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Dynamics of progesterone, estradiol, cortisol, triiodothyronine and indicators of adaptive immunity concentrations in female dogs during estrus

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Abstract

In different periods of postnatal ontogenesis in dogs, as well as in other animals, there are critical physiological periods, the adequacy of which depends on immunoendocrine homeostasis. The estrus in bitches is a critical period because it lasts longer than in other mammals and has specific endocrine and immune changes in the body. In this study authors demonstrated the dynamics of changes in hormone content and indicators of adaptive cellular immunity in bitches during the estrus. Determination of these indicators does not require expensive laboratory equipment and is available in veterinary practice. Taking into account the fact that estrus in dogs lasts long enough, it is not clear enough what indicators and in what terms should be determined for predicting possible dysfunctions of the reproductive system and, as a result, premature impossibility of reproduction in bitches. The peak concentration of progesterone occurs on the 20th day of estrus, the reaction of the immune system, which is manifested by the maximum decrease in the phagocytic activity of neutrophils, should be noted. An increase in the concentration of estradiol on the 5th day of estrus, in contrast to progesterone, had the opposite effect on phagocytic activity of neutrophils, namely, the ability of neutrophils to phagocytosis increased. Further, we conducted clinical animals' observations for three months after estrus for the possibility of inflammatory processes in the reproductive system development. According to the obtained indicators, no complications were found.

Keywords: Estrus, Phagocytic activity of neutrophils, Progesterone, Estradiol, Cortisol

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1. Introduction

Canine reproductive physiology is unique compared to other mammalian species. In particular, the reproductive cycle of female canids is characterized by long periods of proestrus and estrus, followed by obligatory diestrus and prolonged inactivity of the ovaries (anestrus) (Nagashima & Songsasen, 2021).

Undoubtedly, estrous cycle is a critical period in the ontogenesis of bitches and involves physiological changes in the organism aimed at folliculogenesis, ovulation, sexual intercourse and fertilization with subsequent pregnancy. The restructuring takes place in the endocrine system because hormones through the hypothalamic pituitary system trigger metabolic and behavioral changes in the body. Before the onset of proestrus, there is an increase in gonadotropin-releasing hormone impulses from the hypothalamus, which, in turn, stimulates the release of follicle-stimulating hormone and luteinizing hormone (LH) from the anterior lobe of the pituitary (Concannon, 2009). An increase in the pulses of pituitary hormones initiates the growth of follicles, which stimulates gonadal steroidogenesis (Concannon et al., 2009). Prolonged elevation of estradiol during proestrus causes a surge of LH, which is followed by ovulation approximately 60 hours later in dogs (Farstad et al., 1989). Bitches are unique in aspect that concentration of progesterone begins to rise immediately before or at the beginning of the LH surge, which is associated with the luteinization of the follicles before ovulation and includes

the metaplasia of granulosa cells into large and small luteal cells (Groppetti et al., 2015). The dynamics of the sex steroids content during estrus is sufficiently studied and the determination of their concentration is used to determine the time of ovulation and, accordingly, fertilization. Thus, an increase in the concentration of progesterone in the blood serum (>1.5–2 ng/ml) is widely used to estimate when the LH surge occurs (Wildt et al., 1978). Ovulation is estimated to occur approximately two days after the LH surge (Groppetti et al., 2015). In the scientific literature, there are not enough discussions regarding the researches of estrus as a physiological stressor for the bitches, because unlike other animals (cows, sheep, pigs), it runs for a longer period. Data from laboratory, clinical, and epidemiological studies of this process suggests that strong and chronic psychogenic stress affects animal and human health, including susceptibility to infections (Glaser & Kiecolt-Glaser, 2005; Kemeny & Schedlowski, 2007) and delayed wound healing (Detillion et al., 2004; Vitalo et al., 2009). The importance of understanding the stressor of estrus is also related to the frequent development of inflammatory processes in the uterus after the estrus and the premature impossibility of reproduction in bitches. In some countries, this disease develops in almost 25% of bitches under the age of 10 (Lövebrant, 2013).

The normal course of the sexual cycle is also affected by the functional state of the thyroid gland (Chastain, 1990). Thyroid hormones also have a documented effect on the secretion of hormones involved in the reproduction and maintenance of pregnancy. The action of thyroid hormones is explained by the presence of thyroid-stimulating hormone and thyroxine receptors in human ovarian tissue (Thuróczy et al., 2016). Thyroid hormone receptors were identified in oocytes, and their deficiency may affect fertilization performance (Zhang et al., 1997).

There are many factors that lead to violations of the sexual cycle in bitches (Risvanli et al., 2016). Such factors also include adequate interaction between the links of the local (mucous membrane of the reproductive organs) and general immunity in the body of the bitches and between the immune and endocrine systems (Schlafer & Foster, 2016).

Although it is well known that ovarian hormones, in addition to direct effects on the reproductive system, affect the entire body of the bitches in many ways, there is still a lack of data on how different parts of the immune, reproductive and, in particular, the endocrine systems interact (Bartoskova et al., 2014).

The immune system of the uterine mucosa plays an important role during the estrous cycle and pregnancy. This is achieved by maintaining a balance between defense against pathogens and tolerance to allogeneic spermatozoa and hemi-myelogenous embryos. Thus, bitch is adapting to the various events associated with pregnancy (eg, implantation, placentation, parturition) and promoting tissue remodeling. This balance is based on a complex population of immune cells consisting of macrophages, natural killer (NK) cells, B- and T-lymphocytes regulated by local and systemic signaling, including endocrine disruption and changes as the pregnancy progresses (Garcia et al., 2015; Turner et al., 2012; Mold & McCune, 2012; Robertson, 2000; Bulmer et al., 1991). Overall, a comprehensive review of the literature indicates a critical role of sex hormones in diverse populations of immune cells, creating a complex network of interactions between the endocrine and immune systems. A disturbance in this fine-tuned balance can lead to implantation failure and an unwanted pregnancy outcome.

The aim of research was to establish the dependence between the concentration of progesterone, estradiol, cortisol, triiodothyronine in blood serum and indicators of the cellular link of adaptive immunity during estrus in dogs.

2. Material and Methods

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed during conduction experiments on animals, including current legislation of Ukraine (Law of Ukraine, 2012), international bioethical norms - materials of the IV European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Purposes (WHO Chronicle, 1985) and Directive of the European Parliament and of the Council on the protection of animals used for scientific (Directive, 2010).

Eight Labrador bitches aged 3 to 5 years were involved in the experiment. The animals were kept in one cattery and fed with dry feed. Infectious diseases were excluded from the animals' studied. Serum and blood plasma collected from the lateral subcutaneous vein of the forearm on days 1, 5, 10, 15, 20, 25 of the estrus were used for the study. The first day of the cycle was considered the day when bloody discharge appeared from the bitch's vagina. To obtain serum, blood was transferred into vacuum tubes of Vacutest® (Italy) with a blood coagulation activator (SiO_2) followed by centrifugation at 1500 rpm. within 10 min. To obtain plasma, part of the collected blood was transferred to a test tube with EDTA. The content of estradiol, progesterone, cortisol and triiodothyronine were determined in the blood serum. The absolute content of leukocytes, lymphocytes, monocytes, neutrophils and the phagocytic activity of neutrophils (PAN) were determined in the stabilized blood.

Determination of the concentration of hormones in blood serum by the ELISA method

Quantitative assessment of the concentration of hormones in blood serum was determined by the method enzyme-linked immuno sorbent assay (ELISA) using the Multiskan FC analyzer (Thermo Scientific, Finland) and using standard test systems of the LLC "Hema" (Ukraine). The tested sample and the conjugate (labeled with horseradish peroxidase) were introduced into the well of the tablet with

the immobilized antigen. The hormone from the sample competed with the conjugate for binding to the antigen on the surface of the well. After washing, the activity of the enzyme bound on the surface of the well of the tablet is manifested by the addition of the substrate, and is measured at a wavelength of 450 nm (nm). The intensity of the color reaction is inversely proportional to the amount of hormone in the sample. The tested sample and the conjugate (labeled with peroxidase) were introduced into the well of the tablet with the immobilized antigen. The hormone from the sample competed with the conjugate for binding to the antigen on the surface of the well. After washing, the activity of the enzyme (bounded on the surface of the well of the tablet) is manifested by the addition of the substrate, and is measured at a wavelength of 450 nm. The intensity of the color reaction is inversely proportional to the amount of hormone in the sample. Units of measurement of hormone content in blood serum are nmol/l. Physiological limits of progesterone content in bitches during anestrus <0.5-6.0; in the follicular phase – 10.0-80.0; luteal phase – 25.0-60.0. Physiological limits of estradiol content in bitches during anestrus <0.073; luteal phase – 0.073-0.22; follicular phase >0.22. Physiological limits of cortisol content in bitches are 25-250, triiodothyronine content is 0.5-2.8 (physiological limits are presented according to the instructions of the test systems, provided by the manufacturer).

Determination of the absolute number of leukocytes, neutrophils, monocytes and lymphocytes

Determination was carried out with the help of a hematological analyzer BC-2800Vet of the MINDRAY company. Cells were counted and measured by the impedance method. This method is based on determining the electrical resistance that occurs when particles pass through an aperture. The unit of measurement of the presented data is G/l.

Determination of phagocytic activity of neutrophils (PAN)

The reaction for determining the phagocytic activity of neutrophils is carried out in 96-cell plates for immunological reactions with cells with a capacity of 0.2 ml and a round bottom. The phagocytosis test is carried out with the addition of 0.06 ml of a 0.1% suspension of baker's yeast cells, previously killed by heating. In the samples the number of phagocytic neutrophils per 50 neutrophils is counted. A cell - neutrophil that swallowed 1 or more yeast cells - was considered phagocytizing (Vlizlo et al., 2012).

Statistical processing of the obtained data in series of experiments was carried out using Stata 13.1 software, differences were considered reliable at $p<0.05$. Data is presented as arithmetic mean and error of the mean value (M ± m).

3. Results

Results of the study of the progesterone concentration dynamics in blood serum during estrus

The analysis of the concentration of progesterone in the blood serum of bitches had regular changes during estrus (Figure 1). Thus, on the 1st day of the estrus, its concentration on average was 2.13±0.11, which is typical for anestrus. During the next five days, a slight increase in progesterone content was noted to 3.27±0.69.

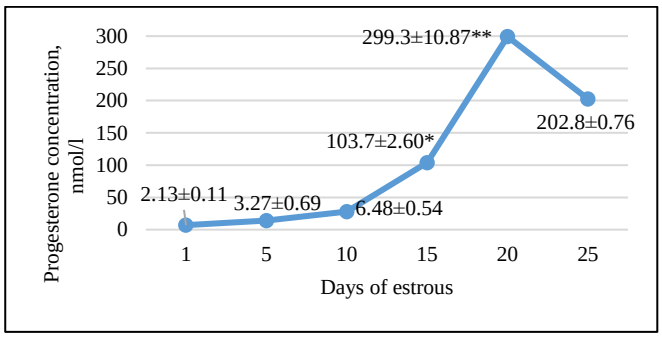

Figure 1. Dynamics of progesterone content in blood serum during estrus (*$p<0.05$ and **$p<0.001$ compared to the first day of estrus)

On the 10th day of estrus, the hormone content increased significantly ($p<0.05$) to 6.48±0.54, this concentration is typical for the initial stage of the luteal phase of estrous cycle. Determination of progesterone concentration in blood serum on the 15th day of estrus showed a high (16-fold) significant ($p<0.05$) increase compared to the 10th day. The tendency to increase the concentration was preserved in the next five days of observation and already on the 20th day, this indicator was 299.3±10.87 ($p<0.05$). After the 20th day of estrus, we note a decrease in progesterone serum concentration, and already on the 25th, it decreased to 202.8±0.76.

Results of the study of the dynamics of estradiol concentration in blood serum during estrus

Determination of the concentration of estradiol in the blood serum of bitches during estrus showed the changes, which are shown in Figure 2.

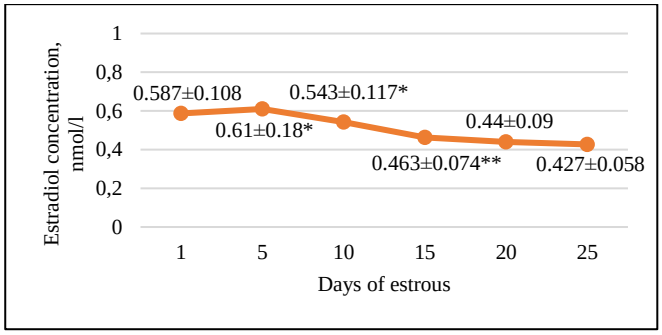

Figure 2. Dynamics of estradiol content in blood serum during estrus (*$p<0.05$ and **$p<0.001$ compared to the first day of estrus)

Thus, on the first day, the hormone content was 0.587±0.108, which is typical for the follicular phase of the sexual cycle. On the 5th day, an unreliable trend of increasing the concentration to 0.61±0.18 was noted, and on the 10th, a decrease to 0.543±0.117. Subsequently, with a sharp increase in progesterone concentration, the content of estradiol continued to gradually decrease and on the 15th day decreased to 0.463±0.074, on the 20th to 0.44±0.09, and on the 25th to 0.427±0.058.

Results of the study of the dynamics of cortisol concentration in blood serum during estrus

Analysis of cortisol concentration dynamics in blood serum during estrus (Figure 3) showed that this indicator was within physiological limits. Thus, from the 1st to the 10th day, an increase in concentration was observed from 113.94±34.42 to 138.3±38.64. Starting from the 15th day, there is a significant decrease in the concentration of this hormone in blood serum to 68.5±21.95. From the 15th to the 25th day, the cortisol content tended to increase, but within 10 nmol/l.

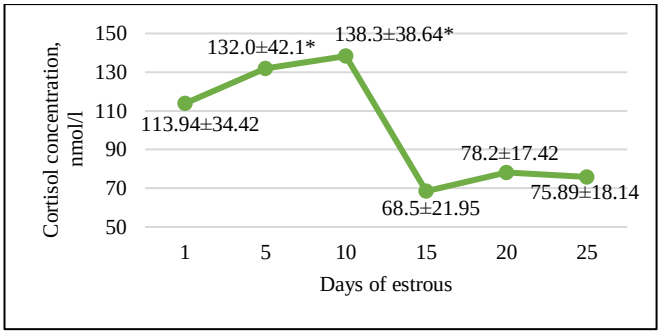

Figure 3. Dynamics of cortisol content in blood serum during estrus (*p<0.05 compared to the first day of estrus)

Results of the study of the triiodothyronine (T3) dynamics concentration in blood serum during estrus

On the first day of estrus, the concentration of T3 in blood serum was 3.057±0.94, which is slightly more than physiological limits (Figure 4). Further determination showed is a gradual tendency to decrease of this hormone in blood serum during estrus. A slight increase in hormone concentration is noted on the 10th day of the cycle.

Results of the study of the phagocytic activity of neutrophils (PAN)

Physiological limits of PAN in dogs vary between 55-65% of the absolute number of these cells. The ability of neutrophils to phagocytosis changed during the estrus is shown in Figure 5. Thus, on the 5th day, this indicator increased significantly (p<0.05) by 13% compared to the first day and amounted 4.11±0.46 G/l. The analysis of the subsequent dynamics shows a tendency to decrease the PAN, and in the period from 15 to 20 days, a significant decrease, up to 16%, was observed. Over the next 5 days of observation, PAN increased from 2.9 ± 0.81 to 3.13 ± 0.39 G/l.

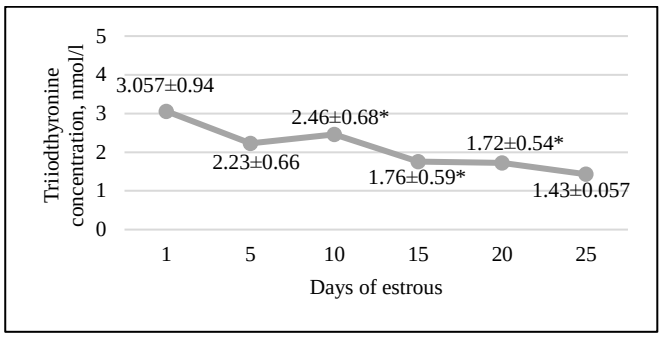

Figure 4. Dynamics of triiodothyronine content in blood serum during estrus (*p<0.05 compared to the first day of estrus)

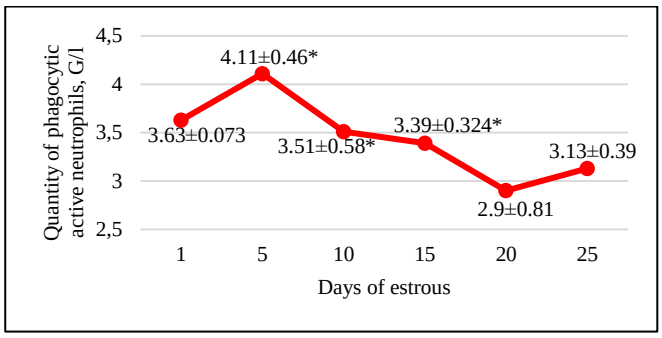

Figure 5. Dynamics of phagocytic activity of neutrophils during estrus (*p<0.05 compared to the first day of estrus)

Results of the dynamics of the absolute number of leukocytes (ANL), neutrophils, lymphocytes and monocytes study during estrus

During estrus, ANL dynamics tended to decrease. So, from day 1 to day 25, this indicator decreased by 2.6 G/l (21%) (Figure 6.). However, the populations of different cells had different regularities. Thus, against the background of the general trend of ANL decreasing on 5th day, the content of neutrophils tended to increase and lymphocytes to decrease. A slight decrease in the monocyte population was also observed. On the 10th day of estrus, all the populations of the studied cells had a tendency to decrease, and on the 15th day, the number of lymphocytes increased by 13%, and the tendency of the decrease in neutrophils remained. A slight increase in ANL on the 25th day of estrus occurs against the background of monocytes.

Although the number of neutrophils had a slight (2%) tendency to decrease on the 25th day, at the same time the PAN, on the contrary, increased by 7%.

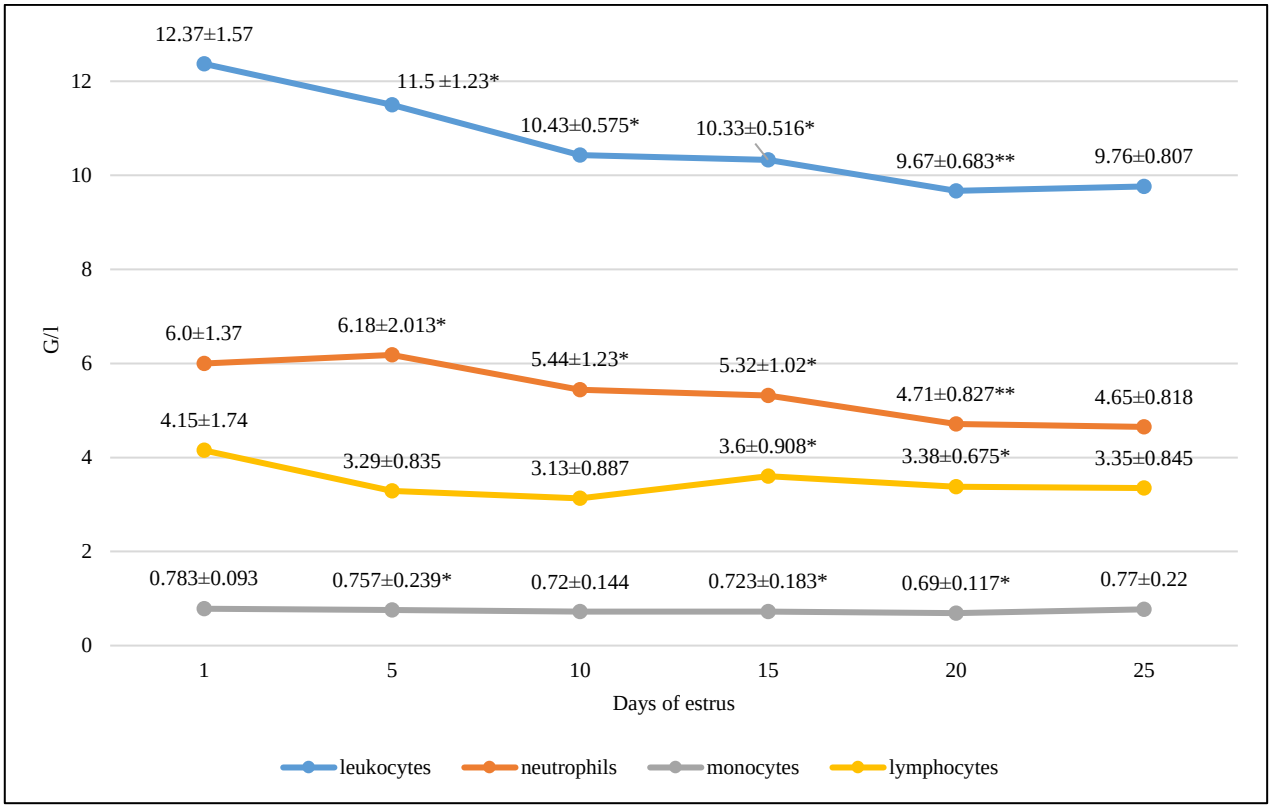

Figure 6. Dynamics of the absolute number of leukocytes (ANL), neutrophils, lymphocytes and monocytes during estrus (*p<0.05 and **p<0.001 compared to the first day of estrus)

4. Discussion

In this research, authors demonstrated the dynamics of changes in hormone content and indicators of adaptive cellular immunity in bitches during the estrous cycle. Determination of these indicators does not require expensive laboratory equipment and is available in veterinary practice. Taking into account the fact that estrus in dogs lasts long enough, it is not clear enough what indicators and in what terms should be determined for predicting possible dysfunctions of the reproductive system and as a result of premature impossibility of reproduction in female dogs. In our research, authors observed the animals for three months after estrus for the possibility inflammatory processes in the reproductive system development. It is an established fact that reproductive problems in dogs are often associated with immuno-endocrine disorders of the endometrium (Dhaliwal et al., 1999).

The average concentration of progesterone in all female dogs in our study begins to rise from 5 to 10 days, and the indicator of 6.48±0.54 nmol/l indicates that on the 10th day there was a surge of luteinizing hormone (LH), which is necessary for follicle ovulation. This finding is consistent with a large number of previous studies in which ovulation occurs approximately two days after the LH surge (Groppetti et al., 2015; Kutzler et al., 2003; Onclin et al., 2002; Jurczak & Janowski, 2018) and is associated with a sharp increase in progesterone concentration (Steckler et al., 2013; Thomassen et al., 2006), reflecting the increased level of luteinization associated with ovulation of many follicles.

The peak concentration of progesterone occurs on the 20th day of estrus. The reaction of the immune system, which is manifested by the maximum decrease in the phagocytic activity of neutrophils, should be noted. This fact points to the immunoregulatory function of progesterone, which obviously has physiological significance and consists in preparation for possible pregnancy. In the dog, implantation occurs around the 17th day of embryonic life, and the balance between pro- and anti-inflammatory reactions in fetal-maternal communication is crucial for the onset of pregnancy. Understanding of such immune mechanisms in canine reproduction is still insufficient (Tavares et al., 2021).

To the best of our knowledge, there is a paucity of data on the relationship between serum progesterone concentrations and PAN during the estrus. Progesterone has also been shown to suppress immune function within the uterus, possibly increasing its susceptibility to bacterial infection (Sugiura et al., 2004; Faldyna et al., 2001). Progesterone normally suppresses inflammatory innate immune responses (Hall & Klein, 2017).

An increase in the concentration of estradiol on the 5th day of estrus, in contrast to progesterone, had the opposite effect on PAN, namely, the ability of neutrophils to phagocytosis increased. Neutrophils are phagocytic innate

immune cells required to destroy bacteria by activating a wide range of effector reactions and generating large amounts of reactive oxygen species (ROS) (Zeng et al., 2019).

Somewhat different results were obtained in earlier studies (Bartoskova et al., 2014). The authors show that progesterone and estradiol-17β cause a decrease in the activity of neutrophils due to inhibition of phagocytosis-induced oxidative burst. Although the results did not show a clear dose-dependent decrease in neutrophil activity. Physiologically, increase of PAN and concentration of estradiol-17β on the 5th day, in our opinion, is related to the need to rehabilitate the reproductive tract and prevent the possible development of inflammatory processes. It is believed that the pro-inflammatory role of estrogens (in particular due to their effect on the innate immune system) contributes to the strengthening of immune reactions that develop in women (Markle & Fish, 2014).

Analysis of the dynamics of the absolute number of lymphocytes content during estrus showed the opposite effect of the sex steroids influence, in contrast to PAN. Thus, with an increase in the concentration of estradiol-17β on the 5th day, the content of lymphocytes decreases. This may indicate the suppressive effect of this hormone on adaptive immunity, namely on the most reactogenic cells of the body, in order to prevent autoaggression. The protective effects of estrogen in autoimmune conditions, such as multiple sclerosis and rheumatoid arthritis, are thought to be due to the combined effect of estrogen-mediated T-lymphocytes expansion and activation (Polanczyk et al., 2004; Polanczyk et al., 2003; Offner & Polanczyk, 2006)

In our study, the specific dynamics of cortisol during estrus was observed. This is expressed in the fact that during the follicular phase of the sexual cycle (up to 10th day), the concentration of this hormone increases, and on the 15th day, authors note its sharp decrease from 138.3±38.64 to 68.5±21.95 (2 times). Stress-like increases of glucocorticoids in plasma inhibit gonadotropin secretion and may disrupt ovarian cycling. Studies in ewes have shown that prolonged cortisol stress suppresses the GnRH pulse rate in ewes in the follicular phase, and this may be dependent on the presence of ovarian steroids (Oakley et al., 2009).

Therefore, the data regarding the dynamics of cortisol obtained in our experiment may indicate that estrus is a stressful factor for the bitches' body and also has a regulatory effect on the release of GnRH and, accordingly, the regulation of the transition from the follicular phase of the cycle to the luteal one. A clear dependence of the effect of cortisol concentration on the dynamics of innate adaptive immunity has not been established. Against the absolute number of lymphocytes (acquired adaptive immunity) tended to decrease. Several mechanisms have been proposed and documented to explain stress-induced lymphopenia, including both reduced lymph node outflow and decreased proliferative and activating cytokines (eg, IL-2) for lymphocytes (Stockham & Scott, 2008). Elevated cortisol levels due to activation of the hypothalamic-pituitary-adrenal (HPA) axis may also affect thyroid function and thyroid hormone metabolism by affecting the release of stimulating factors by the hypothalamus and pituitary gland and by reducing the deiodination of thyroxine (T4). After all, it may affect to the metabolically active triiodothyronine (T3) in peripheral tissues (Corder-Ramos et al., 2019; Daminet & Ferguson, 2003). The somewhat high concentration of T3 in the dogs in our studies on the first day of the sexual cycle is probably physiological, since low levels of this hormone lead to reproductive disorders such as prolonged anestrus, silent estrus, prolonged proestrus and problems with ovulation (Risvanli & Kalkan, 2016).

Perhaps the increased level of T3 at the beginning of estrus is necessary for the start of folliculogenesis. Thus, at each stage of follicular development, factors of autocrine, endocrine and/or paracrine origin act directly or indirectly on follicular cells, directing their differentiation, either for follicle growth or for atresia (Salvetti et al., 2009). Among these factors are T3 and T4, which have been identified in the follicular fluid of human ovarian follicles (Wakim et al., 1993). A decrease in the concentration of thyroid hormones inhibits the development of follicles, and therefore ovulation may be unsuccessful. Like leptin and insulin, thyroid hormones are well-known regulators of metabolism and energy balance, including during pregnancy (Ladyman, 2008). An increase in the concentration of T3 on the 10th day of the estrus was also noted and is probably associated with the provision of luteinization of postovulatory follicles. There are data demonstrating that thyroid dysfunction affects not only luteolysis in cyclic and pregnant rats, but also lutein vascularization (Silva et al., 2018). These results indicate that hormone and metabolite concentrations change during pregnancy to meet the energy demands of both the fetus and maternal tissues. Physiological hyperleptinemia, hyperinsulinemia, and changes in cortisol and thyroid hormones indicate pregnancy-induced adaptation of metabolic functions (Menchetti et al., 2020). Hormonal influence on the dynamics of monocytes in bitches during estrus generally had a tendency to decrease within 20 days from the beginning of the estrus, but on the 25th day their absolute number increased to the level of the 1st day of this process. Monocytes are "professional" phagocytes. The ability to phagocytosis is more pronounced in them than in other leukocytes. To recognize foreign agents, monocytes use a number of lectin receptors, as well as receptors for the C3b component of complement and Fc-fragments of antibodies (Kazmirchuk et al., 2012).

The authors` data may indicate that monocytes have a significant role in the regulation of immunophysiological functions related to pregnancy. If it does not occur, the population of these cells increases. Several studies provide

evidence that monocyte recruitment, differentiation into macrophages and reproductive tract function are modulated by pregnancy-related hormones (Tonello & Poli 2007). Hormonal influence can be achieved by direct binding to the corresponding hormone receptors expressed on human and murine macrophages (Salem, 2004; Zhang et al., 2003; Bukovsky et al., 2003), or indirectly by modulating the levels of cytokines and growth factors that target resident macrophages and affect their secretory profile.

Thus, further studies of the immunomodulatory function of pregnancy-related hormones will improve our understanding of endocrine-immune interactions before and during pregnancy and may help to develop selective strategies for the treatment of infertility and pregnancy complications.

5. Conclusion

Authors' results of research show that the phagocytic activity of neutrophils changes depending on the concentration of sex steroids. An increase in the concentration of estradiol on the 5th day synchronously increases the PAN, starting from the 10th day due to an increase in the concentration of progesterone PAN sufficiently decrease. The effect of progesterone on PAN in the luteal phase of the sexual cycle proves that immunosuppression is obviously physiologically justified in this period. In addition, it was found that the concentration of cortisol in blood serum during the first 10 days of estrus had a tendency to increase. And from the 10th to the 15th days, there was a decrease of more than 2 times. The above also indicates a decrease in the organism's protective properties. Thus, further research is needed to clarify the changes that may occur in the immune system of female dogs in the period of estrus during the use of pharmacological means of immunotropic action.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this study, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed during conduction experiments on animals, including current legislation of Ukraine (Article 26 of the Law of Ukraine 5456-VI dated 16.10.2012 "On the Protection of Animals Cruelty Treatment") and the "General Ethical Principles of Animal Experiments" adopted by the First National Congress on Bioethics (Kyiv, 2001), international bioethical norms (materials of the IV European Convention on the Protection of Vertebrate Animals Used for Experimental and Other purposes (Strasbourg, 1985)) and Directive 2010/63/EU of the European Parliament and of Council of 22 September 2010 on the protection of animals used for scientific.

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