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PHYSIOLOGICAL ANTIOXIDATIVE/OXIDATIVE STATUS IN BOVINE COLOSTRUM AND MATURE MILK

KANKOFER MARTA and LIPKO-PRZYBYLSKA JUSTYNA

Department of Animal Biochemistry and Physiology, Faculty of Veterinary Medicine, Agricultural University, Poland

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As colostrum provides the initial substrate supply, of immunological compounds and antioxidants to the neonate, the aim of the present study was to determine the antioxidative/oxidative status of bovine colostrum and milk. Colostrum was collected from healthy dairy cows (n=15) immediately after parturition, 6, 18, 36 h and 7 days later. The total antioxidant capacity (TAC) expressed as μmol/g protein was determined spectrophotometrically at 532 nm based on the reduction of tri-pyridyl-s-triazine (TPTZ). The indirect analysis of the intensity of lipid peroxidation – LPI, (expressed as μmol/g protein), corresponding to oxidation of peroxides, was measured spectrophotometrically at 505 nm. The TAC values have gradually increased from the 6th hour to day 7 (36h vs. 0h and day 7 vs. 0h; $p<0.01$) and were maximal in mature milk (day 7) (day 7 vs. 36h; $p<0.05$) whereas LPI values slightly fluctuated within the same period reaching maximal values at 36h (36h vs. 0h, 6h, 18h; $p<0.05$). In parallel, the ratio TAC/LPI has progressively increased from 71.6 (0h) to 177.6 (day 7). No overall positive correlation between TAC and LPI values was evidenced during the studied period. These results demonstrate dynamic changes of antioxidant systems while LPI remained stable, providing efficient neutralisation of radical oxygen species.

Key words: colostrum, milk, total antioxidant capacity, lipid peroxidation, cow

INTRODUCTION

Colostrum is one of the first and most important suppliers of natural host defense systems against different kinds of biologically active compounds for the neonate.

Numerous papers already described the presence and characteristics of antimicrobial factors (Clare *et al*., 2003) in the colostrum, as well as the necessary nutrients for the suckling newborn. There is however lack of data about antioxidative properties of colostrum.

Due to a sudden increase in oxygenation during and after parturition, the neonate can be exposed to oxidative stressful conditions. Higher concentrations of malondialdehyde, one of the products of lipid peroxidation, were found in the umbilical blood of neonates, compared to the peripheral blood of adults (Zhao *et al*., 2004). This may confirm the presence of oxidative stress conditions immediately after parturition and may indicate the need of developing protective systems against harmful effects of reactive oxygen species (ROS) (Mc Cord, 2000).

Colostrum itself can be a source of redox reactions, because of the presence of unsaturated fatty acids (Lindmark-Mansson and Akesson, 2000), but also because bacteria are killed by ROS. The balance between production and neutralization of ROS is maintained by antioxidant defence systems. They contain enzymatic and non-enzymatic compounds (Sies, 1993).

The activities of antioxidant enzymes were already described in milk, but such data for colostrum is scarce. Based on experiments, the antioxidant defense of milk consists of antioxidant enzymes such as: glutathione peroxidase, superoxide dismutase, catalase, lactoferrin, and vitamins E, A and C (Korycka-Dahl *et al*., 1979; Debski *et al*, 1987; Hivri and Griffiths, 1989; Andresson and Oste; 1994).

The antioxidative status of biological samples can be analysed by the determination of single components of the system or by measurement of total antioxidant capacity (TAC). Due to the fact that antioxidative systems act synergistically, a single analysis may not reflect the total potential. The analysis of the intensity of peroxidation may help to elucidate the effectiveness of the antioxidant systems.

The aim of the present study was to determine antioxidative/oxidative status in bovine colostrum by comparison of the total antioxidant capacity (TAC) as well as the intensity of lipid peroxidation (LPI) processes in samples collected several times after parturition.

MATERIAL AND METHODS

Animals and protocol design

Colostrum was collected from 15 clinically healthy Holstein – Friesian, 3-5 years old cows. Animals were kept at the same farm, fed a standard diet containing sugar beet pulp, barley straw, grass silage and mineral-vitamin supplementation. Samples were taken immediately after spontaneous parturition (time 0) and after 6h, 18h, 36h and then after 7 days respectively. The 7^{th} day samples were considered as mature milk. Samples were immediately frozen (–20^{o}C) until analysis.

Colostrum was subjected to defatting by centrifugation at 2500 x g for 15 min (4^{o}C) and the supernatant was used for the determination of TAC, LPI and of protein concentration.

Biochemical methods

The TAC was estimated according to the method of Benzie and Strain (1996) based on the reducing ability of colostrum, but with some modifications. The changes in absorbance were directly related to the "total" reducing capacity of the electron donating antioxidants present in examined colostrum samples.

The working reagent consisting of: (1) 300 mmol/L acetate buffer (pH 3.6), (2) 10 mmol/L 2,4,6-tri-pyridyl-s-triazine (TPTZ, Sigma, Poznan, Poland) solution in 40 mmol/L HCl and (3) 20 mmol/L $FeCl_3$ x $6H_2O$ solution in H_2O dest, mixed in the ratio of 10:1:1, was prepared immediately before use.

Working reagent (2250 μL) was mixed with 25 μL of supernatant and absorbance was measured at 593 nm against the working reagent alone, serving as the control sample. After exactly 10 min of incubation at room temperature, the absorbance was read again. The difference in absorbance at zero and at 10 min was compared with the standard curve prepared with 10 different dilutions of Fe (II) between 0 and 1000 μmol/l. The results were expressed as μmol/g protein in the supernatant (mean ± S.E.M.). Intra-assay 8.8% (n = 10) and inter-assay 8.5% (n = 10) coefficients of variation were established.

The method for measuring the intensity of lipid peroxidation (Alberti *et al*., 2000) is based on the estimation of radical cation formed in the reaction of alkoxy and peroxy radicals derived from the hydroperoxides by use of N,N,diethyl-para-phenylene diamine (DEPPD). Incubation mixture contained 1 mL of acetate buffer (pH 4.8), 10 μL of aqueous solution of DEPPD (0.37 mol/L) and 20 μL of the supernatant. After 1.5 h incubation at 37°C absorbance was read at 505 nm against distilled water. In the control sample, 20 μL of distilled water replaced the supernatant. Calculations were based on a standard curve prepared with 6 different dilutions of H_2O_2. The results were expressed as μmol/g protein of supernatant (mean ± S.E.M.). Intra-assay 9.1% (n=10) and inter-assay 8.9% (n=10) coefficients of variation were established.

The protein content of supernatants was determined according to the method of Lowry (1951) with bovine serum albumin as standard.

Statistical analysis

Results in duplicate were averaged and compared using Student's t-test. A probability $p<0.05$ was considered as significant. Statistical analysis of correlation was performed with software Statistica 6.0.

RESULTS

The results of TAC determinations are presented graphically in Figure 1. The TAC values were similar at t = 0h and 6 h later (3.58±0.58 μmol/g protein and 3.42±0.51 μmol/g protein, respectively), thereof they increased at 18 h (5.02±0.84 μmol/g protein), but not significantly compared to the initial values. They dramatically increased at 36 h (6.55±0.74 μmol/g protein) and on the 7th day (8.88±0.92 μmol/g protein) compared to previous values (36 h or 7th day vs. 0 h, vs. 6 h, vs. 18 h; $p<0.01$). Besides, the TAC value on day 7 was highest (day 7 vs. 36 h; $p<0.01$).

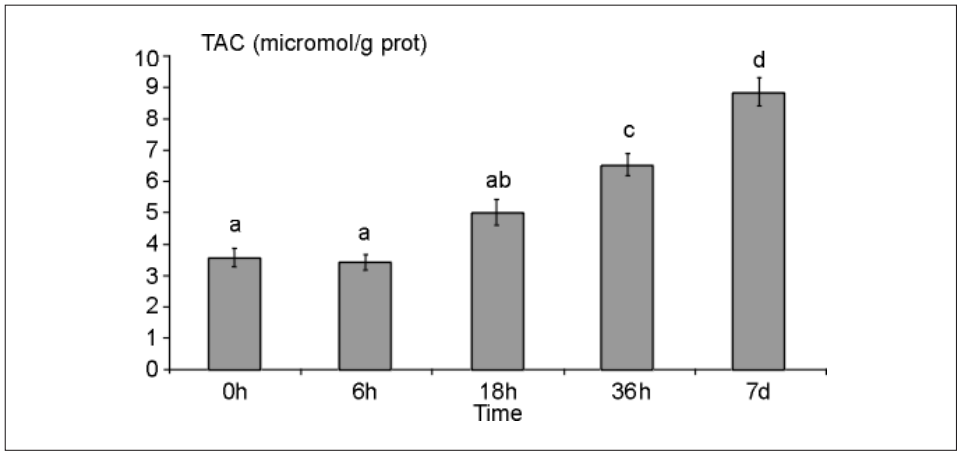

Figure 1. Total antioxidant capacity (μmol/g protein) in colostrum sampled immediately, 6, 18 and 36 hours after parturition, and 7 day milk of healthy dairy cows (mean ± S.E.M, n = 15)
[a,b] Histograms with different superscripts differ significantly ($p<0.05$)

The initial LPI determined immediately after parturition was high (0.050 ± 0.004 μmol/g protein) and slowly declined 6 h later. Thereafter, hydroperoxide concentrations increased again until 36 h (18h vs. 6h: $p<0.05$, and 36 h vs. 6h or 18h: $p<0.05$) and finally decreased not significantly on day 7 (Figure 2).

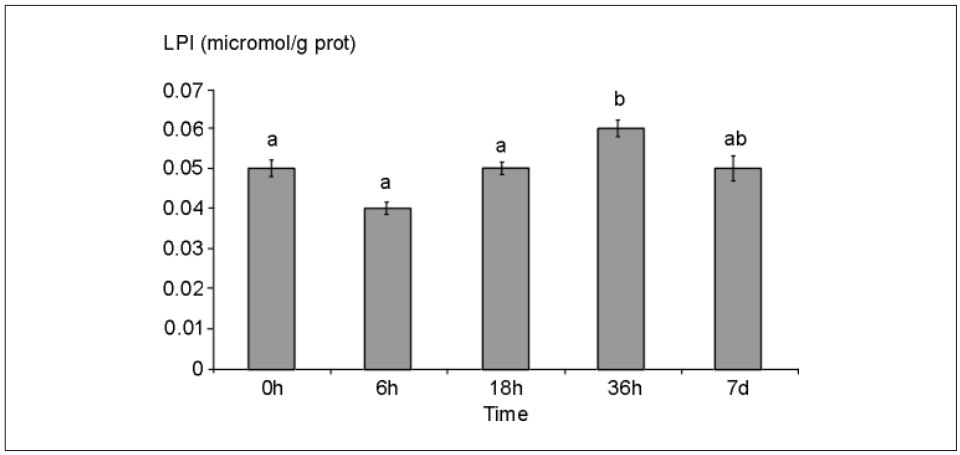

Figure 2. Lipid peroxidation intensity (μmol/g protein) in colostrum sampled immediately, 6, 18 and 36 hours after parturition, and 7 day milk of healthy dairy cows (mean ± S.E.M, n = 15)
[a,b] Histograms with different superscripts differ significantly ($p<0.05$)

The fluctuation of LPI values resulted in the lack of a clear correlation between TAC and LPI values on the overall considered period (from parturition to day 7). Nevertheless, TAC and LPI values positively correlated at 0h ($r = 0.52$; $p<0.05$), and at 6h ($r = 0.51$; $p<0.05$) indicating the relationship between these two parameters. The ratio TAC/LPI increased progressively from 71.6 at t=0 h to 177.6 on day 7 after parturition (Figure 3). TAC correlated negatively with protein content at day 7 ($r = -0.55$; $p<0.05$).

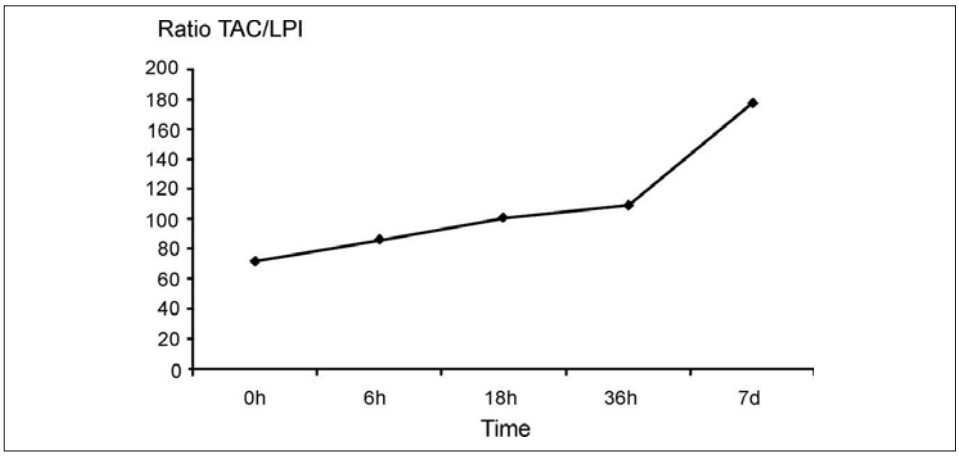

Figure 3. TAC / LPI ratio in bovine colostrum and milk sampled immediately, 6, 18 and 36 hours after parturition, and 7 day milk of healthy dairy cows (mean ± S.E.M, n = 15)

The colostrum protein concentrations were the highest immediately after parturition (164 g/L) and then gradually decreased according to time (147, 85, 59 and 49 g/L respectively).

DISCUSSION

Since colostrum is the first food for the newborn and influences the initial postnatal substrate supply, hence it requires a closer examination. Feeding colostrum during the first few hours after parturition brings to the neonate not only nutrients, but also protective substances against microorganisms, as well as antioxidants, because of the opened barrier between neonate intestinal cells and blood (Blum, 2006).

The results of the present study show that TAC values increased according to time and were highest in mature milk, suggesting that the antioxidative potential changes dynamically and/or that ROS fluctuations in the colostrum stimulate antioxidant systems.

It is not clear whether components of antioxidant defense of colostrum are of blood origin, or are directly synthesized in the mammary gland. That is why the

relationship between availability of particular components of antioxidant systems from the diet or blood and the efficiency of each system should be evaluated. Indeed, LPI fluctuated from parturition to the 7th day and was not dramatically modified (either increased nor decreased) within this period, probably because of an adequate antioxidative protection in the colostrum. In this way, a progressive increase of the ratio TCA/LPI was also observed within the studied period, indicating that antioxidant systems showed a dynamic status, whereas hydroperoxide concentrations remained roughly stable.

Unsaturated fatty acids present in the colostrum are influenced by the diet (Fidler and Koletzko 2000) and are susceptible to peroxidative damage. Among them, conjugated linoleic acid obtained from linoleic acid ruminal biohydrogenations exhibits some antioxidative properties (Parodi, 1997; Molkentin, 2000; Peterson *et al*., 2002).

The content of colostrum and mature milk can be influenced by breed, dietary regimen, as well as season and health status of the dam (Kelly, 2003). Catalase activity in cow milk has been found to show seasonal variations (Hivri and Griffiths, 1989). Colostrum and milk have to provide necessary antioxidants and efficiency of antioxidative defence systems against ROS. The proper balance between production and neutralisation of ROS is necessary both for health and adequate development of the neonate, but also the influence the quality of milk for human consumption.

Zhao *et al*., (2004) compared colostrum and blood TAC values in neonates immediately after birth and in adults. TAC was significantly higher in colostrum than in blood, and a lower TAC in the umbilical blood of neonates than in the peripheral blood of adults was detected as well, while malondialdehyde concentrations were higher in the umbilical blood than in the peripheral blood of adults.

Since many compounds by different modes of action constitute the antioxidative defence mechanisms, their coordination is very important. It is probable that the evaluation of a single antioxidant compound may not reflect the total properties of the studied biological system. This was the main reason why methods able to evaluate the total antioxidant capacity were developed. They certainly do not cover the whole spectrum of antioxidants, due to their varied chemical properties, but they could be good markers for the comparison of dynamic changes in the examined samples (Janaszewska and Bartosz, 2002). The system for TAC determination used in the present study was based on 2,4,6-tri-pyridyl-s-triazine (TPTZ) reduction. The major disadvantage of this method is the low pH of the incubation mixture which disturbs adequate determination of thiol groups. The aim of the present study, however, focused rather on the comparison of the TAC values according to time and analysed under equal conditions, than on the estimation of real TAC values of colostrum and milk. Korpela *et al*. (1995) described the presence of a peroxyl radical and of a superoxide radical in bovine milk. The oxygen radical absorbance capacity (ORAC) method showed slightly higher values in colostrum and transitional human milk than in mature milk. Positive correlations were found between

antioxidant vitamin intake during pregnancy and ORAC values in human colostrum (Alberti-Fidanza *et al*., 2002).

Available literature contains scarce information about the comparison of TAC between bovine colostrum and milk. There are some reports on single antioxidants mainly concerning milk rather than colostrum. Khackik *et al*. (1997) identified 34 carotenoids in human milk and Hidiroglou (1989) reported that α tocopherol concentrations were higher in bovine colostrum than milk at 4^{th} day of lactation. Glutathione peroxidase activity was similar in human and bovine milk (Debski *et al*., 1987), but this enzyme activity, as well as selenium content in milk, decreased within lactations in women (Hojo, 1986). In the same way, the superoxide dismutase activity in cows was about 100 times lower in milk than in the blood (Hoolbrook and Hicks, 1978). In women (Kiyosawa *et al*., 1993), this enzyme activity was reported to be increased in mature milk compared to colostrum.

High values of TAC in colostrum/milk protect the neonate against uncontrolled ROS increase and consequently, against peroxidative damage to biologically active macromolecules such as immunoglobulins or enzymes present in these biological fluids. Elements of the antioxidative system may also protect the mammary gland from the development of inflammation, as well as from peroxidative damage to mammary gland cells. Any unbalanced increase in LPI would result not only in peroxidative damage to the cell membrane, but also in alterations of important biochemical pathways by inactivation of enzymes (Halliwell, 2006).

In conclusion, the present study clearly demonstrates that the total antioxidant capacity shows dynamic changes in colostrum and milk from parturition up to day 7, whereas slight fluctuations of LPI were observed within the same period. Whether these changes are genetically controlled, or simply answer a current challenge of the mammary tissue requires further studies. The coupled determinations of TAC and LPI in colostrum and milk would allow evaluation of the potential beneficial effects on the neonate of dietary supplementation with antioxidant compounds of pregnant cows.

Address for correspondence:
Kankofer Marta
Department of Animal Biochemistry and Physiology,
Faculty of Veterinary Medicine,
Agricultural University,
20-033 Lublin, ul. Akademicka 12,
Poland
E-mail: marta.kankofer@ar.lublin.pl

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FIZIOLOŠKI ANTIOKSIDATIVNO – OKSIDATIVNI STATUS KOLOSTRUMA I MLEKA KRAVA

KANKOFER MARTA i LIPKO-PRZYBYLSKA JUSTYNA

SADRŽAJ

Kako kolostum obezbeđuje supstrate, imunske komponente i antioksidanse novorođenčetu na početku života, cilj ovog rada bio je da odredi oksidativni/antioksidativni status kravljeg kolostruma i mleka. Kolostrum je sakupljen od zdravih mlečnih krava (n=15) odmah nakon teljenja, a potom nakon 6, 18, 36 časova i 7 dana. Ukupni antioksidativni kapacitet (TAC) izražen u μmol/g proteina određen je spektrofotometrijski na 532 nm, na osnovu redukcije tri-piridil-S-tiazina (TPTZ). Indirektna analiza intetnziteta lipidne peroksidacije (LPI), izražena u μmol/g proteina, koja odgovara oksidaciji peroksida, merena je spektrofotometrijski na 505 nm. Vrednosti TAC su postepeno rasle od 6. časa do 7 dana nakon teljenja (36 časa i 7 dana $p<0.01$ u odnosu na 0. čas) i dostigle su maksimum u zrelom mleku 7. dana ($p<0.05$ u odnosu na 36. čas), dok su vrednosti LPI malo varirale u istom periodu, dostigavši maksimalnu vrednost 36. časa ($p<0.05$ u odnosu na 0., 6., i 18. čas). Odnos TAC/LPI je progresivno rastao od 71.6 (0. čas) do 177.6 (7. dan). U posmatranom periodu nije utvrđena ukupna pozitivna korelacija između vrednosti TAC i LPI. Ovi rezulati ukazuju na dinamičke promene u okviru antioksidativnog sistema, dok LPI ostaje stabilan, što obezbeđuje efikasnu neutralizaciju reaktivnih formi kiseonika.