Paper

Relationships between oxidative stress, haematology and iron profile in anaemic and non-anaemic calves

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The aim of this study was to investigate the relationships between oxidative stress, haematology and iron profile in neonatal dairy calves. Serum and haemolysate malondialdehyde (MDA), serum total antioxidant capacity, thiol groups, iron, total iron binding capacity, transferrin saturation and red blood cell (RBC) parameters were assessed in two groups: anaemic calves (n=14) and non-anaemic calves (n=16). Blood samples were collected from all of the calves within 24–48 hours after birth and at 7, 14, 21 and 28 days of age. A significant decrease in serum iron amount and transferrin saturation value (P<0.05) and a significant increase in haemolysate MDA concentration (P<0.05) in the anaemic calves were observed, when compared with non-anaemic calves. Total antioxidant capacity and thiol groups showed a significant positive correlation with iron profile and RBC parameters (haematocrit and haemoglobin) in the anaemic calves at day 21 (P<0.05). On the other hand, the concentration of haemolysate MDA was inversely correlated with the value of serum total antioxidant capacity (P<0.05). The results of the present study revealed that anaemic calves showed more severe oxidative stress than non-anaemic calves. In addition, iron insufficiency may be linked to the impairment of antioxidant defence system and oxidative damage of erythrocytes in the neonatal calves.

Introduction

In physiological conditions, there is a balance between the generation of reactive oxygen species (ROS) and antioxidant defences in the body. Oxidative stress refers to a condition that antioxidant defence is unable to detoxify ROS formed and/or the generation of ROS exceeds the antioxidant capacity of the cell.[1–3] As a stressor, birth can lead to changes in the generation of ROS and body's antioxidant defences. Therefore, birth in humans and animal is associated with oxidative stress that occurs mainly due to the induction of pulmonary respiration.[4 5] Neonates are particularly susceptible to oxidative stress because of exposure to the hypoxic-hyperoxic challenge due to the transition from the hypoxic intrauterine environment to extrauterine life, susceptibility of neonates to infection and limited antioxidant protection.[6 7] Reduced antioxidants and increased malondialdehyde (MDA) have been revealed in neonates of humans and animals, which indicates the occurrence of oxidative stress after birth.[8 9] ROS concentration in the blood of newborn calves was higher than in cows.[9] The concentration of MDA in the erythrocytes of calves was the highest at birth and following the first solid feed intake at the third week.[9] Inanami and others[8] hypothesised that susceptibility of calves to oxidative stress during the neonatal period might be explained by the immature defence system against superoxide radicals as they established higher concentration of thiobarbituric acid reactive substances (TBARS) and lower antioxidant activities in the serum of calves.

Iron is an essential element in living cells. It is generally accepted that the iron requirements of young calves are higher than those of adults and thought to be about 100 parts per million.[10] Cows' milk contains low concentrations of iron; therefore, neonatal calves can easily become iron deficient as they grow.[11] Knowles and others[12] and Egli and Blum[13] stated that serum iron levels were lower in calves than those in adults. A declining trend in haemoglobin and haematocrit (HCT) values due to iron deficiency has been reported in neonatal calves during the first weeks of life.[14 15] Iron deficiency would be expected to give a decrease in the antioxidant enzymes of erythrocytes and plasma total antioxidant capacity.[16–18] In addition, increased oxidant activity has been reported in patients with iron deficiency anaemia.[18] The erythrocytes of patients with iron deficiency are lysed faster than normal erythrocytes following exposure to hydrogen peroxide (H_2O_2), which indicate some defects in the protective mechanism of red blood cell (RBC) with iron deficiency against oxidative damage.[18 19] Therefore, in addition to decreased haemoglobin production, a decrease in the lifespan of erythrocytes in circulation would occur in iron deficiency that further exacerbates the anaemic condition.[20] So it seems that iron deficiency along with the strong imbalance between pro-oxidants and antioxidants could be expected to contribute to the oxidative damage of erythrocytes in neonatal calves. Although it has been suggested that oxidative stress occurs in neonatal calves,[8 9] the relationship of this process with the changes of erythrocyte parameters and iron profile in neonatal calves has not been evaluated. The objectives in the present study were to (1) compare the changes of oxidative stress

Veterinary Record (2017) doi: 10.1136/vr.104179

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Provenance and peer review Not commissioned; externally peer reviewed.

Received October 29, 2016
Revised May 2, 2017
Accepted June 4, 2017

markers and iron profile in neonatal anaemic and non-anaemic calves and (2) to investigate the relationship of oxidative stress markers with iron profile and RBC parameters in neonatal anaemic and non-anaemic calves.

Materials and methods

The study was conducted in a dairy herd (Astan Quds Razavi) at Mashhad suburb (northeast of Iran). The herd consisted of pure bred animals of Holstein breed. The herd was totally confined in free-stall housing without access to pasture. Dry cows were fed with alfalfa hay (20.08 kg), concentrate (1.25 kg) containing barley, cotton seed, bran, beetroot and 1 per cent dry matter (DM) supplement and corn silage (11.4 kg). The ration was balanced according to NRC[10]). Cows were dried two months before expected time of parturition and transferred to a separate stall. As the time of parturition approached, the cows were moved to straw-bedded maternity pens. Following parturition, the umbilicus of each calf was treated with povidone iodine and the calf was weighed and transferred to individual pens. Within the first six hours of life, 4 kg of dam's colostrum was fed by nipple bottle and colostrum feeding was continued every 12 hours for three days. Then, herd milk was replaced for feeding twice daily (4 kg every 12 hours) until 60 days of life for male and until 75 days of life for female calves. Calf starter (started from five to six days of life) containing concentrate (90 per cent DM) and high-quality alfalfa (10 per cent DM) and also water offered free choice after transferring to individual pen.

Animals

Thirty Holstein calves were selected for the experiment and assigned into two different groups. The animals were classified as anaemic (n=14) and non-anaemic calves (n=16), according to the values of RBC parameters including HCT, haemoglobin and erythrocyte count. The anaemic calves had erythrocyte parameters below the reference intervals reported for clinically healthy cattle during the first month of life (HCT 21–30 per cent, haemoglobin 8.4–12.0 g/dl, erythrocyte count 4.9–7.5×10^6/μl; Wood and Quiroz–Rocha[21]).

Blood sampling

Ten millilitres of jugular blood was taken from all calves 24–48 hours after birth and at 7, 14, 21 and 28 days of age for measuring haematological and biochemical parameters. Two millilitres of blood was anticoagulated with EDTA for haematological analysis. Seven millilitres of blood without anticoagulant was centrifuged at 1800×*g* for 10 minutes. Serum was collected and stored at −20°C until processing. All animal procedures were approved by the Animal Welfare Committee of the School of the Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

For preparation of erythrocyte haemolysate, anticoagulated blood samples were centrifuged at 800×*g* for 15 minutes at 4°C. The plasma and buffy coats were removed by aspiration. The sediment containing blood cells was washed three times by resuspending in isotonic PBS, followed by recentrifugation and removal of the supernatant fluid and the buffy coats. One volume of the packed red cells was lysed in nine volumes of ice-cold distilled water to prepare a 10 per cent erythrocyte haemolysate. The resulting haemolysate was used for measuring of MDA concentration.

Biochemical and haematological analysis

Anticoagulated blood was analysed shortly after collection for haematological parameters including number of RBC, haemoglobin, HCT, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) by an automatic veterinary haematology cell counter (Nihon Kohden, Celltac α, Tokyo, Japan). The serum samples were used for determination of iron profile (iron and total iron binding capacity [TIBC] amounts by commercial kits [Pars Azmoon, Tehran, Iran]) and oxidative stress parameters including total thiol groups, total antioxidant capacity and MDA. The concentration of MDA was also measured in the erythrocyte haemolysate.

The transferrin saturation was also calculated according to the serum iron levels and TIBC as (Iron / TIBC) * 100. The concentration of MDA in serum and haemolysate samples was determined as TBARS according to Placer and others[22]. The concentration of total thiol groups was determined in the serum sample using dithionitrobenzoic acid method.[23] The total antioxidant capacity of the serum samples was measured using ferric reducing ability of plasma (FRAP) assay, which depends on the reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH.[24]

Statistical analysis

Statistical analysis was conducted using SPSS for Windows (release V.16, SPSS) with a P value of ≤0.05 as statistically significant. Data were expressed as mean±se. Repeated measures analysis of variance (ANOVA) was used for comparison of measured parameters between trial groups. Independent sample *t* test was used for comparison between groups at each sampling time. For determination of the relationship of oxidative stress markers with iron profile and erythrocyte parameters, Pearson's method was performed on the paired data obtained by the individual cases in two different groups.

Results

Changes in iron profile and oxidative stress parameters in anaemic and non-anaemic calves

The values (mean±se) of iron profile and oxidative stress parameters in anaemic (n=14) and non-anaemic (n=16) calves are presented in table 1. Repeated measures ANOVA revealed that time had significant effects (P<0.05) on the amounts of serum MDA (F=18.660), haemolysate MDA (F=3.403), thiol groups (F=21.856), iron (F=7.093), TIBC (F=9.204) and transferrin saturation (F=6.058). Group had significant effect (P<0.05) on the iron (F=4.237), transferrin saturation (F=4.578) and haemolysate MDA (F=3.403). A significant decrease in iron (F=4.237, P≤0.05) and transferrin saturation (F=4.587, P<0.05) in the anaemic calves was observed when compared with non-anaemic calves. Animals of the anaemic group presented a significant increase in haemolysate MDA (F=3.403, P<0.05) concentrations when compared with non-anaemic group.

Comparison of iron profile and oxidative stress parameters at different times

The results of iron profile and oxidative stress parameters at different times are shown in table 2. The amounts of haemolysate MDA were higher in the anaemic calves than in the non-anaemic calves at first 24–28 hours of life (F=1.202, T=2.011, P≤0.05)

Table 1: Mean±se of iron profile and oxidative stress parameters in anaemic and non-anaemic calves

	Anaemic calves	Non-anaemic calves
Serum iron (μg/dl)*	121.60±5.10	135.28±4.30
TIBC (μg/dl)	311.70±6.00	326.70±5.20
Transferrin saturation (%)*	38.60±0.90	41.60±0.70
Serum MDA (nmol/l)	0.54±0.04	0.60±0.03
Haemolysate MDA (nmol/l)*	0.82±0.03	0.73±0.03
Total thiol groups (mmol/l)	1.20±0.04	1.23±0.03
FRAP (mmol/l)	0.46±0.04	0.51±0.03

*Significant difference between anaemic and non-anaemic calves (P<0.05).
FRAP, ferric reducing ability of plasma; MDA, malondialdehyde; TIBC, total iron binding capacity.

Table 2: The results (mean±se) of iron profile and oxidative stress parameters in anaemic and non-anaemic calves in different times

	24–48 hours	Day 7	Day 14	Day 21	Day 28
Serum iron (µg/dl)					
Anaemic calves	117.40±5.60	109.50±7.70*	121.30±8.30	123.30±6.30*	137.50±7.00*
Non-anaemic calves	123.50±5.60	125.10±2.80*	131.70±6.10	146.30±5.40*	161.10±9.40*
TIBC (µg/dl)					
Anaemic calves	311.70±6.60	290.10±9.90*	312.10±8.30	313.50±8.00*	331.40±8.20*
Non-anaemic calves	318.40±8.10	308.70±3.60*	323.60±6.90	338.50±6.70*	356.00±10.30*
Transferrin saturation (%)					
Anaemic calves	37.30±1.04	37.30±1.40*	38.50±1.60	39.10±1.10*	41.10±1.10*
Non-anaemic calves	38.30±1.080	40.40±0.60*	40.30±1.20	43.00±0.80*	44.80±1.30*
Haemolysate MDA (nmol/l)					
Anaemic calves	0.61±0.03	0.90±0.14	0.77±0.08	0.67±0.05	1.10±0.08*
Non-anaemic calves	0.51±0.03	0.80±0.08	0.76±0.08	0.82±0.09	0.68±0.04*
Serum MDA (nmol/l)					
Anaemic calves	0.44±0.08	0.41±0.12	0.42±0.07	0.74±0.06	0.79±0.07
Non-anaemic calves	0.47±0.03	0.43±0.06	0.43±0.04	0.78±0.06	0.85±0.08
FRAP (mmol/l)					
Anaemic calves	0.49±0.07	0.45±0.07	0.44±0.05	0.46±0.05	0.49±0.03
Non-anaemic calves	0.45±0.03	0.53±0.03	0.50±0.03	0.53±0.04	0.56±0.06
Total thiol (mmol/l)					
Anaemic calves	1.24±0.04	1.17±0.05	1.23±0.05	1.18±0.04	1.18±0.03
Non-anaemic calves	1.27±0.04	1.24±0.03	1.22±0.03	1.19±0.03	1.19±0.04

*Significant difference between anaemic and non-anaemic calves at that time ($P<0.05$).
FRAP, ferric reducing ability of plasma; MDA, malondialdehyde; TIBC, total iron binding capacity.

and day 28 ($F=4.732$, $T=4.154$, $P<0.05$). The anaemic calves also presented a significant decrease in serum iron, TIBC and transferrin saturation concentrations when compared with non-anaemic calves at days 7, 21 and 28 ($F=3.078$, $T=-2.342$, $P<0.05$ for serum iron; $F=3.448$, $T=-2.067$, $P<0.05$ for TIBC and $F=1.868$, $T=-2.511$, $P<0.05$ for transferrin saturation at day 7; $F=0.498$, $T=-2.756$, $P<0.05$ for serum iron; $F=0.371$, $T=-2.419$, $P<0.05$ for TIBC and $F=2.050$, $T=-3.082$, $P<0.05$ for transferrin saturation at day 21; $F=0.001$, $T=-2.056$, $P<0.05$ for serum iron and $F=0.233$, $T=-2.170$, $P<0.05$ for transferrin saturation at day 28).

Relationship of oxidative stress markers with iron profile and erythrocyte parameters

Pearson's correlation (r) analysis of the paired data obtained by the individual anaemic calves revealed the existence of a significant positive correlation between serum FRAP concentration and the levels of serum iron ($r=0.793$, $P=0.002$), TIBC ($r=0.762$, $P=0.004$), transferrin saturation ($r=0.802$, $P=0.002$), HCT ($r=0.644$, $P=0.024$) and haemoglobin ($r=0.596$, $P=0.041$) at day 21. In addition, total thiol groups showed a significant positive correlation with serum iron ($r=0.836$, $P=0.001$), TIBC ($r=0.775$, $P=0.003$) and transferrin saturation ($r=0.864$, $P=0.000$). On the other hand, the concentration of haemolysate MDA was inversely correlated with the value of serum FRAP ($r=-0.568$, $P=0.05$). No significant correlations were observed between the measured parameters in the non-anaemic calves.

Discussion

Heidarpour Bami and others[16] and Mohri and others[25] suggested iron supplementation during first month of life in dairy calves prevented the decrease in RBC parameters. The significant decrease in iron and transferrin saturation concentrations ($P<0.05$) in the anaemic calves observed in the present study confirmed the significance of iron insufficiency in the progressive reduction of RBC parameters during the first weeks of life in calves. Iron requirement for domestic animals is influenced by age, growth rate and availability of dietary iron source. It is generally accepted that the iron requirements of young animals are higher than those of mature ruminants. The lowest value of iron in anaemic group was observed at day 7. After that, a slow and gradual increase in serum iron concentration was observed. This is due to the fact that studied calves were only fed with milk until the age of six days and, therefore, showed the least amount of iron up to seven days. From the age of six days, along with milk, the concentrates were freely given to the calves studied, and as the calves' diet contained iron, the iron levels increased up to 28 days. However, decreased serum iron and transferrin saturation in anaemic calves persisted at days 21 and 28 when compared with the non-anaemic calves. Therefore, it seems that the diet did not adequately meet the iron requirement of the anaemic calves.

Reduced antioxidant and increased lipid peroxidation have been identified in neonatal calves, which indicate the occurrence of oxidative stress after birth.[8,9] Trace elements such as iron are essential components of the body's antioxidant defence that play an important role in the prevention of free-radical-induced damage to tissues.[26] In the present study, a significant decrease in iron profile and a significant increase in haemolysate MDA ($P<0.05$) concentration were observed in calves of the anaemic group when compared with non-anaemic group. Oxidative damage to iron-deficient erythrocytes has been related to the generation of free radicals and increased lipid peroxidation.[27] The antioxidant defence system is impaired in erythrocytes of patients with iron deficiency, which would increase their susceptibility to oxidative damage.[14,17,18,28]

In the present study, significant positive correlations between the levels of iron profiles (serum iron, TIBC and transferrin saturation) and antioxidants (FRAP and total thiol) were observed in the anaemic calves. The relationship of iron profile with antioxidants suggested that iron insufficiency may, in part, have a role in the impairment of antioxidant defence system in the anaemic calves. Iron is an essential component of the body's antioxidants such as catalase. Catalase is a haem-containing enzyme that appears to be important in protecting erythrocytes against high levels of H_2O_2. Catalase destroys

hydrogen peroxide by conversion to water and oxygen. Except in dogs, mammalian erythrocytes generally have high catalase activities. In addition to its benefit to erythrocytes, the presence of catalase in RBCs may help protect somatic cells exposed to high levels of hydrogen peroxide, such as in sites of active inflammation.[29] It seems that iron supplementation to neonatal calves could improve haematological parameters and could enhance the antioxidant defence system.[16] Yoo and others[18] reported that oxidant activity in patients with iron deficiency anaemia was significantly higher than controls, while total antioxidant and catalase activity was significantly lower. After iron supplementation, oxidant, antioxidant and catalase activity reached the levels of the control group, and no significant differences were observed among groups.

A significant positive correlation between the amount of FRAP and erythrocyte parameters (HCT and haemoglobin) was observed in the anaemic calves. The relationship of oxidative stress with the severity of anaemia suggested that oxidative stress may, in part, have a role in the pathogenesis of anaemia in the studied anaemic calves. Aslan and others[30] reported a positive correlation between total antioxidant capacity and haemoglobin levels in patients with iron deficiency anaemia. On the other hand, the haemolysate MDA concentration was inversely correlated with the value of serum FRAP. Failure to increase the body antioxidant capacity commensurate with the occurrence of oxidative stress in the first weeks after birth suggested that the antioxidant system did not meet the body needs, and therefore, increased lipid peroxidation was observed in the studied anaemic calves. Due to abundance of polyunsaturated fatty acids and presence of powerful transition-metal catalyst, erythrocytes are one of the most susceptible cells to peroxidative damage.[31] Failure to increase the antioxidant capacity of the body during the first weeks of life will make the erythrocytes more susceptible to oxidative stress. These findings suggest that supplementation of iron and antioxidants such as vitamin C (based on its antioxidant properties and its role in iron metabolism[3 29];) by neonatal calves would be beneficial in improving the antioxidant defence system and combating the adverse effects of oxidative stress during the first weeks of life.

Conclusion

The present study is the first investigation in which the changes in oxidative stress markers along with haematological parameters and iron profile as well as their relationship in anaemic and non-anaemic dairy calves were studied. The present study indicated that iron insufficiency may be linked to the impairment of antioxidant defence systems in the neonatal calves. Moreover, impaired antioxidant capacity may induce oxidative damage in erythrocytes of neonatal calves which was manifested by enhanced lipid peroxidation.

Acknowledgements

The authors wish to thank the technicians who kindly helped them for sample collection of this study.

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Veterinary Record

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Veterinary Record 2017 181: 265 originally published online August 3, 2017
doi: 10.1136/vr.104179