

# Effect of In Vitro Copper and Zinc Supplementation on Neutrophil Phagocytic Activity and Lymphocyte Proliferation Response of Transition Dairy Cows

Kalyan De<sup>1</sup> · Shashi Pal<sup>2</sup> · Joydip Mukherjee<sup>3</sup> · Shiv Prasad<sup>4</sup> · Ajay Kumar Dang<sup>5</sup>

Received: 31 March 2015 / Accepted: 31 August 2015 / Published online: 9 November 2015  
© NAAS (National Academy of Agricultural Sciences) 2015

**Abstract** Three experiments were conducted to assess the effect of in vitro copper (Cu) and zinc (Zn) supplementation on neutrophil phagocytic activity (NPA) and lymphocyte proliferation response (LPR) of transition dairy cows. In the first experiment, six multiparous crossbred (Karan-Fries: Tharparkar × Holstein-Friesian) cows were selected; the neutrophils and lymphocytes were isolated from the blood samples of different peripartum days of each cow. To determine the effect of in vitro Cu and Zn supplementation on the NPA and LPR, 2.360 μM Cu and 9.175 μM Zn were added separately into the culture media of neutrophils and lymphocytes. It was found that the addition of Zn in the culture media increased the NPA and B lymphocyte LPR significantly ( $P < 0.05$ ) as compared to non-supplemented neutrophils and lymphocytes. In the second experiment, another six multiparous crossbred cows were selected and fed with 20 ppm Cu/cow/day individually from 45 days pre-calving to 45 days post-calving. Their blood samples were collected during different peripartum days and 2.360 μM Cu was incorporated separately into the culture media of NPA and LPR to know whether there is need for extra Cu after Cu supplementation. Addition of Cu in the culture media of lymphocyte of Cu fed animals increased B lymphocytes LPR significantly ( $P < 0.05$ ). On the day of calving, the B and T lymphocyte LPR increased significantly ( $P < 0.05$ ) and phagocytic activity increased by 15.38 %. In the third experiment, six more multiparous crossbred cows were selected and fed with 80 ppm Zn/cow/day individually from 45 days pre-calving to 45 days post-calving. Their blood samples were collected during different peripartum days and 9.175 μM Zn was added separately into the culture media of NPA and LPR to know whether there is need for extra Zn after Zn supplementation. A significantly ( $P < 0.05$ ) higher LPR (T and B) was found on 3 days before parturition with in vitro addition of Zn in the culture media and it remained higher up to 7 days post-partum. Peripartum days had ( $P < 0.05$ ) significant effect on both in vitro NPA and LPR in all three experiments. It is concluded that extra Cu and Zn supplementation around peripartum period has beneficial effects on in vitro NPA and LPR.

**Keywords** Copper · Zinc · Phagocytic activity · Lymphocyte proliferation response · Peripartum · Dairy cow

## Abbreviations

ADF: Acid detergent fiber; Con A: Concanavalin A; CP: Crude protein; Cu: Copper; DM: Dry matter; EE: Ether extract; FCS: Fetal calf serum; GLM: General linear model; kg: Kilogram; LPR: Lymphocyte proliferation response; LPS: Lipopolysaccharide; mm: Millimeter; NDF: Neutral detergent fiber; NDRI: National Dairy Research Institute; NPA: Neutrophil phagocytic activity; PMN: Polymorphonuclear leukocytes; ppm: Parts per million; rpm: Revolutions per minute; SOD: Superoxide dismutase enzyme; Zn: Zinc; μM: Micro molar;

✉ Kalyan De  
kalyande2007@gmail.com

<sup>1</sup> Physiology and Biochemistry Division, ICAR-Central Sheep and Wool Research Institute, Avikanagar, Rajasthan, India

<sup>2</sup> Livestock Production and Management Section, ICAR-National Dairy Research Institute, Karnal, India

<sup>3</sup> Department of Veterinary Physiology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, India

<sup>4</sup> Inian Council of Agricultural Research, New Delhi, India

<sup>5</sup> Dairy Cattle Physiology Division, ICAR-National Dairy Research Institute, Karnal, India

## Introduction

In dairy cows, major metabolic and physiological changes occur during transition of pregnancy to lactation [17]. Immunological capacity remained lower from 1 month before calving to 1 month after calving [1, 19]. During this period, mostly animals remained in negative energy balance [6] which causes physiological stress of the animals and ultimately leads to immune suppression that adversely affects the animal health and production.

The immune suppression during the transition period increases the susceptibility to diseases of dairy cows [18]. In this period, important neutrophil functions are impaired and lymphocyte proliferation is reduced [12]. The respiratory burst activity of polymorphonuclear leukocytes (PMN) decreased 1 week before parturition and started to increase from 3rd week of lactation [7]. Lymphocyte proliferation in vitro has been used as an indirect measure of immune responsiveness. Concanavalin A (Con A) mitogens are used for stimulating T lymphocyte proliferation in vitro [23] and lipopolysaccharide (LPS) for B lymphocyte proliferation [9].

Among different measures taken to avoid the periparturient immune suppression, supplementation of antioxidant micronutrients considered to be most effective in overcoming this problem. Copper (Cu) is an important part of the superoxide dismutase enzyme. This enzyme safeguards the cells from the deleterious effects of oxygen metabolites produced during phagocytosis [26]. Zinc (Zn) is a structural component of the superoxide dismutase enzyme (SOD) and almost 300 other metabolic enzymes. It is also involved in cell growth, cell replication, cell-mediated immunity and generalized host defense [25].

There is a paucity of information on the effect of in vitro Cu and Zn supplementation on NPA and LPR around peripartum period and it is not known whether there is need for extra Cu and Zn in Cu and Zn fed cows for increasing NPA and LPR around the calving period. Therefore, the present study was carried out to assess the effect of in vitro Cu and Zn supplementation on NPA and LPR in transition dairy cows.

## Materials and Methods

### Study Site

The present study was carried out at the Cattle Yard of National Dairy Research Institute (NDRI), Karnal, Haryana; located at 29°42'N latitude and 72°02'E longitudes at an altitude of 250 m above the mean sea level. The average annual maximum and minimum ambient temperature

ranges between 45 and 1.4 °C in this area. The mean annual relative humidity ranges between 41 and 85 % and the annual rainfall from 760 to 960 mm. The experiment was carried out during August to April.

### Animal Selection

The experiment was conducted on Karan-Fries cows, developed for higher milk production at NDRI, Karnal, developed through crossing native Tharparkar and exotic Holstein-Friesian breeds. Eighteen pregnant crossbred (Tharparkar × Holstein-Friesian) cows of average body weight around 450 kg were selected from the Institute's experimental herd during their late gestation 45 days before the expected date of calving.

### Feeding

Individual cows were offered with a normal feeding regime i.e., ad libitum green fodder (*Trifolium alexandrinum/Zea mays/Avena sativa*) and 4 kg concentrate mixture daily. Concentrate mixture composed (90.51 % DM, 19.81 % CP, 8.62 % total ash, 3.7 % EE, 58.97 % NDF, and 13.4 % ADF) of maize, groundnut cake, mustard cake, wheat bran, rice bran, and common salt. After calving, the animals were offered *ad libitum* green fodder (23.19 % DM, 8.18 % EE, 9.61 % total ash, 1.14 % EE, 49.79 % NDF, and 26.45 % ADF), and 1 kg concentrate mixture was given for every 3 kg milk production.

### Experimental Procedures

To assess the effect of in vitro Cu and Zn supplementation on NPA and LPR in transition cows, in the first experiment, six Karan-Fries cows were selected and kept in normal conventional feeding regime. Their blood samples were collected from the jugular vein in 10 ml heparinized vacutainer on 30th, 15th, 7th, 3rd day before calving, on the day of calving and 3rd, 7th, 15th, 30th day after calving. Neutrophil phagocytic activity and LPR were evaluated from the collected blood samples with in vitro supplementation of Cu and Zn in the culture media individually.

In the second experiment, another six Karan-Fries cows were selected and provided with additional 20 ppm Cu/kg DM/day individually from 45 days prepartum to 45 days postpartum. Their blood samples were collected from the jugular vein in 10 ml heparinized vacutainer on 30th, 15th, 7th, 3rd day before calving, on the day of calving and 3rd, 7th, 15th, 30th day after calving. Neutrophil phagocytic activity and LPR were evaluated from the collected blood samples with the addition of Cu in the culture media.

In the third experiment, another group of six Karan-Fries cows were selected and provided with additional 80 ppm/kg DM/day Zn individually. Their blood samples were collected from the jugular vein in 10 ml heparinized vacutainer on 30th, 15th, 7th, 3rd day before calving, on the day of calving and 3rd, 7th, 15th, 30th day after calving. Neutrophil phagocytic activity and LPR were evaluated from the collected blood samples with the addition of Zn in the culture media.

#### Estimation of In Vitro Phagocytic Activity of Blood Neutrophils

Neutrophils were isolated from blood samples by hypotonic lysis of erythrocytes and adjusted to  $5 \times 10^6$  live cells/ml in the culture media (RPMI 1640) containing 10 % fetal calf serum (FCS). 200  $\mu$ l of the cell suspension was placed per well in triplicate in a 96-well flat-bottomed tissue culture plate. Zymosan (650  $\mu$ g/ml) and nitroblue tetrazolium (NBT) (250  $\mu$ g/ml) were added to the cell suspension and incubated at 37 °C in a humidified CO<sub>2</sub> incubator (95 % air and 5 % CO<sub>2</sub>) for 2 h. NBT is yellow in color, but it gets changed to blue after phagocytosis, which can be measured spectrophotometrically [2].

#### Estimation of In Vitro Lymphocyte Proliferation Response

The blood samples were centrifuged at 3000 rpm for 30 min, then buffy coat was collected. 1:1 v/v Dulbecco's phosphate saline (DBPS) was added to re-suspend the buffy coat. The suspended buffy coat was carefully layered on lymphocyte separation medium (Histopaque 1077) at concentration of 4:1 v/v in sterile 15 ml polypropylene centrifuge tube and centrifuged at 2500 rpm for 30 min at room temperature to separate the lymphocytes. After separation of the lymphocytes, culture media (RPMI 1640) with 10 % FCS was added to the lymphocyte suspension to adjust at  $5 \times 10^6$  live lymphocytes/ml. 200  $\mu$ l of the cell suspension was placed per well in triplicate in a 96-well flat-bottomed tissue culture plate. The mitogen Concanavalin A (5  $\mu$ g/ml) and Lipopolysaccharide (50  $\mu$ g/ml) were used to stimulate T and B lymphocytes, respectively. The cells were allowed to proliferate with and without mitogen, for determining the difference between cell proliferations. All cultures were incubated at 37 °C in a humidified CO<sub>2</sub> incubator (95 % air and 5 % CO<sub>2</sub>) for 24 h. The proliferative response of lymphocyte was estimated using the colorimetric MTT (tetrazolium) according to the procedure given by Mosmann [14].

#### Supplementation of Cu and Zn in the Culture Media

Normal plasma level of Cu and Zn was 12.234  $\mu$ M and 9.442  $\mu$ M, respectively. Before conducting this experiment, different doses of Cu and Zn were supplemented in the culture medium to see their influence on the NPA for standardization of the best suitable supplementation dose. Micronutrients were added in seven different doses, i.e., 5, 10, 25, 50, 75, 100, and 150 % of the plasma level in the culture media where 100 % is equal to the level found in plasma of normal lactating Karan-Fries cows. Culture media, which were without any extra micronutrient supplementation served as control. The maximum effect was found with in vitro supplementation of 25 % (2.360  $\mu$ M) of Cu of normal plasma level and 75 % (9.175  $\mu$ M) of Zn of normal plasma level [4]. Therefore, we supplemented 2.360  $\mu$ M Cu and 9.175  $\mu$ M Zn in the culture media of NPA and LPR of transition cows during the peripartum period.

#### Statistical Analysis

The data were analyzed using the GLM procedure by analysis of variance which includes the effect of in vitro Cu and Zn supplementation on NPA and LPR. A post hoc analysis was performed using Tukey's tests for pairwise comparisons. Data were presented as mean  $\pm$  SEM, and statistical analysis was carried out using SPSS software, version 14.0. The level of statistical significance was set at  $P < 0.05$ .

#### Results and Discussion

Table 1 describes the effect of in vitro micronutrient supplementation in the culture media on NPA. It was found that in vitro supplementation of micronutrient had significant ( $P < 0.05$ ) effect on the NPA. Addition of Zn in the culture media increased the NPA significantly ( $P < 0.05$ ) as compared to non-supplemented neutrophils whereas addition of Cu increased the NPA (10 %). Neutrophil phagocytic activity increased by 38.95 % with the addition of Zn in the culture media on day of the calving. There was increase in NPA from calving to 30 days postpartum with the addition of Cu and Zn. Peripartum days had significant ( $P < 0.001$ ) effect on the NPA while interaction between peripartum days and micronutrients had no significant effect on the in vitro NPA.

Table 2 describes the effect of in vitro micronutrient supplementation in the culture media on T lymphocyte LPR. No significant difference was found with in vitro micronutrient supplementation in the culture media in T

**Table 1** In vitro phagocytic activity of blood neutrophils after in vitro Cu and Zn supplementation during different peripartum days in crossbred cows

Groups	Prepartum days			Calving	Postpartum days			Overall mean		
	-30	-15	-7		-3	0	3		7	15
Cont	0.566 <sup>c</sup> ± 0.031	0.384 ± 0.031	0.276 ± 0.031	0.208 ± 0.031	0.126 ± 0.031	0.131 ± 0.031	0.161 ± 0.031	0.248 ± 0.031	0.324 ± 0.031	0.270 <sup>a</sup> ± 0.0123
Cu (2.360 μM)	0.712 <sup>b</sup> ± 0.037	0.453 ± 0.041	0.265 ± 0.041	0.158 ± 0.041	0.152 ± 0.041	0.135 ± 0.041	0.177 ± 0.047	0.271 ± 0.037	0.347 ± 0.047	0.297 <sup>ab</sup> ± 0.0143
Zn (9.175 μM)	0.689 <sup>b</sup> ± 0.037	0.499 ± 0.041	0.29 ± 0.041	0.164 ± 0.041	0.174 ± 0.041	0.155 ± 0.037	0.202 ± 0.041	0.301 ± 0.041	0.459 ± 0.058	0.326 <sup>b</sup> ± 0.015

Data are presented as Mean ± SE

Cont, control (without any extra supplementation); Cu (2.360 μM), addition of 2.360 μM Cu in the culture media; Zn (9.175 μM), addition of 9.175 μM Cu in the culture media

Means with similar superscripts letters within a column do not differ significantly ( $P < 0.05$ ) with each other neutrophils were isolated from blood of cows without any supplementation  
Neutrophils were isolated from blood of without any supplemented (fed) group of cows

O.D. at 540 nm

**Table 2** In vitro lymphocyte stimulation index of T lymphocytes after in vitro Cu and Zn supplementation during different peripartum days in crossbred cows

Groups	Prepartum days			Calving	Postpartum days			Overall mean		
	-30	-15	-7		-3	0	3		7	15
Control	1.588 <sup>a</sup> ± 0.127	1.424 <sup>ab</sup> ± 0.127	1.130 ± 0.110	1.049 ± 0.139	1.009 ± 0.094	0.902 ± 0.099	1.101 ± 0.094	1.163 ± 0.110	1.470 ± 0.110	1.204 ± 0.038
Cu (2.360 μM)	1.66 <sup>ab</sup> ± 0.110	1.334 <sup>a</sup> ± 0.110	1.200 ± 0.127	1.124 ± 0.156	1.035 ± 0.094	0.868 ± 0.110	1.048 ± 0.110	1.151 ± 0.110	1.633 ± 0.127	1.228 ± 0.042
Zn (9.175 μM)	1.971 <sup>b</sup> ± 0.099	1.751 <sup>b</sup> ± 0.110	1.085 ± 0.110	1.062 ± 0.156	1.034 ± 0.127	0.861 ± 0.127	1.168 ± 0.094	1.234 ± 0.110	1.680 ± 0.110	1.315 ± 0.042

Data are presented as Mean ± SE

Cont, control (without any extra supplementation); Cu (2.360 μM), addition of 2.360 μM Cu in the culture media; Zn (9.175 μM), addition of 9.175 μM Cu in the culture media

Means with similar superscripts letters within a column do not differ significantly ( $P < 0.05$ ) with each other neutrophils were isolated from blood of cows without any supplementation  
Lymphocytes were isolated from blood of without any supplemented (fed) group of cows

Measurement was taken dual wavelength measuring system, at a test wavelength of 503 nm and a reference wavelength of 630 nm

**Table 3** In vitro lymphocyte stimulation index of B lymphocytes after in vitro Cu and Zn supplementation during different peripartum days in crossbred cows

Groups	Prepartum days			Calving			Postpartum days			Overall mean
	-30	-15	-7	-3	0	3	7	15	30	
Cont	1.415 ± 0.149	1.236 ± 0.129	0.969 ± 0.129	0.928 ± 0.163	1.056 ± 0.110	0.816 ± 0.115	1.074 ± 0.110	1.078 ± 0.129	1.476 <sup>b</sup> ± 0.129	1.117 <sup>a</sup> ± 0.043
Cu (2.360 μM)	1.516 ± 0.149	1.368 ± 0.149	0.993 ± 0.149	0.994 ± 0.182	0.990 ± 0.115	0.814 ± 0.129	1.020 ± 0.129	1.114 ± 0.129	1.629 <sup>ab</sup> ± 0.129	1.160 <sup>ab</sup> ± 0.051
Zn (9.175 μM)	1.506 ± 0.149	1.685 ± 0.149	1.032 ± 0.129	1.001 ± 0.182	1.260 ± 0.149	0.907 ± 0.149	1.324 ± 0.129	1.078 ± 0.129	1.961 <sup>b</sup> ± 0.129	1.306 <sup>b</sup> ± 0.051

Data are presented as Mean ± SE

Cont, control (without any extra supplementation); Cu (2.360 μM), in vitro supplementation of 2.360 μM Cu in the culture media; Zn (9.175 μM), in vitro supplementation of 9.175 μM Cu in the culture media

Means with similar superscripts letters within a column do not differ significantly ( $P < 0.05$ ) with each other neutrophils were isolated from blood of cows without any supplementation Lymphocytes were isolated from blood of without any supplemented (fed) group of cows

Measurement was taken dual wavelength measuring system, at a test wavelength of 503 nm and a reference wavelength of 630 nm

**Table 4** Effect of in vitro Cu supplementation in the culture media on PA and LPR of Cu fed cows

Activity	Groups	Prepartum days			Calving			Postpartum days			Overall mean
		-30	-15	-7	-3	0	3	7	15	30	
In vitro NPA	Cu	0.429 ± 0.032	0.362 ± 0.032	0.313 ± 0.032	0.139 ± 0.032	0.117 ± 0.032	0.080 ± 0.032	0.157 ± 0.032	0.204 ± 0.032	0.358 ± 0.032	0.240 ± 0.032
	Cu (2.360 μM)	0.456 ± 0.053	0.328 ± 0.036	0.267 ± 0.048	0.073 ± 0.059	0.135 ± 0.037	0.082 ± 0.048	0.185 ± 0.037	0.217 ± 0.042	0.338 ± 0.037	0.231 ± 0.015
In vitro LPR of T lymphocyte	Cu	1.352 ± 0.122	1.294 ± 0.086	1.184 ± 0.122	1.094 <sup>a</sup> ± 0.122	1.063 <sup>a</sup> ± 0.086	1.118 ± 0.086	1.170 <sup>a</sup> ± 0.086	1.314 <sup>a</sup> ± 0.086	1.481 ± 0.086	1.230 ± 0.033
	Cu (2.360 μM)	1.335 ± 0.122	1.294 ± 0.086	1.179 ± 0.122	1.363 <sup>b</sup> ± 0.122	1.293 <sup>b</sup> ± 0.100	1.174 ± 0.086	1.422 <sup>b</sup> ± 0.100	1.181 <sup>b</sup> ± 0.086	1.425 ± 0.086	1.296 ± 0.034
In vitro LPR of B lymphocyte	Cu	1.059 ± 0.139	1.105 <sup>a</sup> ± 0.098	1.033 ± 0.139	1.042 ± 0.139	0.985 <sup>a</sup> ± 0.098	1.014 ± 0.098	1.070 ± 0.098	1.169 ± 0.098	1.024 <sup>a</sup> ± 0.098	1.056 <sup>a</sup> ± 0.038
	Cu (2.360 μM)	1.315 ± 0.139	1.451 <sup>b</sup> ± 0.098	1.242 ± 0.139	1.169 ± 0.139	1.365 <sup>b</sup> ± 0.113	1.181 ± 0.098	1.154 ± 0.113	1.331 ± 0.098	1.330 <sup>b</sup> ± 0.098	1.282 <sup>b</sup> ± 0.039

Data are presented as Mean ± SE

Cu (2.360 μM), in vitro supplementation of 2.360 μM Cu in the culture media

Means with similar superscripts letters within a column do not differ significantly ( $P < 0.05$ ) with each other

Neutrophils and lymphocytes were isolated from blood of Cu fed (20 ppm/cow/day) cows

NPA Neutrophil phagocytic activity, LPR lymphocyte proliferation response

For NPA O.D. was 540 nm and, LPR was measured in dual wavelength measuring system, at a test wavelength of 503 nm and a reference wavelength of 630 nm

**Table 5** Effect of in vitro Cu supplementation in the culture media on PA and LPR of Zn fed cows

Activity	Groups	Prepartum days					Calving	Postpartum days					Overall mean
		-30	-15	-7	-3	0		3	7	15	30		
In vitro NPA	Zn	0.585 ± 0.029	0.358 ± 0.029	0.178 ± 0.029	0.184 ± 0.029	0.163 ± 0.029	0.162 ± 0.029	0.246 ± 0.029	0.226 ± 0.029	0.411 ± 0.029	0.279 ± 0.010		
	Zn (9.175 µM)	0.521 ± 0.033	0.399 ± 0.034	0.177 ± 0.052	0.152 ± 0.036	0.151 ± 0.033	0.141 ± 0.036	0.240 ± 0.036	0.153 ± 0.039	0.423 ± 0.031	0.262 ± 0.012		
In vitro LPR of T lymphocyte	Zn	1.439 ± 0.092	1.338 ± 0.083	1.282 ± 0.131	1.218 <sup>a</sup> ± 0.092	1.107 ± 0.083	1.109 ± 0.092	1.218 ± 0.107	1.482 ± 0.092	1.515 ± 0.083	1.301 ± 0.032		
	Zn (9.175 µM)	1.346 ± 0.092	1.253 ± 0.083	1.163 ± 0.185	1.532 <sup>b</sup> ± 0.107	1.194 ± 0.092	1.233 ± 0.092	1.277 ± 0.107	1.471 ± 0.083	1.312 ± 0.092	1.309 ± 0.036		
In vitro LPR of B lymphocyte	Zn	1.052 <sup>a</sup> ± 0.115	1.210 ± 0.103	1.220 ± 0.163	1.308 <sup>a</sup> ± 0.115	1.203 ± 0.103	1.167 ± 0.115	1.237 ± 0.133	1.506 ± 0.103	1.361 ± 0.103	1.252 ± 0.040		
	Zn (9.175 µM)	1.287 <sup>b</sup> ± 0.115	1.127 ± 0.103	1.246 ± 0.231	1.498 <sup>b</sup> ± 0.133	1.216 ± 0.115	1.172 ± 0.115	1.348 ± 0.133	1.307 ± 0.103	1.359 ± 0.115	1.284 ± 0.045		

Data are presented as Mean ± SE

Zn (9.175 µM), in vitro supplementation of 9.175 µM Cu in the culture media

Means with similar superscripts letters within a column do not differ significantly ( $P < 0.05$ ) with each other

Neutrophils and lymphocytes were isolated from blood of Zn fed (80 ppm/cow/day) cows

For NPA O.D. was 540 nm and, LPR was measured in dual wavelength measuring system, at a test wavelength of 503 nm and a reference wavelength of 630 nm

NPA Neutrophil phagocytic activity, LPR lymphocyte proliferation response

lymphocyte blastogenic response with mitogen (Con A). T lymphocyte LPR increased (9.22 %) with Zn supplementation in the media as compared to non-supplemented media. Cu supplementation increased the LPR of T lymphocyte by 2 %. It was found that the days around peripartum period had significant ( $P < 0.001$ ) effect on LPR of T lymphocyte.

Table 3 describes the effect of in vitro micronutrient supplementation in the culture media on B lymphocyte LPR. There was significant ( $P < 0.05$ ) influence of in vitro micronutrient supplementation on mitogen (LPS)-induced B lymphocyte blastogenic response. The result showed that in vitro Zn supplementation in the culture media significantly ( $P < 0.05$ ) increased the B lymphocyte LPR as compared to control groups. The increase in LPR of B lymphocyte was 16.92 % in in vitro Zn supplemented group as compared to the control group. On 30th day postpartum, mitogen (LPS)-induced lymphocyte blastogenic response was significantly ( $P < 0.001$ ) higher in Zn supplemented group as compared to the other groups. It was found that different days of peripartum period had a significant ( $P < 0.001$ ) effect on the B lymphocyte LPR.

In dairy cattle, a few weeks before calving dry matter intake starts reducing [13] and at the same time there is a demand for nutrient to initiate milk synthesis. Therefore, a marked decline in serum concentrations of Cu and Zn at calving occurred. Drop in the serum concentrations of these nutrients is associated with impaired immune functions. The drop in serum concentrations of Cu and Zn is largely due to colostrum formation, changes in dry matter intake and ruminal metabolism [10].

Spears [20] observed that phagocytic activity is reduced in Cu deficiency and it can be changed with Cu supplementation. Similarly, in the present study, NPA was increased by 10 % with in vitro supplementation of Cu in the culture media of neutrophils (Table 1) around the periparturient period. Our results are also in agreement with the findings of Dang et al. [3] who also reported increase in NPA during the prepartum and postpartum period with Cu supplementation. A significant increase in NPA was found with in vitro Zn supplementation in the culture media (Table 1). De et al. [5] reported increase in NPA in dairy cattle with extra Zn feeding during the peripartum period. In the present study, the LPR of B lymphocyte increased significantly ( $P < 0.05$ ) with in vitro supplementation of Zn in the culture media as compared to non-supplemented lymphocytes (Table 3). Weiss and Spears [22] reported that Zn affects the cell proliferation, replication and immunity. Zinc is essential for lymphocyte replication [21] as indicated by higher B lymphocyte LPR observed with in vitro Zn supplementation in the lymphocyte culture of dairy cows without extra Zn supplementation (experiment 1).



In vitro Cu supplementation in the culture media on NPA and LPR of neutrophils and lymphocytes isolated from Cu fed cows showed no increase in NPA (Table 4) of the Cu fed cows. However, on the day of calving, NPA increased by 15.38 % as compared to control only Cu fed cows. Lymphocyte proliferation response of T lymphocyte increased significantly ( $P < 0.05$ ) on 3 days prepartum, on the day of calving and 7 days postpartum with in vitro supplementation of Cu in the culture media of lymphocyte from Cu supplemented animals. On the day of calving the increase was 21.64 %. In vitro Cu supplementation in the lymphocyte culture media of Cu fed cows, increased the B lymphocyte LPR significantly ( $P < 0.05$ ). The increase was 21.40 %. Almost all the peripartum days showed higher LPR of B lymphocyte with in vitro supplementation of Cu in the culture media of lymphocyte of Cu fed animals. On the day of calving the increase was 38.58 %. Our study showed that, B lymphocyte LPR significantly increased with in vitro Cu supplementation in the culture media of lymphocyte of Cu fed animals (Table 4). Senthilkumar et al. [15] also reported higher LPR in Cu supplemented lambs. Solaiman et al. [16] also reported higher lymphocyte proliferation ( $P < 0.05$ ) in kids supplemented with 100 mg Cu/day.

Table 5 gives the result of the effect of in vitro Zn supplementation in the culture media on NPA and LPR isolated from Zn fed cows. There was no significant variation in NPA with in vitro supplementation of Zn in the culture of neutrophils of Zn fed cows. There was also no significant change in mitogen (Con A) induced T lymphocyte LPR with in vitro supplementation of Zn in the culture media of Zn fed cows. However, a positive response in T lymphocyte LPR was found from 3 day prepartum to the 7 day postpartum with in vitro supplementation of Zn in the culture media of lymphocyte of Zn fed cows. Our result showed a significant ( $P < 0.001$ ) increase in the LPR of T lymphocyte on 3rd day prepartum with in vitro supplementation of Zn in the culture media of lymphocytes. The increase was 25.78 %. On the day of calving and 3rd day after calving the increase in mitogen (Con A) induced LPR of T lymphocyte was 7.86 and 11.18 %, respectively, with in vitro addition of Zn in the culture media of lymphocyte of Zn fed cow. A similar trend was also found in B lymphocyte LPR. A positive response in B lymphocyte proliferation was observed from 7th day prepartum to the 15th day postpartum with in vitro supplementation of Zn in the culture media of Zn fed cows. There was a significant ( $P < 0.001$ ) increase in LPR of B lymphocyte on 3 days before calving with in vitro supplementation of Zn in the culture media of lymphocyte of Zn fed animal. The increase was 14.52 %. However, peripartum days had significant ( $P < 0.001$ ) influence on the changes of NPA and LPR.

In the present study, there was no overall significant difference in NPA and LPR with in vitro Zn supplementation in

culture media of neutrophils and lymphocyte of Zn fed cows (experiment 3). It may be attributed to the fact that the cows have got a sufficient amount of Zn with extra Zn feeding during their transition period to maintain NPA and LPR.

It was observed that the NPA and LPR changes significantly during the transition period in all the three experiments. It is gradually decreased toward parturition and again increased gradually as postpartum days progressed. Similar finding was previously reported by Dang et al. [3]. It may be due to diversion of micronutrients towards fetal growth and colostrum formation [3].

In the present study, it was found that the effect of in vitro supplementation of Cu and Zn on NPA of neutrophils and LPR was more pronounced in close to calving periods in all the experiments. Earlier it was indicated that the feed intake was reduced and most of the micronutrients were diverted towards fetal growth, milk and colostrum formation [3], which may lead to insufficiency of micronutrients for immune activity of blood cells. The plasma Cu level was decreased before calving because of drainage to the fetal liver [24]. That causes inadequate availability of Cu for NPA and LPR during very close to calving. Therefore, when in vitro supplementation of Cu was done in the culture media, it increased the NPA and T lymphocyte LPR of Cu non-fed cows (experiment 1) and, NPA and LPR of both T and B lymphocyte of Cu fed cows (experiment 2) on the day of calving and post-calving days. Similarly, the level of Zn was reduced significantly around calving and post-calving periods [11] that also leads to inadequacy of Zn for NPA and LPR around calving period. As a consequence, in vitro Zn supplementation in the culture media increased the NPA, B lymphocyte LPR of Zn non-fed cows (experiment 1) and T lymphocyte LPR of Zn fed cows on the calving day and post-calving period (experiment 3). Ibs and Rink [8] had already reported that Zn is important in B cell activation. It also affects the phagocytic activity of neutrophils.

## Conclusions

We found an increase in NPA and LPR on the day of calving and early postpartum (7 days postpartum) period with in vitro supplementation of Cu and Zn in the culture media of Cu and Zn fed and non-fed cows. Keeping this view, it is concluded that extra micronutrient supplementation (Cu and Zn) can improve immunity on the day of calving and postpartum period by increasing NPA and LPR.

**Acknowledgments** This research was supported by the Department of Biotechnology (Grant Number: BT/PR8404/AAQ/1/548/2013 DATED 20/06/2014), Ministry of Science and Technology, Government of India.

## Compliance with Ethical Standards

**Conflict of interest** We hereby disclose that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

## References

- Castillo C, Hernandez J, Bravo A, Lopez-Alonso M, Pereira V, Benedito JL (2005) Oxidative status during late pregnancy and early lactation in dairy cows. *Vet J* 169:286–292
- Choi HS, Kim JW, Cha YN, Kim C (2006) A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *J Immunoassay Immunochem* 27:31–44
- Dang AK, Prasad S, De K, Pal S, Mukherjee J, Sandeep IVR, Mutoni G, Pathan MM, Jamwal M, Kapila S, Kapila R, Kaur H, Dixit S, Mohanty AK, Prakash BS (2013) Effect of supplementation of vitamin E, copper and zinc on the in vitro phagocytic activity and lymphocyte proliferation index of peripartum Sahiwal (*Bos indicus*) cows. *J Anim Physiol Anim Nutr* 97(2):315–321
- De K, Prasad S, Kaur M, Pal S, Mohanty AK, Dang AK (2012) Effect of in vitro supplementation of different doses of micronutrients on the phagocytic activity of blood neutrophils isolated at calving from crossbred cows. *Indian J Dairy Sci* 65(5):375–381
- De K, Pal S, Prasad S, Dang A (2014) Effect of micronutrient supplementation on the immune function of crossbred dairy cows under semi-arid tropical environment. *Trop Anim Health Prod* 46(1):203–211
- Grummer RR (2007) Strategies to improve fertility of high yielding dairy farms: management of the dry period. *Theriogenology* 68:S281–S288
- Hoeben D, Monfardini E, Opsama G, Dosogne H, DeKuif A, Beckers JF, Burvenich C (2000) Chemiluminescence of bovine polymorphonuclear leukocytes during the periparturient period and relation with metabolic markers and bovine pregnancy associated glycoproteins. *J Dairy Res* 67:249–259
- Ibs KH, Rink L (2003) Zinc-altered immune function. *J Nutr* 133(5 Suppl 1):145
- Mamchak AA, Hodgkin PD (2000) Regulation of lipopolysaccharide-induced B-cell activation: evidence that surface immunoglobulin mediates two independently regulated signals. *Immunol Cell Biol* 78(2):142–148
- Meglia GE, Johannisson A, Petersson L, Persson Waller K (2001) Changes in some blood micronutrients, leukocytes and neutrophil expression of adhesion molecules in periparturient dairy cows. *Acta Vet Scand* 42:139–150
- Meglia GE, Holtenius K, Petersson L, Öhagen P, Waller KP (2004) Prediction of vitamin A, vitamin E, Selenium and Zinc status of periparturient dairy cows using blood sampling during the mid dry period. *Acta Vet Scand* 45:119–128
- Meglia GE, Johannisson A, Agenäs S, Holtenius K, Persson Waller K (2005) Effects of feeding intensity during the dry period on leukocyte and lymphocyte sub-populations, neutrophil function and health in periparturient dairy cows. *Vet J* 169(3):376–384
- Melendez P (2006) Nutritional management of the transition period to optimize fertility in dairy cattle. In: Proceedings 3rd Florida & Georgia dairy road show, Tifton, GA, USA, March 7, pp 1–50
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival; Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Senthilkumar P, Nagalakshmi D, Ramana Reddy Y, Sudhakar K (2009) Effect of different level and source of copper supplementation on immune response and copper dependent enzyme activity in lambs. *Trop Anim Health Prod* 41:645–653
- Solaiman SG, Craig TJ Jr, Reddy G, Shoemaker CE (2007) Effect of high levels of Cu supplement on growth performance, rumen fermentation and immune response in goat kids. *Small Rumin Res* 69:15–123
- Sordillo M, Aitken SL (2009) Impact of oxidative stress on the health and immune function of dairy cattle. *Vet Immunol Immunopathol* 128:104–109
- Sordillo LM, Contreras GA, Aitken SL (2009) Metabolic factors affecting the inflammatory response of periparturient dairy cows. *Anim Health Res Rev* 10(1):53–63
- Sordillo LM (2005) Factors affecting mammary gland immunity and mastitis susceptibility. *Livest Prod Sci* 98:89–99
- Spears JW (2000) Micronutrients and immune function in cattle. *Proc Nutr Soc* 59:587–594
- Tomlinson DJ, Socha MT, DeFrain JM (2008) Role of trace minerals in the immune system. In: Proceedings of the Pennsylvania state dairy cattle nutrition workshop, Grantville, PA, pp 39–52
- Weiss WP, Spears JW (2006) Vitamin and trace mineral effects on immune function of ruminants. In: Sejrnsen K, Hvelplund T, Nielsen MO (eds) Ruminant physiology. Wageningen Academic Publishers, Utrecht, pp 473–496
- Wilkinson JM, Dyck MK, Dixon WT, Foxcroft GR, Dhakal S, Harding JC (2012) Transcriptomic analysis identifies candidate genes and functional networks controlling the response of porcine peripheral blood mononuclear cells to mitogenic stimulation. *J Anim Sci* 90(10):3337–3352
- Xin Z, Waterman DF, Hemken RW, Harmon RJ (1993) Copper status and requirement during the dry period and early lactation in multiparous Holstein cows. *J Dairy Sci* 76:2711–2716
- Yang FL, Li XS (2015) Role of antioxidant vitamins and trace elements in mastitis in dairy cows. *J Adv Vet Anim Res* 2(1):1–9
- Yang FL, Li XS, He BX (2011) Effects of vitamins and trace elements supplementation on milk production in dairy cows: a review. *Afr J Biotechnol* 10:2574–2578