The aim of the study was to evaluate the oxygen metabolism of neutrophils in peripheral blood of mares in relation to intensity of endometrium inflammations. The study involved 36 half-breed mares, aged 4-22 years, showing fertility disturbances. In 26 mares neutrophils were found in uterine smears, which indicated endometritis (15 – moderate inflammation and 11 – severe inflammation). In the rest mares, cytological examination excluded inflammation. Blood samples were evaluated in terms of neutrophils chemiluminescence without stimulation (CL-WS) and with stimulation by opsonised zymosan (CL-OZ). The study demonstrated (only in case of CL-WS) an increase in chemiluminescence of cells in mares with a severe inflammation of the endometrium. The increased chemiluminescence activity was accompanied by a decrease in activation index (OZ/WS) of neutrophils, suggesting some imbalance between production and elimination of reactive oxygen species (ROS). The correlation analysis demonstrated a statistically significant relation between the intensity of the uterus inflammation, which was verified by cytological examination and CL-WS of peripheral blood neutrophils, as well as their activation index. The obtained results suggest that activated neutrophils are an important source of ROS which can play a role in the pathogenesis of endometritis in mares.

Key words: mares, endometritis, neutrophils, chemiluminescence.

In mares, the most common cause of fertility disturbances are uterine diseases resulting from bacterial infections (2, 15, 33). Endometrium infections with absolutely pathogenic bacteria occur relatively seldom. More commonly facultative pathogenic bacteria, such as Streptococcus sp., Staphylococcus sp. and coliform bacteria, are isolated from the uterus (5, 21, 26, 29). Cytological examination of the endometrium smears, based on detection of neutrophils and their count is accepted as a quick test to examine the intensity of endometritis (5, 8, 11, 19, 22, 23, 30). Germicidal potential of these fastest reacting and dominating cells is mainly conditioned by formation of reactive oxygen species (ROS) (17). It is believed that disturbances in the prooxidative-antioxidative balance may play a role in pathogenesis of a number of uterine diseases such as cervicitis, uterine myoma (10), uterine lymphatic cysts (4), endometritis (36). It was also demonstrated that phagocytic and chemotactic activity of polymorphonuclear cells (PMN) in the uterus and periphery is different in mares susceptible and resistant to chronic uterine infection - CUI (3, 13, 28). On the other hand, Washburn et al. (31) showed that concentration of sex hormones (oestrogens, progesterone) in blood can modulate the oxygen metabolism of leukocytes in peripheral blood of ovariectomised mares and experimentally induced bacterial (Str. equi subsp. zooepidemicus) endometritis.

The aim of the study was to evaluate the oxygen metabolism of neutrophils in peripheral blood of mares in relation to the intensity of endometrium inflammations.

Material and Methods

Animals. The study involved 36 half-breed mares, aged 4-22 years, showing fertility disturbances. The animals belonged to different breeding centres in Poland, and were subjected to normal reproductive examinations.

Sample collection, bacteriological and cytological analyses. Samples for bacteriological and cytological analyses were collected from mares’ uterus using the set for collecting endometrium smears (IMV, France). The samples were transported to laboratory within 24 h from the collection using transport swabs AMIES (Eurotubo, Spain). For general analysis, the collected material was placed on Columbia basis media in aerobic and anaerobic environment, as well as on McConkey medium with crystal violet. The identification of bacteria was performed using API-tests and a computer programme API LABb Plus
(BioMérieux, France). Cytological smears were stained with May-Grünwald and Giemsa (MGG) stains and were evaluated microscopically under oil immersion (100x). A minimum of 20 fields were evaluated on each slide. The obtained results were graded as: no inflammation (0–2 neutrophils/field), moderate inflammation (2–5 neutrophils/field), and severe inflammation (>5 neutrophils/field) (22).

Analysis of chemiluminescence of neutrophils. The evaluation of oxygen metabolism of neutrophils was done in whole blood collected into tubes containing lithium heparin using chemiluminescence (CL) with luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). The luminol was diluted in 0.4% solution of NaOH to a concentration of 28 µmol/mL. The assessment of the chemiluminescence was performed by kinetic method using Luminometer BioOrbit 1251 (Pharmacia LKB, Finland). The analysis was done for 40 min at 38.0 ± 0.1°C, during which the CL measurements were taken at 5-min intervals. The results were presented as the value of CL integration, which is an area under the curve of emission against the function of time (mV/min). Both spontaneous luminol dependent (without stimulation – WS) and stimulated chemiluminescence was determined. The following CL stimulators were used: 100 µg of opsonised zymosan (OZ) suspended in PBS (10 ml) and equine plasma (10 ml). The examined samples contained 200 µl of PBS or 100 µl of PBS + 100 µl of stimulator, 100 µl of luminol and 150 µl of whole blood collected into a tube with heparin. The tests started immediately after the addition of blood to the prepared reagents. All measurements were repeated three times during one test and arithmetic mean value was calculated. Due to the fact that CL value is inversely proportional to the concentration of haemoglobin (Hb), whose spectrum of light absorption weakens chemiluminescence, and directly proportional to the number of neutrophils (N) in the sample, the obtained results were corrected relating CL value to 1,000 cells. In the blood collected into tubes containing K₂(EDTA), Hb concentration was determined, together with white blood cell count (WBC) and percentage of N. The assay of Hb and WBC was performed by automatic method using haematological analyser ABCVet (Horiba ABX, France), whereas the percentage of N was determined by microscopic analysis of blood smears using MGG stain. Optimisation of the results was done according to the presented formula, taking into account the volume of the blood sample (14):

\[
\text{CL calculated} = \frac{\text{CL measured} \times \text{Hb}}{\text{WBC} \times N \times 150/100}
\]

Hb – haemoglobin content in % (in relation to the mean value calculated for the particular group of horses), WBC (×10³/µL) – absolute value of white blood cell count, N – percentage of neutrophils in the WBC picture, 150 – volume of blood in microlitres.

The results of measurements of CL of neutrophils were presented in the form of activation index (OZ/WS), that is ratio between OZ-induced and spontaneous ROS generation by blood cells.

Statistical analysis. The obtained results are presented as arithmetic mean (x̄) and standard deviation (±SD). The significance of the differences between mean values was verified using Tukey test assuming the differences to be significant if their probability was below 5%. Correlation between the variables was assessed using Pearson correlation coefficients (r). All statistical analyses were performed using Statistica v. 6.0 StatSoft software.

Results

Table 1 shows the results of chemiluminescence activity of peripheral blood neutrophils compared to the results of clinical examinations of mares and bacteriological and cytological examinations of uterine smears. It turned out that spontaneous chemiluminescence of neutrophils (CL-WS) was the highest (statistically significant difference in comparison with group 0 – no inflammation of endometrium) in mares from group 2, characterised by an intensive endometritis (purulent inflammation in 82% of cases) resulting from the infection with Streptococcus equi subsp. zooepidemicus (36.3%), Streptococcus dysgalactiae subsp. equisimilis (18.2%), haemolytic Escherichia coli (18.2%), Staphylococcus sp. (9.1%), Str. equi subsp. zooepidemicus + Klebsiella sp. (9.1%) and Str. equi subsp. zooepidemicus + Str. dysgalactiae subsp. equisimilis (9.1% of cases). Relatively high spontaneous CL activity characterised also mares from group 1, which included animals with a moderate inflammation and endometrium infection caused by Staphylococcus sp. (34%), haemolytic E. coli (20%), Str. equi subsp. zooepidemicus (20%), Str. dysgalactiae subsp. equisimilis (13%), and E. coli + Str. equi subsp. zooepidemicus (13% of cases). However, the statistical analysis did not confirm significant differences in comparison with the other groups (0 and 2) of animals. Mean values of chemiluminescence of neutrophils stimulated with opsonised zymosan (CL-OZ) were similar in all groups. The results of calculated activation index (OZ/WS) confirmed the relationship between endometritis and oxygen metabolism of neutrophils in peripheral blood. It was noted that the mean values of this index were significantly (P<0.05) lower in both groups of sick mares (1 and 2) than in healthy animals (group 0).

Correlation analysis demonstrated a statistically significant relationship between the intensity of the uterine inflammation verified by cytological examination and spontaneous chemiluminescence of neutrophils in peripheral blood (Fig. 1) and the activation index (Fig. 2). No significant relationship was found between the result of cytological examination and oxygen metabolism of neutrophils stimulated in vitro with opsonised zymosan.
Characteristics of reproductive tract, uterine bacterial flora, and mean values ( $\bar{x} \pm SD$) of spontaneous and stimulated chemiluminescence of neutrophils in peripheral blood in the studied mares

<table>
<thead>
<tr>
<th>Group of mares</th>
<th>Clinical status (%)</th>
<th>Type/species of bacteria (participation of isolates in %)</th>
<th>CL (mV/min)</th>
<th>Activation index (OZ/WS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>WS</td>
<td>OZ</td>
</tr>
<tr>
<td>0 (n=10)</td>
<td>- normal uterus, no exudation (80%), - exudation in the uterus (20%)</td>
<td>E. coli (20%)</td>
<td>360 ± 238</td>
<td>3,369 ± 1,835</td>
</tr>
<tr>
<td></td>
<td>- enlarged uterus, exudation in the uterus (60%), - enlarged uterus, exudation in the uterus and mucus-purulent uterine secretion (20%), - enlarged uterus, exudation in the uterus and mucus-purulent uterine secretion + vulvar perviousness (13.3%)</td>
<td>Staphylococcus sp. (20%), Str. equi subsp. zooepidemicus (10%)</td>
<td>b 1,144 ± 934</td>
<td>3,344 ± 2,042</td>
</tr>
<tr>
<td>1 (n=15)</td>
<td>- enlarged uterus, exudation in the uterus (18.2%), - enlarged uterus, exudation in the uterus and mucus-purulent or purulent uterine secretion (45.4%), - enlarged uterus, exudation in the uterus and mucus-purulent uterine secretion + vulvar perviousness (36.4%)</td>
<td>Str. equi subsp. zooepidemicus (36.3%), Str. dysgalactiae subsp. equisimilis (18.2%), Str. dysgalactiae subsp. equisimilis (9.1%), Str. equi subsp. zooepidemicus + Klebsiella sp. (9.1%), Str. equi subsp. zooepidemicus + Str. dysgalactiae subsp. equisimilis (9.1%)</td>
<td>b 1,406 ± 777</td>
<td>3,791 ± 1,818</td>
</tr>
<tr>
<td>2 (n=11)</td>
<td>- enalarged uterus, exudation in the uterus (20%), - exudation in the uterus and mucus-purulent uterine secretion (36.4%)</td>
<td>Lack of growth (50%)</td>
<td>a 360</td>
<td>3,369</td>
</tr>
</tbody>
</table>

$\bar{x}$ - arithmetic mean; SD – standard deviation; n – number of mares; 0 – no inflammation; 1 – moderate inflammation; 2 – severe inflammation; CL - area under the curve of light emission in the function of time (40 min); WS - without stimulation; OZ - opsonised zymosan; OZ/WS – ratio between opsonised zymosan stimulated (OZ) to spontaneous (WS) chemiluminescence; $^{ab}$ - mean values differ significantly for $P<0.05$ (refer to difference between groups of mares)

**Discussion**

The most often isolated bacteria from mare’s uterus are $\beta$-haemolytic streptococci (Str. equi subsp. zooepidemicus, Str. dysgalactiae subsp. equisimilis) and bacteria from coli group (haemolytic strains E. coli). Absolutely pathogenic bacteria of Pseudomonas (P. aeruginosa), Klebsiella (K. pneumoniae), and Taylorella (T. equigenitalis), anaerobic bacteria, fungi as well as mycoplasma and ureaplasma are found more seldom (2, 5, 9, 22, 26). Similar results were obtained in this study. The species of isolated bacteria in sick and healthy mares were similar; however, it is worth noting that their prevalence was higher in animals with clinically and cytologically diagnosed endometritis.

The basic criterion for classification of animals to particular groups (0, 1, 2) were cytological examinations consisting of the evaluation of neutrophil count in uterine smears. These cells are the main leukocytes involved in the uterus defence mechanisms against bacterial infections. Migration of neutrophils from peripheral blood, initiated by chemotactic factors released at the site of inflammation, results in a significant increase in their number in the uterine, discharged within a few hours from the infection and remaining elevated for a few next days (27). It is believed that the presence of neutrophils in cytological uterine smears is the best index of endometritis (1) and their number is directly proportional to the intensity of the inflammation (5, 11, 22, 35). It was demonstrated that cytological examination allows diagnosing two times more mares with endometritis than analysis of uterine bacterial flora (22, 35), although there is a mutual correlation between the results of these studies (5, 11, 29). It is worth emphasising that $\beta$-haemolytic Streptococcus is associated with a higher number of positive cytology than coliforms (22).

Bactericidal potential of neutrophils is connected mainly with their oxygen metabolism. Since creation of toxic ROS is accompanied by light emission, the measurement of CL of neutrophils enables to estimate the level of their activation and ability to eliminate pathogens (34). An interest in the functional activity of neutrophils (including bactericidal connected with generating ROS) in the course of endometritis in mares was expressed in relatively few studies and the obtained results were not always unequivocal. For example, according to some researchers the susceptibility to CUI may be connected with weakening of the local immune potential, which is manifested by dysfunction of migration and phagocytic and bactericidal activity of uterine neutrophils (16, 32).
However, contradictory results were obtained by Troedsson et al. (28), who demonstrated in the in vitro studies a higher chemotactic and phagocytic activity of uterine neutrophils in mares susceptible to CUI in comparison with resistant mares. A partial explanation for these discrepancies may be a well-documented influence of the heat cycle on the function of neutrophils. It was demonstrated that functions of uterine cells in mares resistant to CUI are more strongly expressed during oestrus than during dioestrus (3). Thus, it seems that the observed suppression of phagocytic activity of uterine neutrophils could be the result of insufficient opsonisation in the mucous uterine discharge rather than the suppression of functional activity of these cells (28). Moreover, it was shown that stimulation of neutrophils in the course of endometritis is not limited only to the population of uterine cells. A similar reaction was demonstrated also in case of peripheral blood cells (24, 28), which can suggest their priming with cytokines released from activated immunocompetent cells (7).

Our study, evaluating the activation state of peripheral blood neutrophils in a stimulated and non-stimulated state, demonstrated a selective (only spontaneous chemiluminescence) intensification of oxygen-dependent bactericidal activity of these leukocytes in mares with severe inflammation and increased infection of the endometrium in comparison with healthy animals. These results correspond with the study by Asbury and Hansen (3), who found a significantly higher, oxygen-dependent bactericidal activity of neutrophils in mares with bacterial endometritis in comparison to the healthy ones. A similar relationship was demonstrated in case of CL activity of uterine cells. These observations are especially important since they confirm the usefulness of evaluation of the intensity of ROS generation by peripheral blood cells in case of local inflammatory states independently of their etiology.

The obtained results suggest that neutrophils of sick mares were activated proportionally to the intensity of the local inflammation. The involvement of “overproduction” of ROS in the pathogenesis of many diseases does not raise any doubts nowadays, while special attention is necessary in case of the diseases involving disturbances of immunological processes and chronic inflammations (17, 20). The results of numerous studies suggest that the main cellular source of ROS during inflammation are the activated phagocytic cells, both migrating as well as those located in the site of inflammation; at the same time there is a positive correlation between the production of ROS and the intensity of inflammation (3, 25, 28).

An increased generation of ROS by blood cells of sick mares was accompanied by a decrease in the ratio between zymosan stimulated (CL-OZ) and non-stimulated chemiluminescence (CL-WS). This index, referred to as the activation index, is characterised by the level of induction of neutrophils. According to Safronova et al. (24) lower values of this index can result from the lack of balance between production and elimination of ROS and can be used as a criterion of oxidative stress. It is a commonly known fact that the intensification of oxidative reactions in the course of inflammation is accompanied by an insufficient antioxidative protection of the organism. Unfavourable effects of this phenomenon, manifested mainly by tissue damage, also in the area of the reproductive system of a female, were an impulse for numerous studies (4, 6, 10).

Interesting results were obtained by Yaralioglu-Gurgoze et al. (36), who demonstrated a significantly lower erythrocytic glutathione peroxidase (GPx) activity in mares with endometritis in comparison to the healthy ones. This result can suggest an insufficient elimination of ROS in sick animals, the more so that this phenomenon was accompanied by a clear increase in malondialdehyde (MDA) content, which is one of the final products of lipid peroxidation in cellular membranes. Similar results suggesting oxidative stress that accompanies endometritis were obtained also in
research performed in women (24), cows (12), and camels (18).

Postulated engagement of ROS in the pathogenesis of uterus diseases finds its justification also in the results of the presented study, although it is still unclear whether oxidative stress is the cause or the consequence of these states.

References
33. Wawron W.: Puerperal endometritis in mares:

