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Effect of Poly-herbal mixture and butyric acid feeding on immune parameters of postpartum Murrah buffaloes

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ABSTRACT

The present study was conducted in 24 Murrah buffaloes to study the effect of supplementation of poly-herbal mixture and butyric acid with the objective to reduce parturition stress and to improve immunity. In this study, control group i.e T_0 (n=6) was offered ration as per NRC requirement plus 10 % and the rest of three supplemented groups T_1, T_2 and T_3 (n=6 for each group) were offered ration as per control group along with three different supplementations, (T_1= poly-herbal supplementation for seven day post partum, T_2= poly-herbal supplementation for seven day post partum + 200 ml butyric acid supplementation (30 day prepartum and 30 day postpartum) and T_3= 200 ml butyric acid supplementation (30 day prepartum and 30 day postpartum). Total leukocyte count (TLC) values in all groups showed decreasing trend as observed on 1st, 2nd and 5th days of postpartum. On the 1st day of postpartum, blood neutrophil percentage was found significantly lower ($p<0.05$) in T_2 and T_3 group as compared to T_0 group. On the 5th day of postpartum, blood neutrophil percentage were found significantly lower ($p<0.05$) in T_1, T_2 and T_3 groups as compared to T_0 group. On day one postpartum, the phagocytic activity (PA) and myeloperoxidase activity (MA) was significantly higher ($p<0.05$) in the T_2 and T_3 group of buffaloes in comparison to T_0 group. On the 5th day of postpartum PA and MA were found to be significantly higher ($p<0.05$) in the T_1, T_2 and T_3 group of buffaloes in comparison to T_0 group. These results indicate that supplementation of poly-herbal, poly-herbal + butyric acid and butyric acid during the transition period reduces the stress and improves immunity in Murrah buffaloes. Out of all the supplemented groups, maximum improvement was observed in the poly-herbal+butyric acid supplemented group.

Key words: Blood parameter, Butyric acid, Murrah buffaloes, Myeloperoxidase activity, Poly-herbal.

INTRODUCTION

The transition from gestation to lactation is a period of great metabolic stress for buffaloes. This period is characterized by severe negative energy balance and low serum or plasma concentrations of several minerals and vitamins, suggesting lowered health status (Goff, 2006). During this period metabolism shifts from demand of pregnancy to those of lactation. This leads to chain of physiological and biochemical reactions, i.e. increases the production of reactive oxygen species (ROS) which causes immunosuppression (Sordillo, 2005). Oxidative stress occurs when the production of ROS exceeds the antioxidant defense mechanisms present in the body. Immune cells are particularly sensitive to oxidative stress because (1) their membranes contain high concentrations of polyunsaturated fatty acids that are very susceptible to peroxidation and (2) they produce large amount of ROS when stimulated. A number of components of host defense system are altered during this period including neutrophil function, lymphocyte responsiveness to mitogen stimulation, antibody responses and cytokine production by immune cells (Kehrli *et al.*, 2006). Increased challenge to pathogenic microorganisms and decreased animal resistance renders animal more susceptible to various infectious and non-infectious diseases during periparturient period (Goff, 2000).These diseases have considerable negative impact on the profitability of dairy farms.

Buffaloes are the major milk producer in India and contribute to 51% (Basic Animal Husbandry and Fisheries Statistics, 2014) of total milk production, therefore there is a need to strengthen the immunity of buffaloes around calving and this can be done by giving various immune modulators to them. Various studies conducted for understanding the effect of butyrate, a SCFA on human health revealed the involvement of SCFA in immune processes, mostly as anti-inflammatory agents and in modulating inflammatory cytokine expression (Zapolska-Downar and Naruszewicz, 2009). The present study was taken with an aim to check the immuno modulatory effect of poly-herbal mixture comprising of about six herbs traditionally being used postpartum in cattle and buffaloes for improving reproductive health by farmers of India. Butyrate, being an immune modulator, having anti-inflammatory effects and an incredible source

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of energy (Jianping, 2013), was chosen as supplement for the present study.

MATERIALS AND METHODS

Ethical approval: Necessary approval from the Institutes Animal Ethics Committee was taken for carrying out this study.

Selection of buffaloes : The present study was conducted on 24 multiparous Murrah buffaloes maintained at LRC, ICAR-NDRI; Karnal, Haryana, India, having 2-6 parity and age group of 55 to 120 months. The average age at calving was 42-44 months. All the buffaloes were in their last month of pregnancy. The buffaloes were divided into 4 groups *viz*.; T_0 control (n=6) without any supplementation, T_1 poly-herbal (n=6), T_2 poly-herbal + butyric acid (n=6) and T_3 butyric acid (n=6) buffaloes.

Feeding of buffaloes: Control group i.e T_0 (n=6) was offered ration as per NRC requirement plus 10 % and the three supplemented groups T_1, T_2 and T_3 (n=6 for each group) were offered ration as per control group and different supplements, (T_1= poly-herbal supplementation for seven day post partum, T_2= poly-herbal supplementation for seven day post partum + 200ml butyric acid (99%) supplementation 30 day prepartum and 30 day postpartum, T_3= 200ml butyric acid (99%) supplementation 30 day prepartum and 30 day postpartum). The Poly-herbal mixture supplemented /day was made by mixing 25 gms each of following six herbs :i) Foeniculum vulgare (Saunf); ii) Trachyspermum ammi (Ajwain); iii) Trigonella foenum-graecum (Methi); iv) Zingiber officinale (Sundh); v) Anethum graveolens (Sowa) and vi) Elettaria cardamomum (Cardamom), along with that 25 gram black salt (Kala Namak) was also added. This mixture was boiled for about 20-30 minutes till half of water remains and then 250 grams of Jaggery (Gur) were added and heated for 5-10 minutes. The prepared supplement was fed immediately after parturition, after mixing with small quantity of concentrate mixture to the buffaloes. Thereafter, daily for another six days, i.e. total seven days the above supplement was given in the morning hours to the buffaloes.

Blood sampling: Blood samples were collected on 1st, 2nd and 5th days of post partum from all the Murrah buffaloes and analyzed for TLC, differential leucocyte counts (DLC), type of neutrophil (immature band nuclei, and mature segmented nuclei). Neutrophil were isolated and studied for their phagocytic and myeloperoxidase activity as indicated below

Analysis : Blood TLC was measured by counting the white blood cells on a hemocytometer under a microscope. For estimating blood DLC, a blood smear was prepared on a clean glass slide. The stained blood smear was examined under an oil immersion objective for accurate cell identification. All the leucocytes were classified. About 100 cells were counted to determine the % of different leucocytes.

All materials and reagents used for the isolation of blood polymorphonuclear neutrophil (PMN) were sterile. Isolation of PMN from peripheral blood was performed using hypotonic lysis of erythrocytes. Briefly, 10 ml of ethylenediamine tetraacetic acid mixed blood was poured into the Falcon tubes and centrifuged (2000 × g, 15 min., 4°C); the plasma layer, buffy coat and top layer of the blood-packed cells were discarded. About 3 ml of the blood-packed cell was lysed by adding 6 ml of double distilled water and gently mixed for 45 s. using a magnetic stirrer. After restoration of the isotonicity by the addition of 3 ml of 2.7% NaCl with gentle mixing for 60 s., the suspension was centrifuged (2000 × g, 10 min., and 4°C). For the second lysis procedure, after resuspending of the pellets in 3 ml of Dulbecco s phosphate buffered saline (PBS) (without $CaCl_2$ and $MgCl_2$), 6 ml of double distilled water was added and gently mixed for 30 s, then 3 ml of 2.7% NaCl was added, gently mixed for 60 s, and centrifuged (2000 × g, 5 min., 4°C). The remaining cell pellet was washed 4 times in PBS (500 × g, 10 min., 4°C) and the final cell pellet was resuspended in RPMI media for further analysis.

Estimation of PA was based on the principle that phagocytic cells produce O_2^- anions. These anions reduce the yellow colored water-soluble nitroblue tetrazolium (NBT) to water insoluble blue or purple colored formazan crystals. The OD of the reduction product was determined at 540 nm (Chai *et al*., 2005). For estimating PA, 1 million viable cells were taken in each well of 96 well culture plates. Two different volumes of the same concentration of zymosan and NBT were tried. Five treatments well of ELISA plate contained 10 µl NBT along with 10 µl zymosan and cells. Five other treatments well contained 20 µl NBT along with 20 µl zymosan and cells. Five blank well contained only cells. Their final volume was adjusted to 200 µl by adding media. It was incubated for 3 hr in CO_2 incubator at 5% CO_2 and 95% air level. OD was taken at 540 nm.

The activity of Myeloperoxidase enzyme was measured by ELISA kits (Uscn life science, USA). For preparing paralysate of neutrophil, the isolated neutrophil were dissolved in 1 ml PBS. Glass beads were added to neutrophil suspension and shock was given for 25 seconds by bead beater (Unigenetics Instrument Pvt. Ltd., India). Put it on ice for 1 min, then again shock was given for 25 seconds. It was centrifuged at 1000 xg for 10 min. Supernatant was taken in 2 ml eppendorf tubes and was stored at -20°C till further estimation. All the kit components were brought in room temp (18-25°C) before use. The standard was reconstituted with 1 ml of standard diluents, gently shaken and kept for 10 min at room temp. The concentration of the standard in stock solution was 200 ng/ml. The stock solution was diluted to 100 ng/ml which served as a high standard. Prepared seven tubes with 5ml standard diluents and used

to prepare a double dilution series dilution (500 µl from stock dilution was dispensed in first tube followed by 500 µl of first tube mixture to next and dilution carried out to 6 tubes last tube containing only standard diluents). Tubes were mixed properly before next transfer. Assay diluents A and assay diluents B: Diluted 6 ml assay diluents A and B concentration (2X) with 6 ml of distilled water. Detection Reagent A and B: The stock reagent A and B was spinned and diluted with working assay diluent A and B respectively (1:100). Wash solution: 20 ml of wash solution was diluted with 580 ml deionised and distilled water to prepare 600 ml of wash solution (1X). Wells were determined for diluted standard, blank and sample (7 well for standard and 1 well for blank). 100 µl each of dilution of standard, blank and samples were added into the appropriate wells. All the liquid from each well was removed and added 100 µl detection reagents. A working solution to each well and incubated for 1 hr at 37°C after covering it with plate sealer. Aspirated the solution and washed with 350 µl of 1X wash solution to each well using multichannel pipette (3times).The remaining liquid was removed from well. Added 100 µl of detection reagent B working solution to each well. Incubated for 30 min at 37°C after covering it with plate sealer. Repeated the wash process 5 times as step 4. Added 90 µl of substrate solution to each well and cover with plate sealer, protected from light and incubated for 20 min at 37°C. Added 50 µl stop solution to each well and mix by tapping the side of plate. The reading was taken at 450 nm immediately through ELISA reader (Dang *et al.*, 2007).

Statistical analysis: The data were analyzed by analysis of variance using SPSS 17.0 statistical software package.

RESULTS AND DISCUSSION

The results of blood TLC, neutrophil, lymphocyte, monocyte, PA and MA of control, poly-herbal, poly-herbal + butyric acid, and butyric acid group of Murrah buffaloes has been presented in Table 1and 2 respectively.

Table 1: Effect of Poly-Herbal Mixture and Butyric Acid Supplementation on the total and differential blood cell counts (Mean ±SE) in postpartum Murrah buffaloes

Treatment	Days	TLC ($\times10^5$)	Neutrophil			Lymphocytes	Monocytes
			Total (%)	Band (%)	Segmented (%)	(%)	(%)
Control(T_0)	1	9.5±0.56	41^{a}±1.52	3.83^{Aa}±0.064	96.17^{Aa}±0.06	49.72±1.56	4.0^{a}±0.06
	2	8.99±0.45	41.03^{a}±1.54	3.67^{Aa}±0.092	96.33^{Aa}±0.10	49.98^{a}±1.60	3.8^{a}±0.09
	5	7.63±0.45	35.02^{a}±1.61	3.24^{Ba}±0.067	96.76^{Ba}±0.05	56.87^{a}±1.70	3.2^{a}±0.08
Poly-herbal (T_1)	1	9.45±0.74	41^{Aa}±1.52	3.78^{Aa}±0.085	96.22^{Aa}±0.09	49.98±1.61	3.8^{a}±0.05
	2	8.6±0.47	38.5^{Ab}±1.97	3.58^{Aa}±0.068	96.42^{Aa}±0.07	53^{a}±2.04	3.5^{b}±0.09
	5	6.7±0.31	29.03^{Bb}±0.96	2.47^{Bb}±0.062	97.53^{Bb}±0.06	63.27^{b}±1.07	2.8^{b}±0.106
Poly-herbal +	1	8.4±0.43	35.98^{Ab}±1.56	2.89^{Ab}±0.150	97.11^{Ab}±0.15	55.27±1.61	3.6^{b}±0.04
Butyric acid (T_2)	2	7.3±0.25	33.02^{Ab}±1.61	2.74^{Ab}±0.143	97.27^{Ab}±0.14	58.63^{b}±1.65	3.3^{b}±0.04
	5	6.6±0.15	28.47^{Bb}±1.25	2.41^{Ab}±0.107	97.60^{Ab}±0.11	63.93^{b}±1.32	2.8^{b}±0.11
Butyric acid (T_3)	1	8.4±0.39	36.1^{Ab}±1.51	2.89^{Ab}±0.129	97.11^{Ab}±0.13	55.15±1.52	3.6^{b}±0.045
	2	7.5±0.35	34^{Ab}±1.42	2.82^{Ab}±0.122	97.09^{Ab}±0.19	57.52^{b}±1.44	3.38^{b}±0.037
	5	7.1±0.25	30.17^{Bb}±1.32	2.53^{Ab}±0.115	97.47^{Ab}±0.11	62.97^{b}±1.3	2.9^{b}±0.04

Means bearing Capital letter within days and Means bearing small letter within treatment groups differ significantly ($p<0.05$), TLC=Total leukocyte counts,

Table 2: Effect of Poly-Herbal Mixture and Butyric Acid Supplementation on the PA and MA (Mean ±SE) in postpartum Murrah buffaloes

Treatment	Days	PA	MA
Control(T_0)	1	0.57^{Aa}±0.08	2.21^{a}±0.17
	2	0.68^{Aa}±0.07	3.54^{a}±0.21
	5	0.94^{Ba}±0.09	5.78^{a}±0.23
Poly-herbal (T_1)	1	0.64^{Aa}±0.029	2.41^{a}±0.14
	2	0.78^{Ba} ± 0.036	3.87^{a}±0.17
	5	1.28^{Cb} ± 0.028	7.78^{b}±0.23
Poly-herbal +Butyric acid (T_2)	1	0.88^{Ab}±0.034	3.13^{b}±0.09
	2	1.04^{Bb}±0.042	4.76^{b}±0.23
	5	1.36^{Cb}±0.036	8.07^{b} ±0.24
Butyric acid (T_3)	1	0.84^{Ab}±0.033	3.01^{b}±0.06
	2	0.96^{Bb}±0.035	4.66^{b}±0.17
	5	1.16^{Cb}±0.05	7.28^{b}±0.44

Means bearing Capital letter within days and Means bearing small letter within treatment groups differ significantly ($p<0.05$), PA=Phagocytic activity, MA=Myeloperoxidase activity.

Number, PA and MA of blood cells : Values of blood TLC on the 1st day postpartum in both control and treatment groups of buffaloes were within the normal range. The increase in TLC level on 1st day postpartum may be due to higher recruitment of leukocytes by bone marrow and higher cortisol level during acute stress and inflammation at the time of calving (Mormède *et al*., 2007). However, on subsequent days (2nd and 5th) TLC values were lower in all groups as compared to1st day postpartum, but the difference was non significant ($p<0.05$) and the decrease in TLC on 2nd and 5th day may be due to increase in migration of leukocytes to the uterine lumen followed by migration to the mammary alveolar lumen and subsequently in milk.

On 1st, 2nd and 5th day of postpartum, total neutrophil and band neutrophil percentage were significantly lower ($p<0.05$) in T_2 and T_3 group as compared to control group of buffaloes, whereas, blood neutrophil and band neutrophil percentage were also significantly lower ($p<0.05$) in T_1 group as compared to control group on 5th day only. On 1st, 2nd and 5th day of postpartum, level of segmented (mature) neutrophil percentage was significantly higher ($p<0.05$) in T_2 and T_3 group as compared to control group of buffaloes, however segmented (mature) neutrophil percentage was significantly lower ($p<0.05$) in T_1 group as compared to control group on 5th day only. Decreasing trend of neutrophilic activity from 1st day postpartum till 5th day was noted in all groups in the present study, similar trend was observed by Dosogne *et al*., (2003) also in buffaloes. Lower levels of blood neutrophil percentage in treatment groups as compared to control group was probably due to lower stress levels or lesser inflammatory problems. Serhan and Savill (2005) reported that neutrophils are the first cells to migrate from blood into an inflamed area after initiation of inflammation. The main function of neutrophil is phagocytosis and intracellular killing by engulfing bacteria by two distinct mechanisms i.e. respiratory burst and digestion by lysosomal enzymes. Rise in neutrophil count is also associated with an excessive rise in cortisol level (Mormède *et al*., 2007) around calving due to endocrinological, physiological and psychological stresses (Burton *et al*., 2005). Better performance of the treatments groups is due to anti-inflammatory and immunomodulator action of poly herbal mixture, whereas, butyrate acts as immunomodulator in presence of inflammatory stimuli through regulation of neutrophil function and modulation of inflammatory cytokine expression (Zapolska-Downar and Naruszewicz, 2009).

On 1st day of postpartum, lymphocyte percentage was non significantly different among the treatments and control groups. On 2nd and 5th day of postpartum, lymphocyte percentage was significantly ($p<0.05$) higher in T_2 and T_3 group as compared to T_0 group of buffaloes, further lymphocyte percentage was also significantly higher ($p<0.05$) in T_1 group but on 5th day only. On 1st, 2nd and 5th day of postpartum, monocytes percentage was significantly lower ($p<0.05$) in T_2 and T_3 group as compared to T_0 group of buffaloes, whereas, monocyte percentage was significantly lower ($p<0.05$) in T_1 group on 2nd and 5th day.

On 1st, 2nd and 5rd day of postpartum PA and MA of blood neutrophil was significantly higher ($p<0.05$) in the T_2 and T_3 group as compared to T_0 group of buffaloes, whereas PA and MA was significantly higher ($p<0.05$) in T_1 group only on 5th day. The improvement in PA of blood neutrophil in treatment groups was probably due to synergistic anti-inflammatory and immunomodulatory effect of poly herbal mixture; its use has increased significantly the number of matured neutrophil influencing the neutrophillic PA. In control group lower level of PA was due to more proportion of immature neutrophil release around parturition as immature nutrophils do not have proper structure to fight with the infection or perform normal function resulting in immunosupression (Meglia *et al*., 2001; Dang *et al*., 2013). Higher myeloperoxidase activity in the treatment groups is the indication of presence of more number of mature nutrophils. Generally, butyrate regulates neutrophil function and modulates inflammatory cytokine expression, especially in the presence of inflammatory stimuli (Zapolska-Downar and Naruszewicz, 2009). The lower myeloperoxidase activity in control group was due to presence of more immature neutrophil and their poor ability to release the granular enzyme (Pathan *et al*., 2015), which was the reflection of beneficial effect of poly-herbal and butyrate supplementation in treatment groups.

CONCLUSION

Supplementation of poly-herbal, poly-herbal + butyric acid and butyric acid during periparturient period improved the immunity level of Murrah buffaloes. Lower percentage of neutrophil and higher PA and MA of blood neutrophil in the supplemented groups of Murrah buffaloes indicates the better immunity status in these buffaloes to fight with the periparturient stress. Among all the supplemented groups, maximum benefits were observed in poly-herbal+butyric acid group buffaloes. Therefore, during periparturient period feeding of 425g poly-herbal mixture for seven days of postpartum and 200 ml of butyric acid for 30 days pre and postpartum in buffaloes may reduce post partum stress and increase their productivity.

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