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BLOOD SERUM AND FOLLICULAR FLUID RELAXIN: A PILOT STUDY OF THE HORMONE EFFECTS ON OVARIAN FUNCTION AND FERTILIZATION EFFICIENCY

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▪ ***Hypothesis/aims of study.*** To date, one of the most important avenues of research in the field of reproductive medicine is the searching for new biochemical markers of oocyte quality and the prediction of the effectiveness of *in vitro* fertilization (IVF) protocols. The aim of this study was to assess the effect of relaxin levels in blood serum and follicular fluid on the efficiency of ovulation stimulation, fertilization, and characteristics of the embryos.

Study design, materials and methods. This prospective randomized cohort study included 11 patients undergoing infertility treatment in a superovulation stimulation protocol using gonadotropin-releasing hormone antagonists. Age, body mass index, hormonal status, ovarian response, endometrial thickness and structure, the number and quality of oocytes and embryos, as well as fertilization efficiency were assessed. The level of relaxin in blood serum and follicular fluid samples was determined on the day of transvaginal follicle puncture using enzyme immunoassay.

Results. A correlation between follicular fluid relaxin levels and body mass index, age, the number of oocytes, and their fertilization efficiency ($p < 0.05$) was established. Changes in follicular fluid relaxin level were revealed depending on the gonadotropin preparations ($p < 0.05$) and triggers of final maturation of oocytes ($p < 0.05$). The tendency of the effect of gonadotropin doses on circulating relaxin levels, and of the hormone itself on endometrial thickness and the quality of oocytes was determined.

Conclusion. Determination of the relaxin concentration can be considered as a promising method for predicting the result of ovarian stimulation and the efficiency of fertilization in IVF protocols.

▪ **Keywords:** relaxin; *in vitro* fertilization; follicular fluid; oocyte; fertilization efficiency; endometrium.

РЕЛАКСИН СЫВОРОТКИ КРОВИ И ФОЛЛИКУЛЯРНОЙ ЖИДКОСТИ: ПИЛОТНОЕ ИССЛЕДОВАНИЕ ВЛИЯНИЯ ГОРМОНА НА ФУНКЦИЮ ЯИЧНИКОВ И ЭФФЕКТИВНОСТЬ ОПЛОДОТВОРЕНИЯ

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▪ ***Актуальность.*** Одним их важнейших направлений исследований в области репродуктивной медицины является поиск биохимических показателей качества ооцитов и прогнозирование эффективности протоколов экстракорпорального оплодотворения.

Цель — оценить влияние уровней релаксина в сыворотке крови и фолликулярной жидкости на эффективность стимуляции овуляции, оплодотворения, характеристики полученных эмбрионов.

Материалы и методы исследования. В проспективное когортное рандомизированное исследование вошли 11 пациенток, проходивших лечение бесплодия в протоколе стимуляции суперовуляции с применением антагонистов гонадотропин-рилизинг-гормона. Оценивали возраст, индекс массы тела, гормональный статус, овариальный ответ, толщину и структуру эндометрия, количество и качество ооцитов, эмбрионов, эффективность оплодотворения. Уровень релаксина в образцах сыворотки крови и фолликулярной жидкости определяли в день проведения трансвагинальной пункции фолликулов методом иммуноферментного анализа.

Результаты исследования. Установлена корреляция между уровнем релаксина в фолликулярной жидкости и индексом массы тела, возрастом, количеством ооцитов, эффективностью их оплодотворения ($p < 0,05$). Отмечено изменение уровня релаксина в фолликулярной жидкости в зависимости от применяемых препаратов гонадотропинов ($p < 0,05$), а также триггеров финального созревания ооцитов ($p < 0,05$). Выявлена тенденция влияния доз гонадотропинов на уровни циркулирующего релаксина и самого гормона — на толщину эндометрия и качество ооцитов.

Выводы. Определение концентрации релаксина можно рассматривать в качестве перспективной методики прогнозирования результата стимуляции яичников, эффективности оплодотворения в протоколах экстракорпорального оплодотворения.

▪ **Ключевые слова:** релаксин; экстракорпоральное оплодотворение; фолликулярная жидкость; ооцит; эффективность оплодотворения; эндометрий.

Background

According to modern scientific concepts, one of the leading criteria for the success of assisted reproductive technology (ART) programs is the quality of germ cells, the so-called oocyte factor, which determines the potential of fertilization, *in vitro* embryo development, implantation, and early pregnancy [1, 2]. Oocyte growth and maturation are directly related to the microenvironment of the developing follicle and depend on the composition of the follicular fluid (FF), including blood plasma components that have passed through the follicular barrier and products of the secretory activity of granulosa and thecal cells of the follicle [3]. At the same time, FF aspiration during transvaginal follicle puncture (TFP) is a routine and integral part of the procedure, which makes it an accessible medium to search for biochemical predictors of oocyte quality and predict the effectiveness of *in vitro* fertilization (IVF) protocols [4]. To date, more than 37 such markers have been described, including reactive oxygen species, gonadotropic and sex steroid hormones, and several others, as well as cytokines, growth factors, amino acids, prostaglandins, inhibins, vitamins, and vitamin-dependent proteins [5, 6].

One of the potentially promising markers is the relaxin hormone, a pleiotropic polypeptide of the insulin superfamily with a molecular weight of about 5–6 kDa, which has a multifactorial effect on the functions of various systems and organs. One of the most studied forms of the hormone, which is involved in the functioning of the reproductive system, is relaxin-2 [7]. In women, relaxin is secreted by the ovarian corpus luteum and granulosa and theca cells of the follicle. It is also produced in the endometrium and placenta during pregnancy [8]. Relaxin is involved in the maturation of oocytes and affects the development rate and quality of embryos [9]. Thus, relaxin receptors (relaxin/insulin-like family peptide receptor [RXFP] 1) are expressed in granulosa cells of primary and secondary human follicles [10]. *In vitro* expression of RXFP1 and RXFP2 receptors and the hormone itself were determined in oocytes, cumulus cells, and preimplantation embryos (at all developmental stages, including blastocysts). The addition of relaxin to the culture medium increases the content of mature oocytes and promotes the development of pig and primate embryos, improving their in vitro quality [11–13].

The correlation between the folliculogenesis processes and relaxin concentration increase in

FF was established [14]. The correlation between the blood serum relaxin concentration and oocyte count obtained is of interest [15]. In view of the above, some authors have considered relaxin as a predictor of successful embryo transfer. For example, relaxin levels >800 pg/mL produced by luteinizing granulosa cell culture were associated with implantation and prolongation of pregnancy after an IVF cycle, whereas levels <200 pg/mL were associated with protocol failure [16].

Studies examining relaxin at preimplantation stages and in ART protocols are sporadic and ambiguous. Thus, the determination of relaxin concentrations may be a relevant avenue in predicting the ovarian response to stimulation and fertilization process in IVF protocols and reflecting their effectiveness.

Aim. This study aimed to assess the effect of relaxin levels in blood serum and FF on the ovarian response, the count and quality of oocytes, the efficiency of fertilization, and the number and quality of embryos obtained by superovulation stimulation in IVF protocols.

Study design, materials, and methods

The prospective cohort randomized study included married couples with infertility ($n = 9$) and patients participating in the delayed motherhood program ($n = 1$) and in the oocyte donation program ($n = 1$), who applied to the Department of Assisted Reproductive Technologies of the D.O. Ott Research Institute of Obstetrics and Gynecology. A total of 11 patients were examined according to the inclusion criteria, namely, patients who were aged 20 to 43 years and who provided voluntary informed consent to participate in the study. (The consent form was approved by the Ethics Committee of the D.O. Ott Research Institute of Obstetrics and Gynecology.) The exclusion criteria were IVF contraindications, large uterine myoma, body mass index (BMI) >35 kg/m^2, endometrial hyperplastic processes, infectious and systemic autoimmune diseases, type 1 or 2 diabetes mellitus, and any localized malignant neoplasms.

Stimulation of ovulation

All patients underwent standard examination before entering the IVF protocol. Ovarian stimulation was performed from day 3 of the menstrual cycle using a fixed protocol and recombinant gonadotropins (rFSH Gonal-F, Merck Serono, Italy, and rFSH + rLH Pergoveris, Merck Serono, Switzerland) or human menopausal gonadotropins (hMGT-Meriofert, IBSA Institut Biochimique, S.A., Switzerland) and a gonadotropin-releasing hormone (GnRH) antagonist (ganirelix 0.25 mg, Vetter Pharma, Organon, Germany). The starting dose was adjusted depending on the ovarian reserve, age, BMI, and previous stimulation protocol outcomes. If necessary, the dose was adjusted according to the ovarian response. Recombinant human chorionic gonadotropin Ovitrelle at a dose of 250 μg (Merck Serono, Italy) or GnRH agonist Diphereline at a dose of 0.2 mg (Ipsen Pharma Biotech, France) was used as ovulation triggers ($n = 7$ and 4, respectively).

All patients underwent ultrasound monitoring of ovarian stimulation (number of growing follicles and diameter) and endometrium assessment (structure and thickness). Doses of gonadotropin preparations were prescribed individually. The criterion for the final oocyte maturation trigger was the presence of 3 or more follicles with a diameter of >17 mm. The TFP was performed under general anesthesia with ultrasound guidance 36 h after the trigger injection.

During TFP, the number of punctured follicles and the number and maturity of the resulting oocyte-cumulus complexes (OCCs) were recorded. The follicles were not washed. The quality of oocytes was assessed using a fertilization method of intracytoplasmic sperm injection (ICSI) and was determined as the ratio of oocytes at the metaphase stage of the second meiotic division (*MII*) to the number of OCCs. In the standard IVF technique, the fertilization efficiency was determined as the ratio of zygotes with two pronuclei on day one of the development to the number of OCCs obtained. In ICSI fertilization, the efficiency was estimated as the ratio of two nuclear zygotes on day one of the development to the number of oocytes at the *MII* stage on the day of puncture. Embryos were cultured until day five on Vitrolife media (Sweden). Embryos were assessed after five days according to Gardner (1999). Blastocysts ≥3BB were considered good-quality embryos. Embryos were transferred to three patients on day five of culturing.

The luteal phase of the cycle was supported by progesterone preparations from the day of follicle puncture.

Collection of blood serum samples and aspiration of FF

Blood samples were obtained from female patients in the study group immediately before the follicular TFP procedure and before the anesthetic agents were administered. The samples were then centrifuged for 20 min at 1500 g. FF was aspirated by puncturing each follicle (with obligatory assessment of OCCs and without further capacity separation). FF samples were centrifuged at 1500 g for 20 min. The resulting material was stored for 1–2 months at −80°C according to the guidelines.

Determination of relaxin levels in blood serum and FF

The relaxin level in the material under study was determined using a commercial kit for quantitative determination of relaxin-2 by sandwich enzyme immunoassay according to the manufacturer's instructions (SED868Hu enzyme-linked immunosorbent assay kit for relaxin 2, USA). The range of the determined concentrations was 3.1–500 pg/mL. According to the guidelines, there were no differences in the methods used to determine FF and blood serum relaxin.

Statistical analysis

Statistica 10 software package (StatSoft, Inc., USA) was used for data processing. The normality of distribution was checked using the Shapiro–Wilk test. The nonparametric Mann–Whitney criterion and Kruskal–Wallis test were used to compare the parameters being studied. Data are presented as median (25^{th} to 75^{th} percentile). A multivariate factor analysis was performed to comprehensively describe the objects: it allows to describe the real correlation between study attributes and assess the reliability and accuracy of conclusions based on the obtained data. The factor loadings (*a*) were calculated and interpreted as correlations between the corresponding study indicators and individual factors (hypothetical, not directly measurable, latent attributes, and to some extent related to the measured indicators). The factors were identified by principal component analysis. The data obtained for the analysis were normalized. Factor analysis was also performed to assess correlations. When assessing the correlation between the study indicators, the Spearman's rank correlation coefficient r_s was used. The correlation strength was assessed according to the following values: 0–0.3, very weak; 0.3–0.5, weak; 0.5–0.7, average; 0.7–0.9, high; 0.9–1, very high. *P* values <0.05 were considered statistically significant.

Results

Blood plasma and FF relaxin concentrations were assessed in infertile patients ($n = 11$) treated in the IVF protocol with ovulation stimulation. In connection with the cryopreservation of obtained oocytes ($n = 1$), cancelation of embryo transfer due to ovarian hyperstimulation syndrome ($n = 1$), and segmentation of cycle ($n = 5$), we failed to perform a correct statistical data processing to assess the correlation between treatment efficiency and relaxin levels in FF and blood serum. Pregnancy was confirmed by pelvic ultrasound examination in two patients.

The study revealed a significant correlation between FF relaxin and age ($r = -0.65$), BMI ($r = -0.67$), number of punctured follicles ($r = 0.66$) and oocytes obtained ($r = 0.62$), and high correlation strength with the efficiency of fertilization ($r = -0.85$). Significant correlations between the study indicators and blood relaxin were absent. The observed correlation between blood relaxin and some of the study parameters, including particular endometrial thickness, oocyte quality, and total gonadotropin dose in the protocol, was a tendency (Fig. 1).

A significant difference in FF relaxin levels was demonstrated in patients who used rFSH/rFSH + rLH compared with those taking hMGT ($Z = 2.279$; $p < 0.05$). No significant difference was found for relaxin levels in blood serum depending on the gonadotropin preparations used. The decrease in blood relaxin levels with the use of menotropins was only a tendency (Fig. 2, *a*).

A difference was found between the indicators of FF relaxin levels depending on triggers used for final oocyte maturation. There was no significant difference for relaxin levels in blood serum depending on the indicated preparations ($Z = -2.051$, $p < 0.05$; Fig. 2, *b*).

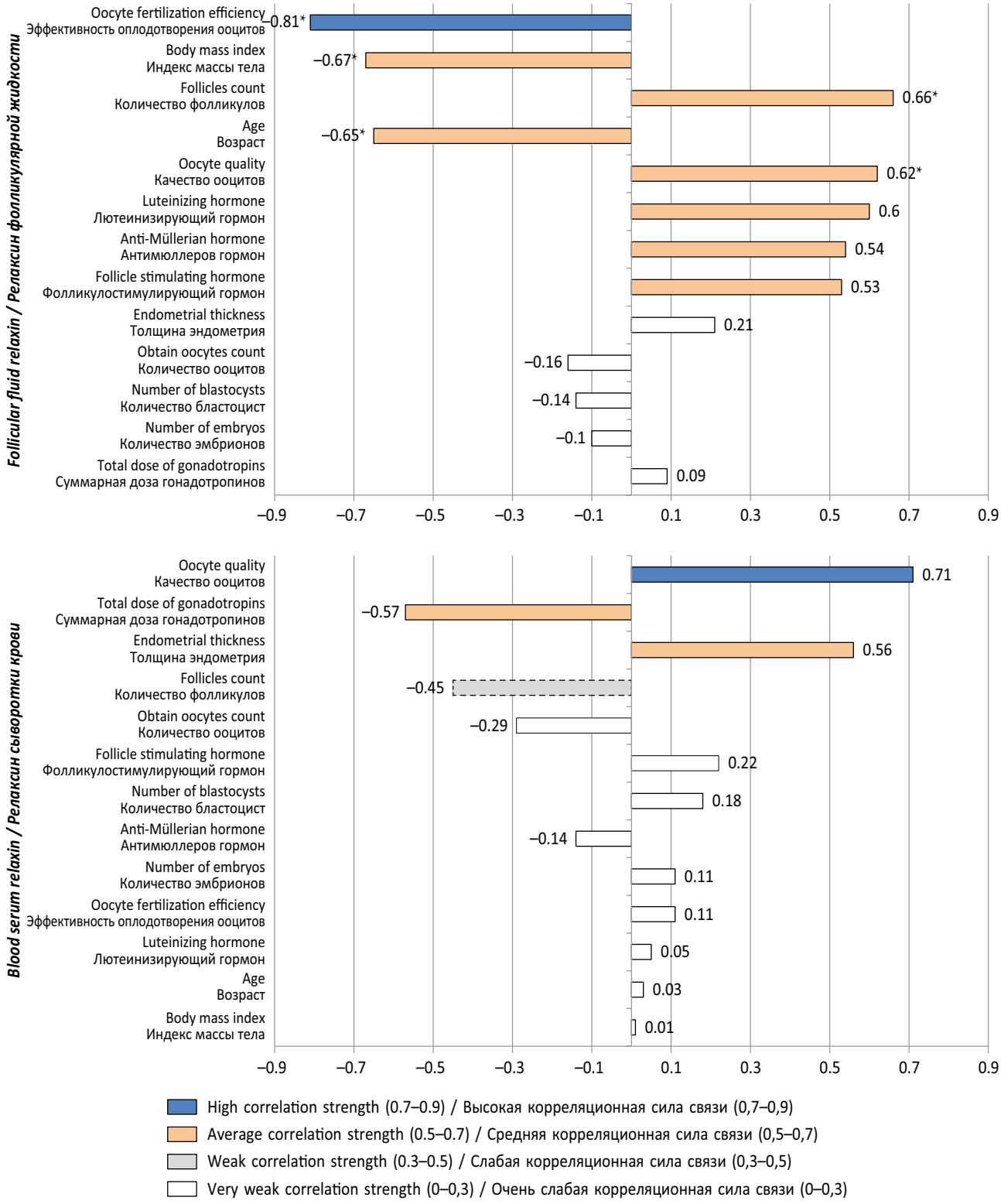

Fig. 1. Correlation between blood serum and follicular fluid relaxin levels and the studied parameters (* $p < 0.05$)

Рис. 1. Корреляция между уровнем релаксина в фолликулярной жидкости и сыворотке крови и исследуемыми показателями (* $p < 0,05$)

Analysis with load assessment revealed two factors. The first factor was associated with FF relaxin ($a = 0.96$), age ($a = -0.87$), AMH level ($a = 0.99$), BMI ($a = -0.87$), TFP day ($a = 0.99$), number of punctured follicles punctured ($a = 0.99$) and oocytes obtained ($a = 1$), and fertilization efficiency ($a = -0.99$). The second factor has the highest association with blood

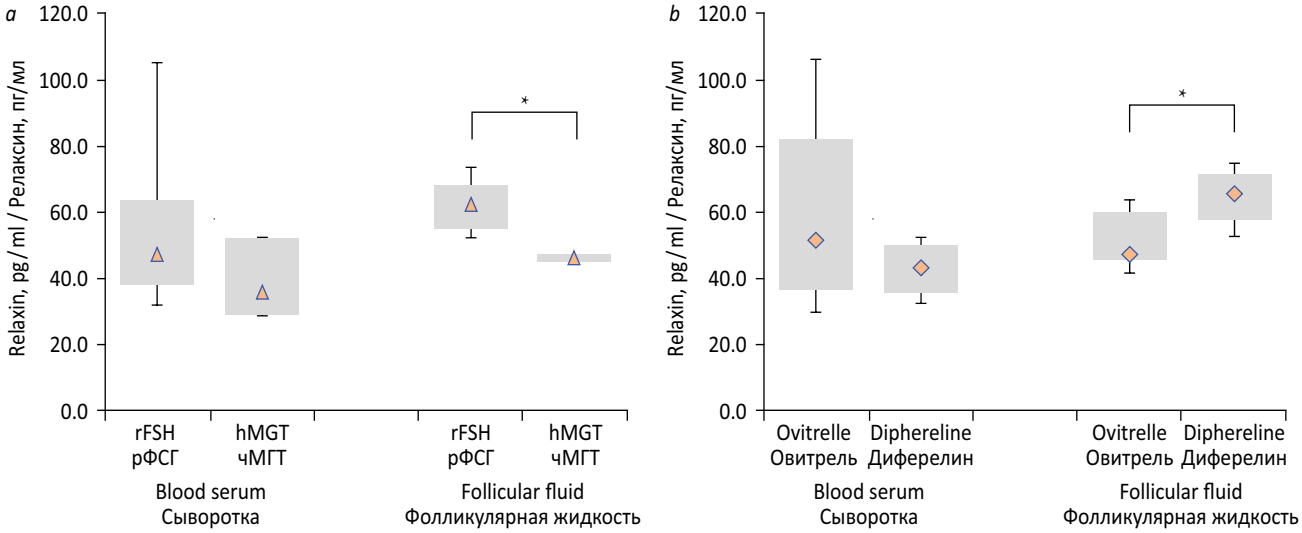

Fig. 2. *a*. Blood serum and follicular fluid relaxin levels with the use of gonadotropin preparations in the study groups; *b*. Blood serum and follicular fluid relaxin levels with the use of triggers of final oocyte maturation in the study groups (* $p < 0.05$)

Рис. 2. Содержание релаксина в сыворотке крови и фолликулярной жидкости в исследуемых группах пациентов при применении различных препаратов гонадотропинов (*a*); содержание релаксина в сыворотке крови и фолликулярной жидкости в исследуемых группах пациентов при применении различных триггеров финального созревания ооцитов (*b*) (* $p < 0,05$)

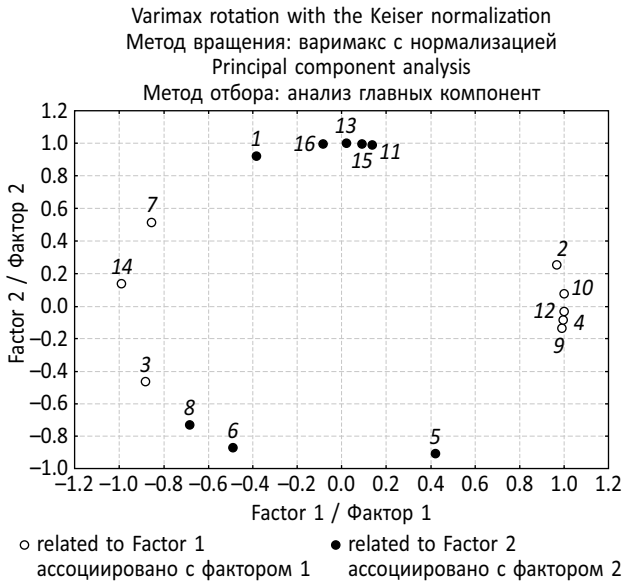

Fig. 3. Two-dimensional graph of factorial loads of the studied indicators. Graph numbering: indicators associated with Factor 1: *2*, follicular fluid relaxin level; *3*, age; *4*, blood serum anti-Müllerian hormone level; *7*, body mass index; *9*, day of puncture; *10*, number of punctured follicles; *12*, number of oocytes; *14*, fertilization efficiency; indicators associated with Factor 2: *1*, blood serum relaxin level; *5*, blood serum luteinizing hormone level; *6*, blood serum follicle-stimulating hormone level; *8*, ∑ dose of gonadotropins; *11*, endometrial thickness; *13*, quality of oocytes; *15*, number of blastocysts on day 5; *16*, number of good quality embryos on day 5

serum relaxin ($a = 0.9$), total gonadotropin dose ($a = 0.84$), oocyte quality ($a = 0.99$), endometrial thickness ($a = 0.99$), and number ($a = 0.99$) and quality ($a = 0.99$) of embryos on day 5 of their development. This factor is inversely related to the luteinizing ($a = -0.89$) and follicle stimulating ($a = -0.88$) hormones (Fig. 3).

No correlation was found between relaxin levels in FF and blood serum and the duration of infertility, number of pregnancies and labor, and history of ovarian surgery. No correlation was also found between the FF values and blood relaxin concentrations and the number and quality of the embryos obtained in the patients of this group.

Рис. 3. Двухмерный график факторных нагрузок исследуемых показателей: показатели, связанные с фактором 1: *2* — уровень релаксина в фолликулярной жидкости, *3* — возраст, *4* — уровень антимюллерова гормона в сыворотке крови, *7* — индекс массы тела, *9* — день проведения пункции, *10* — количество пунктированных фолликулов, *12* — количество ооцитов, *14* — эффективность оплодотворения; показатели, связанные с фактором 2: *1* — уровень релаксина в сыворотке крови, *5* — уровень лютеинизирующего гормона в сыворотке крови, *6* — уровень фолликулостимулирующего гормона в сыворотке крови, *8* — суммарная доза гонадотропинов, *11* — толщина эндометрия, *13* — качество ооцитов, *15* — количество бластоцист на 5-е сутки, *16* — количество эмбрионов хорошего качества на 5-е сутки

Discussion

According to the literature, the pool of circulating relaxin is determined by the level and duration of corpus luteum function during the luteal phase of the cycle and in pregnancy, whereas FF relaxin levels reflect the secretory activity of granulosa and theca cells [8, 17]. In this study, relaxin levels in the bodily fluids were assessed only on the day of follicular TFP, which probably explains the lack of correlation between relaxin levels in FF and blood.

In vitro studies revealed an increase in FF relaxin concentration with the growth of the follicle [18, 19]. An association was found between relaxin levels in blood serum and follicle and oocyte counts obtained in IVF protocols [20]. In our study, we found a correlation between the concentration of FF relaxin and the number of punctured follicles and oocytes obtained, which partly agrees with the results of previous studies. This may be explained by the production of relaxin both by steroid-producing follicle cells with their naturally increased number during ovarian stimulation in IVF cycles and by OCCs and the oocytes themselves. Relaxin may be involved in the signaling interaction between the oocyte and surrounding somatic cells, in the processes of folliculogenesis, and oocyte growth and maturation [17]. However, in some studies, no correlation was found between FF relaxin levels and the oocyte count obtained [21].

Morphofunctional maturity of the oocyte is the basis for successful fertilization and is important for developing the embryo in the early stages. Some *in vitro* studies demonstrate the expression of relaxin receptors and the hormone itself by oocytes, OCCs, as evidenced by the favorable effect of relaxin in addition to the cell culture medium. In this study, only a tendency for the effect of relaxin levels in blood serum on oocyte quality was observed. However, various individual factors, including genetic ones, were not taken into account. We also found a negative correlation between FF relaxin concentrations and oocyte fertilization efficiency, which should be further studied in a larger sample.

We obtained a significant inverse correlation between FF relaxin, age, and BMI of the patients. No data were found in the available literature on the correlation between blood relaxin or FF levels and age. We demonstrated a decrease in FF relaxin levels with increasing age of patients, which is presumably associated with physiological processes of decreased relaxin production by follicle cells with age-related changes in the ovaries (decreased ovarian reserve, increased follicle atresia rate, and reduced oocyte quality) [22].

Current literature suggests negative effects of increased BMI on female fertility, particularly on oocyte quality, embryo development, and abnormal metabolites in FF. One of the mechanisms for the realization of the adverse effect of this factor may be the lack of production of the relaxin hormone and the disruption of its signaling pathways [23, 24].

No studies describing changes of relaxin levels in blood and FF depending on the preparations used in the IVF protocol were found. Thus, we found during the study that FF relaxin secretion was higher with the use of recombinant gonadotropin (Gonal-F, Pergoveris) compared with the urinary (Meriofert) preparations, which should also be studied in more detail. At the same time, the effect of the total dose of gonadotropins on the hormone indicators in FF was not revealed, but a tendency toward a negative correlation with the blood relaxin level was observed. The difference in FF relaxin concentrations was determined depending on the triggers used for the final maturation of oocytes; the hormone level was higher when using the GnRH agonist Diphereline. Probably, the results obtained are explained by the duration of exposure to the preparations used.

Relaxin changes the processes of phase transformation, endometrial decidualization, implantation, and trophoblast invasion, which must be taken into account when considering the effect of blood serum relaxin on indicators of endometrial thickness.

The LGR-7 relaxin receptor is expressed in the stromal and glandular cells of the human endometrium; RXFP1 was found in the uterine myometrium. The hormone itself is secreted by endometrial cell structures during both the proliferative and secretory phases of the menstrual cycle, as well as during the implantation window [25]. Relaxin stimulates the expression of endometrial secretory proteins (glycodelin, an insulin-like growth factor binding protein-1), which

confirms its participation in implantation [9, 26]. Relaxin enhances the production of vascular endothelial growth factor in stromal and glandular endometrial cells, especially during the secretory phase of the cycle. *In vitro* studies have shown that treatment with relaxin preparations plays an important role in stimulating endometrial angiogenesis, increases the proliferation of endothelial cells in the uterine blood vessels and the number of endometrial arterioles, which leads to endometrial thickening, increases the probability of implantation, and affects the invasion of trophoblasts [27, 28].

Conclusion

Based on the pilot study, the level of relaxin in FF and blood serum may be a potential marker for predicting ovarian response to stimulation, the count and quality of oocytes, and the efficiency of their fertilization. Further studies are needed to assess the role of relaxin in the processes of oocyte maturation, fertilization, embryo development, and implantation. The data obtained will allow us to determine the effects of relaxin production and its signaling pathways on the efficiency of ART programs.

Additional information

Conflict of interest. The authors declare no conflict of interest.

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Contribution of authors

O.N. Bespalova, the concept and design of the study.

V.A. Zagainova, E.A. Lesik, E.A. Komarova, collection of the material.

N.N. Tkachenko, V.L. Borodina, use of laboratory research methods.

V.A. Zagainova, material processing.

Yu.P. Milyutina, statistical data processing.

V.A. Zagainova, O.V. Kosyakova, I.D. Mekina, text writing.

O.N. Bespalova, A.M. Gzgzian, I.Yu. Kogan, text editing.

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