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ORIGINAL RESEARCH

Heat shock protein 70, oxidative stress, and antioxidant status in periparturient crossbred cows supplemented with α-tocopherol acetate

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Abstract The study was conducted to investigating the effect of α-tocopherol acetate on heat shock protein 70 (Hsp70), oxidative stress, and antioxidant status during periparturient period in medium body condition score crossbred cows. Twenty crossbred Karan Fries cows with confirmed pregnancy were selected 2 months before expected date of calving. The cows were randomly distributed in to two groups: 10 cows were kept as control and 10 were supplemented with α-tocopherol acetate during dry period for 2 months. Blood samples were collected at −20, −10, −5, 0, 5, 10, and 20 days in relation to the expected date of calving. Superoxide dismutase, catalase, and total immunoglobulin were significantly higher ($P<0.01$) in treatment as compared to control cows. Heat shock protein 70 and thiobarbituric acid reactive substance levels were significantly lower ($P<0.01$) in the treatment cows than their counterpart. Treatment with α-tocopherol acetate during dry period resulted in reduced oxidative stress, heat shock protein Hsp70 levels, improved antioxidant, and improved immunity status indicating beneficial effect of α-tocopherol acetate treatment.

Keywords Heat shock protein 70 · Oxidative stress · Periparturient period · α-Tocopherol acetate

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Abbreviations

SOD	Superoxide dismutase
CAT	Catalase
TBARS	Thiobarbituric acid reactive substance
Hsp70	Heat shock protein 70
ROS	Reactive oxygen species

Introduction

Transition period is especially critical for health and subsequent performance of dairy cows (Castillo et al. 2005). Dairy cattle are more susceptible to a variety of metabolic and infectious diseases during the transition period compared with peak lactation (Sordillo et al. 2007; Sharma et al. 2011). Physiological changes during transition period associated with rapid differentiation of secretory parenchyma, intense mammary gland growth, and the onset of copious milk synthesis and secretion are accompanied by a high-energy demand and an increased oxygen requirement (Gitto et al. 2002). This increased oxygen demand augments the production of oxygen-derived reactants, collectively termed reactive oxygen species (ROS). Excessive production of free radicals and concomitant damage at cellular and tissue levels are controlled by cellular antioxidant defense systems. When ROS are produced faster than they can be safely neutralized by antioxidant mechanisms, oxidative stress results (Trevisan et al. 2001). There are growing evidences that oxidative stress is a threat to transition period and an increase in its level may lead to calving-related complications in both man and animals (Orhan et al. 2003; Castillo et al. 2005; Dimri et al. 2010).

Expression of heat shock protein (HSP) may vary in certain physiologic conditions, such as pregnancy, besides being a response to stressful stimuli. High-producing dairy cows commonly suffer from metabolic stress, and if this is

combined with other stressful situations, such as oxidative stress, hot environment, and infectious diseases, it may contribute to increasing heat shock protein 70 (Hsp70) concentrations in cells. Under stressful conditions, there is evidence of increased concentrations of Hsp70 in several types of cells in cattle (De Maio 1999; Lacetera et al. 2006). Heat shock proteins are as molecular chaperones that regulate intracellular processes to maintain homeostasis during cell proliferation/differentiation and are essential for the maintenance of normal cell function (Haslbeck 2002). They play an important role in protein–protein interactions such as folding and assisting in the establishment of proper protein conformation (shape) and prevention of unwanted protein aggregation. By helping to stabilize partially unfolded proteins, Hsp70 aids in transporting proteins across membranes within the cell (Walter and Buchner 2002; Borges and Ramos 2005).

Antioxidants can be broadly defined as any substance that delays, prevents, or removes oxidative damage to target molecules (Halliwell and Gutteridege 2007). α-Tocopherol functions as an antioxidant that terminates the chain of events of oxidative processes by donation of its phenolic hydrogen to chain propagating lipid peroxyl radicals, resulting in the enhanced formation of the less reactive α-tocopheroxyl radical (Putman and Comben 1987; Zhang and Omaye 2001). Excess ROS production is considered to be one factor causing apoptosis. Inhibition of ROS production by vitamin E should therefore delay this process. In support of that, Colitti et al. (2000) found that α-tocopherol in sheep mammary gland controls the expression of bcl-2, a factor involved in the inhibition of apoptosis by the prevention of lipid peroxidation induced by ROS.

However, to the best of our knowledge, there is no literature available on the effect of vitamin E supplementation on Hsp70, oxidative stress, and antioxidant status during transition period in medium body condition score (BCS) crossbred cows so far. The present study was planned to explore the effect of vitamin E supplementation on Hsp70 levels, oxidative stress [thiobarbituric acid reactive substance (TBARS)], and antioxidant status [superoxide dismutase (SOD) and catalase] during transition period.

Material and methods

The experiment was approved by the Institutional Animal Ethics Committee constituted as per the article number 13 of the CPCSEA rules, laid down by the Government of India.

Experimental design and treatment

The experiment was conducted at NDRI, Karnal (29°43′ North latitude and 76°58′ East longitude) Haryana, India. Twenty crossbred Karan Fries (Holstein Fresian × Tharparkar) cows (450±9.59 kg body weight) and medium body condition score (2.5±0.06 BCS) were utilized in this experiment. The cows were randomly distributed in to two groups, 10 cows in each. The cows of both groups were in similar parity (2.7±1.8 vs 2.9±1.9). All cows of two groups were fed as per standard feeding practices followed at the National Dairy Research Institute, India. Each cow was offered 6 kg of green fodder [dry matter (DM) basis], 1 kg of silage (DM basis), and 4 kg of concentrate mixture daily (Table 1). Concentrate mixture contained (in grams per kilogram DM), groundnut cake (150), maize (180), barley (140), wheat bran (200), mustard cake (120), rice polishings (120), deoiled cottonseed cake (60), mineral mixture (20), and common salt (10). The cows in the control group were supplemented with no vitamins, and in the treatment group, they were given 1,000 IU of tocopherol acetate per cow per day from 60 days prepartum to calving. The cows were kept under open housing system throughout the experiment, but 14 days before the expected date of calving, they were moved to a calving pen. Five days after parturition, the cows were moved to the paddock meant for lactating animals. Green fodder was offered ad libitum and 1 kg of concentrate mixture was given for every 3 kg of milk produced.

Sampling and sample analysis

Blood samples were collected from jugular vein of animals on −20, −10, −5, 0, 5, 10, and 20 days in relation to expected date of calving with use of Vacutainer tubes (BD Franklin, USA) containing heparin as anticoagulating agent at 7:30 a.m. in the morning. Samples were brought to the laboratory in chilled ice boxes soon after collection and centrifuged at 1,200×*g* at 4 °C for 20 min to separate the plasma from packed erythrocytes. Plasma samples were stored at −20 °C until analysis of Hsp70, TBARS, total immunoglobulin, and α-tocopherol. Packed erythrocytes were used for estimation of superoxide dismutase and catalase activity. The BCS of cows was established at the beginning of the study and then on the days of blood collection by the same person using a five-point

Table 1 Approximate daily α-tocopherol acetate (in milligrams) intake of two groups of cows

Daily intake	Groups	
	Control	Treatment
6 kg mixed green fodder	170	170
1 kg silage	5	5
4 kg concentrate mixture	40	40
α-tocopherol acetate supplement (IU/day)	0	1,000
Total	215	1,215

scale (ADAS 1986). Daily milk yield up to 15 days of lactation was recorded.

An enzyme-linked immunosorbent assay was used to determine the relative concentration of Hsp70 in plasma samples. The assay was performed using a modified method of Gutierrez and Guerriero (1991). Minimum detection limit was 3.32 ng/100 μl. Intra- and inter-assay coefficients of variation were 6.54 and 9.26 %, respectively.

The activity of SOD in 1 % erythrocyte lysate was determined by the method of Marklund and Marklund (1974). The assay is based on the ability of SOD to exhibit the autooxidation of pyrogallol. The values were expressed as units per gram of hemoglobin (Hb). The plasma catalase (CAT) activity was measured as per method described by Aebi (1983). In brief, 20 μl of 1 % erythrocyte lysate was incubated in 1.0 ml of 30 mM H_2O_2 at 37 °C and a decrease in absorbance was noted every 10-s interval for 1 min at 240 nm in a UV spectrophotometer (Specord 200 Double Beam UV–Visible Spectrophotometer, Germany). The catalase activity was expressed as micromoles of H_2O_2 decomposed per minute per milligram Hb using 36 as a molar extinction coefficient of H_2O_2. The extent of lipid peroxidation, an index of oxidative stress, was measured as thiobarbituric acid reactive substances formed. Lipid peroxides were measured by TBA test method of Asakawa and Matsushita (1979). Immunoglobin in the plasma sample was estimated by zinc turbidity method (McEwan and Fisher 1970).

The estimation of vitamins was done on a Waters HPLC Model 510 (Milford, MA, USA) fitted with a tunable absorbance detector model 486, a μ-Bondapak C-18 column of 3.9×300 mm size (Waters, USA), a model 510 pump, a rheodyne injector with 20 μl loop, and Millennium software. The Solvent system consisted of acetonitrile and HPLC water in the ratio of 95:5 at the flow rate of 1.7 ml/min (Chawla and Kaur 2001). α-Tocopherol was separated at 290 nm wavelengths for 3.25 min.

Statistical analysis

Values were expressed as means ± standard error (Snedecor and Cochran 1994). Data for all measured variables were analyzed as repeated measures using the MIXED procedure of SPSS version 19. The model included the main effects of vitamin E treatment (groups), days around calving, and their interactions. The pair-wise comparison of means was carried out using "Tukey's multiple range test." Catalase, SOD, and Hsp70 were correlated with Pearson's correlation method.

Results

Control cows showed higher ($P<0.01$) BCS reduction from late pregnancy (day 20 prepartum) to early lactation (day 20 postpartum) than treatment cows (Fig. 1). In control animals, Hsp70 level in plasma on each observation day up to calving was significantly ($P<0.05$) higher than the preceding level (Table 2). However, following parturition, it declined up to day 20 postpartum. Towards calving, Hsp70 level increased albeit slowly in treatment in comparison to control. Values on each observation day were significantly ($P<0.05$) lower than corresponding control values. Hsp70 was negatively correlated with SOD ($P<0.01$, $r=-0.42$) and catalase ($P<0.01$, $r=-0.34$).

SOD (Table 2) and CAT (Table 3) activity in erythrocytes in control animals declined towards calving and the value further declined after parturition. In treatment animals, SOD and CAT activity also followed same patterns as in control, but their activity on all observation days was significantly ($P<0.05$) higher than corresponding values in control animals.

In control animals, TBARS level in plasma on each observation day up to day 5 (postpartum) was nonsignificantly higher than the preceding level (Table 3). However, following parturition, it also increased and was significantly higher on day 20 postpartum from its levels on each day. As calving comes near, TBARS level increased albeit slowly in group II in comparison to control. Values on days −5 (prepartum), 0 (calving), 5, 10, and 20 postpartum were significantly ($P<0.05$) lower than corresponding values in control cows.

Plasma total immunoglobulin level in control animals decreased steadily up to day 5 (postpartum), and after that, value increase was noted on days 10 and 20 (postpartum) (Table 4). In treatment animals, total immunoglobulin followed a similar pattern as that in the control; however, its value on all day was significantly ($P<0.05$) higher than corresponding values in their counterpart.

Changes of α-tocopherol acetate of two groups of cows are reported in Table 4. In the control, α-tocopherol acetate levels were significantly higher on days −20, −10, and −5 prepartum

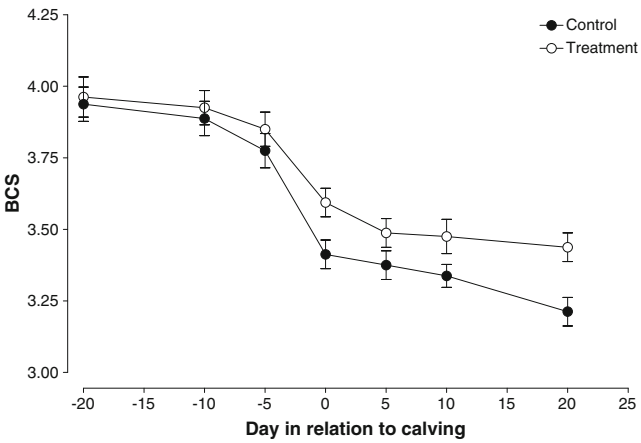

Fig. 1 Changes of BCS in periparturient dairy cows. Data are reported as mean ± SE of BCS of control and treatment group cows in relation to calving

Table 2 Mean ± SE of heat shock protein 70 (Hsp70) and superoxide dismutase (SOD) in control and treatment group cows during periparturient period

Day in relation to calving	Hsp70 (ng/ml)		SOD (units/g Hb)	
	Control	Treatment	Control	Treatment
−20	$60.79\pm1.38^{a, A}$	$54.31\pm0.81^{b, A}$	$3,093.10\pm74.14^{a, A}$	$3,837.83\pm93.81^{b, A}$
−10	$67.22\pm0.82^{a, B}$	$62.24\pm0.76^{b, B}$	$2,848.32\pm77.06^{a, B}$	$3,478.12\pm93.32^{b, B}$
−5	$77.88\pm0.79^{a, D}$	$70.42\pm1.12^{b, D}$	$2,741.60\pm53.28^{a, B}$	$3,264.84\pm62.01^{b, C}$
0 (calving)	$90.26\pm0.81^{a, F}$	$82.08\pm0.53^{b, F}$	$2,519.64\pm105.52^{a, C}$	$3,083.89\pm67.00^{b, D}$
5	$85.51\pm1.22^{a, E}$	$79.48\pm1.10^{b, F}$	$2,297.55\pm65.47^{a, D}$	$2,789.63\pm70.30^{b, A, E}$
10	$83.25\pm0.88^{a, E}$	$77.02\pm1.02^{b, E}$	$2,013.48\pm75.20^{a, E}$	$2,752.29\pm108.15^{b, E}$
20	$70.28\pm0.52^{a, C}$	$66.87\pm0.90^{b, C}$	$1,962.56\pm46.63^{a, E}$	$2,540.13\pm78.57^{b, F}$

Values (mean ± SE) with different superscript small letters in a row and capital letters in a column differ significantly ($P<0.05$)

in comparison to α-tocopherol acetate levels on days 0 (calving), 5, 10, and 20 postpartum. In the treatment, changes in α-tocopherol acetate level followed similar pattern as that in control; however, values on each observation day were significantly ($P<0.05$) higher than corresponding control values. Daily milk yield averaged 12.00 and 14.70 kg in control and treatment, respectively, which showed 22.50 % increase in milk yield in treatment in comparison to control group.

Discussion

In the present experiment, BCS change was 0.2 from calving to 20 days postpartum in control group and 0.15 in treatment group of cows indicating the higher BCS loss in control cows. Body condition score maintained significantly in treatment in comparison to control group of cows indicates that BCS loss was less in α-tocopherol-treated cows. Transition cows with high BCS lose more body weight and body condition than thinner cows (Bernabucci et al. 2005; Aggarwal et al. 2008). The average BCS change was −0.2±0.1 in the dry period (weeks −5 to −1) and average BCS loss was 0.53±0.13 in early lactation (calving to week 5) (Andersen et al. 2008).

To our knowledge, this is the first study to evaluate the Hsp70 concentrations in plasma during periparturient period in crossbred cows. Heat shock causes the intracellular expression of a specific group of proteins called HSPs that have broad cytoprotective properties. The first demonstration of HSP-mediated cytoprotection involved the phenomenon of thermotolerance, whereby a brief heat shock conferred protection. Subsequent studies demonstrated that the induction of HSPs also protected cells and whole organs against nonthermal cytotoxic agents such as oxidants, tumor necrosis factor-α, and endotoxin (Wong et al. 1997). Oxidative stressors such as nitric oxide and reactive oxygen intermediates are known to be major mediators of cell damages during inflammation (Wong et al. 1996). Exposure to superoxide anion in the presence of the free radical scavenging enzymes, superoxide dismutase, and catalase improved cell survival and prevented Hsp induction in human (Omar and Pappolla 2005). Cows during periparturient period are under oxidative stress and α-tocopherol acetate has been found to reduce the oxidative stress (Chandra and Aggarwal 2009). Oxidative stress is one of the factors, which increase Hsp70 levels. In female dairy cattle, the age and stage of lactation affect the plasma Hsp72 level (Kristensen et al. 2004). After calving, the SOD and catalase levels declined in both the groups. Bernabucci et al. (2005) also reported decreased SOD activity towards calving, and after calving, SOD activity rapidly declined. The declined of SOD activity, around calving, might be the result of the altered homeostatic control. These variations might have induced an imbalance between the production

Table 3 Mean ± SE of catalase (CAT) and thiobarbituric acid reactive substance (TBARS) in control and treatment group cows during periparturient period

Day in relation to calving	CAT (μmoles of H_2O_2 decomposed/min/mg Hb)		TBARS (nmole/ml)	
	Control	Treatment	Control	Treatment
−20	$141.60\pm0.52^{a, A}$	$152.22\pm0.84^{b, A}$	$2.72\pm0.14^{a, A}$	$2.46\pm0.13^{a, A}$
−10	$138.72\pm0.82^{a, B}$	$147.54\pm0.84^{b, B}$	$2.75\pm0.15^{a, A}$	$2.48\pm0.13^{a, b, A}$
−5	$138.01\pm1.05^{a, B}$	$144.28\pm0.71^{b, C}$	$3.17\pm0.16^{a, A}$	$2.58\pm0.14^{b, A}$
0 (calving)	$134.98\pm0.64^{a, C}$	$142.63\pm0.92^{b, D}$	$3.77\pm0.17^{a, B}$	$2.92\pm0.15^{b, A, B}$
5	$133.97\pm1.05^{a, C, D}$	$139.09\pm0.64^{b, E}$	$4.04\pm0.18^{a, B}$	$3.15\pm0.17^{b, B, C}$
10	$132.56\pm0.89^{a, D}$	$135.07\pm0.87^{b, F}$	$5.02\pm0.22^{a, C}$	$3.52\pm0.19^{b, C, D}$
20	$129.67\pm0.65^{a, E}$	$136.28\pm0.86^{b, F}$	$6.58\pm0.33^{a, D}$	$4.04\pm0.31^{b, D}$

Value (mean ± SE) with different superscript small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 4 Mean ± SE of total immunoglobulin and α-tocopherol in control and treatment group cows during periparturient period

Day in relation to calving	Total immunoglobulin (mg/ml)		α-tocopherol (μg/ml)	
	Control	Treatment	Control	Treatment
−20	$30.33\pm0.75^{a, A}$	$37.53\pm0.42^{b, A}$	$2.75\pm0.04^{a, B}$	$3.24\pm0.16^{b, A}$
−10	$27.70\pm0.82^{a, B}$	$34.49\pm0.54^{b, B}$	$2.65\pm0.04^{a, B}$	$3.37\pm0.15^{b, A}$
−5	$24.58\pm0.68^{a, C}$	$30.25\pm0.74^{b, C}$	$3.08\pm0.15^{a, A}$	$3.35\pm0.17^{b, A}$
0 (calving)	$23.45\pm0.36^{a, C}$	$29.87\pm1.01^{b, C}$	$1.94\pm0.11^{a, C}$	$2.73\pm0.12^{b, B}$
5	$20.23\pm0.74^{a, D}$	$26.45\pm0.82^{b, D}$	$2.03\pm0.12^{a, C}$	$2.23\pm0.09^{b, C}$
10	$23.24\pm0.54^{a, C}$	$23.56\pm0.24^{a, E}$	$1.84\pm0.17^{a, C}$	$2.16\pm0.10^{b, C}$
20	$26.22\pm0.67^{a, B}$	$29.58\pm0.41^{b, C}$	$1.88\pm0.13^{a, C}$	$2.17\pm0.11^{b, C}$

Value (mean ± SE) with different superscript small letters in a row and capital letters in a column differ significantly ($P<0.05$)

of reactive oxygen metabolites and their safe disposal (reduction of antioxidants), and the condition might indicate a loss of homeostatic control in the postpartum period. After calving, the catalase levels declined. Since SOD activity increases H_2O_2 production, protection from ROS would only be conferred by a coordinate increase of catalase and GSH-Px-E activities (Kehrer and Smith 1994; Aitken et al. 2009). In support of this conjecture, catalase activity was found to be increased in cows in the present study. The decomposition of H_2O_2 or its interaction with $^{\cdot}O_2^{-}$ would generate hydroxyl radicals ($OH^{\cdot}$). Hydroxyl radicals can attack all biological molecules, including membrane lipids, and can result in initiation of lipid peroxidation (Balasinska 2004). A positive and significant correlation has also been reported between catalase activity and SOD activity (Frei 1994; Kehrer and Smith 1994; Sharma et al. 2011).We also observed a positive correlation between SOD and catalase activity in our study. Reduced activity of these enzymes after calving may be due to reduction in the availability of copper, zinc, and iron in the early postpartum period or the reduction in antioxidant enzymes may be due to susceptibility of these enzymes to the oxidative reactive molecules (Sordillo et al. 2007).

TBARS is indicative of lipid peroxidation and increased levels after calving indicates the imbalance between oxidants and antioxidants (Bernabucci et al. 2005). The increase in TBARS level after calving was in accordance with reports given by Saleh et al. (2007); they used TBARS values as a marker of lipid peroxidation in cattle. The increase of TBARS before and after calving indicates that periparturient cows are under oxidative stress. Oxidative stress in cows is a contributory factor to increase disease susceptibility (Sordillo 2005; Sharma et al. 2011), since metabolic demands associated with late pregnancy, parturition, and initiation of lactation would be expected to increase the production of ROS, resulting in oxidative stress. In the present study, TBARS concentration was lower in vitamin E treatment groups than the control cows. The lower levels of TBARS in vitamin E-treated group were supported by Bouwstra et al. (2008, 2010).

Pre- and postpartum decreases in plasma immunoglobulin concentrations observed in this study were in agreement with previous reports in dairy cattle (Miller et al. 1993; Maurya 2011). Immunosuppression during the periparturient period has been reported (Mallard et al. 1998; Huzzey et al. 2009) which is responsible for mastitis and incidence of metritis. Oxidative stress during the transition period particularly during parturition may be a major underlying cause of inflammatory and immune dysfunction in dairy cattle (Sordillo and Aitken 2009). Immune cells are particularly sensitive to oxidative damage because their cell membrane contains high concentration of polyunsaturated fatty acids and are more susceptible to peroxidation (Spears and Weiss 2008), so immunoglobulin production from immune cells decreased in the control. Vitamin E, an antioxidant, protects immune cells from lipid peroxidation, resulting in increased immunoglobulin levels in the treatment cows (Politis et al. 2004; Spears and Weiss 2008).

Weiss et al. (1997) reported plasma vitamin E levels with daily supplementation of 1,000 IU vitamin E in periparturient cows. Campbell and Miller (1998) reported as low as 0.73 and 1.15 μg/ml of plasma vitamin E in 0 and 1,000 IU vitamin E-supplemented cows during dry period. Vitamin E and β-carotene supplementations to cows during the dry period improve their plasma vitamins A and E and β-carotene status after parturition as well as to improve milk production (Chawla and Kaur 2004). In agreement with other studies (Chandra and Aggarwal 2009; Bouwstra et al. 2010), extra daily supplementation of vitamin E resulted in higher vitamin E blood levels. Also, in accordance with other studies (Chandra and Aggarwal 2009; Bouwstra et al. 2010; Maurya 2011), cows showed a decreasing vitamin E level around calving, starting from 4 weeks before calving and lowest level was measured on the day of calving. Chawla and Kaur (2004) reported that the beneficial effect of daily supplementation of 1,000 IU vitamin E to dry cows on plasma α-tocopherol concentration at the time of parturition was evident and helped to regain normal plasma vitamin E status of 3.0 μg/ml within 12 days after parturition in the treated group, which supports our results.

The findings of the present experiment are consistent with the available literature and support the beneficial effect

of supplementing vitamin E to dry cows, which resulted in increased plasma α-tocopherol concentration at the time of parturition and helped to regain normal plasma vitamin E status for better immune function. In general, all the fat-soluble antioxidant vitamins decreased at parturition. This decrease might be due to various factors such as udder engorgement, transfer of vitamins into colostrum and milk, change in vitamin intake, hormonal changes, and so on. Goff and Stabel (1990) hypothesized that udder engorgement between 7 to 14 days prepartum may also be responsible for the decrease in plasma vitamin levels.

An increase in milk yield to the extent of 13.8 % on supplementation of vitamin E at 9,000 IU/kg concentrate mixture has been reported (Sinek et al. 2000). Chawla and Kaur (2004) also reported around 20 % increase in the milk production in cows fed with vitamin E at 1,000 IU per day during dry period.

Plasma Hsp70 was found to be negatively correlated with SOD and catalase enzymes and treatment with α-tocopherol acetate during dry period resulted in increased antioxidant status. Hsp70 levels were found to be significantly decreased around calving indicating the beneficial effect of α-tocopherol acetate treatment.

Conclusion

The results of this study indicated that oxidative stress supervenes during transition period in crossbred cows. Supplementation of antioxidants like vitamin E has beneficial effects in improving the antioxidant activity, immunoglobulin level, and decrease in plasma Hsp70 level.

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