Trop Anim Health Prod
DOI 10.1007/s11250-012-0319-6

REGULAR ARTICLES

Amelioration of altered oxidant/antioxidant balance of Indian water buffaloes with subclinical mastitis by vitamins A, D_3, E, and H supplementation

Umesh Dimri · Mahesh Chandra Sharma · Shanker K. Singh · Pankaj Kumar · Ricky Jhambh · Bishwambhar Singh · Samiran Bandhyopadhyay · Med Ram Verma

Accepted: 21 November 2012

Abstract The effect of vitamins A, D_3, E, and H supplementation on oxidative stress indices in Indian water buffaloes suffering from subclinical mastitis was investigated. Changes in the total oxidant capacity (TOC), total antioxidant capacity (TAC), nitric oxide (NO), and activities of glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) in milk were evaluated before and after the supplementation of vitamins A, D_3, E, and H. The buffaloes suffering from subclinical mastitis revealed remarkable alterations in the milk oxidants/antioxidants balance shifted towards oxidative status. The buffaloes with subclinical mastitis revealed significantly ($P \leq 0.01$) higher TOC, NO contents, and CAT activity, while TAC content and GSH-Px activity were significantly ($P \leq 0.01$) lower in comparison with the healthy controls. However, SOD activity did not show any significant change. Supplementation of vitamins A, D_3, E, and H to these animals revealed significant ($P \leq 0.01$) reduction in TOC, NO, and CAT, while a significant ($P \leq 0.01$) increase in TAC and GSH-Px activity was also evident. From the present study, it may be concluded that supplementation of these vitamins can help ameliorate the altered milk oxidants/antioxidants balance towards normalcy and, thus, ensue recovery from subclinical mastitis in the Indian water buffaloes.

Keywords Buffaloes · Oxidative stress · Subclinical mastitis · Vitamin supplementation

U. Dimri (✉) · M. C. Sharma · S. K. Singh · P. Kumar · R. Jhambh · B. Singh · S. Bandhyopadhyay · M. R. Verma
Division of Medicine, Indian Veterinary Research Institute, Izatnagar, 243122 Bareilly, UP, India
e-mail: udimrimesh@rediffmail.com

B. Singh · M. R. Verma
Livestock Economics and Statistics, Indian Veterinary Research Institute, Bareilly, India

S. Bandhyopadhyay
Eastern Regional Station, Kolkata, India

and Animal Husbandry, DUVASU, Mathura 281001, UP, India

Published online: 13 December 2012

Introduction

Mastitis is characterized by the inflammation of the mammary gland and is almost always caused by a bacterial infection. Approximately 60 to 70 % of new infections that develop during lactation do not cause obvious clinical signs and are often unrecognized (Deluyker et al. 2005). Subclinical mastitis is the main form of mastitis in modern dairy herds, exceeding 20 to 50 % of cows in given herds (Pitkala et al. 2004). These subclinical infections are a serious concern for dairy farmers because of decreased milk production, reduced milk quality, and increased transmission of pathogens that cause mastitis (Tesfaye et al. 2010; Mweu et al. 2012). The occurrence of the disease is an outcome of the interplay between the infectious agents and management practices stressing the defense of the udder (McDougall et al. 2009; Schukken et al. 2012; DeVliegher et al. 2012). The ability to control the degree of oxidative stress can be effective in ameliorating the severity of several proinflammatory-based diseases, such as mastitis. Failure to adequately control the accumulation of reactive oxygen species within metabolically active tissues is well-established that certain antioxidant micronutrients,

Table 3 Repeated-measures analysis of TOC (in micromoles H_2O_2 equivalent per liter), TAC (in micromoles Trolox equivalent per liter), concentration of NO (in micromoles per liter), and activities of GSH-Px (in units per gram), CAT (in micromoles H_2O_2 per milligram per minute), and SOD (in units per milligram) in milk of healthy and mastitic water buffaloes (least squares means)

Health status	Parameter	Treatment status		Overall
		Nontreated (control)	Treated	
Healthy	TOC	14.8231	14.7634	14.7933A
	TAC	0.5076	0.5127	0.5101B
	NO	3.7955	3.8037	3.7996A
	GSH-Px	35.9738	36.3845	36.1792B
	CAT	82.2837	82.9127	82.5982A
	SOD	2.6022	2.6035	2.6078
Infected (mastitis)	TOC	21.5856b	18.5381a	20.0618B
	TAC	0.3354a	0.4602b	0.3978A
	NO	10.4457b	6.1094a	8.2775B
	GSH-Px	19.8700a	28.5922b	24.2311A
	CAT	154.6171b	123.8301a	139.2236B
	SOD	2.6007	2.6075	2.6041

Significant difference ($P \leq 0.01$) between treated and nontreated (control) groups of the same health status (small letters within the same row)

Significant difference ($P \leq 0.01$) between healthy and infected (mastitis) animals (overall) (capital letters within the same column)

Kankofer 2012). A decreased level of GSH-Px in subclinical mastitic buffaloes' milk with higher SCC count can be the consequence of the consumption of this cytosolic enzyme to scavenge the overproduced free radicals. This implies that the mastitic buffaloes' milk might have remarkably increased PMNs with exhausted GSH-Px. The finding of the present study concords with previous scientific reports demonstrating significantly lower GSH-Px activity in milk cell supernatant isolated from mastitic animals (Mukherjee 2008; O'Rourke 2009). While in contrary to our finding, Andrei et al. (2011) reported the positive correlation between SCC and GSH-Px activity in dairy cows suffering from subclinical mastitis. The current study demonstrates the amelioration of GSH-Px activities towards normalcy post vitamins A, D_3, E, and H supplementation, albeit the SCC value decreased remarkably. This implies the recovery from subclinical mastitis by vitamin supplementation and subsequent restoration of GSH-Px homeostasis.

In this study, the SOD was not affected. Hicks (1980) found that the SOD activity is not correlated with the somatic cells of milk and not influenced by an elevated cellular numbering (Lipko-Przybylska and Kankofer 2012) and Rizzo et al. (2012) examined SOD in bovine milk and reported that the enzyme was identical, in respect to its [illegible] in milk. They also reported that all SOD activities were located in the milk's serum phase because no activity was associated with the fat fraction. It seems that reactions of dismutations achieved by SOD are weakly dependent on SCC, suggesting that PMNs have minor, if any, release of the superoxide anion (O^{2-}). Therefore, the oxidative stress generated with elevated somatic cell number seems to weakly touch the first antioxidant barrier and the formed free radicals do not require SOD intervention. This pleads in favor of the formation of H_2O_2 by immunity cells, free radicals not requiring a dismutation and playing an important role in intercellular immunities signals that come with the elevated SCC. According to a study conducted in vitro by Boulanger et al. (2002), the PMNs activated by *Escherichia coli* can damage mammary epithelial cells by the oxygenated radicals. SOD incorporation did not prevent the cytotoxic effects of these radicals. These imply the essence of the other antioxidants, such as CAT and GSH-Px.

Altered oxidant/antioxidant status signifies that buffaloes with subclinical mastitis are in a state of significant oxidative stress and an altered antioxidant defense mechanism is under operation. States of oxidative stress have been demonstrated earlier in various infectious diseases of animals including mastitis (Atakisi et al. 2010; Dimri et al. 2010). Under certain disease conditions, the increase in oxidants and decrease in antioxidants cannot be prevented, and the oxidative/antioxidative balance shifts towards the oxidative status (Cemek et al. 2006). Results of the current studies revealed that subclinical mastitis affecting buffalo's oxidant/antioxidant balance shifts towards the oxidative status. This may be the result of the overproduction of free radicals by the inflammatory cells recruited to combat the infection and exhaustion of the buffalo's antioxidant system.

Antioxidant vitamins generally enhance various aspects of cellular and noncellular immunity. Vitamin E is an important antioxidant that has been shown to play an important role in immunoresponsiveness and health in dairy cows (Spears and Weiss 2008). As a hydrophobic antioxidant that incorporates into lipid environments, vitamin E significantly decreases lipid peroxidation in different organs and body fluids (Kara et al. 2008). β-Carotene has significant antioxidant activities and can effectively quench the free radicals (Sharma and Sharma 2009). Potential antioxidants include either natural free radical scavenging antioxidant enzymes or the agents which are capable of augmenting the activity of these enzymes which includes GSH-Px, glutathione *S*-transferase, SOD, and CAT. In the current study, vitamins A, D_3, E, and H treatment had shown potential enhancement of antioxidant defense parameters.

It may be possible that low biotin levels may be favoring [illegible] antioxidant. Treatment of subclinical mastitic buffaloes with

Glutathione peroxidase activity in milk

Glutathione peroxidase (GSH-Px) activity in milk was assayed by estimating the content of oxidized glutathione formed by the action of GSH-Px, as described by Lipko-Przybylska and Kankofer 2012, but modified using H_2O_2 as substrate with the presence of 5,5′-dithiobis(2-nitrobenzoic acid). One unit of activity catalyzes the oxidation by H_2O_2 of 1.0 μmol of reduced glutathione (GSH) to oxidized glutathione per minute at pH 7.0 at 25 °C.

Catalase activity

Catalase (CAT) was measured according to Silanikove et al. (2012). Briefly, the assay mixture consisted of 100 mM of phosphate buffer (pH 7.0), 500 mM of H_2O_2, and 10 μl of milk in a final volume of 1.0 ml. The reaction started by adding H_2O_2, and its decomposition was monitored by following the decrease in absorbance at 240 nm for 1 min. The enzyme activity was calculated using an extinction coefficient of 0.043 mM/cm. CAT activity was calculated in terms of micromoles of H_2O_2 consumed per milligram of protein per minute of incubation.

Superoxide dismutase

Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) (Lipko-Przybylska and Kankofer 2012). Milk sample (10 μl) was added with 50 mM of phosphate buffer (pH 7.8), 39 mM of methionine, 2.6 mM of NBT, and 2.7 mM of ethylenediaminetetraacetic acid. Lastly, riboflavin was added to obtain a concentration of 0.26 mM; changes in absorbance at 560 nM were recorded after 20 min. In this assay, the activity was expressed in relative units per milligram of protein. One unit of SOD activity is defined as the amount of protein that inhibits the rate of NBT reduction of 50 %.

Statistical analysis

The SCC in milk was transformed to $\log_{10}$ to follow a normal distribution. Furthermore, the data were analyzed using the repeated measures design by taking health status (healthy, infected) and treatment (treated, nontreated) as additional variables in the model. Two-way analysis of variance was used to analyze the daywise data (mean±standard error) among the various groups. Statistical analysis was

<0.01 were considered significant.

Results

Changes in SCC in milk during the experimental period are depicted in Fig. 1. SCC in the HC and HT groups remained within the normal limits and did not differ from each other. In the MT group, the count was significantly ($P \leq 0.01$) higher at day 4 in comparison to the day 0 value, but on subsequent sampling, values decreased steadily and, at day 21, it reached within the normal limit. In the MC group, again at day 4, SCC was significantly higher than the day 0 value. Furthermore, a nonsignificant decrease in SCC was observed at days 7, 14, and 21.

Levels of TOC and TAC are presented in Table 1. Concentration of NO and activities of GSH-Px, CAT, and SOD are depicted in Table 2. On day 0, the values of TOC and NO were significantly ($P \leq 0.01$) higher in buffaloes with subclinical mastitis (groups MC and MT) than the values of healthy controls (groups HC and HT). In group MT, there was a significant ($P \leq 0.01$) decrease in values of NO and TOC from day 4 posttreatment, whereas in group MC, a significant ($P \leq 0.01$) decrease in values of NO and TOC were not found on successive days.

TAC values of MT and MC groups were significantly ($P \leq 0.01$) lower than the values of HC and HT groups on day 0. In the MT group, there was a significant ($P \leq 0.01$) increase in TAC values from day 4 posttreatment. GSH-Px activity of the MT and MC groups was significantly ($P \leq 0.01$) lower than the values of the HC and HT groups before treatment. There was a significant ($P \leq 0.01$) increase in GSH-Px activities of the MT group from day 7 posttreatment, whereas there was a gradual decrease in GSH-Px activities of the MC group. However, CAT activities of the MT and MC groups were significantly ($P \leq 0.01$) higher than the values of the HC and HT groups before treatment. CAT activity in the MT group decreased significantly ($P \leq 0.01$) from days 4 up to 21 posttreatment, whereas in the MC group, it was enhanced significantly ($P \leq 0.01$) from days 4 up to 21 posttreatment. SOD activity remained unaffected in all the groups from days 0 to 21 posttreatment.

Analysis by repeated-measures method revealed significant ($P \leq 0.01$) differences between healthy and infected groups, treatments within groups, and days of treatment with respect to the values/activities of TOC, TAC, NO, GSH-Px, and CAT (Table 3). Further analysis of data revealed that the values of TOC and NO (considering health status (healthy, infected) and treatment (treated, nontreated) as additional variables) (Table 3) exhibited a significant ($P \leq 0.01$) decrease in the MT group as compared to the MC group. The TAC values and GSH-Px activities were significantly higher ($P<0.01$) in the MT group as compared to the

significant alteration in the SOD activity was revealed

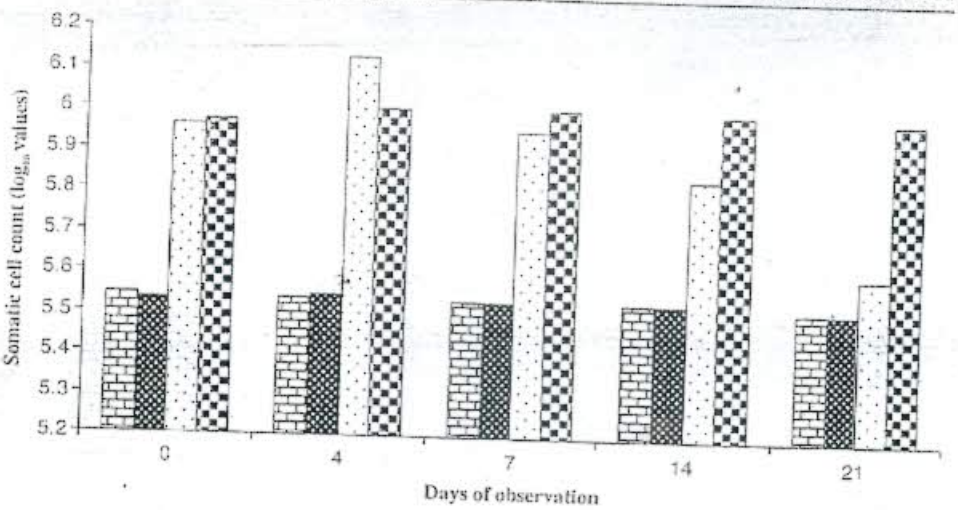

Fig. 1 SCC in milk of various groups at different time periods. *HC* healthy control, *HT* healthy treated, *MT* mastitis treated, *MC* mastitis control. ▦ HC, ▩ HT, ▭ MT, ▨ MC

between MT and MC groups. There were no significant differences in the values/activity of all considered oxidant/antioxidant parameters between HC and HT groups.

Discussion

Oxidative stress resulting from increased production of free radicals and decreased antioxidant defense leads to the disruption of normal metabolism and physiology and contribute to health disorders in lactating animals (Ranjan et al. 2005; Lykkesfeldt and Svendsen 2007; Zhao and Lacasse 2008; Salman et al. 2009). It is known that inflammatory cells are increased as a result of inflammation; recruited neutrophils and macrophages produce reactive oxidants, such as H_2O_2, hypochlorite, and oxygen radicals, and these reactive oxygen substances produced by cells of the immune system show potent cytotoxic effects on pathogenic organisms (Celi 2011). Free radicals are produced as a result of pathogen phagocytosis when mastitis occurs, which may result in lesions of the mammary epithelial cell and decreased milk secretion (Barbano et al. 2006).

Measuring TAC is considered a valuable tool for determining the overall antioxidative potential of cells or the whole organism, provided there was repeated sampling during certain time periods (Kankofer et al. 2010). In current study, TOC was higher in milk from mammary quarter with subclinical mastitis than in healthy mammary quarter of buffaloes, whereas TAC was lower in milk from mammary quarter with subclinical mastitis compared to those without

Table 1 TOC (in micromoles H_2O_2 equivalent per liter) and TAC (in millimoles Trolox equivalent per liter) in the milk of various groups of water buffaloes

Groups	Parameters	Day of observations				
		0	4	7	14	21
HC	TOC	14.83±0.66	14.80±0.71	14.82±0.68	14.83±0.51	14.84±0.49
	TAC	0.51±0.03	0.50±0.02	0.51±0.02	0.51±0.02	0.51±0.02
HT	TOC	14.41±0.77	14.62±0.79	14.87±0.61	14.92±0.56	14.99±0.65
	TAC	0.50±0.03	0.51±0.02	0.51±0.03	0.52±0.03	0.52±0.03
MT	TOC	21.63±0.71[a]	19.71±0.82[a, b]	18.22±0.68[a, b]	17.62±0.87[a, b]	15.51±0.75[b]
	TAC	0.37±0.02[c]	0.41±0.02[c, d]	0.46±0.02[c, d]	0.47±0.03[c, d]	0.59±0.02[d]
MC	TOC	20.90±0.66[a]	21.12±0.68[a]	21.73±0.73[a]	21.87±0.74[a]	22.31±0.64[a]
	TAC	0.38±0.02[c]	0.37±0.02[c]	0.33±0.02[c]	0.31±0.02[c]	0.29±0.02[c]

HC healthy control, *HT* healthy treated, *MT* mastitis treated, *MC* mastitis control)

[a] Significantly higher at $P \leq 0.01$ when compared with groups HC and HT

[b] Significantly lower at $P \leq 0.01$ when compared with day 0 values of the same group

[c] Significantly lower at $P \leq 0.01$ when compared

Table 2 Concentration of NO (in micromoles per liter) and activities of GSH-Px (in units per gram), CAT (in micromoles H_2O_2 per milligram per minute), and SOD (in units per milligram) in the milk of various groups of water buffaloes

Groups	Parameters	Day of observations				
		0	4	7	14	21
HC	NO	3.79±0.31	3.80±0.36	3.81±0.54	3.78±0.49	3.80±0.51
	GSH-Px	35.17±4.17	36.94±3.53	36.53±4.97	36.14±4.54	35.09±3.91
	CAT	82.37±7.64	84.53±8.19	81.23±7.09	80.49±9.71	82.79±8.33
	SOD	2.60±0.22	2.61±0.23	2.60±0.23	2.70±0.24	2.66±0.22
HT	NO	3.76±0.42	3.78±0.33	3.80±0.45	3.83±0.37	3.85±0.49
	GSH-Px	36.11±4.28	36.63±4.37	36.52±4.81	36.41±3.88	36.25±4.06
	CAT	84.63±9.14	83.29±8.22	82.88±9.23	82.17±8.42	81.60±7.54
	SOD	2.59±0.23	2.58±0.24	2.58±0.23	2.59±0.24	2.57±0.23
MT	NO	9.43±0.43[a]	7.13±0.57[a, b]	5.11±0.61[b]	4.79±0.73[b]	4.08±0.67[b]
	GSH-Px	21.34±4.59[c]	24.19±4.63[c]	30.54±4.15[c, d]	31.65±3.76[c, d]	35.24±3.92[d]
	CAT	153.17±13.93[a]	148.67±12.68[a, b]	129.31±13.44[a, b]	102.12±12.51[a, b]	85.89±13.24[b]
	SOD	2.60±0.25	2.59±0.22	2.62±0.21	2.63±0.23	2.61±0.24
MC	NO	9.71±0.63[a]	9.89±0.55[a]	10.92±0.71[a]	10.54±0.68[a]	11.17±0.59[a]
	GSH-Px	20.81±4.05[c]	20.57±4.35[c]	20.41±3.68[c]	19.55±4.15[c]	18.01±4.55[c]
	CAT	149.63±11.17[a]	152.22±10.91[ad]	153.71±11.64[ad]	157.83±12.42[ad]	159.71±13.71[ad]
	SOD	2.59±0.21	2.60±0.21	2.61±0.23	2.62±0.27	2.60±0.21

HC healthy control, *HT* healthy treated, *MT* mastitis treated, *MC* mastitis control

[a] Significantly higher at $P \leq 0.01$ when compared with groups HC and HT

[b] Significantly lower at $P \leq 0.01$ when compared with day 0 values of the same group

[c] Significantly lower at $P \leq 0.01$ when compared with groups HC and HT

[d] Significantly higher at $P \leq 0.01$ when compared with day 0 values of the same group

subclinical mastitis. This implies that subclinical mastitis could increase TOC, leading to the increase in formation of free radicals in milk. Elevated TOC and NO in subclinical mastitis affected buffaloes also imply the lipid peroxide-mediated pathogenesis. NO is a potent effecter radical which regulates many biological functions apart from a complex role in inflammatory response (Atakisi et al. 2010). During inflammation, NO increases and reacts with superoxide anions, leading to the formation of peroxynitrite radicals. Peroxynitrite radical is quite reactive and oxidizes long chain fatty acids in cell membrane, leading to the increase in lipid peroxidation and formation of free radicals (Leterrier et al. 2012). Administration of vitamins A, D_3, E, and H had successfully normalized the altered TAC, TOC, and NO levels of subclinically mastitic buffaloes, indicating its potential antioxidant activity against subclinical mastitis.

In this study, CAT activity was significantly higher in subclinical mastitic buffaloes than in healthy buffaloes. But CAT activity was significantly reduced in the MT group administered with vitamins A, D_3, E, and H. Silanikove et al. (2012) concluded that CAT plays a central role in milk sensitivity and specificity. Additionally, bacterial contaminants may be a potential contributor for CAT in milk. CAT has been reported to scavenge H_2O_2 and is a part of intracellular defense systems against oxidation. The high CAT activity in relation with SCC might be an adaptive mechanism to the superfluous H_2O_2 released by polymorphonuclear neutrophils (PMNs) in high milk SCC. The intervention of this antioxidant is crucial to reduce mammary cell damage (Boulanger et al. 2002). However, some concern may be raised because many of the common bacterial contaminants produce higher concentrations of CAT (Silanikove et al. 2012) and could interfere with the analysis of the native enzyme in milk. In present study, GSH-Px activity was significantly lower in subclinically mastitic buffaloes than in healthy buffaloes. However, the GSH-Px activity enhanced significantly in the MT group following vitamins A, D_3, E, and H administration.

SOD, CAT, and GSH, along with GSH-Px, are the most important intracellular antioxidant defense systems to combat the impending oxidative injuries triggered by free radicals during inflammation or other inciting agents. In milk

clinical mastitis is yet to be established in terms of

H_2O_2 in the presence of glutathione (Lipko-Przybylska and