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Effect of rumen protected methionine and choline supplementation on leukogram profile, oxidative stress, inflammatory and immunomodulatory responses of Surti buffaloes during transition period

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ABSTRACT

Present study was conducted to observe effect of supplementing rumen protected methionine and choline on leukogram profile, oxidative stress, inflammatory and immunomodulatory responses during transition period in Surti buffaloes. Twenty-seven pregnant Surti buffaloes were selected and divided into three groups of nine animals each with following diet regime: Group I (Control)-basal diet, Group II (RPM)-basal diet+rumen protected methionine and Group III (RPM+RPC)-basal diet+rumen protected methionine+rumen protected choline. Supplementation of RPM @ 10 g/buffalo/day and RPC @ 50 g/buffalo/day was done from -15 d prepartum to 30 d postpartum. Blood samples were collected at start of experiment, 1st, 3rd and 6th week postpartum. At 1st and 3rd week postpartum, significantly higher level of GSH, SOD as well as TAS and lower level of LPO were observed in Group II and III as compared to control. Group III had highest SOD as well as TAS and lowest LPO levels. TNF-α and haptaglobin during postpartum period were significantly lower in Group II and III. Group III had lowest levels of TNF-α at 1st and 3rd week and haptoglobin at 1st, 3rd and 6th week postpartum. *In vitro* neutrophil phagocytic activity and lymphocyte proliferation were significantly higher in Group III followed by Group II and control during entire postpartum period. It was concluded that supplementation of rumen protected methionine and choline during transition phase of Surti buffaloes reduces oxidative stress as well as inflammatory tendencies and increases antioxidant status as well as immune response. Beneficial effects of supplementing both are more than supplementing rumen protected methionine alone.

Keywords: Rumen protected choline, Rumen protected methionine, Surti buffaloes, Transition period

Transition period is a stressful phase for dairy animals. Rapid foetal growth (NRC 2001) and onset of lactation (Zhou *et al.* 2017) have high nutrient demands. So, low DMI likely causes negative energy balance. During this period, liver and reproductive organs are highly susceptible to infection and tissue stress contributes to inflammatory responses. Inflammatory process initiates synthesis of positive acute phase protein to alter liver function for re-establishing homeostasis (Batisel *et al.* 2017). Excess free radicals during oxidative stress cause cellular damage and chronic inflammatory responses further accentuates physiological stress jeopardizing animal's health and production (Trevisi *et al.* 2016).

High quality protein, essential amino acid and antioxidants are necessary to prevent stress. First limiting amino acid for dairy animals, Methionine (NRC 2001) is precursor for other sulphur containing amino acids (Brosnan and Brosnan 2006). Choline, a component of acetylcholine, acts as a lipotropic factor and is involved in one carbon metabolism (i.e. Folate). Without any role as cofactor, choline still is often referred as a vitamin. In spite of endogenous synthesis, it still is required in larger amounts than other vitamins. Its deficiency may result in suppressed growth, renal dysfunction and fatty liver.

Methionine minimizes risk of fatty liver and ketosis by enhancing fat metabolism in liver, curtails inflammatory processes and reduces oxidative stress by synthesizing a natural antioxidant glutathione. Apart from having lipotropic and immunomodulatory effects, methionine and choline reduces inflammatory responses during transition period. Dietary availability of these methyl donors (e.g. methionine and choline) is limited owing to extensive microbial degradation in rumen (Zhou *et al.* 2016). To prevent ruminal degradation, amino acids are encapsulated. Supplementing rumen protected methionine and choline (RPM, RPC) may help to meet daily demand of methyl group and improve milk production as well as health of buffaloes by improving liver function and combating oxidative stress during transition period. Considering dearth of such studies and benefits of dietary supplementation

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of RPM and RPC, present study was therefore planned to observe effect of supplementation of rumen protected methionine and choline on leukogram profile, oxidative stress, inflammatory and immunomodulatory responses during transition period in Surti buffaloes.

MATERIALS AND METHODS

The present study was approved by Institutional Animal Ethics Committee (IAEC, No: 066-VCN-VPY-2018). Climate of the location of study is typical of tropical and coastal region. During the experimental period, minimum and maximum mean values of dry bulb temperature (°C) was 22.8 and 34.8, relative humidity (%) was 36.00 and 85.00 and temperature humidity index (THI) was 65.06 and 87.77, respectively.

Selection of animal, grouping and dietary supplementation: Twenty-seven advanced pregnant multiparous Surti buffaloes were selected and divided into three groups based on parity (2-5), previous lactation yield (1000-1527 kg/lactation) and body condition score (BCS; 3-4). Feeding was done as per ICAR feeding standard, 1998. The dietary supplemental treatment was as follows: Group I (Control)-basal diet, Group II (RPM)-basal diet+rumen protected methionine and Group III (RPM+RPC)-basal diet+rumen protected methionine+rumen protected choline. Supplementation of RPM @ 10 g/buffalo/day and RPC @ 50 g/buffalo/day was done from -15 d prepartum to 30 d postpartum.

Feed sample collection and proximate analysis: The feed sample was collected at the beginning of experiment and during the study period. Representative samples as per seasonal feed supply, were collected and kept in labelled polythene bags. The collected samples were analysed for their proximate composition. Collected samples were analysed for dry matter by hot air oven (Tempo Instruments & Equipment's Pvt. Ltd., Mumbai) following a standard protocol (AOAC 2007). The oven-dried samples were ground to 1 mm particle size for analysis of their proximate principles such as crude protein (CP), ether extract (EE) or crude fat, crude fibre (CF) and total ash (AOAC 2007). The nitrogen free extract (NFE) was calculated using the formula:

$$\text{Nitrogen free extract (NFE) \%} = \text{100 - (\% Crude protein + \% Ether extract + \% crude fibre + \% Total ash)}$$

Blood sample collection and analysis: Blood samples were collected in the morning (7:30 to 8:30 AM) in K_3EDTA and clot activated vacutainers at 15 days prepartum and 1st, 3rd and 6th week of calving. Whole blood collected in K_3EDTA vacutainers was used for leukogram profile, i.e. determining total and differential leukocyte count (TLC and DLC), for preparation of hemolysate to estimate oxidative stress parameters such as reduced glutathione (GSH), superoxide dismutase (SOD) and lipid peroxidation (LPO) in terms of malondialdehyde (MDA) produced. Whole blood was also used for *in vitro* assessment of lymphocyte proliferation and phagocytic activity of neutrophils. Blood with and without anticoagulant was centrifuged (3000 rpm for 15 min) to separate plasma and serum respectively and stored at -20°C in deep freeze until further analysis. Blood plasma was used for analysis of total antioxidant status (TAS). Serum was used for analysing the levels of tumor necrosis factor-α (TNF-α) and haptaglobin.

Leukogram profiling: Total leucocyte count (TLC) was estimated by using fully automated haematology cell counter machine (MEDONIC CA 620/530 VET). Differential leucocyte count (DLC) was estimated by preparing blood smear on glass slides, staining with Leishman's stain and observing under 100× objective (oil immersion) of microscope.

Analysis of oxidative stress parameters: Estimation of reduced glutathione (GSH) levels in RBC hemolysate was estimated as per the method cited by Moron *et al*. (1979). Superoxide Dismutase (SOD) was estimated as per method described by Madesh and Balsubramaniyam (1998) and the membrane peroxidative damage (lipid peroxidation - LPO) in erythrocytes was determined in terms of Malondialdehyde (MDA) production by the method suggested by Rehman (1984). Plasma total antioxidant status (TAS) was estimated with the help of method described by Miller *et al.* (1993).

Estimation of inflammatory biomarkers: Bovine specific ELISA assay kits were used for analysing inflammatory biomarkers such as tumor necrosis factor-α (TNF-α) (Cloud-Clone Corp) and haptaglobin (MyBioSource) concentration in serum.

In vitro neutrophil phagocytic activity and lymphocyte proliferation index: Neutrophils from blood were isolated by method described by Mehrzad *et al.* (2004). Modified colorimetric nitro blue tetrazolium (NBT) reduction assay method was used to determine phagocytic activity of neutrophils (Choi *et al.* 2005).

Lymphocyte proliferation assay: Freshly collected whole blood samples were centrifuged at 3000 rpm for 15 min. Buffy coat layer was collected and resuspended in 1:1 v/v Dulbecco's phosphate saline (DBPS) solution. Buffy coat mixed with DBPS solution was carefully layered on Histopaque 1077 (lymphocyte separating medium) at 4:1 v/v concentration in 15 ml sterile polypropylene centrifuge tube. It was centrifuged for 2000 rpm for 20 min to separate lymphocytes. Lymphocytes were washed thrice with 1:1 v/v DBPS solution by centrifuging at 2000 rpm for 10 min. The washed cells were resuspended in RPMI 1640 media (Sigma) containing 10% FCS and centrifuged at 2000 rpm for 5 min. Lymphocyte rich pellet was resuspended in 2 ml of growth medium after final wash. Trypan blue exclusion method was used to determine the proportion of viable cells in the separated lymphocytes and were adjusted to 5×10^6 live lymphocyte/ml. Wells with and without mitogen in triplicate containing 10^6 viable lymphocytes/well in RPMI media with 10% fetal bovine serum (FBS) (HiMedia Laboratories, India) were maintained in 96 well flat bottom tissue culture plate. Mitogen Concanavalin A was used @ 5 µg/ml of the final culture volume (200 µl). Wells kept as blank contained only 200 µl of culture media. Culture

plate was incubated at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2) for 2 days. Colorimetric MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay given by Mosmann (1983) was used to observe the proliferative response of lymphocyte.

Statistical analysis: Results obtained were analysed statistically by one way ANOVA using DMRT for interpreting effect of different groups for the parameters under study. DMRT was used for mean separation at 5% level of significance (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

The results for chemical composition of feed, leukogram profile, changes in oxidative stress parameters, changes on inflammatory and immunomodulatory responses are mentioned in Tables 1, 2, 3 and 4 respectively.

Proximate composition of feed samples: The values obtained were within normal range for Indian feedstuffs (ICAR 2013).

Leukogram profiling: Total leucocyte count, percentage of neutrophil, lymphocyte, eosinophil, basophil and eosinophil did not differ significantly between the groups. However, in all the groups, TLC was higher at 1st week postpartum and decreased thereafter with advancing lactation. Lowest TLC level were observed at -15 d in all the groups. Similar to our study, Movaliya *et al.* (2013) found non-significant effect of rumen bypass methionine and lysine supplements on Jaffarabadi heifers.

Oxidative stress parameters: Levels of reduced glutathione (GSH) and superoxide dismutase (SOD) at 1st and 3rd week postpartum were significantly higher ($p<0.05$) in Group II and III than control. SOD level was the highest in Group III. Similarly, postpartum TAS level was higher ($p<0.05$) in Group III and II than control. RPM+RPC supplemented Group during 1st week postpartum had the highest TAS level. Lipid peroxidation (LPO) at 1st and 3rd week postpartum was significantly ($p<0.05$) lower in Group III followed by Group II as compared to control. Higher level of LPO and lower level of SOD, GSH and TAS in control group animals indicates the animals were under oxidative stress during the transition period. Generally, during transition, animals are in negative energy balance due to reduced feed intake and increased demand of nutrients because of stress of parturition and lactation. Stress is also responsible for increased free radical production. During transition, there is also increased production of free radicals because of the hepatic NEFA oxidation. Enzymatic (GSH, SOD and catalase) and non-enzymatic (Vitamin E, C and glutathione) antioxidant barrier in animals helps in oxidative stress alleviation by scavenging free radicals (Tspilakou *et al.* 2017).

GSH is the most abundant endogenous antioxidant. Synthesis of GSH requires three amino acids, viz. glycine, gamma glutamine and cysteine. Methionine and choline act as methyl donor and are important source of intracellular antioxidant namely GSH and taurine (Brosnan and Brosnan 2006). Supplementation of methionine and choline reduced free radical production by enhancing antioxidant status during transitional stress period in Surti buffaloes. In agreement to our findings, Sun *et al.* (2016) observed higher ($p<0.05$) total antioxidant capacity and GSH and lower MDA concentration in methionine, choline and methionine plus choline supplemented transition dairy cows. Methionine supplemented ewes had higher (36.5%) glutathione level as compared to control ewes (Tspilakou *et al.* 2017). Similar results for TAS (Tspilakou *et al.* 2017) and GSH (Zhou *et al.* 2016, Batisel *et al.* 2017) had also been reported.

Inflammatory biomarkers: Tumor necrosis factor-α (TNF- α) and haptaglobin during postpartum period were significantly ($p<0.05$) lowered in Group III and II than control. Group III had lowest ($p<0.05$) TNF-α at 1st and 3rd week. During entire postpartum duration as compared to control, RPM supplemented group had lower haptaglobin levels, whereas RPM+RPC supplemented group had the lowest levels. Present results of lowered TNF-α during the postpartum period are in agreement with the finding of Sun *et al.* (2016) and for haptaglobin by Batisel *et al.* (2017).

Liver is highly susceptible to injury that leads to infection and inflammatory response at calving. TNF-α is a proinflammatory marker and cytokine derived from macrophages and monocytes. Inflammation during transition causes release of proinflammatory cytokines such as TNF-α thereby increasing its concentration in blood. Simultaneous to TNF-α, there may also be release of positive acute phase protein (Sun *et al.* 2016, Batisel *et al.* 2017). Supplementation of methionine and choline during the transition period in the present study might have reduced the inflammatory responses and therefore, their concentration was lowered in these supplemented groups. Changes in proinflammatory cytokines are also linked to PMNL and immunity related activity. Hence, their

Table 1. Chemical composition of feed (% DM basis)

		Dry mater	Crude protein	Ether extract	Crude fibre	Total ash	NFE
Concentrate		91.07	20.2	2.4	12	6.93	58.47
Green fodder	Hybrid Napier	27.57	8.3	1.3	29	13.15	48.23
	Jowar (Green)	25.43	7.3	1.28	37	8.36	45.51
	Lucerne	25.26	18.1	1.79	27	12.15	40.95
	Green grass (Para Grass)	25.52	6.7	1.66	33	12.09	46.51
Dry fodder	Paddy straw	89.76	2.3	1.52	40	17.28	38.94
	Jowar hay	89.6	7.4	1.52	39	8.34	43.69

Table 2. Leukogram profile (Mean±SE) in different supplemental groups at -15 day, 1st, 3nd and 6th week of parturition in Surti buffaloes

	Control					RPM					RPM + RPC				
	Pre partum	Postpartum				Pre partum	Postpartum				Pre partum	Postpartum			
	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N = 27)	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N = 27)	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N =27)
TLC (10^3×µl)	8.89 ±0.07	11.30 ±0.07	10.44 ±0.07	9.26 ±0.06	10.33 ±0.07	8.88 ±0.08	11.30 ±0.08	10.44 ±0.07	9.25 ±0.07	10.33 ±0.07	8.91 ±0.10	11.32 ±0.10	10.46 ±0.09	9.27 ±0.09	10.35 ±0.09
Neutrophils (%)	29.56 ±1.12	28.11 ±1.32	27.11 ±1.03	28.44 ±1.07	27.89 ±0.76	29.56 ±1.12	28.33 ±1.30	27.00 ±1.09	28.00 ±1.03	27.78 ±0.75	29.67 ±1.11	28.33 ±1.30	27.33 ±1.09	28.44 ±1.03	28.04 ±0.62
Lymphocyte (%)	63.78 ±0.68	63.11 ±0.77	64.22 ±0.70	63.44 ±0.78	63.59 ±0.19	63.56 ±0.67	62.22 ±0.76	63.33 ±0.73	63.44 ±0.73	63.00 ±0.43	63.67 ±0.67	63.11 ±0.73	64.33 ±0.73	63.33 ±0.76	63.59 ±0.39
Monocyte (%)	4.44 ±0.34	4.56 ±0.29	4.44 ±0.29	4.67 ±0.24	4.56 ±0.16	4.56 ±0.38	4.44 ±0.24	4.33 ±0.29	4.67 ±0.24	4.48 ±0.10	4.56 ±0.34	4.56 ±0.29	4.33 ±0.29	4.44 ±0.24	4.44 ±0.21
Basophils (%)	0.11 ±0.11	0.22 ±0.15	0.11 ±0.11	0.00 ±0.00	0.11 ±0.06	0.11 ±0.11	0.22 ±0.15	0.11 ±0.11	0.00 ±0.00	0.11 ±0.06	0.11 ±0.11	0.22 ±0.15	0.11 ±0.11	0.00 ±0.00	0.11 ±0.06
Eosinophils (%)	3.33 ±0.29	3.44 ±0.29	3.56 ±0.29	3.67 ±0.29	3.56 ±0.19	3.44 ±0.29	3.44 ±0.29	3.56 ±0.29	3.56 ±0.29	3.52 ±0.19	3.56 ±0.29	3.56 ±0.29	3.56 ±0.29	3.56 ±0.29	3.56 ±0.15

Different upper-case superscripts within row shows significant difference between the groups at $p<0.05$.

Table 3.Changes in oxidative stress parameters (Mean±SE) in different supplemental groups in Surti buffaloes

	Control					RPM					RPM + RPC				
	Pre partum	Postpartum				Pre partum	Postpartum				Pre partum	Postpartum			
	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N = 27)	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N = 27)	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N =27)
GSH (mg/dl)	9.63 ±0.44	7.19^{A} ±0.30	7.51^{A} ±0.30	9.31 ±0.30	8.00^{A} ±0.30	9.65 ±0.26	8.34^{B} ±0.32	8.66^{B} ±0.32	9.46 ±0.32	8.82^{AB} ±0.32	9.64 ±0.42	8.81^{B} ±0.27	9.13^{B} ±0.27	9.53 ±0.27	9.16^{B} ±0.27
SOD (U/L)	3.92 ±0.24	2.36^{A} ±0.11	2.80^{A} ±0.12	3.91 ±0.11	3.02^{A} ±0.11	3.95 ±0.22	2.85^{B} ±0.11	3.31^{B} ±0.11	3.92 ±0.10	3.36^{AB} ±0.11	3.98 ±0.19	3.36^{C} ±0.15	3.85^{C} ±0.16	3.98 ±0.15	3.73^{B} ±0.15
TAS (µM)	315.6 ±25.56	198.6^{A} ±13.97	224.7^{A} ±26.63	226.9^{A} ±26.64	216.7^{A} ±18.10	314.8 ±31.79	264.0^{B} ±10.98	293.9^{B} ±22.25	299.4^{B} ±21.10	285.8^{B} ±17.36	315.0 ±24.99	308.5^{C} ±6.72	314.9^{B} ±19.72	321.5^{B} ±21.29	314.9^{B} ±15.16
LPO (nM)	4.19 ±0.32	8.90^{C} ±0.46	8.12^{C} ±0.36	4.12 ±0.36	7.05^{C} ±0.30	4.07 ±0.38	6.32^{B} ±0.28	5.78^{B} ±0.33	4.08 ±0.33	5.40^{B} ±0.26	4.04 ±0.41	4.74^{A} ±0.38	4.10^{A} ±0.29	4.00 ±0.29	4.28^{A} ±0.22

Different upper-case superscripts within row shows significant difference between the groups at $p<0.05$.

Table 4. Changes in inflammatory and immunomodulatory responses (Mean±SE) in different supplemental groups in Surti buffaloes

	Control					RPM					RPM + RPC				
	Pre partum	Postpartum				Pre partum	Postpartum				Pre partum	Postpartum			
	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N = 27)	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N = 27)	-15 day (n = 9)	1 week (n = 9)	3week (n = 9)	6week (n = 9)	Overall (N =27)
TNF- α (pg/ml)	130.58 ±3.50	339.75^{C} ±6.52	206.52^{C} ±1.48	165.01^{B} ±1.48	234.50^{C} ±5.82	130.56 ±1.97	259.72^{B} ±6.79	194.20^{B} ±1.57	157.22^{A} ±2.29	206.31^{B} ±3.07	131.69 ±3.74	222.74^{A} ±3.31	179.66^{A} ±2.15	158.96^{A} ±1.98	187.12 ^{A}±2.32
Haptaglobin (µg/ml)	24.10 ±0.59	44.60^{C} ±2.73	35.79^{C} ±2.27	28.29^{C} ±0.52	36.23^{C} ±1.43	23.24 ±0.75	30.12^{B} ±1.25	25.62^{B} ±1.06	23.36^{B} ±0.75	26.37^{B}	23.71 ±0.60	24.67^{A} ±0.43	18.28^{A} ±0.58	20.60^{A} ±1.25	21.19^{A} ±0.47
Lymphocyte proliferation index	2.49 ±0.03	1.19^{A} ±0.03	1.24^{A} ±0.02	1.33^{A} ±0.02	1.25^{A} ±0.01	2.48 ±0.01	1.32^{B} ±0.02	1.40^{B} ±0.01	1.46^{B} ±0.01	1.39^{B} ±0.02	2.50 ±0.02	1.42^{C} ±0.02	1.48^{C} ±0.02	1.54^{C} ±0.01	1.48^{C} ±0.02
Neutrophil phagocytic activity	0.28 ±0.01	0.12^{A} ±0.02	0.24^{A} ±0.02	0.24^{A} ±0.01	0.20^{A} ±0.00	0.28 ±0.01	0.14^{B} ±0.02	0.26^{B} ±0.02	0.26^{B} ±0.01	0.22^{B} ±0.00	0.28 ±0.01	0.16^{C} ±0.02	0.28^{C} ±0.02	0.28^{C} ±0.01	0.24^{C} ±0.00

Different upper-case superscripts within row shows significant difference between the groups at p<0.05.

measurement gives an overall view of the immune system of animals (Sun *et al.* 2016).

Immunomodulatory responses: *In vitro* neutrophil phagocytic activity and lymphocyte proliferation were significantly ($p<0.05$) highest in Group III followed by Group II as compared to control during entire postpartum period. These results are in agreement with the findings of Osorio *et al.* (2013), Zhou *et al.* (2016) and Batisel *et al.* (2017) for *in vitro* neutrophil phagocytic activity, and Sun *et al.* (2016) for *in vitro* lymphocyte proliferation index.

Neutrophil phagocytosis and lymphocyte proliferation in peripheral blood is widely accepted as an indicator of immune status. The key feature of transition period is immune dysfunction that is characterized by impaired neutrophil phagocytosis and lymphocyte proliferation, which results into increased risk of bacterial infections of dairy animals (Osorio *et al.* 2013). There is absolute requirement of methionine for proliferation of human lymphocyte (Hall *et al.* 1986). Furthermore, methionine and choline supplementation enhance the neutrophil phagocytosis and oxidative burst capacity by increasing intracellular Ca^{++} flux into neutrophil as intracellular Ca^{++} is key base for neutrophil activation. Further, supplementation of methionine and choline also contributes to increased phagocytosis by increasing GSH availability to neutrophils (Zhou *et al.* 2016).

In the present study, due to supplementation, leukogram profile did not reveal any significant difference for neutrophil and lymphocyte in DLC between groups but the potential of neutrophil for phagocytosis and lymphocytes for proliferation was increased. It suggests that supplementation of RPM and RPM+RPC during transition period in Surti buffaloes improves immune response and amongst them it is better for RPM+RPC supplementation.

It was concluded from the present study that supplementation of rumen protected methionine and choline during transition phase of Surti buffaloes reduces oxidative stress as well as inflammatory tendencies and increases antioxidant status as well as immune response. Beneficial effects of supplementing both are more than supplementing rumen protected methionine alone.

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