Indian Journal of Animal Sciences **83** (12): 1261–1266, December 2013/Article

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

MUNEENDRA KUMAR[1], HARJIT KAUR[2], PHONDBA B T[3], RIJUSMITA SARMA DEKA[4], GULAB CHANDRA[5], VEENA MANI[6] and NEELAM GUPTA[7]

National Dairy Research Institute, Karnal, Haryana 132 001 India

Received: 3 April 2012; Accepted: 28 August 2013

ABSTRACT

The study was conducted to observe adverse effects of lead (Pb) treatments and protective effect of zinc on lymphocyte proliferation and to quantify the expression levels of superoxide dismutase (Cu/ZnSOD) genes involved in antioxidant's defenses in periparturient Karan Fries (Tharparker × Holstein-Friesian) cow. Blood samples were collected from peripartum Karan Fries cow at 30 and 15 days prepartum, at day of calving (0 day) and 15 and 30 days postpartum for estimation of lymphocytes proliferation and SOD expression. A fixed number of cells (2×10^6) were grown in culture for 72 h with different levels of Pb (10^{-4}, 10^{-5}, 10^{-6} M) and their adverse effects were counteracted by Zn (50, 55, 60 μM) and analysed for the lymphocyte proliferation (MTT assay) and for the expression level of Cu/ZnSOD using the real-time PCR technique with light upon extension (LUX) fluorogenic primers. Pb dosage had no adverse effect and the overall mean proliferation values indicated that 60μM Zn may be optimum for maximizing lymphocyte proliferation. The Cu/ZnSOD mRNA expression in lymphocytes was higher at higher dosage of Pb in comparison to lower dosage in all the 3 (50, 55, 60 μM) Zn treatments. There was no significant difference at different levels of Zn on SOD expression. Cu/ZnSOD mRNA expression decreased from –30 days to 0 days but increased from 0 to 30 days after calving. The results suggested that the zinc may have an ameliorative effect on lead exposed oxidative stress on lymphocytes proliferation and Cu/ZnSOD mRNA expression through modulation of superoxide dismutase expression

Key words: Lead, Lymphocyte, Periparturient, Superoxide dismutase (SOD), Zinc

Lead induced oxidative stress, reduced the antioxidant defense system of cells via depleting superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase (Ahamed *et al*. 2005). Lead exposure interfering with some essential metal, inhibiting sulfhydryl dependent enzymes or antioxidant enzymes activities and /or increasing susceptibility of cells to oxidative attack by altering membrane integrity and fatty acid composition (Hande and Naran 2000). Reactive oxygen species (ROS) generated due to lead exposure reduces the immunity status of animals by affecting cell-mediated immunity and neutrophil function (Rada 2008). Lead exposure suppresses B-cell, helper T-cell or macrophage 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). Heavy metal also alters the nucleic acid concentration (Das *et al*. 2001). Besides heavy metal exposure periparturient period also increases production of free radicals and reactive oxygen species which leads to oxidative stress resulting in peroxidative damage of lipids and other macromolecules (Toyokuni 1999).

Zinc, an important component of biomembranes and an essential co-factor in a variety of enzymes (Soylak and Kidnap 2001), is involved in the activation of B-cells and thus can be helpful in maintenance of cellular and humoral immunity. In India, commonly fed feeds contain zinc content below the critical level of 40 ppm as recommended by National Research Council (2001) for dairy cattle. However, for optimum immune response in stressed animals, the zinc requirements are suggested to be 10–20 times higher than the normal recommendations (NRC 2001). In the present research the aim is to evaluate change in mRNA expression of antioxidant enzymes, Cu/ZnSOD in lead and zinc treated leukocytes of periparturient cows and to determine whether zinc treatment in lead exposed leukocytes can modulate their expression.

Present address: [1,3,4,5] Ph. D. Scholar (muneendra82@gmail.com, bhupendravet@gmail.com, riju_sharma2002@yahoo.com, gulabdrvet@gmail.com), [2,6]Principal Scientist (harjit 1955 @gmail.com, Veenamani1@yahoo.com), Dairy Cattle Nutrition Division. [7]Principal Scientist (neelamgnbagr@gmail.com), National Bureau of Animal Genetic Resources, Karnal.

MATERIALS AND METHODS

Blood was collected at 7.00 AM from Karan Fries cow on days -30, -15, 0, 15 and 30 days of periparturient (– indicates the expected day before calving, + indicates days after calving and day 0 being day of calving). Blood was collected before the morning meal from the jugular vein lithium- heparin vacuum tubes, kept in box containing ice and immediately delivered to the laboratory for analysis. The experiment was carried out in accordance with state and local laws and ethical regulation.

Lymphocyte proliferation assay: Immediately after collection, the tubes were transported to the laboratory in ice for further processing and lymphocytes were separated. A fixed no. of cells (2×10^6) were grown in culture medium for 72 h with different levels of lead (10^{-4} M, 10^{-5} M, 10^{-6} M) (Mosmann 1983). To counteract the adverse effect of heavy metals on cell proliferation, different levels of zinc (50 µm, 55 µm, 60 µm), were supplemented to the lymphocytes. The protective effect of different levels of zinc supplementation to lead exposed lymphocytes was seen on cell proliferation.

Mitogen 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.

Superoxide dismutase (SOD) mRNA expression: Superoxide dismutase mRNA expression level was estimated from cultured lymphocyte using the real-time PCR technique with fluorogenic primers.

Primers: Real time PCR primers were designed to monitor SOD expression. 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/ZnSOD gene, and 96 bp for β-actin reverse keeping gene.

Complementary DNA synthesis: Complementary DNA was synthesized from cultured lymphocyte by using a cell to cDNA II kit. Total RNA was isolated from bovine blood leukocytes according to the manufacturer's instructions. Real-time PCR was performed on an Mx3000p stratagene system (US) 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 house keeping gene β-actin. 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:

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

Data computation and statistical analysis: Effect of treatments on lymphocyte proliferation was analyzed by one-way ANOVA using SPSS 17.0.0, according to Snedecor and Cochran (1994). Differences with probabilities ($P<0.050$) were considered significant. Results of the reverse transcription real–time PCR were represented as Ct values. The Δ was computed as the difference of the Ct values derived from the target gene and the β- actin (reference gene). The —ΔCt was computed as the difference between the paired samples, calculated as

—ΔCt = Δ Ct of basal sample - Δ Ct of sampling time

The n- fold differential expression in a target gene of sampling time compared to the basal counterpart was expressed as $E^{-\Delta Ct}$. Differences were considered to be significant at $P\geq0.05$.

RESULTS AND DISCUSSION

Lymphocyte proliferation: Different levels of lead treatment did not have any effect on lymphocyte proliferation (Table 1). However, proliferation in lead treated groups were significantly lower in comparison to control, zinc and both, Zn+Pb treated groups. In present findings, proliferation of lymphocytes were significantly higher ($P<0.05$) in zinc treated groups in comparison to control group. Supplementation of zinc showed ameliorative effect over lead exposed lymphocyte, and amelioration was maximum in 60 µm zinc treated group. Significantly higher ($P<0.05$) proliferation in zinc treated and lead exposed lymphocyte indicated that 60µm zinc may be taken as optimum dose for maximizing lymphocyte proliferation.

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 cadmium. Mishra *et al*. (2003) reported that low exposure to lead was reported to exert immunostimulating effects in contrast to higher exposure, which usually leads to immunosuppression. Rajiv (2001) found that in periparturient cows, stimulation index for cell-mediated immunity (CMI) decreased by 47% at parturition from 1 week pre-partum values in crossbred cows and increased significantly at 1 week postpartum. A significant decrease in lymphocyte proliferation at calving

Table 1. Effect of different levels of zinc on proliferation of lead exposed lymphocyte

Days of calving	Control	10^{-4} M Pb	10^{-5} M Pb	10^{-6} M Pb	50 µm Zn	55 µm Zn	60 µm Zn	50 µm Zn + 10^{-4} M Pb	50 µm Zn + 10^{-5} M Pb	50 µm Zn + 10^{-6} M Pb	55 µm Zn + 10^{-4} M Pb	55 µm Zn + 10^{-5} M Pb	55 µm Zn + 10^{-6} M Pb	60 µm Zn + 10^{-4} M Pb	60 µm Zn + 10^{-5} M Pb	60 µm Zn + 10^{-6} M Pb
–30	1.42	1.02	1.00	1.07	1.65	1.68	1.78	1.49	1.44	1.592	1.48	1.52	1.60	1.65	1.68	1.73
	±0.03	±0.03	±0.06	±0.03	±0.12	±0.045	±0.23	±0.11	±0.04	±0.08	±0.10	±0.20	±0.17	±0.06	±0.12	±0.05
–15	1.32	1.07	1.09	1.13	1.58	1.58	1.62	1.21	1.38	1.43	1.41	1.44	1.50	1.46	1.60	1.59
	±0.04	±0.05	±0.04	±0.07	±0.09	±0.021	±0.029	±0.05	±0.03	±0.04	±0.08	±0.05	±0.09	±0.02	±0.09	±0.04
0	1.28	1.11	1.11	1.18	1.35	1.53	1.59	1.20	1.25	1.32	1.32	1.44	1.47	1.37	1.48	1.50
	±0.01	±0.06	±0.06	±0.04	±0.03	±0.029	±0.045	±0.01	±0.09	±0.15	±0.05	±0.11	±0.04	±0.03	±0.04	±0.02
+15	1.35	1.13	1.22	1.25	1.36	1.53	1.62	1.20	1.316	1.33	1.37	1.47	1.52	1.49	1.52	1.54
	±0.03	±0.08	±0.02	±0.03	±0.03	±0.01	±0.04	±0.03	±0.05	±0.03	±0.02	±0.06	±0.06	±0.05	±0.03	±0.06
+30	1.42	1.22	1.30	1.30	1.56	1.61	1.65	1.462	1.50	1.50	1.50	1.51	1.54	1.54	1.53	1.60
	±0.07	±0.09	±0.09	±0.05	±0.09	±0.05	±0.05	±0.03	±0.01	±0.01	±0.05	±0.01	±0.03	±0.04	±0.04	±0.01
Mean±SE	1.36 [b]	1.12 [a]	1.15 [a]	1.19 [a]	1.50[c]	1.59 [c]	1.65 [d]	1.31 [b]	1.38 [b]	1.44 [c]	1.42 [c]	1.48 [c]	1.53 [d]	1.51 [d]	1.57 [d]	1.60 [e]
	±0.03	±0.03	±0.05	±0.04	±0.06	±0.03	±0.03	±0.07	±0.04	±0.05	±0.03	±0.02	±0.02	±0.05	±0.04	±0.04

Mean bearing different superscripts differ significantly (P<0.05).

was also reported by Daniel *et al.* (1991). In buffaloes, Panda (2006) recorded a decrease in the stimulation index from 2.33 to 1.40 on the day of parturition from 15 days prepartum similar to the present findings. Moreover, a generalized reduction of blood mononuclear 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 samples might be due to increased oxidative stress at that time. Zinc 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 zinc level influence T cell development as well as T cell functions, whereas granulocytes are only influenced by high dosage of zinc or profound zinc deficiency. Zinc required for biological activity of thymulin (a thymus dependant hormone) and receptors for thymulin are located on the surface of T-cells and thus, zinc is involved in the immune cell proliferation and maintenance of cell-mediated immunity. Kundu (1993) found that supplementation of both copper and zinc in milk fed calves enhanced the cellular immune response.

Superoxide dismutase (SOD) mRNA expression

Linearity and sensitivity of the reverse transcription real–time PCR: The cycling numbers of real-time RT-PCR reactions in the 10-fold serially diluted samples assayed for Cu/ZnSOD are shown in Table 2.

The calculated coefficient for determination were 0.952 and 0.994 for the Cu/ZnSOD and β-actin, indicating that the RNA expression is adequately represented by the Ct value. From the linearity data, we determined that the assay for Cu/ZnSOD gene was sensitive to 10 ng of RNA.

Determination and distribution of Cu/ZnSOD : The ΔCt value and the relative expression (*n*- fold) are presented in Table 3.

Table 2. Mean Ct value for target (Cu/ZnSOD) and reference (b-actin) genes

Cu/ZnSOD ng mRNA	Target	Reference	ΔCt
100.0	20.46±0.37	13.27±0.38	6.48±0.48
10.0	22.65±0.41	16.47±0.35	6.03±0.93
1.0	25.94±0.38	19.46±0.41	5.37±0.46
0.1	31.29±0.44	24.17±0.63	6.38±0.58

Table 3. Regression equations and RT-PCR efficiency of target (Cu/ZnSOD) and reference (ß-actin) genes

	Cu/ZnSOD		
	Target	Reference	ΔCt
Slope	–0.282	–0.285	0.114
Constant	4.846	3.077	6.741
R^2	0.952	0.994	0.048
E ($10^{-1/slope}$)	1.919	1.931	

ΔCt, difference of the Ct values derived from the target gene being assayed and the ß-actin, considered as reference gene; E,PCR efficiency.

Table 3.

The ΔCt value and *n*-fold change in expression of Cu/ZnSOD ranged from 5.13 to 5.58 and 1.67 to 3.68, respectively in control group at different expected days of calving. In leukocytes of periparturient cows, expression of Cu/ZnSOD changed significantly, however, a significant interaction (P<0.01) with *in vitro* treatment was observed (Table 4a,b). It is evident that SOD expression was lowest at the day of calving in all control, lead, zinc and both lead and zinc treated group, which increased after calving and was maximum on the 30 day after calving in all groups. Expression of Cu/ZnSOD mRNA expressed as ΔCt and *n*-fold and their interaction are presented in Tables 4a, b. Cu/

Table 4 a. Mean ΔCT values and relative expression (*n*-fold) of the target genes

	Days of calving	Control	10^{-4} M Pb	10^{-5} M Pb	10^{-6} M Pb	50 µm Zn	55 µm Zn	60 µm Zn	50 µm 10^{-4} M Pb	50 µm 10^{-5} M Pb	50 µm 10^{-6} M Pb	Zn55 µm 10^{-4} M Pb	Zn55 µm 10^{-5} M Pb	Zn55 µm 10^{-6} M Pb	Zn60 µm Zn 10^{-4} M Pb	Zn60 µm Zn 10^{-5} M Pb	Zn60 µm Zn 10^{-6} M Pb
ΔCt Cu/	–30	5.58	5.16	5.21	5.27	5.55	5.57	5.65	5.28	5.37	5.39	5.30	5.37	5.43	5.49	5.53	5.57
ZnSOD	–15	5.45	5.14	5.21	5.26	5.49	5.49	5.62	5.23	5.25	5.25	5.28	5.30	5.32	5.38	5.41	5.41
	0	4.89	4.09	4.28	4.42	4.92	4.98	5.17	4.19	4.30	4.44	4.21	4.34	4.36	4.76	4.82	4.85
	+15	5.13	4.78	4.84	4.88	5.29	5.21	5.23	4.76	4.86	4.88	4.79	4.86	4.92	4.96	5.10	5.12
	+30	5.35	4.98	5.18	5.21	5.31	5.41	5.48	4.99	5.18	5.25	5.11	5.24	5.29	5.16	5.29	5.31
Cu/ZnSO,	–30	1.97	1.74	1.78	1.82	1.99	2.03	2.28	1.76	1.79	1.87	1.82	1.85	1.87	1.88	1.94	1.96
n- fold	–15	1.88	1.67	1.72	1.72	1.92	1.96	1.98	1.73	1.74	1.77	1.75	1.77	1.78	1.79	1.81	1.85
	0	1.67	1.56	1.59	1.62	1.70	1.78	1.82	1.55	1.60	1.63	1.57	1.62	1.64	1.59	1.64	1.66
	+15	2.09	1.78	1.83	1.85	2.09	2.18	2.28	1.79	1.83	1.86	1.82	1.89	1.93	1.85	1.96	1.98
	+30	3.68	2.48	2.59	2.67	3.63	3.71	3.78	2.49	2.61	2.69	2.52	2.67	2.72	2.56	2.86	2.98

ΔCt, difference of the Ct values derived from the target gene being assayed and the β – actin, considered as reference gene; *n*-fold, differential expression in a target gene of sampling (–30, –15, 0, 15 and 30 days from calving).

Table 4b. Contrast analysis of group × sampling time interaction of the target genes

	Between subject MSE	Group	Sampling	Group × Sampling
ΔCt Cu/ZnSOD	6.611	0.275	0.000	0.022
Cu/ZnSOD, *n*- fold	1.693	0.016	0.001	0.015

Contrast analysis of group × sampling time interaction was significant for Cu/ZnSOD relative mRNA expression at + 30 days ($P<0.05$).

ZnSOD mRNA expression decreased as the days of pregnancy advanced among all treatments (Table 4a, b). Decrease in Cu/ZnSOD mRNA expression was minimum in zinc treated groups, however decrease was maximum in different levels of lead treated groups. Adverse effect of lead treatments on Cu/ZnSOD mRNA expression was ameliorated to a certain extent by zinc supplementation and expression was similar to control. In control and zinc treated groups, Cu/ZnSOD expression increased significantly ($P<0.01$), peaking at 30 days from calving.

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 an imbalance between ROS and antioxidants, which is referred to as oxidative stress (Gabai *et al.* 2004). The efficacy of this neutralization system is dependent on the genome for the enzymatic defence system, such as SOD, glutathione peroxidase, glutathione reductase catalase, and on nutrition for the vitamins and minerals. Some strategies aimed at reducing oxidative stress- related alterations were tried in laboratory animals (Agrawal and Rao 2000), but, to the best of our knowledge, appropriate investigations still lacking in ruminants (Colitis *et al*. 2000, 2002). For this study, lead as heavy metal and zinc as protective mineral were chosen. The relative expression of Cu/ZnSOD in the lead treated group was significantly lower compared to that of both lead and zinc supplemented group and control.

However, increase in the SOD expression after calving in zinc treatment was higher as compared to lead treated group but lower as compared to control. It is reported that plasma zinc level of 0.90 µg/ml at 15 days prepartum decreased to 0.64 µg/ml on the day of parturition in dairy buffaloes, which increased to normal values after 15 days postpartum (Panda 2006). This might be the reason that zinc supplementation at 10 or 20% higher levels in the present experiment resulted in increased expression of SOD. Increase in lead concentration in the culture resulted in increased SOD expression. Lead supplemented 50 µm zinc groups showed higher expression ($P\leq0.05$) at –15,0 and 15 days, but, 55 µm zinc groups showed higher expression at 0 day only and 60 µm zinc groups showed no increase on all days of cultures.

In erythrocytes, from the workers exposed occupationally to lead, the activities of the antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase were remarkably lower than the non-exposed workers (Sugawara *et al*. 1991). The decreased SOD activity in battery manufacturing workers (BMW) group is probably due to interaction of lead with copper molecule. As SOD is a zinc-copper containing enzyme, hence lead-exposure induced copper deficiency resulted in decreased SOD activity (Mytroie 1986). Recent studies have also demonstrated protective effect of zinc against oxidative stress and DNA damage (Wolfgang 2007, Song *et al*. 2009). Prasad (2007) demonstrated that zinc is needed for some DNA binding proteins. Zinc 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 zinc dependant enzyme, which is involved in DNA replication (Shankar and Prasad 1998). Evidence suggested that

metallothionein (MT), a protein involved in zinc homeostasis and its mRNA abundance is increased when zinc is supplemented to zinc deficient animals (Shay and Cousins 1993). Zinc 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.

The reverse- transcription comparative real-time PCR method provides precise and sensitive quantitative results, detecting mRNA, expression in nanogram or picogram 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.

Our results showed that lymphocyte proliferation and Cu/ZnSOD expression were suppress as the days of pregnancy advances, dietary intake of lead further reduce immune response and antioxidant status of periparturient cows. Lymphocyte proliferation and expression of Cu/ZnSOD were recovered in zinc treated groups.

ACKNOWLEDGEMENTS

We are thankful to the National Dairy Research Institute, Karnal and Indian Council of Agricultural Research, Pusa, New Delhi, India for the fellowship award for carrying out this research work. We also acknowledge the research facilities provided by the National Bureau of Animal Genetic Resources, Karnal, India.

REFERENCES

Agarwal S and Rao A V. 2000. Carotenoids and chronic diseases. *Drug Metabolism and Drug Interactions* **17**: 189–210.

Ahamed M, Verma S, Kumar A and Siddiqui M K J. 2005. Environmental exposure to lead and its correlation with biochemical indices in children. *Science of the Total Environment* **346**: 48–55.

Ariza M E, Bijur G N and Williams M V. 1998. Lead and mercury mutagenesis: role of H_2O_2, superoxide dismutase, and xanthine oxidase. *Environmental and Molecular Mutagenesis* **31**: 352–61.

Barnett J B, Hamer D H and Meydani S N. 2009. Low zinc status: a new risk factor for pneumonia in the elderly? *Nutrition Reviews* **68**: 30–37.

Colitis M, Stradioli G and Stefanon B. 2000. Effect of vitamin E deprevation on the involution of mammary gland in sheep. *Journal of Dairy Science* **83**: 345–50.

Colitis M, Stradioli G and Stefanon, B. 2002. Regulation of mRNA expression of Phase I and Phase II enzymes in blood following administration of Tomato and grape extract in sheep. *Proceeding of the first European Symposium on Bioreactive secondary plants products. Veterinary Medicine* **32**. Wien University of Veterinary Medicine.

Daniel L R, Chew B P, Tanaka T S and Tjoelker, L W. 1991. *In vitro* effects of ß-carotene and vitamin A on paripartum bovine peripheral blood mononuclear cell proliferation. *Journal of Dairy Science* **74**: 911–15.

Das K K, Das S N and Dasgupta S. 2001. The Influence of ascorbic acid on nickel induced hepatic lipid peroxidationin rats. *Journal of Basic and Clinical Physiology and Pharmacology* **12**: 187–94.

De Guise S, Bernier J and Dufresne M M. 1996. Immune functions in Beluga' whales *(Delphinapterus leucas):* evaluation of mitogen-induced blastic transformation of lymphocytes from 'peripheral blood, spleen and thymus. *Veterinary Immunology and Immunopathology* **50**: 117–26.

Ewers U, Stiller W R and Idel H. 1982. Serum immunoglobulin, complement C3 and salivary IgA levels in lead workers. *Environmental Research* **29**: 352–57.

Gabai G, Testoni S, Piccinini R, Marindli L and Stradaioli G. 2004. Oxidative stress in primiparous cows in relation to dietary starch and the progress of lactation. *Animal Science* **79**: 99–108.

Hande G and Naran E. 2000. Can antioxidants be beneficial in the treatment of lead poisoning? *Free Radical Biology & Medicine* **29**: 927–45.

Hennig B, Meerarani P, Toborek M and Mc-Clain C.1999. Antioxidant-like properties of zinc in activated endothelial cells. *Journal of the American College of Nutrition* **18**: 152–58.

Kankofer M. 2002. Placenta release/retention in cows and its relation to peroxidative damage of macromolecules. *Reproduction in Domestic Animals* **37**: 27–30.

Kundu M S. 1993. 'Dietary zinc and copper levels influencing immune status of calves.' Ph D Thesis. NDRI (Deemed University), Karnal, India.

Meunier N, O'Connor J M, Maiani G, Cashman K D, Secker D L, Ferry M, Roussel A M and Coudray C. 2005. Importance of zinc in the elderly. *European Journal of Clinical Nutrition* **59**: 1–4.

Mishra K P, Singh V K, Rani R, Yadav V S, Chandran V, Srivastava S P and Seth P K. 2003. Effect of lead exposure on the immune response of some occupationally exposed individuals. *Toxicology* **188**: 251–59.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assay. *Journal of Immunological Methods* **65**: 55–63.

Mytroie A A. 1986. Erythrocyte SOD activity and other parameter of copper status in rats ingesting lead acetate. *Toxicology and Applied Pharmacology* **82**: 512–20.

Nonnecke B J, Kimura K, Goff J P and Kehrli M E. 2003. Effect of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. *Journal of Dairy Science* **86**: 2359–68.

NRC. 2001. *Nutrient Requirements of Dairy Cattle.* 7th Revised edn. Washington D C, National Academy Press.

Panda N, Kaur H and Mohanty T K. 2006. Reproductive performance of dairy buffaloes supplemented with varying levels of vitamin E. *Asian - Australasian Journal of Animal Sciences* **19** (1): 19–25.

Prasad A S. 2007. Clinical, immunological, anti-inflammatory and antioxidant roles of zinc. *Experimental Gerontology* **43**: 370–77.

Rada B and Leto T L. 2008. Oxidative innate immune defenses by

Nox/Duox family NADPH oxidases. *Contributions to Microbiology* **15**: 164–87.

Rajiv. 2001. 'Influence of ß-carotene and vitamin E supplementation on udder health and immunocompetence in dairy cattle.' Ph D Thesis, NDRI (Deemed University), Karnal.

Shankar A H and Prasad A S. 1998. Zinc and Immune function: the biological basis of altered resistance to infection. *American Journal of Clinical Nutrition* **68**: 447–63.

Shay N F and Cousins R J. 1993. Cloning of rat intestinal mRNAs affected by zinc deficiency. *Journal of Nutrition* **123**: 35–41.

Snedecor C W and Cochran W G. 1994. *Statistical Methods.* Iowa State University Press. Ames, Iowa.

Song Y, Leonard S W, Traber M G and Ho E. 2009. Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses, and DNA repair in rats. *Journal of Nutrition* **139**: 1626–31.

Sugawara E, Nakamura K, Miyake T, Fukumura A and Seki Y. 1991. Lipid peroxidation and concentration of glutathione in erythrocytes from workers exposed to lead. *British Journal of Industrial Medicine* **48**: 239–42.

Soylak M and Kidnap M. 2001. Serum copper and zinc concentrations of patients with rheumatoid arthritis from Kayseri-Turkey. *Fresenius Environmental Bulletin* **10**: 409–10.

Toyokuni S. 1999. Reactive oxygen species-induced molecular damage and its application in pathology. *Pathology International* **49**: 91–102.

Tuerk M J and Fazel N.2009. Zinc deficiency. *Current Opinion in Gastroenterology* **25**: 136–43.

Wolfgang M. 2007. Metallothionein redox biology in the cytoprotective and cytotoxic functions of zinc. *Experimental Gerontology* **43**: 363–69.