CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.2643089

Available online at: http://www.iajps.com Research Article

COMPARISON OF ESTRADIOL VALERATE AND 17B-ESTRADIOL EFFICACY IN ART PROGRAMS WITH APPLICATION OF VITRIFIED EMBRYOS AT PATIENTS WITH PREVIOUS MULTIPLE IMPLANTATION FAILURES

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Article Received: February 2019 **Accepted:** March 2019 **Published:** April 2019

Abstract:
Aim. This article is devoted to the problem of multiple implantation failures of the thawed embryos and the endometrium preparation with the application of HRT, which include estrogen medicines. The variety of estrogen medicines and the lack of information about their comparative effectiveness in ART programs prompted us to conduct a study aimed at finding the most optimal and effective estrogen medicine. For comparative analysis, we selected the oral forms of EV and 17β–E due to their ease of use and dosing.
Materials and methods. The objects of the study were different according to the chemical structure of the oral forms of estrogen-containing medicines (EV and 17β–E) for HRT. Both are used in the process of endometrium preparation for the transfer of thawed embryos in patients with previous repeated implantation failures. The subjects of the study were the levels of expression of α-ER and markers of proliferative activity of cells (Ki-67) in the endometrium, echographic characteristics of the endometrium, and the amount of serum estradiol in the blood and the effectiveness of thawed embryo transfer performed using different forms of estrogen.
Results. Significant differences were revealed ($p<0.001$), when comparing the levels of α-ER expression in the endometrium and the marker of endometrium proliferative activity Ki-67 under the influence of various forms of estrogen in the study groups. Analysis of proliferative changes in the endometrium based on the ultrasound examination also revealed statistically significant differences in the study groups ($p<0.001$). The difference was likewise noted in its echostructure. The analysis of the influence of various estrogen medicines on estradiol serum levels did not reveal any significant differences in the estradiol blood level at patients of the analyzed groups in any days of MP ($p>0,1$). The statistical analysis revealed significant differences in the frequency of positive outcomes (clinical pregnancy) in the study groups ($\chi 2 = 6,40$, $p = 0,041$, the number of degrees of freedom $df = 2$) with the best indicators in Group 2.
Conclusions. Based on the above provided data, it can be concluded that in the framework of hormone replacement therapy in the preparation of the endometrium for the thawed embryo transfer at patients with previous multiple implantation failures, it is preferable to use the oral form of 17β–E, as compared to the oral form of EV.
Keywords: *implantation failures, frozen embryo transfer protocols, hormone replacement therapy, 17β-estradiol, immunohistochemistry.*

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Please cite this article in press Anna Sulima et al., ***Comparison of Estradiol Valerate and 17β-Estradiol Efficacy in Art Programs with Application of Vitrified Embryos at Patients with Previous Multiple Implantation Failures,*** *Indo Am. J. P. Sci, 2019; 06(04).*

INTRODUCTION:

Multiple disorders of reproductive function in patients with multiple implantation failures of the thawed embryos highlight the need for endometrium preparation with application of hormone replacement therapy (HRT), which contains estrogen medicines [1-8]. The variety of estrogen medicines and lack of information about their comparative effectiveness in ART programs prompted us to conduct a study which aims at finding the optimal and most effective estrogen medicine. For the purposes of our comparative analysis, we selected the oral forms of estradiol valerate (EV) and 17β-estradiol (17β–E) due to their ease of use and dosing.

Micronized 17β-E is an estrogen identical in its chemical structure to endogenous estradiol, which is the most active estrogen and exceeds the activity of estrone in 5-15 times. Estron is a result of hepatic metabolism of 17β-E, the ratio of estradiol to estron is 0.7. When ingestion of 2 mg 17β-E per day, its plasma concentration fluctuates between 293-550 pmol/l for several hours and decreases 146-183 pmol/l after 24 hours. The equilibrium concentration of estradiol in the blood plasma occurs after about 5 days, with daily oral administration of 17β–E [9, 10]. EV is an ether of crystalline form 17β-E. After absorption in the intestine, EV is metabolized during the first passage through the liver to 17β-E, so it can be considered as a precursor of natural estrogen. In the course of the second passage through the liver, estron and estron sulfate are formed, and the ratio of estradiol to estron is 0.17. The maximum serum concentration of estradiol achieved with oral EV is approximately 110.2 pmol/l and is usually achieved within 4-9 hours after taking the drug [9].

Based on the above data, we suggested the more effective preparation of the endometrium for the transfer of thawed embryos with the use of oral forms of the drug containing 17β–E in comparison with the medicine containing EV, in view of its bioidentity to a natural estrogen and the characteristics of its metabolism.

MATERIAL AND METHODS:

The oral forms of estrogen-containing medicines - estradiol valerate (EV) and 17β–E – are the object of the study. The latter two differ from each other according to their chemical structure and are used for hormone replacement therapy (HRT) while preparing endometrium for the transfer of thawed embryos in patients with previous repeated implantation failures.
The subjects of the study comprised: levels of expression of α-estrogen receptors (α-ER) and markers of proliferative activity of cells (Ki-67) in the endometrium, echo graphic characteristics of the endometrium, and the amount of serum estradiol in the blood and the efficacy of cryotransfer with application of different forms of estrogen.

Instrumental, morphological, morphometric, immunohistochemical, enzyme immunoassay and statistical methods were utilized.

The study included 100 patients aged 24 to 45 years with two or more implantation failures of a thawed embryo in the history. The patients were divided into 2 groups:

1) Group 1 – 50 patients who received an oral form of estradiol valerate (EV) in a dose of 4 mg from 2nd day of menstrual period (MP) during endometrium preparation for the transfer of one thawed embryo;
2) Group 2 - 50 patients who received an oral form of 17β-estradiol (17β–E) in a dose of 4 mg from 2nd day of MP during endometrium preparation for transfer of one thawed embryo.

To assess the effect of estrogen medicines on the state of the endometrial receptor apparatus, all patients underwent an endometrial biopsy in the period between the 9^{th} and 11^{th} day of MP preceding the cycle with the transfer of the thawed embryo using a manual vacuum aspirator Ipas MVA Plus® Aspirator (USA) [11].

Preservation of materials was carried out in the 10.0% buffered formalin solution for the purpose of maintaining integrity of tissues and cells. Subsequently, the histological materials were exposed to alcohols of the increasing concentration (50.0%, 60.0%, 70.0%, 80.0%, 96.0% and absolute alcohol), and were clarified in xylol. Finally, the materials were enclosed in paraffin as well as manufactured into paraffin blocks. Cuts of 4-5 microns were taken from paraffin blocks and colored with hematoxylin and eosin [12].

The immunohistochemical research (IHC) was conducted following a standard technique [12]. It comprised the application of paraffin blocks, reactants of the DAKO company, Mouse Monoclonal Antibodies to α-ER (a clone 1D5 DAKO, Denmark, RTV), Ki-67 Antibodies (a clone of MIB-1 of DAKO, Denmark, RTV) and grouping of CD138 plasmocytic (sindekan-1 DAKO, Denmark, RTV). To analyze the results of IHC reactions, the following quantitative method was used: Histochemical score index (H-score) was calculated using the formula HS=1a +2b+3c, where a is the percentage of poorly

painted cells, b corresponds to the percentage of moderately painted cells; c is equal to the percentage of strongly painted cells; 1, 2, 3 indicate the intensity of coloring expressed in points.

The level of α-ER expression was estimated based on the H-score: a result of 0 to 10 points is considered to be negative, a number of 11to 100 points is observed as low, an outcome of 101–200 is classified as moderate, and a development of 201+ points is categorized as high [12]. The morphometric study also measured the percentage of Ki-67 positive cells in the glands and a stroma using a microscope at a magnification of 400x within the Software DP-SOFT program with subsequent statistical processing. Photos were made with the OLYMPUS C 5050Z digital camera OLYMPUS CX41 fixed on the microscopes with a video eyepiece of DCM-130E SCOPE.

Blood level of estradiol was measured on the 2^{nd}-3^{rd}, 6^{th}-7th, 10^{th}-11^{th}, 14^{th}-16^{th} day of the menstrual period and on the day of embryo transfer (Days 19 to 21) by the enzymoimmunoassay (EIA) method with application of "DRG EIA Estradiol" reagent (closed joint stock company "DRG of Tekhsistems, Russia) ranging from 60 pmol/l up to 1655 pmol/l. Following the working standards and clinical recommendations developed for women of reproductive age in accordance with the menstrual period phase, normal indexes were set within the following framework: the follicular phase — 68.0-1269.0 pmol/L, the ovulatory phase — 131.0-1655.0 pmol/l, the luteal phase — 91.0-861.0 pmol/l [13].

Ultrasound assessment (ultrasound) of endometrial thickness and structure was performed using a series of longitudinal and transverse sections on the Sonix SP (Canada) device by convex linear vaginal multi–frequency sensors (3.5-7.5 MHz) in the scanning mode in a two-dimensional gray scale (B-mode) on the 2^{nd}-3^{rd}, 6^{th}-7^{th}, 10^{th}-11^{th}, 14^{th}-16^{th} days of MP and on the day of embryo transfer (Days 19 to 21).

For statistical data processing, the following programs were used: IBM SPSS 19.0, Matlab 7. The sampling mean (M), and the standard deviation of residuals (σ) were computed for the qualitative data presented in form of descriptive statistics. The latter value was calculated if the qualitative data distribution diverged from the normal values. The values were categorized using the Shapiro-Wilk test for normality. For pairwise statistical comparisons of qualitative data sets, with each set not varying from the normal distribution, the Student's criterion with a two-tailed critical region was used. To compare two empirical distributions of categorical data, the Pearson chi-squared test ($\chi 2$) with the designation of degrees of freedom (df) was applied. Additionally, the Fisher's angle transform criterion supplemented by the Yates' amendment (designated as T) was utilized for the comparison of qualitative dichotomizing data. In all cases, the level of statistical significance was designated as "p". The following scale was developed to measure significance:

$p<0.05$ significant
$p<0.01$ very significant
$p<0.001$ highly significant [14].

RESULT AND DISCUSSION:

Significant differences were revealed ($p<0.001$), when comparing the levels of α-ER expression in the endometrium and the marker of endometrium proliferative activity Ki-67 under the influence of various forms of estrogen in the study groups. In Group 2 there was a more prominent expression of α-ER (207.2±1.13 points). Furthermore, markers of cells proliferative activity Ki-67 in the glands and stroma of the endometrium (37.2±0.41 and 21.1±0.49 respectively) were observed (see Table 1).

Table 1. α-estrogen receptors' expression and Ki-67 expression in the endometrium (own representation).

Group of patients	α-estrogen receptors' expression M±m	Ki-67 expression in the endometrium gland M±m	Ki-67 expression in the endometrium stroma M±m
1 (50)	123,6±0,69	22,8±0,35	7,7±0,18
2 (50)	207,2±1,13	37,2±0,41	21,1±0,49
Value of the criterion and statistical significance of the differences	t=63,10 p <0,001	t=26,70 p <0,001	t=25,50 p <0,001

Analysis of proliferative changes in the endometrium (thickness, structure and growth dynamics) derived from the ultrasound revealed statistically significant differences in the study groups ($p<0.001$). The endometrial thickness was 4.1 ± 0.03 mm on Days 6-7 of MP in Group 1, and in Group 2 – 6.4 ± 0.04 mm. The tendency observed during the first ultrasound examination continued in later: on Days 10-11 and 14-16, the endometrial thickness in Group 1 was 6.8 ± 0.04 mm, and 7.3 ± 0.03 mm, respectively, and was significantly lower compared to Group 2 - 8.2 ± 0.04 mm, and 9.2 ± 0.03 mm, respectively (see Table 2). The difference was noted not only in the endometrium thickness, but also in its echo structure. In Group 2, the endometrium constituted an expressed three-layer structure on the Day 14 of MP upon the application of 17β-E, while in Group 1, which was using EV, these changes were less pronounced.

Table 2. Ultrasound assessment of endometrium thickness in dynamics (own representation).

Day of menstrual period	Endometrium thickness (mm) M±m		Significance of the differences
	Group 1	Group 2	
2–3	2,15 ±0,03	2,35 ±0,04	t = 4,17, p < 0,001
6–7	4,13 ±0,03	6,37 ±0,04	t = 44,50, p < 0,001
10–11	6,84 ±0,04	8,19 ±0,04	t = 25,00, p < 0,001
14–16	7,27 ±0,03	9,20 ±0,03	t = 42,70, p < 0,001
19–21	8,40 ±0,03	9,86 ±0,03	t = 31,60, p < 0,001

The analysis of the influence of various estrogen drugs on estradiol serum levels did not reveal the significant differences in the estradiol blood level at patients of the analyzed groups on any days of the menstrual period ($p>0,1$) (see Table 3).

Table 3. Assessment of estradiol blood level in dynamics (own representation).

Day of menstrual period	Estradiol blood level, pmol/l		Significance of the differences
	Group 1 M±m	Group 2 M±m	
2–3	76,14 ± 0,06	71,43 ± 0,13	p = 0,672
6–7	325,94 ± 0,74	323,87 ± 1,1	p = 0,345
10–11	661,18 ± 0,70	635,25 ± 0,93	p = 0,631
14–16	1476,53 ± 0,96	1465,78 ± 0,87	p = 0,795
19–21	793,21 ± 0,67	778,33 ± 0,79	p = 0,573

The efficacy of thawed embryo transfer was evaluated on Day 21 after an embryo transfer by transvaginal ultrasound. The evaluation was performed in order to determine the presence and localization of the fetal egg. The statistical analysis revealed significant differences in the frequency of positive outcomes (clinical pregnancy) in the study groups ($\chi 2 = 6,40$, $p = 0,041$, the number of degrees of freedom $df = 2$) with the best indicators in Group 2 (see Table 4). Uterine pregnancy was diagnosed in 16 patients in Group 2 (32.0%) who received the oral hormone replacement therapy with 17β–E on Day 21 after the embryo transfer. InGroup 1, uterine pregnancy was diagnosed only in 12.0 % (6) of patients treated with HRT by oral EV (see Table 4).

Table 4. The thawed emryo transfer outcomes (own representation).

The embryo transfer outcome	Group 1	Group 2
Implantation failure	42 (84,0 %)	31 (62,0 %)
Biochemical pregnancy	2 (4,0 %)	3 (6,0 %)
Clinical pregnancy	6 (12,0 %)	16 (32,0 %)

CONCLUSION:

Based on the above provided data, it can be concluded that in the framework of hormone replacement therapy during the preparation of the endometrium for the thawed embryo transfer at patients with previous multiple implantation failures, it is preferable to use the oral form of 17β–E, compared with the oral form of EV. The obtained results are probably related to the peculiarities of the chemical structure and metabolism of the analyzed drugs.

List of symbols and abbreviations:

EIA - enzymoimmunoassay
EV – estradiol valerate
HRT – hormonal replacement therapy
IHC - immunohistochemistry
MP – menstrual period
α-ER - α-estrogen receptor
Ki-67 - marker of endometrium proliferative activity
17β–E - 17β–estradiol

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