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Follicular fluid

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SUMMARY

Follicular fluid is an immediate environment of a developing oocyte. Therefore, determining its composition provides a good tool for evaluation of oocyte quality and its capability to be fertilized as well as for assessment of the course of early pregnancy. The review of the available literature concerning this subject proves that there is a need for further studies on the composition and role of follicular fluid in order to learn about yet unknown mechanisms responsible for reproduction failures and pathologies of early embryonic and fetal development.

Key words: follicular fluid; follicular maturation; oocyte

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INTRODUCTION

Folliculogenesis is a process whereby a primordial ovarian follicle matures and differentiates into an antral and then preovulatory follicle, which, when fully mature, is capable of releasing an oocyte, ready to be fertilized, during ovulation. In females, the process of development and maturation of ovarian follicles, from the primordial stadium to mature, tertiary Graafian follicles capable of ovulation, lasts approximately one year. The fate of follicles is controlled both by peptide and steroid hormones as well as by intraovarian growth factors. Follicle recruitment takes place in the first days of the menstrual cycle, but the whole period of preparation for ovulation takes approximately 85 days and is not regulated by hormones [1].

COMPOSITION AND ROLE OF FOLLICULAR FLUID

The first sign of the beginning of tertiary follicle formation is the appearance of an antrum around granulosa cells. The antrum fills with follicular fluid which is derived from both plasma transfer and secretory activity of granulosa and thecal cells. The exact mechanism regulating the early phase of antrum formation remains unknown. It seems to be associated with autocrine/paracrine action of factors, such as activin, KIT ligand or gap junction Cx 37 protein.

The basic hypothesis on the formation of follicular fluid suggests that granulosa cells produce hyaluronic acid and chondroitin sulfate, proteoglycans that generate osmotic gradient leading to fluid transfer from blood vessels of the thecal layer. Moreover, inter-alpha-trypsin inhibitor and versican (Vcan) are also present in follicular fluid. They create complexes with hyaluronic acid and additionally increase retention of these molecules in the antrum. Follicular fluid creates an environment in which granulosa cells and oocyte can find regulating molecules essential for their further development [2].

Starting from the antral phase (antral follicle), its further development is dependent upon pituitary hormones. LH and FSH control final stages of folliculogenesis, but local growth factors may modulate their action.

The biochemical analysis of follicular fluid composition may deliver significant information about oocyte quality and maturation. This is achieved by analysis of single parameters considered to be oocyte quality predictors as well as by metabolomics, i.e. evaluation of chemical processes associated with oocyte maturation and proteomic analysis revealing gene products associated with this process. The proteomic analysis of follicular fluid shows the presence of various proteins from different functional groups, e.g. insulin-like growth factors, receptors, anti-apoptotic proteins or metalloproteinases. Seventeen proteins have been identified whose expression in follicular fluid has changed upon HCG administration, which makes them biomarkers that show how the follicular microenvironment changes depending on infertility conditions [3].

The chemical components of follicular fluid that are significant for the development of follicles and oocytes can be categorized in the following way: a) hormones, b) growth factors, c) reactive oxygen species that are free radicals, d) anti-apoptotic factors, e) proteins, peptides and amino acids, f) sugars, and g) prostanoids.

Pituitary gonadotropins: FSH and LH are present in follicular fluid, and their quantities are affected by their circulating concentrations. There is a correlation between concentrations of pituitary gonadotropins in both environments: serum and follicular fluid [4,5].

The average intrafollicular FSH concentration is slight and increases approximately 10 hours after LH surge, reaching 50% of its serum value [6]. As with FSH, the LH level is low at the beginning of the cycle and increases between 10 and 20 hours after LH surge, reaching the highest values between 20 and 30 hours (40% of its serum concentration) [6]. Of interest is the observation that 20 hours after LH surge the condition of the follicular basement membrane deteriorates and the morphological and functional luteinization process begins. At the same time, the follicular fluid level of estradiol decreases abruptly [6].

Administration of gonadotropins in controlled ovary hyperstimulation used in *in vitro* fertilization is an intervention that changes the intrafollicular environment. Recently published studies have shown that hyperstimulation with the use of gonadotropins alters intrafollicular cytokine quantity, which may disrupt oocyte maturation and embryo competence. All these negative phenomena are not observed in modified natural cycles [7]. As growth hormone, gonadotropins affect granulosa cell production of a range of substances significant for oocyte maturation, e.g. hyaluronic acid. By exerting a synergistic action with estradiol, through cyclic AMP secretion, they increase oocyte cytoplasmic maturation and control meiosis [5]. Follicular fluid from follicles containing oocytes that were successfully fertilized, contains higher HCG and FSH levels compared with the surroundings of oocytes that were not fertilized, which supports the significant role of gonadotropins in this process [8].

Growth hormone (GH) enhances FSH-dependent estradiol production by granulosa cells and formation of LH and FSH receptors in these cells. Although the presence of GH in follicular fluid has been proven, its relationship with potential pregnancy and its development remains unknown [5].

Prolactin is present in follicular fluid and, by contrast with pituitary gonadotropins and steroid hormones, its levels in this environment do not correlate with its circulating concentration [4]. The intrafollicular prolactin level is significantly lower in the late postmenstrual phase compared with other phases of the menstrual cycle and is relatively stable during LH surge [6]. However, the presence of intrafollicular sources of this hormone should be excluded since prolactin is not detectable in follicular fluid of patients treated with bromocriptine [6].

As estradiol, progesterone and androstenedione levels, the intrafollicular prolactin concentration shows an association with differences in oocyte maturation as well as with its capability to be fertilized and to cleave after *in vitro* fertilization [9]. High intrafollicular prolactin levels are associated with successful development of pregnancy, which suggests a significant role of this hormone in the process of oocyte maturation [8].

Estradiol and progesterone are detectable in follicular fluid in various quantities after spontaneous LH surge and after HCG administration in non-stimulated and stimulated cycles. In the postmenstrual phase, estradiol levels in the fluid of large follicles (over 8 mm) are considerable, whereas in the premenstrual phase, it decreases while progesterone levels rise [4].

Before and approximately at the time of LH surge, estradiol levels in the follicular fluid of

preovulatory follicles are slightly higher than in the remaining follicles, and subsequently decrease. Shortly before LH surge, the progesterone concentration increases abruptly. This rise continues for the next 25 hours, after which the level slowly decreases. Changes in the progesterone concentration indicate the occurrence of ovulation. The levels of both hormones and their reciprocal proportions facilitate the evaluation of oocyte capability to be fertilized [6]. In the literature, there are various opinions on the role of progesterone in follicular fluid. Some authors claim that high progesterone concentrations or a slight proportion between estradiol and progesterone levels can predict implantation and pregnancy. Others believe that they are associated with abnormal fertilization resulting in an increase in multipronuclear embryos. It seems that optimal exposure of an oocyte to progesterone, i.e. the determination of a threshold which, when exceeded, results in oocyte damage, is currently impossible [5].

Follicular fluid also contains androgens. Concentrations of androstenedione and testosterone in this environment are higher in the preovulatory phase, but they are significantly lower than estradiol levels. Higher intrafollicular androgen concentrations are noted before LH surge. This supports the claim that these hormones are significant elements of estradiol biosynthesis [10]. Higher androgen values are associated with lower oocyte quality and lower number of zygote cleavages after fertilization. A low estradiol/testosterone index is linked with early follicular atresia, lower chances for fertilization and limited development of pregnancy. It is commonly acknowledged that certain androgen quantities in follicular fluid are crucial for their normal growth despite being responsible for follicular atresia.

It is assumed that corticosteroids have a positive influence on the final stages of follicle maturation and embryo implantation. Cortisol inhibits steroidogenesis in the ovary and has a positive effect on oocyte maturation. During LH surge, the level of cortisol in follicular fluid increases. It is higher in mature follicles compared with immature ones. 11â hydroxysteroid dehydrogenase, an enzyme that catalyzes the conversion of cortisone to cortisol, is found in human ovaries. The measurement of its activity provides indirect information about intrafollicular cortisol and cortisone levels. Its activity in follicular fluid aspirates after a three-day incubation is inverse to oocyte fertilization success in *in vitro* procedures. That is why, it is proposed that this enzyme should be considered as a predictor of successful fertilization [11].

Levels of 21-hydroxylase-derived steroids (11-deoxycortisol and 11-deoxycorticosterone) along with precursors of 17-hydroxyprogesterone in follicular fluid correlate positively with lipid content in luteinized granulosa cells. These compounds, acting through mineralocorticoid receptor, promote granulosa cell luteinization. The physiological relevance of this correlation for folliculogenesis and oocyte maturation has not been fully explored thus far [12].

Anti-Müllerian hormone (AMH) is produced by granulosa cells of preantral follicles and small antral follicles. It is believed to inhibit primordial follicle development and be responsible for follicle recruitment and selection during further stages of folliculogenesis. This hormone is also present in follicular fluid and its concentration allows evaluation of embryo quality. It is thought to be a reliable marker of oocyte viability [13].

Adenosine 3',5'-cyclic monophosphate (cAMP) is an element of signal transduction and can be used in LH action on target cell membrane receptors as one of secondary messengers. This nucleotide performs a significant role in oocyte maturation in numerous species. cAMP is present in human follicular fluid in high concentrations before LH surge with subsequent decrease after 20 hours. The cause of this abrupt decline in the fluid of older follicles is unknown, but it can result from increased phosphodiesterase activity [6].

Inhibins are produced by follicular granulosa cells and their intrafollicular level represents the activity of granulosa cells of single follicles. A high level of inhibins A and B is probably associated with the number of oocytes obtained during *in vitro* fertilization procedures, but not with their quality and capability to be fertilized [14]. Lau observed a positive correlation between oocyte quality and activin A concentration in follicular fluid with no similar correlation in the case of inhibins A and B [15].

Bone morphogenetic protein 15 (BMP-15) affects oocyte maturation. Its intrafollicular concentration positively correlates with estradiol levels, and higher concentrations of this protein are observed in cases of successful fertilization and oocyte cleavage rather than in cases of fertilization failure [16].

Insulin-like growth factors I and II (IGF-I and IGF-II), polypeptides that promote tissue proliferation and differentiation, are also pre-

sent in follicular fluid. Various studies support their significant role in normal ovary functioning and follicular maturation, thus confirming their importance in fertility [17].

Certain studies prove that IGF-1 is fundamental for follicular development, up to the phase dependent upon gonadotropins. Animal tests have revealed that IGF-1 inactivation blocks follicular growth without the possibility of its repair by gonadotropins. It must be emphasized, however, that normal GH and IGF-1 levels are not absolute conditions for preserving fertility [18]. Laron's syndrome, in which normal ovulation can occur despite low IGF-1 levels, is an example [19].

Androgens cause a marked increase in the number of basic follicles, which is accompanied by a three-fold increase in IGF-1 concentration and a five-fold increase in IGF receptor production in primordial follicles [20]. It has also been shown that IGF-1 stimulation in *in vitro* settings results in an increase in preantral follicle diameters and estradiol production [21]. IGF-1 affects both granulosa and thecal cells causing, as evidenced in *in vitro* studies, an increase in granulosa cell proliferation and estradiol production, increased sensitivity of granulosa cells to FSH, rise in secretion of inhibin A, activin A and follistatin by granulosa cells and androgen synthesis support in thecal cells [22]. In humans, IGF-1 stimulates VEGF production by granulosa cells, but the major influence on autocrine functions in thecal cells and paracrine functions in granulosa cells is exerted by IGF-2.

The synergistic action of IGF-1 and FSH on enhancing proliferation and steroidogenesis in animals is well-known [23]. In the presence of FSH, IGF-1 increases the synthesis of LH receptors in thecal and granulosa cells [24].

IGF-binding proteins play a key role in IGF bioavailability regulation by selective IGF binding and preventing them from binding to their receptors. IGFBP are inhibitors of gonadotropin-induced follicular growth and differentiation, which results in its inhibition at the level of target cells. Changes of intrafollicular IGFBP levels lead to changes in IGF bioavailability and regulation of gonadotropin action on follicles. Some studies show that IGF bioavailability itself, rather than its concentration, changes during follicular growth and atresia. This is associated with changes in IGFBP mRNA expression and changes in their proteolytic degradation [25]. As with concentrations of protein binding insulin-like growth factors, high intrafollicular levels of insulin-like growth factors significantly correlate with oocyte fertilization rates and embryo morphological score. Furthermore, it has been shown that a combination of high intrafollicular concentrations of IGFBP-3 and IGFBP-4 with low concentrations of pregnancy-associated plasma protein A (PAPP-A) shows similar correlations [26].

PAPP-A, a glycoprotein synthesized in the trophoblast, which can be used for evaluating placental efficiency and the risk of Down syndrome, also seems to play an important role in follicular maturation [27]. It has been shown that this glycoprotein, whose levels in the follicular fluid rise until childbirth, acts as a protease and eliminates one of IGF-binding proteins, thereby affecting follicular development. PAPP-A can also take part in determining the dominant follicle and perhaps even in ovulation itself [28].

Amphiregulin is a cytokine that belongs to the epidermal growth factor (EGF) family. It probably mediates the effect of HCG on oocyte maturation. It has been detected in follicular fluid where its levels demonstrate an inverse correlation with fertilization rates, but do not affect embryo quality [29]. Intrafollicular proinflammatory cytokines are derived from plasma and their local ovarian synthesis. This group of cytokines includes interleukins (IL). It is believed that IL-1 beta of follicular fluid affects oocyte maturation and fertilization, but does not perform any role in embryo development after fertilization. IF-2 and IF-10 correlate with specific hormone levels in follicular fluid, but no relationship has been found with outcomes of assisted reproductive technology [5]. High levels of IF-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in follicular fluid have been observed in the case of embryos with high implantation potential. However, no significant relationship has been observed between intrafollicular IF-2, TNF-alpha and leukotriene B4 levels and oocyte maturation, fertilization and development of pregnancy. Nevertheless, the proportions between IL-1 alpha/ TNF alpha and IL-1 alpha/LTB4 (leukotriene B4) vary significantly between patients that managed and did not manage to get pregnant [30]. No significant differences in intrafollicular IGF-1, IL-6, IL-8, EGF and GM-CSF levels have been found between patients with good and poor response to sperm microinjection. However, PDGF (platelet derived growth factor) levels are high in this environment in good responders [31]. Follicle vascu-

larity, intrafollicular oxygen content and mitochondrial activity are factors that guarantee optimal oocyte development [5]. Reactive oxygen species (ROS) play a significant role in cellular signaling and homeostasis. However, during oxidative stress, the level of ROS can rise markedly and be harmful to oocytes. The exact influence of ROS on oocyte maturation requires further elucidation. A positive correlation between the ROS level in follicular fluid and oocyte maturity parameters has been observed. On the other hand, physiological ROS levels damage embryos and affect reproduction. It should be agreed that the optimal balance between oocyte access to oxygen and antioxidants is fundamental for the formation of normal meiotic spindle and an appropriate number of chromosomes. Free radical scavengers or endogenous antioxidants can counteract harmful effects of ROS. Follicular fluid contains superoxide dismutase and selenium-dependent glutathione peroxidase. A high level of the former is associated with oocyte fertilization disorders while a high level of the latter is linked with oocyte capability to be fertilized, but its low level is related to fertilization defects [32]. Total antioxidant capacity of follicular fluid is significantly higher in follicles containing an oocyte that is capable of being fertilized and shows a positive correlation with embryo quality and the number of pregnancies obtained using assisted reproductive technology [33].

The fluid in follicles containing mature oocytes that are capable of being fertilized and forming normal embryos contains lower amounts of nitrates and nitrites than in the case of embryos with low implantation potential. Moreover, the nitric oxide (NO) concentration is significantly higher in patients with endometriosis and hydrosalpinx, i.e. diseases associated with lower fertilization potential [9]. It seems then that intensive NO production in the oocyte microenvironment is unfavorable and leads to embryo development disorders [34].

The level of vascular endothelial growth factor (VEGF) in follicular fluid is a negative marker of oocyte quality since its synthesis is associated with hypoxia within the cumulus-oocyte complex. However, this marker has not been used in practice thus far [5].

Apoptotic changes in the follicular environment are associated with low oocyte quality. It is believed that an apoptotic antigen, serum soluble Fas (sFas), regulates follicular atresia and oocyte maturation. This factor is present in follicular fluid [35]. A low concentration of sFas and a high concentration of sFas-L (sFas ligand) indicate a high level of apoptosis and low oocyte quality [36]. A comprehensive analysis of data concerning the intrafollicular sFas level has shown that it is not a reliable marker of the quality of oocytes and embryos that develop from them [5].

Follicular fluid contains various proteins derived from serum filtrate and produced by granulosa and thecal cells. The identification of proteins that could be considered markers of good follicular development is technically complicated. That is why the understanding of the role of individual proteins in follicular growth and maturation is still limited [37]. Changes in alpha-fetoprotein, CEA and CA-125 antigens in follicular fluid seem to be insignificant in oocyte quality assessment [37]. Follicular fluid contains CD44 antigen, a glycoprotein engaged in intercellular interactions. Its concentration is lower in follicles containing oocytes capable of being fertilized and forming a normal embryo. It remains unknown, however, whether or not CD44 levels have a negative effect on oocytes [38]. It has been shown that leptin levels in follicular fluid correlate in a positive way with the fertilization rate and have a weak correlation with embryo morphological score. It is thought that leptin levels in this environment do not accurately represent oocyte quality and cannot be used for their selection [39].

Endothelin is a peptide composed of 21 amino acids and secreted by vascular endothelial cells. It plays a crucial role in vascular homeostasis. Endothelins have three isoforms: endothelin-1, endothelin-2 and endothelin-3. The concentration of endothelin-2 is significantly higher in follicles containing oocytes that can be fertilized and cleave [40]. The follicular concentration of this peptide correlates with IGF-1, which indicates their possible synergistic action promoting intrafollicular FSH activity [40].

Before oocytes become mature and able to be fertilized, the follicular fluid concentration of ovarian maturation inhibitor (OMI) declines. Follicles with high OMI activity contain immature oocytes, which means that OMI is a negative marker of oocyte quality [41].

It is believed that the concentration of certain amino acids in follicular fluid may be of predictive value, for instance D-asparagine acid, the presence of which correlates with the percentage of oocytes with good morphology [42]. Hyaluronic acid, the main component of the

extracellular matrix, is also present in follicular fluid, and its concentration is positively correlated with granulosa cell apoptosis and is higher in follicles containing oocytes that are incapable of being fertilized [43]. The level of hyaluronic acid in follicular fluid is low in cases of ineffective implantation and in hormonal disorders, which suggests that the production of this compound has an impact on embryo implantation potential [44].

Myo-inositol, a cyclic polyhydroxy alcohol containing six carbon atoms, is present in follicular fluid. Its concentration is positively correlated with estradiol levels as well as with the quality of oocytes and embryos that develop from them [45]. Myo-inositol supplementation improves the quality of oocytes obtained after ovarian stimulation in *in vitro* fertilization procedures [46].

CONCLUSION

Follicular fluid is the immediate environment of a developing oocyte. That is why, determining its composition is a good tool for assessing oocyte quality, its capability to be fertilized and further course of early pregnancy. The knowledge of factors associated with the highest oocyte quality facilitates rationalization of methods used in assisted reproductive technology and contributes to the understanding of the causes of early miscarriages after natural fertilization. The review of the available literature proves that further investigation in the composition and role of follicular fluid is necessary. It will contribute to better understanding of yet unknown mechanisms responsible for reproduction failures and pathology of early embryonic and fetal development.

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