cd exposed lymphocyte culture improved lymphocyte proliferation and relative abundance of SOD mRNA expression and increase was reported highest in 40  $\mu$ M Cu treated lymphocytes. These results indicated that Cu can ameliorate adverse effect of Cd on lymphocyte proliferation and SOD expression in transitional dairy cows.

**Key words:** Cadmium, Copper, Lymphocytes proliferation, mRNA expression, Superoxide dismutase

The dramatic increase in energy requirements needed for the onset of lactation in peripartum cows is often accompanied by a decrease in voluntary dry matter intake that causes a negative energy balance (Bell 1995). Negative energy balance, increased reactive oxygen species (ROS) production and elevated blood cortisol around parturition were postulated to be responsible for reduced antioxidant status, periparturient immunosuppression (Burton *et al.* 1995) and increased susceptibility towards infectious diseases (Maurya 2011). Besides periparturient period, heavy metals exposure also suppresses antioxidant status, cell mediated as well as humoral immune responses of animals (Aboud 2010).

Cadmium (Cd)-related cytotoxicity is closely associated with oxidative stress, which is induced by the generation of ROS and the disturbance of anti-oxidative enzymes, such as SOD, catalase and glutathione peroxidase (Poli *et al.* 2004). To protect the cells from the damaging effects of

ROS, mammals have several anti-oxidative enzymes, such as SOD, catalase and glutathione peroxidase (Furukawa *et al.* 2004, Roberts *et al.* 2006). Oxidative stress is responsible for reduced mRNA expression of antioxidant enzymes like SOD and immune responses (Fisher *et al.* 2011). Subtle changes in the mitogen responsiveness of lymphocytes (Koller *et al.* 1979, Koller and Roan 1980) or shifts in lymphocytes subpopulations (Oshawa and Kawai 1981) subsequent to Cd exposure may also reflect an altered immune status. In view of its key role in countering the oxidative stress, SOD is considered as the first line of defense against ROS. The aim of present study is to evaluate changes in lymphocyte proliferation and mRNA expression of SOD in Cd exposed leukocytes from periparturient crossbred cows and to determine whether Cu treatment in Cd exposed lymphocytes can modulate their proliferation and SOD mRNA expression.

### MATERIALS AND METHODS

Animal care procedures were approved and conducted under the established standard of the Institutional Animal Ethics Committee (IAEC).

*Animal management and experimental design:* Effect of different levels of Cd and Cu on lymphocyte proliferation and SOD expression was studied in periparturient Karan Fries cow (Tharparker  $\times$  Holstein-Friesian) maintained at cattle yard of the institute. The nutrient requirements of cows were met by feeding concentrated mixture, wheat

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straw, and available fodder (NRC 2001) and had free access to drinking water. Blood samples were collected at 0700 h in heparinized vacuutainer tubes by venipuncture of anterior vena cava at -30, -15, 0, 15 and 30 days around calving. “-” indicates the expected day before calving and day “0” being day of calving. Collected blood samples were used for determination of mitogenic response of lymphocytes and relative abundance of SOD mRNA expression.

**Lymphocyte stimulation index:** Lymphocytes were isolated from whole blood samples with the help of lymphocyte separation medium and a fixed number of cells ( $2 \times 10^6$ ) 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  $10^{-3}$  M,  $10^{-4}$  M,  $10^{-5}$  M, and  $10^{-6}$  M Cd. To counteract the adverse effect of Cd on cell proliferation and SOD expression, 30, 35, and 40  $\mu$ M Cu were added into the culture medium.

**MTT assay:** The proliferative response of lymphocyte 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 lymphocyte 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 GAC GTG GAA) and primers for  $\beta$ -actin i.e. forward primer (GTACGA 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.

**First strand cDNA synthesis and quantitative real-time PCR:** The reverse-transcription comparative real-time PCR method provides precise and sensitive quantitative results, detecting mRNA expression in nanogram or pictogram quantities of total RNA, available from small size samples. Data analysis using the comparative Ct method has advantages because it eliminates the need to construct a standard curve, allowing simple quantification of the relative gene expression of paired samples.

Total RNA was isolated from cultured lymphocyte and cDNA was synthesized by using a cells-to-cDNA II kit. Real-time PCR was performed on an Mx3000p stratagene system 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  $C_t$  values. The  $\Delta C_t$  was computed as the difference of the  $C_t$  values derived from the target gene being assayed and the  $\beta$ -actin, considered as reference gene. The  $\Delta\Delta C_t$  was computed as the difference between the paired samples, calculated as:

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

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

Generated data for lymphocyte 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 Cd and Cu), sampling times (-30, -15, 0, 15 and 30), and the interaction of treatments and sampling times.

## RESULTS AND DISCUSSION

**Mitogenic response of lymphocyte:** Effect of days in relation to calving and different levels of Cd and Cu on lymphocyte proliferation is given in Tables 1, 2. As the day of calving advanced, mitogenic response of lymphocyte decreased ( $P < 0.001$ ) and was reported lowest on the day of calving. Lymphocyte proliferation showed negative correlation with Cd levels and proliferation index was reported lowest with  $10^{-3}$  M concentration of Cd ( $P < 0.05$ ). Whereas, Cd exposed lymphocyte showed positive correlation with Cu levels on proliferation index and index was reported highest in 40  $\mu$ M Cu treated groups ( $P < 0.05$ ). Significant interaction ( $P < 0.05$ ) was noted in between treatment and days of parturition.

Decrease in lymphocyte count towards calving from 15 d before calving, the lowest on the day of calving and then increase in levels 15 d after calving, were also reported by Deka *et al.* (2015) in periparturient buffaloes. Panda *et al.* (2006) recorded a decrease in the stimulation index from 2.33 to 1.40 on the day of parturition from 15 days prepartum, which is in accordance with our findings. Our results are in agreement with Daniel *et al.* (1991) who reported a significant decrease in lymphocyte proliferation at calving. Moreover, a generalized reduction of blood leukocyte functions during the periparturient period was observed, due to the physiological demands imposed on

Table 1. Effect of different levels of Cd on mitogenic response of lymphocyte from periparturient crossbred cows

Day of sampling	Level of Cd <sup>a</sup>						P-value <sup>b</sup>		
	0 M	10 <sup>-3</sup> M	10 <sup>-4</sup> M	10 <sup>-5</sup> M	10 <sup>-6</sup> M	SEM	Treatment (T)	Period (P)	T×P
-30	2.21	1.14	1.18	1.38	1.68	0.03	<0.001	0.039	0.043
-15	2.09	0.96	1.03	1.24	1.54	0.01	0.018	0.044	0.018
0	1.89	0.83	0.98	1.18	1.25	0.05	0.028	<0.001	0.029
15	2.09	1.04	1.12	1.49	1.72	0.01	0.033	0.046	0.031
30	2.28	1.68	1.72	1.85	1.92	0.01	0.017	0.038	0.049

<sup>a</sup> In treatment groups, lymphocyte from periparturient cross bred cows were cultured with 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup> molar concentration of Cd for 72 h. <sup>b</sup>P<0.05 denotes significant difference from the control.

Table 2. Effect of different levels of Cu and Cd on mitogenic response of lymphocyte from periparturient crossbred cows

Day of sampling	Treatment <sup>a</sup>												P-value <sup>b</sup>					
	30 µM Cu					35 µM Cu					40 µM Cu					Treatment (T)	Period (P)	T×P
	10 <sup>-3</sup> M	10 <sup>-4</sup> M	10 <sup>-5</sup> M	10 <sup>-6</sup> M	SEM	10 <sup>-3</sup> M	10 <sup>-4</sup> M	10 <sup>-5</sup> M	10 <sup>-6</sup> M	SEM	10 <sup>-3</sup> M	10 <sup>-4</sup> M	10 <sup>-5</sup> M	10 <sup>-6</sup> M	SEM			
-30	1.25	1.75	2.04	2.10	0.01	1.63	2.19	2.27	2.50	0.01	1.70	2.30	2.60	2.64	0.04	0.018	0.394	0.889
-15	1.21	1.73	2.08	2.05	0.05	1.61	1.91	2.33	2.42	0.009	1.65	2.21	2.54	2.61	0.02	<0.001	0.562	0.779
0	1.10	0.64	1.80	1.91	0.02	1.41	1.87	1.96	2.32	0.03	1.40	1.74	2.06	2.13	0.06	0.042	0.016	0.048
15	1.22	1.74	2.03	2.06	0.01	1.59	1.98	2.38	2.43	0.01	1.67	2.27	2.53	2.59	0.01	0.038	<0.001	0.059
30	1.60	2.16	2.38	2.39	0.02	1.96	2.21	2.51	2.63	0.06	2.06	2.21	2.53	2.64	0.09	0.035	0.029	0.049

SEM, standard error of the mean; “-” indicate days before calving and “0” indicate day of calving. <sup>a</sup>In treatment groups, effect of 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup> molar concentration of Cd on mitogenic response of lymphocyte was counteracted by 30, 35, and 40 µM Cu. <sup>b</sup>P<0.05 denotes significant difference from the control.

the dairy cow by the lactating mammary gland (Nonnecke *et al.* 2003).

Besides transitional stress, De Guise (1996) reported that bovine leukocytes are susceptible to the immunomodulatory effects of *in vitro* exposure to heavy metals such as mercury, lead and Cd. Aboud (2010) noted decreased percentage of phagocytosis in *T. nilotica* fish exposed to lead acetate, mercuric chloride and cadmium chloride indicating that heavy metals have a suppressive effect on cellular immune functions. Reduced lymphocytic phagocytic activity in heavy metal exposed fish was also reported by Anderson *et al.* (1999), Ward and Neumann (1999), Canli and Atli (2003). Marth *et al.* (2001) found that Cd inhibit L-4/aCD40 induced proliferation of purified B cells and peripheral blood mononuclear cells in a dose dependent fashion. A dose-dependent suppression of the humoral immune response against sheep red blood cells was observed in mice exposed to Cd.

In present study, adverse effect of transitional stress and Cd exposure on mitogenic response of lymphocyte was counteracted by Cu supplementation. Results showed that lymphocyte culture with 40 µM of Cu had maximum proliferation. Bartoskewitz *et al.* (2007) supplemented 200 ppm Cu to deer maintained in captivity and observed improved lymphocyte proliferation. Bala *et al.* (1991) showed suppressed proliferative response to T cell mitogens in Cu-deficient animals. Cu is required for maintenance of both both humoral and cell-mediated immunity (Lukasewycz and Prohaska 1983). Mononuclear cells from

heifers receiving the low Cu diet produced less interferon when stimulated with con A than cells isolated from cows supplemented with Cu (Weiss and Spears 2006). Cu deficiency also reduces lymphoid organ weights and lymphocyte proliferative responses (Mulhern and Koller

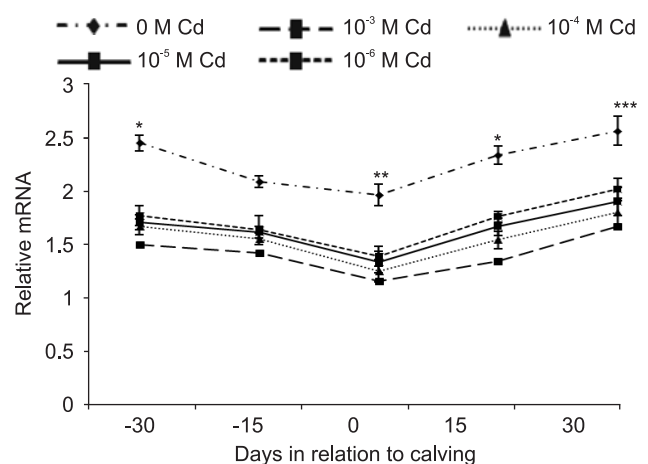


Fig. 1. Effect of different levels of Cd on *in vitro* SOD expression in cultured lymphocytes. Single (\*), double (\*\*) and triple (\*\*\*) asterisks indicate significant effect of treatment, days in relation to calving and treatment and day interaction respectively. “-” indicate days before calving and “0” indicate day of calving. Differences between the treatments, days and treatment and day interaction were considered significant at P<0.05.

1988) and immunoglobulin M concentrations (Windhauser *et al.* 1991).

**SOD mRNA expression:** The relative expression (*n*-fold) of SOD in Cd and Cu treated lymphocytes is presented in Figs 1, 2. mRNA expression of SOD reduced in all treatments as the day of calving advanced and decrease was noted maximum at the day of parturition. Culturing of leukocytes with different levels of Cd and Cu showed significant effect on SOD expression ( $P < 0.05$ ). Group with  $10^{-3}$  M Cd showed minimum mRNA expression of SOD. Addition of Cu in the Cd exposed lymphocyte improved mRNA expression of SOD ( $P < 0.05$ ) and increase was reported highest in 40  $\mu$ M Cu treated lymphocytes. Days of sampling and their interaction with treatment also showed significant effect ( $P < 0.05$ ) on mRNA SOD expression.

**Levels of Cd and Cu:** Dairy cows experience an abrupt change in metabolic status around the time of calving and the beginning of lactation (Kankofer 2002) that can lead to oxidative stress (Gabai *et al.* 2004). The efficacy of the antioxidants neutralization system is dependent on the genome for the enzymatic defense systems, such as SOD and Catalase. It is well recognized that Cd-related cytotoxicity is closely associated with oxidative stress, which is induced by the generation of ROS and the disturbance of anti-oxidative enzymes, such as catalase and GSH-Px (Chen *et al.* 2010). Bertin and Averbek *et al.* (2006) reported that exposure to cadmium chloride significantly decreased the expression of SOD in a dose-dependent manner which is in accordance to our findings. SOD activity declined from day 7 until day 11 for 50  $\mu$ g/l Cd and day 14 for 100 or 200  $\mu$ g/l Cd in clam *Mactra veneriformis* in responses to sublethal Cd (Fang *et al.* 2010). In this study, we demonstrated that Cu/Zn-SOD decreased by cadmium chloride exposure at mRNA and protein levels. This situation may be either due to damage to the protective system caused by the large amount of ROS generated during periparturient period or Cd exposure or to stimulation of other antioxidants to overcome ROS toxicity; these possible processes need to be studied further. SOD activity returned

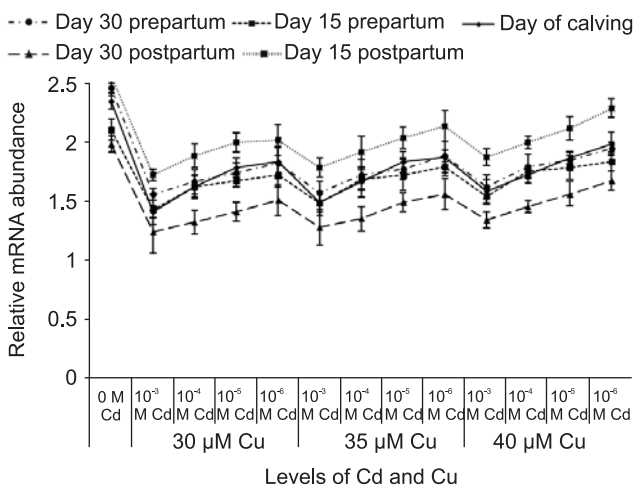


Fig. 2. Effect of different levels of Cd and Cu on *in vitro* SOD expression in cultured lymphocytes.

to control level in the 40  $\mu$ M Cu treated group. These results indicated that Cu is required for biological activity of Cu-Zn SOD and loss of Cu results in complete inactivation of Cu-Zn SOD and induces many diseases in human and animals (Noor *et al.* 2002). Panemangalore and Bebe (1996) observed a significant linear relationship between dietary Cu levels and SOD activity. Similarly, Colitis *et al.* (2002) demonstrated that natural antioxidants counteract the adverse effect of oxidative stress in periparturient cows 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 among all treatments. Dosing of lymphocyte culture with Cd further reduced lymphocyte proliferation and mRNA SOD expression and decrease was reported maximum in treatment containing highest Cd level. Adverse effect of transitional stress and Cd exposure on antioxidant activity and lymphocyte proliferation was returned near to control level in the 40  $\mu$ M Cu treated group indicating that more than 40  $\mu$ M Cu may be required.

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