ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA

VOL. LXIV (1) SECTIO DD 2009

Department of Pathophysiology, University of Life Sciences in Lublin
Akademicka str. 12, 20-033 Lublin
e- mail: joanna.wessely@up.lublin.pl

JOANNA WESSELY-SZPONDER, MARZENA OLSZEWSKA

The relation between neutrophil action, acute phase proteins, and ascorbic acid concentration in plasma of calves in the course of BRD

Zależność pomiędzy aktywnością neutrofili, poziomem białek ostrej fazy i stężeniem kwasu askorbinowego w osoczu krwi w przebiegu zespołu oddechowego u cieląt

Summary. Neutrophils, apart from their role in immunodefence, are also connected with lung injury, because of excessive release of enzymes such as elastase and myeloperoxidase (MPO), as well as nitric oxide (NO) generation. The purpose of this study was the relation between release of neutrophil products, acute phase proteins level (APP) and ascorbic acid (AA) concentration in the course of BRD in calves. The study was carried out on 10 healthy and 10 BRD calves. Neutrophil enzymes were assessed spectrophotometrically, NO level was determined by Griess reaction. Fibrinogen level was estimated by the heat precipitation method. Haptoglobin was assessed by the Owen's method. AA analysis was done after reaction with dinitrophenyhydrazine. We estimated that elastase (71.97 ± 3.89%) and MPO (29.7 ± 5.88%) release from neutrophils isolated from BRD calves was significantly higher than from healthy animals (54.23 ± 5.66% and 19.18 ± 8.3%, respectively, $p < 0.05$). Moreover, our experiment revealed the positive correlation between secretory action of neutrophils and APP. We also observed that increased secretory action correlated negatively with plasma concentration of AA.

Key words: neutrophil, BRD, elastase, ascorbic acid

INTRODUCTION

The respiratory disorders including bovine respiratory disease (BRD) are very important economical problem in cattle farms [Van der Fels-Klerx *et al*. 1995]. Neutrophils play a crucial role in this disease, because of the release of some enzymes such as elastase and myeloperoxidase (MPO) and nitric oxide (NO) generation. All these factors can lead to lung injury. Elastase mediates tissue destruction by degrading of elastin, collagen, and proteoglycan leading to vascular and trans-alveolar protein leakage and dys-

function of lung [Stockley 1995, Coomber *et al*. 2001]. MPO, in conjunction with HO^- and Cl^-, generated hypochlorous acid (HOCl), regarded as a strong bactericidal agent, which also leads to tissue damage [Sahoo *et al*. 1998]. Nitric oxide (NO) generated by neutrophils during inflammation, modulates both acute and chronic inflammatory reactions, and is involved in the tissue damage by lipid peroxidation, DNA oxidation, or inactivation of enzymes and proteins especially in the airway inflammation. Moreover superoxide anion, rapidly reacting with NO, yields peroxynitrite [Muijsers *et al*. 1997, Robbins and Grisham 1997, Abu-Sound and Hazen 2000, Misso *et al*. 2000, Wessely-Szponder and Bobowiec 2005]. The release of these compounds may be a strong weapon against invading microorganisms, but excessive release of elastase, MPO, and NO is an important factor in the tissue destruction during inflammatory process and may lead to lung injury during BRD in calves [Muijsers *et al*. 1997, Misso *et al*. 2000, Wessely-Szponder and Bobowiec 2005]. The acute phase proteins (APP) are a group of blood proteins that are connected with inflammation [Humblet *et al*. 2004]. Fibrinogen is used in cattle as a reliable indicator of the presence of inflammation. It is involved in hemostasis, providing a substrate for fibrin formation, and tissue repair [Murata *et al*. 2004]. On the other hand it takes part in rising changes in lung in the course of infection with *Mannheimia haemolityca* and it is important factor in occurrence of fibrous lesions in BRD [Wessely-Szponder and Bobowiec 2005]. Haptoglobin an alpha-globulin constituent binds free haemoglobin, which is toxic and proinflammatory in the plasma and reduces oxidative damage associated with haemolysis. In cattle its circulating level is negligible in normal animals, but increases markedly on immune stimulation. This parameter is clinically useful for measuring the occurrence and severity of inflammatory responses in cattle [Owen *at al*. 1960, Godson *et al*. 1996, Heegaard *et al*. 2000]. Moreover α4-Integrin expression on bovine peripheral blood neutrophils is linearly related to this protein level in inflammation of the respiratory system [Soethout *et al*. 2003]. Ascorbic acid (AA) is synthesised in the liver of calves after 3 weeks of age and this is essential for immune function [Sahinduran and Albay 2004]. The objective of this study was the relation between release of neutrophil products, APP level and AA concentration in the course of BRD in calves.

MATERIALS AND METHODS

The study was carried out on 10 healthy and 10 BRD calves between two and three months of age without dietary supplementation with vitamin C. Physical examination of each calf was performed before the collection of blood. Neutrophils were isolated from peripheral blood according to the method of Mottola [Hoeben *et al*. 1997, Wessely-Szponder and Bobowiec 2005]. The obtained cell pellet was resuspended in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM-Sigma) with addition of bovine serum. After isolation, viability of neutrophils was determined by trypan blue exclusion. After the counting and differentiation, the cells were adjusted to a final concentration of $2 \cdot 10^6$ cells/ml. Neutrophil degranulation was assessed by elastase and MPO release. 100% enzyme content was estimated by incubating cells in the presence of 0.5% CTAB (hexadecyltrimethylammonium bromide-Sigma), since CTAB results in complete cell lysis and release of all granule enzymes. Elastase activity was measured with azocasein

(Sigma) as a substrate after 10 min. incubation at room temperature. MPO release was measured by spectroscopy after 10 min incubation at room temperature with equal volume of o-phenylendiamine (OPD-Sigma). The elastase and MPO reactions were stopped by the addition of TCA and H_2SO_4, respectively. Absorbance was measured on ALAB-PLATE READER ELISA at 492 nm. All samples were assayed in duplicate [Coomber *et al*. 1997, Galligan and Coomber 2000]. Nitric oxide level was determined by Griess reaction: 50 µl of supernatant were mixed with 200 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylendiamine dihydrochloride and 2.5% H_3PO_4). Absorbance at 545 nm was measured after 10 min. incubation with Griess reagent and compared with a standard. Obtained values were expressed as a concentration of nitrite, the stable product of NO, which accumulates in medium [Nims *et al*. 1995, Roy *et al*. 1996, Ruchaud-Sparagano *et al*. 1998, Ridnour *et al*. 2000]. Fibrinogen level was estimated by the heat precipitation method [Gando *et al*. 2005]. Haptoglobin level was assessed by the method described by Owen et al. AA analysis was done spectrometrically at 520 nm after reaction with dinitrophenylhydrazine [Wei *et al*. 1996].

Examined values were compared using analysis of variance and Student's t-test and differences were considered as significant at $p < 0.05$.

RESULTS

The elastase release was greater in cultures isolated from BRD calves (71.97 ± 3.89%) than from healthy animals (54.23 ± 5.66%, $p < 0.05$). MPO was also released in higher degree from neutrophils in BRD group (29.7 ± 5.88%) in comparison with neutrophils obtained from healthy calves (19.18 ± 8.3%, $p < 0.05$) (Fig. 1). Mean fibrinogen level in BRD calves reached 2.0 ± 0.14 g/l and the high positive correlation ($r = 0.65$) between elastase

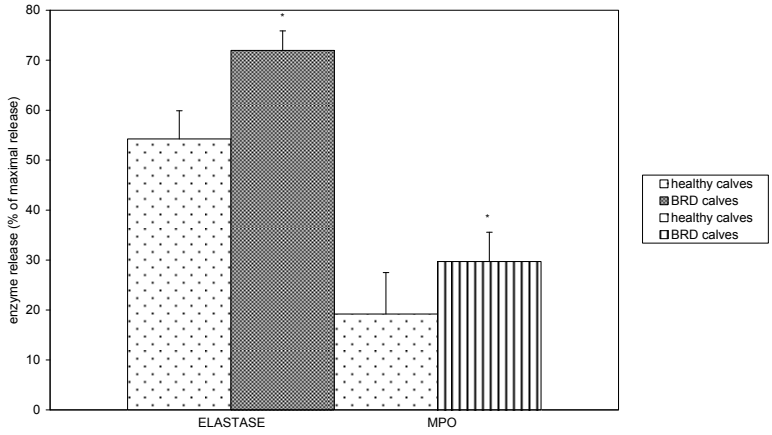

Fig. 1. Release of elastase and MPO by neutrophils from healthy and BRD calves. * $p < 0.05$ versus neutrophils from healthy calves (mean ± SD)

Rys. 1. Uwalnianie elastazy i MPO przez neutrofile uzyskane od zdrowych cieląt i w przebiegu BRD, * $p < 0,05$ w stosunku do neutrofili od zdrowych cieląt

release from neutrophils isolated from BRD animals and the level of plasma fibrinogen was shown (Fig. 2). The mean haptoglobin level in BRD calves was 58.31 ± 20.41 mg/dl, whereas in healthy animals was negligible. In BRD group the mean plasma level of AA was 0. 44 ± 0.16 mg/100 ml, whereas in healthy calves it reached 0.55 ± 0.18 mg/100 ml. Obtained results indicated that relation between elastase release and haptoglobin plasma level was r = 0.74 in BRD calves (Fig. 3). The MPO release correlated (r = 0.44) with plasma fibrinogen level and plasma haptoglobin level (r = 0.59). In BRD group the negative correlation was noted between plasma ascorbic acid concentration and elastase release was (r = -0.30), between plasma ascorbic acid and MPO release (r = -0.42) (Fig. 4), and between plasma ascorbic acid and NO generation was r = -0.44 (Fig. 5). In the group of healthy calves these relations were without statistical significance.

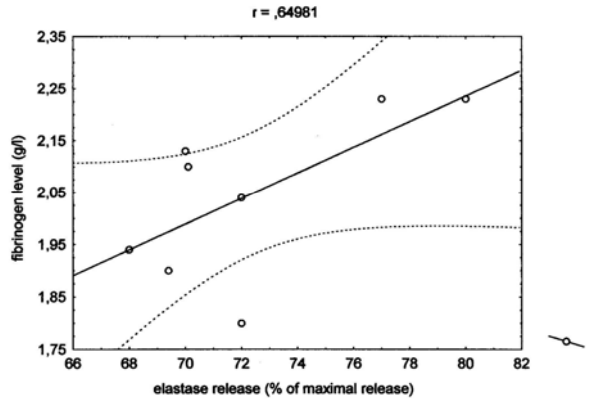

Fig. 2. Correlation between elastase release (% of maximal elastase release) by neutrophils and fibrinogen plasma level from BRD calves

Rys. 2. Zależność pomiędzy uwalnianiem elastazy przez neutrofile a poziomem fibrynogenu w osoczu cieląt w przebiegu BRD

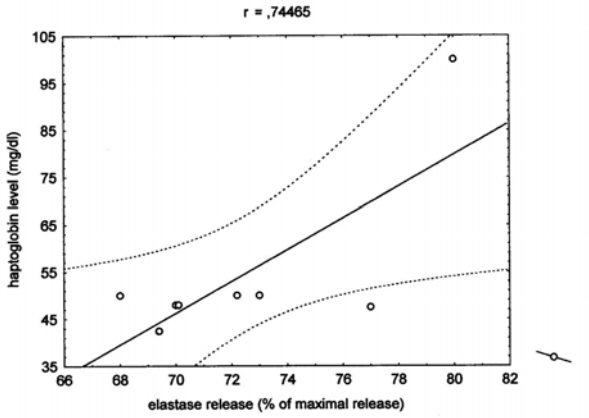

Fig. 3. Correlation between elastase release (% of maximal elastase release) by neutrophils and haptoglobin plasma level from healthy and BRD calves

Rys. 4. Zależność pomiędzy uwalnianiem elastazy przez neutrofile a poziomem haptoglobiny w osoczu cieląt w przebiegu BRD

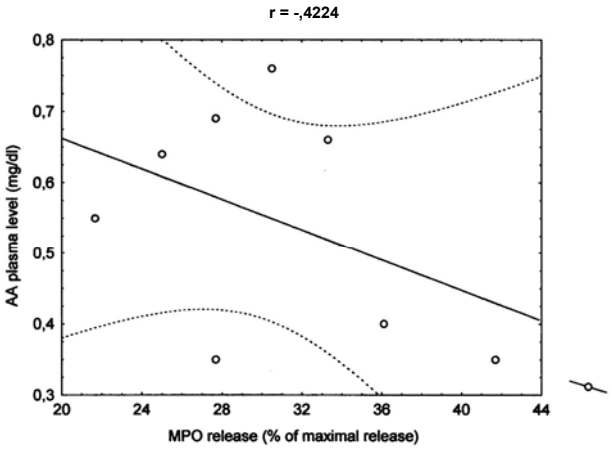

Fig. 4. Correlation between MPO release (% of maximal MPO release) by neutrophils and ascorbic acid plasma level from BRD calves.
Rys. 4. Zależność pomiędzy uwalnianiem MPO przez neutrofile i poziomem kwasu askorbinowego w osoczu cieląt w przebiegu BRD

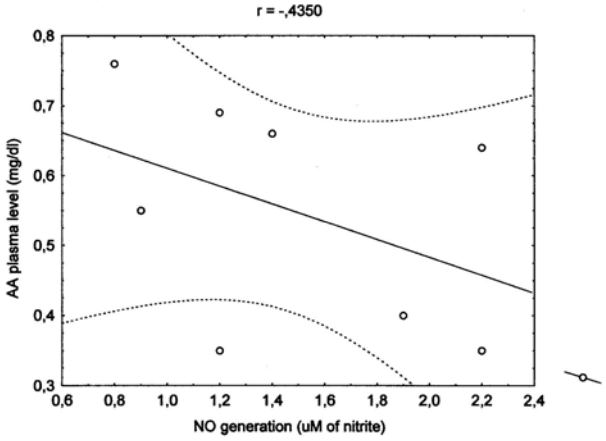

Fig. 5. Correlation between NO generation by neutrophils and ascorbic acid plasma level from BRD calves
Rys. 5. Zależność pomiędzy wytwarzaniem NO przez neutrofile i poziomem kwasu askorbinowego w osoczu cieląt w przebiegu BRD

DISCUSSION

We observed that elastase and MPO release from neutrophils isolated from BRD calves was significantly higher than from healthy animals ($p < 0.05$). NO generation was also greater in this group. Moreover, our experiment revealed the positive correlation

between secretory action of neutrophils and APP. We also observed that increased secretory action correlated negatively with plasma concentration of AA.

Previous experiments shown, that neutrophil secretory action was increased in heifers in the course of BRD. Elastase, as well as MPO and NO leading to lung tissue destruction during respiratory tract infections and were estimated as markers of lung injury [Coomber *et al*. 2001, Wessely-Szponder *et al*. 2004, Wessely-Szponder and Bobowiec 2005]. Hover, there was lack of studies about relationship between neutrophil secretory action, APP level, and AA concentration in calves during respiratory disease.

Within APP the role of fibrinogen is clearly elucidated. Fibrinogen specifically binds to CD11/CD18 integrins on the cell surface of phagocytes, triggering a cascade of intracellular signals that lead to enhancement of degranulation, phagocytosis, antibody-dependent cellular cytotoxicity and delay of apoptosis [Murata *et al*. 2004]. Therefore its elevated level in experiment was connected with inflammatory process during infection in BRD in calves and with enhanced degranulation from neutrophils.

According to Horadagoda *et al*. [1999] haptoglobin level in cattle is a useful marker for discrimination between acute and chronic inflammation. Serum haptoglobin was also previously used as an indicator of acute phase response in bovine respiratory disease [Godson *et al*. 1996]. Moreover, it was estimated that concentration of this APP correlated with severity of clinical signs in *mastitis*, experimental infection with bovine syncytial virus, and *Haemophilus sommus* [Heegaard *et al*. 2000]. Our study revealed that haptoglobin plasma level correlated with enzymes release from neutrophils of BRD calves, as well as with generation of NO.

AA is needed for recovery of all tissues and it is necessary to immune function. According to Kleczkowski *et al*. [2005] clinical signs in *mastitis* in cows were associated with considerable decrease of the AA level in serum. Infection may stimulate the inflammatory process and release of oxygen free radicals, which deplete the endogenic AA. Moreover, the decrease of serum AA concentration can reduce its synthesis, increase the uptake by cells, and increase the oxidative processes. Especially AA in neutrophils can be oxidized by ROS. Moreover, a negative correlation between AA and interleukin-6, and TNF was observed [Kleczkowski *et al*. 2005]. Dietary vitamin C deficiency reduced lymphocyte mitogenesis in guinea pigs. Dietary vitamin C supplementation, in turn, increased concentration of blood immunoglobulins in dairy calves and it is necessary in maintaining normal primary and secondary antibody responses [Hidiroglou *et al*. 1995, Chew 1996, Hirvonen and Pyorala 1998]. Our experiment results are in accordance with Jagos *et al*. [1997] who estimated low plasma levels of vitamin C in young calves suffering from respiratory infection. Moreover, decrease of AA level may be connected, apart others, with increased neutrophil function during inflammatory process in calves in the course of BRD without dietary supplementation with vitamin C.

REFERENCES

Abu-Sound H., Hazen S. 2000. Nitric oxide is a physiological substrate for mammalian peroxidases. J. Biol. Chem. 275, 37524–37532.

Chew B. 1996. Importance of antioxidant vitamins in immunity and health in animals. Anim. Feed. Sci. Tech. 59, 103–114.

Coomber B.L., Galligan C.L., Gentry P.A. 1997. Comparison of in vitro function of neutrophils from cattle deficient in plasma factor XI activity and from normal animals. Vet. Immunol. Immunopathol. 58, 121–131.

Coomber B., Nyarko K.A., Noyes T.M., Gentry P.A. 2001. Neutrophil-platelet interactions and their relevance to bovine respiratory disease. Vet. J. 161,41–62.

Galligan C., Coomber B. 2000. Effects of human IL-8 isoforms on bovine neutrophil function in vitro. Vet. Immunol. Immunopathol. 74,71–85.

Gando S., Kameue T., Matsuda N., Hayakawa M., Hoshino H., and Kato H. 2005. Serial changes in neutrophil-endothelial activation markers during the course of sepsis associated with disseminated intravascular coagulation, Thromb. Res.,116, 91–100.

Godson D., Campos M., Attah-Poku S., Redmont M., Cordeiro D., Sethi M., Harland R., Babiuk L. 1996. Serum haptoglobin as an indicator of acute phase response in bovine respiratory disease. Vet. Immunol. Immunopathol., 51, 277–292.

Heegaard P., Godson D., Toussaint M., Tjornehoj K., Larsen L., Viuff B., Ronsholt L. 2000. The acute phase response of haptoglobin and serum amyloid A in cattle undergoing experimental infection with bovine respiratory syncytial virus. Vet. Immunol. Immunopathol. 77, 151–159.

Hidiroglou M., Ivan M., Batra TR. 1995. Concentration of vitamin C in plasma and milk of dairy cattle. Ann. Zootech. 44, 399–402.

Hirvonen J., Pyorala S. 1998. Acute-phase response in dairy cows with surgically-treated abdominal disorders Vet. J., 155, 53–61.

Hoeben D., Dosogne H., Heyneman R., Burvenich C. 1997. Effect of antibiotics on the phagocytic and respiratory burst activity of bovine granulocytes. Eur. J. Pharm. 332, 289–297.

Horadagoda N.U., Knox K.M., Gibbs H.A., Reid S.W., Horadagoda A., Edwards S.E.R., Eckersall P.D. 1999. Acute phase proteins in cattle: discrimination between acute and chronic inflammation. Vet. Rec. 17, 437–441.

Humblet M., Coghe J., Lekeux P., Godeau J-M. 2004. Acute phase protein assessment for an early selection of treatments in growing calves suffering from bronchopneumonia under field conditions. Res. Vet. Sci. 77, 41–47.

Jagos P., Bouda J., Dvorak R. 1977. Hladiny kyseliny askorbove pri bronchopueumonii telat. Vet Med. (Praha) 22, 133–136.

Kleczkowski M., Kluciński W., Shaktur A., Sikora J 2005. Concentration of ascorbic acid in the blood of cows affected with mastitis. Bull. Vet. Inst. Pulawy 49, 203–207.

Misso N., Peacock C.D., Watkins D.N., Thompson P.J., 2000. Nitrite generation and antioxidant effects during neutrophil apoptosis. Free Rad. Biol. Med. 28, 934–943.

Muijsers R., Folkerts G., Henricks P., Sadeghi-Hashjin G., Nijkamp F. 1997. Peroxynitrite: A two-faced metabolite of nitric oxide. Life Sci. 60, 1833–1845.

Murata H., Shimada N., Yoshioka M. 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet. J. 168, 28–40.

Nims R., Darbyshire J.F., Saavedra J.E., Christodoulou D., Hanbauer I., Cox G.W., Grisham M., Krishna M., Wink D., 1995. Colorimetric methods for the determination of nitric oxide concentration in neutral aqueous solutions. Comp. Meth. Enzymol. 7, 48–54.

Owen J.A., Better F.C., Hoban J. 1960. A simple method for determination of serum haptoglobins J. Clin. Path. 13, 163–164.

Ridnour L., Sim J., Hayward M., Wink D., Martin S., Buetter G., Spitz R. 2000. A spectrophotometric method for the direct detection and quantitation of nitric oxide, nitrite, and nitrate in cell culture media. Anal. Biochem. 281, 223–229.

Robbins R., Grisham M. 1997. Nitric oxide. Int. J. Biochem. Cell Biol. 29, 857–860.

Roy S.C., More T., Pati U.S., Srivastava S.K. 1996. Effect of *P. multocida* vaccination on buffalo polymorphonuclear hydrogen peroxide and nitric oxide production Vet. Immunol. Immunopathol. 51, 173–178.

Ruchaud-Sparagano M-H., Ruivenkamp C.A., Riches P., Poxton I.R., Dransfield I. 1998. Differential effects of bacterial lipopolysaccharides upon neutrophil function. FEBS Letters 430, 363–369.
Sahinduran S., Albay M. 2004. Supplemental ascorbic acid and Prevention of neonatal calf diarrhoea. Acta Vet. (Brno) 73, 221–224.
Soethout E.C., Rutten V., Houwers D., Groot H., Antonis A., Niewod, Muller A. 2003. α4-Integrin expression on bovine peripheral blood neutrophils is related to inflammation of the respiratory system. Vet. Immunol. Immunopathol. 93, 21–29.
Sahoo G., More T., Singh V.K. 1998. A comparative study on certain enzymes of the granulocyte from different ruminant species. Comp. Immunol. Microbiol. Infect. Dis. 21, 319–325.
Stockley R. 1995. Role of inflammation in respiratory tract infections. Am. J. Med. 29, 8–10.
Van der Fels-Klerx H.J., Saatkamp H.W., Verhoeff J., Dijkhuizen A.A., 2002. Effects of bovine respiratory disease on the productivity of dairy heifers quantified by experts. Livestock Prod. Sci. 75, 157–166.
Wei Y., Ota R., Bowen H., Omaye S. 1996. Determination of human plasma and leukocyte ascorbic acid by microtiter plate assay. J. Nutr. Biochem. 7, 179–186.
Wessely-Szponder J., Bobowiec R. 2005. Znaczenie czynników endogennych, w tym neutrofili, w patogenezie stanów zapalnych układu oddechowego u bydła. Życie Wet. 5, 274–278.
Wessely-Szponder J., Bobowiec R., Martelli F., Wójcik M., Kosior-Korzecka U., 2004. Assessment of neutrophil components as markers of lung injury in the course of bovine respiratory tract infection. Polish J. Vet. Sci. 7, 157–161.

Streszczenie. Neutrofile, pełniące w organizmie ważną funkcję obronną, są również odpowiedzialne za powstawanie uszkodzeń płuc spowodowanych nadmiernym uwalnianiem enzymów m.in. elastazy i mieloperoksydazy (MPO), jak również tlenku azotu. Celem pracy było zbadanie zależności pomiędzy uwalnianiem produktów neutrofilowych, poziomem białek ostrej fazy i stężeniem kwasu askorbinowego w osoczu cieląt w przebiegu BRD. Badanie przeprowadzono na 10 zdrowych i 10 chorych na BRD cielętach. Uwalnianie enzymów neutrofilowych oceniono spektrofotometrycznie, wytwarzanie tlenku azotu metodą Griessa, poziom fibrynogenu metodą precypitacji termicznej, a stężenie haptoglobin metodą Owena. Poziom kwasu askorbinowego zmierzono po reakcji z dinitrofenylohydrazyną. Stwierdzono, że uwalnianie elastazy (71,97 ± 3,89%) i MPO (29,7 ± 5,88%) z neutrofili od chorych cieląt było istotnie wyższe niż od zdrowych (odpowiednio 54,23 ± 5,66% i 19,18 ± 8,3%, $p < 0,05$). Ponadto wykazano dodatnią korelację pomiędzy uwalnianiem produktów neutrofilowych a poziomem białek ostrej fazy i ujemną korelację aktywności wydzielniczej i stężenia kwasu askorbinowego w osoczu.

Słowa kluczowe: neutrofil, BRD, elastaza, kwas askorbinowy