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Comparison of antioxidant defence parameters in colostrum and milk between Berrichon du Cher ewes and Uhrusk ewes

Justyna Lipko-Przybylska, Edyta Albera and Marta Kankofer*

Department of Animal Biochemistry and Physiology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20–033 Lublin, ul. Akademicka 12, Poland

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The aim of the study was to evaluate the profile of antioxidant parameters in ewes' colostrum and milk in relation to breed during 5 d post partum. Total antioxidant capacity (TAC) was analysed and the activity of the enzymic antioxidants, glutathione peroxidase (GSH-Px) and glutathione transferase (GSH-Tr), as well as the concentration of the non-enzymic antioxidants, vitamin C, vitamin A and β-carotene, were measured. Samples were collected from healthy animals belonging to two ewe breeds: Berrichon du Cher (n=15) and Uhrusk (n=15) kept in the Podlasie Province (Poland). Colostrum was sampled directly after parturition, as well as after 12, 24 and 48 h later and milk was sampled 5 d after parturition. Colostrum and milk for the evaluation of all parameters except for vitamin A and β-carotene were centrifuged, and the supernatant was used for further analysis. Spectrophotometric methods were used for biochemical measurements. The results showed dynamic changes of antioxidative parameters within the time period examined. TAC values and GSH-Px activity increased significantly during the experiment. GSH-Tr activity showed a similar tendency in Uhrusk ewes but an opposite relationship in Berrichon du Cher. Concentrations of examined vitamins followed the increasing trends noticed in the activities of antioxidative enzymes. Moreover, differences between breeds in the evaluated parameters were detected; these differences were not unequivocal however. The results are also a source of not previously published physiological antioxidant profile in colostrum and milk of ewes over the post-partum period.

Keywords: Antioxidants, colostrum, ewes.

Colostrum is a yellowish, thick fluid which is secreted by glands of female mammals in the last period of pregnancy and over the first days after parturition. It is the complete nutrient for newborns, whose organisms are not yet fully developed. It also contains immunoglobulins and other biologically important components such as growth factors, cytokines, nucleosides, antibodies and antioxidants, which not only protect the newborn organism against environmental factors, but also make possible proper functioning outside mother's environment (Blum, 2006).

It is believed that physicochemical properties of colostrum and milk depend on the characteristic composition of each species and reflect the needs of newborns of each species. Ewes' colostrum is characterized by high concentrations of the main nutrients (fat, 9·0 g/100 ml; protein, 4·7 g/100 ml; and lactose, 5·8 g/100 ml) and high concentrations of vitamins and minerals (Park et al. 2007). Taurine, a derivative of amino acids which contains sulphur and is characteristic of ewes' milk, plays an important metabolic role, similarly to carnitine in human milk.

It is claimed that during the periparturient period an uncontrolled increase in production and ineffective neutralization of reactive oxygen species (ROS) may take place, which may cause disturbances of pro- and antioxidative balance and may lead to oxidative stress in both mother and newborn (Zhao et al. 2004). Therefore the antioxidative defence in colostrum may represent the first line of defence in the young organism in new environmental conditions, especially owing to enteral absorption which is open for 36–48 h after parturition (Blum et al. 1997). Moreover it may also protect mammary gland itself from infection.

Cells of a living organism possess two defence systems against excessive ROS which are able to scavenge them by different mechanisms. The first one (enzymic) uses the activity of enzymes, such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and glutathione transferase

*For correspondence; e-mail: marta.kankofer@up.lublin.pl

(GSH-Tr). The other one (non-enzymic) is based on antioxidative properties of different substances, among which are vitamins C, E and A, as well as β-carotene and glutathione (Storey, 1996). Antioxidative systems protect macromolecules from peroxidative damage which may disturb biochemical pathways and exert toxic effects. Cell membranes, under the influence of ROS excess, may change permeability leading to altered intercellular metabolism (Halliwell & Gutteridge, 1995).

Literature data claim the presence of both antioxidative defence systems in colostrum and milk of women (Kiyosawa et al. 1993; Kasapovice et al. 2005; Savice et al. 2005), cows (Hoolbrook & Hicks, 1978; Przybylska et al. 2007; Kankofer & Przybylska, 2008), swine (Snitynskyj et al. 1996; Pinelli-Saavedra & Scaife, 2005) as well as other species (Mohamed et al. 2005). However, there is little information on the antioxidative profile in colostrum and milk of ewes, and especially on the influence of breed on these parameters.

The aim of the study was to describe the profile of antioxidant parameters based on measurements of the total antioxidant capacity (TAC), activity of the enzymic antioxidants, GSH-Px and GSH-Tr, as well as concentrations of the non-enzymic antioxidants, vitamin C, vitamin A and β-carotene, in colostrum and milk of ewes during the 5 d after parturition, and to compare the parameters between the two ewe breeds, Berrichon du Cher and Uhrusk.

Material and Methods

The experiment was approved by the local Ethical Committee for Experiments on Animals. The ewes came from two farms in Podlasie Province. The animals were traditionally kept in pastures over the summer and in farm buildings on litter over the winter. Nutritional supplements, routinely used for periparturient ewes, included hay, barley meal and salt lick as well as an addition of vitamins and minerals. Dry matter intake per animal ranged between 1·7 and 2·1 kg/d depending on animal status and it increased during lactation in comparison to pregnancy, ME intake ranged between 15·1 and 16·7 MJ/d, crude protein ranged between 180 and 250 g/d. Nutrient intakes were similar in both farms and samples were collected at the same breeding season, so allowing a comparison of results.

Ewes were divided into two groups depending on breed: Group 1, Berrichon du Cher (n=15); and Group 2, Uhrusk ewe (n=15). The French ewe breed Berrichon du Cher is characterized by a high yield of meat, but poor wool growth, whereas the Polish Uhrusk ewe is bred for high yields of wool.

Colostrum was sampled from clinically healthy animals, always from the same half of the mammary gland, at the following times: immediately after parturition; 12 h after parturition; 24 h after parturition; 48 h after parturition; and 5 d after parturition (assumed to be mature milk). Directly after sampling, colostrum and milk were aliquoted, frozen and kept at −20 °C until biochemical analysis. Whole colostrum and milk were used for the determination of vitamin A concentration. For the other determinations samples were centrifuged at 1500 ***g*** at 4 °C for 10 min in order to remove fat.

Biochemical analysis

TAC was measured according to Benzie & Strain (1996). The working reagent consisted of 300 mM-acetate buffer, 2,4,6-tripyridyl-s-triazine (TPTZ, 10 mM in 40 mM-HCl) and 20 mM-$FeCl_3 \cdot 6H_2O$ mixed together directly before use in ratio 10 : 1 : 1. The incubation mixture contained the working reagent (2·25 ml) and supernatant (0·025 ml). The absorbance in relation to the working reagent alone was measured at 593 nm at time 0 and after 10 min of incubation. Calculations were based on a standard curve prepared for different dilutions of Fe(II) between 0 and 1000 μM. TAC was expressed in μmol/g of supernatant protein.

GSH-Px (E.C.1.11.1.9) activity was determined according to Paglia & Valentine (1967). The method is based on changes in absorbance connected with transformation of NADPH to NADP at 340 nm wavelength. The incubation mixture contained: 2·58 ml 0·05 M-phosphate buffer (pH 7·0), 0·1 ml 8·4 mM-NADPH, 0·1 ml glutathione reductase (100 U/mg protein), 0·1 ml 1·125 M-sodium azide, 0·1 ml 0·02 M-glutathione, 0·1 ml supernatant 2-fold diluted with buffer and substrate (0·1 ml 0·022 M-H_2O_2). Measurements were carried out between the 2nd and 4th minute of the reaction in a spectrophotometer, Ultrospec 2000 (Pharmacia, Uppsala, Sweden). Results were recalculated per protein concentration and expressed in picokatals per protein content of sample (pkat/mg protein).

GSH-Tr (E.C.3.1.2.7) activity was determined according to Rice-Evans et al. (1991). GSH-Tr catalyses reactions between glutathione and 1-chloro-2,4-dinitrobenzene (CDNB); a coloured conjugate 2,4-dinitrophenyl-5-glutathione is formed. The incubation mixture contained: 1·7 ml 0·1 M-phosphate buffer (pH 6·5), 0·1 ml 20 mM-glutathione, 0·1 ml CDNB (20 mM in ethanol) and 0·1 ml supernatant 5-fold diluted with buffer. Measurements were carried out at 340 nm in a spectrophotometer, Ultrospec 2000. Results were recalculated per protein concentration and expressed in pkat/mg protein.

Vitamin C concentration was determined according to Omaye et al. (1979). The method is based on the reaction with 2,6-dichlorophenolindophenol. The supernatant was mixed with a 10% solution of HPO_3 and centrifuged; the fluid above the sediment was used for measurements. To 0·6 ml of the sample were added 0·3 ml citrate–acetate buffer (pH 4·15) and 0·3 ml dichlorophenolindophenol (0·1 g/l); the absorbance was measured at 520 nm. Then a few crystals of ascorbate were added until decolouration

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Table 1. Antioxidative profile of colostrum and milk in Berrichon du Cher (Group 1, $n=15$) and Uhruska ewes (Group 2, $n=15$) immediately after parturition and 12, 24, 48 h and 5 d later

	Groups	Time 0	Time 12 h	Time 24 h	Time 48 h	Time 5 d
†TAC, μmol/g protein	1	4·74 (1·60) A, a	7·73 (2·76) A, b	9·28 (2·06) A, bc	10·56 (3·0) A, cd	9·73 (4·5) A, bde
	2	4·78 (1·70) A, a	6·63 (2·6) A, b	9·81 (2·42) A, c	7·72 (2·53) B, bd	6·69 (2·85) B, be
GSH-Px, pkat/mg prot	1	23·62 (5·7) A, a	37·54 (9·1) A, b	45·8 (7·8) A, c	60·0 (17·0) A, d	51·8 (16·0) A, cde
	2	29·63 (5·3) B, a	38·87 (8·8) A, b	49·0 (8·1) A, c	57·0 (19·0) A, cd	49·3 (17·0) A, ce
GSH-Tr, pkat/mg prot	1	93·0 (26·0) A, a	71·0 (23·0) A, b	71·0 (19·0) A, bc	58·0 (17·0) A, bde	50·0 (17·0) A, e
	2	37·0 (12·0) B, a	57·0 (13·0) A, b	69·0 (20·0) A, c	61·0 (16·0) A, bcd	63·0 (13·0) A, bce
Vitamin C, μmol/g prot	1	0·52 (0·19) A, a	0·39 (0·14) A, a	0·64 (0·14) A, ab	0·78 (0·26) A, b	0·82 (0·26) A, bc
	2	0·54 (0·20) A, a	0·59 (0·19) B, a	0·93 (0·26) B, b	0·78 (0·23) A, bc	0·98 (0·24) A, bd
β-carotene, μg/g prot	1	0·54 (0·19) A, a	1·17 (0·3) A, b	1·69 (0·52) A, c	1·92 (0·64) A, cd	1·50 (0·49) A, bce
	2	0·56 (0·18) A, a	1·31 (0·44) A, b	1·62 (0·54) A, c	1·39 (0·58) A, bcd	1·43 (0·56) A, bce
Vitamin A, μg/g prot	1	15·15 (3·7) A, a	25·38 (4·9) A, b	24·58 (6·2) A, bc	22·19 (6·0) A, bd	21·7 (6·7) A, be
	2	15·61 (4·2) A, a	26·94 (7·4) A, b	26·88 (6·1) A, bc	24·1 (6·2) A, bd	22·0 (7·4) A, de

†TAC, total antioxidative capacity; GSH-Px, glutathione peroxidase; GSH-Tr, glutathione transferase
a, b, values without a common lower case superscript are significantly different ($P<0·05$) between particular times of sample collection
A, B, different upper case superscripts denote significant differences ($P<0·05$) between examined breeds at the same time of sample collection

and the absorbance was remeasured. Results were expressed in μmol/g protein.

Vitamin A and β-carotene concentration were determined according to Suzuki & Katoh (1990). Colostrum was mixed with ethanol in a 1 : 1 ratio, extracted with 3 ml hexane after 30-min shaking, and then centrifuged at 800 ***g***. The absorbance in relation to hexane was measured at 453 nm (β-carotene) and 325 nm (retinol and β-carotene) wavelength. The concentration of retinol was calculated on the basis of the difference between concentrations of measurements at two wavelengths. Results were given in μg/g protein.

Protein concentration was determined according to the biuret method using a kit (Cormay, Lublin, Poland).

Statistical analysis

Means from two repeats were averaged and subjected to statistical analysis for significance of differences between separate samples, as well as between the breeds. P value $<0·05$ was considered as significant. Kołmogorow-Smirnow's test showed that experimental data did not have normal distribution. Anova Friedman and Man-Whitney tests were used for further analysis of variance by means of Statistica 5.0 software.

Results

Results are presented in Table 1. TAC values, given in μmol/g protein in colostrum and milk of ewes of both analysed groups differed significantly ($P<0·05$) within the time period examined. Moreover, the comparison between breeds confirmed significant differences ($P<0·05$) at 48 h and 5 d post partum.

GSH-Px activity, measured in pkat/mg protein in colostrum and milk of ewes from the two groups increased significantly during the experimental time period. When both breeds were compared, the activity was significantly ($P<0·05$) higher in Group 2 ewes than in Group 1 ewes directly after parturition. In the next samples similar values were maintained.

GSH-Tr activity, measured in pkat/mg protein, in colostrum and milk of ewes from Group 1 decreased significantly ($P<0·05$) during the experimental period, while Group 2 showed an opposite relationship.

The comparison between two breeds showed significantly ($P<0·05$) higher values directly after parturition and significantly lower ($P<0·05$) values in the fifth sample for Group 1 ewes than for Group 2 ewes. However, over the next hours, the activities remained at a similar level.

The concentration of vitamin C, measured in μmol/g of protein, in colostrum and milk of ewes from both groups

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increased significantly ($P<0·05$) within the experimental period. When both ewe breeds were compared, the concentration of vitamin C was statistically ($P<0·05$) higher in Group 2 than in Group 1 at 12 h and 24 h post partum; other values were similar.

The concentration of β-carotene, measured in μg/g of protein, in colostrum and milk of ewes from Group 1 increased significantly ($P<0·05$) within 48 h after parturition and decreased 5 d post partum. In Group 2 ewes a similar decrease was seen at 48 h post partum.

The concentration of β-carotene in both breeds of ewes was similar. Only at 48 h after labour the values were significantly ($P<0·05$) higher in Group 1 ewes than in Group 2 ewes.

The concentration of vitamin A, measured in μg/g of protein, in colostrum and milk of ewes from both groups was lowest directly post partum and increased significantly ($P<0·05$) within first 24 h after parturition. Further values were on similar level. No statistically significant differences in the concentration of vitamin A were detected between breeds.

Mean protein concentrations in Group 1 were: 189 (±15) mg/ml, 141 (±12), 95 (±8), 109 (±11), 109 (±10) respectively immediately after parturition as well as 12, 24, 48 h and 5 d after parturition. In Group 2 respective values were: 234 (±18) mg/ml, 146 (±13), 115 (±10), 122 (±12), 117 (±11) and did not differ between groups.

Discussion

Pregnancy, parturition and lactation are periods of special metabolic demands and changes in the concentrations of steroid hormones which may be susceptible to alterations and energetic deficiencies (Bertoni et al. 1984) leading to the imbalance between production and neutralization of ROS and in consequence to symptoms of disease (Halliwell, 2006) not only in mother but also the newborn.

The present results confirmed dynamic changes with time in the antioxidative profile in ewes of both breeds during the post-partum period, which were significantly marked during 24–48 h after parturition, and which might be connected with the response to temporal imbalance between the production and neutralization of ROS, as well as to the process of regaining this balance.

Antioxidative/oxidative balance, which is maintained by enzymic and non-enzymic systems, allows for proper functioning of cells and cell membranes and protects from oxidative damage to macromolecules which is the main reason for metabolic alterations (Halliwell, 2006).

Determination of TAC and comparison between different time points is a good marker of overall antioxidative potential which in living cells consists of both antioxidative enzymes and non-enzymic antioxidants. The activities and concentrations of single antioxidants are not simply the sum of TAC owing to different dependencies and regulations. That is why the determinations of single antioxidants yield also additional information about the efficiency of this system and eventual dysfunction.

Different levels of antioxidant capacity in blood of periparturient ewes are reported by Fürll et al. (2003). These authors, who compared single and twin pregnancies in two breeds (Merino and Black-head) relate these differences to food intake. In present study, however, food rations were similar.

Piccione et al. (2008) reports an increase of oxidative stress intensity in blood of ewes starting from parturition to 200 d of lactation which was confirmed in our results by the increase in TAC as well as the increase in other single antioxidants measured.

Activity of antioxidative enzymes is related to ROS imbalance but it can be also influenced and regulated by steroid hormones (Al-Gubory et al. 2008; Pajovic & Saicic, 2008). During the periparturient period intensive changes in the concentration of steroid hormones occur which might be another reason for fluctuations in enzyme activities noticed also in the present study.

Measurement of superoxide dismutase (SOD) activity in cows confirms breed-dependent changes in its activity (Hoolbrook & Hicks, 1978) which may suggest similar relations also for the other antioxidative enzymes. Higher levels of GSH-Px activities in ewes than in cows are described by Hojo (1986), Dębski et al. (1987) and Bhattacharya et al. (1988).

In our studies on ewes we found changes only in GSH-Px and GSH-Tr activities in samples taken directly after labour and 5 d later (for transferase). This is not a direct confirmation, but may indicate differences in antioxidative activities during the postparturient period.

All examined values were expressed per protein content. This is mainly important for enzymic antioxidants where the activity depends partly on protein content, but for better and easier comparisons between examined breeds, non-enzymic antioxidants were expressed similarly.

The comparison of individual values of antioxidative parameters between the chosen breeds Berrichon du Cher and Uhrusk showed ambiguous differences. They referred to differences in activities and concentrations of individual studied parameters at some sampling times. Only the concentration of vitamin A remained unchanged in both groups of animals during the whole experimental period.

Literature data concerning antioxidative profile in colostrum and milk of ewes, including differences between breeds, are limited, therefore the results obtained will be discussed also with results for other breed-related parameters or other species.

Several papers report breed-dependent differences in some milk parameters in ewes, e.g. fat, protein, minerals, amino acids (Fantova & Zikova, 1988; Fadel et al. 1989; Antunovic et al. 2001; Kracmar et al. 2005). Kracmar et al. (2005) conclude that all differences (i.e. both higher and/or lower data) are probably caused by differences in mineral nutrition, composition of diets, soil conditions of the

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individual regions and average data reported by different authors, without specifications of the season, month or day of lactation, serial number of lactation etc. There is however no direct proof that the changes in antioxidative profile are nutrition-dependent. Moreover, breeds differ in genomic profile; whether it includes antioxidative parameters remains to be elucidated but the present results suggest the possibility of a breed-specific profile of antioxidative pattern.

In our studies we confirmed differences in the concentration of vitamin C in the first 24 h between the two ewe breeds. The reason for it is difficult to interpret but similar breed-dependent differences were noticed in three breeds of camels in colostrum and milk, and also in plasma (Mohamed et al. 2005).

Literature data indicate small amounts or even no β-carotene, but some retinol in milk of ewes. It influences the colour of milk. Plasma concentrations show a similar relation (Yang et al. 1992). According to Yang & Tume (1993) the activity of enzymes responsible for metabolizing β-carotene to retinol is higher in ewes than in cows or goats. Differences in β-carotene concentrations in milk of different cow breeds are reported. These may result not only from genetic conditions, but also from the diet, season or individual properties (Morris et al. 2002; Noziere et al. 2006).

Our results show that the concentration of vitamin A was similar in both breeds, both in colostrum and milk, while the concentration of β-carotene was statistically different on the second day for the two breeds.

Capper et al. (2005) investigated the effect of maternal vitamin E and fatty acid supplementation prepartum and post partum on lamb antioxidant status and suggest that antioxidative/oxidative status may be manipulated by supplementation of the ewe during pregnancy and lactation. It may also mean that the efficiency of whole antioxidative system can be supported by appropriate supplementation, which might improve morbidity of periparturient ewes and their newborns.

In summary, although uniform changes in activity of the antioxidative system between Berrichon du Cher and Uhruska ewes post partum were not clearly detected, the results bring new knowledge about physiological values of antioxidants in these species. The results obtained for both breeds followed a similar trend in the examined period of time, confirming the general profile of antioxidative system in post-partum ewes. Moreover, these results highlight the need for further studies on the properties and regulation of antioxidative systems as well as peroxidative processes in periparturient animals.

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