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Effect of vitamin C supplementation on immune status and oxidative stress in pregnant Murrah buffaloes during thermal stress

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ABSTRACT

The experiment was conducted to study the effect of vitamin C on oxidative stress and immune status in buffaloes during summer. Primiparous Murrah buffaloes (12) in late gestation were used for this study. The buffaloes were divided into 2 groups of 6 animals each. Group 1 was served as control and group 2 was supplemented with ascorbic acid @ 10g/animal/day from day –45 to day 0 (day of parturition). Blood samples were collected on days –45, –30, –7 and 0. Lymphocyte proliferation index (LPI) and neutrophil phagocytic activity (PA) were analyzed in the whole blood. Plasma was analyzed for, antioxidant enzymes (SOD, CAT and GPx). The PA decreased and the decline was significant on day –7 and day 0 in both groups compared to day –45. The PA was significantly (lower in group 2). LPI was significantly lower on day 0 compared to day –45 in both groups but the variations between groups were not significant. SOD, CAT and GPx activity increased significantly from day –45 to day 0 in both groups. However, the increase in their activity was significantly lower in group 2 compared to group 1. Plasma TAS decreased 12.3 and 6.8% in group 1 and group 2, respectively, from day –45 to day 0 and overall levels were significantly higher in group 2 than group 1. TBARS increased with advancement of pregnancy and the levels were significantly higher on day –7 and 0 in both groups. The levels of TBARS were significantly higher in group 1 compared to group 2. The results indicated that the deviations in immune status and oxidative stress caused due to thermal stress restored towards normalcy by supplementation of vitamin C.

Key words: Buffalo, Immune status, Oxidative stress, Thermal stress, Vitamin C

Free radical reactions are the integral part of normal metabolism. Oxidative stress is experienced by living organisms from both exogenous and endogenous sources (Lesser 2006). Body employs antioxidants to quench these free radicals. Antioxidants fall in 2 broad categories: enzymatic and non enzymatic (Agarwal and Prabhakaran 2005). The enzymatic antioxidants act by scavenging both intra-cellular and extra-cellular superoxide radicals and preventing lipid per oxidation of plasma membranes. Nonenzymatic antioxidants (vitamin C, E, albumin and glutathione) play important role in combating oxidative stress. Antioxidants also enhance immunity by maintaining the structural and functional integrity of the important immune cells. A compromised immune system results in reduced animal production efficiency through increased susceptibility to diseases thereby leading to increased animal morbidity and mortality (McDowell 2002).

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Heat stress is one of the factors which causes ROS mediated oxidative stress in farm animals (Bernabucci *et al.* 2002). A major strategy to reduce the effects of heat stress on farm animals is to alter the micro-environment (West 2003 and Mader *et al.* 2006). The effective strategy to reduce heat stress through the use of sheds, fans and evaporative cooling (Bucklin *et al.* 1991) are capital intensive and are of limited use for small and medium size daries. Anti-oxidants such as vitamin C and E are free radical scavengers, which protect the body defense system against excessive produced free radicals during thermal stress and stabilize health status of the animals. Vitamin C has 2-fold importance: (i) it spares vitamin E (Frey 1991) and (ii) it helps in reduction of tocopheroxyl radicals back to its active vitamin E (Packer *et al.* 1979). Although ruminants can synthesize vitamin C (McDowell 1989), a large reduction in plasma vitamin C concentration was reported in calves stressed by housing conditions (Cummins and Brunner 1991) and heat stressed cows (Padilla *et al.* 2006). Oral supplementation of vitamin C effectively alleviated stress in sheep (Ghanem *et al.* 2008) and goats (Ayo *et al.* 2006). Anderson and Lukey (1987) reported that ascorbate might have a protective role in the

immune system as a scavenger of free radicals generated by phagocytic cells. Careful perusal of the literature revealed an absence of sufficient information regarding the role of vitamin C during summer stress in buffaloes. In the present study the effect of vitamin C supplementation on immune status and oxidative stress in pregnant buffaloes is reported during thermal stress.

MATERIALS AND METHODS

Healthy primiparous Murrah buffaloes (12) in their late gestation were used for this study. The animals were divided into 2 groups of 6 animals each:

Group 1: control group of 6 primiparous Murrah buffaloes

Group 2: treatment group of 6 primiparous Murrah buffaloes supplemented with vitamin C @ 10 g / animal/day throughout the experiment.

All the experimental animals were maintained in an open housing condition yard. The yard has shed with roof made of asbestos sheets. All the animals were offered ration consisting of roughages (maize or *jowar*) and concentrate mixture 2.0 kg / animal / day. The concentrate composed of maize 30%, GNC 21%, mustered cake (oiled) 12%,wheat bran 20%, deoiled rice bran 11%, mineral mixture 2% and common salt 1% with 20% DCP and 70% TDN. The treatment group was supplemented with ascorbic acid @ 10 g/animal/day in the morning by mixing with 100 g concentrate for each animal separately. Blood samples were collected from jugular vein in sterile heparinised vacutainer tubes on days –45, –30, –15, –7 and 0 (the day of parturition). The blood samples were kept in an ice box and transported to the laboratory. All the blood samples were centrifuged at 3000 rpm for 30 min and plasma was separated and used for estimation of different enzymes. Neutrophils from peripheral blood were isolated using hypotonic lysis of erythrocytes (Vishnoi *et al.* 2007). The cell suspension of neutrophils was adjusted to 5×10^6 live cells per ml by the culture media containing 10% FCS; 200µl of the diluted cell suspension per well in triplicate in a 96-well, flat-bottomed tissue culture plate. The cells were allowed to proliferate with zymosan (650 µg/ ml) and nitroblue tetrazolium (NBT, 250 µg/ml) concentrations. All cultures were allowed to incubate at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2) for 2 h. Amount of zymosan phagocytosed was used as an indicator of PA. Nitroblue tetrazolium assay was used to determine the production of superoxide anion (O_2^-) in the neutrophils. Nitroblue tetrazolium is yellow in color, but is changed to blue formazan after phagocytosis, which can be measured spectro-photometrically (Choi *et al.* 2006). OD was taken at 540 nm using multiwell scanning spectrophotometer.The proliferative response of lymphocytes was estimated as per Mosmann (1983). The plasma superoxide dismutase (SOD), glutathione peroxidase (GP_X), catalase (CAT), total antioxidants status (TAS) and thiobarbituric acid reactive substances were estimated by the kits. The results were analyzed and subjected to test of significance one way analysis by Sigma Plot 11.0 software package.

RESULTS AND DISCUSSION

The study was carried out during the hot humid season from July to September and the climatic variables recorded during the experiments are given in Table 1. The THI was > 80 throughout the experimental period.

The mean phagocytic activity (PA) in groups 1 and 2 was 2.19±0.03 and 2.14±0.03 on day –45 and the activity decreased to 1.00±0.01 and 1.27±0.04, respectively, on day 0. The decrease in PA was 54.3% and 40.6%, respectively, in groups 1 and 2 from day –45 to day of parturition. Within the groups, the mean PA decreased with the advancement of gestation period in both groups and the levels were significantly lower on day –7 and day 0 in group 1 as well as group 2 compared to day –45. The decline in PA of group 2 during the experiment was slower and PA of group 2 was significantly higher ($P<0.05$) compared to group 1.

Cummins and Brunner (1991) and Salageanu *et al.* (1971) reported that plasma ascorbate concentration decreased in calves subjected to housing or disease stress. Dietary supplementation of calves with ascorbic acid increased plasma ascorbate concentration and concurrently improved antibody response to an antigen (Cummins and Brunner 1991). Ascorbate may also play a protective role in the immune system as a scavenger of free radicals generated by phagocytic cells (Anderson and Lukey 1987). Cattle treated with dexamethasone showed evidence of decreased neutrophill function, which was alleviated by subcutaneous ascorbic acid injections (Roth and Kaeberle 1985). Stimulation of neutrophill phagocytosis decreased neutrophill ascorbic acid content (Oberritter *et al.* 1986). It is suggested that high levels of ascorbic acid may be necessary to prevent

Table 1. Meteorological variables recorded during the period of experiment

Month	Maximum temperature (°C)	Minimum temperature (°C)	Relative humidity (%)	Dry bulb temperature (°C)	Wet bulb temperature (°C)	Temperature humidity index
July, 2011	33.1	26.3	71	31.7	27.1	82.95
August	32.1	25.7	73	31.6	27.6	83.28
September	32.3	24.5	81	28.7	26.4	80.27

Table 2. Proliferative response and phagocytic activity in pregnant Murrah buffaloes during summer supplemented with vitamin C

Parameter	Groups	Gestation days					Overall average
		–45*	–30*	–15*	–7*	0 (calving)	
Lymphocytes stimulation index (con A)	1	$0.96^{a}\pm0.06$	$0.93^{ab}\pm0.05$	$0.86^{a}\pm0.04$	$0.76^{bc}\pm0.04$	$0.67^{c}\pm0.04$	0.84±0.05
	2	$0.97^{a}\pm0.04$	$0.93^{ab}\pm0.03$	$0.85^{a}\pm0.03$	$0.75^{bc}\pm0.04$	$0.63^{c}\pm0.02$	0.82±0.05
Lymphocytes stimulation index (LPS)	1	$1.06^{a}\pm0.02$	$1.00^{a}\pm0.02$	$0.80^{b}\pm0.04$	$0.70^{bc}\pm0.04$	$0.61^{c}\pm0.04$	0.83±0.08
	2	$1.02^{a}\pm0.02$	$0.97^{a}\pm0.02$	$0.78^{b}\pm0.04$	$0.70^{bc}\pm0.04$	$0.61^{c}\pm0.02$	0.82±0.07
Phagocytic activity	1	$2.19^{a}\pm0.03$	$2.03^{ab}\pm0.02$	$1.91^{b}\pm0.02$	$1.42^{cX}\pm0.05$	$1.00^{dX}\pm0.01$	$1.71^{X}\pm0.20$
	2	$2.14^{a}\pm0.03$	$2.07^{ab}\pm0.03$	1.99b±0.02	$1.62^{cY}\pm0.02$	$1.27^{dY}\pm0.04$	$1.82^{Y}\pm0.15$

Observation with different superscripts (a,b,c,d) differ significantly within the group; observations with different superscripts (X,Y) differ significantly between the groups; * represents the mean (±2) of gestation days.

Table 3. Plasma SOD, CAT, GP_X, TAS and TBARS concentrations in pregnant murrah buffaloes during summer supplemented with vitamin C

Parameter	Groups	Gestation days					Overall average
		–45*	–30*	–15*	–7*	0 (calving)	
Superoxide dismutase (SOD,U/ml)	1	$2.50^{a}\pm0.02$	$2.80^{a}\pm0.02$	$3.00^{bX}\pm0.02$	$3.19^{cX}\pm0.03$	$3.37^{dX}\pm0.04$	$2.97^{x}\pm0.14$
	2	$2.46^{a}\pm0.04$	$2.69^{ab}\pm0.04$	$2.78^{bcY}\pm0.04$	$2.80^{cY}\pm0.04$	$2.81^{cy}\pm0.04$	$2.71^{Y}\pm0.06$
Catalase (CAT,nmol/min/ml)	1	$47.26^{a}\pm0.57$	$50.57^{aX}\pm0.56$	$56.69^{bX}\pm0.57$	$64.62^{cX}\pm0.59$	$74.74^{dX}\pm0.58$	$58.78^{X}\pm4.44$
	2	$46.00^{a}\pm0.35$	$49.04^{ab}\pm0.33$	$50.48^{bY}\pm0.33$	$56.73^{cY}\pm0.38$	$60.00^{dY}\pm0.31$	$52.45^{Y}\pm2.30$
Glutathione peroxidase (GP_X, nmol/min/ml)	1	$74.00^{a}\pm0.83$	$100.81^{b}\pm0.80$	$126.81^{cX}\pm0.80$	$138.78^{dX}\pm0.82$	$208.25^{eX}\pm0.84$	$129.73^{X}\pm2.02$
	2	$75.54^{a}\pm0.69$	$100.33^{b}\pm0.73$	$121.84^{cY}\pm0.71$	$131.34^{dY}\pm0.73$	$197.63^{eY}\pm0.73$	$121.53^{Y}\pm1.98$
Total antioxidant status (TAS, mM)	1	$1.62^{a}\pm0.02$	$1.59^{aX}\pm0.03$	$1.53^{bX}\pm0.02$	1.49cX±0.02	$1.42^{dX}\pm0.03$	$1.53^{X}\pm0.03$
	2	$1.61^{a}\pm0.02$	$1.57^{bY}\pm0.02$	$1.55^{bY}\pm0.05$	$1.54^{bY}\pm0.04$	$1.45^{cY}\pm0.02$	$1.57^{Y}\pm0.02$
Thiobarbituric acid reactive substances (TBARS, nM)	1	$5.58^{a}\pm0.11$	$5.66^{a}\pm0.06$	$5.83^{a}\pm0.08$	$6.63^{bX}\pm0.09$	$7.94^{cX}\pm0.07$	$6.33^{X}\pm0.20$
	2	$5.55^{a}\pm0.03$	$5.61^{a}\pm0.02$	$5.72^{ab}\pm0.04$	$5.93^{bY}\pm0.05$	$6.90^{cY}\pm0.02$	$5.94^{Y}\pm0.22$

Observation with different superscripts (a,b,c,d) differ significantly within the group; observations with different superscripts (X,Y) differ significantly between the groups;* represents the mean (±2) of gestation days.

peroxidative damage, and is also important in leucocytes, because immune function, and in particular phagocytic function, is linked to the release of O_2 radicals that participate in the microbicidal activity of macrophages. Thus, higher PA observed in group 2 compared to group 1 may be a response to vitamin C supplementation in group 2.

The mean lymphocyte proliferation index (LPI) to concanavalin A (Con A) and lipo-polysaccharide (LPS) on day –45 was 0.96±0.06; 0.97±0.04 and 1.06±0.02; 1.02±0.02 in groups 1 and 2, respectively. LPI decreased gradually and the decrease was not significant from day –45 to day –15 in both the groups. But the LPI was significantly lower on day –7 and 0 compared to day –45 in both the groups. The LPI was not significant between groups 1 and 2 throughout the experiment. The overall means of LPI to Con A and LPS were 0.84±0.05; 0.82±0.05 and 0.83±0.08; 0.82±0.07 in groups 1 and 2, respectively.

Lymphocyte proliferation response (LPI) is a representative of cellular immunocompetence. This measure can potentially be used as an indicator of an individual's ability to mount an immune response to specific pathogens or immunomodulators. Elvinger *et al.* (1991) evaluated proliferation of lymphocytes stimulated with mitogens (PHA, pokeweed or con A) *in vitro* at incubated temperatures of 38.5°C and 42°C, and *in vivo* in lactating Holstein cows subjected to heat stress and reported that proliferation of lymphocytes was reduced in cells subjected to heat stress. High incubation temperature had little effect on viability of cells, but there was decrease in lymphocyte proliferation *in vitro* at high temperature in cells obtained from heat stressed cows. It is documented that high cortisol concentration in animals had immunosuppressant effect (Parillo and Fauci 1979). Corticoid concentrations in animals exposed to thermal stress (THI > 72) increased significantly. Therefore, low response of lymphocytes to mitogens might be a cumulative result of both high ambient temperature and high plasma cortisol concentrations (Parillo and Fauci 1979). Prolonged exposure to severe heat stress was responsible for the decline of immune cells' reactivity (Lacetera *et al.* 2006), which might contribute to the higher occurrence of infections during summer (Cook *et al.* 2002).

Several studies (Soper *et al.* 1978, Elvinger *et al.* 1991,

Lacetera *et al.* 2002) elucidated the relationships between heat stress and immune cell function in bovines. However, the results of these studies are contradictory. Tyler and Cummins (2003) observed that dietary ascorbyle-2-PO_4 reduced proliferation of multinuclear leucocytes (MNL) stimulated with PWM (P<0.01) in Holstein heifers after transportation stress. Another study reported that lower quantities of added ascorbate enhanced the response to PHA (Standefer *et al.* 1987). Stressors have been associated with increased circulatory concentration of glucocorticoids and decreased functioning of the cells of the immune system. In the present study, the LPI to concanavalin A (Con A) decreased from day –7. Blecha and Baker (1986) also found that LPI to Con A was reduced in cattle when they were exposed to stressful conditions. In such conditions, the administration of antioxidants proved useful for improvement of immune functions (Victor *et al.* 1999).

The mean superoxide dismutase (SOD) activity in group 1 and group 2 on day –45 was 2.50 ±0.02 and 2.46 ± 0.04 U/ml, respectively, and the difference was not significant. The SOD activity increased significantly (P<0.01) with the advancement of gestation in group 1 as well as group 2 and this increase was higher from day –15 in both the groups. The increase was 25.8% and 11.4% from day –45 to day 0 in groups 1 and 2, respectively. Between the groups SOD was significantly lower in group 2 compared to group 1 indicating lower increase in group 2 than in group 1. The role of intracellular SOD is to scavenge the superoxide ($\bullet O_2^-$) that is produced by a number of reaction mechanisms, including several enzyme systems, as a part of normal cellular functions (Fee *et al.* 1975). There is an increased production of superoxide radicals from threshold concentration due to stress conditions imposed either by thermal stress or physiological status (pregnancy/lactation) of an animal. In the present study the increase in SOD activity might be a response to both thermal stress and pregnancy stress. The increase in SOD activity with the advancement of pregnancy during summer in this study was corroborated by several workers (Chandra and Agarwal 2009, Pathan *et al.* 2010, Bernabucci *et al.* 2005, Sharma *et al.* 2011). Bernabucci *et al.* (2005) and Sharma *et al.* (2011) observed increased (P<0.05) SOD activity during the last 3 weeks of pregnancy and after calving the activity declined rapidly. Pathan *et al.* (2010) observed an increasing trend of plasma SOD activity towards parturition and the concentration was significantly higher (3.03±0.13 U/ml) on the day of calving compared to day 30 before parturition (2.13±0.19 U/ml). Chandra and Agarwal (2009) also reported an increase in SOD activity with the advancement of pregnancy during summer. An increase in SOD levels during summer in prepartum cows with peaks around calving was reported by Bernabucci *et al.* (2002) and these authors suggested that this increase in SOD around calving might be the result of a possible homeostatic control (Bernabucci *et al.* 2005). Manish *et al.* (2011) also reported significantly (P<0.05) higher erythrocyte SOD levels in heat stressed goats during the summer compared to pre-summer months. The lower SOD activity observed in group 2 compared to group 1 in this study indicated a reduced oxidative stress in animals supplemented with vitamin C. Kumar *et al.* (2011a) and Kumar *et al.* (2011b) also reported that SOD activity decreased in buffaloes supplemented with vitamin C during thermal stress.

The mean plasma catalase (CAT) activity of group 1 and group 2 was 47.26 ± 0.57 and 46.00±0.35 nmol/min/ml, respectively, on day –45 and increased significantly (P<0.01) to 74.74±0.58 and 60.00±0.31 nmol/min/ml on day 0. The increase was statistically significant (P<0.01) from day –15 in both groups and the increase was 36.8% and 23.3% in groups 1 and 2, respectively from day –45 to day 0. Between the groups CAT activity was significantly lower (P<0.01) in group 2 than group 1 from day –15 to day 0.The results are corroborated by Kumar *et al.* (2007), Lallawmkimi (2009), Kumar *et al.* (2010), Kumar *et al.* (2011). Hydrogen peroxide production increased due to increased SOD activity during heat stress (Bernabucci *et al.* 2002) and this in turn resulted in a coordinated increase in plasma catalase and glutathione peroxidase concentrations (Clemens and Waller 1987, Frei *et al.* 1989, Kehrer and Smith 1994). Thus a positive and significant correlation exists between catalase and SOD activities. Bernabucci *et al.* (2002) reported an increase in catalase activity in cows during summer with the peak reaching around calving time. Chandra and Agarwal (2009) also reported significantly higher catalase activity in pregnant crossbred cows during summer compared to that in winter. Significantly higher catalase activity in buffaloes was also reported during summer compared to that of winter by Lallawmkimi (2009).

The catalase activity was significantly lower (P<0.01) on days –15, –7 and 0 in group 2 supplemented with vitamin C compared to non supplemented group 1. Kumar *et al.* (2010) and Kumar *et al.* (2011a) also reported significantly lower catalase activity in buffaloes supplemented with vitamin C compared to that of non supplemented group subjected to thermal stress.

The mean plasma glutathione peroxidase (GP_X) activity of groups 1 and 2 were 74.00±0.83 and 75.54±0.69 nmol/min/ml on day –45 and increased to 208.25±0.84 and 197.63±0.73 nmol/min/ml, respectively, on day 0. The increase in GPx activity of groups 1 and 2 was 64.5% and 61%, respectively, from day –45 to day of calving. The GPx activity increased continuously with advancement of gestation period and the levels were significantly higher on day –30, –15, –7, and reached its peak on day of calving in both groups. Between the groups, GPx activity was significantly lower in group 2 compared to group 1 on days –15, –7 and 0.

The results are in agreement with studies of Pathan *et al.* (2010), Bernabucci *et al.* (2002), Kumar *et al.* (2010) and

Kumar *et al.* (2011b) who have also reported that GPx increased during stressful conditions. Pathan *et al.* (2010) observed an increasing trend in concentration of erythrocytic GSH-PX towards parturition in buffaloes and the levels were significantly higher on the day of parturition compared to day 30 before parturition. Bernabucci *et al.* (2002) also, found an increase in GPX levels during summer in prepartum cows with peaks reaching around calving time. Lallawmkimi. (2009) reported higher GPX activity in summer than winter in buffaloes. Manish *et al.* (2011) reported significantly (P<0.05) higher erythrocyte GPx levels in stressed goats during the summer compared to pre-summer months.

The GPx activity was lower in group 2 compared to group 1 indicating reduced oxidative stress in buffaloes supplemented with vitamin C. Kumar *et al.* (2010) also reported that supplementation of vitamin C lowered GPx activity in buffaloes exposed to heat stress in climatic chamber. The lower levels of GPx in our study may be a response to minimize cell damage caused due to oxidative stress during summer in buffaloes supplemented with vitamin C.

The mean plasma ferric reducing antioxidant power (FRAP) of groups 1 and 2, respectively, was 1.62±0.02 and 1.61±0.02 mM on day –45 and it decreased to 1.42±0.03 and 1.45±0.02mM, respectively, on the day of parturition. The decrease in FRAP was 12.3 and 6.8% in groups 1 and 2, respectively, from day –45 to day 0. The decrease in FRAP was significant from day –15 and the lowest levels reached on day 0. The overall mean of group 1 and group 2 (1.53±0.03 *vs.* 1.57±0.02) was statistically different with significantly higher (P<0.01) values in group 2. Between the groups, the average FRAP was similar and was not significant on day –45 in both groups 1 and 2. The average FRAP was significantly higher in group 2 compared to group 1 on day –15, –7 and 0.

All living organisms, including mammals have developed a complex antioxidant network to counteract the effects of reactive species. Non-enzymatic antioxidants constitute an important aspect of this network. The results of this study are in agreement with the work of several workers. Prior and Cao (1999) reported that increased production of reactive species due to high ambient temperatures resulted in a decrease in total antioxidant capacity *in vivo.* Similarly, significant reduction in TAS was reported by Aengwanich *et al.* (2011) in no shade animals compared to animals kept under shade during summer. Miller *et al.* (1993), Brezezinska-Slebodzinska (2001), Chatterjee *et al.* (2003) reported higher antioxidant status during parturition in vitamin E supplemented crossbred cows. Chandra and Agarwal (2009) also reported lower FRAP in prepartum cows during summer. Higher FRAP values observed in group 2 compared to group 1 in our study might be due to the antioxidant protective role of vitamin C supplementation.

The mean plasma thiobarbituric acid reactive substances (TBARS) concentration of groups 1and 2, respectively, was 5.58±0.02 and 5.55±0.03 nM day –45 and it increased to 7.94±0.07 and 6.90±0.02 nM, respectively, on day 0.The increase in TBARS concentration of groups 1and 2 was 29.7% and 19.6%, respectively, from day –45 to day 0. The increase in TBARS levels were significant at day –7 and reached highest on day of calving in both groups 1 and 2. The overall mean of group 1 was significantly higher (P<0.01) than group 2 (6.33±0.20 vs. 5.940.22 nM). Statistically significant (P<0.01) differences were observed between the groups on day –7 and day 0. There was an increase in TBARS concentration with advancement of gestation period in both groups 1 and 2, with significantly lower values in group 2.

Stress induced lipid peroxidation of biological membranes is commonly measured in terms of TBARS. The increased production of oxygen radicals during thermal stress leads to the formation of lipid hydro-peroxides through the Fenton's reaction (Trevisan *et al.* 2001). This resulted in TBARS induced reduction of membrane fluidity and increased erythrocyte membrane fragility (Chen and Yu 1994). The results of this study are in agreement of earlier works (Bernabucci *et al.* 2002, Tanaka *et al.* 2007, Kumar *et al.* 2007 and Chandra and Agarwal 2009). The increase in TBARS concentration was reported in heat stressed Holstein heifers by Bernabucci *et al.* (2002). Kumar *et al.* (2007) also observed the increase in TBARS concentration in cattle and buffaloes subjected to thermal stress. Higher TBARS concentration in pregnant crossbred cows during summer (6.230.02nm) compared to winter (5.37±0.07nmol) was reported by Chandra and Agarwal (2009). The increase of thiobarbituric acid reactive substances (TBARS) immediately before and after calving confirmed that cows during the transition period were under oxidative stress (Bernabucci *et al.* 2002). The lower TBARS concentration observed in group 2 compared to group 1, in this study may be a response of lower oxidative stress in vitamin C supplemented pregnant buffaloes. This is due to the fact that ascorbic acid acts as a chain blocker of lipid peroxidation. Vitamin C protects biomembranes from lipid peroxidation damage by eliminating peroxyl radicals in the aqueous phase before the later can initiate peroxidation (Frei *et al.* 1989). The decrease in TBARS concentration of group 2 compared to group 1 was corroborated by other workers (Tanaka *et al.* 2007, Kumar *et al.* 2011a, Kumar *et al.* 2011b).

Heat stress is the problem of great concern in tropical countries. In the present experiment, significant alterations occurred in enzyme activities, oxidative stress and immune status in pregnant Murrah buffaloes during summer. Supplementation of vitamin C resulted in improvement in immune status and oxidative stress indicating that vitamin C may be used as dietary supplement to reduce oxidative stress and to maintain immune status of pregnant buffaloes during summer stress.

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