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Production and Characterization of Nanoparticles Lipid Carrier (NLCs) Loaded with Red Clover Isoflavones Extract and Their effect on Serum Lipid profile in Postmenopausal Period

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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 , to assess the effect of phytoestrogens (PEs) for treating hyperlipidemia that associated with menopause period using nanoparticles lipid carrier (NLCs) loaded with red clover isoflavones extract (RCIE) compared with hormone replacement therapy (HRT). 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 preformed, the first experiment included 20 mice divided into two groups 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 mice have injected IP daily 160 mg/kg of VCD and divided into three groups (C, D and E), (10 / group) then treated with dermal sticker for six weeks: Group C: treated with dermal sticker saturated with 10ug/ kg B.W of estradiol benzoate (EB) diluted with virgin coconut oil (VCNO). Group D: treated with dermal sticker saturated with 0.1 of VCNO. Group E: treated with dermal sticker saturated with 0.1ml of RCIE-NLCs. The results of statistical analysis showed a significant increase in the level of TC, TG, LDL, and VLDL in group VCD. While RCIE-NLCs had a clear effect and significant decreased in the TG, TC, LDL and VLDL cholesterol compared to groups VCD and EB, and significant increase in the HDL-C in group RCIE-NLCs compared to groups VCD and EB. The results of this study revealed that administration of RCIE-NLCs shows an effective to regulation of lipids metabolism disorders due to hormonal changes that associated with menopausal transition via impact of VCD.

Keywords: Red clover, *Trifolium pratense* , Menopause, NLCs, Hyperlipidemia, Estrogen benzoate.

Introduction

Menopause is clinically recognized when a woman has not menstruated for a year owing to ovarian follicular activity reduction, which generally occurs between the ages of 45 and 55. (1). Due to hormonal changes, such as low production of estrogens and elevated levels of circulating androgens, different lipid metabolic disorders emerge during the menopausal transitional phase, which may lead to the formation of metabolic syndromes, such as type 2 diabetes and cardiovascular disease.(2). The creation of excess fatty acids, adipocytocines, reactive oxygen species and pro-flammatory cytokines that induce lipid peroxidation and lead to insulin resistance, abdominal adiposity and dyslipidemia is a fundamental element of lipid metabolism and excessive adipose tissue. (3). In the present study, treatment of female mice with VCD to stimulate perimenopause complicated with hyperlipidemia served as the model of perimenopause. Hormone replacement therapy (HRT) was effectively utilized to treat symptoms of menopause for several decades. Long-term HRT consumption nevertheless related with a higher risk of ovarian and endometrial hyperplasia. In connection to cardiovascular and breast cancer.(4) This is why researchers started investigating alternatives to HRT, such phytoestrogenics (PEs). The isoflavonoids are classified into coumestans and isoflavones. (5) Isoflavones can be discovered as traditional medicines to cure many conditions in Red Clover (*Trifolium pratense*), the native species in the Central Asian region, Europe and North Africa. (6). RC is likely to have beneficial benefits not just on symptoms of menopause, but also on the skeletal system, as well as cardiovascular and hypolipidemic impacts (7). RC has a positive impact on perimenopausal and postmenopausal

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women's lipid profiles. (8) Therefore, is an alternative to traditional hormone treatment derived from several food supplements accessible in markets. (9,10) RC is structurally similar to estrogen compounds but is non-steroidal, and is believed to have the ability to bind to two subtypes, the selective estrogen receptor (ER), and therefore does not pose a risk to the breast and endometrium. (11). Also, do not increase postmenopausal women's incidence of clotting, making PEs a good option to HRT (12, 13) Nanoparticles (NPs), with diameters of 1 - 100 nm, are described as particles. Nanoparticles Lipid carriers (NLCs) are colloidal with increased stability and efficiency in drug loading of the newest generation. The lipids employed for NLC production typically are physiological lipids (biodegradable and biocompatible) to enable medicines to be administered with a decreased, chronic and acute toxicity to the appropriate place of action. (14). NLCs enable the treatment of deep skin layers or even blood circulation to be targeted to make them a high-tech, state-of-the-art technology. A broad range of drug supply methods is referred to by NLCs. The most typical lipid vesicles are known to carry lipophilic or hydrophilic active substances. (15) However, to date, lack or no studies using red clover isoflavones extract (RCIE) as NLCs have reported effects on menopausal status.

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.

Preparation of Red clover isoflavones extract (RCIE)

The figure 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.

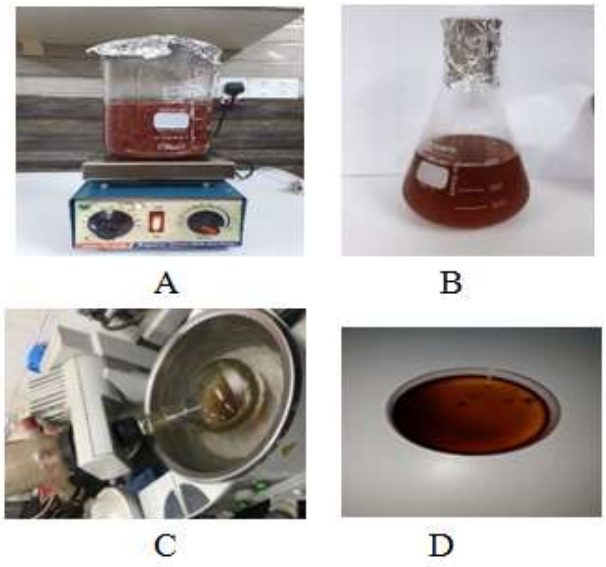

Figure 1: Extraction of RC isoflavones (A) RC soaked in Methanol, (B) RC after filter, (C) Methanol removal, (D) dried RC extract

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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. (16) 6 mg of isoflavones extract (17) 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. 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. (16)

Experimental design

Two experiments were preformed, 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. (18)

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 according to (19) 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 0.1ml of RCIE-NLCs.

Results and Discussion

The figure 2 showed concentrations of genistein, glycitein 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. Figure 3 shows concentration of standards.

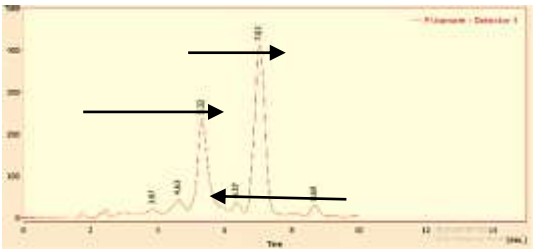

Figure 2: chromatographia HPLC of RCIE, A: Glycitein, B: Genistein, C: Malonate.

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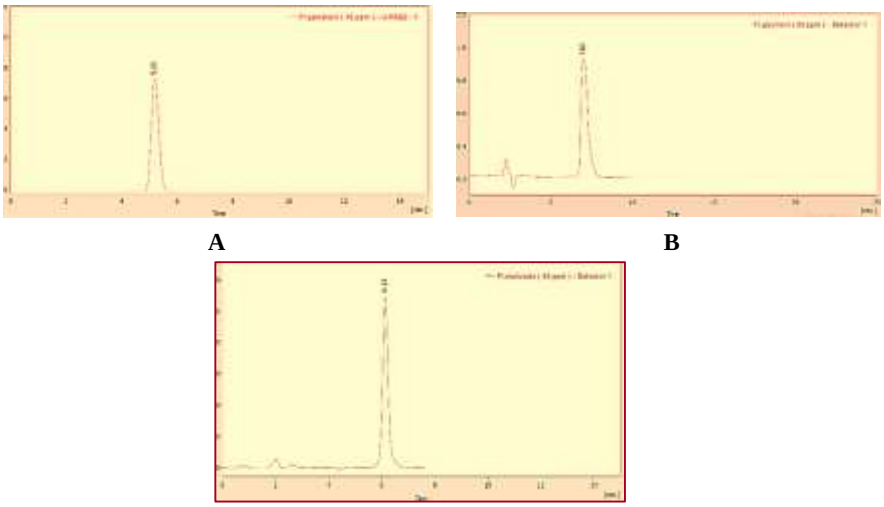

A **B**

C

Figure 3: chromatographic HPLC of standards: A: Genistein, B: Glycitein, C: Malonate respectively.

Characterization of NLC Formulation

Figure 4 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.

Figure 4: Nanostructured lipid carrier loaded with red clover extract

Atomic force microscopy (AFM)

Figure 5, A and B shows images at two and three dimensions of producing sample RCIE-NLCs. From Figure 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, Figure 5, C shows the histogram of grain size distribution on the surface.

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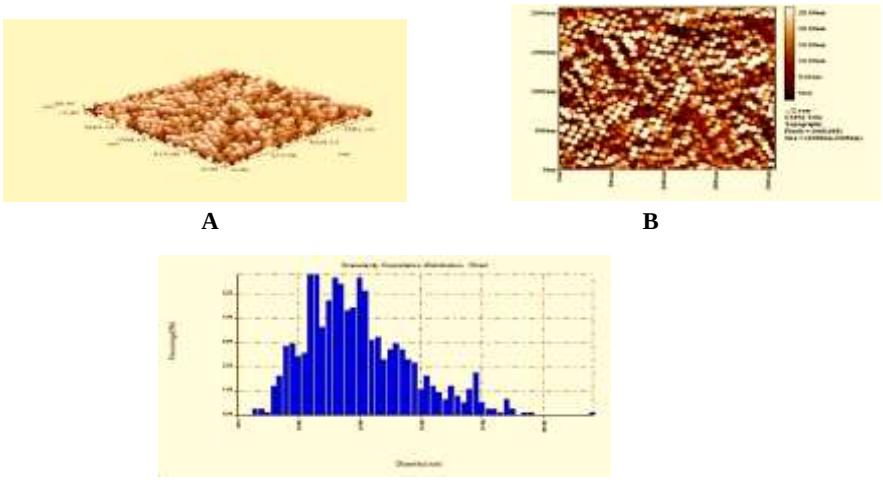

A **B**

C

Figure 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)

Figure 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 figures notice that the prepared RCIE-NLCs were highly dispersed and this indicates the quality of the prepared nanomaterial.

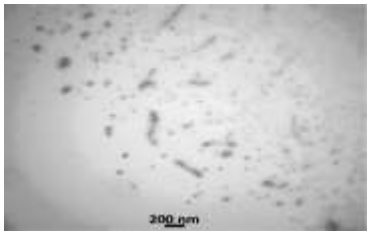

Figure 6: Transmission electron microscopy image of RCIE-NLCs formulation, with X34000.

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 may as show in Figure 7. It confirmed the stability of the colloidal system which is high enough to keep the particles aside and prevent the aggregates formation (20). Stepping up stability with higher surfactant levels. In general, ZP values larger than ±30 mV were good indications of static dispersion system stability. (21)

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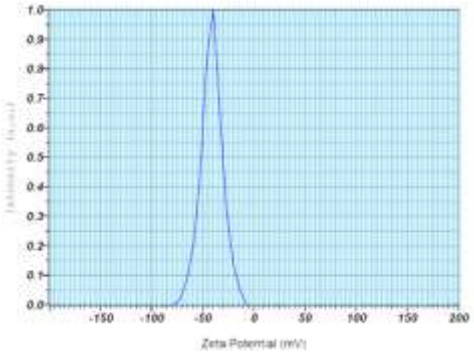

Figure 7: Zeta potential of RCIE-NLCs

Encapsulation Efficiency (EE)

The Concentration of the loaded RC extract in the NLC was calculated according to the following equation. EE (%) = (Total concentration of drug content- free drug / Total drug concentration of content) × 100

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. Due to their better trapping effectiveness and greater formulation stability, NLCs were selected over the SLNs. In comparison with the solid lipids alone, liquid lipids in the formulation may transport longer drugs. Increased liquid lipid content might improve the EE of the formulation. (22). The proportion of oil phase to water phase exhibited a significant influence on the EE of NLC, which resulted in an increase in the EE of NLC .This might be because the particles were less aggregated in a wider area. Increased surfactant concentration also led to an increase of EE. Smaller surfactant concentrations and greater lipid concentrations will produce increased viscosity of formulation, leading to increased viscosity sensitivity to a shear force that prevents the production of nanodroplets and less drug volumes, and will ultimately lead to a decline in the EE lipid matrix. (23)

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

Menopause is marked by hormonal changes from the reproductive to the non-reproductive stage of life. Table (1) shows the effect of VCD which caused a decreased in estrogen level compared with group control, and shows highly significant increase in ES levels in group EB compared to VCD group. Group VCNO had non-significant decrease in ES level compared with group EB. While group RCIE-NLCs had significant increase in ES level compared with group VCD. The VCD mouse model of menopause has been required repeated daily intraperitoneal injections of VCD causes loss of primordial and primary ovarian follicles, which generate estrogen hormone by acceleration of atresia, by alters the expression and distribution of the Bccl-2 family of proteins which regulate apoptosis (24) Decreasing the ovarian mRNAs, proteins and/or enzyme activity of estradiol generation and the sex steroid hormones precursor and VCD depleted all primordial follicles by 14 days of the end of daily dose through direct suppression of autophosphorylation by the survival receptors c-kit, situated on the oocyte plasma membrane. During this time frame, ovarian failure begins the cycle length increases, oestrogen levels vary to very low levels and therefore simulate human perimenopause. (25)

EB is an oestrogen medicine that is used in hormone treatment for menopause and low levels of estrogens in women, and is a drug-friendly ester for Estradiol, an endogenous natural hormone in the body. (26), Estradiol is the most powerful version and the main feminine sex hormone of all mammalian estrogen steroids. EB thus exerts the same downstream effects by attaching the estrogen receptor (ER) to different tissues, including in the breasts, cervics, ovaries, skin, prostate, bone, fat

and brain, including ERα and ERβ subtypes. (27). Estradiol is relatively low in its own oral bioavailability (2-10%). The metabolism of the first-pass gut and liver destroys the estradiol molecule fast before it is able to enter the systemic circulation and its oestrogenic effects (28). Phytoestrogens are structurally similar to natural estrols because they exhibit a phenolic ring with a radical carboxylate hydroxyl attached to carbon three, and give them the ability to engage in selectively high-intensity binding with oestrogen receptors. Phytoestrogens are still the most common types of phytoestrogens (29). Isoflavones are either estrogenic or anti-estrogenic depending on their concentration, gender and interaction with ERs on their unique target organ. (30). Compared to 17-β-estradiol, about 1/1000, Compared to 17-β-estradiol, about 1/1000, isoflavones have an estrogenous potential lowhave an estrogenous potential low (11). Isoflavones may use other methods than ER to exercise their biological effects. , for example, may be used to control gene expression of estrogen-regulated products, by using tyrosine kinases and other peptide receptors on the plasma membrane of specific cells, isoflavones might bundle to the same ERs. The cell cycle modulation and antioxidant effects also comprise the putative modes of action of isoflavones. Intact nanoparticles larger than 100 nanometers do not penetrate the skin surface due to their diameters and hardness (31). As skin-rich epidermal lipids, lipid nanoparticles attached to the surface of the skin would permit lipid exchanges between the surface of the skin and nanoparticles. (32). NLs are capable of supplying drugs via follicles (33). Moreover, each follicle is linked to sebum-releating drums, which provide a lipid-enriched environment. This setting is useful for NL trapping. Some NLCs may speed up entry in follicles/sabaceous glands via glyceride lipids (34). And this is agreeing with our formulation RCIE-NLCs.

Effect of VCD, EB, Coconut oil and RCIE-NLCs on Lipids profile

Hyperlipidaemia is a frequent symptom in women with menopause and is related with ovarian dysfunction. (35), VCD accelerate progression of metabolic syndrome. Table (2) shows effect of VCD which cause hyperlipidaemia in mice model for menopause, the statistical analysis showed highly significant increase in the TG, TC, LDL and VLDL compared with control group. VCD caused highly significant decreased in HDL level compared with control group, and non-significant decreased of EB effect on lipids profile compared with group VCD. Also group coconut oil had non-significant effect on lipids profile compared with group EB. While group RCIE-NLCs have positive significant effect on lipid profiles compared with groups VCD and EB. Increased fatty visceral tissue is a sign of metabolic syndrome. High levels of TG, TC, LDL-C, and VLDL-C are part of the metabolic syndrome. (36) The rise in plasma TG, TC, LDL-C, and VLDL-C has been seen following the discontinuation of menses, whereas HDL-C in women postmenopausal has been decreased. There are substantial changes also after menopause, which may cause cardiovascular conditions, such as an atherogenic lipid profile due to an increase of the overall level of cholesterol and LDL and decreased levels of HDL-C. (2)

As transdermal ES is not digested in the liver, passive skin diffusion enters the blood vessels, (37) The influence on the lipid profile of the first transit in the liver appears to be crucial. So this form of HRT is of no effect on the ES administration via the transdermal route, when compared to the oral method, and therefore has a lower influence on the lipid profile. (38) It delays and somewhat diminishes the impact of the transderm on the lipid profile. The transdermal route effects are thus only evident during extended treatment on lipids. (39) In this study, the transdermal administration of EB did not recorded a significant effect on the lipid profile, while in another study, researchers found that transdermal HRT had no effect on the hepatic enzyme . These results suggest that the initial liver passage's hepatic impact via the transdermal route is lower. (40) The method of ES delivery might further alter the quality of cholesterol molecules. Serum amyloid A (SAA) is an acute-phase liver-produced protein linked with the development and inflammation of atherosclerosis. (41). Coconut oil,

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made up of 92% saturated fatty acids, the majority of which (approximately 70%) are fatty acids of the lower chain, known as medium chain fats (MCFAs). HDL in the VCNO group, MCFAs increased in no mean way (in triglyceride form), compared to group EB (MCFAs), and the ATP A1-binding cassette transporter, preferentially up-regulates the intestinal production of ApoAI (ABCA1). The interaction of apoA-I and ABCA1 facilitates an efflux of cell cholesterol to lipid-free apoA-I, facilitating the transport and synthesis of HDL particles in reverse cholesterol. (42). The present results of non- significantly change for TG, TC, LDL and VLDL compared with group EB may be because not all saturated fatty acids produce the same cholesterol-raising effects. Red clover-obtain isoflavones are beneficial for lipid profiles (43), particularly if they are in the form of nanoparticles. It has been demonstrated that genistein binds directly to ERβ and modulates endothelial cells, prompting nitric oxide production with activation of ERβ, which in turn induces potentially significant vascular anti-inflammatory and antiatherogene effects. (44) Isoflavones lowers LDL-C and enhance hepatic LDL receptors via inhibition of 7α-hydroxylase to reduce endogenous synthesis of cholesterol. (45). Isoflavone have many mechanisms, including prevention of cholesterol biosynthesis and esterification. The main lipoprotein of LDL particles is much less HepG2 release of apolipoprotein B (46), isoflavones enhance the abundance of sterol Regulatory Element Binding Protein (SREBP) and Sterol Regulatory Element (SRE) expression in HepG2 cells and, in fact, these increases lead to increased LDL-receptor surface expression. (47) Relevant observations include an elevation in HDL-C and a drop in TG levels in the menopausal mice model. The efficacy mechanism of isophlavones might be based on the phytoestrogenic characteristics of the red clover isoflavones, enhance the gene expression of apoprotein AI and result in higher in HDL hepatic composition and therefore in serum HDL levels. Isoflavones down control the quantity of mature SREBP-1, the downstream target gene of AMPK is a key transcription factor in the development of fat synthesis, which leads to lower expression of lipogenic genes such as FAS and a concomitant reduction of serum hepatic TGs. (48)

Conclusion

Due to decreased oestrogen production, menopause can lead to different alterations in lipid metabolism. These modifications include an improved fat mass and lower fat-free mass which affects the basic metabolic rate. This study has been used by RCIE-NLCs to assess the effectiveness and safety of a new Nano Transdermal Protective Protocol for the control of lipid metabolism founded on a nanostructured formula of isoflavones. The results of this study revealed that administration of RCIE-NLCs for six weeks as transdermal treatment shows significantly decrease the serum LDL, TC, TG levels and increase serum HDL level, and shows an effective in compensating for estrogen deficiency resulting from loss of primordial and primary follicles in the ovary via impact of VCD.

Table (1): Comparison between control, VCD, EB, Coconut oil and RCIE-NLCs groups in ES level:

Mean ± SE	Groups					
	Control	VCD	EB	VCNO	RCIE-NLCs	LSD
ES(ng/ml)	33.154±2.735 b	2.127±23.767 D	36.444±3.371 a	24.728±2.775 d	30.008±2.809 c	2.001 **

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Table (2): Comparison between control, VCD, EB, Coconut oil and RCIE-NLCs groups on Lipids profile:

Groups	Mean ± SE (mg/dl)				
	TG	TC	LDL	HDL	VLDL
Control	54.20±5.40 A	80.00±2.92 A	50.36±1.28 a	23.00±2.92 a	10.84±0.48 A
VCD	79.40±4.78 B	101.94±6.97 b	66.88±2.17 b	18.80±1.64 b	16.62±0.22 B
EB	76.25±3.29 B	97.80±3.98 b	61.97±1.59 b	17.19±2.518 b	15.84±0.29 B
Coconut oil	75.53±3.33 B	97.22±4.39 b	65.99±1.52 B	19.36±1.45 B	15.96±0.39 B
RCIE-NLCs	60.88±5.04 D	86.03±6.42 d	49.71±0.83 Ac	22.99±3.15 a	12.16±0.50 C
LSD value	4.357 **	5.463 **	5.355 **	2.254 **	1.144 **

Means having with the different letters in same column differed significantly, ** (P≤0.01).

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إنتاج وتوصيف جزيئات الدهن النانوية المحملة بايزوفلافونات البرسيم الاحمروتأثيرها على مستوى الدهون في مرحلة سن اليأس

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الخلاصة

الجسيمات النانوية الصالحة للأكل المشتقة من النبات (PDNPs) عبارة عن حويصلات غشائية بحجم النانو تطلقها النباتات الصالحة للأكل. فهي غير سامة ولها خصائص استهداف خاصة بالأنسجة ويمكن إنتاجها بكميات كبيرة ، تقييم تأثير فيتويستروغنز (PEs) لعلاج فرط شحميات الدم المرتبط بفترة انقطاع الطمث باستخدام حاملة الدهون النانوية (NLCs) المحملة بمستخلص الايسوفلافون البرسيم الأحمر (RCIE) مقارنة مع العلاج بالهرمونات البديلة(HRT) ، أجريت الدراسة باستخدام نموذج خمسين أنثى من الفئران البالغة لانقطاع الطمث باستخدام 4- فينيل سيكلوهكسين ثنائي أكسيد (VCD) وتم التعامل معها على النحو التالي لمدة 6 أسابيع. تم إجراء تجربتين ، تضمنت التجربة الأولى 20 فأرًا مقسمة إلى مجموعتين المجموعة أ: المجموعة الضابطة حقنت 0.1 مل دي. داخل الصفاق (IP) يوميًا. المجموعة ب: حقنت هذه المجموعة IP يوميًا 160 مجم / كجم من وزن الجسم من .VCD شملت التجربة الثانية 30 فأرًا حقنت IP يوميًا 160 مجم / كجم من VCD وقسمت إلى ثلاث مجموعات C) و D و (E ، (10 / مجموعة) ثم عولجت بملصق جلدي لمدة ستة أسابيع: المجموعة :C عولجت بالجلد ملصق مشبع بـ 10 ميكروجرام / كجم من وزن الجسم من استراديول بنزوات (EB) مخفف بزيت جوز الهند البكر .(VCNO) المجموعة د: تعامل بملصق جلدي مشبع بـ 0.1 من .VCNO المجموعة هـ: تعامل بملصق جلدي مشبع بـ 0.1 مل من.RCIE-NLCs ، أظهرت نتائج التحليل الإحصائي زيادة معنوية في مستوى TC و TG و LDL و VLDL في مجموعة .VCD بينما كان لـ RCIE-NLCs تأثير واضح وانخفاض كبير في كولسترول TG و TC و LDL و VLDL مقارنة بمجموعات VCD و EB ، وزيادة كبيرة في HDL-C في مجموعة RCIE- NLCsمقارنة بمجموعات VCD وEB ، كشفت نتائج هذه الدراسة أن إعطاء RCIE-NLCs يظهر فاعلية في تنظيم اضطرابات التمثيل الغذائي للدهون بسبب التغيرات الهرمونية المرتبطة بمرحلة انقطاع الطمث من خلال تأثيرVCD .

الكلمات المفتاحية: البرسيم الأحمر ، سن اليأس ، NLCs فرط شحميات الدم ، بنزوات الاستروجين.