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Total antioxidant capacity, neutrophil profile, *in vitro* phagocytic activity, myeloperoxidase (MPO) activity and IL-8 status in uterine infected Murrah buffaloes during peripartum period

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ABSTRACT

The present experiment was carried out with the objective to investigate the antioxidant status of peripartum Murrah buffaloes by assessing total antioxidant capacity (TAC), neutrophil profile, *in vitro* phagocytic activity, myeloperoxidase (MPO) activity, IL-8. Based on uterine fluid scoring, white side test buffaloes (n=24) were classified into healthy (n=11) and uterine infected buffaloes (n=13). Blood samples were collected 7 day before calving (day 7), on day of calving (day 0) and after calving (day 7, 14, 21 and 35) for estimation of the mentioned parameters and uterine fluid samples were collected during postpartum period, i.e. day 7, 14, 21, 35 after calving for grouping of buffaloes. Results showed that TAC concentration decreased on day of calving and elevated afterwards whereas the percentage of neutrophils was significantly higher on 14th, 21th and 35th day after calving in uterine infected buffaloes compared to healthy group. Phagocytic activity was lower during peripartum period and IL-8 concentration was significantly higher on 21 day after calving in uterine infected buffaloes as compared to healthy buffaloes. Pearson correlation between serum TAC with uterine score gave a non-significant correlation of –0.33. From the above research it can be concluded that lower concentration of TAC, decreased phagocytic activity of neutrophils in uterine infected buffaloes indicate poor anti-oxidant status to combat against oxidative stress making animals susceptible to infections. Therefore, proper nutritional management with additives should be provided during peripartum period to reduce the incidence of uterine infections as well as for better welfare.

Keywords: IL-8, *In vitro* phagocytic activity, Murrah buffaloes, Myeloperoxidase activity, Total antioxidant capacity (TAC)

Buffaloes known as the black gold are generally found in the Indian subcontinent which is the major source of milk production. They contribute 55% in India's total milk production whereas in Asia and world it is 38 and 12.1% respectively (FAO STAT 2007). Peripartum period is the most critical period for dairy animals generally extending from 3 weeks prior to 3 weeks after parturition (Smith and Risco 2005) during which animal has to go through physical, physiological stress which takes a great role in compromising the immune response making them susceptible to various uterine infections. Annual incidence of uterine infections in postpartum dairy buffaloes ranges from 20 to 75% (Usmani *et al.* 2001) Excess generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) results damage of macromolecules during peripartum. Oxidative stress is a considerable factor in compromised immune responses which amplifies the sensitivity of individuals to different health hazards (Sordillo 2005, Wilde 2006). Low level of plasma antioxidant status together with total antioxidant capacity, ascorbic acid, and diminished glutathione concentration is associated with mastitis, subclinical and clinical endometritis has been demonstrated by many studies (Hanafi *et al*. 2008, Heidarpour *et al*. 2012). The measure of TAC includes evaluation of collective action of all the antioxidants present in plasma (Ghiselli *et al*. 2001). Latest study showed that low level of serum TAC, high level of lipid peroxidation and nitric oxide production during peripartum period influence the endometrial expression of antioxidative genes that compromise the uterine health during postpartum period in Sahiwal cows (Baithalu *et al*. 2017). However literature regarding changes in TAC level in buffaloes during peripartum period are not reported. Neutrophils, the first line of cellular defence play a vital role in onset of disease during peripartum period and IL-8 is the major cytokine which attracts neutrophils to the site of infection (Kehrli *et al*. 1989) and this expect need to be explored in case of Murrah buffaloes. However very few investigations were carried out regarding TAC, neutrophils

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Table 1. Uterine fluid scoring of animals (Sheldon *et al.* 2009)

Score	Characteristics of uterine fluid
0	Clear
1	Flakes of pus
2	Mucopurulent
3	Purulent

profile, phagocytic activity, myeloperoxidase activity and IL-8 concentration during peripartum period in relation to occurrence of uterine infection in Murrah buffaloes. With this overview, the present study was undertaken to elucidate the oxidative stress and physical status of Murrah buffaloes by understanding the TAC, MPO activity and IL-8 level fluctuations.

MATERIALS AND METHODS

Location of the study: The study was carried out in Livestock Research Centre located in ICAR-NDRI, Karnal, Haryana, India. The latitude, longitude and elevation of the experimental area is 29.685629 (29° 41′ 8.2644″N), 76.990547 (76° 59′ 25.9692″E) and 252.37m respectively. The prevailing climate in Karnal is known as a local steppe climate. The average annual temperature is 24.4°C and average annual rainfall is around 617mm in Karnal.

Experimental design: For the experiment 24 Murrah buffaloes of 2nd parity were taken. Optimum conditions were maintained for all experimental animals and were kept in general herd in open housing system and the guidelines of institute ethical committee were followed throughout the research period. Blood samples were collected on 7 days before calving (-7th), on the day of calving (0th) and 7th, 14th , 21st , 35th day after parturition from all experimental and serum was separated by centrifugation at 3,000 rpm for 10 min at 4° C and stored at –20°C. It was analysed for estimation of TAC, neutrophil profile, phagocytic activity, MPO activity and IL-8 concentration whereas uterine fluid was collected on day 7th, 14th, 21st and 35th after calving using a blue sheath (IMV technology, France) fitted in Universal AI gun after disinfecting the perineal region with 70% alcohol were scored according to colour, odour and consistency for presence of uterine diseases.

Animal classification: Scoring was done as per Sheldon *et al.* (2009) given in Table 1. Animals showing score 1, 2, 3 were considered as uterine infected buffaloes and animals with score 0 without any postpartum complications were classified as healthy buffaloes. For detection of subclinical endometritis white side test was conducted. 1 ml of uterine fluid was heated with equal volume of 5% sodium hydroxide up to boiling point and after cooling the intensity of colour changes were studied and graded as normal (turbid or no colour), mild infection (light yellow colour), moderate infection (yellow colour) and severe infection (dark yellow colour). Animals coming in category of mild, moderate and severe are taken as infected animals and rest of the animals showing turbid or no colour changes are taken as healthy animals. From total experimental Murrah buffaloes (n=24), 11 animals were turned healthy without uterine infection whereas 13 animals developed uterine infection.

Estimation of total antioxidant capacity (*TAC*) *using ELISA kit:* Standards were prepared, optical densities of samples were taken and TAC was calculated as per the protocol given in the ELISA kit (CAYMAN total antioxidant assay kit).

Estimating neutrophils percentage in peripheral blood: By using Field's stain method differential neutrophil counts were estimated from the blood sample of Murrah buffaloes. Blood smear was prepared, air dried and fixed with ethanol (absolute 99.9%) followed by staining with Field's stain. After drying they are examined under oil immersion objective to determine the % of neutrophils and other leucocytes by counting around 200 cells.

Estimation of in vitro *phagocytic activity of blood neutrophils:* Hypotonic lysis of RBC method by Mehrzad *et al.* (2004) was used for neutrophils isolation from peripheral blood. Phagocytic cells produce O_2^- anions. These O_2^- reduces the yellow coloured water soluble nitrobluetetrazolium (NBT) to water insoluble blue or purple coloured formazan crystals. The OD of the reduction product was determined at 540 nm. The concentration of total viable neutrophils was calculated and final concentration was adjusted to 5×10^6 cells per millilitre of cultured medium (RPMI 1640 with 50 mM Hepes, 0.1mM sodium pyruvate, streptomycin 100 µg/ml, penicillin 100 IU/ml). Cell suspension of 200 µl per well in duplicate was placed in a 96 well flat bottomed tissue culture plate. The cells were allowed to proliferate with zymosan (1 mg/ml) and NBT 0.5 mg/ml. The blank well consisted of 200 µl of culture medium along with same concentrations of NBT and zymosan. The plate was allowed to incubate at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2) for 5 h and the optical density (OD) of the reduced product was determined at 540 nm using multi-well scanning spectrophotometer (TECAN, Seestrasse 103, Switzerland).

Estimation of MPO activity of neutrophils: The concentration of myeloperoxidase (MPO) in the neutrophils samples was determined by comparing the O.D. of the samples to the standard curve. Procedure of assay was followed as per the protocols provided by the manufacturers (Myeloperoxidase Omnikine ELISA Kit).

Estimation of interleukin-8 (*IL-8*) *by ELISA kit:* The concentration of IL-8 in the serum sample was estimated by comparing the OD of the samples to the standard curve prepared. Procedure of assay was followed as per the protocols provided by the manufacturers (Thermofisher Scientific IL-8 human ELISA kit).

Statistical analysis: Descriptive statistics were calculated for different blood parameters for both healthy and uterine infected group and the results were expressed as mean± SE. Within group comparisons were performed using independent sample T test. One way ANOVA was used to compare between groups. Group wise multiple comparisons were performed using Tukey's post hoc test. The difference of means was considered significant at $P<0.05$. All the

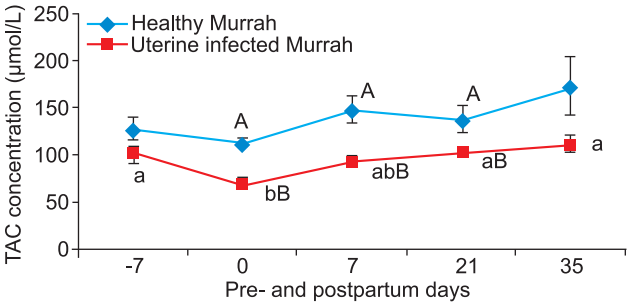

Fig. 1. Serum TAC in multiparous Murrah buffaloes during different days of peripartum period. Values expressed as mean±SEM. Values with different superscript are significantly (P<0.05) different.

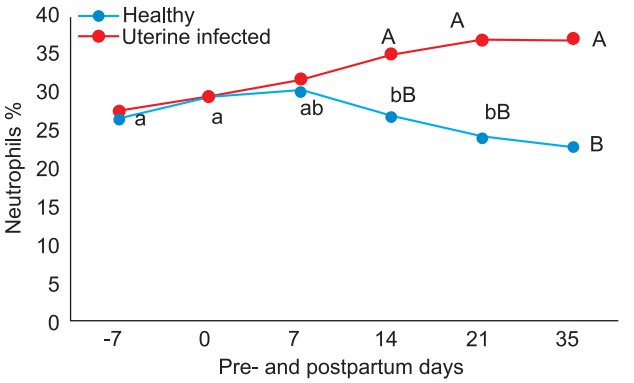

Fig. 2. Blood neutrophil per cent in multiparous Murrah buffaloes during different days of peripartum period. Values expressed as mean±SEM. Values with different superscript are significantly (P<0.05) different.

analysis was performed using IBM SPSS Statistics 22, Prism.

RESULTS AND DISCUSSION

Total antioxidant capacity (TAC) using ELISA kit: The mean serum TAC concentration is depicted in Fig. 1. which shows significantly (P<0.05) higher TAC level was observed on day of calving and 7th, 21st day post calving in healthy buffaloes when compared with uterine infected buffaloes. When comparison was made across the days, in healthy group, serum TAC concentration decreased (P>0.05) on day of calving but remain elevated throughout the postpartum period. However, in uterine infected buffaloes, TAC concentration was significantly decreased (P<0.05) on day of calving from that of 7th day prior to calving and concentration elevated afterwards and maximum concentration was obtained on 35th day post calving. When the correlation was made between serum TAC with uterine score, a non-significant correlation of -0.33 was obtained.

Ghiselli *et al.* (2001) reported the decreased levels of TAC and provided information regarding the dynamic equilibrium between pro-oxidant and antioxidant molecules in the plasma. Reduction in the level of antioxidants makes the system incapable to protect the cellular components resulting in compromised immunity and onset of various infections (Miller *et al*. 1993, Sordilo and Atiken 2009). In our study, healthy buffaloes exhibiting higher TAC level as compared to uterine infected group which signifies that presence of oxidative stress in uterine infected animals reduces the ability to detoxify the system and compromises immunity as well as increases the susceptibility to get infected. The pattern of changes observed in the present study in TAC concentration in buffaloes is in close agreement to the observations made by earlier researchers (Castillo *et al*. 2005, Baithalu *et al*. 2017). In the present study, lowest level of TAC was recorded at parturition with gradual rise in further postpartum period in both groups. This is in close agreement with Brezezinska *et al.* (1994), Chawla and Kaur (2004), Khatti *et al.* (2017) and it might be due to high free radical burden during calving that reduces the concentration of SOD and CAT leads to overall reduction in the TAC. Altogether, results indicated that high concentration of TAC in healthy buffaloes depicted better anti-oxidant status to combat against oxidative stress and lower TAC concentration in infected buffaloes had signified damaging effect on uterine health due to oxidative stress as well as lactation stress.

Neutrophil profile in peripheral blood: The percentage of neutrophils count during pre and post-partum period in both group are depicted in Fig. 2. The percentage of neutrophils was significantly higher (P<0.05) in uterine infected group on 14th, 21st and 35th day postpartum when compared with healthy buffaloes. When comparison was made across the days, significantly higher (P<0.05) percentage of neutrophils was observed on 7 day prior to calving and day of calving compared to 21st and 35th day post calving in healthy buffaloes. The percentage of neutrophils in circulation has shown a increasing trend from 7 day prior to calving to 35 days post calving in uterine infected buffaloes although difference was non-significant (P>0.05).

The blood leucocytes count and neutrophils counts reflect the immune status of animal (Mohapatra and Dang 2018). Neutrophils are known as first line defence; they migrate first from blood into an inflamed area for phagocytosis and intracellular killing by engulfing bacteria with two distinct mechanisms, the respiratory burst and by digestion through lysosomal enzymes (Jain 1986). Increase in TLC on the day of calving is a normal phenomenon of parturition (Mateus *et al.* 2002, Sheldon *et al.* 2009) and was mainly contributed by increase in neutrophil count. Different stressors at the time of parturition like endocrinological, physiological and psychological stresses during peripartum period (Hussain and Daniel 1992, Burton *et al.* 1995) impairs the migration of neutrophils and other leucocytes from peripheral circulation, resulting in brief increase in circulating counts of TLC and neutrophils on the day of calving (Islam 2014, Preisler *et al.* 2000). The increase in the neutrophil percent postpartum in uterine infected animal compared to healthy animal as observed in our study might be due to the persistence of infection leading to increase in stimulus for production of neutrophils to

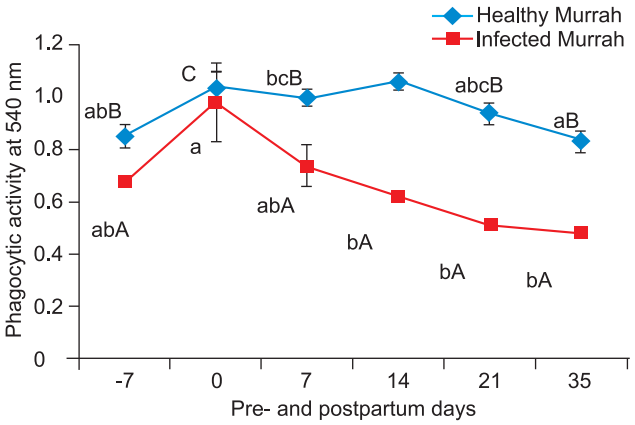

Fig. 3. *In vitro* phagocytic activity of neutrophils in multiparous Murrah buffaloes during different days of peripartum period. Values expressed as mean±SEM. Values with different superscript are significantly (P<0.05) different.

eliminate the infection present in the uterus (Kim *et al.* 2005). Higher neutrophil percentage on the day of calving in healthy buffaloes may be due to antipartum rise in cortisol level which is in close agreement with the findings of Mohapatra and Dang (2018). However, TLC decreases during postpartum period and it is coupled with migration and recruitment of blood neutrophils towards uterine lumen and mammary tissues (Preisler *et al.* 2000).

In vitro *phagocytic activity of blood neutrophils: In vitro* phagocytic activity of neutrophils in Murrah buffaloes is presented in Fig. 3. Significantly (P<0.05) higher phagocytic activity was observed on all sampling days except on the day of calving in healthy buffaloes compared to uterine infected group. Phagocytic activity followed a decreasing trend following parturition upto 35th days post calving in both healthy and uterine infected buffaloes. However, the significantly higher (P<0.05) activity was observed on day of calving when compared to 14th, 21st and 35th days post calving in uterine infected buffaloes. In healthy buffaloes, significantly higher (P<0.05) activity was observed on day of calving and 14th day post calving when compared with 7 days prior to calving and 35th days post calving.

Analysis of *in vitro* phagocytic activity of neutrophils provides a very effective tool for the study of innate immune response of animals. Reduced neutrophil functions and compromised host defence mechanisms during peripartum period in dairy animals have also been observed by Meglia *et al.* (2001) and Dang *et al.* (2012). Poor activity of neutrophils may be due to more number of immature neutrophils which are coming in circulation which have no proper machinery to fight or phagocytose against infection. The suppression in the phagocytic activity may be due to an increase in the cortisol levels during peripartum period. Parturition reflex causes higher plasma cortisol level that causes hyper stimulation of red bone marrow for the faster release of neutrophils. As a result of this, there is release of more number of immature band neutrophils and a less number of matured segmented neutrophils. That is why the phagocytic activity of neutrophils decreases as evident in our study (Paape *et al*. 2003, Pathan *et al.* 2017). The results in the present study in Murrah buffaloes were in close agreement with the study conducted by Kehrli *et al.* (1989), who observed that blood PMN function begins to decline prior to parturition, reaches a peak shortly after parturition, and slowly returns to prepartum levels by about 4 weeks postpartum. According to two researchers peripheral blood neutrophil function of periparturient dairy cattle is impaired relative to nonparturient cattle (Kehrli *et al*. 1989, Cai *et al.* 1994). The mechanisms responsible for PMN function impairment in periparturient dairy are very poorly understood. Kehrli *et al.* (1989) found high ingestion activity of neutrophils during parturition and it decreased after parturition. In the present study, the phagocytic activity of healthy animals were higher as compared to uterine infected group which is similar with the observations of Kim *et al.* (2005), which might be due to presence of higher cortisol level and relatively more no of targets which exhausts immune system and PMN cells to fight against the pathogens in uterine infected buffaloes as compared to the healthy group of buffaloes.

MPO activity of Neutrophils: MPO activity (ng /ml) of neutrophils in both groups of Murrah buffaloes is depicted in Fig. 4. In case of healthy Murrah buffaloes, higher MPO activity was observed on all days of sampling when compared with uterine infected buffaloes however significantly (P<0.05) higher MPO activity was obtained on 7 days prior to calving in healthy buffaloes. When comparison was made across the days in case of infected Murrah buffaloes, the MPO activity was significantly (P<0.05) higher on 7 days prior to calving and on day of calving compared to other days of sampling, i.e. 7, 21 and 35 days post calving whereas in healthy animals MPO activity was higher on 7 days prior to calving and then it decreases gradually.

During any disease condition the levels of neutrophil enzymes increased, which provide immunity to the animals (Haddadi *et al*. 2006). During calving when animals go

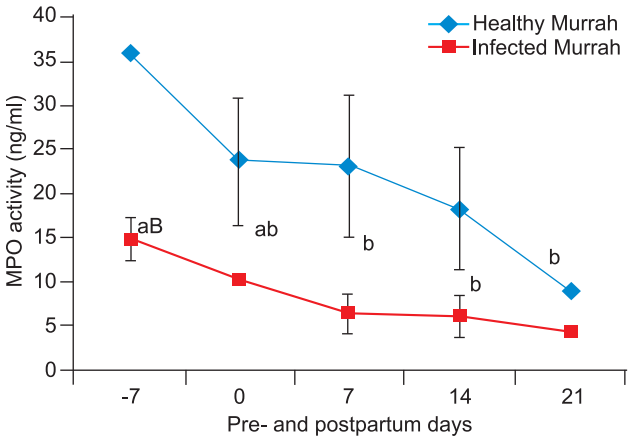

Fig. 4. MPO activity of neutrophils in multiparous Murrah buffaloes during different days of peripartum period. Values expressed as mean±SEM. Values with different superscript are significantly (P<0.05) different.

down in concentration of these enzymes due to higher level of cortisol make animal more susceptible to postpartum diseases. Decreased neutrophil enzyme levels during calving may be because at calving more immature neutrophils are released that are poor in synthesis of granular enzyme as well as due to high cortisol are not able to release granular enzyme (Pathan *et al.* 2017). The results of PMN (Polymorphonuclear) myeloperoxidase activity found in this study are partially similar to studies by Hammon (2006) and Cai *et al.* (1994). As a reliable measure, PMN myeloperoxidase activity was used to determine the ability of PMN to kill pathogens. In this study, PMN myeloperoxidase activity decreased prior to parturition in healthy animals as reported by Hammon *et al.* (2006) and in other cases PMN myeloperoxidase activity declined only after parturition as reported by Cai *et al.* (1994). In periparturient dairy animals, the mechanisms responsible for PMN function impairment are poorly understood. The onset of lactation, metabolic challenges associate with late gestation and psychological stress could be responsible in part for PMN function impairment during this crucial period (Kimura *et al.* 2006).

Interleukin-8 (IL-8) by ELISA kit: The mean serum concentration of IL-8 (pg/ml) in serum during pre and post-partum period in buffaloes is depicted in Fig. 5. IL-8 concentration was significantly (P<0.05) higher on 21 days post calving in uterine infected buffaloes when compared with healthy Murrah buffaloes. When compared across the days, IL-8 concentration was significantly lower on 21 days post calving compared to all other days in healthy buffaloes. However, in uterine infected Murrah buffaloes, there was increase in IL-8 concentration from day 0 to day +21, however, the increase was not significant (P>0.05).

IL-8 is a potential chemoattractant factor for neutrophil which mediate transendothelial migration of neutrophils to tissue spaces to destroy bacterial pathogens (Kehrli and Harp 2001). IL-8 regulates the recruitment of neutrophils as well as T-lymphocytes to the site of infection (Wang *et al.* 2007). Activation of neutrophils during inflammation is a

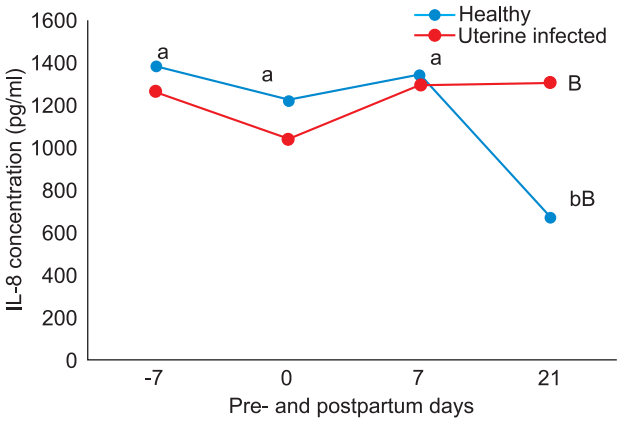

Fig. 5. Serum IL-8 concentration in multiparous Murrah buffaloes during different days of peripartum period. Values expressed as mean±SEM. Values with different superscript are significantly (P<0.05) different.

key event which is mediated by IL-8 (Galligan and Coomber 2000). IL-8 is produced by a wide range of cells including monocytes, activated neutrophils, endothelial, and epithelial cells (Caswell *et al.* 1999). The pattern of changes in IL-8 concentration was similar to that of neutrophils suggesting its role in chemotaxis to increase the recruitment of neutrophils to protect uterine health from invading pathogen after parturition.

From the above study it can be concluded that higher concentration of total antioxidant capacity in healthy buffaloes indicate better anti-oxidant status whereas in uterine infected ones the lower concentration of serum total antioxidant capacity signify poor antioxidant mechanism to combat oxidative stress exposing them to uterine infections and exhibiting damaging effects on uterine health. Further it may be concluded that decreased phagocytic activity of neutrophils as well as myeloperoxidase concentration make animal more susceptible to infections. However, these results will help in understanding the antioxidant status of buffaloes around peripartum period and will help to develop strategies to improve the immune functions for better management and animal welfare purpose. Also, further studies are required in this field to explore more about the neutrophil world in buffaloes at molecular level for a better understanding.

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