Evaluation of effectiveness of a non-hormonal succinate-based composition on the levels of estradiol, follicle-stimulating and luteinizing hormones in women's blood during menopause.

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ABSTRACT

The repeated statistical analysis of the primary digital data from the previous placebo-controlled clinical study on the effects of a succinate-containing composition on the climacteric syndrome was carried out. Nonparametric statistics were significantly more informative than the previously used parametric Student's t-test, since there was no normal distribution in the obtained samples. Among the most informative was paired sign test. In addition to the previously observed increase in estradiol levels, we observed a statistically significant increase in the concentrations of LH and FSH. It was found that the correction of climacteric syndrome with the metabolic composition has not disturbed the physiological ratios between these hormones. In the experimental and control groups, higher levels of estradiol corresponded to lower concentrations of FSH and LH in the blood. Impact of the metabolic composition on the hormonal and psycho-emotional status of women depended on the initial hormonal and psycho-emotional state before the metabolic correction. The effects of metabolic composition were more pronounced when initial blood estradiol levels were low. In the absence of normal distributions in the samples of the analyzed clinical data, a standard use of parametric criteria masked the real picture and led to incorrect conclusions.

Keywords:

Climacteric syndrome. metabolite correction, estradiol, follicle stimulating hormone, luteinizing hormone, effectiveness of nonparametric statistics.

INTRODUCTION

Analysis of the results of the experimental and placebo-controlled clinical trials published earlier [1] demonstrated that the use of a non-hormonal succinate based compositions causes significant correction of the estral cycle and slowing down of osteoporosis development in female rats of post-reproductive age; and also causes significant alleviation of the climacteric syndrome

(CS) symptoms in women of 40-60 years of age (such as anxiety, sleep disturbances, fatigue, depression etc. – that are found in 90% of menopause cases) [19]. The reason to revisit the data from the previous clinical trial is to evaluate causes of «stability» of FSH and LH levels, that do not correspond to a more that 4x increase in the blood estradiol concentration in women after 3-week course of the composition. In order to answer this question, we decided to re-analyze the primary digital data of the previously conducted clinical study.

Published description of the clinical part of the study was based on the results that came from using parametric Student t-test to analyze the primary digital information. Such analysis is widely used in most biomedical studies without considering that the fact that the Student t-test requires normal distribution in the samples to be analyzed [2, 8, 11]. Limited use of non-parametric tests prior to 2003 was most likely due to the fact that regulatory documents at the time viewed the use of non-parametric statistical tests to evaluate preclinical and clinical data only as additional procedures “in order to test a hypothesis about differences (or similarities) between averages”. Meanwhile, the canonical statistical analysis dictates that this alternative is strictly determined by the lack of the normal distribution in the analyzed samples [2, 8, 11]. Artificial normalization of the sample is not a simple problem in biological [8], let alone clinical studies. Experiments with animals of the same species, sex, similar ages, weight and conditions, sensitivity to a stimulus under study, strict regulation of the numbers in the control and experimental groups produce data that are well described by normal distribution in over 50% of the time. In clinical studies, despite large samples, it is extremely difficult to form groups of patients that are similar in individual parameters, even after careful selection of anthropometric characteristics, sex, age, and multiple inclusion and exclusion criteria of the study that are approved by an ethics committee. Accordingly, the presence of normal distributions in clinical data is most likely a rare event rather than a real possibility.

The published article [1], as in most biomedical studies, makes no mention of the type of distribution of the obtained data. Therefore, we deemed it necessary to check the distribution of the primary digital data. We hoped that a more adequate statistical analysis would help illuminate the relationships between the hormones of the hypothalamus and the ovaries: follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol. It was important to establish whether there are any imbalances between hormones that are descriptive of the state of the neuro-hormonal regulation along the hypothalamus-pituitary-ovarian axis. Misregulation of the physiological relationships between hormones is an unavoidable consequence of the hormone replacement therapy (HRT). Currently, HRT is considered a standard treatment for the alleviation of unpleasant and pathological manifestation of the CS [14]. However, HRT does not always slow down climacteric progression of cardio-vascular diseases, neurological and cognitive dysfunctions [13].

In addition, HRT carries increased risk of the development of hormone-dependent tumors [10, 12]. Because of the increased risk of such complications, it is recommended to use various modifications of HRT and, if possible, altogether alternative methods to alleviate pathological manifestations of the CS [4, 17, 19, 21]. This is the basis of scientific and practical inquiries into non-hormonal metabolic remedies to treat CS.

Finding made by V.M. Dilman, V.A. Anisimov and M.N. Kondrashova about the ability of exogenous succinate to restore the sensitivity of the hypothalamus to estrogen in female rats with ovariectomies became the stimulus to study the influence of the succinate-containing composition on CS [7]. Loss of hypothalamic sensitivity to the hormonal signals is a natural sign of aging [5, 6]. The fact that succinate-containing “metabolic therapy” restored hypothalamic sensitivity to small doses of estrogens, which act as negative feedback signals, was assessed by the authors of the study as a sign of rejuvenation. We viewed these results in light of the current knowledge about possible effects of succinate on the hypothalamus [3]. Special consideration is placed on the effects of small concentrations of succinate as a ligand for orphan receptor QPR91 [9] and stabilizer of the hypoxia-inducible factor (HIF) [20].

The results mentioned above initiated an inquiry (based on existing clinical data) into whether the physiological relationship between estradiol, FSH, and LH remains intact in menopausal women who are treated with the metabolic succinate-containing composition.

Thus, the aim of the research was to analyze the primary digital material, produced earlier in the placebo-controlled clinical study, in order to detect changes in the relationships between levels of estradiol, FSH and LH in women during menopause undergoing a course of the non-hormonal metabolic succinate-containing composition.

MATERIALS AND METHODS

The material is the primary digital documentation from the previously conducted and described [1], placebo-controlled clinical study on evaluation of the succinate-containing composition effects on menopause. Primary documents in a Microsoft Access database were kindly provided by A.B.Peskov, Ph.D and M.L.Uchitel.

In order to explain the gist of the existing data, we will remind the readers the most important, in our opinion, aspects of the previously conducted study [1].

The composition of non-hormonal metabolic compound was approved for clinical use by The Nutrition Institute of the Russian Federation.

All the ingredients of the composition were packaged in two fast-dissolving gelatin capsules so that ammonium succinate was isolated from other substances. Such separation of the

ingredients is due to the ammonium succinate's ability to reduce the shelf life of other components, if kept together.

Table 1. The ingredients of the non-hormonal metabolic composition

Component	Quantity
Content of white capsule	
Ammonium succinate	200 mg
Content of colored capsule	
Vitamin E (tocopherol acetate solution in oil 92%)	8 mg
Zinc fumarate acid hydrate - aqua chelating dimeric form	8 mg
Calcium succinate acid- aqua chelating dimeric form	92 mg
Magnesium succinate 4-aqueous - aqua chelating dimeric form	20 mg
Glycine	32 mg
Sodium L - Glutamate 1-hydrate	40 mg
Total mass of substances in both capsules is 400 mg	

Design and methods of the earlier study:

Type of study: investigative with the use of randomization, placebo-control and double blind control; control points – surrogate.

Goal of the clinical study: to evaluate the possibility of using substrate composition (Table 1) for the relief of CS without the use of any hormonal or other pharmacological agents.

Aims of the study

- To randomly select 90 people (70 people to be included in the main group and 20 in the control group) among the volunteers who wished to participate in the study, who are diagnosed with the CS and met specified below inclusion and exclusion criteria.
- To evaluate initial clinical status of the selected patients (including *status objectivus communis*, gynecological status, psychological status), using numerically expressed indicators adapted for the evaluation of the CS.
- To study the initial subjective patients' conditions using standard questionnaires, including Kupperman scale.
- To study the initial levels of the sex hormones in the patients selected for the study (blood concentrations of estradiol, follicle stimulating hormone (FSH), and luteinizing hormone (LH)).
- To study the initial levels of the cholesterol metabolism markers, glucose, prothrombin.
- To conduct a 3-week course of the metabolic composition (Table 1) in the main group, in the control – a course of potato starch (placebo), packaged in the same way as the metabolic composition.

- To study the dynamic of the above-mentioned parameters, including clinical, laboratory and using various instruments.

Timeline of the study

- Patients are observed prior to administration of the non-hormonal metabolic composition – 14 days.
- Dynamic observations of the patients as they take the non-hormonal metabolic composition – 21st day.
- Observations of the patients after the end of the non-hormonal metabolic composition course – 14 days.
- Administration regiment: 1 white and 1 colored capsule 2 times per day (morning and evening, with a meal).

Inclusion criteria:

- Verified diagnosis of CS.
- 40-60 years of age.

Exclusion criteria:

- Severe extragenital somatic pathology.
- Oncological conditions.
- Psychiatric conditions.
- Taking medication to treat CS.

Patients' visits:

- Prior to taking the non-hormonal metabolic composition.
- During the first week of taking the non-hormonal metabolic composition (tolerance control).
- During the second-third weeks of taking the non-hormonal metabolic composition (preliminary results).
- At the end of the 21-day course of treatment with the non-hormonal metabolic composition.

Study's protocol

Complete evaluation program (first and last visits):

.1 Evaluation of clinical status using Kupperman methods as modified by E.V. Uvarova, including exams by a gynecologist and a psychiatrist.

.2 Levels of actual anxiety (AA) were evaluated using Spielberger-Hanin test.

.3 Laboratory tests:

- General blood panel
- General urinalysis

• Biochemical blood panel. Blood serum levels of total cholesterol, A-cholesterol, triglycerides, low-density betalipoproteins (LDL), and very low-density lipoproteins (VLDL) were determine using BIOM-01M analyzer. Total protein and glucose – using Hitachi analyzer, levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol – using kits «GonadotropinIFA – FSH», «GonadotropinIFA – LH», «Micropale Steroid Estradiol»). The prothrombin index was determined at the same time.

• Blood serum levels of hormones (FSH, LH, estradiol) were determined using kits «GonadotropinIFA – FSH», «GonadotropinIFA – LH», «Micropale Steroid»)

.4 Tests using various equipment:

- ECG was done once, during the initial evaluation.
- Ultrasound (US) of uterus and ovaries.
- US of breast tissues was done once, during the initial evaluation

Additional evaluations (second and third visits):

- Evaluation by a physician, recording results in the patients' charts, checking patients' diary.

Primary documentation of the study

- patients' diary
- patients' medical charts
- Protocols of the laboratory tests and tests using various instruments
- All of the primary documentation was included in the Microsoft Access database.

Ages in the groups: from 40 to 45 years of age – 9 patients; form 45 to 50 years of age – 28; form 50 to 55 years of age – 25; from 55 to 60 years of age – 5; 60-61 years of age – 3 patients.

All data collected were analyzed using parametric criteria in Microsoft Excel. The results were presented as arithmetic means ± confidence interval, given p=0.05.

Thus, the description presented in the article [1] is based on the results of the parametric analysis of the data.

In order to re-analyze the same data, the current work used Sigma plot program and non-parametric statistical tests, specifically sign test - z [2, 11].

RESULTS AND DISCUSSION

Checking the distribution

Several methods are used to check for normal distribution in a sample: visual – graphic method, χ^2 test, Kolmogorov-Smirnov test, and others. For the ease of interpretation, we chose the graphic method and used the following well-known rules: «the most important characteristics of a normal distribution are gradual symmetric decrease in the frequency as one moves away from the center of the distribution», as shown in Figure 1, and «equivalency of the arithmetic mean, median, and mode» [2].

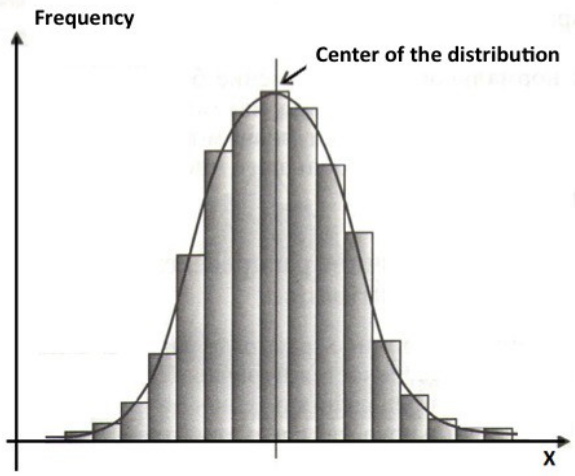

Figure 1. Frequency histogram (displayed as intervals of X) given normal distribution [2, 8, 11].

Next, using standard calculations we derived statistical mean ($\overline{X}$), median (M), standard deviation (SD), and standard error (SE) from the data on concentrations of estradiol, FSH, and LH in the blood of the women in the main group before and after the course of the non-hormonal metabolic composition or placebo, as if normal distribution was present in the analyzed samples.

As you can see from Tables 2 and 3, the calculations do not correspond to those of a normal distribution. The standard deviations are large and begin to approach averages. Small values of the standard errors should not be a source of confusion, since they are calculated given a relatively large n, (n= 63) according to the formula

$SE = SD/\sqrt{n}$.

The bigger the n, the smaller SE, but this does not improve the sample. It is specifically SE that is usually mentioned in most biomedical studies (despite canonical statistical methods), while they remaining silent about the lack of normal distribution. Seemingly, this leads to the good calculations of the means and the errors. In the data we are re-analyzing, such methods allowed to detect an increase (with high level of significance) in the concentration of the estradiol after the course of the non-hormonal metabolic supplement, but, did not detect any significant changes in

the blood concentrations of FSH and LH. In the control group the course of placebo resulted in a small tendency toward decrease in the blood concentrations of estradiol and therefore, an increase in concentrations of FSH and LH.

Table 2.

Concentration of estradiol, FSH and LH in the blood of women in the main group before and after the course of the non-hormonal metabolic composition.

Statistical parameters	Initial level			Final level		
	[Estradiol] pmol/L	[FSH] mIU/L	[LH] mIU/L	[Estradiol] pmol/L	[FSH] mIU/L	[LH] mIU/L
$\bar{X}$	76.54	43.15	20.75	274.22	50.28	25.15
M	44.57	35.21	16.70	150.00	49.91	21.08
SD	80.23	32.28	15.75	318.43	37.64	18.77
SE	10.11	4.07	1.98	43.74.	4.74	2.36

Table 3.

Concentration of estradiol, FSH and LH in the blood of women in the control group before and after the course of placebo.

Statistical parameters	Initial level			Final level		
	[Estradiol] pmol/L	[FSH] mIU/L	[LH] mIU/L	[Estradiol] pmol/L	[FSH] mIU/L	[LH] mIU/L
$\bar{X}$	68.54	43.40	19.31	57.44	53.18	28.05
M	19.41	35.93	14.61	54.05	61.25	23.34
SD	78.44	34.10	14.98	41.08	33.02	16.99
SE	18.00	7.62	3.27	10.27	7.78	4.00

A graphic comparison (Tukey diagrams) of the data before and after the non-hormonal metabolic composition course also shows an increase only in the blood concentration of estradiol, and does not give any clear idea of change in the concentrations of FSH and LH (Figure 2).

Subsequent check for the presence of normal distributions in the analyzed parameters gave unsatisfactory results. Histogram distributions for blood concentrations of estradiol, FSH, and LH (Figure 3) in the surveyed women before the course of the non-hormonal metabolic composition are far from normal distributions shown on Figure 1.

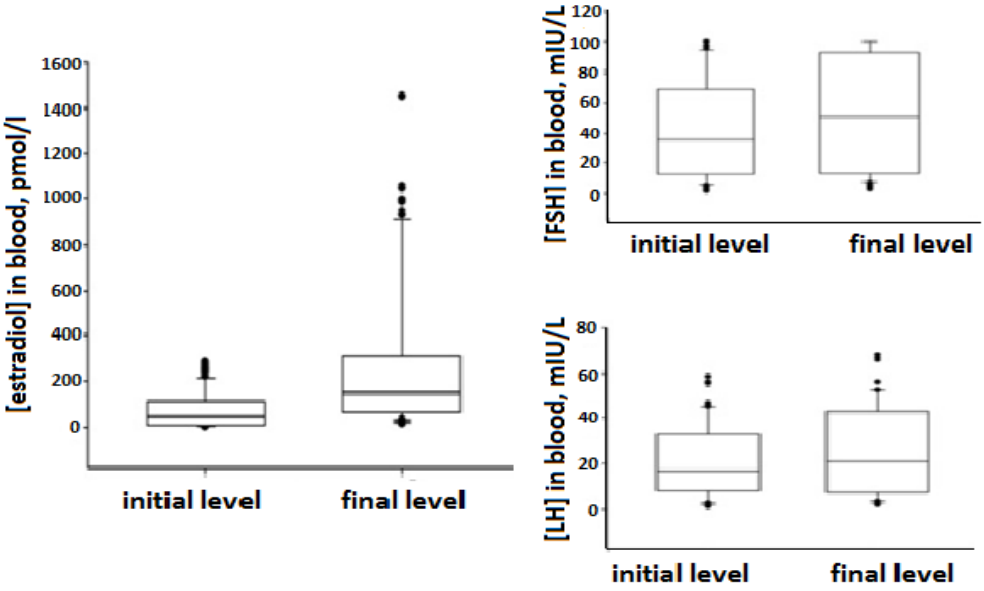

Figure 2. Comparison of the Tukey charts for concentrations of estradiol, FSH and LH in the blood of the main group of women before and after a course of the non-hormonal metabolic composition.

As follows from the histograms shown in Figure 3, the initial data of the blood content of estradiol, FSH, and LH in the main group of surveyed women does not at all correspond to normal distribution. The same goes for the data shown in Table 2 - blood concentrations of estradiol, FSH, and LH in the control group to prior to receiving placebo (Table 3, Figure 4) are also far from normal distribution.

Thus, none of the analyzed samples at the initial period show normal distribution. Therefore, the use of Student t-test for evaluating and comparing the initial parameters with the subsequent data is counter-indicated and incorrect. Based on the presented initial data we do not expect appearance of normal distributions in the analyzed samples after a course of the non-hormonal metabolic composition or placebo. This indeed turned out to be true (Figures 5, 6).

A graphical comparison (Tukey diagrams) of the data before and after the course of the non-hormonal metabolic composition only shows an increase in the blood estradiol levels, but it does not give any clear idea of changes in the concentrations of FSH and LH and demonstrates the absence of any dynamics after the placebo course (Figure 7, 8).

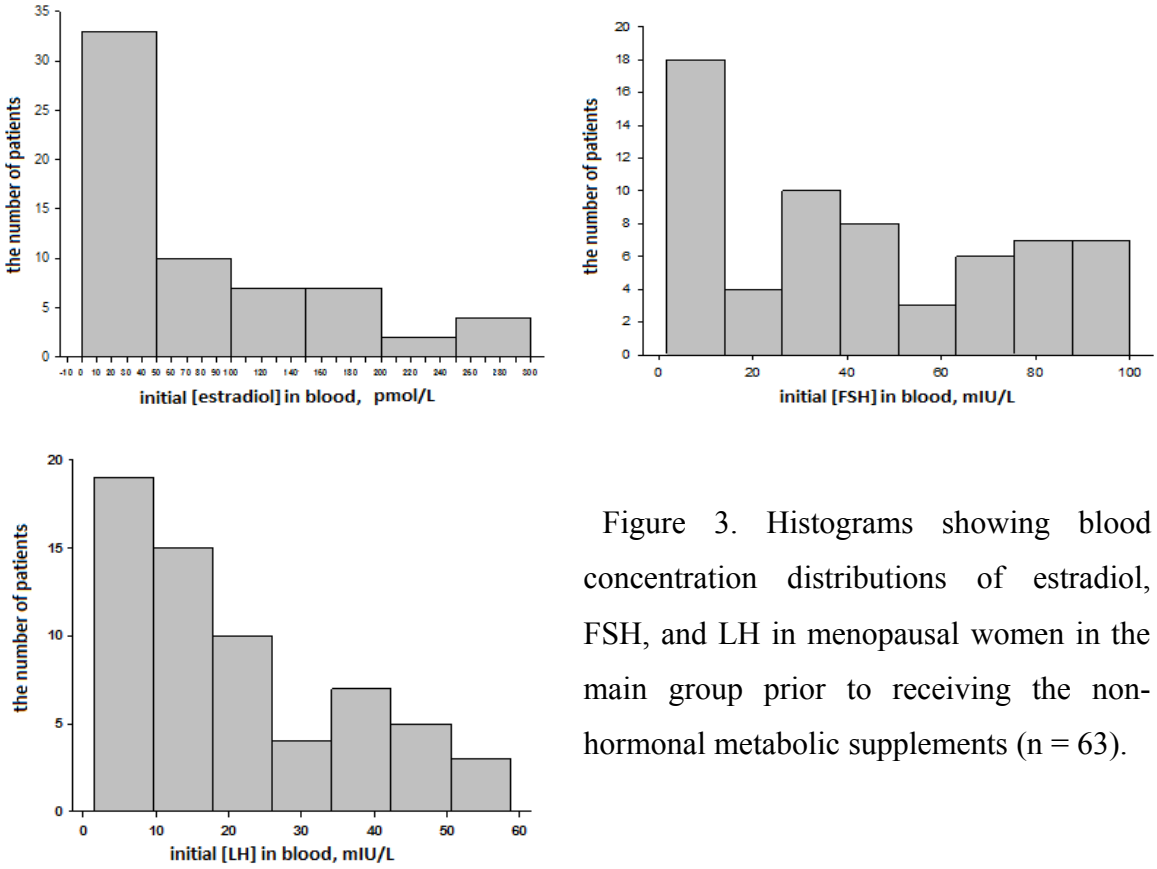

Figure 3. Histograms showing blood concentration distributions of estradiol, FSH, and LH in menopausal women in the main group prior to receiving the non-hormonal metabolic supplements (n = 63).

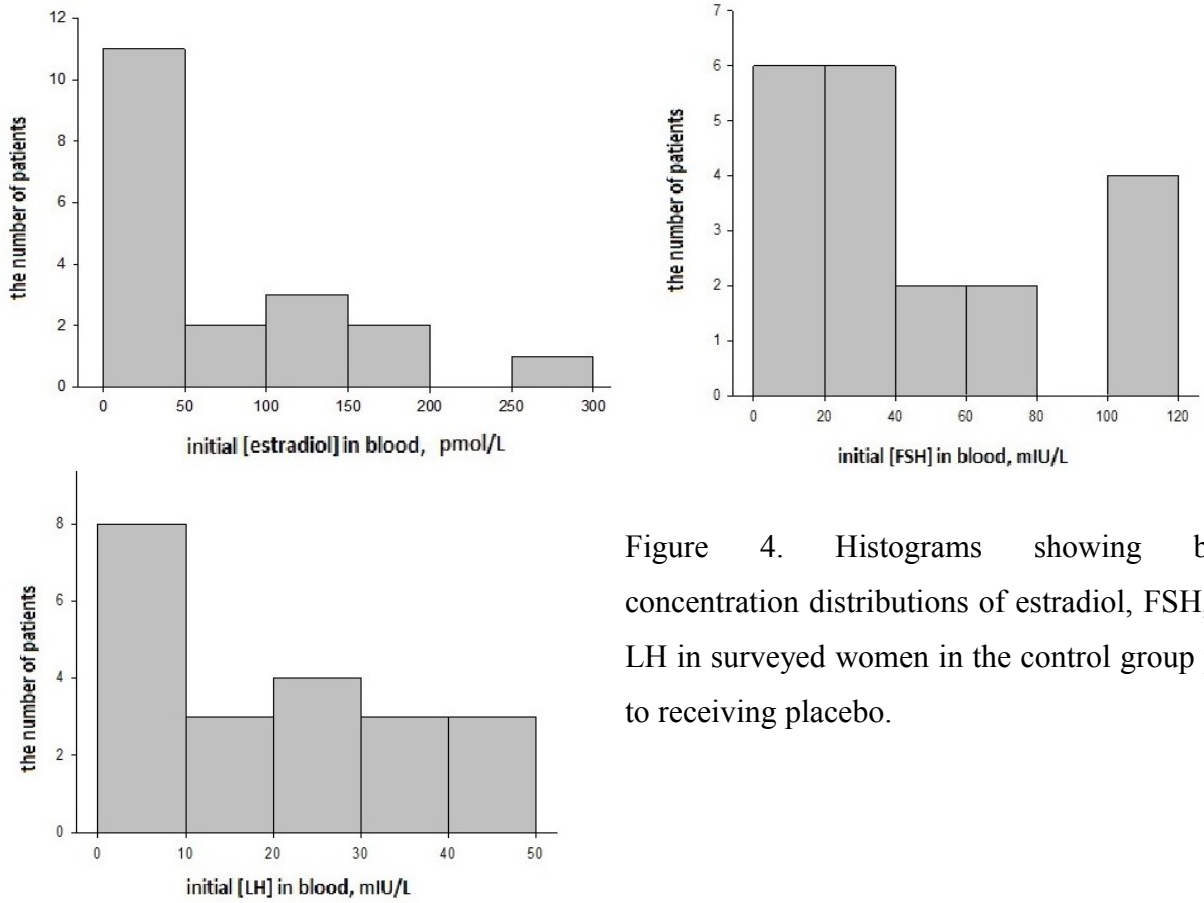

Figure 4. Histograms showing blood concentration distributions of estradiol, FSH, and LH in surveyed women in the control group prior to receiving placebo.

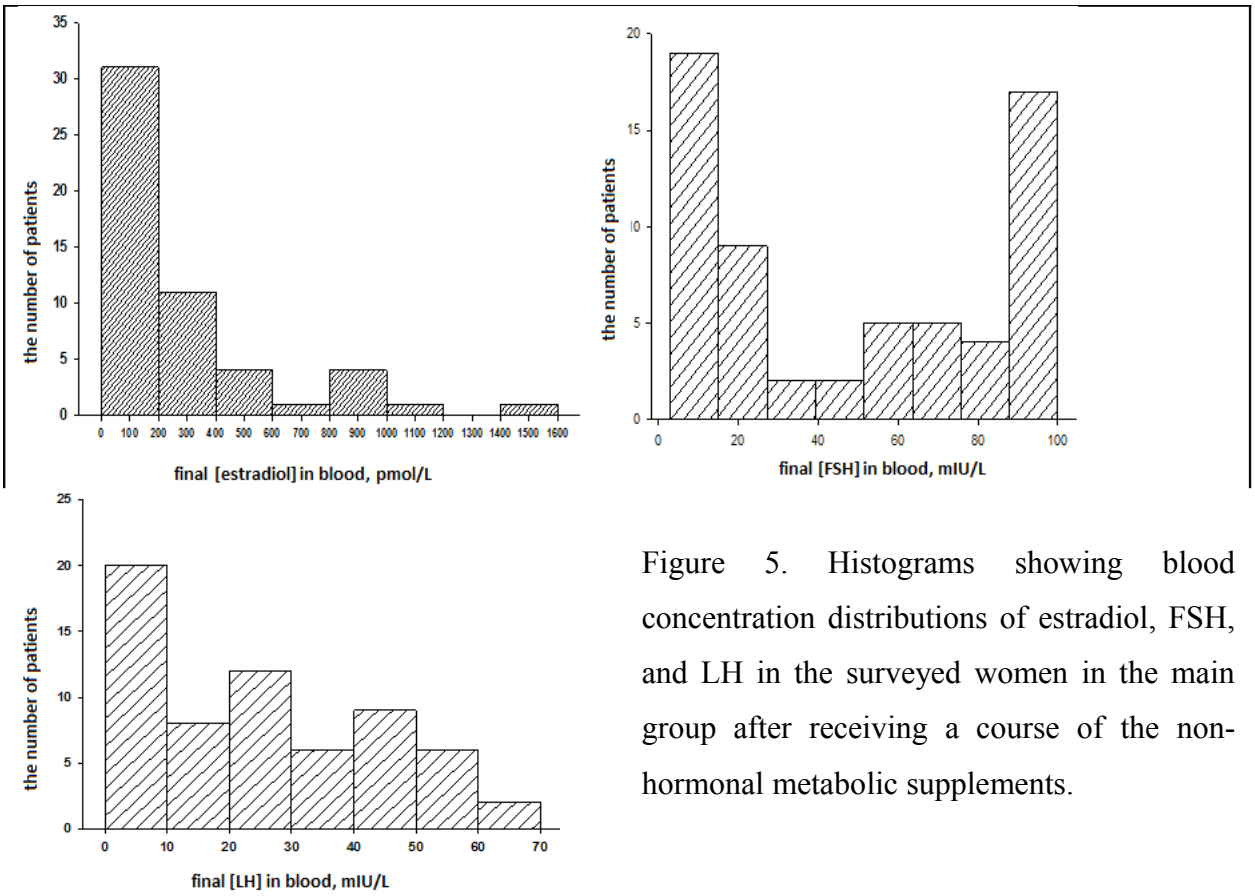

Figure 5. Histograms showing blood concentration distributions of estradiol, FSH, and LH in the surveyed women in the main group after receiving a course of the non-hormonal metabolic supplements.

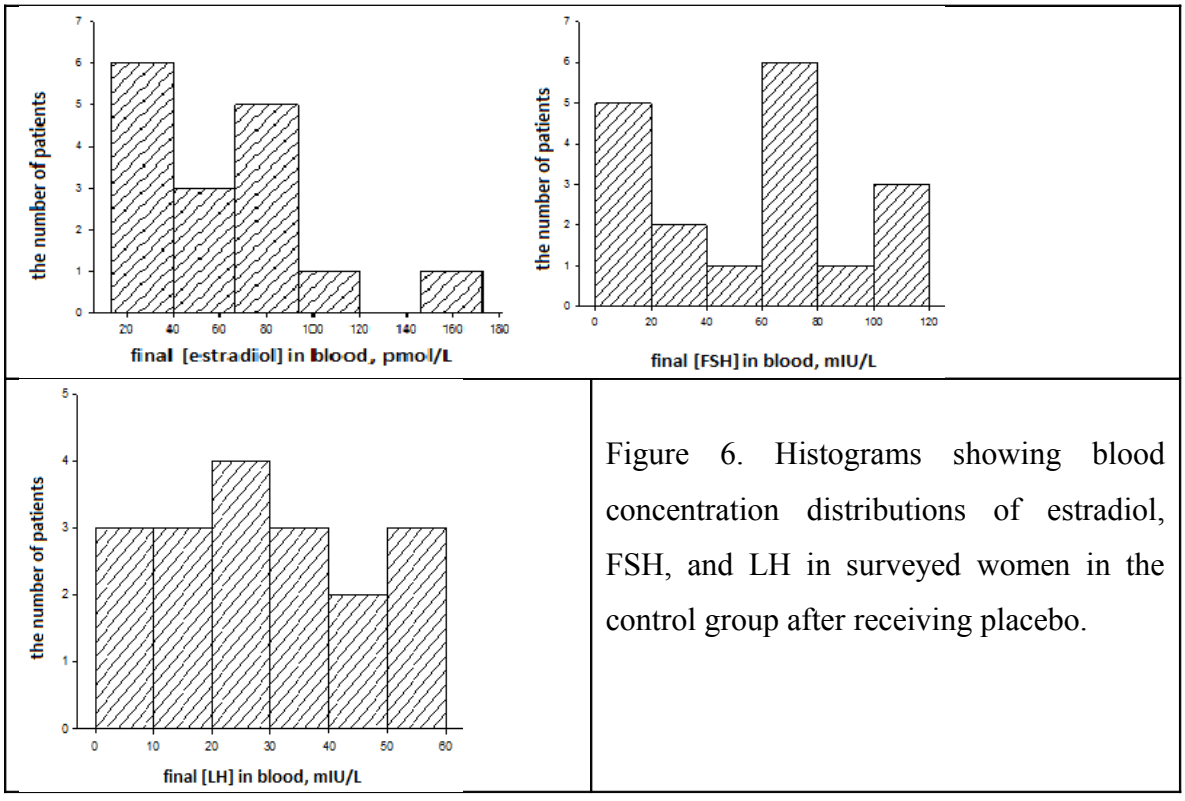

Figure 6. Histograms showing blood concentration distributions of estradiol, FSH, and LH in surveyed women in the control group after receiving placebo.

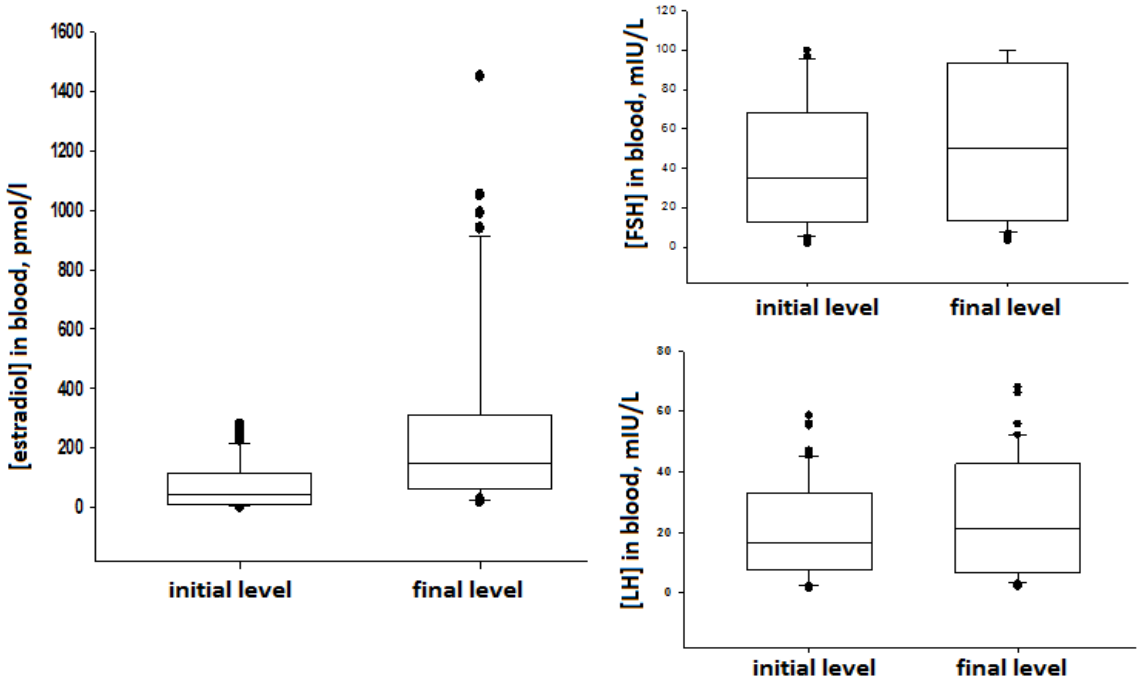

Figure 7. Tukey diagrams comparison of blood concentrations of estradiol, FSH, and LH in the main group of women before and after the course of the non-hormonal metabolic composition.

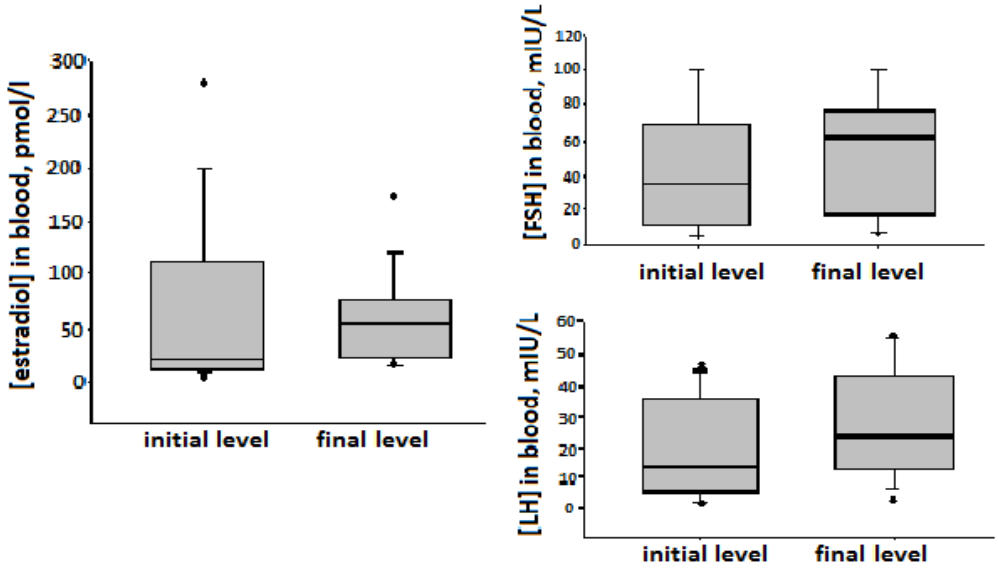

Figure 8. Tukey diagrams comparison of blood concentrations of estradiol, FSH, and LH in the control group of women before and after the course of placebo.

Thus, neither parametric calculations nor graphic analysis allow for the correct (according to the statistical rules) evaluation of FSH, LH, and estradiol dynamics. Although, noted increase in

the estradiol levels after the non-hormonal metabolic composition course is not called into question.

Comparison of the digital data using non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks test also shows that the difference in estradiol concentration is statistically significant **(**$P<0.001$), given the minimal P value of <0.05. Changes in the concentrations of FSH and LH using this test are not significant: P values are 0.247 and 0.210, respectively. After placebo course all changes in the concentrations of estradiol, FSH and LH also turned out to be not statistically significant; the p values were 0.446; 0.348 and 0.079, respectively.

For the subsequent analysis, along with the incorrect (for this study) parametric Student t-test, we used non-parametric test - Sign Test - z, which is used in pairwise comparisons of related samples (Table 4). The application of the Sign Test guarantees that statistically directed reactions to the stimulus under study for each patient in the pairwise samples will be considered. Turns out that, unlike analysis using parametric Student t-test and non-parametric Kruskal-Wallis test, the use of a more sensitive non-parametric Sign test allows for an easy detection of not only an increase in the estradiol concentration, but also significant increase in FSH and LH concentrations after the course of the non-hormonal metabolic composition. This occurs despite the negative feedback mechanism where a large increase in estradiol levels could completely halt any increase in the levels of FSH ad LH [15, 18], as it occurs in HRT when exogenous estradiol is administered.

To analyze quantitative interdependence of individual changes in the concentrations of estradiol, FSH, and LH in each patient, we calculated the rank correlation coefficient ρ using Spearman method (Spearman rank correlation coefficient) before and after the course of the non-hormonal metabolic composition (Table 4). It can be seen that estradiol, FSH, and LH, both in the initial period and after the course of the non-hormonal metabolic composition (in the table this group is abbreviated NMC) show a high degree of statistically significant negative correlation described by the coefficient **ρ**. In other words, higher levels of estradiol corresponds to lower levels of FSH and LH in all samples.

In turn, we always see a significant positive correlation between FSH during and after the course of the metabolic therapy. We want emphasize that such correlation remained after the course of the non-hormonal metabolic composition. That means that the therapy did not disturb the natural relationships and, probably, physiological regulation between the ovaries and the central link in the hypothalamus-pituitary-ovarian axis. This differs from the HRT, where high doses of exogenous hormones inevitably disturb these physiological relationships between endogenous FSH, LH and artificially increased estradiol concentration.

Table 4.

Comparison of changes in the concentrations of estradiol, FSH and LH in the blood of women in the main group after a course of the non-hormonal metabolic composition (NMC) according to Student's t test and signs test. Rank correlation coefficient ρ was calculated.

Time of analysis. Type of hormone	$\bar{x}$ ± SE, (n –number of patients) The range of values: (min. ÷ max.)	Type of test and comparison		Correlation coefficient (**ρ**) with comparing to the concentration of another hormone
		Student's t test, Compared to the Initial level.	Signs test: deviations relative to the initial level	
[Estradiol], pmol/L Initial level.	76.54 ± 10.11 (n=63) 4.5 ÷ 278.8			Initial levels FSH - 0.756; p<0.0005; and LH - 0.683; p<0.001;
[Estradiol], pmol/L After the course of the metabolic composition:	274.22 ±43.74 (n=53) 21.1÷1449	p =0.0003	Comparing to the initial level of Estradiol: (n=53) +40; -13; Increment: p<0.01	-
[FSH], mIU/L Initial level.	43.15 ±4.07 (n=62) 1.65÷100			LH initial level - 0.752; p<0.0005
[FSH] mIU/L After the course of NMC	50.28 ±4.74 (n=63) 3.04÷100	p >0.1	Comparing to the initial level of FSH (n =63) +37; - 17; Increment: p=0.01	Estradiol after NMC - 0.760; p<0.0005
[LH] mIU/ Initial level.	20.75 ±1.98 (n=62) 1.42÷56.05			Estradiol initial -0.683; p<0.001
				FSH initial + 0.752; p<0.0005
[LH] mIU/L After the course of NMC	24.15 ±2.91 (n=63) 2.97÷68.0	p>0.1	Comparing to the initial level of LH (n=63) + 37; -17 Increment: p<0.01	Estradiol after NMC - 0.673; p<0.001
				FSH after NMC + 0,739; p<0.0005

In conclusion, we want to point out that the discovery of the lack of normal distributions in samples of initial and "end" concentrations of estradiol, FSH and LH levels in both groups of patients have prompted us to use non-parametric tests according to laws of statistics. This approach revealed a clear dependence of the magnitude and direction of changes in the concentrations of estradiol, FSH and LH and the maintenance of physiological quantitative relationships between the hormones. In addition, the analysis of distributions initiated the

identification of dependency in the shifts of some adverse symptoms associated with the development of menopause, taking into account individual dynamics and the initial level of estradiol. Recall that from the data presented in Table 2 and histograms in Figures 3 and 4, it is clear that the entire sample of the main group is divided by the median at the estradiol concentration of 44.57 pm/l. It turned out that for patients with the initial concentration of estradiol less than the median value (to the left of the median), in 95% of the cases there is an increase in estradiol concentrations after the course of the metabolic therapy. Patients with a higher initial concentrations of estradiol – to the right of the median – show either an increase or decrease in the concentration of estradiol with equal probability after taking the non-hormonal metabolic composition. Patients with low initial level of estradiol typically show 5-7 CS symptoms, which are alleviated to a large degree after the metabolic therapy. According to the Spielberger-Hanin test, the actual anxiety on average decreased in 43% of patients among 70 - it is on the verge of significance. While with the low initial level of estradiol (to the left of the median) reduction in anxiety occurred in 31 patients with a high level of significance ($P < 0.01$). It is interesting to note that the subjective assessment of anxiety did not decrease in all patients, but none of the patients indicated increase in the anxiety. However, a large decrease in anxiety after the course of the metabolic therapy was observed in the background of the initially high level of anxiety. Analysis showed that for the 15 patients with high initial level of anxiety, the metabolic therapy had a positive effect in all cases.

Thus, it is clear that parametric statistical tests may be completely unusable for analysis of clinical data, if there is no normal distribution. In such situations, it is necessary to use more appropriate nonparametric tests. Experience showed that it is necessary to pay attention not only to the initial distribution, mean, and median, but also to the dependency of the patients' reaction to the studied stimulus on the position of the patients' subgroup in the distribution histogram. At the end, such statistical approach allows for a more complete characterization of the direction and dependency of the reactions of the patients from their initial status.

CONCLUSIONS

1. Additional statistical analysis of the primary digital data from the previous placebo-controlled clinical study to evaluate of the effects of succinate-containing composition on the climacteric syndrome revealed (by using non-parametric tests) that in addition to previously discovered significant increase in estradiol levels, there were also significant increase in the concentrations of LH and FSH. The metabolic therapy did not disturb physiological correlations between hormones: increase in the estradiol levels in the study's groups, both before and after the

course of the non-hormonal metabolic composition, corresponded to a lower concentrations of FSH and LH.

2. Use a non-hormonal metabolic succinate-containing composition during menopause contributed to changes in the hormonal and physiological status of women. The impact of the metabolic composition was dependent on the initial hormone levels and psycho-emotional state of the surveyed women. Lower initial level of estradiol resulted in its larger increased and more pronounced alleviation of a number of psycho-emotional symptoms of the CS.

3. Our experience of applying additional statistical analysis to the data calls for a wider use of non-parametric tests, consideration of individual status of the patients during the course of the clinical study; it also points to how relatively non-informative parametric tests turn out to be in the absence of normal distribution in the obtained samples.

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