Original Article

# Inflammation and Apoptosis Factors in Mares' Follicular Fluid Regarding Seasonal Cyclicity

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## ABSTRACT

**Background:** The in vitro production of human and animal embryos rapidly has been developed in recent years. The year-round practice of obtaining oocytes from valuable mares does not account for seasonal changes in the oocyte microenvironment or their potential impact on oocyte quality.

**Objectives:** This study aimed to assess levels of inflammation and apoptosis markers in the follicular fluid (FF) of mares during the transitional anovulatory period and the breeding season with normal estrous cycles.

**Methods:** The experimental samples included clinically healthy crossbred mares aged 6-12 years. The expression of inflammation and apoptosis factors (interleukin [IL]-1α, IL-1β, Tumor necrosis factor alpha (TNF-α), nuclear factor kappa B [NF-kB], B-cell lymphoma-2 [BCL2], FAS, Bcl-2–associated X protein (Bax), caspase 3) in FF samples from the transitional and ovulatory phases was assessed by Western Blotting.

**Results:** The inflammation and apoptosis developed in large FFs (≥31-35 mm) in both periods, evidenced by an increase in the levels of IL-1α, IL-1β, TNFα, FAS, and caspase-3. In luteinizing follicles during the transitional period, anti-apoptotic markers NF-kB and BCL2 reached their peak level, while in large estrous follicles, the level of these markers significantly decreased. This indicated that apoptosis in large FFs is not triggered by TNFα. In hormonally stimulated preovulatory follicles, the level of all inflammation and apoptosis markers decreased except for NF-kB and TNFα. On the contrary, the TNFa reached a maximum level, which could indicate preparation for follicle rupture and the release of a mature oocyte (ovulation).

**Conclusion:** The findings indicate the presence of specific regulatory pro- and antiapoptotic mechanisms involved in the preparation of mature follicles for ovulation.

**Keywords:** Apoptosis, Follicular fluid (FF); Inflammation, Mares, Reproduction

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## Introduction

The unique biochemical composition of follicular fluid (FF), which provides a vital microenvironment for each stage of oocyte development up to ovulation, makes this fluid an ideal candidate for better understanding folliculogenesis. Analysis of FF components is important for identifying potential biomarkers associated with follicle development and oocyte maturation, thereby improving assisted reproductive technologies (ART). In monovular species, including humans, horses, and cattle, phases of follicular growth occur regularly throughout the reproductive life of the female. Initially, all follicles grow at the same rate. Once a follicle (usually the largest) in the mare's ovary reaches a critical size (~22.5 mm), a phenomenon known as deviation begins, when the largest follicle continues to grow as the dominant follicle. In contrast, all other (subordinate) follicles stop growing and undergo atresia (Ginther et al., 2001).

The most important specific feature of horses is the seasonality of reproduction. The sexual periodicity of mares is regulated mainly by the photoperiod, i.e. the length of daylight, which determines the presence of two main phases of sexual activity: First, the winter acyclic, anovulatory period of sexual rest (anestrus), and second the active sexual phase with normal estrous cycles and ovulation during the breeding season (early spring - summer - early autumn). There are two transition periods (spring and autumn) between them, during which the normal functioning of the hormonal "hypothalamic-pituitary-gonads" axis is established or extinguished, respectively. During these periods, the development of several follicular waves in the ovaries does not finish with ovulation, and the leading follicles are luteinized without ovulation. However, modern reproductive biotechnologies are focused on year-round oocyte extraction from the ovaries of valuable mares, without accounting for seasonal variations in FF composition, primarily due to differences in hormonal backgrounds. The influence of season on follicular activity in mares has been studied. Differences have been recorded between ovulatory and anovulatory periods, as well as between the two halves of the ovulatory season (Donadeu & Watson, 2007). Since both growing and regressing follicles can coexist in the equine ovary, their developmental conditions may differ, potentially affecting oocyte quality and ART outcomes. However, the mechanisms of follicle development in horses remain poorly understood. In particular, unlike in other domestic species, the influence of local inflammation and apoptosis on follicle development in FF has not been assessed in mares.

Several studies have demonstrated multiple autocrine, paracrine, and endocrine interactions between the immune and reproductive systems. Cytokines can stimulate or inhibit cell growth, regulate cell proliferation, induce chemotaxis, and modulate the expression of other cytokines. Cytokine production in the ovaries, their accumulation in FF, and their influence on folliculogenesis suggest that cytokines may be important autocrine and paracrine regulators of ovarian function (Pan et al., 2024; Sarapik et al., 2012). The regulatory influence of cytokines can be observed at all stages of folliculogenesis, from the development of the primordial follicle to oocyte maturation. Cytokine levels in FF are proposed as markers of embryo quality and as prognostic criteria for in vitro fertilization (IVF) outcomes. In particular, interleukin (IL)-1β levels in FF were higher in patients with a negative IVF outcome (Andreeva et al., 2017).

In eukaryotic cells, NF-kB (nuclear factor kappa-light-chain-enhancer of activated B) is an important nuclear transcription factor that regulates the expression of many cytokines, including proinflammatory cytokines (Sung et al., 2024). Disruption of NF-κB function can lead to apoptosis suppression, persistent cell replication, and increased angiogenesis, which are observed in cancer cells (Bahrami et al., 2024).

Tumor necrosis factor-alpha (TNF-α) is one of the most studied cytokines and participates in inflammatory processes. TNF-α is involved in the regulation of physiological processes, including follicular development, steroidogenesis, ovulation, and corpus luteum function. It has also been reported that TNF-α can regulate granulosa cell differentiation and apoptosis, depending on the developmental stage (Silva et al., 2020). In particular, TNF-α interacts with insulin-like growth factor (IGF) receptors on granulosa cells, initiating their proliferation (Andreeva et al., 2017).

The IL-1 family is a large and complex network of pleiotropic proinflammatory cytokines that play a key role in regulating immune cells and inflammatory processes. IL-1β has been shown to regulate follicular development by promoting granulosa cell proliferation and preventing premature differentiation. This factor also influences apoptosis in ovarian granulosa cells and appears to be involved in several ovulation-related processes, including protease synthesis, regulation of plasminogen activity, and prostaglandin production (Brännström, 2004; Silva et al., 2020).

Apoptosis is a form of programmed cell death involved in follicle development and atrophy in the ovaries of all mammals: In oogenesis, folliculogenesis, oocyte loss/selection, and atresia. A delicate balance between pro- and anti-apoptotic factors determines the final fate of follicular cells. Apoptosis plays a critical role in oocyte maturation by disrupting the communication between granulosa cells and oocytes (Yadav et al., 2018; Regan et al., 2018). Although the specific signals, receptors, and intracellular signaling pathways that lead to apoptosis in granulosa cells are not fully defined, several pro- and anti-apoptotic factors have been identified, including Fas, caspases, TNF, IL-1β, Bcl-2 (B-cell lymphoma-2), and Bax (BCL2-associated X protein). The type of apoptosis and signaling pathway depends on the stage of follicle development and the origin of the internal or external trigger. Large amounts of granulosa cell apoptosis in the granulosa membrane lead to follicle death (Regan et al., 2018).

Caspases are known as the main "executors" of apoptotic cell death. Caspases can activate each other, forming a caspase cascade. This cascade can be initiated in two different ways; in both cases, the final effector caspase is caspase-3. It has been demonstrated that only in antral, especially in fully formed follicles, and atretic follicles, an active apoptotic process accompanying follicular atresia occurs (Albamonte et al., 2019).

Proapoptotic proteins, Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer), alter mitochondrial permeability, promoting the formation of pores in the outer mitochondrial membrane and the release of various factors involved in apoptosis, including cytochrome c and apoptosis-inducing factor. Bax is mainly associated with follicular atresia (Klimentova et al., 2021; Hussein, 2005).

Bcl-2 is an important anti-apoptotic protein. It is expressed on the mitochondrial membrane, the nuclear membrane (to a lesser extent), and the cell surface (Senichkin et al., 2020). Anti-apoptotic proteins of the Bcl-2 family mainly regulate apoptosis at the level of MOMP (mitochondrial outer membrane permeabilization). They impede the increase in mitochondrial outer membrane permeability induced by Bax and Bak. Bcl-2 is found mainly in the developing primordial/primary follicles, and it is involved in follicular selection/loss. Luteolysis is also associated with altered expression of Bcl-2, Bax, and TNF (Hussein, 2005).

Fas/APO-1 protein, also called CD95, belongs to the TNF receptor family. The interaction between Fas and its ligand, FasL, regulates numerous physiological and pathological processes mediated by programmed cell death. It was shown that the Fas/FasL system acts mainly in follicular atresia and luteal regression (Albamonte et al., 2013).

Thus, various active biochemicals identified in FF have been associated with follicle development and degradation in different species. Considering the rapidly developing reproductive biotechnologies in horse breeding, it seems sensible to find additional factors characterizing the state of the follicle and developing oocyte, and in particular, during different seasons of sexual activity of mares. Therefore, this study aimed to assess levels of inflammation and apoptosis markers in the FF of mares during the transitional anovulatory period and the breeding season with normal estrous cycles.

## Material and Methods

The work was conducted at the experimental stable of «The All-Russian Research Institute for Horse Breeding named after Academy Member V.V. Kalashnikov» (Ryazan Region, Russian Federation). The experiments involved crossbred, clinically healthy, barren mares aged 6-12 years, with no signs of inflammation or sclerosis of the ovarian tissue after OPU (ovum pick-up) procedures. They were kept outdoors year-round and demonstrated well-defined seasonal variation in reproductive function. The care and feeding conditions of the animals complied with accepted zootechnical and veterinary standards.

Aspiration of FF from follicles was performed during two phases of mares' sexual activity: In the spring transition (T) period with anovulatory cycles (February-March) and in the active ovulatory (O) phase (breeding season) with normal estrous cycles and ovulation (May-June). The number and size of follicles in the mares' ovaries were determined using an EXAGO ultrasound scanner (ECM, France). Large follicles (≥31 and ≥35 mm in diameter for the T and O periods, respectively) were selected for the experiments, considering the echostructure of the intra-FF. Large follicles with a homogeneous black echostructure in the spring transition period were considered equally capable of reaching ovulation or luteinizing (group 1). The first signs of luteinization were considered to be the appearance of echogenic inclusions in the follicle cavity (group 2) (Figure 1).

During the period of normal physiological estrus in the ovulatory phase, large follicles were aspirated closer to ovulation based on the accompanying physiological signs (pronounced uterine edema, change in the shape and consistency of the mature preovulatory follicle)

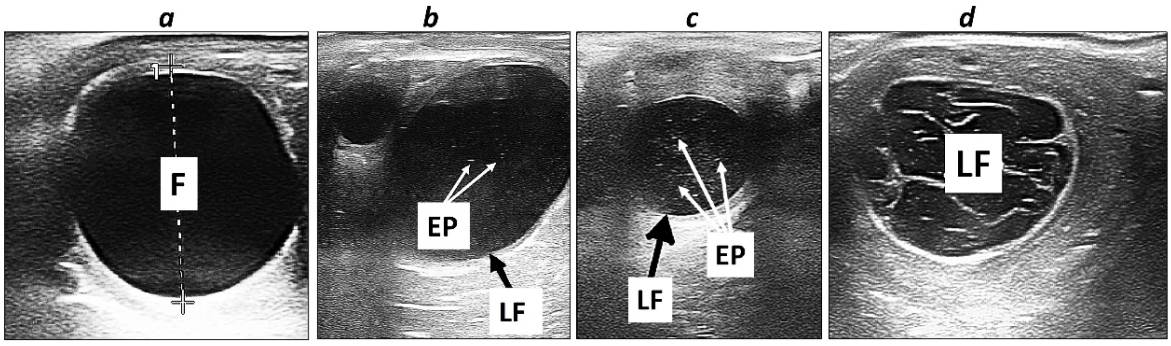


**Figure 1.** Ultrasound images of follicle luteinization stages in mares

a) Non-luteinized follicle (F), b) Initial stage of follicle luteinization (LF) – appearance of echogenic particles (EP) in the follicle cavity, c) Progressive stage of luteinization; d) Luteinized follicle

(group 3). In group 4, mares were treated with 1 intramuscular injection of the drug "Surfagon" (analogue of gonadotropic releasing hormone [GnRH], ZAO Mosagrogen, Moscow), 50 µg, for induction of ovulation. Follicles were punctured 24 hours after the injection, i.e, in the preovulatory phase. In total four groups of follicles were formed: Group 1, large (≥31 mm) follicles in the spring transition period, without signs of luteinization (T); group 2, large (≥31 mm) follicles in the spring transition with signs of luteinization (Tl); group 3, large estrous follicles (≥35 mm) in the ovulatory period without hormonal treatment (Oe); Group 4, large estrous follicles (≥35 mm) in the ovulatory period after hormonal treatment (Oh).

Before the procedure of follicle puncture from ovaries (OPU), the mares were administered flunixin meglumine (Norbrook Laboratories Limited, UK) (300-500 mg) and the antibiotic vetbicin-3 (benzylpenicillin, OAO Sintez, Russia) (10000-15000 U per 1 kg of animal weight). Animals were sedated by administering detomidine hydrochloride (Domosedan, Zoetis-Pfizer, USA) at 0.2-0.5 mg intravenously, with additional doses administered as needed throughout the procedure. Additionally, epidural anesthesia with 2% lidocaine (5-7 mL) was administered after the first injection of Domosedan.

FF was extracted from the mares' ovaries transvaginally using a 12-G, double-lumen needle of 65 cm length (Minitube, Germany). The procedure was performed under ultrasound rectal control using an EXAGO ultrasound scanner (ECM, France) equipped with a modified endovaginal unit and a microconvex probe. A COOK Medical K-MAR-5200 vacuum pump (William A. Cook Australia PTY. LTD) was used for FF aspiration. Needles, conducting systems, and vessels for FF were specific for each follicle. The same mares (n=3) were used in the experiments in two seasons (transitional and ovulatory), and FF was obtained from follicles of different groups from the same mare. During the transitional phase, the OPU procedure was performed when follicles ≥31 mm were found in the follicular wave in the mare's ovaries after winter anestrus. Two OPU procedures were performed in each of the two mares, with a 2- and 3-week interval between sessions. In the third mare, FF samples for groups 1 and 2 were obtained in a single OPU procedure from two follicles. During the ovulatory period, OPU sessions were performed in each of the three mares in two consecutive normal estrous cycles. The interval between OPU sessions was 2-3 weeks. Information on follicle size and stages of cyclicity in mares is presented in the Table 1. Oocyte collection was not the aim of these experiments. A total of 12 FF samples (3 per group) were obtained to assess transcription factor levels. FF samples were centrifuged for 20 min at 3000 rpm, packaged in 1.5 mL Eppendorf tubes, frozen, and stored at -25 °C before the analysis.

## Analysis of transcription factor levels in FF by Western blotting

The analysis of inflammation and apoptosis factors in mares' FF samples was performed at the certified biochemistry laboratory of The Ryazan State Medical University named after Academician I.P. Pavlov (Central region, Ryazan, Russian Federation).

FF was preliminarily purified of cells and debris by centrifugation at 3000 g, at 4°C, for 15 min. Then, protein concentration was determined using the Bradford spectrophotometric method, and dilutions of 5, 10, 20, 50, and 100 µg/well were prepared to determine the linear detection range. As a result, a single bright band corresponding to the molecular weight of the target protein was observed on the blot.

**Table 1.** The stage of cyclicity and follicular size in mares at the moment of FF aspiration (OPU sessions)

| Mare | Spring Transitional (Non-ovulatory) Period | | | | Ovulatory Period | | | |
|---|---|---|---|---|---|---|---|---|
| | Groups of Follicles | | | | | | | |
| | 1 (T) (≥31 mm) | | 2 (TI) (≥31 mm) | | 3 (Oe) (≥35 mm) | | 4 (Oh) (≥35 mm) | |
| | Foll. Size (mm) | Stage of Cyclicity | Foll. Size (mm) | Stage of Cyclicity | Foll. Size (mm) | Stage of Cyclicity | Foll. Size (mm) | Stage of Cyclicity |
| 1 | 36 | Second FW* | 33 | Third FW | 45 | Estrus oedema 2** | 44 | Estrus oedema 2 |
| 2 | 34 | First FW | 37 | Third FW | 42 | Estrus oedema 1 | 39 | Estrus oedema 2 |
| 3 | 37 | Third FW oedema 2 | 36 | Third FW oedema 2 | 43 | Estrus oedema 1 | 45 | Estrus oedema 1 |

*Follicular wave after winter anestrus; **The degree of uterine oedema (1-4).

Analysis of serial dilutions of the pool at 5, 10, and 20 µg/well demonstrated linearity, and a correlation coefficient ($R^2$)> 0.95 for all proteins was considered acceptable. In the 50-100 µg/well concentration range, a plateau was observed, and these concentrations were considered unacceptable for analysis. Thus, a dilution of 20 µg/well was optimal.

A total of 20 µg of proteins was subjected to electrophoresis using the TGX Stain-Free FastCast acrylamide kit (Bio-Rad) in the Laemmli buffer system (Bio-Rad) for analysis. Before loading, the samples were processed according to the Bio-Rad protocol. They were mixed with Laemmli sample buffer (Bio-Rad) containing 2.5% 2-mercaptoethanol (Bio-Rad) at a 1:3 ratio and incubated for 5 min at 70 ˚C. Electrophoresis conditions were 100 V for 90 min. Proteins were transferred from the gel to the membrane using the semi-dry method with Trans-Blot Turbo (Bio-Rad, USA). Proteins on the membrane were blocked with 1% EveryBlot Blocking Buffer (Bio-Rad, USA) containing 0.1% Tween for 30 min at room temperature. For the analysis of transcription factors, the following primary rabbit polyclonal antibodies were used at a dilution of 1:500:

Fas, apoptosis marker (AF5342 FAS Antibody, Affinity, China); Bcl-2, apoptosis marker (AF6139 Bcl-2 Antibody, Affinity, China); Bax, apoptosis marker (AF0120 Antibody, Affinity, China); Cleaved-Caspase 3, apoptosis marker (AF7022 Antibody, Affinity, China); IL-1β, interleukin 1 beta – (AF5103 IL1 beta Antibody, Affinity, China); IL-1α, interleukin 1 alpha – (DF6893 IL1 alpha Antibody, Affinity, China); TNF- a – tumor necrosis factor a (AF7014 TNF alpha Antibody, Affinity, China); NF-kB p65, transcription factor involved in inflammation (AF5006 NF-kB p65 Antibody, Affinity, China).

Visualization of primary antibodies was performed using secondary goat antibodies (Goat anti-rabbit IgG [H+L] cross-adsorbed secondary antibody, HRP, Invitrogen, USA) at a dilution of 1:4000 and incubation for 1 hour at room temperature. Chemiluminescence was recorded using ChemiDoc XRS+ (Bio-Rad, USA). The intensity of the resulting bands was analyzed densitometrically using ImageLab software (Bio-Rad, USA). The molecular weight of the analyzed proteins was confirmed by comparison with precision plus protein standards dual color (Bio-Rad, USA). The protein content was estimated relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primary antibodies GAPDH loading control monoclonal antibody [GA1R], DyLight 68 [Invitrogen, USA], dilution 1:1000, secondary rabbit antibodies - rabbit-anti-mouse IgG [H+L] secondary antibody, HRP [Invitrogen, USA], dilution 1:4000).

## Statistical analysis

The analysis and graphical representation of the results were performed using GraphPad Prism software, version 9.5.0. Data in the graphs are presented as Mean±SD. To assess statistical significance, analysis of variance (ANOVA) with the post hoc Tukey test was used. The mean value of each column was compared with the mean of every other column at significance levels of $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$.

The number of samples per group in this study limits statistical power and increases error in effect estimation. Therefore, the obtained P values should be interpreted with caution. The Tukey test we used is a robust method for pairwise comparisons and controlling for overall error. The obtained significant differences confirm the identified trends, which can emerge even with a small number of samples in the compared groups. This work is

considered a pilot study, generating hypotheses for further testing on a larger number of samples.

## Results

The minimum level of IL-1α in FF was noted in large follicles of group 1. This indicator increased in group 2 and group 3, exceeding group 1 by 24.4% (P=0.0141) and 37.9% (P=0.0009), respectively, and in group 4 (Og), it decreased, relative to group 3 (P=0.0506) (Figure 2a).

Similar results were obtained when measuring IL-1β levels. The minimum level in the FF was noted in group 1. In groups 2 and 3, it exceeded those by 23.3% and 33.3%, respectively (P=0.0094). The amount of IL-1β in group 4 significantly (P=0.0506) decreased compared with group 3, closer to that of group 1 (Figure 2b).

The minimum level of TNF-α in the FF was also noted in group 1, it slightly increased in group 2 and reached maximum in groups 3and 4, exceeding the values of group 1 by 16.6% (P=0.057), 48.4% (P=0.0001) and 75.4% (P≤0.0001), respectively (Figure 2 c).

The level of the transcription factor NF-kB in the FF of mares was the same in groups 1 and 4. In group 2, it exceeded that of group 1 by 24.6% (P=0.35). In group 3 (Oe), it was significantly lower by 24.2% (P=0.026) (Figure 2d).

When analyzing apoptosis markers in FF of mares, the following results were obtained.

The amount of Bax in FF in animals of all groups did not differ significantly (Figure 2i).

The level of anti-apoptotic protein Bcl2 in the transition period in follicles of mares with signs of luteinization (group 2) slightly increased, compared to group 1. In the ovulatory period, this indicator significantly decreased in both groups 3 and 4 compared to group 2 by 15.4% (P=0.0167) and 14.6% (P=0.0249), respectively (Figure 2f).

The level of proapoptotic protein Fas in FF of groups 2 and 3 increased significantly, exceeding the values of animals of group 1 by 65.0% and 63.9%, respectively (P≤0.0001). In group 4, it was sharply reduced to the level of group 1 (P≤0.0001) (Figure 2g).

A similar distribution pattern was observed when assessing effector caspase-3 levels. It was also minimal in FF animals of groups 1 and 4 and increased in groups 2 and 3, exceeding the indicators of group 1 by 35.4% (P=0.0045) and 31.6% (P=0.0174), respectively. A more noticeable decrease in caspase-3 levels occurred in group 4 relative to groups 2 (P=0.001) and 3 (P=0.015) (Figure 2h).

## Discussion

Inflammation and tissue remodeling are considered systemic events in ovarian physiology, regulating folliculogenesis and fertility (Duffy et al., 2019; Santos et al., 2020). Moderate concentrations of proinflammatory cytokines in the FF are necessary to enhance granulosa cell proliferation, stimulate apoptosis, and follicular atresia (Sarapik et al., 2012). In our studies, the lowest levels of the inflammatory markers IL-1α, IL-1β, and TNF-α were observed in large (≥31 mm) follicles without signs of luteinization during the spring transition period (group 1). In luteinizing follicles in the spring transition period (group 2), these indicators increased (P≤0.05). The maximum inflammation intensity was observed in the follicles of group 3 – large estrous follicles (≥35 mm) during the ovulatory period without hormonal treatment. Hormonal stimulation of estrous follicles (group 4) reduced the intensity of the inflammatory reaction. At the same time, the NF-kB level increased significantly only in the FF of group 2.

It is known that IL-1 enhances LH receptor expression and stimulates androgen production (Adashi, 2000). However, elevated TNF-α levels in FF inhibit oocyte maturation and increase the frequency of chromosomal abnormalities (Liang et al., 2023). It was also found that with excessive production of IL-1 and TNF-α, premature luteinization of follicles occurs, which correlates with a low fertilization rate and increases the likelihood of premature termination of pregnancy in women (Chen et al., 1995). In this study, the development of an inflammatory reaction was accompanied by activation of apoptosis, as evidenced by increased caspase-3 levels in FF from groups 2 and 3.

Two main apoptotic pathways have been described in the literature: External (when specific molecules bind to cell membrane death receptors) and internal (or mitochondrial) (Cassimeris et al., 2010; Creagh, 2014; Jurcau & Ardelean, 2021). However, in both cases, the final link in the apoptotic reaction cascade is the activation of caspase-3. In our work, we studied the mechanism of programmed cell death activation and showed that the extrinsic pathway triggers it via the Fas ligand.

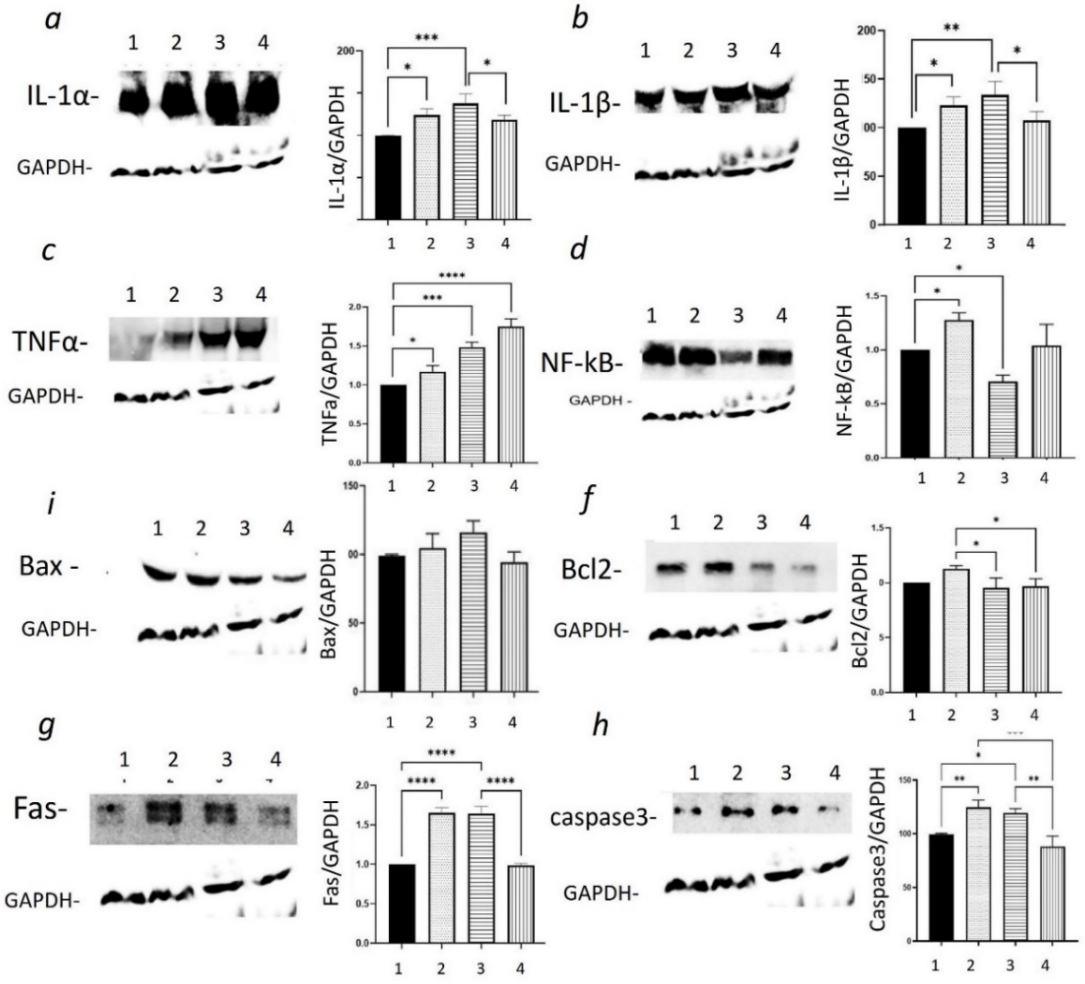


**Figure 2.** Levels of inflammation (IL-1α, IL-1β, TNF-α), Pro- and anti-apoptotic (Bax, NF-kB, Bcl2, Fas, caspase-3) factors in mares' FF, aspirated from follicles of 4 groups

IL: Interleukin; TNF-α: Tumor necrosis factor-alpha.

Note: 1: Follicles ≥31 mm in spring transition period (T); 2: follicles ≥31 mm with signs of luteinization in spring transition period (Tl); 3: Estrous follicles ≥35 mm in ovulatory period (Oe); 4: Estrous follicles ≥35 mm in ovulatory period 24 hours post hormonal stimulation of ovulation (Oh). Data in the graphs are presented as Mean±SD. Each group contained 3 replicates (n=3). $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.0001$, $^{****}P\leq0.00001$.

In follicles without signs of luteinization during the spring transition period (group 1), a relative balance of pro- and anti-apoptotic factors is observed, with a minimum value compared to other groups of follicles. When determining the further phase of development along the luteinization pathway (group 2), an inflammatory reaction developed (IL-1α, IL-1β), the level of NF-kB sharply increased ($P\leq0.05$), which activated the production of TNF-α, and it in turn triggered apoptosis along the external "receptor" pathway (through the Fas ligand), which was expressed in a sharp increase in the level of effector caspase-3 ($P\leq0.01$).

Our results are consistent with the data of White et al., who reported that stimulation with proinflammatory cytokines leads to activation of NF-kB complexes, which regulate the expression of key genes controlling apoptosis, angiogenesis, and cell proliferation (White et al., 2009). In large estrous follicles during the ovulatory period without hormonal treatment, a similar pattern of inflammation and apoptosis was observed (levels of IL-1α, IL-1β, TNF-α, Fas, and caspase-3 increased). Still, the trigger was not NF-kB, whose levels were significantly lower than in all other groups ($P\leq0.05$). Accordingly, the Bcl2 indicator, an apoptosis inhibitor targeted by NF-kB, was reduced, suggesting the establishment of

an anti-apoptotic mechanism and the possibility of further follicular development up to ovulation.

The most interesting results were obtained in group 4. The dynamics of the studied parameters in this group showed less pronounced signs of inflammation (IL-1α and IL-1β) and apoptosis (Fas and caspase-3) than in groups 2 and 3 ($P \leq 0.05$). At the same time, the TNFa indicator was highest in group 4, with NF-kB levels similar to those in non-luteinized follicles of the transitional period (group 1). It should be clarified that the follicles of group 4, stimulated by GnRH 24 hours before FF puncture, were closer to ovulation compared to the follicles of group 3. It can be assumed that the decrease in the inflammatory response, in parallel with low levels of Fas and caspase-3, and the activation of TNF-α before ovulation are associated with preparation for follicle rupture. Our results are consistent with other studies, which reported high levels of caspase-dependent apoptosis in antral follicles but did not detect these signs in preovulatory follicles (Yuan & Giudice, 1997; Glamoclija et al., 2005).

It should be clarified that our work focused on FF. However, the studied inflammatory and apoptotic factors are also present in the oocyte itself and in the thecal and granulosa cells of the follicle (Silva et al., 2020). In particular, no differences in Bcl2 or caspase-3 mRNA levels were observed in granulosa cells from small, medium, and large antral follicles in mares. Still, a positive correlation was observed between these two indicators (Samie et al., 2025). In another study examining mRNA levels of these genes in equine follicles, follicle maturation was characterized by increased Bcl2 transcription and decreased caspase-3 transcription (Wischral et al., 2022).

Data have also been obtained demonstrating that IL-1β inhibits in vitro maturation of equine oocytes, thereby regulating the resumption of meiosis in oocyte-cumulus complexes (COCs) (Martoriati et al., 2003). Maximum IL production was observed in granulosa and theca cells of preovulatory follicles after gonadotropin treatment (Ingman & Jones, 2008).

Thus, the maturation stage of large antral follicles in the ovaries of mares in two seasons of sexual activity (transitional [anovulatory] and ovulatory) is accompanied by different dynamics of the levels of inflammation and apoptosis factors, reflected in the composition of the FF and ensures the creation of an optimal environment for further oocyte development and ovulation, or luteinization of the follicle. In hormonally-stimulated estrous cycles, a special balance of pro- and anti-apoptotic factors was observed in mares' FF before ovulation. Due to the widespread use of hormonal stimulation in horse breeding, studying this process is of great importance. The obtained results open up prospects for further scientific research in this area, possibly toward expanding the molecular spectrum of FF and analyzing intercellular communication between the oocyte, granulosa, and theca cells, mediated by molecular signaling pathways.

## Conclusion

During the transitional and ovulatory seasons, levels of inflammatory and apoptotic factors increase in mares' FF from large antral follicles (31-35 mm). In this study, the highest levels of inflammatory and proapoptotic factors are observed in the FF of luteinizing and large estrous follicles. Apoptosis during luteinization occurs via the external pathway (via Fas ligand), while TNF-α does not trigger apoptosis in estrous follicles. 24 hours after hormonal stimulation of ovulation, the levels of almost all indicators of inflammation and apoptosis factors (IL-1α, IL-1β, Fas, Bax, Bcl2, caspase-3) in FF decreased, except for NF-kB and TNF-α. The latter reached its peak value, which may reflect preparation for follicle rupture and the release of a mature oocyte. The data obtained in our study indicate that, before ovulation, the classical apoptotic cascade is not triggered, confirming the uniqueness of the biochemical profile of FF at this stage. In this regard, a more thorough study of the components and the dynamics of changes in the FF composition is required, given the growing need to improve the effectiveness of ART programs in horse breeding.

## Ethical Considerations

### Compliance with ethical guidelines

This study was approved by the Bioethics Commission, the All-Russian Research Institute of Horse Breeding, Divovo, Russia (Protocol No.: 8-23 dated 11.28.2023).

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### Authors' contributions

Conceptualization, methodology, funding acquisition, and resources: Lyudmila Fedorovna Lebedeva and Elena Vladimirovna Solodova; Data collection and data

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## Conflict of interest

The authors declared no conflict of interest.

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