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Research Article

Pharmaceutical and Pharmacological Evaluation of the Effect of Nano-Formulated Spironolactone and Progesterone on Inflammation and Hormonal Levels for Managing Hirsutism Experimentally Induced in Rats

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Abstract. Hirsutism is a dermatological condition that refers to the excessive growth of hair in androgen-sensitive areas in women. Recently, the enhancement of the visible signs of a hairy female has taken special concern that affected the quality of life. The present study was developed to compare the follicular targeting effect of topical spironolactone (SP) or progesterone (PG)-loaded nanostructured lipid carrier (NLC) on the management of hirsutism. Four NLC formulations were prepared using cold homogenization techniques and pharmaceutically evaluated. SP-NLC and PG-NLC topical hydrogels were prepared to explore their pharmacological effect on letrozole induced polycystic ovarian syndrome (PCOS) in rats. Inflammatory mediators, antioxidant, and hormonal parameters were assayed. Additionally, histopathological examination was carried out to confirm the successful induction of PCOS. Results confirmed that all NLC formulations have a spherical shape with particle size ranged from 225.92 ± 0.41 to 447.80 ± 0.66 nm, entrapment efficiency > 75%, and zeta potential (− 31.4 to − 36.5 mV). F1 and F3 NLCs were considered as selected formulations for SP and PG, respectively. Female Wistar rats treated with F1 formulation for 3 weeks displayed better outcomes as manifested by the measured parameters as compared to the other tested groups. A significant reduction in hair follicle diameter and density was observed after topical application of SP or PG nano-gels. Finally, the outcomes pose a strong argument that the development of topically administered SP-NLC can be explored as a promising carrier over PG-NLC for more effectual improvement in the visible sign of hirsutism.

KEY WORDS: Hirsutism; Hydrogel; Spironolactone; Polycystic ovary Syndrome; Nanostructure lipid carriers.

INTRODUCTION

Hirsutism refers to the excessive growth of hair in androgen-sensitive areas in women. Growing hair is usually dark, thick, and coarse. The commonly affected areas are the upper lips, chin, and central chest (1). This condition is one of the most distressing and embarrassing conditions for a woman. The perception of hirsutism is widely subjective, and the quality of life of the hairy females can be adversely affected by this phenomenon. Hence, the present study will offer a new possible solution for this problem by the use of topically applied nano-formulated drugs.

A hyper-androgenic condition such as polycystic ovary syndrome (PCOS) is considered the most common cause of hirsutism representing approximately 70% of hirsutism cases (2). PCOS is usually accompanied by weight gain, infertility, and insulin resistance (3). The latter leads to hyperinsulinemia which will cause the ovaries to increase the production of androgen, leading to anovulation (4). Additionally, the relative alteration in LH/FSH levels, which are two major pituitary hormones, is another pathophysiological perturbation in PCOS. Luteinizing hormone (LH) stimulates the production of androgen substrates, which are converted for the production of sex hormones such as testosterone and estrogen. On the other hand, follicle-stimulating hormone (FSH) stimulates the maturation of the ovarian follicle before being released. LH and FSH need to be in a precise balance for normal physiological function (5).

Spironolactone (SP) is a lipophilic drug which belongs to a class of medications known as potassium-sparing diuretics (6). Food and Drug Administration approved spironolactone

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(SP) for clinical use as an antihypertensive agent (7). SP has been reported in dermatological therapy due to its anti-androgenic effect resulting from dual mechanisms, namely, reduction of androgen production and competitive blocking of androgen receptor in target tissues (8). Numerous clinical studies have demonstrated the efficacy of oral SP by the doses 50–200 mg per day for 6 months on hirsute women. The observed results in multiple studies showed improvement of all factors measured. Yet, this oral regimen is commonly associated with dose-dependent adverse effects (9).

Progesterone (PG) is a natural lipophilic steroid hormone secreted primarily by the corpus luteum of the ovary and the placenta. PG acts on a wide range of tissues, and it is involved in milk production, aging, and hormonal disorder in menopausal women, besides its role as an ovulation inhibitor (contraceptive hormones) (10). The first line of treatment for hirsutism is oral contraceptive (OCP) hormonal medication, especially in those women desiring contraception. Estrogen/progesterone combinations act by suppressing FSH and LH levels. Also, they reduce gonadotropin secretion leading to a decrease in ovarian androgen production (11). Many side effects of PG such as breast tenderness, irregular vaginal bleeding, mood changes, and mild fluid retention were reported after the administration of OCP medication.

Nanostructure lipid carriers (NLCs) have attracted growing attention in pharmaceutical research for dermal and transdermal delivery enhancement. This colloidal drug delivery system is produced using blends of solid lipid (long chain) and liquid lipid (short chain) (12). NLCs nowadays are widely used in topical cosmetic or dermatological follicular targeting preparations as it offers the benefits of reducing both the drug dose and the systemic side effects accompanied by oral delivery of medication (13). The increase in drug distribution to the target site within the hair follicles may result from the small size of the lipid particles that assures close connection to the stratum corneum, thus promoting drug penetration into the hair follicle (14). On the other hand, NLCs were developed as promising drug delivery carriers through the skin due to its lipid nature and biocompatibility. Due to the high ability of NLCs to entrap medications, they have great contribution in resolving the insolubility problem of lipophilic drugs upon reaching systemic circulation (15). Additionally, NLCs are characterized by large surface area which enables longer contact time of the drug with the skin for maximum penetration (16). Generally speaking, the aim of the current study is to formulate SP-loaded NLC and PG-loaded NLC as a topically applied hirsute medication to achieve dual effects, locally on the hair follicles and systemically on the hormonal level.

MATERIALS AND METHODS

Materials

SP was kindly supplied by Eipico (Cairo, Egypt). Progesterone and Tween 80 were purchased from Sigma-Aldrich Chemical Co. (ST Louis, MO, USA). Stearic acid and oleic acid (OA) were donated by Gattefossé (Saint-Priest, France). Poloxamer 188, tri-ethanolamine, and Carbopol 934 were obtained from El-Nile Pharmaceutical Co. (Cairo, Egypt). Letrozole® was obtained from Novartis pharmaceuticals (El Amiria, Cairo, Egypt); other solvents used were of analytical reagent grades.

Preparation of SP-NLCs and PG-NLCs

Four different formulations of NLCs are prepared by the cold homogenization technique Table I. Firstly, solid lipid (stearic acid) was indirectly heated (up to 65 °C), and then, liquid lipid (oleic acid) was added in two different ratios 8:2 and 6:4 w/w and then mixed. Next, either SP or PG was dissolved in an aqueous surfactant solution (Tween 80 and Poloxamer 188). After that, the previous aqueous solution was added to the melted lipid phase while stirring with subsequent rapid cooling in ice. Homogenization was done using a high-speed homogenizer (AH-2010; ATS Engineering Inc., Suzhous Branch, Jiangus, China) at 12000 rpm for 20 min with subsequent probe sonication at 90 W for 5 min, whereas they were still immersed in the ice-bath (17). The resultant NLCs formulations were stored at room temperature for further investigation.

Characterization of SP-NLCs and PG-NLCs

Physicochemical Characterization

NLCs of either SP or PG were characterized in terms of the average particle size (PS), polydispersity index (PI), and zeta potential (ZP). The previous parameters were estimated using a Malvern particle size analyzer (Zeta seizer 4000S, Japan) at 25 °C after proper dilution of the samples with double distilled water. The recorded results in Table II are the means ± standard deviations (SD) of three determinations.

Transmission Electron Microscopy (TEM)

Surface morphology of SP-NLC and PG-NLC were observed using transmission electron microscopy JEOL 1010 (JEOL Ltd, Tokyo, Japan) at 200 kV. One drop of each NLC was diluted to 50-fold with pure water before dropping onto a Formvar/Carbon 230 Mesh copper grids (Zhongjingkeyi Technology Co. Ltd., Beijing, China), air-dried at room temperature of about 25 ± 2 °C for 24 h, and then negatively stained with (1% w/v) phosphotungstic acid for approximately 20 min before observation (18).

Entrapment Efficiency (EE%)

To calculate the amount of either SP or PG entrapped inside the prepared nano-carrier system, about 100 mg of NLCs equivalent to a known amount of each drug was subjected to centrifugation for 20 min at 25,000 rpm, filtered through disk filter (pore size: 0.45 μm, Sigma-Aldrich, USA). Finally, the amount of either SP or PG in each supernatant (free drug) was determined spectrophotometrically at $ʎ_{max}$220 nm and 240 nm for SP and PG, respectively. Drug entrapment efficiency (EE%) was finally calculated using the following equation:

$$EE\% = \frac{\text{Wt.of added drug} - \text{Wt.of free drug}}{\text{Wt.of added drug}} \times 100$$

where *wt. of added drug is* the initial weight of either SP or PG added in the formulations, and *Wt. of free drug* is the amount of each drug in the supernatant (19).

Table I. Formulation of spironolactone and progesterone nanostructure lipid carrier

Ingredients	NLCs Formulations			
	F1	F2	F3	F4
Spironolactone (mg)	30	30	———	———
Progesterone (mg)	———	———	100	100
Stearic acid (gm)	1.8	2.4	1.8	2.4
Oleic acid (gm)	1.2	0.6	1.2	0.6
Tween 80 (mg)	750	750	750	750
Poloxamer 188 (mg)	750	750	750	750
H_2O	QS	QS	QS	QS

Four different formulations of NLCs containing SP and PG were prepared by the cold homogenization technique. Stearic acid used as a solid lipid while liquid lipid (oleic acid) was added in two different ratios 8:2 and 6:4 w/w then mixed. Finally; either SP or PG was dissolved in an aqueous surfactant solution (Tween 80 and Poloxamer 188). *QS* quantity sufficient, *NLCs* nanostructured lipid carriers

Ex vivo Permeation Study

Ex vivo release studies of SP and PG from the selected NLC formulations (F1 and F3), respectively, across hairless rat skin, were carried out using Franz diffusion cells (20). Firstly, the skin section was washed with water and kept for about 30 min in phosphate buffer pH 7.4 to ensure complete saturation (21). Then, the skin section was positioned between receptor and donor compartments. About 2 ml of each NLCs dispersion was located in the donor compartment. The receptor medium (15 ml) consisted of a mixture of phosphate-buffered (PBS) pH .4: ethanol (4:1) (v/v). The addition of ethanol to the receptor fluid was done to enhance the solubility of both SP and PG upon release in the aqueous buffer (22, 23). The temperature was maintained at 37 ± 1 °C. Drug release from different nano-carrier formulations was assessed for 24 h by intermittently sampling the receptor compartment (5 ml) and fresh mixture of PBS: ethanol solution within the same ratio as previously mentioned was replaced. Samples were filtered, and the amount of either SP or PG released was determined using UV spectrophotometer (Shimadzu, UV-Vis 1601 PC spectrophotometer, wavelength range of 200-1000 nm, Tokyo, Japan)

FT-IR Analysis

Fourier transform infrared (FTIR) analysis was performed on the lyophilized form of the selected SP-NLC and PG-NLC formulations along with pure SP and PG. The samples were mixed with KBR (IR grade) at a ratio of 100:1 and scanned over a wave range of 4000–400 cm^{-1} (Shimadzu IR/FTIR spectrophotometer (435 U-O4), Japan. Each data point was recorded in three replicates using absorbance mode to facilitate quantitative analysis (24).

DSC Analysis

Differential scanning calorimetry (DSC) measurements were done for pure SP and PG, and their nano-formulations using (PerkinElmer DSC Calorimeter, Waltham, MA, USA), both selected SP-NLC and PG-NLC formulations were lyophilized before the investigation and accurately weighed for 3 mg and then sealed in an aluminum pan. Finally, the experimental parameters were programed to reach 400 °C with a heating rate of 10 °C/min in a dry nitrogen environment. An empty pan, sealed in the same way as the sample, was used as a reference (25).

Preparation of SP-NLC and PG-NLC Hydrogels

The selected SP-NLC and PG-NLC formulations (F1 and F3) were formulated as a topical hydrogel using 1% w/v Carbopol 934 as a gelling agent. Carbopol 934 (1% w/v) was added to the nano-carrier dispersion under overhead stirring at 300 rpm. Stirring was continued for 1 h until the Carbopol got dispersed. The gel dispersion was then neutralized using tri-ethanolamine and then left for 24 h for complete swelling and equilibration of Carbopol. The final concentrations of SP and PG in the NLC gels were maintained at 2% and 8%, respectively.

Table II. Physicochemical characterization of different spironolactone and progesterone nanostructure lipid carrier formulations

NLCs Formulations	Particle size (nm) ± SD	PDI (%) ± SD	Zeta potential (mv)	% Entrapment Efficiency ± SD
F1	225.92 ± 0.41	0.428 ± 0.01	− 36.5 ± 0.6	89.99 ± 0.45
F2	318.75 ± 0.36	0.712 ± 0.09	− 31.4 ± 0.8	70.35 ± 0.56
F3	270.10 ± 0.12	0.393 ± 0.11	− 36.3 ± 0.9	90.91 ± 0.82
F4	447.80 ± 0.66	0.539 ± 0.12	− 33.1 ± 0.6	73.70 ± 0.55

Results of particle size, PDI, zeta potential measurements using a Malvern particle size analyzer and entrapment efficiency of different SP-NLC and PG-NLC formulations. Values were represented as mean of triplicate ± standard deviation (Mean ± SD, $n = 3$). *PDI* polydispersity index

Evaluation of SP-NLC and PG-NLC Hydrogels

SP- and PG-loaded NLC hydrogels were examined for their physical appearance, pH, and rheological properties using rotational Brookfield viscometer (HBDV-III, USA) (26).

Pharmacological Evaluation

Animals

Adult female Wistar albino rats weighing 120–150 g. were purchased from the National Institute of Ophthalmology, Giza, Egypt. The animals were kept in the animal house of the faculty of pharmacy, MSA University. They were housed under constant environmental conditions of 12/12 h dark/light cycles and a temperature of 25 ± 2 °C. The animals were fed commercially available rat normal pellet diet and water *ad libitum* and were left 7 days for acclimatization. Animal experiments were conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's expectations for animal care and use/ethics committees. The study was approved by the ethics committee of the Faculty of Pharmacy, MSA University (PH2/Ec2/2018PD).

Experimental Design

Twenty-four Wistar rats were evenly divided into 4 groups; the first group received a topical drug-free formulation to serve as a normal control group, while the second group received letrozole 1 mg/kg P. O using an oral tube for 21 days and served as a PCOS positive control group. The third and fourth groups received letrozole 1 mg/kg P. O using an oral tube for 21 days and were simultaneously treated by either topical SP-NLC or PG-NLC hydrogels, respectively, for 21 days (27).

Pharmacological Study

At the end of the treatment period, rats were anesthetized, and blood was collected from the jugular vein and centrifuged (4000 rpm, 4 °C) for 10 min to separate sera. ELISA technique was carried out using the corresponding ELISA kit for the assessment of serum LH, FSH, estrogen, PG, and testosterone in addition to levels of TNF-α and IL-6. All ELISA tests were based on sandwich method, which measures the amount of antigen (analyte) between 2 layers of antibody (i.e., capture and detection antibodies).

Histopathological Examination

Rats' ovaries and skin were removed and preserved in 10% paraformaldehyde. After fixation in formalin, the ovary specimens were dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax. Paraffin blocks were sectioned and stained with Hematoxylin and Eosin (H&E) for histopathological examination by a light microscope (Olympus BX50, Tokyo, Japan) under a magnification of × 40 for histopathological examination. Additionally, hair follicle mean diameter was measured under the microscope, and density was determined using Born-Viewer Image Analyzer.

Statistical Analysis

Values are expressed as mean ± SE of 6 rats, and the difference between groups was tested for significance using analysis of variance (ANOVA), followed by Tukey's multiple comparison test as the *post-hoc* test. The level of statistical significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Preparation of Nanostructured Lipid Carrier

In the present study, four different formulations of NLCs loaded with SP and PG were successfully prepared using cold homogenization technique. This technique is selected during this study to avoid accelerated degradation of PG (thermolabile hormone) due to the elevated temperature of the lipid mixture throughout the preparation (28), as shown in Table I. Stearic acid was selected as a solid lipid due to its ability to solubilize lipophilic drugs (29) as the higher solubility of the drug in the solid lipid is a very critical issue for the NLCs formulation. Oleic acid was chosen as liquid lipid due to its ability to uniform the monondisperse systems; in addition, it was reported in some publications that oleic acid vesicle-loaded medications enhanced the epidermal accumulation of drug (30) with the subsequent deep localized effect of our tested medication on the hair follicle. Tween 80 was selected among various surfactants due to its approved regulatory status and success in preparing various NLCs (31). Additionally, poloxamer 188 was chosen as a second non-ionic surfactant for its ability to increase the mechanical stability of the formed NLC (32). Furthermore, Iti Chauhan *et al*. (33) stated the ability of hydrophilic polymers like poloxamines or poloxamers in forming a coating layer around the lipid particles; thus, they have a great contribution in increasing the residence time of drug molecules in the systemic circulation.

Physicochemical Characterization

The small size of nanoparticles makes the dispersion kinetically stable against sedimentation. In addition, particle size can be used as an indicator of instability (34). The particle size of all NLCs formulations (F1–F4) is presented by the z-average diameter was between 225.92 ± 0.41 and 447.80 ± 0.66 nm, as shown in Table II. It was observed that the particle size of SP-NLC and PG-NLC formulations was mainly affected by the liquid lipid concentrations. As on increasing the oleic acid concentration up to 40% of the total lipid content, the mean particle size of NLCs was consecutively decreased. The decrease in size of the particles with a higher amount of OA might be due to the incompatible mixing between OA and stearic acid; as a result, the free OA might form nano-emulsion with an additional surfactant which results in the formation of smaller particles. Furthermore, the presence of non-ionic surfactants in all formulations may result in stabilization of the lipid-based vesicles (35).

The dispersity index of either SP-NLC or PG-NLC is in the range between 0.393 ± 0.11 and 0.712 ± 0.12, as shown in Table II, which indicates the uniformity of the prepared samples with respect to the particle size as well as the homogeneity and stability of the prepared nanoparticles. Hu *et al*. (36) and Agrawal *et al*.'s (31) studies conducted that the PI of NLCs formulations was decreased by increasing the oleic acid content. Their previous conduction was significantly observed in F1 and F3 formulations. ZP values of all NLCs are in the range of − 31.4 ± 0.8 to − 36.5 ± 0.9 mV Table II. The higher electrostatic repulsions between the particles reflect higher stability. All NLCs formulations showed negatively charged values which were favorable since it indicates long-term physical stability and particle adhesion properties (37). Additionally, it was observed that increasing the liquid lipid concentration in F1 and F3 formulations was associated with an increase in ZP, probably due to the increase in the number of ionized carboxylic groups of oleic acid present at a higher liquid lipid concentration.

Transmission Electron Microscopy

Transmission electron micrograph (TEM) of either SP-NLC or PG-NLC formulations portrays that the particles were spherical in shape with smooth morphology and no aggregated particles were observed. Regardless of the concentration of solid lipid: liquid lipid used, NLCs particles were nanometer-sized with a proper size distribution (181.99–307.48 nm). The round shapes observed assure the malleability of the formed colloidal vesicles. The stabilized spherical particles were formed during homogenization after the addition of Tween 80, which plays a significant role in reducing interfacial tension and thus reduced particle aggregation. Likewise, the incorporation of poloxamer as a hydrophilic surfactant in both formulations initiated a significant reduction in the particle size as a result of its steric stabilization effect (32). The average particle size observed in the TEM images is in good agreement with the size obtained from the PS analyzer, as shown in Fig. 1.

Entrapment Efficiency (EE%)

The effect of oleic acid (OA) on drug entrapment efficiency in NLCs is investigated in Table II. It has been observed that the drug entrapment efficiency of NLCs had increased to (89.99% and 90.91%) by increasing the percentage of oleic acid from 20 to 40% w/w in SP-NLCs (F1) and PG-NLCs (F3), respectively. This observation might be due to the incorporation of liquid lipid OA into solid lipids (stearic acid), which have led to massive crystal order disturbance. Greater imperfections in the crystal lattice leave enough space to accommodate drug molecules, which subsequently improved drug-loading capacity and drug entrapment efficiency (38). Higher entrapment in all NLCs formulations containing 40% w/w oleic acid indicates higher solubility of either SP or PG in oleic acid, compared to stearic acid.

To sum up the results of the previous pharmaceutical experiments, it was observed that F1 and F3 shown minimum particle size, maximum ZP value, and highest entrapment efficiency %. Accordingly, they had been chosen as a selected NLC formulation to carry out the further investigations.

Ex vivo Permeation Study

The *ex vivo* release profiles of SP-NLC (F1) and PG-NLC (F3) formulations prepared by binary lipid phase (10%) in comparison with their drug suspensions containing the same drug concentration are evaluated using Franz diffusion cell as shown in Fig. 2. The prepared nanoparticles showed initial burst release within 30–40 min of 30.05% for SP and 36.20% for PG, followed by slow diffusion of both medications out of the core. The initial burst of drugs might be due to the presence of oleic acid on the outer shell of nanoparticles, while the slow diffusion pattern of both medications after the initial burst might be due to the penetration of the diffusion medium into the hydrophobic lipid core. Additionally, there was an observed reduction in the drug release at the end of diffusion that reflecting the depletion of the SP and PG from the core lipid matrix, resulting in a reduced concentration gradient. Drug suspension (used as references) release about 45% for SP compared with 85% from SP-NLC formulation and about 33% PG in comparison with 76% from PG-NLC formulation at the end of 24 hours release study using the same experimental conditions.

FT-IR Analysis

FT-IR spectra of SP (pure), PG (pure), and their selected NLCs formulations are illustrated in Fig. 3. The spectrum of

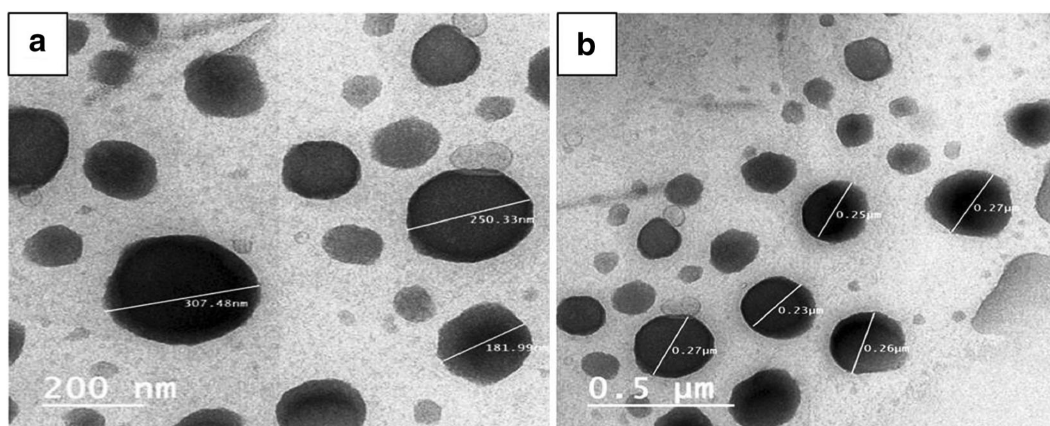

Fig. 1. Transmission electron micrographs of **a** SP-NLCs and **b** PG-NLCs

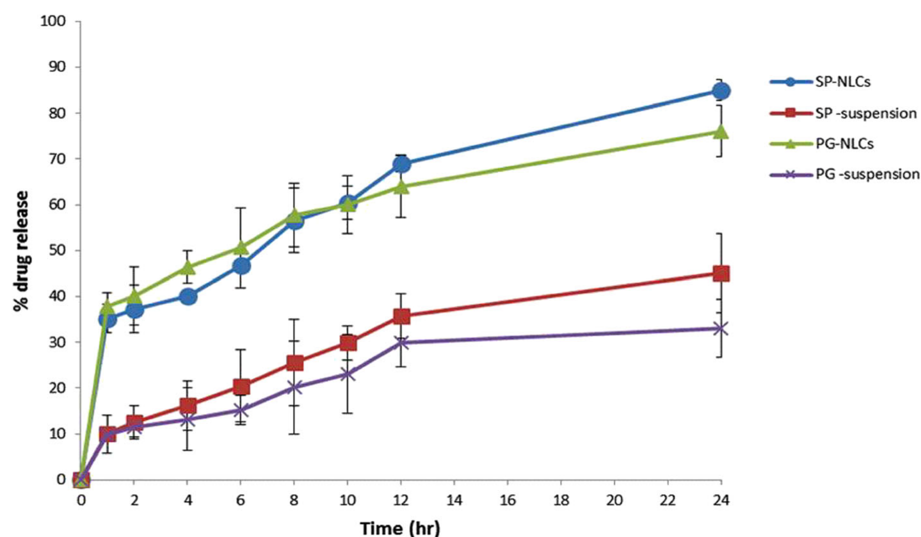

Fig. 2. *Ex vivo* permeation study of SP-suspension, PG-suspension, SP-NLC, and PG-NLC optimized formulation in PBS of pH 7.4

SP (pure) indicated the presence of C-H aliphatic bands at 2926 and 2857 cm^{-1}. The sharp absorption band of lactone carbonyl group C=O appeared around 1769 cm^{-1}, while the retinoic c=o groups appeared at 1686 cm^{-1}. The spectrum of SP-NLC revealed the presence of absorption bands at 2949, 1770, and 1682 cm^{-1} corresponding to C-H aliphatic and C=O groups, respectively. Shifting of carbonyl groups confirms the possibility of interaction between SP and nano-lipid vehicles via intermolecular hydrogen bonding (39). The FT-IR spectrum of PG (pure) indicated the presence of C-H aliphatic bands at 2938 and 2853 cm^{-1} along with C=O absorption band at 1697and 1662 cm^{-1}, while the spectrum of PG-NLC showed no change in the wavenumbers related to carbonyl or aliphatic C-H, enlightening that there are no signs of interaction between the PG and the NLC components.

DSC Analysis

Generally, DSC is a widely used tool to characterize raw materials used in lipid-based drug delivery systems (40). In this study, DSC has been carried out to investigate the melting and recrystallization behavior of material that has crystalline structures and allows us to evaluate the compatibility of drugs with the lipid excipients used (41). The DSC thermogram of SP (pure), PG (pure), and both their NLCs lyophilized dried form is shown in Fig. 4. The thermal curves of SP (pure) showed a characteristic sharp endothermic peak at 205.39 °C; this characteristic peak became less intense and shifted to 217.72 °C in SP-NLCs, indicating the entrapment of SP in the lipid matrix. A similar event has been detected in the thermogram of PG-NLC as the characteristic sharp endothermic peak of PG (pure) was clearly visualized at 125 °C; this peak was shifted in the DSC apparatus in PG-NLCs to 119.65 °C. The most probable reason for the shifting in the characteristic peaks of both SP and PG in their formulations could be due to the higher hydrophobic nature of both which enhances their dissolution in the molten stearic acid (42). On the other hand, the presence of the characteristic peak of both SP and PG in their lipid nano-formulations proved the absence of any interaction between both drugs and the chosen lipids.

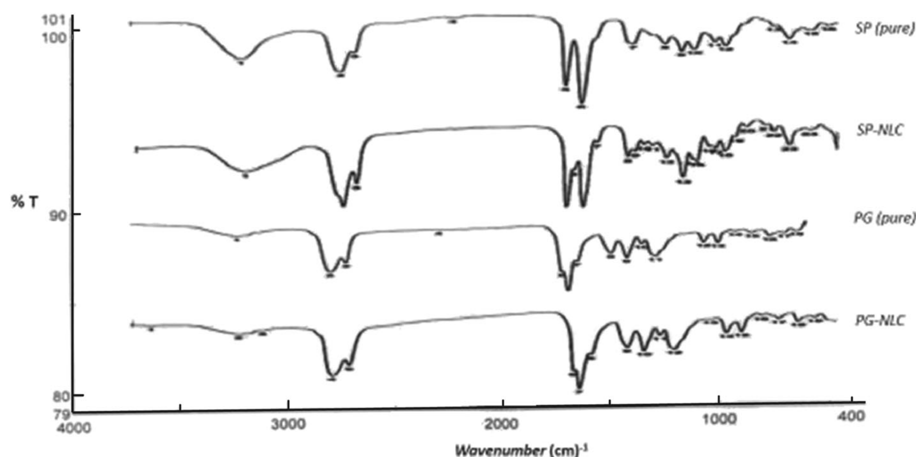

Fig. 3. FT-IR spectra of SP (pure), SP-NLCs, PG (pure), and PG-NLCs

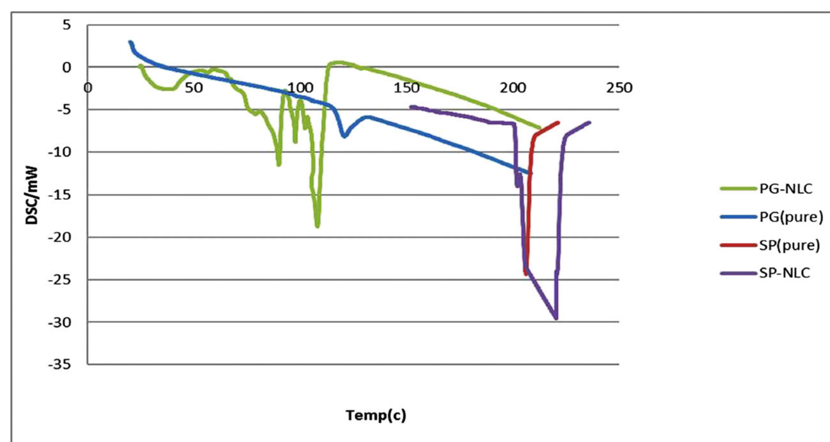

Fig. 4. DSC thermograms of SP (pure), SP-NLCs, PG (pure), and PG-NLCs

Evaluation of SP-NLC and PG-NLC Topical Hydrogels

To investigate the dermatological effect of both SP-NLC and PG-NLC formulations on the treatment of hirsutism, topical hydrogels were prepared using 1% w/v Carbopol 934 as a gelling agent. Visual inspection of the prepared hydrogels indicates a suitable homogeneity and consistency with the absence of lumps. pH values of the developed hydrogels were

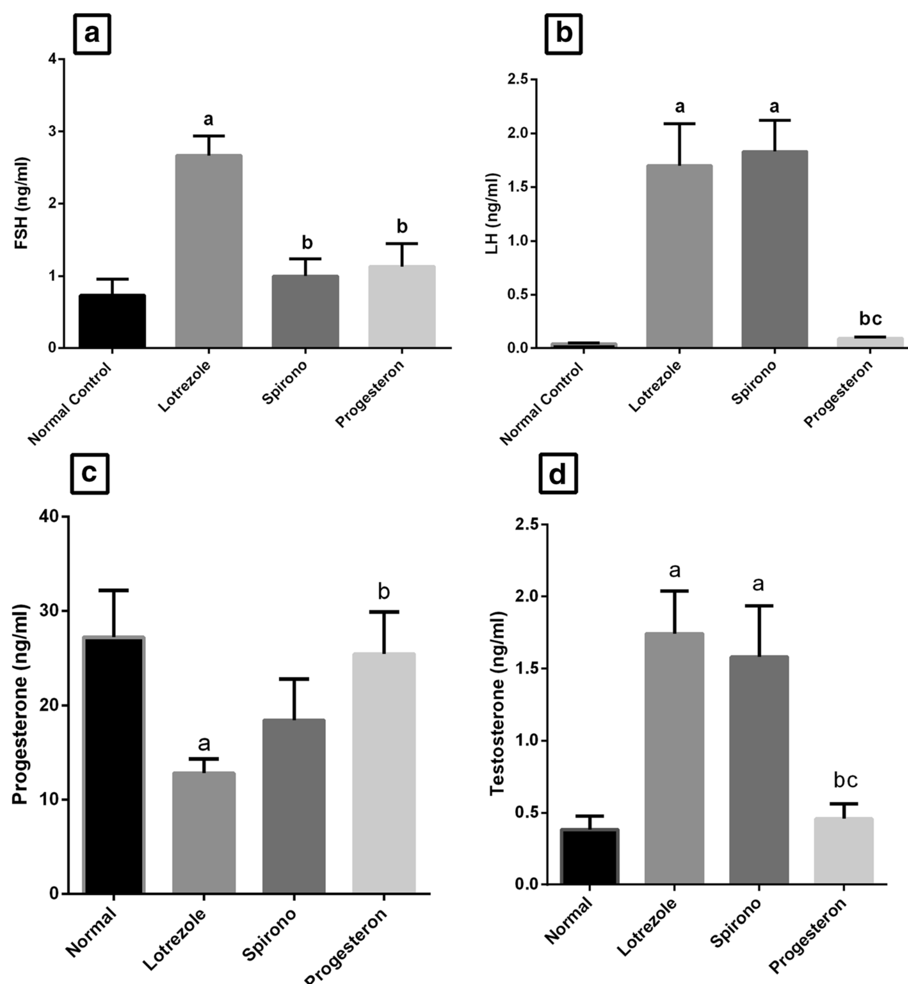

Fig. 5. Effect of Letrozole and 21 days administration of SP-NLC and PG-NLC topical hydrogels on serum **a** FSH, **b** LH, **c** PG, and **d** testosterone. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test ($P < 0.05$). Values are mean ± SE of (6 animals) as compared with normal control (a), letrozole (b), SP-NLC hydrogel (c), and PG-NLC hydrogel (d) treated groups

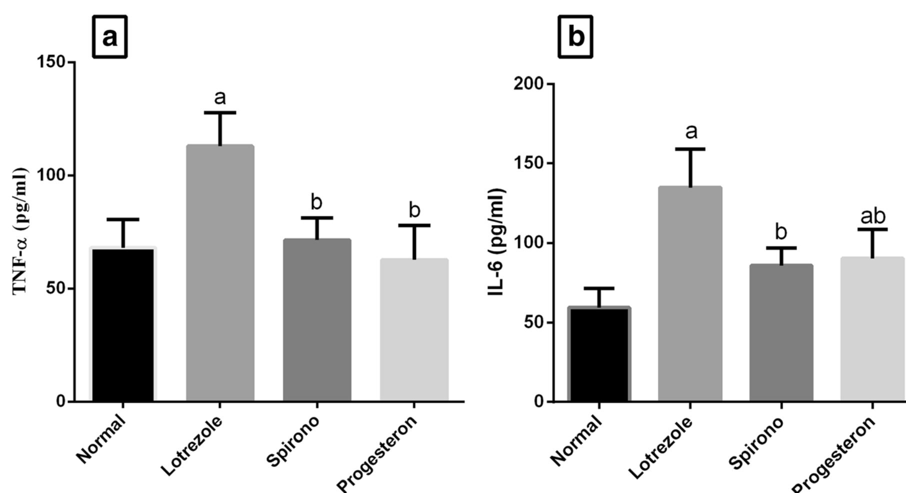

Fig. 6. Effect of Letrozole and 21 days administration of SP-NLC and PG-NLC topical hydrogels on serum **a** TNF-α and **b** IL-6. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test ($P < 0.05$). Values are mean ± SE of (6 animals) as compared with normal control (a) and letrozole (b) treated group

ranged from 5.50 ± 0.14 to 6.4 ± 0.60 for SP-NLC and from 5.95 ± 0.20 to 6.25 ± 0.15 for PG-NLC, which are considered an acceptable value to avoid the risk of irritation after skin application. The rheological parameter of both NLC gel formulations exhibited shear thinning flow pattern with a viscosity value (1570 ± 33.67 and 1743.75 ± 13.15 cps) for SP-NLC and PG-NLC gels, respectively, thus, indicating the non-Newtonian pseudoplastic behavior of the examined gels.

Pharmacological Evaluation

Hormonal Plasma Assays

In the current study, PCOS was induced by daily oral administration of letrozole (1 mg/kg), a non-steroidal aromatase inhibitor, to female Wistar rats for 21 consecutive days. Deficiency in aromatase activity plays an important role in PCOS, affecting steroidogenesis and triggering ovarian failure (43). Aromatase enzyme catalyzes the synthesis of estrogen from androgens. Its deficiency results in hormonal imbalance with decreased estrogen and increased androgen level. The circulating androgens, together with excess intra ovarian androgens, cause polycystic ovaries (44). This condition has also been associated with abnormal follicular development (45).

In the present study, 21 days of daily oral administration of letrozole (1 mg/kg) brought about a significant increase in serum FSH (263.7%), LH (3853.5%), and testosterone (357%). Meanwhile, it reduces serum PG levels (53%) as presented in Fig. 5. These results are in agreement with previous reports where letrozole in a dose-dependent manner decreased serum PG level, increased LH and testosterone, and markedly elevated FSH level in the higher doses of letrozole (0.5 and 1 mg/kg) (27). Furthermore, Fig. 6 shows that letrozole was able to increase both serum levels of TNF-

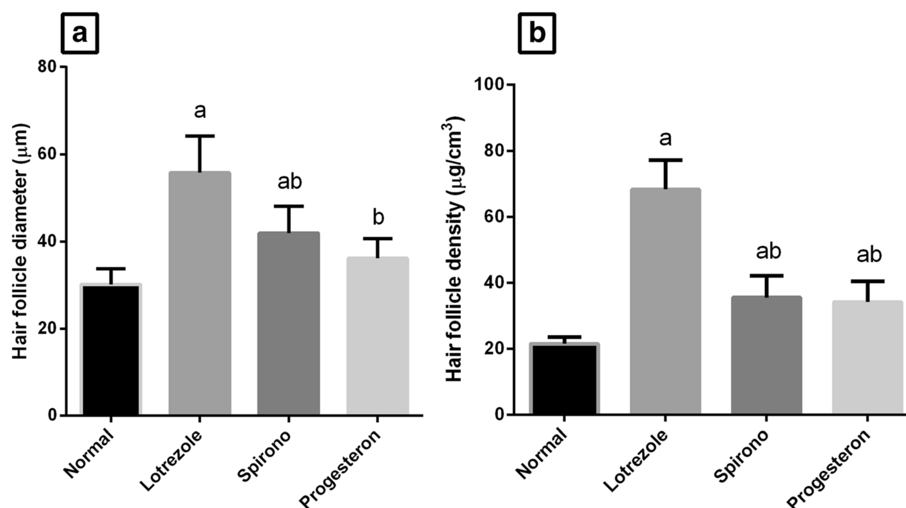

Fig. 7. Effect of Letrozole and 21-day administration of SP-NLC and PG-NLC topical hydrogels on hair follicle **a** diameter and **b** density. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test ($P < 0.05$). Values are mean ± SE of (6 animals) as compared with normal control (a) and letrozole (b) treated group

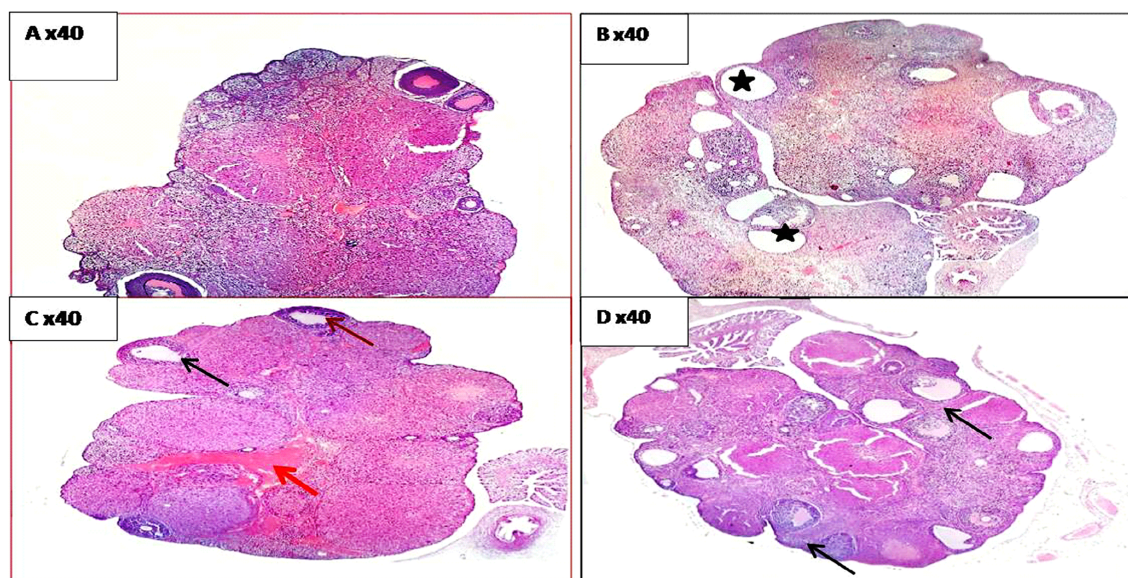

Fig. 8. Effect of Letrozole and 21 days administration of SP-NLC and PG-NLC topical hydrogels on histopathological examination in the ovary. **A** Normal control group. **B** Letrozole-positive control group. **C** and **D** Treated by topical SP-NLC and PG-NLC hydrogels, respectively

α (66.2%) and IL-6 (126.8%) as indicators for inflammation. These inflammatory-inducing effects of letrozole were also previously observed in other models of PCOS (46, 47). In the current study, all biochemical parameters were assessed in plasma using the ELISA technique.

SP is an aldosterone antagonist that is used in PCOS because it blocks testosterone receptors, thus terminating its actions (48). Daily application of SP-NLC topical hydrogel reversed FSH level but had no effect on LH, PG, or testosterone levels. A similar result was obtained by a previous study documenting no change in testosterone or LH levels in women with PCOS (49); this might be attributed to the fact that SP acts on the receptor level and does not affect hormonal production. In contrast, the application of PG-NLC hydrogel restored the levels of all hormones, perhaps due to a negative feedback mechanism. Meanwhile, serum TNF-α and IL-6 levels are significantly reduced in rats treated by either SP-NLC or PG-NLC hydrogels Fig. 6.

Hair Follicles Parameters

All of the rats in the treated groups showed a considerable moderated rate of hair growth on the areas in which both topical nano-gel applied which was reflected on the duration of time needed for shaving since the hair follicles became looser and more comfortable to pluck, the hair follicles' diameter and density (Fig. 7). Additionally, it was noticed that no allergic reaction to the topical medication or skin eruption in the treated areas in which the hydrogels were applied in all tested groups.

Histopathological Examination

The induction of PCOS was also confirmed by histopathological examination of the rats' ovaries, which revealed the presence of numerous follicular cysts. Figure (8) shows (A) ovary of rats in normal control group showing a normal histological structure in the proestrus with different stages of growing follicular, (B) ovary of letrozole-treated rats showing numerous follicular cysts (★), (C) ovary of SP-NLC-treated rat showing few outer growing follicles (→) and many corpora lutea with intramedullary congested BVs and extravasation of blood (red arrow), and on the contrary, (D) ovary of PG-NLC-treated rats showing normal histological structures with many growing follicles (→) and intact stromal tissue.

CONCLUSION

In the present study, SP and PG nanostructured lipid carriers were successfully prepared by cold homogenization technique using various solid lipid: liquid lipid ratios. All NLC formulations exhibited nanometer size, stable polydispersity index, and also acceptable zeta potential negative charge. Regarding the pharmacological effect of the NLC formulations, the null hypothesis was rejected; the pharmacological evaluation indicated that the increase in the hair follicle diameter and density of rat-induced PCOS model was significantly decreased after topical application of both SP-NLCs and PG-NLCs nano-gels for successively 21 days. The effect of the topically administered NLCs on hirsutism might be attributed to their local action on the hair follicle resulted from interaction of the lipid-based nano-carrier with the lipophilic sebum present inside the follicular ducts and systemic action on the hormonal level.

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