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Original paper

THE EFFECT OF HORMONAL THERAPY ON HEMOSTATIC PARAMETERS IN *IN VITRO* FERTILIZATION

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Summary: Hemostatic system, as an integral part of biological homeostatic mechanism in humans, is subject to changes due to effect of hormonal therapy used in preparation for *in vitro* fertilization. The objective of the study was to analyze the impact of hormonal changes on homeostatic parameters during four cycles of preparation, according to long-term protocol for *in vitro* fertilization. The following parameters were determined in the study: prothrombin time, activated partial thromboplastin time, fibrinogen, antithrombin III, plasminogen, α_2-antiplasmin and plasminogen activator inhibitor. In the period of ovarian hyperstimulation and in postovulatory period, significantly lower PT, APTT, AT III, PAI-1 and α_2-APL values were obtained ($p<0.05$), while fibrinogen concentration was significantly higher ($p<0.05$), in relation to the first two periods. PLG activity was significantly decreased in postovulatory period in relation to former three periods ($p<0.05$). Data obtained by Pearson's correlation analysis show that there is significant negative correlation in postovulatory period between PLG and α_2APL, as well as between APTT and PAI-1 ($R_p>0.404$, for $p<0.05$). These changes are manifested as more pronounced procoagulant forms, which are suppressed by simultaneous activation of fibrinolytic system as the opposite response, what keeps the balance in homeostatic system and prevents the development of adverse thrombotic complications.

Key words: in vitro fertilization, hormones, hemostatic parameters

Introduction

In the last few years, *in vitro* fertilization (IVF), as refined method of the assisted reproduction, has been used for treatment of infertility. For achieving the ovarian stimulation and with a view to produce as many functional follicles as possible, protocols using different hormonal combinations have been implemented (1). Most commonly, so-called long-term protocol has been used, which includes the application of gonadotropin releasing hormone (GnRH) analogue, with the intent to induce, by multiple doses of GnRH agonist, reversible blockade of gonadotropic and ovarian function, that is, the suppression of endogenous gonadotropic secretion (2). Further application of exogenous humane menopausal gonadotropins (HMG) produces superovulation and larger number of functional follicles. Since the suppressive effect of GnRH analogue extends to the second phase of the cycle, stimulation or substitution therapy (human chorion gonadotropin hCG, or progesterone) is an imperative, which simulates the luteal phase, because GnRH analogue suppresses the secretion of endogenous LH. The application of these hormonal agents leads to production of multiple number of follicles and rapid rise of endogenous estradiol and progesterone concentrations what sometimes may produce side-effects, primarily on hemostatic system (3).

Hemostasis, as one of the most important integral parts of biological homeostatic mechanism in humans, is indirectly involved in the maintenance processes of functional body integrity. Recent studies, both in the field of normal hemostasis and in the domain of hemostatic disorders, have been based on foundations of biochemical regulatory mechanisms, molecular biology and genetic engineering.

The role of hormones in development of higher risk of thromboembolic complications is well-known, as much in pregnancy as in application of hormones for therapeutical reasons, administered in the form of oral contraceptives and substitution therapy (4, 5).

The objective of the study was to analyze the impact of hormonal changes on hemostatic parameters, during the four periods of preparation for IVF, when the woman's body is initially brought into the

state of hypoestrogenism followed by extremely high level of serum estradiol. The following parameters were measured for evaluation of hemostatic system: coagulation parameters (prothrombin time-PT, activated partial thromboplastin time-APTT, fibrinogen-FIB), physiological coagulation inhibitor antithrombin III-AT III, fibrinolytic plasminogen parameter-PLG, and physiological fibrinolysis inhibitors (α_2-antiplasmin α_2-APL, plasminogen activator inhibitor - PAI-1).

Material and Methods

The study included 25 women in the protocol of *in vitro* fertilization, 28 to 42 years of age. During the therapeutical IVF program, the ovulation was stimulated by combined application of GnRH analogue (Suprefact) and sole or combined gonadotropins, such as FSH (Metrodin) and/or HMG (Humegon). GnRH agonists were applied from day 19 or 21 of former cycle to the second day of menstrual cycle. The agonists were used until adequate hormonal concentrations were achieved: LH < 5 IU/L; estradiol < 50 pg/mL and progesterone < 1 ng/mL, as well as sonographic verification of the absence of follicles, what resulted in reversible hypogonadotropic hypogonadism. Gonadotropins (Metrodin and/or Humegon) were applied from the time of the achieved hormonal and ultrasound parameters, in the way that the doses were increased in relation to the growth of follicles and other biological indicators. The stimulation of follicular growth was controlled by monitoring the estradiol concentrations, and ultrasound verification of follicular size, i.e., monitoring of average growth of follicles in each ovarium and the thickness of endometrium. The ovulation was induced by h-CG administration (Pregnyl), when the size of follicles was 10 24 mm and mean endometrial thickness 10 13.5 mm, as well as the estradiol concentration was 500 1000 pg/mL. Programmed aspiration of oocytes from stimulated follicles was carried out 36 hours after h-CG application.

The sampling was carried out in four time periods:

Period I basic value, before the beginning of therapy;

Period II suppression of hypophyseal function, minimum estradiol concentration, before HMG (Humegon•) administration;

Period III ovarian hyperstimulation, maximum estradiol concentration, just before hCG (Pregnyl•) adminsitration;

Period IV postovulatory period, after hCG administration just before embryonal transfer.

The following parameters were measured in plasma samples: PT, APTT, FIB, AT III, PLG, PAI-1 and α_2-APL. Plasma was used for measuring the hemostatic parameters. Blood samples were taken from 7 to 9 morning hours. Sodium citrate was used as anticoagulant.

Coagulometry was used for PT, APTT and FIB measurements, while spectrophotometry was applied for AT III, PLG; PAI-1 and α_2-APL, along with commercial Behring tests using the Behring Coagulation Timer coagulometer (6).

The following statistical methods were used in the study: Student's t-test and Pearson's correlation analysis (Rp Pearson's correlation coefficient) (7).

Results

In order to evaluate the effect of hormonal therapy on hemostatic parameters in *in vitro* fertilization, their values were measured in four different periods of preparation for IVF. The changes of determined parameters were compared between these periods and reciprocally.

Table I illustrates $\bar{x}$ and SD for each determined parameter during four periods of measurements as well as their referential values.

Comparison of the values of these parameters between different periods of preparation for *in vitro* fertilization showed the following results: PT and APTT values were found to be significantly lower while FIB concentrations were significantly higher in periods III and IV in relation to periods I and II; significant de-

Table I Clinical evaluation of hemostatic parameters during the period of preparation for *in vitro* fertilization

Parameter	I	II	III	IV	Reference values
PT, s	13.6 ± 1.27	13.2 ± 1.23	12.5 ± 1.22*	12.7 ± 0.88*	11 15
APTT, s	36.7 ± 5.56	36.1 ± 4.01	34.2 ± 3.15*	34.5 ± 3.60	26 36
FIB, g/L	2.6 ± 0.51	2.6 ± 0.44	3.4 ± 0.58*	3.9 ± 0.63*	1.8 3.5
AT III, %	99 ± 11.9	95 ± 8.9	90 ± 7.2*	86 ± 7.2*	75 120
PLG, %	100 ± 10.9	99 ± 10.9	97 ± 9.1	92 ± 6.2*	75 120
α_2-APL, %	97 ± 9.6	96 ± 6.9	92 ± 8.4*	90 ± 7.3*	80 120
PAI-1, U/mL	3.1 ± 0.99	3.4 ± 0.83*	2.8 ± 0.70*	2.6 ± 0.77*	0.3 3.5

* significant value in relation to basic ($p<0.05$)

crease of AT III and PAI-1 levels was found in periods III and IV in relation to periods I and II, and significant difference was also found between period I and II; significant decrease of PLG activity was found in period IV in comparison with other three periods; significant α_2-APL decrease was found in periods III and IV in relation to periods I and II, and significant difference was also found between period III and IV.

Reliability of correlation between values of measured hemostatic parameters was analyzed in all four observed periods. Considering that the most significant changes were found in hyperstimulatory and postovulatory periods, Pearson's correlation test was carried out only for periods III and IV. Data obtained from this analysis revealed significant correlation ($R_p > 0.404$, $p < 0.05$) between PLG and α_2-APL ($R_p = 0.524$), and APTT and PAI-1 ($R_p = 0.459$) in period IV.

Discussion

Hemostasis is the result of balance between coagulation, i.e. formation of fibrin coagulum responsible for stasis of hemorrhage, and fibrinolysis, which promotes decomposition of fibrin and elimination of thrombus. Hormonal therapy in the form of oral contraceptives or hormonal substitution therapy in postmenopausal women interferes with hemostatic system in view of side-effect. Large number of studies suggests the possibility of procoagulant conditions frequently causing thromboembolic complications due to application of such therapy (8, 9). Extremely high effect has been reported in application of third-generation oral contraceptives.

In vitro fertilization, as state-of-the-art assisted reproduction, uses protocols with different hormonal combinations to achieve ovarian stimulation and produce as many functional follicles as possible. Rapid rise of endogenous estrogen concentration as the result of such therapy affects hemostasis as well (10 12).

Determination of *prothrombin time* is the measure of activity of coagulation factors of extrinsic pathway of coagulation (II, V, VII, X, fibrinogen), while *partial thromboplastin* time is the indicator of the status of coagulation factors of intrinsic pathway of coagulation (VIII, IX, XI, XII), prekallikrein, high-molecular-weight kininogen and factors of both intrinsic and extrinsic pathways of coagulation cascade (fibrinogen, II, V, X). Relatively shorter PT and APTT noted in studies by Kodama and associates (3) is explained by the increase of concentrations of particular coagulation factors due to hemoconcentration, what is manifested by higher risk of thrombosis. Lox and Canez (14) also interpreted such shorter PT and APTT as the consequence of increased concentration of factors II, V, VII, IX and X, what was hypothetically explained by higher activity of liver during more marked biodegradation of larger amounts of estrogen. Our study reported significantly shorter prothrombin time in phases of ovarian hyperstimulation and postovulatory period in comparison with basic period, while significantly shorter partial thromboplastin time was seen in hyperstimulation period in relation to basic one (*Table I*). These changes remained within referential values in all patients.

Fibrinogen, being the most prevalent coagulation factor, is susceptible to changes of concentration under the activity of hormonal drugs, what is suggested by numerous studies dealing with effects of oral contraceptives and hormonal substitution therapy on parameters of hemostasis (15). Higher fibrinogen concentration during hormonal preparation for IVF is explained by estrogen stimulation of liver for fibrinogen synthesis (16), decreased fibrinogen clearance (17), or presence of fibrinogen as the protein of the acute phase of inflammatory process which participates in ovulatory process (7). Our study reveals significant increase of fibrinogen concentration in periods III and IV in relation to the first two periods, when such increase exceeds the normal limits and indicates the shift of balance to coagulation process (*Table I*). Fibrinogen together with PT and APTT measurements, as the principle regular tests for evaluation of coagulation factor changes, may be used for monitoring of procoagulant oscillations during the preparation for IVF.

Antithrombin III is the most important inhibitor of blood coagulation. Its major function is the inactivation of thrombin and F Xa, while it neutralizes, to a lesser degree, the activity of IXa, XIa and XIIa coagulation factors, as well as the activity of kallikrein and plasmin. The mechanism of thrombin inhibition is expressed by its bonding to AT IIIa reactive center and formation of stable and irreversible thrombin-antithrombin (TAT) complex. The decrease of concentration of this coagulation factor is present in hormonal therapy both because of administration of oral contraceptives (18) and implementation of different IVF protocols (7, 16). Such drop of activity is believed to be the result of the increased consumption of antithrombin III due to developed hypercoagulation (17), provoked by large amounts of endogenous estradiol. During the examinations, a significant decrease of AT III level was noted in periods III and IV in relation to the first two periods (*Table I*). Changes of AT III were within reference values.

Major enzyme of fibrinolytic system degrading the fibrin is plasmin. According to its structure and characteristics, plasmin is serine protease and it is found in plasma as inactivated form proenzyme plasminogen. Plasmin expresses its proteolytic activity by degradation of arginine and lysine bonds primarily in fibrin and fibrinogen molecules. Multiple heterogenous compounds have the capacity of converting the plasminogen into plasmin. They are known as plasminogen activators. The most important *plasminogen activators* are the following: tissue plasminogen activator (t-PA 1 and t-PA 2) and urokinase-plasminogen activator type (u-PA). The studies dealing with the

effect of hormonal therapy on fibrinolytic system show the increase of plasminogen concentration and, most commonly, the increase of concentration of its activators (12, 9), what corroborates the hypothesis that the activation of fibrinolysis is also involved in balance process along with the activation of coagulation. During the IVF preparations, our subjects manifested significant fall of plasminogen activity in period IV in comparison with former three ones. Considering that the values changed within normal limits, the change of plasminogen concentration only failed to give sufficient information on resulting hypofibrinolytic processes (*Table I*).

Several inhibitors are involved in the inhibition of fibrinolytic process, and their activity is expressed either via direct plasmin inhibition (antiplasmins) or through plasminogen activator inhibition (PAI).

α_2-antiplasmin (α_2APL) is the most important plasmin inhibitor. The inhibition of fibrin degradation under the effect of plasmin is accomplished by α_2APL in three ways: very rapid inhibition of free plasmin in plasma, inhibition of plasminogen bonding to fibrin, and cross-linking with fibrin α-chains during blood coagulation. *Plasminogen activator* inhibitors are plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). They belong to serine proteases. PAI equally inhibits both t-PA and u-PA, but has no effect on prourokinase activity. Lower fibrinolytic activity is the most frequent impairment of hemostatic system causing the thrombophilia, while the increase of PAI-1 concentration is the most common cause of this condition. It has been proved that hormones interfere with the activation of fibrinolytic process just by having the effect on its inhibitors. Higher estrogen level predisposes the increase of fibrinolytic potential due to reduced activity of PAI-1 and α_2-APL (12). The increase of t-PA concentration, as endothelial component of fibrinolysis, along with concurrent decrease of PAI-1 concentration lead to activation of fibrinolysis as equilibrium response to activated coagulation. Estrogens are considered to reduce the synthesis of PAI-1 or to increase its clearance, what results in higher t-PA activity (19). These fibrinolytic effects are diminished in postmenopausal women who are not covered by hormonal substitution therapy, and consequently, they are at higher risk of cardiovascular disorders. The obtained results showed significantly lower PAI-1 concentrations in periods III and IV in relation to periods I and II, and also significantly lower activity of α_2-APL in periods III and IV in comparison with earlier periods as well as between periods III and IV (*Table I*). Although all changes of fibrinolysis inhibitors remained within referential values, they supported the fact that reduced activity of fibrinolysis inhibitors produced the activation of fibrinolytic system itself, what was highly significant for opposing the procoagulant processes and preventing the thrombosis.

In postovulatory period, where maximum level of endogenous estradiol is followed by rise of progesterone concentration due to application of humane chorion gonadotropin, the correlation of plasminogen and antiplasmin may be noted, suggesting the development of hypofibrinolysis, which is simultaneously compensated with the fall of concentration of its inhibitors, favoring the fibrinolytic process as opposing to activated coagulation. This is supported by the correlation between coagulation parameters (APTT) and inhibitors of fibrinolysis (PAI). Correlation coefficient between PLG and α_2-APL was 0.524, while between APTT and PAI-1 was 0.459, what was statistically significant in both cases.

These changes are manifested as more pronounced procoagulant forms, which are being suppressed by simultaneous activation of fibrinolytic system as the antagonistic response, what keeps the balance in hemostatic system and prevents the development of adverse thrombotic complications. Our group of patients had no such complications.

Given the course of changes occurring in hemostatic system, predictive testing of hemostatic parameters is recommended in women who are scheduled for IVF preparation, both in those patients predisposed to thrombotic incidences due to genetic deficits of some hemostatic parameters as well as in others for detection of probable deficits of coagulation and fibrinolytic factors. Such screening allows for early detection of pathological changes in hemostatic system as well as making diagnosis, treatment and, accordingly, evasion of thromboembolic complications.

UTICAJ HORMONSKE TERAPIJE NA PARAMETRE HEMOSTAZE U *IN VITRO* FERTILIZACIJI

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Kratak sadržaj: Hemostazni sistem, kao integralni deo biološkog homeostatskog mehanizma čoveka, je podložan promenama usled dejstva hormonske terapije korišćene u pripremi za *in vitro* fertilizaciju. Cilj ovog rada je bio ispitivanje uticaja hormonskih promena na hemostatske parametre u toku četiri ciklusa pripreme, prema dugotrajnom protokolu za *in vitro* fertilizaciju. U radu su određivani parametri: protrombinsko vreme, aktivisano parcijalno tromboplastinsko vreme, fibrinogen, antitrombin III, plazminogen, α_2-antiplazmin i plazminogen aktivator inhibitor. U periodu hiperstimulacije ovarijuma i u postovulacionom periodu dobijeno je značajno sniženje PT, APTT, AT III, PAI-1 i α_2 ($p<0,05$), i značajni porast koncentracije fibrinogena ($p<0,05$), u odnosu na prva dva perioda. Aktivnost PLG je bila značajno snižena u postovulacionom periodu u odnosu na prethodna tri perioda ($p<0,05$). Podaci dobijeni Pearson-ovom korelacionom analizom pokazuju da postoji značajna negativna korelacija u postovulacionom periodu izmedu PLG i α_2APL, kao i između APTT i PAI-1 ($Rp>0,404$, za $p<0,05$). Ove promene se manifestuju kao izraženije prokoagulantne forme, koje bivaju suprimirane istovremenim aktiviranjem fibrinolitičkog sistema kao suprotnog odgovora, čime se održava ravnoteža u hemostaznom sistemu i sprečava nastanak neželjenih trombotičkih komplikacija.

Ključne reči: in vitro fertilizacija, hormoni, hemostatski parametri

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