Production and characterization of nanoparticles lipid carrier (NLCs) loaded with red clover isoflavones extract for menopause therapy

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Abstract
Plant-derived edible nanoparticles (PDNPs) are nano-sized membrane vesicles released by edible plants. They are non-toxic, have tissue-specific targeting properties, and can be mass-produced. Our aim is to assess the effect of phytoestrogens (PEs) for menopause therapy using nanoparticles lipid carrier (NLCs) loaded with red clover isoflavones extract (RCIE). Study was conducted using fifty adult female mice model for menopause using 4-Vinylcyclohexene dioxide (VCD) and handled as follows for 6 weeks. Two experiments were performed, the first experiment included 20 adult female mice it lasted two weeks and divided into two groups (10 mice/group) Group A: Control group has injected 0.1 ml D.W intraperitoneal (IP) daily. Group B: This group has injected IP daily 160 mg/kg B.W of VCD. The second experiment was included 30 adult female mice have injected IP daily 160 mg/kg of VCD for inducing menopause for two weeks, then shaved at the last third of the back and divided into three groups (C, D and E), 10 mice in each one, then treated with dermal sticker for six weeks: Group C: The group was treated with dermal sticker saturated with 10ug/ kg B.W of estradiol benzoate (EB) diluted with virgin coconut oil (VCNO). Group D: Control group was treated with dermal sticker saturated with 0.1 of VCNO. Group E: The group was treated with dermal sticker saturated with 0.1 ml of RCIE-NLCs. A red clover extract isoflavones standardized by HPLC to contain (2.7, 6.6 and 1.04) mg/g of genistein, glycitein, and malonate respectively. The characterization of RCIE-NLCs was done by transmission electron microscopy (TEM), Scanning probe microscopy (SPM), Zeta potential. TEM images showed that shape of particles was mostly spherical and few cylindrical with average diameter (53.70) nm. SPM images showed the average grain size was 38.78 nm. Encapsulation Efficiency (EE) was 83.3%. Zeta potential was -41.1 Mv. The results of statistical analysis showed a significant decrease (P≤0.01) in the level of estrogen in group B (23.767) ng/ml compared with group A (33.154) ng/ml, no significant decrease (P≥0.01) compared with group D (22.728) ng/ml, and significant increase (P≤0.01) in the levels of FSH and LH in group B (4.0688 and 3.0839) mIU/ml compared with groups A (2.9206 and 2.1469) mIU/ml respectively, and no significant change (P≥0.01) compared with group D (3.9974 and 3.1049) mIU/ml respectively, and significant increase (P≤0.01) in the level of estrogen in groups C, E (36.444 and 30.008) ng/ml respectively compared with groups B and D (23.767 and 22.728) ng/ml respectively, and significant decrease (P≤0.01) in the levels of FSH and LH in group C (2.5556 and 0.0485) mIU/ml and group E (3.2116 and 2.4825) mIU/ml respectively, compared with group B (4.0688 and 3.0839) mIU/ml and group D (3.9974 and 3.1049) mIU/ml respectively. The results of this study revealed that administration RCIE-NLCs shows an effective in compensating for estrogen deficiency resulting from loss of primordial and primary follicles in the ovary via impact of VCD, compared to EB. This study strongly suggests that RCIE-NLCs have potential as alternative to HRT for menopause therapy.

Keywords: Red clover, Trifolium pratense, Menopause, NLCs, isoflavones, Estrogen benzoate,
Introduction

During the recent decades, the following of medical progress and the consequent increase in life expectancy, more women reach menopause each year. [1] Menopausal women are interested in alternative therapy to reducing the severity of menopausal symptoms and its complications. Such as, vasomotor symptoms (hot flushes, night sweating and palpitations), loss of libido, vaginal dryness, urogenital atrophy, osteoporosis, insomnia and cardiovascular diseases [2]. The emergence of these symptoms and risk factors may be a direct result of estrogen depletion because of ovarian failure [3, 4]. Therefore, hormone replacement therapy (HRT) has been successfully used for several decades to treat these menopausal symptoms. However, the longtime use of HRT associated with an increased risk of endometrial hyperplasia and ovarian cancer [5] and in relation to breast cancer and cardiovascular events. [6] For this reason, researchers began to investigate alternative to HRT, such as phytoestrogens (PES), they are plant secondary metabolites divided into two main subgroups, isoflavonoids and lignans. The isoflavonoids are divided into isoflavones and coumestans, [7]isoflavones are found in red clover (RC) [Trifolium pratense], a native plant of central Asia, Europe and northern Africa used as traditional medicine to treat several ailments [8]. RC can probably have desirable effects not only on relieving the menopausal symptoms, but also on skeletal systems and cardiovascular [9]. RC have a beneficial effect on the lipid profile of perimenopausal and postmenopausal women. [10] Therefore, it is a source of several dietary supplements available in the market administered as an alternative to conventional hormone therapy. [11, 12]

RC is non-steroidal compounds structurally similar to natural estrogen, they are considered selective estrogen receptor (ER) modulators as they can bind with two subtypes: ERα and ERβ, the advantage of this PE does not harm the breast and endometrium. [13] Also, do not elevate the risk of clotting in postmenopausal women; this make PE to be a good alternative to HRT [14, 15]. Nanoparticles (NPs) are characterized as particles with sizes (1 - 100) nm. NPs exist in the natural environment because of physical, biological and chemical processes. [16] Nanoparticles Lipid Carrier (NLCs) are the latest generation colloidal nanoparticles with stability improved and drug loading efficiency. The lipids used in preparation of NLCs are usually physiological lipids (biocompatible and biodegradable) so, that drugs can be delivered at the required location of action with the controlled release with reduced acute and chronic toxicity. [17]

During the past decades, NLCs are gaining momentum with their multiple advantages for the skin diseases management. NLCs enable to target the therapeutic loading to deep skin layers or even to reach the blood circulation making them up a promising cutting-edge technology. NLCs refer to a large panel of drug delivery systems. Lipid vesicles are the most conventional, known to be able to load lipophilic and hydrophilic active agents [18]. However, to date, lack or no studies using red clover isoflavones extract (RCIE) as NLCs have reported effects on menopausal status. The present study was designed to assess the efficacy and safety of a RCIE-NLCs for treating signs and symptoms related to the symptomatology in female mice model for menopause and evaluate its effects on levels of steroid hormones.

Materials and Methods

Newly dried RC was purchased from local markets of Dohuk province, Iraq. Then, RC was classified by the University of Baghdad grassland, 4-vinylcyclohexene diepoxide (VCD), standards of genistein, glycitein and Malonate, Tween 80 (Sigma Aldrich, Germany), Lecithin Soybean (Santa Cruz, USA), Glycerol (Merck, Germany), Estradiol benzoate (Cosina, Chicago), Estrogen- ELISA –Kit, FSH- ELISA- Kit, LH- ELISA –Kit (Fine Test, China), Virgin coconut oil (Abideen, Pakistan).
Preparation of Red clover isoflavones extract (RCIE)
The fig 1 shows preparation of RCIE. 100g of RC was crushed to fine powder by Grinding (Braun GmbH). Grinded RC powder was separated to be fine to pass through sieve size (75–100) μm. Then added to 500ml methanol (80%) and mixed with Magnetic stirrer for 2 hrs. at room temperature. This procedure was repeated two times. The extract was filtered through no.1 filter paper (Whitman International Ltd., Kent, UK) using a Buchner funnel, then the filtrate was concentrated with a rotary evaporator under reduced pressure.

![Fig 1: Extraction of RC isoflavones (A) RC soaked in Methanol, (B) RC after filter, (C) Methanol removal, (D) dried RC extract.](image)

Identification and Estimation of Genistein, Glycitein and malonatee in the RC extracts by HPLC technique
The quantity of isoflavones were analyzed by HPLC technique (SYKAWN), Equipped with UV detector in combination with Ezchrome software. Reverse-Phase column C18-OD5 has been employed in this technique. A mixture of Acetonitrile and double deionized water in the ratio 70:30 has been used as the mobile phase. The solvent flow rate was kept as 1mL/min and the UV detection wavelength was set at 270 nm. The instrument is set as per the chromatographic condition described above. The Sample was centrifuged at 10000rpm for 10 min, filtered by 0.45μm filter. By means of a suitable syringe, 100μl of standard solution is injected and subjected to HPLC. The chromatogram was recorded. The sample was also subjected to HPLC analysis. The qualitative and quantitative evaluation of isoflavones in the sample was performed on the basis of retention time and chromatographic behavior with reference to that of authentic standards. From the peak areas of the graph, the percentage of isoflavones was calculated. Chromatograms obtained at 280 nm for isoflavones for the authentic as well as the samples gave the best compromise between sensitivity and baseline noise. [19]

Preparation of NLCs
One hundred mg of solid lipid (glyceryl monostearate) and liquid lipid (virgin coconut oil) range from 60% to 40%, w/w were dissolved in 8.5 ml of Dichloromethane (DCM) were blended and melted at 40°C to form a uniform and clear lipid phase. [20] 6 mg of isoflavones extract [21] subsequently added to the lipid phase and ensures heating temperature always maintained at 10°C above melting temperature of solid lipid. Meanwhile,
the aqueous phase was prepared by blending 200 mg of Tween 80 and 80 mg of soy lecithin were prepared in 50 ml D.W.\textsuperscript{22} Immediately, the aqueous mixture was added onto lipid mixture. The pre-emulsion was homogenized using Homogenizer at 11000 rpm for 15 minute. The emulsions were ultrasonicated using probe sonicator for (5 to 20) min. durations at 40 amplitudes. To evaporate of DCM, the obtained nanoemulsion was stirred at 400 rpm for 3 hrs. NLC was cooled in ice water bath to room temperature and stored at 4°C. \textsuperscript{20}

Characterization of RCIE-NLCs

1- Atomic Force Microscope (AFM)
The granularity accumulation distribution of RCIE-NLCs by Scanning probe microscope (SPM)AA3000. \textsuperscript{23}
2- Transmission Electron Microscopy (TEM)
The surface morphology of the formulation was investigated using transmission electron microscopy (TEM, FEI TECNAI G220 TWIN MODEL 94320522121).
3- Zeta Potential (ZP): ZP was determined by using Particle size analyzer (Delsa Nano C Beckman Cutler). \textsuperscript{20}
4- Encapsulation Efficiency (EE): The EE of RCIE-NLCs was estimated after separation of free plant extractand lipids from the aqueous phase by ultrafiltration. The concentration of loaded plant extract in the aqueous phase were then evaluated by HPLC. The concentration of the loaded RC extract in the NLC was calculated according to the following equation. \textsuperscript{24}
\[
EE(\%) = \frac{\text{Total concentration of drug content - free drug}}{\text{Total drug concentration of content}} \times 100
\]

Experimental animals
A total 50 female albino mice were used in this study were albino at the age of (8-10) weeks and average weight (21±6 g), the mice were housed in polypropylene cages under controlled conditions at temperature (25-28) C° with a 12/12 hr light/ dark cycle. Mice were acclimatized condition for 7 days before commencement of the experiment.

Experimental design
Two experiments were performed, the first experiment for inducing menopause, included 20 adult female mice for two weeks and divided into two groups 10 mice in each one:

Group A: Control group has injected 0.1 ml D.W intraperitoneal (IP) daily.
Group B: This group has injected IP daily 160 mg/kg B.W of VCD. \textsuperscript{25} The second experiment was included 30 adult female mice have injected IP daily 160 mg/kg of VCD for inducing menopause for two weeks, then shaved at the last third of the back and divided into three groups (C, D and E), 10 mice in each one, then treated with dermal sticker for six weeks:

Group C: The group was treated with dermal stickers saturated with 10ug/ kg B.W according to\textsuperscript{26} of estradiol benzoate
Group D: Control group was treated with dermal sticker saturated with 0.1 ml of virgin coconut oil.
Group E: The group was treated with dermal sticker saturated with 0.1 ml of RCIE-NLCs

Blood samples: After six weeks, animals were sacrificed by cervical dislocation. Then open the inverted T-shaped cavity and draw blood directly from the heart by stabbing the heart to get as much blood as possible. One mL of blood was collected from each mouse in test tubes. Serum separated from coagulated blood sample by centrifugation at 2500 rpm for 15 min and kept it by freezing at -20 C until used. \textsuperscript{27}

Statistical analysis: The Statistical Analysis System\textsuperscript{28} was used to affect different factors in study parameters. Least significant difference (LSD) test at P<0.05 was used to significant compare between means in this study.
Results and Discussion
The fig. 2 showed concentrations of genistein, glycine and malonate in RCIE by HPLC technique were (2.7, 6.6 and 1.04) mg/g respectively, in retention time (5.196, 7.023 and 6.133) respectively. Fig 3 shows concentration of standards.

Fig. 2: chromatographia HPLC of RCIE.A:Glycine, B: Genistein, C: Malonate.

Fig.3: chromatographic HPLC of standards:A: Genistein,B: Glycine, C: Malonate respectively.

Characterization of NLC Formulation
Fig4 shows RCIE-NLCs with milky white solution, the proportions used in the preparation of RCIE-NLCs were increased volume of aqueous phase, increase in drug content of particles and high concentration of surfactant.
Atomic force microscopy (AFM)

Fig. 5, A and B shows images at two and three dimensions of producing sample RCIE-NLCs. From Figure, we can note that the shape of particles was a mix between spherical and cylindrical. The grain size distribution of surface was about from (18 to 62) nm at average 38.78 nm. Fig. 5, C shows the histogram of grain size distribution on the surface.
**Fig 5:** Images of (AFM) for RCIE-NLCs (A) two dimensions, (B) three dimensions, (C) histogram of the distribution of grain size.

**Transmission electron microscopy (TEM)**

Fig. 6, shows the image of TEM obtained from RCIE-NLCs. It can be shown the shape of particles was mostly spherical and few cylindrical with average diameter (53-10) nm and maximum distribution 40. Also the figure notices that the prepared RCIE-NLCs was highly dispersed and this indicates the quality of the prepared nanomaterial.
Fig 6: Transmission electron microscopy image of RCIE-NLCs formulation, with X34000.

Zeta potential (ZP) determination
Zeta potential of the formulation was determined to study the stability behavior of the formulation in vitro and in vivo which was found to be -41.1 mV as shown in Fig. 7. It confirmed the stability of the colloidal system which is high enough to keep the particles aside and prevent the aggregates formation. Also, the negative charge of the nanoparticles will delay their protein binding and thereby results in longer circulation half-life of the nanoparticles. Stability increased with increasing concentrations of surfactant. Values of ZP greater than ±30 mV were generally good indicators of static stabilization of the dispersion system.

Fig 7: Zeta potential of RCIE-NLCs

Encapsulation Efficiency (EE)
The current study showed that concentration of genistein, glycitein and malonate were (1.4, 2.8 and 0.8) mg/g respectively, in the RCIE-NLCs, when 6 mg of RC extract was used to load the NLC, approximately 1 mg of free RC extract was detected in an aqueous RCIE-NLCs dispersion, suggesting that 5 mg (83.3%) of RC extract was successfully encapsulated into the NLCs. NLCs were preferred over the SLNs because of their higher entrapment efficiency and more stability of the formulation. The liquid lipids present in the formulation were able to carry more drug as compare to solid lipids alone. Increasing the liquid lipid content in the formulation could enhance the EE of the formulation. The ratio of oil phase and aqueous phase showed a great impact on the EE of NLC increase aqueous phase volume results in an increase in EE. This could be due to lesser aggregation of the particles in a largerspace. Also, increase in concentration of surfactant resulted in increase in EE.

Lesser surfactant concentration and higher lipid concentration will cause increase in viscosity of the formulation which will result in higher viscous resistance against a shear force which will hinder the...
formation of nanodroplets and also the lesser amount of drug will get solubilized into viscous lipid matrix which ultimately results into decrease EE. Increase in drug content is expected to raise the EE by providing more space to incorporate the drug. Increment of the lipid content also reduces the escaping of drug into the external phase. On the other way, higher amount of organic solvent will lead to leaching of the drug from the lipid core which ultimately will decrease the EE of the formulation.

Effect of VCD on Estrogen, FSH and LH Levels

Table (1) shows the effect of VCD which caused a decreased in estrogen level and increased in FSH and LH levels in group B compared with group A. The results of statistical analysis showed a significant decrease \((P \leq 0.01)\) in the level of estrogen in group B \((23.767)\) ng/ml compared to groups A \((33.154)\) ng/ml and showed significant increase \((P \leq 0.01)\) in the levels of FSH and LH in group B \((4.0688\) and \(3.0839)\) mIU/ml respectively, compared to groups A \((2.9206\) and \(2.1469)\) mIU/ml respectively. The VCD mouse model of menopause has been required repeated daily intraperitoneal injections of VCD to cause loss of primordial and primary follicles in the ovary that produces estrogen hormone via accelerating atresia in primordial and primary ovarian cells by altering the expression and distribution of the Bcl-2 family of proteins that regulate apoptosis and decreasing ovarian mRNA, protein, and/or activity of the enzymes responsible for generating estradiol and its precursor sex steroid hormones and via the direct inhibition of auto phosphorylation of the survival receptor c-kit, located on the plasma membrane of the oocyte, within 14 days after the cessation of daily dosing, VCD has depleted all primordial follicles. During this time frame of impending ovarian failure, there is an increase in cycle length, estrogen levels fluctuate until they reach very low levels, and FSH and LH levels increase as the inhibitory effects of estrogen are removed, thus mimicking perimenopause in humans. 

Effect of EB, VCNO and RCIE-NLCs on Estrogen, FSH and LH Levels

Table (2) shows increased in estrogen level and decreased in the levels of FSH and LH after treated with Estradiol benzoate (EB) and RCEI-NLCs in groups C and E compared with groups B and D. The results of statistical analysis showed a significant increase \((P \leq 0.01)\) in the level of estrogen in group C \((36.444)\) ng/ml compared to groups B, D and E \((23.767, 22.728\) and \(30.008)\) ng/ml respectively, EB is an estrogen medication which is used in hormone therapy for menopausal signs and low estrogen levels in women is a pro-drug ester of Estradiol, a naturally occurring hormone that circulates endogenously within the human body the most potent form of all mammalian estrogenic steroids is estradiol, and acts as the major female sex hormone. Therefore, EB has the same downstream effects within the body through binding to the Estrogen Receptor (ER) including ERα and ERβ subtypes, which are located in various tissues such as: ovaries, skin, bone, breasts, ovaries, prostate, fat, and brain. Estradiol has very low oral bioavailability on its own \((2-10)\) %. First-pass metabolism by the gut and the liver quickly degrades the estradiol molecule before it gets a chance to enter circulation system and practice its estrogenic effects. And the results of statistical analysis showed a significant decrease \((P \leq 0.01)\) in the level of FSH in group C \((2.5556)\) mIU/ml compared with groups B, D and E \((4.0688, 3.9974\) and \(3.2116)\) mIU/ml respectively, and the results of statistical analysis showed a significant decrease \((P \leq 0.01)\) in the level of LH in group C \((0.0485)\) mIU/ml compared with groups B, D and E \((3.0839, 3.1049\) and \(2.4825)\) mIU/ml respectively, it is known that oral and transdermal estrogen treatments exerts negative feedback effects at the hypothalamic-pituitary level on serum FSH and LH secretion and biosynthesis. This regulatory effect of E2 is exerted at two levels, directly at the pituitary level and indirectly at the hypothalamic level via modulation of GnRH. Estrogen exposure in menopausal women whether for short and mid-term shifts the distribution of isoforms of FSH and LH from acidic to more basic forms, which have a shorter half-life in the circulation. Furthermore, the relative proportion of the more basic FSH and LH isoforms within the pituitary tissue increases with the duration of the exposure to E2. Consequently, the decrease in levels of FSH and LH an increase in more basic FSH and LH isoforms.
As for the RCIE-NLCs, there is a significant increase (P≤0.01) in estrogen level (30.008) ng/ml compared with groups B, D (23.767 and 22.728) ng/ml respectively, and significant decrease (P≤0.01) compared with group C (36.444) ng/ml. Phytoestrogens can improve safety and greater compliance, the most common forms of phytoestrogens are isoflavones, which are non-steroidal compounds similar to natural estrogen structurally, because they exhibit a phenolic ring with a hydroxyl radical attached to carbon three. This structure gives them a ability for high-affinity selective binding to estrogen receptors, thereby vesting them to share in estrogenic activity in human tissues\(^{[47]}\). Isoflavones possess an estrogenic or anti-estrogenic impact depending on their concentration, on endogenous sex steroids, and on the particular target organ in the interaction with the ERs\(^{[48]}\). The estrogenic power of isoflavones is low compared to that of 17-β-estradiol, i.e., approximately 1/1000\(^{[13]}\). Isoflavones may practice their biological effects by means other than ER. For example, isoflavones would work through tyrosine kinase receptors and other peptide receptors which located on the plasma membrane of certain cells, isoflavones can bind to the same ERs, and this enables them to regulate the gene expression of estrogen-regulated products. Other possible action mechanisms of isoflavones include cell-cycle regulation and antioxidant effects. Furthermore, isoflavones play a significant role in preventing menopause-related disturbance and chronic diseases, such as cancer, heart diseases, and diabetes\(^{[49]}\). Intact nanoparticles sized above 100nm are not considered to permeate the skin surface because of their dimensions and rigidity\(^{[50]}\).

Since epidermal lipids are rich in skin surface, lipid nanoparticles attaching to the skin surface would allow lipid exchange between skin surface and the nanocarriers\(^{[51]}\). NLs have the potential to deliver drugs via the follicles\(^{[52]}\). Furthermore, each follicle is associated with sebaceous glands, which release sebum, creating an environment enriched in lipids. This environment is beneficial for trapping of NLs. Some glyceride lipids present in NLs may accelerate the entrance into the follicles/sebaceous glands\(^{[53]}\). And this is agreeing with our formulation RCIE-NLCs. As for the effect of RCIE-NLCs on the levels of FSH and LH, results of statistical analysis showed significant decrease (P≤0.01) in the level of FSH in group E (3.2116) mlU/ml compared with groups B and D (4.0688 and 3.9974) mlU/ml respectively, and showed significant increase (P≤0.01) with group C (2.5556) mlU/ml as for the level of LH the results of statistical analysis showed significant decrease (P≤0.01) level of LH in group E (2.4825) mlU/ml with groups B and D (3.0839 and 3.1049) mlU/ml respectively, and showed significant increase (P≤0.01) with group C (2.0485) mlU/ml. When comparing the effect of RCIE-NLCs on the levels of FSH and LH between groups B and D, noted the estrogenic effect of isoflavones that exert estrogen-like effects at pituitary loci suppressed basal FSH and LH secretion, also reduce total FSH and LH production with increase gonadotrope sensitivity to GnRH\(^{[54]}\).

Like what is known that estrogen decreased normal FSH\(\beta\) and LH\(\beta\) transcription. Estrogen indirectly regulates FSH\(\beta\) and LH\(\beta\) transcription by decreasing activin \(\beta\) and ultimately, decreasing synthesis and secretion of FSH and LH\(^{[55]}\). The current results were compatible with this model. But the effect of isoflavones is a weak compared to that of 17-β-estradiol\(^{[13]}\). So isoflavones have less effect on levels of FSH and LH compared with effect of EB, as is evident from the results. While there was no effect of VCNO on the serum steroid hormone levels, as is evident in the table (2). The results of statistical analysis showed no significant decreased in the levels of estrogen in group D compared with group B reached (22.728 and 23.767) ng/ml respectively, and significant decreased compared with groups C and E (36.444 and 30.008) ng/ml respectively, and no significant decreased in the levels of FSH compared with group B that reached (3.9974 and 4.0688) mlU/ml, with significant increased compared with groups C and E (2.5556 and 3.2116) mlU/ml respectively, and showed no significant increase in the level of LH compared with group B that reached (3.1049 and 3.0839) mlU/ml respectively, with significant increased compared with groups C and E (2.0485 and 2.4825) mlU/ml respectively. These results were indicated that VCNO has no effect on gonadotropic hormones and this is agreeing with\(^{[56]}\) Since VCNO had no effect on FSH and LH that did not effect on estrogen level.
Conclusion
In this study work was used RCIE-NLCs to evaluate the efficacy and safety of a novel protocol of nano-transdermal treatment based on a nanostructured formulation of RC isoflavones as an alternative therapy for HRT. The results of this study revealed that administration of RCIE-NLCs for six weeks atransdermal treatment shows an effective in compensating for estrogen deficiency resulting from loss of primordial and primary follicles in the ovary via impact of VCD, compared to EB, which also was raised estrogen levels, but it is known to be associated with an increases the risk of endometrial hyperplasia and ovarian cancer. These activities could support the continued investigate on of RCIE-NLCs as a potential therapeutic agent in HRT. The results may have an important impact in order to create in a close future an effective agent for use in the government women health programs, thus further studies are warranted to clarify its usefulness in women.

Table (1): Mean value of hormones levels among first experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SE</th>
<th>ES ng/ml</th>
<th>FSH mIU/ml</th>
<th>LH mIU/ml</th>
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</thead>
<tbody>
<tr>
<td>Group A</td>
<td>33.154±2.735 a</td>
<td>3.0206±0.2195 a</td>
<td>2.1469±0.5587a</td>
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<tr>
<td>Group B</td>
<td>23.767±2.127 b</td>
<td>4.0688±0.2526 b</td>
<td>3.0839±0.5616 b</td>
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Table (2): Mean value of hormones levels among second experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SE</th>
<th>ES ng/ml</th>
<th>FSH mIU/ml</th>
<th>LH mIU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>36.444±3.371 c</td>
<td>2.5556±0.3062 c</td>
<td>2.0485±0.2511 c</td>
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<tr>
<td>Group D</td>
<td>22.728±2.775 b</td>
<td>3.9974±0.1468 b</td>
<td>3.1049±0.3667 b</td>
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<tr>
<td>Group E</td>
<td>30.008±2.809 e</td>
<td>3.2116±0.2765 e</td>
<td>2.4825±0.6664 e</td>
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</tbody>
</table>

Note: Similar letters within one column indicate that there are no significant differences in the parameters

References


