Effect of in vitro Supplementation of Different Doses of Micronutrients on the Phagocytic Activity of Blood Neutrophils Isolated at Calving from Crossbred Cows

Kalyan De1, Shiv Prasad2, Shashi Pal3, Mandheer Kaur4, A.K. Mohanty5 and A.K. Dang6

Central Sheep and Wool Research Institute (Avikanagar via Jaipur) 304501

Peripartum period in cows is considered as the most stressful period and maximum suppression in the phagocytic activity (PA) of neutrophils occurs at calving. To study the effect of micronutrients on the in vitro PA of blood neutrophils, blood samples were taken from freshly calved Karan Fries cows and their neutrophils were isolated. Four micronutrients (α-tocopherol, Vitamin A, Copper and Zinc) were supplemented in vitro to the culture of neutrophils and yeast cells/zymosan at the levels of 0 (control), 5, 10, 25, 50, 75, 100 and 150 percent. PA of blood neutrophils was estimated both by microscopic and nitro blue tetrazolium (NBT) assay. Supplementation of α-tocopherol at a concentration of 3.483 μM (75%), Vitamin A (VA) at a concentration of 1.397 μM (50%), copper (Cu) at a concentration of 2.361 μM (25%) and zinc (Zn) at the concentration of 9.176 μM (75%) showed maximum PA. Excess supplementation of Cu at 14.164 μM (150%) significantly (P<0.01) decreased the PA. Our results suggest that micronutrients when added in limited concentration to the culture medium of neutrophils improved their PA.

Keywords: In vitro. Micronutrients. Supplementation. Phagocytic activity. Neutrophils.
metabolites produced during phagocytosis (NRC 2001). Zn is a structural component in superoxide dismutase enzyme (SOD), which aids in quenching free radicals produced from various processes in the body during an immune response (Murray et al. 2000) and has been found to enhance the PA of macrophages and neutrophils (Babior, 1978).

Although supplementation of various vitamins and minerals like VE, VA, Cu, Zn etc in vitro during peripartum period have shown to play an important role in maintaining the health and improving the PA of blood neutrophils (Mehrzad et al. 2002 and Weiss and Spears, 2006). But still their effect during in vitro supplementation is poorly understood. Therefore, the objectives of this study were to determine the effect of supplementation of VE, VA, Cu and Zn on the PA of blood neutrophils isolated on the day of calving and also to determine appropriate concentrations of these micronutrients which can be supplemented in vitro for achieving maximum PA.

MATERIALS AND METHODS

Blood samples were collected from multiparous Karan-Fries (KF) cows on the day of calving. Neutrophils were isolated and their in vitro PA was estimated by two different methods i.e. microscopically and by NBT assay. Phagocytosis is a two-stage process in which particles are first bound to the cell surface and then ingested. Under microscopic assay yeast cells i.e. Saccharomyces cerevisiae were incubated with blood neutrophils. The number of yeast cells phagocytosed or attached to each neutrophils were measured microscopically. Results of microscopic assay have been presented in photographs, whereas, results obtained under NBT assay have been shown in table and figures. For studying NBT assay, neutrophils were allowed to proliferate with Zymosan (650 μg/ml) and NBT (250 μg/ml) concentrations. Amount of zymosan phagocytosed by blood neutrophils was used as an indicator of PA. All cultures were allowed to incubate at 37°C in a humidified CO₂ incubator (95% air and 5% CO₂) for 2h. Nitroblue tetrazolium (NBT) assay was used to determine the production of superoxide anion (O₂⁻) in the neutrophils. NBT is yellow in color, but is changed to blue formazan after phagocytosis which can be measured spectrophotometrically (Choi et al. 2006). OD was taken at 540 nm by using a multiwell scanning spectrophotometer (Microscan MS-5608A).

Levels of VE, VA, Cu and Zn were estimated from normal lactating cows. VE was estimated by HPLC (Chawla and Kaur, 2001), whereas, Cu and Zn were estimated by Hitachi Z 5000 AAS using acetylene as fuel and air as an oxidant. Normal plasma level of VE, VA, Cu and Zn were 4.643 μM, 2.793 μM, 12.234 μM and 9.442 μM respectively. Different dose of vitamins (A and E) and minerals (Cu and Zn) were supplemented in the culture medium to see their influence on the PA of blood neutrophils. Micronutrients were added in 7 different doses i.e. 5%, 10%, 25%, 50%, 75%, 100%, and 150% of the plasma level. Where 100% is equals to the level found in plasma of normal lactating cows. Culture medium which was without any micronutrient supplementation served as control.

As the level of VE found in plasma was 4.643 μM, therefore, accordingly, VE was added in the culture media @ 0.232 μM (5%), 0.464 μM (10%), 1.160 μM (25%), 2.321 μM (50%), 3.482 μM (75%), 4.643 μM (100%) and 6.965 μM (150%). For supplementation of VE, VE acetate (cell culture tested) with molecular weight 472.76 and having a maximum assay of 96% was used. To see the effect of VA supplementation, retinol acetate with molecular weight of 328.50 and having a potency of 475,000 IU/gm, was added at the rate of 0.139 μM (5%), 0.279 μM (10%), 0.698 μM (25%), 1.396 μM (50%), 2.094 μM (75%), 2.793 μM (100%) and 4.189 μM (150%) in the culture media. For studying the effect of Cu on the in vitro PA of blood neutrophils, in vitro cell culture tested Cu sulphate with molecular weight of 249.68 and maximum assay of 99% was added in the concentration of 0.472 μM (5%), 0.944 μM (10%), 2.360 μM (25%), 4.721 μM (50%), 7.082 μM (75%), 9.442 μM (100%) and 14.164 μM (150%) to the culture of neutrophils and zymosan. To see the effect of Zn supplementation, in-vitro cell culture tested Zn sulphate with molecular weight of 287.54 and maximum assay of 99.5% was supplemented @ 0.611 μM (5%), 1.223 μM (10%), 3.058 μM (25%), 6.117 μM (50%), 9.175 μM (75%), 12.234 μM (100%) and 18.351 μM (150%).

For statistical analysis of data the general linear
model (univariate) was applied. The effect of micronutrients was analyzed by one-way ANOVA. Duncan's multiple range test was used to establish significant differences between mean values of the O.D. of different dose of the micronutrients. IBM SPSS statistics 20 was used for all statistical procedures.

RESULTS AND DISCUSSION

Results of microscopic assay of blood neutrophils and yeast cells have been presented in photographs 1-4. Yeast cells (smaller in size) can be seen (Photograph 1) either attached or engulfed by bigger neutrophils (Photograph 2, 4). Optical density (OD) values of formazan crystals using different micronutrient treatments at various doses to the culture media of blood neutrophis and zymosan have been presented from Fig.1 to 4 respectively. Optical density values of VE in the control animals were found to be 0.250 ± 0.047 (Table 1). Supplementation of different doses of VE upto a concentration of 50% in the culture medium did not showed any positive effect on the PA of neutrophils. However, a non-significant increase in PA was seen at 75% (i.e. 3.482 μM) level of VE. From 75% to 150% level of VE, the PA remained above the non supplemented neutrophils. Thereafter, the PA remained almost unchanged and there was no improvement of PA with the increase of additional supplementation of VE doses from 100% and 150%. Improvement in the levels of the VE supplementation causes an improvement in the phagocytic cell activity and function (Heinrichs et al. 2009). Rapid recruitment of neutrophils is critical for maximizing host defense mechanisms and supplementation of 3000 IU of VE/day during the transition period prevented a decline in neutrophil superoxide anion production (Weiss et al. 1997). Feeding still a higher dose of 4000 IU of VE during the last 14 days of the dry period reduced clinical mastitis and new infections at calving (Weiss, 1998). In our study, we also found that with the increase of the dose, there was an increase in the in vitro PA of blood neutrophils, but it remained constant after increasing the supplementation above 75%. Although very higher doses of VE (Weiss, 1998) have been found to have a beneficial effect in cows at calving, but high levels of vitamin E in human diet (>300 mg/d) have been found to decrease the ability of neutrophils to undergo phagocytosis (Boxer,1986) and to kill bacteria and decrease monocyte respiratory burst and IL-1β production (Prasad, 1980).

The mean O.D values of formazan crystals without supplementation of VA were 0.338 ± 0.040 (Table 1). Addition of 5% VA (i.e. 0.139 μM) to the culture medium declined the PA of blood neutrophils cultured in vitro. However, O.D values increased more than the control values at 25% level (0.698 μM) and the highest value of OD i.e. 0.382 ± 0.030 was found at 50% (1.396 μM) level of supplementation of VA. This increase at 50% level in the PA was 13.13% more as compared to the non-supplemented study. Thereafter, the PA gradually decreased with the increase in doses of VA and it remained lower at 150% level. Deficiencies in β-carotene and VA around calving have been associated with lower reproductive performance and higher incidence of intramammary infections (Michal et al. 1994). β-carotene has been found to reduce superoxide formation within the phagocyte and may increase phagocytosis (Sordillo et al. 1997).

Studies carried out, in the dairy cattle have also shown that VA and β-carotene influence

<table>
<thead>
<tr>
<th>Micro-nutrients</th>
<th>Vitamin E</th>
<th>Vitamin A</th>
<th>Copper</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.250 ± 0.047</td>
<td>0.338 ± 0.040ab</td>
<td>0.217 ± 0.014a</td>
<td>0.253 ± 0.020</td>
</tr>
<tr>
<td>5%</td>
<td>0.205 ± 0.040</td>
<td>0.187 ± 0.040b</td>
<td>0.210 ± 0.045ab</td>
<td>0.225 ± 0.027</td>
</tr>
<tr>
<td>10%</td>
<td>0.208 ± 0.039</td>
<td>0.326 ± 0.049ab</td>
<td>0.208 ± 0.011ab</td>
<td>0.274 ± 0.023</td>
</tr>
<tr>
<td>25%</td>
<td>0.208 ± 0.052</td>
<td>0.349 ± 0.048ab</td>
<td>0.233 ± 0.015a</td>
<td>0.287 ± 0.023</td>
</tr>
<tr>
<td>50%</td>
<td>0.225 ± 0.060</td>
<td>0.382 ± 0.030a</td>
<td>0.188 ± 0.022ab</td>
<td>0.278 ± 0.034</td>
</tr>
<tr>
<td>75%</td>
<td>0.289 ± 0.049</td>
<td>0.381 ± 0.050a</td>
<td>0.186 ± 0.014ab</td>
<td>0.308 ± 0.050</td>
</tr>
<tr>
<td>100%</td>
<td>0.271 ± 0.052</td>
<td>0.365 ± 0.012a</td>
<td>0.185 ± 0.025ab</td>
<td>0.290 ± 0.045</td>
</tr>
<tr>
<td>150%</td>
<td>0.270 ± 0.051</td>
<td>0.287 ± 0.008ab</td>
<td>0.148 ± 0.007b</td>
<td>0.284 ± 0.044</td>
</tr>
</tbody>
</table>

Figures with similar superscripts within a column do not differ significantly (P<0.05) with each other.
proliferation, differentiation and function of bovine mononuclear leukocytes (MNL) and neutrophils in vitro (Tjoekler et al. 1988). In vivo and in vitro studies carried out on human leukocytes have shown that carotenoid reactive oxygen species (ROS) quenching capacities control both in vitro and in vivo neutrophils ROS generation and probably protect these cells against DNA, membrane lipid and protein damages during oxidative burst (Walrand et al. 2004). Although VA is available whenever we are feeding green fodder to the animals, but at calving more of this vitamin is going towards colostrums (Bouda et al. 1979), therefore it is less available for other functions and supplementation of this vitamin is needed. Reports on excess supplementation of VA in vivo to dairy cows are not available.

The values of the O.D. obtained after supplementation of Cu in vitro to the culture of blood neutrophils and zymosan have been presented in Table 1. The O.D. values of control samples i.e. without Cu supplementation were 0.217 ± 0.014. There was no significant change in the O.D values with the change in the level of Cu supplementation. But it showed a significantly (p<0.01) lower O.D at 150% level of Cu supplementation when compared to other supplemented and non supplemented neutrophils.

Photograph 1: Culture of yeast cells (Saccharomyces cerevisiae) (400X)

Photograph 2: Culture of blood neutrophils (400X)

Photograph 3: Co incubation of yeast cells and blood neutrophils (1000X)

Photograph 4: Blood neutrophil phagocytosing 7-8 yeast cells (1000X)
Optical density values remained unchanged when Cu was supplemented in the culture medium at 5% and 10% (0.472 μM and 0.944 μM). Maximum O.D values i.e. 0.233 ± 0.015 were found when culture medium was supplemented with 25% (2.360 μM) of Cu. This increase in PA was 7.33% as compared to non-supplemented group. Thereafter, no improvement in PA was seen with the increase of Cu supplementation in vitro. Low Cu status reduced neutrophil phagocytic capacity (Boyne and Arthur, 1981) and administration of Cu to Cu-depleted calves have been found to increase the ability of isolated peripheral blood granulocytes (primarily neutrophils) to kill ingested Candida albicans by over 2-fold (Jones and Suttle, 1981). A significant reduction in PA of neutrophils during inadequate intake of Cu has also been reported by Babu and Failla (1990).

In our study, we observed that there was a significant (p<0.01) reduction in the PA of blood neutrophils when excess of Cu (150%) was added to the culture media. Our in vitro study can be supported by in vivo studies where excess Cu have been found to reduce feed intake which decreases growth and increases mortality in livestock (Perrin, 1990). A reduction in the weight of lymphoid organs such as thymus has also been observed (Underwood and Suttle, 1999).

The O.D values of the in vitro supplementation of Zn have been presented in Table 1. Control culture group values of O.D were 0.253 ± 0.020. These values changed with different doses of Zn supplementation although the effect was not significant. Addition at the rate of 5% level (0.611 μM) of Zn in the culture medium declined the O.D values. Thereafter, with the gradual increase (10%, 25%, 50%, 75%) in the levels of Zn supplementation in the medium, the O.D values also gradually increased upto 75% (9.175 μM) and highest values i.e., 0.308 ± 0.050 were found at 75% level, which is 21.75% higher than the control group. However, further increase of supplementation declined the O.D values. Zn enhances PA of macrophages (Babior, 1978) and neutrophils and this PA occurs through respiratory burst (Chew, 1996). In young calves, the addition of 150 or 300 mg Zn/kg to a control diet containing 65 mg Zn/kg did not affect mitogen-induced blastogenesis or phagocytic and bactericidal activity of isolated neutrophils (Kincaid et al. 1997) which is similar to our findings. However, no beneficial effect was seen when we increase the supplementation dose above 75% in vitro. Although no studies of feeding excess Zn have been reported in cattle, but, giving 300 mg Zn/d for 6 weeks to young adult human subject’s have been found to decrease lymphocyte and phagocyte function (Chandra, 1984). High Zn intakes can also result in Cu depletion, and Cu deficiency impairs immune function (Prohaska and Failla, 1993).

Deficiency of trace minerals or vitamins affects neutrophil function and this effect can be reversed by supplementation as described above. In humans however, it is still not clear whether excesses of micronutrients will enhance or suppress overall innate immunity (Erickson et al. 2000). As literature on the ill effects of feeding of excess micronutrients to dairy cows is limited. Therefore, before feeding micronutrients, feed rations should be evaluated for their concentration, bioavailability and desired requirements depending upon the physiological state of the animal (Yang et al. 2011).

CONCLUSIONS
Our in vitro study carried out on the blood neutrophils isolated on the day of calving substantiates the already available in vivo reports that addition of various micronutrients around calving helps to improve PA. This study shows that under in vitro conditions, the response of stressed neutrophils varied with micronutrient supplementation and the concentration at which PA is maximum indicates that this concentration is sufficient to neutralize the radicals produced by neutrophils during the process of phagocytosis. Further, whenever there is need for supplementation, it should be done only at appropriate doses as additional micronutrients may not have any beneficial effect on the blood neutrophil PA after a particular level.

ACKNOWLEDGEMENTS
The authors are thankful to the Department of Biotechnology, Ministry of Science and Technology, Government of India for providing financial assistance for carrying out this research work.

REFERENCES


Dang, AK; Shiv, K; De, K; Pal, S; Mukherjee, J; Sandeep, IVR; Gilbert, M; Khan, M; Jamwal, M; Kapila, S; Kapila, R; Kaur H; Dixit, S; Mohanty, A; and Prakash, B.S. 2012. Effect of supplementation of vitamin E, copper and zinc on the immune response. *J. Dairy Sci.* **94**:303.


Phagocytic Activity of Blood Neutrophils


