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Effect of zinc treatments on cadmium exposed periparturient bovine lymphocytes *in vitro* on their proliferation and superoxide dismutase (SOD) expression

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ABSTRACT

This study was conducted to evaluate effect of cadmium (Cd) on lymphocyte proliferation and mRNA expression of Cu/Zn superoxide dismutase (SOD) and to determine whether zinc (Zn) treatment in Cd-exposed lymphocytes can modulate lymphocyte proliferation and SOD expression. Blood samples were collected from crossbred transition dairy cow at –30, –15, 0, 15 and 30 days of calving and evaluated for lymphocytes proliferation and SOD expression. Isolated lymphocytes were cultured with 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} molar (M) levels of Cd for 72 h. Adverse effect of transitional stress and Cd on lymphocyte proliferation and mRNA SOD expression was counteracted by 50, 55 and 60 micromolar (μM) Zn. Mitogenic response of lymphocyte and mRNA expression of SOD reduced as the days of parturition advanced. Lymphocyte proliferation and mRNA SOD expression showed negative correlation with Cd levels. Treatment of Zn in the Cd-exposed lymphocyte culture improved lymphocyte proliferation and relative abundance of SOD mRNA expression. In summary, Zn can ameliorate adverse effect of transitional stress and Cd on lymphocyte proliferation and SOD expression in dairy cows.

Key words: Cadmium, Lymphocytes proliferation, mRNA expression, Superoxide dismutase, Zinc

The peripartum period is crucial for health of dairy animals as they undergo endocrine, metabolic and physiological challenges. 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, oxidative stress and elevated blood cortisol around parturition are responsible for reduced antioxidant status, periparturient immunosuppression (Burton *et al*. 1995) and increased susceptibility towards infectious diseases (Deka *et al.* 2015). Besides periparturient period, heavy metals exposure also suppresses antioxidant status, cell mediated as well as humoral immune responses of animals (Aboud 2010). Cd related cytotoxicity was found closely associated with oxidative stress, which is induced by the generation of reactive oxygen species (ROS) and the disturbance of antioxidative enzymes such as SOD, catalase and glutathione peroxidase (Poli *et al.* 2004). Cd binds with the thiol and methyl groups of essential enzymes, phospholipids and nucleic acids, and interferes with oxidative phosphorylation, resulting in accumulation of ROS. To protect the cells from damaging effects of ROS, mammalians have several antioxidative 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. It is a versatile biomarker, especially with respect to the pollution by toxic chemicals, including heavy metals (Cho-Ruk *et al.* 2006).

To minimize adverse effect of transitional stress and Cd exposure on immunity and antioxidant status, various supplements and additives were tried, Zn being one of them. Zn is an important component of biomembranes and an essential co-factor in several enzymes (Soylak and Kidnap 2001) involved in the maintenance of cellular and humoral

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immunity. In India, commonly fed feeds contain Zn content below the critical level of 40 ppm as recommended by NRC (2001) for dairy cattle. However, for optimum immune response in stressed animals, the Zn requirements are suggested to be 10–20 times higher than the normal recommendations (NRC 2001). Hence the present study was undertaken to evaluate change in mRNA expression of SOD in Cd and Zn-treated leukocytes of periparturient cows and to determine whether Zn treatment in lead exposed leukocytes can modulate SOD expression and mitogenic response of lymphocyte.

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 (10^{-3} M, 10^{-4} M, 10^{-5} M and 10^{-6} M) and Zn (50, 55 and 60 µM) on lymphocyte proliferation and SOD expression was studied in periparturient Karan Fries cow (Tharparker × Holstein-Friesian) maintained at Cattle Yard of National Dairy Research Institute, Karnal, India. The nutrient requirements of cows were met by feeding concentrated mixture, wheat straw and available fodder (NRC 2001) and 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. "–" indicates the expected day before calving and day "0" being the day of calving. Collected blood samples were used for determination of mitogenic response of lymphocytes and relative abundance of SOD mRNA expression.

Table 1. Ingredients and nutrient compositions of experimental diet

Composition	Total mixed ration
Ingredients (g/kg DM)	
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[a]	6
Nutrient compositions (g/kg DM)	
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

[a]Premix composition/kg: vitamin A, 500,000 IU; vitamin D_3, 10,000 IU; vitamin E, 100 mg; Ca, 190,000; P, 90,000; Na, 50,000; Cu, 300 mg; Fe, 3,000 mg; Mn, 2,000 mg; I, 100 mg; Co, 100 mg; Se, 1 mg; Mg, 19,000 mg; BHT antioxidant, 3000 mg.

Lymphocyte proliferation index: Lymphocytes were isolated from whole blood samples with the help of lymphocyte separation medium and a fixed number of cells (2×10^6) 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, 50, 55, and 60 µM Zn was 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 µg/ml to the micro cultures. Cells were cultured at 37°C in a 5% CO_2 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 β-actin 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/Zn SOD gene, and 96 bp for β-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 using the SYBR Green qPCR SuperMix which served as a double-stranded DNA-specific fluorescent dye in 25 µl reaction to assess the SOD mRNA expression relative to housekeeping β-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/µl, after which the samples were serially diluted 10–fold for the real-time PCR assay. The PCR efficiencies were calculated according to the equation:

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

Statistical analysis: Results of the reverse transcription real–time PCR, were represented as *Ct* values. The ΔC_t was computed as difference of *Ct* values derived from the target gene being assayed and the β-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 $E^{\Delta\Delta Ct}$. 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 Zn), sampling times (–30, –15, 0, 15 and 30), and the interaction of treatments and sampling times.

Table 2. Effect of Cd on mitogenic response of lymphocyte

Days of calving	Level of Cd[a]					SEM	P-value[b]
	0 M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M		
-30	2.21	1.14	1.18	1.38	1.68	0.03	0.043
-15	2.09	0.96	1.03	1.24	1.54	0.01	0.018
0	1.89	0.83	0.98	1.18	1.25	0.05	0.029
15	2.09	1.04	1.12	1.49	1.72	0.01	0.031
30	2.28	1.68	1.72	1.85	1.92	0.01	0.049

SEM, standard error of the mean; "-", days before calving; "0", day of calving. [a]In treatment groups, lymphocyte from periparturient crossbred cows were cultured with 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M concentration of Cd for 72 h; [b]P<0.05 denote significant difference from the control.

RESULTS AND DISCUSSION

Lymphocyte stimulation index: Effect of days in relation to calving and different levels of Cd and Zn on lymphocyte proliferation is depicted in Tables 2 and 3. 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 Zn levels on proliferation index and index was reported highest in 60 µM Zn-treated groups (P<0.05).

Deka *et al.* (2015) also reported a decrease in lymphocyte count towards calving in periparturient buffaloes. Panda (2006) noted 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 the findings of this study. Rajiv (2001) also reported a substantial decrease in the stimulation index for cell-mediated immunity at parturition from 1 week prepartum values in crossbred cows. Our results are in agreement with findings of 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 the dairy cow by the lactating mammary gland (Nonnecke *et al.* 2003). The reason for reduced lymphocyte 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).

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 lead acetate, mercuric chloride and cadmium chloride exposed *T. nilotica* fish indicated that

Table 3. Effect of Cd and Zn on lymphocyte proliferation

Days of calving	Treatment[a]															
	50 µM Zn				SEM	55 µM Zn				SEM	60 µM Zn				SEM	P value[b]
	10^{-3} M Cd	10^{-4} M Cd	10^{-5} M Cd	10^{-6} M Cd		10^{-3} M Cd	10^{-4} M Cd	10^{-5} M Cd	10^{-6} M Cd		10^{-3} M Cd	10^{-4} M Cd	10^{-5} M Cd	10^{-6} M Cd		
-30	2.2	1.6	1.7	1.9	0.05	1.9	1.9	2.0	2.2	0.04	1.9	2.0	2.0	2.1	0.04	0.014
-15	2.1	1.6	1.6	1.7	0.02	1.7	1.8	1.9	1.9	0.05	1.8	2.0	2.1	2.1	0.03	<0.001
0	1.9	1.2	1.4	1.5	0.03	1.4	1.6	1.7	1.7	0.07	1.6	1.7	1.8	1.8	0.01	0.038
15	2.4	1.8	1.8	2.0	0.06	1.9	1.9	2.1	2.2	0.03	2.0	2.1	2.1	2.2	0.04	0.023
30	2.6	2.0	2.1	2.1	0.01	2.1	2.2	2.2	2.3	0.03	2.2	2.2	2.4	2.5	0.02	0.019

SEM, standard error of the mean; "-", days before calving; "0", day of calving. [a]In treatment groups, effect of 10^{-3} , 10^{-4}, 10^{-5}, and 10^{-6} M concentration of Cd on mitogenic response of lymphocyte were counteracted by 50, 55, and 60 µM Zn; [b]P<0.05 denote significant difference from the control.

heavy metals have a suppressive effect on cellular immune functions. Reduced lymphocytic phagocytic activity in heavy metal exposed fish was also reported by Ward and Neumann (1999), Canli and Atli (2003). Marth *et al.* (2001) found that Cd inhibits 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 several studies (Shenker *et al.* 1977, Lawrence 1981), *in vitro* Cd exposure of about 1×10^{-4} M Cd causes a generalized cytotoxic effect resulting in a reduction of lymphocyte and macrophage activity. Cd may regulate the immune response of the body at its different stages, modifying early and late inflammatory reactions, among others through changing the number of circulating B and T lymphocytes, NK cells and immunological memory cells (Nelson *et al.* 1982).

Zn plays a central role in cellular growth and differentiation for tissues with rapid turnover, including those of the immune system. Even minute alterations in the Zn level influence T cell development as well as T cell functions, whereas granulocytes are only influenced by high dosage of Zn or profound Zn deficiency. Zn required for biological activity of thymulin (a thymus dependant hormone) and receptors for thymulin are located on the surface of T-cells and thus, Zn is involved in the immune cell proliferation and maintenance of cell-mediated immunity. Kundu (1993) found that supplementation of both Cu and Zn in milk fed calves enhanced the cellular immune response.

SOD mRNA expression: The relative expression (*n*-fold) of SOD in Cd and Zn-treated lymphocyte is depicted in Figs 1 and 2. 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

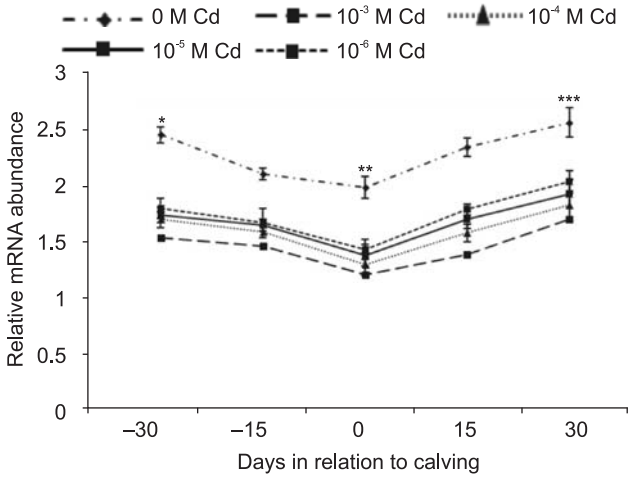

Fig. 1. Effect of Cd on *in vitro* SOD expression in cultured lymphocytes. *, **and *** indicate significant effect of treatment, days in relation to calving and treatment and day interaction respectively. "-", days before calving; "0", day of calving. Differences between the treatments, days and treatment and day interaction were considered significant at P<0.05.

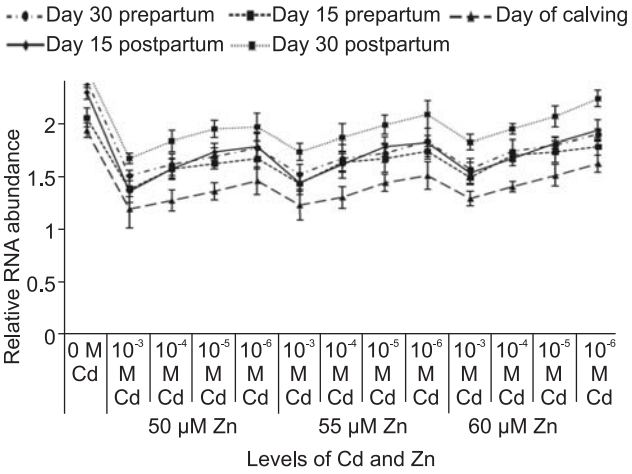

Fig. 2. Effect of Cd and Zn on *in vitro* SOD expression in cultured lymphocytes.

leukocytes with different levels of Cd and Zn showed significant effect on SOD expression (P<0.05). Group with 10^{-3} M Cd showed minimum mRNA expression of SOD. Addition of Zn in the Cd exposed lymphocyte culture improved mRNA expression of SOD (P<0.05) and increased was reported highest in 60 µM Zn-treated lymphocytes.

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, catalase and glutathione peroxidase. 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 Averbeck (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 µg/l Cd and day 14 for 100 or 200 µg/l Cd in clam *Mactra veneriformis* in response to sublethal Cd (Fang *et al.* 2010). In this study, we demonstrated that mRNA expression of Cu/Zn-SOD decreased due to transitional stress and Cd exposure. This situation may be due either 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 to control level in the 60 µM Zn-treated group.

Recent studies also demonstrated protective effect of Zn against oxidative stress and DNA damage (Wolfgang 2007, Song *et al.* 2009). Prasad (2007) demonstrated that Zn is needed for some DNA binding proteins. Zn is able to alter the expression by occupying a site on a transcription factor so as to increase the rate of transcription. The DNA polymerase is the major Zn dependant enzyme, which is

involved in DNA replication (Shankar and Prasad 1998). Evidence suggested that metallothionein (MT), a protein involved in Zn homeostasis and its mRNA abundance is increased when Zn is supplemented to Zn deficient animals (Shay and Cousins 1993). Zn has antioxidant-like properties; thus, it can stabilize macromolecules against radical-induced oxidation *in vitro* as well as limit excess radical production (Hennig *et al.* 1999). 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.

Our results showed that lymphocyte proliferation and SOD expression were suppressed as the days of pregnancy advanced. Dietary intake of Cd further reduces immune response and antioxidant status of periparturient cows. Adverse effect of transitional stress and Cd exposure on lymphocyte proliferation and SOD expression was ameliorated by Zn treatment.

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