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Original article

EVALUATION OF OXIDATIVE AND ANTIOXIDANT STATUS IN DAIRY CALVES BEFORE AND AFTER WEANING

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Summary

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Weaning is a stressful step in calf rearing which can lead to oxidative stress. The purpose of the present study was to investigate oxidative stress and antioxidant status in calves pre and post weaning. A total of 22 clinically healthy female Holstein calves at the same age were selected and their blood samples were examined to measure the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) in red blood cells and serum malondialdehyde (MDA) concentration. Sampling was performed in 4 consecutive stages: 7 days pre-weaning, the day of weaning, 24 and 72 hours after it. The results indicated that the activity of SOD and GPx enzymes increased significantly on the weaning day compared to the previous week. CAT activity increased 24 and 72 hours after weaning and its increase was significant 72 hours after weaning compared to the day of weaning. An increase was observed in MDA level on the day of weaning compared to a week before, and the trend was towards increase after the weaning. Weaning stress leads to disturbances of oxidative system balance and causes oxidative damage in calves. This imbalance can be probably resolved by improving the antioxidant system and supplementing antioxidants such as vitamins E and C against free radicals produced during weaning.

Key words: calf, enzyme antioxidants, oxidative stress, weaning

INTRODUCTION

Oxidative stress is the result of the imbalance between the production of free radicals and active oxygen species on one hand and the antioxidant defense system on the other. In other words, in an aerobic biological system, antioxidant defense mechanisms are designed to counteract free radicals and active oxygen species to neutralise or minimise the harmful effects of these invasive agents (Betteridge, 2000). Some components of this defense system, such as superoxide dismutase

(SOD), glutathione peroxidase (GPx) and catalase (CAT), and molecules containing the thiol group are produced inside the body, but others, such as vitamin E, vitamin C and beta-carotene, should be provided through the diet (Birben *et al.*, 2012). In the oxidative stress condition, many macromolecules are damaged and lipid peroxidation process, DNA oxidation, protein oxidation, and enzymes inactivation occur. It also leads to defect in animal physiological and metabolic processes (Buege & Aust, 1976; Trevisan *et al.*, 2001).

Oxidative stress plays a role in several diseases such as mastitis, acidosis, ketosis, diarrhoea and respiratory diseases (Miller *et al.*, 1993; Lykkesfeldt & Svendsen, 2007). According to previous studies, oxidative stress has been reported at birth, as well as during birth until weaning in calves. Stressors such as weaning stress, are considered as a key factor in possible changes in the immune system (Ranade *et al.*, 2014).

Oxidative stress has been proven during the weaning step in piglets (Zhu *et al.*, 2012). However, no studies have been performed on calves. Considering the importance of the antioxidant defense system and the possible risks of oxidative stress in increasing the potential and incidence of infectious and metabolic diseases in calves, it is necessary to identify any stressor to improve management practices in further research.

Therefore, this study aims to investigate the oxidative stress and antioxidant status before and after weaning in calves.

MATERIALS AND METHODS

Animals and samples

A total of twenty-two clinically healthy female Holstein calves at the same age were selected from a large-scale intensive dairy farm in Tehran province, Iran after thorough clinical examination. Calves were separated from their dams within 1 hour after birth and transported to individual pens.

All calves received 3 to 3.5 liters of high-quality colostrum (≥ 50 mg IgG/mL, estimated by a Brix Refractometer) within two hours after birth and two additional 2 liter meals 12 and 24 hours later.

Whole milk was pasteurised by heating up to 72 °C and then the temperature was reduced to 38 °C using cold-water circulation around the container before feeding to calves. Milk was offered in plastic buckets three times a day in equal portions. Calves were fed milk at about 10% of birth body weight from day 2 until weaning on day 70 of age.

Water and a commercial starter were provided for *ad libitum* intake on day 4. Starter feed composition was as followed: barley grain (ground) 9.8%; corn grain (ground) 47.3%; soybean meal 26%; fish meal 2%; wheat bran 3%; beet pulp 6%; DCP 0.5%; limestone 1.1%; mineral vitamin mix 1.1%; sodium bicarbonate 1.1%; salt 0.5%; zeolite 1.1%. The mineral-vitamin mix contained 1,107 IU/kg vitamin A; 2,106 IU/kg vitamin D_3; 6,000 IU/kg vitamin E; 0.5 g/kg vitamin B_1; 0.5 g/kg vitamin B_2; 48 g/kg Mg; 35 g/kg Zn; 30 g/kg Mn; 23 g/kg Fe; 10 g/kg Cu; 0.6 g/kg I; 0.4 g/kg Co; 0.1 g/kg Se. On day 42, chopped alfalfa (10% of dry matter) was added to the starter ration.

At weaning, allocated milk volume was gradually decreased over a 5-day period. After weaning, calves were kept in their individual pens until the end of the experiment.

All calves were subjected to sequential blood sampling in order to investigate the oxidant/antioxidant status. Sampling was

performed in 4 consecutive stages: stage 1; 7 days pre-weaning, stage 2; the day of weaning, stage 3; 24 hours post-weaning, stage 4; 72 hours post-weaning. Blood samples were collected from the jugular vein of each calf with heparin and without anticoagulant for measuring antioxidant enzyme activities, and for evaluation of lipid peroxidation, respectively. Blood without anticoagulant were centrifuged and the clear, nonhaemolysed sera were separated. Fresh heparinised blood samples were centrifuged (10 min at 3000 rpm) and the plasmas were discarded, erythrocytes were washed three times with normal saline. Packed erythrocytes were haemolysed by adding distilled water. Samples (whole blood, serum, haemolysate) were stored at −70 °C until assayed.

Assays

SOD activity was measured in haemolysate by an iodophenyl nitrophenol phenyltetrazolium chloride modified method (RANSOD Kit, Randox, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl-tetrazolium chloride (INT) to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction. One SOD unit was considered as the amount causing a 50% inhibition of the reduction rate of INT under the assay conditions (McCord & Fridovich, 1969).

GPx activity was evaluated by Paglia and Valentine's method in whole blood, using RANSEL Kit (Randox, UK). GPx catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured (Paglia & Valentine, 1967).

CAT activity was determined in haemolysate according to Aebi's method. The test is based on the determination of the rate constant (k) or the H_2O_2 decomposition rate at 240 nm. Results were expressed as k (s^{-1}) per gram haemoglobin (Aebi, 1984).

Haemoglobin concentration was measured spectrophotometrically by the cyanmethaemoglobin method (Jain, 1986). Twenty microlitres of blood were added to 5 mL of Drabkin's solution (containing potassium ferricyanide and potassium cyanide) in a test tube. In the Drabkin's solution, the red blood cells were haemolysed and the haemoglobin was oxidised by the ferricyanide to methaemoglobin. The cyanide was then converted the methaemoglobin to stable cyanmethaemoglobin. The mixture was allowed to stand for 15 min. After that, 1 mL of the mixture was pipetted into a cuvette. The cuvette was placed in a spectrophotometer set at 540 nm (Jain, 1986).

Measurement of serum MDA was performed in serum by thiobarbituric acid method, for which reactive substances were measured with thiobarbituric acid as peroxidation index. The basis of this measurement is the MDA reaction with thiobarbituric acid in the serum, which results in the formation of pink colour, and is calculated by measuring the intensity of the colour produced at 520 nm (Placer *et al.*, 1966).

Statistical analysis

All data were expressed as mean ± SEM. The statistical comparison between the groups was performed using repeated measures ANOVA. A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

Based on sampling sequence, results were categorised and compared among 4 stages, as followed: stage 1; 7 days pre-weaning, stage 2; the day of weaning, stage 3; 24 hours post-weaning, stage 4; 72 hours post-weaning (Table 1).

Comparison of the mean SOD activity in one week before weaning indicated a significant difference ($P<0.05$) from the other three stages; the lowest level of SOD activity was observed in pre-weaning. It is worth mentioning that, although no significant difference was found in other stages, the mean activity of the enzyme gradually increased from stage 1 to stage 3. In addition, results indicated a decrease in enzyme activity at 72 hours post-weaning compared to 24 hours post-weaning.

According to the results, the average activity of GPx enzyme in stages 2, 3 and 4 was significantly higher than that at stage 1. A significant increase of GPx activity at weaning day compared to that one week before weaning was observed. The activity of the enzyme was reduced 24 hours after weaning.

Evaluation of the mean activity of CAT in the four different stages indicated that its pre-weaning activity was lower than that at the other stages and gradually increased over time so that the level of enzyme activity in 72 hours post-weaning was higher compared to the other stages with significant differences vs stages 1 and 2.

Comparison of the mean concentration of MDA indicated significant differences between the four stages, with mean level of MDA at stages 2, 3 and 4 revealing a gradual increase compared to one week before weaning (stage 1).

DISCUSSION

Under oxidative stress, many macromolecules are damaged, leading to lipid peroxidation, DNA and proteins oxidation, inactivation of enzymes and disturbance in animal physiological and metabolic processes. Oxidative stress plays a key role in the process of multiple diseases. Stressors, such as weaning stress, are considered as an important factor in the potential changes in the immune system and leading to higher susceptibility to infectious diseases.

According to the results, weaning induced oxidative stress in calves, manifested through SOD, GPx and CAT activities elevation, and also increased lipid peroxidation.

Table 1. Concentrations of antioxidant enzymes and malondialdehyde (mean ± SEM, n=22) in calves during different pre- and post-weaning stages

Parameter	7 days pre-weaning (Stage 1)	Weaning (Stage 2)	24 hours post-weaning (Stage 3)	72 hours post-weaning (Stage 4)
SOD (IU/g Hb)	2168.37±88.79 [a]	2637.5±106.79 [b]	2782±69.19 [b]	2619.61±91.78 [b]
GPx (IU/g Hb)	267.07±17.91 [a]	439.49±20.01 [b]	367.82±15.28 [c]	380.16±15.85 [c]
CAT (k/g Hb)	13.05±1.01 [a]	13.06±1.00 [a]	14.09±1.03 [ab]	18.03±1.07 [b]
MDA (µmol/L)	1.60±0.18 [a]	2.25±0.19 [b]	2.57±0.17 [bc]	3.34±0.28 [c]

Different letters in each row indicate significant difference between groups ($P<0.05$).

As the major reactive oxygen species (ROS) produced in aerobic organisms is the superoxide radical which is a highly reactive cytotoxic agent, SOD is a primary line of defense agents against oxygen-derived free radicals, and converts them to peroxide hydrogen which is less harmful than the superoxide radical (Sindhu *et al.*, 2004). A significant increase of SOD activity on the day of weaning compared to the week before was noted in the present study. Increasing activity of SOD during the weaning and the existence of stress resulted from its important enzymatic defense function against superoxide radical generation. About 3 days post weaning and following reduction of the oxidative stress factors, the enzyme activity decreased. Previous reports indicated that when the risk of oxidative damage increases, endogenous antioxidant protection increases too (Bernabucci *et al.*, 2005).

The results of present study revealed that the average activity of GPX enzyme in the last three stages showed a significant increase compared to stage 1. A significant elevation in GPX activity was observed at weaning day vs one week before weaning. Twenty-four hours after weaning, the activity of the enzyme decreased, and this reduction continued until the 72nd hour. Following the increase of SOD activity and the accumulation of hydrogen peroxide, other antioxidant enzymes, including GPx and CAT, became involved. The affinity of GPx for H_2O_2 was stronger than that of CAT, which makes it more efficient at low levels of H_2O_2 (Kalpakcioglu & Senel, 2008). Increasing GPx activity on the day of weaning compared to an week earlier may be attributed to its initial role against H_2O_2. The presence of reduced glutathione is considered necessary to maintain the activity of glutathione peroxidase, so that significantly reduced GPx activity after 72 hours could be a result of GSH depletion at this phase. Previous studies demonstrated that in stress conditions, the GPx activity was elevated coincidentally with increased oxidative stress, and then it decreased gradually due to glutathione reduction (Yasini *et al.*, 2014; Delavari *et al.*, 2017).

Assessment of CAT activity showed that the activity of the enzyme was gradually increased over time, so that its activity was significantly different at 72 hours after weaning. Catalase plays a key role in the removal of H_2O_2 from red blood cells, and there was a linear correlation between catalase activity and hydrogen peroxide concentration (Mueller *et al.*, 1997). In the present study, while decreasing GPx, catalase activity was probably increased to compensate for the protection of red blood cells against dangerous accumulations of H_2O_2. Evaluation of oxidative stress in *Brucella* infected cows suggested that the serum CAT activity increased significantly as compared to healthy cows. A higher catalase activity in the affected animals indicated a greater rate of the hydrogen peroxide formation, and the body's response to the oxidative stress (Kataria *et al.*, 2010).

Some researchers demonstrated that in oxidative stress-associated state such as weaning in pigs, GPx, catalase and SOD concentrations were diminished as a result of the lack of an appropriate compensatory response from the endogenous antioxidant network and down regulation of expression of genes for GPx and catalase (Zhu *et al.*, 2012; Yin *et al.,* 2014). On the other hand some studies showed that the coordinated increase of SOD and GPx in weaned buffalo calves and heat-stressed cows was an indirect compensatory response to increased oxidant challenge

during oxidative status (Bernabucci *et al.*, 2002; Singh *et al.*, 2018).

Based on our study, lipid peroxidation, as determined by MDA concentrations, was significantly higher in stages 2, 3 and 4 compared to stage 1. These results showed that weaning induced ROS that were not totally scavenged by the antioxidant enzymes, which resulted in oxidative stress and lipid peroxidation in calves. Yin *et al.* (2014) showed that piglets suffered lipid oxidative injury at the third day after weaning, and lipid peroxidation then gradually diminished.

In conclusion, weaning stress leads to disturbances of oxidative balance and causes oxidative damage in calves. This imbalance can be probably resolved by improving the antioxidant system by oral and parenteral supplementation with antioxidants such as vitamins E and C against free radicals produced during weaning.

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