STEROID HORMONES OF FOLLICULAR FLUID AND THE OUTCOME OF IN VITRO FERTILIZATION

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STEROIDNI HORMONI U FOLIKULARNOJ TEČNOSTI I ISHOD VANTELESNE OPLODNJE

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ABSTRACT

One of the succes factors of biomedically assisted fertilization is the regular maturation of one or more oocytes. The quality of the oocytes is significantly influenced by the environment in which it is located, the so-called „microenvironment" that includes cumulus cells, follicular fluid in which hormones and growth factors involved in its growth and development are secreted. The main aim was to examine whether the concentration of steroid hormones in the follicular fluid affects the rate of fertilization and the outcome of the in vitro fertilization process itself. The study included 31 patients who were included in vitro fertilization procedure at the Department for Biomedically Assisted Fertilization, Clinic for Gynecology and Obstetrics, Clinical Center Kragujevac. We used follicular fluid as biological material for analysis. Examination of the obtained follicular fluid and collection of oocytes under a stereomicroscope was done in the embryological laboratory at the Department. Biochemical parameters of follicular fluid were analyzed in the Department for Laboratory Diagnostics, Clinical Center Kragujevac. In vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) methods were used as the fertilization method. The criteria of the Istanbul Consensus of Clinical Embryologists were used as a reference framework for embryo quality assessment. Pregnancy was confirmed by a positive serum level of the hormone β-hCG 14 days after embryo transfer. A software package SPSS 20 was used for statistical data processing. The results of the analysis of follicular fluid samples show that there was no statistically significant difference in the concentration of estradiol, progesterone and testosterone in follicular fluid in relation to fertilization rate and the outcome of in vitro fertilization. Based on our results, it can be concluded that the concentration of steroid hormones did not affect fertilization rate and the outcome of in vitro fertilization.

***Keywords**: biomedically assisted fertilization, follicular fluid, oocytes, steroid hormones.*

SAŽETAK

Jedan od faktora uspešnosti biomedicinski potpomognute oplodnje (BMPO) je pravilno sazrevanje jedne ili više jajnih ćelija. Na kvalitet jajne ćelije značajan uticaj ima okruženje u kojem se ona nalazi, tzv. mikrosredina koja uključuje ćelije kumulusa, folikularnu tečnost u kojoj se izlučuju hormoni i faktori rasta uključeni u njen rast i razvoj. Glavni cilj je bio ispitati da li koncentracija steroidnih hormona u folikularnoj tečnosti utiče na stopu oplodnje i na ishod samog procesa vantelesne oplodnje. Istraživanje je obuhvatalo 31 pacijentkinju, koje su bile uključene u postupak vantelesne oplodnje na Odeljenju za biomedicinski potpomognutu oplodnju, Klinika za ginekologiju i akušerstvo, Klinički centar Kragujevac. Kao biološki materijal za analizu koristili smo folikularnu tečnost. Pregled dobijene folikularne tečnosti i sakupljanje jajnih ćelija pod stereomikroskopom urađen je u embriološkoj laboratoriji na Odeljenju BMPO. Biohemijski parametri folikularne tečnosti analizirani su u Službi za laboratorijsku dijagnostiku, Kliničkog centra Kragujevac. Kao metoda fertilizacije korišćeno je vantelesno oplođenje konvencionalni način ili intracitoplazmatska injekcija spermatozoida metoda. Pri proceni kvaliteta embriona korišćeni su kriterijumi Istambulskog konsenzusa kliničkih embriologa, kao referentni okvir. Trudnoća je potvrđivana pozitivnim nalazom serumskog nivoa hormona β-hCG 14 dana posle embriotransfera. Za statističku obradu podataka korišćen je programski paket SPSS 20. Rezultati analize uzoraka folikularne tečnosti pokazuju da ne postoji statistički značajna razlika u koncentraciji estradiola, progesterona i testosterona u folikularnoj tečnosti u odnosu na stopu oplodnje i ishod vantelesne oplodnje. Na osnovu naših rezultata može se zaključiti da kocentracija steroidnih hormona ne utiče na stopu oplodnje i ishod vantelesne oplodnje.

***Ključne reči:** biomedicinski potpomognuta oplodnja, folikularna tečnost, jajne ćelije, steroidni hormoni.*

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ABBREVIATIONS

GV- germinal vesicle
BMI- body mass index
HbsAg- hepatitis B
HCV- hepatitis C virus
HIV- human immunodeficiency virus
FSH- follicle-stimulating hormone
LH- luteinizing hormone
TSH- thyroid-stimulating hormone
FT3- triiodothyronine
FT4- thyroxine
AMH- Anti-Mullerian hormone
HSG- hysterosalpingography
SBER- selective estrogen receptor blockers
HMG- human menopausal gonadotropin
IVF- in vitro fertilization
ICSI- intracytoplasmic sperm injection

INTRODUCTION

One of the most serious problems which couples face in today's world is infertility; our country is not exception to this rule. Infertility affects approximately 10-15% of the world's population and it is constantly growing in the last decade. Globally, one in six/seven couples worldwide has some difficulty conceiving (1). Although the frequency and cause(s) of infertility are not fully characterized, current studies show that approximately 40% of cases can be attributed to the male factor, 40% to the female factor and 20% to combined problems of both sexes (2). Consequnetly, assisted reproductive techniques are developing rapidly and today include many methods for achieving pregnancy (3).

One of the factors for success of biomedically assisted fertilization is proper maturation of one or more oocytes. The main objective of embryologists is to asses the quality of oocyte based on morphology of cumulus-oocyte complex and of the oocyte itself after the removal of the cumulus cells. The oocyte quality is significantly influenced by the environment in which it is located i.e. microenvironment that includes cumulus cells as well as follicular fluid. Hormones are secreted in follicular fluid thus contributing to growth of oocyste and development (4). Therefore, in order to increase the success of biomedically assisted fertilization methods it is necessary to focus on determining critical "microenvironment" parameters that allow us to estimate the quality of oocytes easily and quickly. Any change in follicular fluid composition can affect the oocyte which may potentially affect its development and quality, fertilization and early embryonic development (5, 6, 7, 8).

Estradiol, progesterone and testosterone are the main steroid hormones that play an important role during the follicular and luteal phases of the menstrual cycle. Nevertheless, there are still inconsistencies in the literature regarding the concentrations of estradiol and other steroid hormones in follicular fluids (9). Higher estradiol and progesterone levels were found in follicles from which oocyte with higher fertilization rate were obtained. These observations, however, have not been confirmed by other studies, but it has been proven that the quality of the embryo was not related to the levels of follicular estradiol and progesterone. Regarding pregnancy rates, increased estradiol and progesterone levels in follicular fluid were associated with greater success (10). Lower progesterone concentrations and higher testosterone concentrations were measured in follicular fluid containing germinal vesicle oocytes (*germinal vesicle* - GV) compared to follicular fluid containing MII oocytes (11).

THE AIM

This study focuses on examining the levels of estradiol, progesterone and testosterone in the follicular fluid obtained from stimulated cycles. The main goal is to examine whether the concentration of steroid hormones in follicular fluid affects fertilization rate and the outcome of in vitro fertilization process itself.

METHODS

Patients

The study included 31 patients who were included in the in vitro fertilization procedure at the Department for Biomedically Assisted Fertilization, Clinic for Gynecology and Obstetrics, Clinical Center Kragujevac. All participants were informed about the objectives of the research before joining the research and all of them signed the consent for participation in the research.

The including criteria were: spouses, respectively extramarital partners who have exhausted other possibilities of infertility treatment, spouses or extramarital partners who in the existing community have one child obtained in the IVF procedure, preserved ovarian function, normal body mass index of a woman (BMI). Also, patients had to have the results of the following analyzes and diagnostics:

- microbiological tests (bacterial vaginosis, vaginal and cervical swabs for bacteria, fungi and *Chlamydia trachomatis*), virological tests (*Hepatitis B* (HbsAg), *Hepatitis C virus* (HCV), human immunodeficiency virus (HIV)), hormonal status (2-3 days of menstrual cycle - follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, testosterone, prolactin, thyroid-stimulating hormone (TSH), triiodothyronine hormone (Triiodothyronine- FT3), thyroxine (Thyroxine- FT4), Anti-Mullerian hormone (AMH), cervical screening (Pap smear, colposcopy), ultrasound examination performed with a vaginal probe, hysterosalpingography (HSG).

All results had to be within the reference values.

Criteria for exclusion from the study were: Couples who have not exhausted other options for treating infertility, women who have not preserved ovarian reserve, women who have BMI >30 kg/m², anomalies and benign tumors of the uterus, fallopian tubes and ovaries that prevent the process of in vitro fertilization, the occurrence and pregnancy development, the presence of malignant or suspected tumors of the uterus, fallopian tubes and ovaries, any diseases (internal medicine, immunology, infectology, neurology, psychiatry) if they are without permission to perform the IVF procedure of the appropriate specialist, diseases in which anesthesia or pregnancy would potentially endanger the patient's life. Those patients who suffer from any other endocrine disease that has been confirmed to affect fertility were excluded from the study.

We used follicular fluid as biological material for analysis. Follicular fluid was obtained by puncturing the follicles from 18 mm to 20 mm after controlled ovarian stimulation in the intervention room at the Department for Biomedically Assisted Fertilization. Examination of the obtained follicular fluid and collection of oocytes under a stereomicroscope was done in the embryological laboratory at the Department for Biomedically Assisted Fertilization. Biochemical parameters of follicular fluid were analyzed in the Department for Laboratory Diagnostics, Clinical Center Kragujevac.

Stimulation of ovulation

Stimulation of ovulation is a procedure to stimulate ovulation and controlled ovarian hyperstimulation using various stimulation protocols and the use of gonadotropin drugs, GnRH antagonists and agonists, as well as drugs that belong to the group of selective estrogen receptor blockers (SBER) (Klomifen and Letrozol).

All patients were determined for basal hormonal status, on the second or the third day of the cycle, before being included in the procedure. Serum levels of estradiol, progesterone, follicle-stimulating hormone and luteinizing hormone were determined.

A short stimulation protocol was used according to a certain scheme, depending on the gynecologist's assessment based on the ultrasound findings and hormonal status.

During the stimulation procedure, drugs belonging to the group of selective estrogen receptor blockers (SBER), injections of human menopausal gonadotropin (HMG) such as Merional or Menopur and drugs from the group of antagonists (Cetrotide) were used to counteract the effects of natural pituitary hormones.

During the stimulation, the growth of follicles and the level of sex hormones in the blood were monitored by ultrasound, with successive ultrasound and laboratory examinations.

Stimulation lasted until the leading follicle reached a diameter of 20 mm or two or more follicles with a diameter of 18 mm.

When the increase in serum estradiol concentration corresponded to the presence of two or more follicles > 18 mm, human chorionic gonadotropin (Pregnyl, Organon, the Netherlands) was administered at a dose of 5000 IU, 34-36 hours before oocyte aspiration.

Follicular fluid and oocytes

Oocyte aspiration is a surgical intervention and is performed by ultrasound control while the patient is under general anesthesia. The intervention lasts about 15 to 20 minutes, depending on the number and availability of follicles in the ovaries. After the aspiration of the follicles was completed, the obtained follicular fluid was examined under a microscope in the embryological laboratory and the oocyte number and quality were determined.

Follicular fluids were isolated in which oocytes were found for laboratory analysis. The entire amount of follicular fluid was centrifuged at 3000 rpm for 10 minutes to separate pure follicular fluid, without cellular elements. For the analysis itself, 5 ml of the total amount of follicular fluid was taken.

In vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) methods were used as the fertilization method. After incubation, 16-20 hours after fertilization it is checked whether fertilization has occurred or not. Fertilization has occured if two pronuclei and / or two polar bodies are present.

The fertilization rate is calculated according to the following formula:

$$\frac{Number\ of\ fertilized\ oocytes}{Total\ number\ of\ obtained\ oocytes} \times 100$$

The criteria of the Istanbul Consensus of Clinical Embryologists were used as a reference framework for embryo quality assessment (12). Criteria include assessment of the fragmentation degree and symmetry of the blastomeres. Embryos are divided into class A (excellent, without or 1-10% fragmentation, perfect symmetry), class B (medium, 11-25% fragmentation, moderate asymmetry), class C (poor, > 25% fragmentation, expressed asymmetry). An embryo transfer of a maximum of three embryos was performed under the control of transabdominal ultrasound on the third or the fifth day after oocyte aspiration. From the day of oocyte aspiration, the patients received progesterone depot intramuscularly on the second and the fifth day as a support of the luteal phase. Pregnancy was confirmed by a positive serum level of the hormone β-hCG 14 days after embryo transfer, while clinical pregnancies were confirmed by transvaginal ultrasound findings of a gestational sac with a vital embryo at 6 weeks of gestation.

The implantation rate is calculated according to the following formula:

$$\frac{Total\ number\ of\ pregnancies}{Total\ number\ of\ transfered\ embryos} \times 100$$

The variables measured in the study were: the concentration of hormones (estradiol, progesterone and testosterone) in the follicular fluid.

The values of estradiol, progesterone and testosterone in the follicular fluid were analyzed by chemiluminescent immunoassay on the device UniCel 600, Becman Coulter in the Department for Laboratory Diagnostics, Clinical Center Kragujevac. Follicular fluid samples were diluted to determine estradiol (1: 1000) and progesterone (1: 1000) concentrations, while no follicular fluid dilution was required to determine testosterone concentration.

Statistical data processing

Regularity of the distribution of obtained values was confirmed by using the Kolmogorov-Smirnov test before statistical data processing. Based on the obtained value of p, the test used for statistical analysis was determined (ANOVA parametric test for $p < 0.05$ - samples having normal distribution or nonparametric Mann-Whitney, Kruskal-Wallis and Hi square test test if $p > 0.05$ - samples that do not have a normal distribution). The value of the obtained data was considered statistically significant if $p < 0.05$. The software package SPSS 20 was used for statistical data processing. All values are expressed as a mean value and are presented in a table.

RESULTS

Table 1 presents the relevant results and characteristics of the study group. In total 31 patients were involved, starting from age of 29 till 46. On average 9.1 oocytes were obtained per woman, however, there is a strong deviation between individual participant going from 1 till 20. Only 172 cells were normally fertilized (around 60,5%). It should be pointed out that about 58% of pregnancies were successful. Results show that measured data for estradiol concentration in follicular fluid, progesterone concentration in follicular fluid, and testosterone concentration in the follicular fluid is widely dispersed from the mean.

Table 1. Relevant characteristics and results of the study group

Number of patients	31
Years - mean	36,33 (29-46)
Total number of oocytes	284
Mean value of oocytes per patient	9,16 (1-20)
Number of fertilized oocytes	172 (1-11)
Number of unfertilized oocytes	112 (0-15)
Fertilization rate	60,5%
Achieved pregnancies (β-HCG>5)	18
Implantation rate	31%
Pregnancy rate	58%
Mean estradiol concentration in follicular fluid (ng/L)	1974,65 (115,11-4859,85)
Mean value of progesterone concentration in follicular fluid (µg/L)	11,44 (1,72- 20.48)
Mean value of testosterone concentration in the follicular fluid (ng/ml)	5,53 (1,43-13,33)

High p-values imply that the oocyte fertilization rate is not direclty related to the concentration of estradiol, progesterone and testosterone in the follicular fluid (see, Table 2). Nerevetheless, it should be noted that in case of normally fertilized oocytes in follicular fluid estradiol and progesterone levels were slightly lower, while testosterone levels were slightly higher. In addition, statisitcaly looking the fertilization rate is not related to the outcome of IVF (see, Table 3).

Table 2. Relationship between steroid hormone concentration in follicular fluid and fertilization rate

Steroid hormones of follicular fluid	Hormone concentrations in follicular fluid (Mean 95%)		
	Normal fertilization (172)	Poor fertilization (112)	p*
Estradiol ng/ml	1821,79	1872,07	0,639
Progesterone µg/ml	10,71	11,01	0,673
Testosterone ng/ml	5,27	5,13	0,956

p* Statistical significance

Table 3. Relationship between fertilization rate and realized pregnancies

	Positive pregnancy outcome	Negative pregnancy outcome	*p
Fertilization rate (mean value)	67,34 (±15,15)	61,31 (±19,22)	0,379

p* Statistical significance

Analysis show that there is no statisticaly significant relation between the achieved pregnancies and applied fertilization method (see, Table 4). The pregnancies via IVF were 49% successful, while via the ICSI method 51%. Further,

results indicate that there is no dependency between embryo quality and fertilization methods (p=0,832; p>0,05). Also, there is no statisitcly significant difference between participant age and realized pregnancies within the study group (see, Table 5).

Table 4. Relationship between in vitro fertilization outcomes and oocyte fertilization methods

IVF outcome	Метода		p*
	IVF	ICSI	
Positive outcome (β-HCG>5)	9 (49%)	10 (51%)	0,121
Negative outcome (β-HCG<5)	2 (16,71%)	10 (83,3)	

p* Statistical significance

Table 5. Relationship between mean values of hormone concentrations in follicular fluid and in vitro fertilization outcomes

Steroid hormones of follicular fluid	Hormone concentrations in follicular fluid (Mean 95%)		
	Positive outcome (β-HCG<5)	Negative outcome (β-HCG>5)	p*
Estradiol ng/L	1860,27 (115,11-4857,85)	2133,01 (426,33-4626,82)	0,540
Progesterone µg/L	11,28 (1,93-20,48)	11,67 (1,72-19,56)	0,859
Testosterone ng/ml	5,55 (2,51-13,33)	5,48 (1,43-7,70)	0,708

p* Statistical significance

Results show that there is no statisticly significant deviation between the value of estradiol in follicular fluid of pregnant women and the value of estradiol in the follicular fluid of non-pregnant women (see, Table 5). This also applies to the variation of estradiol values in follicular fluid by age of women (see, Table 6).

The value of progesterone and testosterone in the follicular fluid of pregnant women did not differ statistically from the value of progesterone in the follicular fluid of women who were not pregnant (see, Table 6). Variations in progesterone and testosterone levels in follicular fluid by age of women were also not statistically significant (see, Table 5).

Table 6. Relationship between follicular fluid hormone concentration and age, and between in vitro fertilization outcome and age

Steroid hormones	Mean value of hormone concentration in follicular fluid by age			p*
	30-34	35-39	39-42	
Estradiol (ng/ ml)	1978,97 (957,96-4626,82)	1981,28 (426,33-4857,85)	1959,10 (115,11-2817,11)	0,859
Progesterone (µg/ml)	10,83 (5,96-15,62)	10,99 (1,72-20,48)	12,98 (7,23-17,98)	0,626
Testosterone (ng/ml)	5,77 (2,51-13,33)	5,01 (1,43-7,82)	5,86 (3,52-7,70)	0,598
Positive outcome (β-HCG>5)	8	8	2	0,051
Negative outcome (β-HCG<5)	4	3	6	

p* Statistical significance

DISCUSSION

Approximately 10% of women are unable to naturally become pregnant until the age of 34, and for women between 40 and 45 this is 87%. Although IVF can to a certain extend increase the number of achieved pregnacnies among younger women (i.e. age below 40), these numbers are still relatively low among older women (i.e. age group 40+). All of this is associated with decreased oocyte number and poor oocyte quality with increasing age, along with a higher rate of aneuploidy (13).

It should be pointed out that not all oocytes from all follicles are of the same quality and at the same stage of maturity. Based on the defined monitoring parameters, it is not possible to know in advance which oocyte will be fertilized and provide quality embryos. Embryos of the same patient can be of different quality and still at the end not all embryos will result in pregnancy (10, 14). The various components of the follicular fluid that contains the oocytes can be used as parameters to predict the quality of the oocyte that will be fertilized and give a potentially good embryo resulting in pregnancy. Steroid hormones are important parameters among these components (15).

When comparing the outcome of IVF with the age of women who participated in the study, it can be seen that similar success rates were among women who are in the age group 30–34 and 35–39. On the other hand, the women in the age group 40–42 had the lowest pregnancy success rate. As expected fertility significantly decreases as a woman ages.

Results show that steroid hormone concentrations were not in correlation with age of participants.

In this study, a combination of two methods i.e. ICSI and conventional IVF methods were used for fertilization. The results show that the success of achieving pregnancy does not depend on the method of fertilization, and the quality of the embryo does not depend on the method of fertilization. More IVF were successful after the application of the ICSI method for fertilization of oocytes.

Furthermore, no significant correlation was observed between the concentration of steroid hormones and the rate of fertilization, nor in relation to the outcome of IVF. During the study it was noticed that the concentrations of estradiol and progesterone were slightly lower, while testosterone levels were slightly higher in case of the successful IVF. This was not confirmed in an earlier study (10). Carpintero et al. noticed that elevated levels of estradiol and progesterone in the follicular fluid were associated with an increased chance of pregnancy (10). Estradiol concentrations were slightly elevated in age group 35–39. On the other hand, the concentrations of progesterone were slightly elevated, as well as the concentrations of testosterone in age group 40–42.. In-depth analysis didn't show any statistically significant difference in the concentration of estradiol, progesterone and testosterone in follicular fluid between fertilized or unfertilized oocytes. Caprineto et al. pointed out that testosterone concentrations increased slightly with good fertilization, which was also confirmed in this study. On the other hand, Lamb et al. results show that there was no difference in testosterone concentration in follicular fluid in cases of normal fertilization compared to cases without fertilization (10,16).

Numerous studies have performed 'target analysis' but failed to identify a clinically useful biomarker(s) due to well known limitations within current medical practice. There is a still long way to go to overcome these issues (17, 18,19). One of the major issues is the fact that transfer is done with two embryos. For example, in a situation when only one embryo was successfully implanted, it is difficult to say which embryo was implanted. Furthermore, the process of ovarian stimulation can change the composition of follicular fluid (18). Finnaly, follicular fluid contamination with a follicle flush medium that contains numerous metabolites (e.g. glucose) is possible. Some IVF centers use this method when aspirating follicular fluid. Additionaly, follicular fluid from a previously aspirated follicle can lead to fluid contamination of the next follicle.

Overall, further research is needed to enable more successful in vitro fertilization. A much larger number of follicular fluid samples are needed to more reliably determine which parameters affects the quality of oocytes and to what extent, thus the rate of fertilization i.e. the outcome of IVF.

CONCLUSION

In today's world infertility must be perceived as a complex issue which is challenging to overcome. Even though there are constant breakthroughs within medicine, it is difficult to discover the real cause of infertility. Extra attention is being paid to the composition of follicular fluid as a possible cause of infertility. From the results, it can be concluded that the concentration of steroid hormones does not affect the fertilization rate and the outcome of in vitro fertilization. Consequnetly, the next step in research would be to further study other sex hormones in addition to steroid hormones among larger number of patients. Punctureing each follicle with multiple stitches using a separate needle for each follicle would be more insightful research, however, this is not feasible in clinical practice at the moment.

CONCLUSION

The authors declare that there is no conflict of interest. The authors are solely responsible for the content of this article.

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