Changes in blood lymphocyte subpopulations and expression of MHC-II molecules in wild mares before and after parturition

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Abstract

Introduction: Pregnancy is a physiological state in which the immune system undergoes certain changes. On the one hand, by depleting cell defence mechanisms, it favours development and maintenance of the pregnancy. At the same time cells of the immune system ensure resistance to many risk factors, including infectious agents. **Material and Methods:** The study was carried out on 24 Polish Konik breed mares which were divided into two equal groups. The first group (group I) included mares living in the reserve. The second group (group II) comprised mares maintained under conventional conditions in the stables. The blood samples were collected for the first time in the perinatal period, *i.e*. 2 weeks before parturition (trial 0), then within the first 24 h after delivery, and then on 7th and 21st day after foaling. Flow cytometric analysis of lymphocyte expressing TCD4+, TCD8+, CD2+, and MHC class II antigens was performed. **Results:** Before the delivery, in group I there was a significantly higher CD4:CD8 ratio compared to group II (P ≤ 0.05). Similarly, significantly increased CD4:CD8 ratio in group I was noted within 24 h after parturition (P ≤ 0.001) and it was also observed on 7th day (P ≤ 0.03) and 21st day after foaling (P ≤ 0.02). In the first 24 h after parturition, a significant decline of lymphocytes CD8+ (P ≤ 0.02) was noted. No significant differences in terms of lymphocytes CD2+ and CD3+ were observed. Expression of MHC-II molecules before and after the parturition was higher in group I compared to group II; however, the difference between the groups was not significant. **Conclusion:** The results obtained indicate that mares living in the reserve display higher activity of cell defence mechanisms.

Keywords: mares, pregnancy, immunity, lymphocytes, major histocompatibility complex.

Introduction

Polish Konik breed is a primitive breed originating from wild horses, and one of the oldest varieties in Poland. These horses are characterised by strong resistance to diseases, good fertility, good maternal characteristics, and long life. Moreover, they are exceptionally adaptive to extreme environmental and nutritional conditions. It is generally well known that during pregnancy many physiological changes occur in the body of mares (30). The immune system is subjected to significant modulation and some haematological and biochemical blood parameters are also changed (6, 30, 31). Following the opinion of many authors, these changes may lead to a decrease in immunity, which may result in an increased susceptibility to infections, and in severe cases may lead to pregnancy loss and post-partum disorders (2, 3, 16, 18). Furthermore, it is known that the sex hormones such as oestradiol (E2) and progesterone (P4), which are associated with pregnancy, may differently affect the activity of cellular defence mechanisms of the pregnant mares (12, 17, 20, 22, 24). The literature data also indicate that the activity of the immune system during pregnancy and after delivery is influenced by a variety of factors (27, 28), such as the female's age, nutritional state, previous pregnancies and deliveries, and functional status of

the reproductive and hormonal systems (8, 11, 19). Other significant factors influencing the defence mechanisms include different types of stress and environmental conditions to which pregnant females are exposed (11). Although mares living in the wild are more frequently vulnerable to stress than the mares kept in stables, they are able to adapt to difficult environmental conditions without any severe effects on their reproduction. Literature data reveal that a good indicator of organism immunity in different physiological and pathological states is an evaluation of the cellular defence mechanisms, especially T lymphocytes expressing surface markers: CD4 and CD8, as well as MHC – major histocompatibility complex (4, 5, 13, 14). The CD4+ lymphocytes recognise the MHC antigens of class II, and their decreased amount indicates low response to the antigen stimulation. On the other hand, CD8+ lymphocytes, due to their cytotoxic function, cause damage to the target cells (26, 29). Recent studies conducted on mares indicate that the activity of the cellular defence mechanisms does not differ significantly between pregnant and non-pregnant mares (1, 6, 9). The results of the study by De Mestre *et al*. (10) indicated that although the percentage of CD4+ and CD8+ lymphocytes in pregnant and non-pregnant mares was not significantly different, CD4+ lymphocytes in pregnant mares tend to decrease whereas CD8+ lymphocytes have a tendency to increase. Thus, significant changes are observed only for the CD4:CD8 ratio. Other studies carried out by Agrícola *et al*. (1) demonstrated lack of significant differences between the percentage of CD4+ and CD8+ lymphocytes in the last trimester of the pregnancy period and after delivery. However, significant differences were observed for the overall number of T lymphocytes, helper lymphocytes (Th), and cytotoxic T lymphocytes (Tc).

Due to the lack of available literature describing changes in the immunity of pregnant mares of Polish Konik breed living in natural conditions, the present study aimed to determine the level of cellular immunity in mares during perinatal period on the basis of the analysis of T lymphocyte subpopulations and the expression of MHC- II molecules.

Material and Methods

Experimental animals. The study was conducted at the Roztocze National Park, in the Centre of Conservative Breeding in Eastern Poland, on 24 Polish Konik breed mares, aged 4–14 years, which had previously given birth. Body weight of the mares ranged from 300 to 400 kg. The mares were divided into two equal groups: the first group (group I) included mares living in the reserve; the second group (group II) was maintained under conventional conditions in the stables. Mares of group I living in the reserve did not receive any feed and they only grazed on the vegetation naturally available in the National Park. In turn, the mares of group II were fed in a standard manner; they were given hay and oats, and had water access *ad libitum*. All mares included in the study were dewormed following the programme used in the reserve. Mares living in the wild were in a constant contact with their carer, which greatly facilitated their stress-free examination and blood collection. All mares were naturally mated with stallions of the same breed, and their pregnancies were confirmed by ultrasonography (USG) examination with the (Aloka SD 500 Mitaka-shi, Japan) using a rectal probe with a frequency of 3.5–7 MHz.

Experimental design. The study was conducted during the third trimester of pregnancy and after delivery. Foaling of mares occurred from February to the end of April. At the beginning of the study, the mares were clinically healthy and did not demonstrate any signs of systemic homeostasis disorders. The study involved blood collection and clinical observation, *i.e.* parturition course, leaving the placenta, and appearance of post-foaling oestrus. Blood sampling started in the perinatal period, *i.e.* two weeks before parturition (trial 0), within the first 24 h after delivery, and then at 7th and 21st day after foaling. Blood was collected from the jugular vein into sterile tubes type Vacuatte of 9 mL volume, containing heparin (Greiner Labortechnik, Austria), and delivered to the laboratory within 2 h.

Evaluation of lymphocyte subpopulation and MHC-II molecules. Cytometric analysis of lymphocyte TCD4+, TCD8+, CD2+ subpopulation and MHC class II antigens was performed by flow cytometry (Epics XL flow cytometer, Beckman-Coulter Inc, USA) using a two-step cell labelling in the whole blood. Quality control and self-standardisation of cytometric analysis were performed prior the analysis using Coulter Flow-Check, Coulter Flow-Set preparations, and compensation reagents. The monoclonal antibodies used in this study (Serotec Immunological Excellence, U.K.) were directed against equine surface molecules CD4+, CD8+, and CD2+. Polyclonal antibodies labelled with fluorochromes were used as secondary antibodies for CD4- rabbit anti-mouse IgG- FITC, CD8- rabbit anti-mouse IgG- RPE, and CD2- rabbit anti-rat IgG-FITC, respectively. The isotype control involved the antibodies of rat IgG2a-FITC, mouse IgG1-FITC, and mouse IgG1-RPE (Serotec Immunological Excellence). Molecules of MHC class II antigens were determined using monoclonal antibody mouse anti-horse MHC II, clone CVS20, isotope IgG$_1$ (MyBioSource, USA).

The flow cytometric analysis using monoclonal antibodies involved 100 µL of blood from each collected sample. Ten microlitres of primary monoclonal antibody directed against the surface

molecules CD2+, CD4+, and CD8+ on equine lymphocytes was added to 100 μL of whole blood containing 10^6 cells/mL. After thorough mixing using a vortex mixer, the samples were incubated for 45 min in the dark at room temperature (18–25ºC). Then, the cell suspension was washed three times with 500 μL of PBS. After this time, polyclonal secondary antibodies were added to each sample and after thorough mixing using a vortex mixer; the samples were incubated for 45 min at room temperature (18–25°C). Then, the cell suspension was again rinsed three times with 500 μL of PBS PBS. After that, 500 μL of FACS lysing solution (Becton-Dickinson) was added to each sample, the samples were vigorously mixed and incubated for further 20–25 min at room temperature (18–25ºC). Next, the cell suspension was rinsed two times with PBS. After each rinsing, the samples were centrifuged for 5 min at 250xg at 4°C. After the last centrifugation and decanting, 500 μL of PBS was added to the precipitate obtained, and after thorough mixing the mixture was left for 10 min at room temperature. Control samples were prepared in a similar manner. The ratio of CD4+ to CD8+ was calculated as well. Total lymphocyte T number (CD3+) was calculated as the sum of Th (CD4+) and Tc (CD8+) lymphocytes (9).

Statistical analysis. Statistical analysis was performed using STATISTICA software (version 6.0). The values are presented as arithmetic means (X) and standard deviations ±SD. The differences between the mean values of the examined groups were compared using Student t test for unconnected variables. Probability value of $P \leq 0.05$ was accepted as the limit of statistical significance.

Results

The percentages of T lymphocyte subpopulations and the percentage of cells expressing MHC class II molecules are presented in Figs 1–6. Both before birth as well as 24 h, 7 days, and 21 days after birth, the percentage of lymphocytes CD2+ and CD4+ was higher in group I, but it was not statistically significant. However, the percentage of lymphocytes CD8+ was only slightly higher in group II. A significant decrease in CD8+ cells ($P \leq 0.02$) in group I was observed only at 24 h after birth. In group I the relationship of CD4:CD8 was significantly higher both before birth ($P \leq 0.05$), and 24 h ($P \leq 0.01$), 7 days ($P \leq 0.03$), and 21 days ($P \leq 0.02$) after foaling. In the study on expression of MHC class II antigens, no significant differences were determined between the studied mare groups; however, it was observed that MHC-II expression was higher in mares from group I in all studied periods. The analysis of the results in reference to periods of collection for each group, did not exhibit any significant differences.
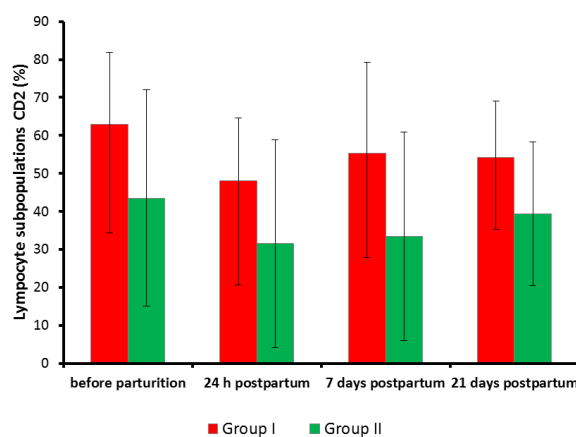

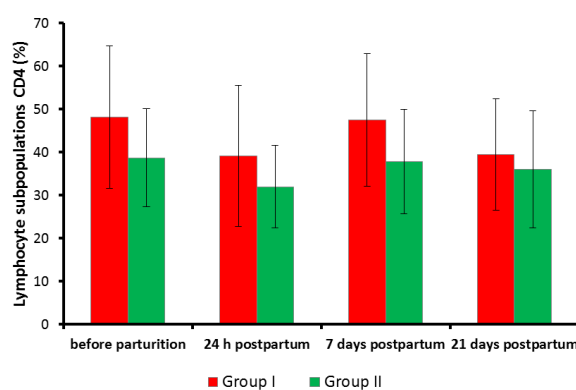**Fig. 1.** Peripheral blood percentage of lymphocyte CD2 from mares during perinatal period

Fig. 2. Peripheral blood percentage of lymphocyte CD4 from mares during perinatal period
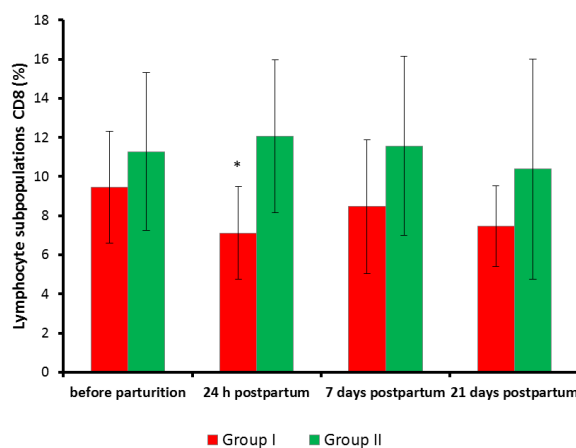

Fig. 3. Peripheral blood percentage of lymphocyte CD8 from mares during perinatal period. * Significant differences between the two groups ($P \leq 0.02$)
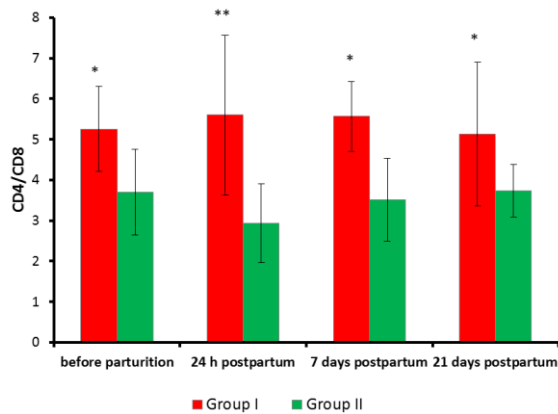
Fig. 4. Peripheral blood lymphocyte CD4:CD8 ratio from mares during perinatal period. Significant differences between the two groups: before parturition (* P ≤ 0.05), 24 h (** P ≤ 0.01), 7 days (* P ≤ 0.03), and 21 days (* P ≤ 0.02) postpartum
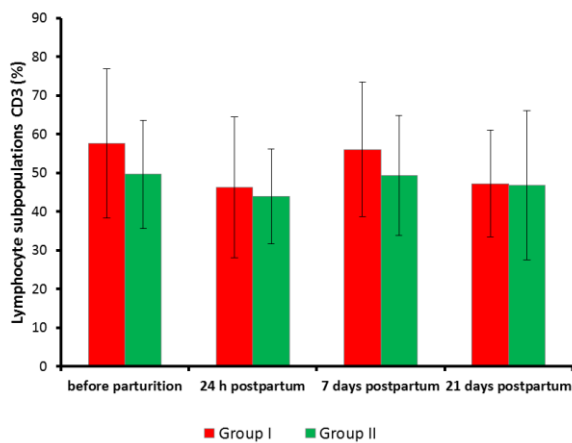

Fig. 5. Peripheral blood percentage of lymphocyte CD3 from mares during perinatal period
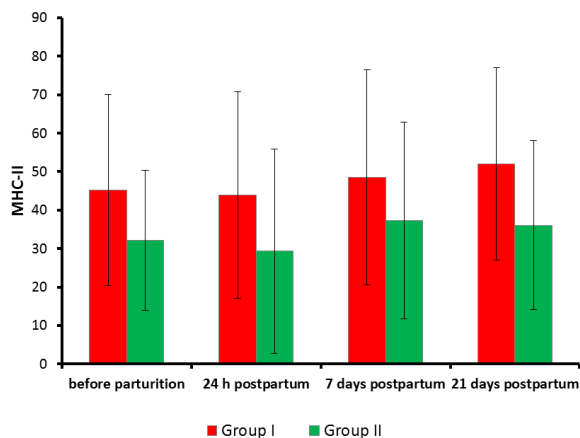

Fig. 6. MHC Class II expression from mares during perinatal period

Discussion

Severe environmental conditions and lack of proper immunity may be the cause of numerous and serious diseases and disorders. Pregnancy is a unique physiological state, during which the immune system is subjected to a certain modulation (23, 25). First and foremost, the cellular immunity mechanisms are weakened, causing a decrease in immunity (14, 24), potentially making the organism of a pregnant mare more susceptible to different infections caused by viruses, bacteria, or fungi (3, 15, 29). Our study was conducted in the third trimester of the pregnancy period and after delivery to evaluate the level of immunity of the studied mares. We were primarily interested whether differences exist in subpopulations of T lymphocytes and in the expression of MHC-II molecules between mares living in the wild and mares living outside the reserve. The results of our study demonstrated certain differences in the evaluated subpopulations of lymphocytes between the studied mare groups. In the group of wild mares, both before parturition and in the following days after delivery, a significantly higher ratio of lymphocytes CD4:CD8 was determined (P ≤ 0.05). This higher ratio resulted from too low a number of CD8+ lymphocytes. In the same mares, an increase, though not statistically significant, in the level of CD2+ and CD3+ lymphocytes was determined. The results of our study are partially consistent with the results obtained by Agrícola *et al*. (1), showing that both in mares from the reserve and from stables, the average percentage of lymphocytes CD2, CD4, CD3, and CD8 as well as the CD4:CD8 ratio was not significantly different before and after foaling. The study by De Mestre *et al.* (10) also indicates the lack of significant differences in the average percentage of CD4 and CD8 between pregnant and non-pregnant mares; however, these authors state that the CD4:CD8 ratio significantly decreases in pregnant mares, as compared to non-pregnant mares. In other studies with cyclic mares no significant differences were found in CD4:CD8 between the follicular and the luteal phases (9). According to Agrícola *et al.* (1) the lymphocyte CD4:CD8 ratio in mares in the last trimester of pregnancy remained on the level of 2.86 ±0.28, whereas after delivery it slightly decreased to 2.57 ±0.32. On the other hand, according to Da Costa *et al.* (9), in healthy horses this ratio amounted to average 2.2:1. However, we determined that in mares living in the reserve, the CD4:CD8 ratio was two times higher and amounted to 5.3 ±1.0 before parturition, and on the first day and 7 days after foaling it was 5.6 ±0.9. On the other hand, in mares kept outside the reserve the ratio was 3.7 ±1.1 and 3.5 ±1.0 in the same periods. These differences between our results and results of other authors may be linked to breed, environmental conditions, or nutritional factors.

The increased expression of MHC-II particles is also worth noting. In the group of wild mares it was higher, but not statistically significant when compared to mares residing outside the reserve. However, no significant changes in the MHC-II expression before

and after foaling were determined. The MHC-II particles play the key role in the presentation of antigens and activation of Th lymphocytes (5, 21). Changes in their expression may influence different immune processes, *e.g.* stimulating B lymphocytes which recognise antigen and lead to formation of correct, specific antibodies. Thus, the MHC-II particles participate in creation of specific immune response. Furthermore, they influence the immune memory of T lymphocytes (5).

In summary, the results obtained indicate that mares living in the reserve display higher activity of cell defence mechanisms, exhibiting slightly higher immunity as compared to the mares residing outside the reserve*, i.e.* in stables. Therefore, it can be hypothesised that the environmental conditions available to mares may influence the efficiency and level of immune response during pregnancy and after delivery.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: The study was approved by the Local Ethical Committee on Experiments on Animals at the University of Life Sciences in Lublin.

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