SELECTED CYTOKINES AND ACUTE PHASE PROTEINS IN HEIFERS DURING THE OVARIAN CYCLE COURSE AND IN DIFFERENT PREGNANCY PERIODS

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Abstract

The studies were done on 30 heifers with synchronized oestrus. IFN-γ was found in sera of 4 heifers in luteal phase and in 1 heifer in follicular phase. Moreover, an increased level of serum haptoglobin (Hp) and serum amyloid component (SAA) was found in 8 heifers in follicular phase. The presence of IFN-γ a proinflammatory cytokine may point to an active inflammation, whereas an increased level of Hp and SAA in oestrus could be connected with an approaching ovulation. It was also found that in pregnant heifers with a detected IFN-γ and TNF-α and an increased level of Hp and SAA retention of placenta and post parturient metritis was diagnosed

Key words: heifers, oestrus cycle, pregnancy, cytokines, acute phase proteins.

A key role in the induction and modulation of the immunological inflammatory response is played by cytokines. They function as positive or negative cell growth factors and inflammatory mediators of the pro-inflammatory and anti-inflammatory activity (5, 13, 19). Through the regulation of the cell growth and maturation, as well as their activation, proliferation, and differentiation, cytokines have a considerable influence on the character, intensity, and duration of the immunological response. They are also a major mechanism regulating the local immunological response, and through their influence on the hypothalamus-hypophysis-adrenal gland axis, as well as other components of the neuro-endocrinal system, they modulate the systemic acute phase response (1).

Cytokines such as IL-1α/β, TNF-α/β, IFN-γ, TGF-β1, IL-4 fulfil a series of significant functions in reproductive processes, beginning with the ovarian follicle growth, ovulation, embryo development, implantation, placenta development, and ending at the delivery (3, 12, 14-17). The local synthesis and cytokines release is regulated by ovarian steroid hormones, specific factors of sperm plasma, and bioactive lipids and proteins produced inside the uterine tissue. Due to such precise regulating mechanisms cytokines play a key role in the regulation of local immunological processes of the uterus, and participate in the wide reconstruction of its tissue, which is necessary for the successful implantation of the embryo. The response of the acute phase starts up in the early pregnancy under the influence of cytokine activity - one of the symptoms of which is the increase in the synthesis of the acute phase proteins, and plays an important role in the reconstruction of the endometrium. On account of their opsonization properties, both proteins intensify the phagocytosis process against the pathogens introduced into the uterus during the fertilization and play an active role in the reconstructing processes of the tissues damaged during the inflammation.

The acute phase protein, which plays an important role in the reproductive processes in cattle, is haptoglobin (Hp). From the studies of Lavery et al. (11) it turns out that the ovaries, uterine tubes, and endometrium already synthesize Hp in the pre-ovulation period. and that the secretion of this protein is regulated by the ovarian steroid hormones. The presence of Hp, especially in the uterine tubes and uterus, and its various functions in the processes of endometrium reconstruction create optimum conditions in the reproductive organs for fertilization and development of early pregnancy. Moreover, the mutual integration of acute phase proteins with the cytokine system ensures a kind of immunological dialogue between the mother and the developing embryo. For the proper development of the pregnancy an appropriate regulation is required and this is ensured by the constant balance between the mechanisms inducing and blocking the increase of the signals provided by the local cytokine net. All factors disturbing this balance can cause pregnancy loss or essentially disturb the course of the perinatal period.

The aim of the studies was to estimate the levels of selected cytokines and acute phase proteins in
the ovarian cycle course and in different pregnancy periods from the aspect of perinatal period disturbances.

Material and Methods

Studies on experimental animals. Studies were conducted on 30 heifers of a Black-and-White breed upgraded with 50% of Holstein-Friesian breed, at the age of 18–22 months, which originated from two farms of dairy cattle breeding profile, closely co-operating with the Veterinary Medicine Faculty in Lublin. The feeding and zoo-sanitary conditions in both farms were identical and did not raise any doubts. The feeding dose included corn silage, meadow hay, and crushed grains of three basic cereals – wheat, barley, and oat. In addition, concentrates for milk cows were used (MZ and Multi-20%), as well as mineral additives. The animals used for the studies were clinically healthy and free from leukosis, brucellosis, and tuberculosis. A properly developed reproductive system and a regular oestrus cycle were confirmed through gynaecological examination.

Oestrus synchronization and artificial insemination. The heifers selected for the studies underwent oestrus synchronization using a prostaglandin PGF₂α analogue -cloprostenol (Estrumate – Schering-Plough Animal Health) at a dose of 0.50 mg, twice in an 11 d period. At the 48th h from the second dose of the preparation all the heifers showed external oestrus symptoms in the form of red mucous membrane of the vaginal vestibule, vulva swelling, and an increased mucus discharge from the vagina. Then, at the 60th h the heifers were inseminated with the frozen sperm and 12 h following the first insemination the next one was conducted. After 6 weeks of artificial insemination, the heifers underwent an ultrasonography examination (USG) in order to confirm the pregnancy. The examination was conducted by ALOKA SSD 500 apparatus with a rectal probe of the frequency 5 MHz. Among 30 inseminated heifers, 25 were pregnant.

Division of the heifers into experimental groups. All the pregnant heifers underwent detailed clinical observation conducted during the whole pregnancy period and then over the course of 14 d after the delivery. On the basis of the received results of clinical examinations (the course of pregnancy and delivery), 25 heifers were divided into two sub-groups: A – heifers with a proper course of delivery and placenta excretion (n=18); and B – heifers with a disturbance in the last delivery phase, i.e. placenta retention (n=7). The retention of foetal membranes was treated after 12 h from the delivery.

Blood collection. The blood was collected from the jugular external vein into a silicone aseptic tube of vacuette 9 ml type (Greiner Labortechnik GmbH, Austria) with a coagulation accelerator. After centrifugation, the serum was frozen at -76 C. The first blood collection took place in the luteal phase (11th d), while the second one in the follicular phase (oestrus) of the ovarian cycle. During pregnancy the blood was collected in the first, second, and third pregnancy trimester (90th, 180th, and 256th d, respectively) and in the last week before the expected delivery, as well as on the 14th d after the delivery.

Determination of serum levels of INFγ, TNFα, and TGF-β1. The cytokines were determined by means of ELISA, using the ready-made kit bovine INF-γ EASIA (Tridelta Development Limited, Ireland), porcine TNF-α (R&D System, USA) and TGF-β1 (R&D System, USA). The determination of specific cytokines was made according to the enclosed procedure.

Determination of serum haptoglobin (Hp) level, serum amyloid component A (SAA), albumin (Alb), and α2 macroglobulin (α2-MG) levels. Hp concentration was determined according to the Jones and Mould method (8), the concentration of SAA by ELISA with a ready-made kit (bovine SAA, by Tridelta Development Limited Company), and the concentration of Alb according to Keay and Doxey method (9). The immunoelectrophoresis method according to Laurell (1972) with an appropriate human antibody (anti-α2 macroglobulin by DAKO, Company, Denmark) was used for the determination of the quantity of α2-MG. The results of the concentration of α2 MG were presented in g/L. On the basis of the received concentrations of the specific acute phase proteins, the acute phase index (API) was calculated according to the formula:

\[
\text{API} = \frac{(\text{Hp mg/mL} + 0.1) \times \text{SAA µg/mL}}{\text{Alb mg/mL} \times \text{α2-MG j/mL}}
\]

The received results underwent statistical computer analysis. The calculations were made by means of Student t-test determining the mean standard deviation and the significance of differences at the level of P<0.01 and P<0.05.

Results

Level of selected cytokines in the blood serum in heifers in the oestrus cycle. The concentration of IFN-γ, TNF-α, and TGBβ1 in the serum of the examined heifers is presented in Table 1. From the data in this table, it turns out that in the case of INF-γ the presence of this cytokine was found in 4 heifers in the luteal phase and in 1 heifer in the follicular phase. The presence of TNF-α was found in only one (the same) heifer in both phases of the cycle, but in the luteal phase, the concentration was considerably higher. Significant differences in the level of TGBβ1 were not found in either phase of the cycle.

Concentration characteristics of selected acute phase proteins and API in heifers in the oestrus cycle. The serum levels of the acute phase proteins (Hp, SAA, Alb, α2MG) and API are shown in Table 2. The received results, determining the presence of Hp in regard to the cycle phase, indicate that its presence is not observed in the luteal phase, while in the follicular phase its presence was found only in 8 heifers, (26.6%). The level of SAA in the follicular phase was significantly
higher than that in the luteal cycle phase. Similarly, the level of Alb was significantly higher in the follicular phase than that in the corpus luteum phase. However, significant differences were not found in the level of α2MG in either cycle phase. The API showing the joint participation of all studied acute phase proteins indicates its higher value in the follicular phase than in the luteal cycle phase.

**Serum levels of selected cytokines in heifers during pregnancy and after delivery.** Concentration results of IFN-γ, TNF-α, and TGFβ1 in the serum of pregnant heifers were presented in Table 3. It was discovered that in the first pregnancy trimester, the level of IFN-γ and TNF-α in both examined groups was undetectable, while TGFβ1 concentration had a similar level. In the second pregnancy trimester, a detectable level of IFN-γ and TNF-α was found in one heifer from group B. During this period, in group A, the level of TGFβ1 increased slightly in relation to group B. In the next two examination periods in group A, the concentration of IFN-γ and TNF-α was still undetectable, while in group B in the third pregnancy trimester, the presence of these cytokines was found in 3 heifers. In the last week before the delivery, the level of these cytokines was detectable in all heifers of this group. With the increase in the concentration of IFN-γ and TNF-α, the level of TGFβ1 significantly lowered. Examining the levels of IFN-γ, TNF-α, and TGFβ1 in primiparous on the 14th d after the delivery, it was found that in group A IFN-γ and TNF-α were present only in one primipara, while in group B, it was present in all the heifers. The level of TGFβ1 significantly decreased in group B in relation to group A.

**Results of the acute phase protein levels and API in pregnancy and after delivery.** The results of the serum levels of Hp, SAA, Alb, α2MG, and API in the course of pregnancy are presented in Table 4. In the first and second pregnancy trimesters, the level of haptoglobin was undetectable in all the examined heifers. The presence of Hp in the serum was found in the third pregnancy trimester in 3 heifers from group B, its mean value equalling 0.6g/L. In the last week before the delivery, the presence of haptoglobin was found in all heifers from this group, with the mean value 0.9g/L. In the particular examination terms, changes in SAA level were observed as well. During the first pregnancy trimester, the concentration of SAA was significantly lower in heifers from group B in comparison with the heifers from group A. In the other two pregnancy trimesters and in the last week before the delivery, the level of SAA in group B increased significantly (20.3 g/L).

During the entire experiment, significant differences in the Alb concentration were not observed. Similarly, the concentration of α2MG in the course of pregnancy in heifers in group A did not change. In group B in the first and second pregnancy trimesters, the level of α2MG reached a level similar to group A. However, in the last pregnancy trimester, the concentration of this protein significantly decreased. Analysing the API indicating the joint participation of all the examined acute phase proteins, it was found that in the third pregnancy trimester and in the last week before the expected delivery in the heifers with placenta retention (group B), the API was significantly higher than that in group A. Comparing the periods before the delivery and 14 d afterwards, it was found that in heifers from group A only one primipara showed the presence of haptoglobin. However, in the heifers with placenta retention (group B) the detectable Hp concentration was found in all cases. Within the same group, a significant increase in SAA level with a simultaneous decrease in that of α2MG was discovered. The API increased as well.
Table 3
Mean serum levels of selected cytokines (IFN-γ, TNFα-, TGFβ-1) in heifers during pregnancy and after delivery

<table>
<thead>
<tr>
<th>Gestation stages</th>
<th>Group</th>
<th>IFN-γ mean OD</th>
<th>TNF-α pg/mL</th>
<th>TGFβ1 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trimester (90 d)</td>
<td>A</td>
<td>≤ 0.159</td>
<td>Nw</td>
<td>7.5 ±4.72a</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>≤ 0.159</td>
<td>Nw</td>
<td>9.9 ±3.39</td>
</tr>
<tr>
<td>Second trimester (190 d)</td>
<td>A</td>
<td>≤ 0.159</td>
<td>Nw</td>
<td>11.4 ±3.73a</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>≥ 0.159 (n=1)</td>
<td>2.92 (n=1)</td>
<td>9.3 ±1.94</td>
</tr>
<tr>
<td>Third trimester (256 d)</td>
<td>A</td>
<td>≤ 0.159</td>
<td>Nw</td>
<td>11.3 ±3.94</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>≥ 0.159 (n=3)</td>
<td>2.98 ±0.24(n=3)</td>
<td>*8.1 ± 2.14</td>
</tr>
<tr>
<td>Week before delivery</td>
<td>A</td>
<td>≤ 0.159</td>
<td>Nw</td>
<td>10.6 ±2.45</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>≥ 0.159 (n=7)</td>
<td>3.83 ±0.43 (n=7)</td>
<td>*7.4 ±2.96</td>
</tr>
<tr>
<td>Two weeks after delivery</td>
<td>A</td>
<td>≥ 0.159 (n=1)</td>
<td>3.21 (n=1)</td>
<td>17.2 ±4.60</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>≥ 0.159 (n=7)</td>
<td>4.32 ±0.27 (n=7)</td>
<td>*13.5 ±4.27</td>
</tr>
</tbody>
</table>

SD; *P≤0.05, **P≤0.01 significance of differences between the groups, Nw – undetectable level; OD – negative control;
A - without placenta retention after the delivery (n = 18); B - with placenta retention (n = 7);
A – significance of differences between the particular heifers in group A; P 0.05; aa P ≤0.01;
B - significance of differences between the particular heifers in group B; P≤0.05; bb P≤0.01.

Table 4
Acute phase index (API) and mean serum levels of Hp, SAA, Alb and α2MG in heifers during pregnancy and after delivery

<table>
<thead>
<tr>
<th>Gestation stages</th>
<th>Group</th>
<th>Hp g/L</th>
<th>SAA µg/mL</th>
<th>Alb g/L</th>
<th>α2MG g/L</th>
<th>API</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trimester (90 d)</td>
<td>A</td>
<td>Nw</td>
<td>2.84±1.02</td>
<td>36.8±1.88</td>
<td>17.7±5.06</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Nw</td>
<td>*1.6±0.70</td>
<td>38.1±0.70</td>
<td>20.0±4.00</td>
<td>0.0002</td>
</tr>
<tr>
<td>Second trimester (190 d)</td>
<td>A</td>
<td>Nw</td>
<td>3.12±1.55</td>
<td>34.7±1.61</td>
<td>18.3±3.35</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Nw</td>
<td>*5.2±4.57bb</td>
<td>34.9±1.44</td>
<td>21.0±3.71</td>
<td>0.0007</td>
</tr>
<tr>
<td>Third trimester (256 d)</td>
<td>A</td>
<td>Nw</td>
<td>2.33±0.47</td>
<td>38.0±1.94</td>
<td>19.4±6.32</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>**0.6±0.01(n=3)</td>
<td>*13.5±1.20b</td>
<td>33.0±2.34</td>
<td>17.6±3.86</td>
<td>**0.004</td>
</tr>
<tr>
<td>Week before delivery</td>
<td>A</td>
<td>Nw</td>
<td>7.50±2.77aa</td>
<td>32.3±2.04</td>
<td>19.8±4.81</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>**0.9±0.01(n=7)</td>
<td>**20.3±8.86bb</td>
<td>32.0±2.00</td>
<td>*14.0±2.76bb</td>
<td>**0.009</td>
</tr>
<tr>
<td>Two weeks after delivery</td>
<td>A</td>
<td>0.24 (n=1)</td>
<td>7.10±2.50</td>
<td>36.6±1.74</td>
<td>15.6±4.68</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>**0.20±0.04(n=7)</td>
<td>**19.5±7.21</td>
<td>33.5±0.75</td>
<td>*12.7±1.45</td>
<td>**0.012</td>
</tr>
</tbody>
</table>

Explanations as in Table 3
Discussion

At present, from the point of view of the proper course of reproductive processes in females, the determination of cytokine profiles Th1 and Th2, and the concentration levels of acute phase proteins are considered to be important diagnostic elements.

Among many cytokines and growth factors, an important role in the reproductive processes is played by IFN-α and TNF-α. These cytokines belong to the group of inflammatory proteins and in the event of their uncontrollable growth in pregnant females, they can cause pregnancy loss (4, 16). Moreover, IFN-γ and TNF-α are inducers of acute phase protein synthesis. An especially interesting role in reproductive processes in cattle is played by TNF-α. Apart from its multidirectional participation in immunological reactions, it also regulates the cell differentiation and renewal and participation in immunological reactions, it also participates in the regulation of luteal phase and transportation through the uterine tube, and has a blocking influence on the release of prostaglandin PGF-2α in early pregnancy.

In the oestrus cycle, its activity can be two-directional, e.g. in the introductory follicular phase it can lead to the ovarian follicle atresia, while in the early luteal phase it becomes a luteotropic factor, and later a luteolytic factor (18). As our own studies indicate, from among 30 heifers on the 11th d of the cycle (luteal phase), the presence of IFN-γ was found in 4 heifers and TNF-α in one heifer. The increased level of TNF-α correlated with the presence of IFN-γ. A similar phenomenon was observed in the same heifer in the follicular phase (oestrus) but it, additionally, had an increased Hp level. Subsequent observations showed that this heifer was in the group of 5 heifers which did not become pregnant after artificial insemination. Increased level of TNF-α and Hp could indicate a mild inflammatory process which could have prevented the fertilization.

During oestrus, an increased mean level of Hp (Table 1) was found in 7 other examined heifers. From the studies of other authors (1, 21), it turns out that the detectable Hp level in the determined phase of the oestrus cycle is connected with the changing hormone profile (the influence of oestrogens and progesterones). It is worth pointing out that the increase in Hp concentration in cattle is also caused by the increased level of cortisol and is a symptomless course of some metabolic diseases (1, 6, 7). The heifers used in our studies did not show any symptoms of metabolic disturbances and the cortisol level in them was maintained within the limits of proper values (unpublished data). Thus we can support and confirm the opinions of quoted authors that the appearance of Hp in the examined heifers was not the result of homeostasis disturbance but apparently resulted from the high oestrogen concentration and approaching ovulation. The fact of the increased SAA level in the follicular phase can be interpreted in a similar way, although its value differed from physiological norms.

Because of its regulating role in the implantation and pregnancy development, an important factor is TGFβ1 (transforming growth factor).

This cytokine is mainly known for a variety of “immunosuppressive” activities on different leukocyte lines, and its deficiency is demonstrated in the heavy inflammatory pathology. Our studies showed that the level of TGFβ1 in both cycle phases was constant. A comparable level of TGFβ1 in both luteal and follicular phases can indicate the lack of the influence of hormones responsible for the regulation of the oestrus cycle on its secretion. The results in different pregnancy trimesters are slightly different. In the group of heifers with placenta retention after the delivery and uterine inflammation (group B), starting from the second pregnancy trimester and after the delivery, the presence of IFN-γ and TNF-α was found and TGF-β1 significantly decreased. In the third pregnancy trimester and in the last week before the expected delivery and after the delivery, the presence of IFN-γ and TNF-α correlated with the increased level of acute phase proteins (Hp and SAA) and the increase in API. The appearance of inflammatory inductors and acute phase proteins in group B proves a disturbed homeostasis in them as a result of the inflammatory process.

The proper course of pregnancy is connected with the determined profile of mother’s cytokine secretion of the Th2 type, while its end can be the result of the movement towards Th1 and the production of pro-inflammatory cytokines. In the case of infection, this response is additionally increased, which is proved by the significant increase in positive acute phase proteins level, pro-inflammatory cytokines, and a clear decrease in the concentration of negative acute phase protein and TGF-β1 factor. The organism homeostasis disturbed in this way during the pregnancy results in complications of the postpartum period in the form of the foetal membrane retention and postpartum uterine inflammations.

References