Antioxidative and oxidative profiles in plasma and saliva of cows in different ages and hormonal status

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Keywords
Antioxidant status,
Oxidative stress,
Saliva,
Cows,
Calves.

Summary
The aim of this study was to evaluate changes in the antioxidant status and oxidative stress parameters in plasma and saliva in order to investigate the physiological conditions of dairy cows. Blood and saliva were collected from clinically healthy female calves (n = 18), sexually mature, non-pregnant cows (n = 19), and pregnant dairy cows (n = 15). Spectrophotometric and spectroflurimetric analyses were carried out in the body fluids of these animals. The level of total antioxidant capacity (TAC) in plasma reached the lowest ($p < 0.05$) value in the group of sexually mature, non-pregnant cows (2.375 ± 0.500 µmol/g). A significant negative correlation (*tau b* = - 0.248, $p < 0.05$) was found between TAC values detected in plasma and saliva of examined animals. The highest ($p < 0.05$) mean values of thiol groups were detected in both plasma (0.007 ± 0.0015 mmol/g) and saliva (0.276 ± 0,116 mmol/g) of mature, non-pregnant cows. Conversely, the highest ($p < 0.05$) levels of formylokinurenine concentration were detected in saliva (11.535 ± 3.785 µg/mg) and plasma (0.133 ± 0.0237 µg/mg) of pregnant dairy cows. A significant positive correlation (*tau b* = 0.255, $p < 0.05$) was also found between the bityrosine content detected in plasma and saliva of the examined cows. In conclusion, although with regards to antioxidant/oxidative parameters saliva reflects the content of plasma only in part, however it shows age-related differences that can be used in the description of the physiological status of cows.

Antiossidanti e ossidanti presenti nel plasma e nella saliva di bovine con diversi profili ormonali

Parole chiave
Status antiossidativo,
Stress ossidativo,
Saliva,
Vacche,
Vitelli.

Riassunto
Scopo di questo studio è stato valutare i livelli di antiossidanti e ossidanti presenti nel plasma e nella saliva di bovine da latte con diversi profili ormonali per determinarne lo stato fisiologico. Sono state condotte analisi spettrofotometriche e spettroflurimetriche su sangue e saliva di vitelle clinicamente sane (n = 18), vacche sessualmente mature non gravide (n = 19) e vacche in gravidanza (n = 15). Il livello più basso (p <0,05) di capacità antiossidante totale (TAC) è stato rilevato nel plasma del gruppo di vacche sessualmente mature, non gravide (2,375 ± 0,500 µmol/g). È stata evidenziata una correlazione negativa statisticamente significativa (*tau b* = - 0,248, $p < 0,05$) tra i valori antiossidanti totali rilevati nel plasma e nella saliva. I valori medi più alti dei gruppi tiolici sono stati trovati sia nel plasma (0,007 ± 0,0015 mmol/g) che nella saliva (0,276 ± 0,116 mmol/g, $p < 0,05$) delle bovine sessualmente mature non gravide mentre la concentrazione di formilchinurenina ha raggiunto livelli più alti ($p < 0,05$) nella saliva e nel plasma delle bovine gravide (11,535 ± 3,785 e 0,133 ± 0,0237 µg/mg). Tra il contenuto di residui di tirosina nel plasma e nella saliva delle vacche è stata invece riscontrata una correlazione positiva (*tau b* = 0,255, $p < 0,05$). In conclusione, per quanto riguarda i livelli di antiossidanti e ossidanti presenti, la saliva rispecchia parzialmente quanto contenuto nel plasma ma mostra differenze legate all'età che possono essere utilizzate nella determinazione dello stato fisiologico delle bovine.

Introduction

Reactive oxygen species (ROS) are constantly produced in the body and are involved in the regulation of certain physiological processes, including cell signalling as secondary messengers that activate transcription factors, and inducing gene expression (Bartosz 2003).

The balance between the formation and neutralisation of ROS is modulated by the body's antioxidant systems. Disturbances in this balance can be the result of the excessive production of ROS or because of the reduced efficiency of antioxidants. This, in turn, leads to oxidative stress and consequently to various pathological conditions (Bansal and Kaushal 2014), including, among others, coronary disease, atherosclerosis, acute myocardial infarction, and rheumatoid arthritis (Čolak 2008).

ROS can also regulate several of the processes that take place in cells affecting the activity of participating substances. In the female reproductive system, ROS acts as essential to signalling molecules involved in, among others, ovarian steroid synthesis, ovulation, corpus luteum formation, luteolysis, and maintenance of pregnancy (Rizzo *et al.* 2012).

Proteins are extremely sensitive to the action of free radicals. Unlike the lipid peroxidation process, the oxidative modification of proteins is faster and linear (with regards to time and concentration). It is therefore a more sensitive indicator of the action of ROS on the cellular components than lipid peroxidation (Čolak 2008). ROS may act on the primary, secondary, and tertiary structures of proteins, and likewise, with amino acids. The amino acid that most frequently undergoes oxidation is sulfamino acid cysteine, by the presence of -SH group in its structure (as a result of the oxidation of this group, disulfide bonds are created) and aromatic amino acids (tryptophan, tyrosine, phenylalanine) and the effects of their modification are formylkynurenine, kynurenine (from tryptophan), and bis-tyrosyl bridges (from phenylalanine and tyrosine).

Any alterations in the protein structure results in the modifications of protein molecules, which leads to changes in the functions and activities of enzymes, and changes in the binding capacity of receptors or other biologically active substances. These changes are reflected in disturbances in metabolic pathways and clinical symptoms are expressed, e.g. retained placenta in cows (Kankofer 2001).

The metabolism of proteins is also hormonally regulated by sex steroids. Metabolism may vary depending on the hormonal status and sexual maturity (Ayres *et al.* 1998, Massafra *et al.* 2000, Pajović and Saicić 2008). Sex steroids also control the synthesis and activity of antioxidant enzymes (Pajović and Saicić 2008).

The use of saliva as a diagnostic material has garnered growing interest from scientists. Veterinarians are also interested in this procedure because saliva collection is a non-invasive procedure (Lamy and Mau 2012). Muthukumar and colleagues (Muthukumar *et al.* 2014) suggested that estrus in buffaloes is indicated by the presence of beta enolase and TLR 4 in saliva.

The aim of our study was to compare the levels of total antioxidant capacity (TAC) and the intensity of protein peroxidation in bovine plasma and saliva collected from sexually immature, sexually mature, non-pregnant, and pregnant cows. The results could provide a biochemical salivary profile in physiological conditions, which may be of use in future diagnoses.

Materials and methods

Plasma and saliva were collected during routine veterinary procedures in accordance with the principles of antiseptics from clinically healthy female calves (aged between 1 and 5-6 months; n = 18), sexually mature, non-pregnant cows (aged 8 months-10 years; n = 19, secretory phase of cycle), and pregnant Polish Black and White dairy cows (aged 4-8 years; n = 15, 3-5 months pregnant). Blood samples were collected via puncture of the jugular vein into tubes with anticoagulant and centrifuged. Plasma samples were portioned and stored at - 20 °C until they were used for analysis.

Saliva was collected at a similar time of day with sponges that were mounted in the space between the teeth and cheeks. Materials were then centrifuged, portioned, and frozen in - 200 °C until they were tested.

Feeding regimes were adjusted according to physiological requirements, and were similar between animals within the examined groups.

Total antioxidant capacity

The total antioxidant capacity (TAC) was measured according to the method described by Benzie and Strain (Benzie and Strain 1996), based on the ferric reducing ability of plasma (FRAP), with some modifications. Changes in absorbance were directly related to the 'total' reducing capacity of the electron donating antioxidants that were present in the examined plasma and saliva samples. The working reagent contained 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tri-pyridyl-striazine (TPTZ, Sigma, Poznań, Poland) solution in 40 mmol/L HCl, and 20 mmol/L $FeCl_3$ x $6H_2O$ solution in H_2Odest, mixed to the ratio of 10:1:1. The working reagent was prepared immediately before use. The working

reagent (2250 µL) was mixed with 25 µL of sample and absorbance was measured at 593 nm. The absorbance of the working reagent alone served as control. After exactly 10 minutes of incubation at room temperature, the absorbance was measured again. The difference in absorbance at 0 and at 10 minutes was compared with a standard curve prepared with 10 different dilutions of Fe (II) between 0 and 1000 µmol/L. The results were expressed as µmol/g protein.

The content of sulfhydryl groups

The concentrations of sulfhydryl residue (SH) in plasma and saliva were measured by spectrophotometry, as detailed by Rice-Evans and colleagues (Rice-Evans *et al.* 1991). A volume of 300 µL 10% (w/v) sodium dodecyl sulphate (SDS, Sigma, Poznań, Poland) in 10 mmol/L sodium phosphate buffer (pH 8.0) was added to 300 µL of sample and mixed precisely. A 2.4 mL of 10 mmol/L sodium phosphate buffer (pH 8.0) was then added. Then 300 µL 20 mg of 5,5-dithiobis-2-nitro benzoate (Sigma, Poznań, Poland) in 50 mL of buffer (DTNB) was added and the aborbance was measured at 412 nm. The control sample contained 300 µL of the same buffer, instead of DTNB. All samples were incubated for 1 hour at 37 °C. After incubation, the absorbance was measured again at 412 nm. The difference in absorbance before and after incubation (after subtracting the respective absorbance of the control) referred to the content of SH groups. The content was calculated using a standard curve prepared with different dilutions of glutathione (GSH, Sigma, Poznań, Poland) ranging from 0 to 1 mmol/L in 10 mmol/L sodium phosphate buffer (pH 8.0) and expressed in mmol/g protein.

The content of formylokinurenine

Formylokinurenine was determined by a spectroflurimetric method described by Rice-Evans and colleagues (Rice-Evans *et al.* 1991). The diluted plasma and saliva samples were excited by light at 360 nm and emission was measured at a 454 nm wavelength. The spectrofluorimeter (Jasco, Tokyo, Japan) was standardised, as described above (Rice-Evans *et al.* 1991). The results were expressed as µg/mg protein.

The content of bityrosine bridges

Bityrosine bridges were determined by a spectroflurimetric method according to Rice-Evans and colleagues (Rice-Evans *et al.* 1991). The diluted plasma and saliva samples were excited by light at 325 nm and emission was measured at a wavelength of 410 mm. The spectrofluorimeter was standardised to 100 deflections with chinine sulphate (0.1 µg/mL in 0.1 mol/ H_2SO_4) at excitation (350 nm) and emission wavelength (445 nm). The results were expressed as µg/mg protein.

Protein content

The protein content of the plasma samples was measured according to the biuret method, using commercial assay kits (Cormay, Łomianki, Poland) based on the method described by Gornal and colleagues (Gornal *et al.* 1949).

The protein content of the saliva samples was measured according to the Bradford method using commercial reagents (Bradford Reagent, Sigma, Poznań, Poland).

Statistical analysis

The biochemical results of duplicates were statistically analysed and compared using SPSS software (SPSS Inc., Chicago, IL). A nonparametric Mann-Whitney U test was used to determine differences among the biochemical variables of all 3 groups. A Kendall's tau-b correlation test was used to determine the association between plasma and saliva oxidative stress parameters. The p-value < 0.05 was considered to be statistically significant.

Results

Analysis of the TAC in plasma (Figure 1A) showed that, among the 3 groups, the highest values were observed in the group of pregnant cows (2.906 ± 0.361 µmol/g). In the group of sexually immature cows, the value was lower (2.872 ± 0.688 µmol/g). The lowest value ($p < 0.05$) was in the group of sexually mature, non-pregnant cows (2.375 ± 0.500 µmol/g).

TAC values in saliva (Figure 1B) were significantly higher as in plasma of all the examined groups of animals when recalculated in relation to the protein content of respective biological fluids. The highest average TAC values in saliva were found in the group of sexually mature, non-pregnant cows (1,154.11 ± 387 µmol/g). In the other groups values reached 957.77 ± 313 µmol/g (in the group of sexually immature cows) and 917.14 ± 256 µmol/g (in the group of pregnant cows). TAC results in saliva did not reveal significant differences between the groups.

A significant negative correlation (*tau b* = - 0.248, $p < 0.05$) was found between the TAC values detected in plasma and saliva of the examined cows. The increase of the TAC values in plasma was accompanied by a decrease of values in saliva.

The mean values of SH groups in plasma (Figure 2A) were similar in immature (0.0065 ± 0.0016 mmol/g

protein), mature non-pregnant (0.007 ± 0.0015 mmol/g protein) and pregnant cows (0.0065 ± 0.0020 mmol/g protein). The U Mann-Whitney test did not show any significant differences.

The concentration of SH groups in saliva (Figure 2B) were significantly higher than that detected in plasma. The highest values were found in mature, non-pregnant cows (0.276 ± 0.116 mmol/g). Significantly ($p < 0.05$) lower values were detected in pregnant animals (0.163 ± 0.072 mmol/g) and in sexually immature cows (0 134 ± 0.094 mmol/g). The last 2 groups did not differ significantly.

The concentrations of formylokinurenine in the plasma (Figure 3A) of immature (0.0805 ± 0.0274 µg/mg protein) and mature, non-pregnant cows (0.0926± 0.0303 µg/mg protein) were similar.

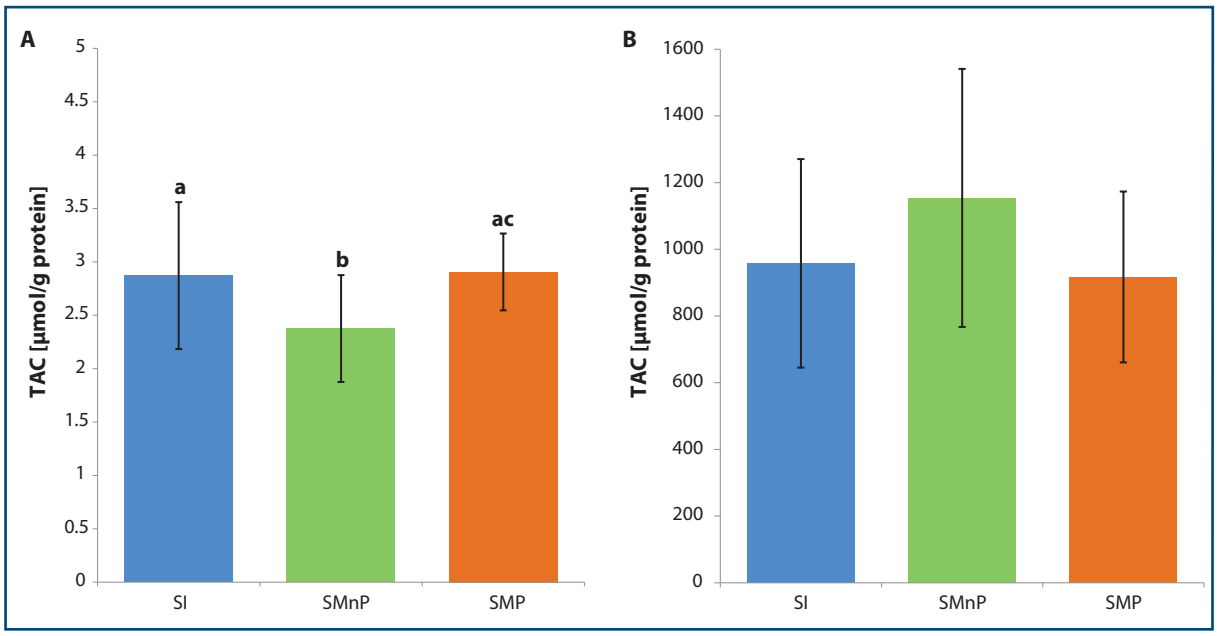

Figure 1. *Total antioxidant capacity (TAC) values in plasma (***A***) and in saliva (***B***) of sexually immature as well as sexually mature non pregnant and pregnant cows.* SI = sexually immature; SMnP = sexually mature, non-pregnant; SMP = sexually mature, pregnant; [a, b] different letters denote significant differences at $p < 0.05$.

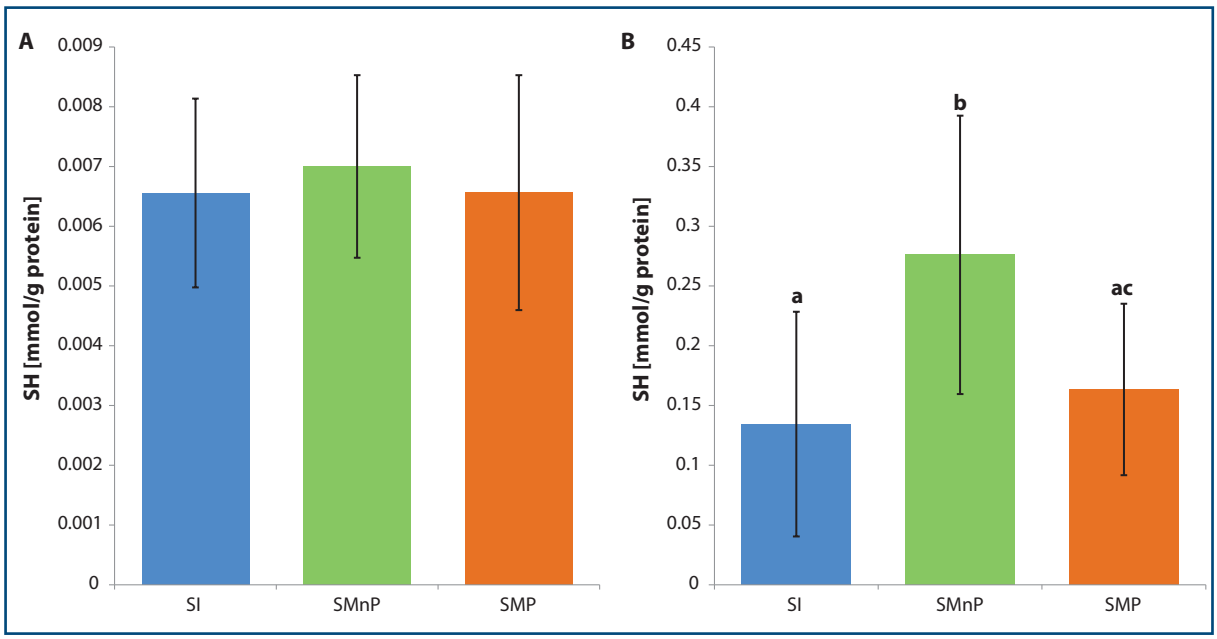

Figure 2. *Sulfhydryl (SH) group content in plasma (***A***) and in saliva (***B***) of sexually immature as well as sexually mature non pregnant and pregnant cows.* SI = sexually immature; SMnP = sexually mature, non-pregnant; SMP = sexually mature, pregnant; [a, b, c] different letters denote significant differences at $p < 0.05$.

Significantly ($p < 0.05$) higher results were detected in pregnant cows (0.133 ± 0.0237 µg/mg protein). The results were also significantly higher in saliva (Figure 3B) for this group, but showed a similar tendency in immature (8.038 ± 5.344 µg/mg protein) and mature, non-pregnant cows (9.387 ± 6.401 µg/mg protein). In pregnant cows, values reached the highest level of (11.535 ± 3.785 µg/mg protein).

The concentrations of bityrosine in plasma (Figure 4A) differed significantly in all examined groups. The lowest values were detected in immature cows (0.249 ± 0.114 µg/mg protein). Values increased to 0.314 ± 0.122 µg/mg in mature, non-pregnant animals and to 0.506 ± 0.084 µg/mg in pregnant cows. The highest values of bityrosine was found in the saliva of pregnant

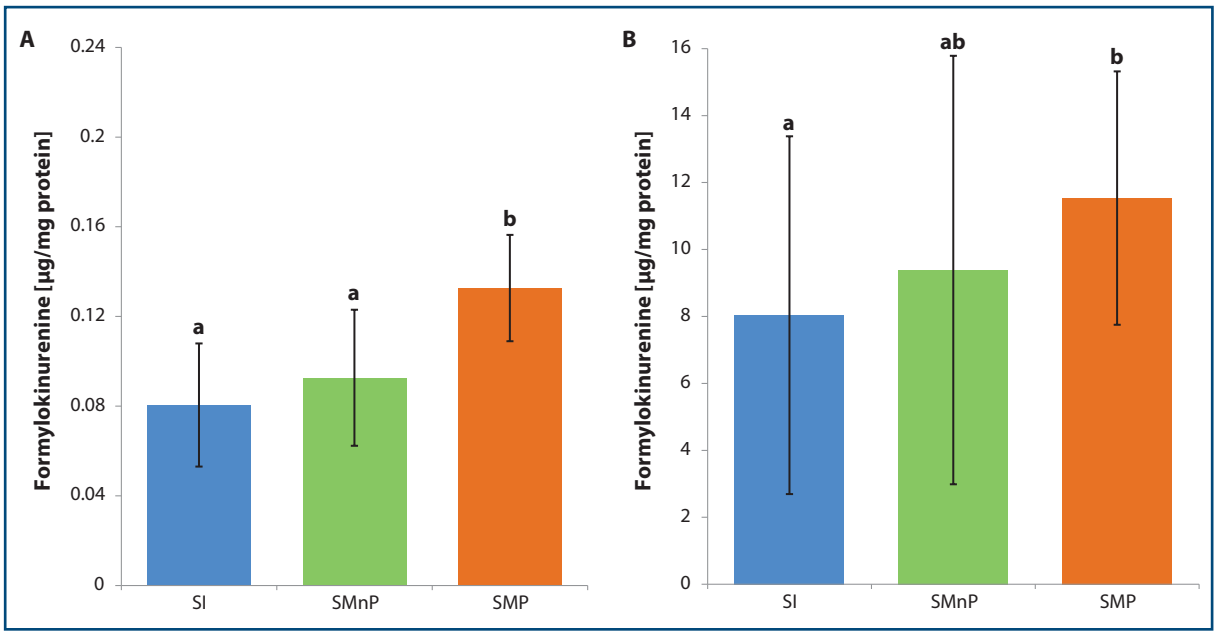

Figure 3. *Formylokinurenine content in plasma (***A***) and in saliva (***B***) of sexually immature as well as sexually mature non pregnant and pregnant cows.* SI = sexually immature; SMnP = sexually mature, non-pregnant; SMP = sexually mature, pregnant; [a, b] different letters denote significant differences at $p < 0.05$.

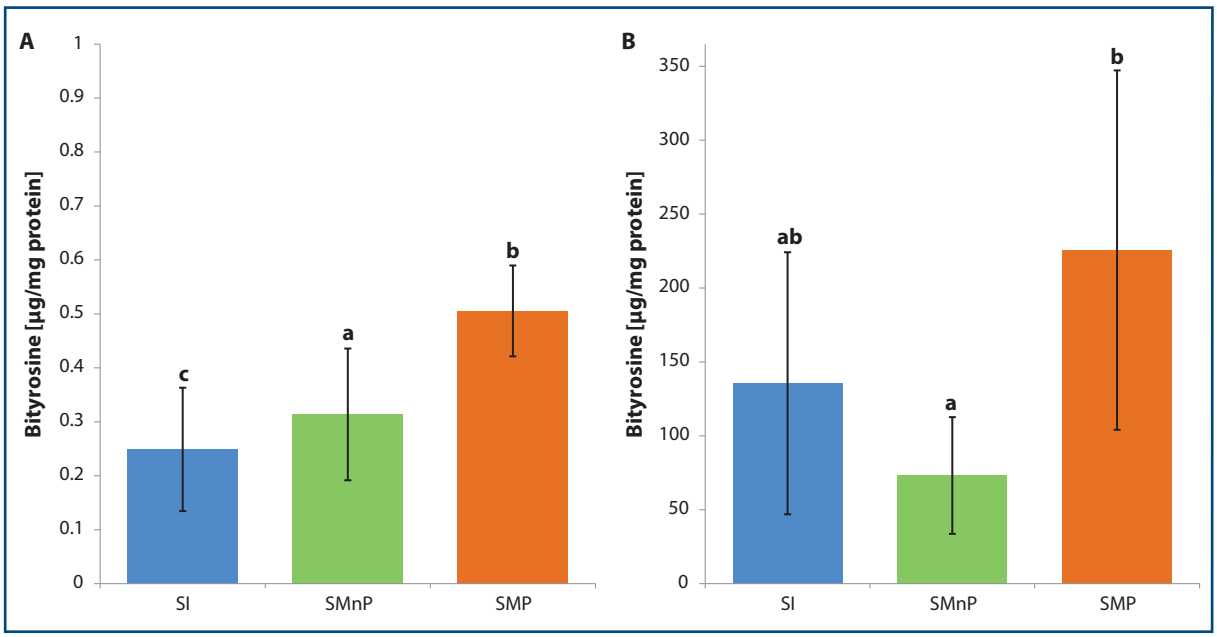

Figure 4. *Bityrosine content in plasma (***A***) and in saliva (***B***) of sexually immature as well as sexually mature non pregnant and pregnant cows.* SI = sexually immature; SMnP = sexually mature, non-pregnant; SMP = sexually mature, pregnant; [a, b, c] different letters denote significant differences at $p < 0.05$.

cows (225.643 ± 121.542 µg/mg protein) (Figure 4B). Concentrations decreased to 135.609 ± 88.644 µg/mg in immature cows, and to 73.179 ± 39.496 µg/mg in mature, non-pregnant cows. Values in pregnant and non-pregnant animals were significantly different ($p < 0.05$).

A significant positive correlation (*tau b* = 0.255, $p < 0.05$) was detected between bityrosine content in plasma and saliva of examined cows.

Discussion and conclusions

The study compared the antioxidant status, represented by the TAC, and oxidative status, represented by the content of protein peroxidative metabolites and SH groups, in plasma and saliva of cows. In this study the animals were sampled according to their different sexual maturity, physiological,and hormonal statuses. Results showed the influence of hormonal status, including sex steroids, on the antioxidant/oxidative status of the examined animals.

Saliva can be defined as a 'reflection of the body' (Lima *et al.* 2010) because its composition contains materials of both local (including salivary glands) and systemic (from blood) origin. This peculiarity makes it a potential source of information about physiological, as well as biological materials, which could be applied in the diagnosis of diseases, including animal diseases. It is worth noting that saliva is easy to obtain and does not cause any stress in animals. Because human saliva proteins have antimicrobial activity and 30% are of blood origin (Schulz *et al.* 2013), saliva composition is dependent on plasma composition.

Together with the somatic development of cows, changes in the concentrations of hormones, especially in sexual hormones like oestrogen, appear at the time of sexual maturation. Changes in hormonal profile are also detected during the course of pregnancy, where the levels of both progesterone and oestrogen fluctuate with significant increase in the third trimester of pregnancy. As these profiles are well defined, this study did not cover the determinations of sex hormones, but only referred to known alterations that appear during sexual maturation and pregnancy.

Unlike all other natural steroids, oestrogens – especially 17β-estradiol (Mooradian 1993) – have a phenolic structure in their molecules (Sugioka *et al.* 1987) that determines their antioxidant ability. One of the mechanisms lying upon their antioxidant activity is probably a stimulating effect on cellular antioxidant enzymes (Bednarek-Tupikowska 2002) as well as scavenging action against free radicals (Ayres *et al.* 1998, Pfohl *et al.* 2002). The regulation of antioxidant enzymes is thought to be influenced by other sex steroid hormones (Massafra *et al.* 2000, Pajović and Saicić 2008), and is reinforced in particular by the effect of age.

Both saliva and plasma are rich in antioxidants. Uric acid (primary salivary antioxidant), albumin, ascorbic acid, glutathione, and antioxidant enzymes are present in saliva (Miricescu *et al.* 2011).

In this study the results of the TAC determinations showed significant differences between pregnant and non-pregnant animals both in plasma and saliva. These differences may indicate a possible antioxidant role of oestrogen, which increases during pregnancy. The difference between the group of sexually immature cows and sexually mature, non-pregnant cows could be related to changes in hormonal profiles. Interestingly, the TAC of saliva was higher than the TAC of plasma, and the lowest levels of the TAC in saliva were found in the group of sexually mature, pregnant cows (in contrast to plasma levels). It should be noted that our results were re-calculated per protein content.

Reyes and colleagues (Reyes *et al.* 2006) demonstrated that, *in vitro*, 17β-oestradiol and oestriol, in addition to its hormonal action, play a role in materno-foetal auto-protection against free radicals. However, *in vivo* studies are necessary to confirm these results. In the same study, Reyes and colleagues claimed that this protection is necessary because pregnancy is a condition that leads to oxidative stress. In other studies, it was found that during the last trimester of pregnancy, human placenta contains enough oestrogen (12.5 µmol/kg wet tissue) to protect cell membranes from being damaged by lipid peroxidation in mild oxidative conditions (Diczfalusky and Mancuso 1969).

The oxidative modification of proteins has a great impact on a variety of cellular functions involving protein receptors, signal transduction mechanisms, transport systems, and enzymes. It may affect the majority of amino acids, leading most often to irreversible alterations based on the introduction of carbonyl or hydroxyl groups to amino acid chains. This may, in turn, result in the fragmentation of the peptide chain, as well as the modification of the amino acid chains, synthesis of inter- and intra-molecular bonds, and the aggregation of molecules (Skrzydlewska and Farbiszewski 1995, Petropoulos and Friguet 2006).

The SH groups, which are present in cysteine and the majority of peptides and proteins, are susceptible to ROS attack and may react with a wide range of ROS and electrophilic compounds. The alterations in this parameter can easily reflect the intensity of protein peroxidative damage as well as the status of proteins possibly damaged by ROS.

This study demonstrated the relatively stable concentrations of SH groups in plasma that are probably protected from peroxidative damage by multifunctional enzymatic and non-enzymatic antioxidant systems. Saliva samples more profoundly reflected the differences in the hormonal status of the examined animals. These differences require further elucidation.

The highest concentrations of protein peroxidation products found in both plasma and saliva of pregnant animals might confirm the possibility of oxidative stress during pregnancy. The increased values of TAC detected in the plasma of this group were probably a consequence of it.

There is evidence that pregnancy is accompanied by an increase in tissue oxygen needs (Öztürk *et al.* 2010). It determines the increase in ROS synthesis and turnover. It might also be related to the oxidation of SH groups and alterations in other examined parameters of protein peroxidation.

Sexual maturation is related to an increase in the size and activity of ovaries as well as their susceptibility to gonadotropins. With the increase of oestrogen secretion, ovaries of 2-month-old calves can react to gonadotropin therapy (Onuma, Hahn, and Foote 1970).

Differences in protein profiles expressed in the endometrium between sexually immature and mature cows have been demonstrated (Giergiel *et al.* 2016). This is why appropriate defences against ROS may assure sex-steroid-dependent physiological processes. Faulkner and colleagues (Faulkner *et al.* 2013) confirmed that particular protein profile is associated with progesterone as well as the stage of cycle.

Apart from the negative effects of ROS on reproductive performance, ROS is associated with several advantageous reactions: sperm capacitation, the regulation of luteal function, the regulation of prostaglandin, and ovarian sex steroid synthesis.

In conclusion, with regards to antioxidant/oxidative parameters, saliva reflects the content of plasma only in part, due to the local metabolism of salivary gland tissues. However, it reveals age-related differences that can be used in the description of the physiological status of cows. Further studies are necessary to define the usefulness of saliva as a biological material in routine laboratory tests.

References

Ayres S., Abplanalp W., Liu J.H. & Subbiah M.T. 1998. Mechanisms involved in the protective effect of estradiol-17beta on lipid peroxidation and DNA damage. *Am J Physiol*, 274 (Pt I): E1002-8.

Bansal M. & Kaushal N. 2014. Introduction to oxidative stress mechanism and their modulation. Springer India, 366 pp.

Bartosz G. 2003. Druga Twarz Tlenu. Wolne Rodniki W Przyrodzie [Second face of oxygen. Free radical in nature]. Warsaw, Wydawnictwo Naukowe PWN.

Bednarek-Tupikowska G. 2002. Antioxidant properties of estrogens. *Ginekologia Polska*, **73**, 61-67.

Benzie Iris F.F. & Strain J.J. 1996. The Ferric Reducing Ability of Plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem*, **239**, 70-76.

Čolak E. 2008. New markers of oxidative damage to macromolecules. *J Med Biochem*, **27** (1), 1-16.

Diczfalusky E. & Mancuso S. 1969. Foetus and placenta (Klopper A. & Diczfalusky E., eds). Blackwell, Oxford.

Faulkner S., Elia G., O'Boyle P., Dunn M. & Morris D. 2013. Composition of the bovine uterine proteome is associated with stage of cycle and concentration of systemic progesterone. *Proteomics*, **13**, 3333-3353.

Giergiel M., Wawrzykowski J. & Kankofer M. 2016. Comparison between endometrial protein profile in Holstein-Friesian heifers and female prepubertal calves. *Vet Ital*, accepted for publishing.

Gornal A.G., Bardawill C.J. & David M.M. 1949. Determination of serum proteins by means of the biuret method reaction. *J Biol Chem*, **177** (2), 751-766.

Kankofer M. 2001. Protein peroxidation processes in bovine retained and not-retained placenta. *J Vet Med A*, **48** (4), 207-212.

Lamy E. & Mau M. 2012. Saliva proteomics as an emerging, non-invasive tool to study livestock physiology, nutrition and diseases. *J Proteomics*, **75** (14), 4251-4258.

Lima D.P., Diniz D.G., Moimaz S.A., Sumida D.H. & Okamoto A.C. 2010. Saliva: reflection of the body. *Int J Infec Dis*, **14**, 184-188.

Massafra C., Gioia D., De Felice C., Picciolini E., De Leo V., Bonifazi M. & Bernabei B. 2000. Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catalase and glutathione peroxidase activities during the menstrual cycle. *J Endocrinol*, **167**, 447-452.

Miricescu D., Greabu M., Totan A., Didilescu A. & Radulescu R. 2011. The antioxidant potential of saliva: clinical significance in oral diseases. *Therapeutics, Pharmacology and Clinical Toxicology*, 15 (2), 139-143.

Mooradian A.D. 1993. Antioxidant properties of steroids. *J Steroid Biochem Mol Biol*, **45** (6), 509-511.

Muthukumar S., Rajkumar R., Rajesh D., Saibaba G., Liao C.C., Archunan G., Padmanabhan P. & Gulyas B. 2014. Exploration of salivary proteins in buffalo: an approach to find marker proteins for estrus. *FASEB Journal*, **28** (11), 4700-4709.

Onuma H., Hahn J. & Foote R.H. 1970. Factors affecting superovulation, fertilization and recovery of superovulated ova in prepuberal cattle. *J Reprod Fertil*, **21**, 119-126.

Oztürk L.K., Akyüz S., Yarat A., Koç S., Gül N. & Dogan B.N. 2010. Salivary lipid peroxidation and total sialic acid levels during healthy gestation and postpartum: a longitudinal study. *Clin Biochem*, **43** (4-5), 430-434.

Pajović S.B. & Saicić Z.S. 2008. Modulation of antioxidant enzyme activities by sexual steroid hormones. *Physiol Res*, **57**, 801-811.

Petropoulos I. & Friguet B. 2006. Maintenance of protein and aging: the role of oxidized protein repair. *Free Radical Research*, **40**, 1269-1276.

Pfohl M., Pfeilschifter J., Ko R. & Schatz H. 2002. Changes in proinflammatory cytokine activity after menopause. *Endocr Rev*, **23**, 90-119.

Reyes M.R., Sifuentes-Alvarez A. & Lazalde B. 2006. Estrogens are potentially the only steroids with an antioxidant role in pregnancy: *in vitro* evidence. *Acta Obstet Gynecol Scand*, **85**, 1090-1093.

Rice-Evans C.A., Diplock A.T. & Symons M.C.R. 1991. Techniques in free radical research. Elsevier, Amsterdam, The Netherlands.

Rizzo A., Roscino M.T., Binetti F. & Sciorsci R L. 2012. Roles of reactive oxygen species in female reproduction. *Reprod Domestic Anim*, **47** (2), 344-352.

Schulz B.L., Cooper-White J. & Punyadeera C.K. 2013. Saliva proteome research: current status and future outlook. *Critical Rev Biotechnol*, **33**, 246-259.

Skrzydlewska E. & Farbiszewski F. 1995. Interakcje Wolnych Rodników Z Białkami. *Postępy Higieny I Medycyny Doświadczalnej*, **49**, 747-766.

Sugioka K., Shimosegawa K. & Nakano M. 1987. Estrogens as natural antioxidants of membrane phospholipid peroxidation. *FEBS Letters*, **210**, 37-39.