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Ex vivo permeation parameters and skin deposition of melatonin-loaded microemulsion for treatment of alopecia

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Abstract

Background: Melatonin (MEL) is a powerful antioxidant molecule with anti-androgenic property. A microemulsion (ME) system loaded with MEL was designed for treatment of androgenic alopecia. Pseudo-ternary phase diagram was constructed, and ME formulae were developed using coconut oil, Tween 80 and PEG 400. In the present study, MEL ME was characterized and evaluated for droplet size, polydispersity index, zeta potential, morphology using TEM imaging. MEL ex vivo permeation study through rat skin followed by tape stripping for stratum corneum (SC) was performed for different ME formulae, to determine skin permeation parameters and detect SC-MEL deposition.

Results: Spherical and uniform particles of MEL-loaded microemulsion were formulated with high stability. In ex vivo permeation study, MEL ME exhibited low steady-state skin flux along with pronounced SC deposition which prevailed a controlled release manner.

Conclusion: The results suggested that MEL ME could be a promising candidate for further permeation and in vivo studies for androgenic alopecia treatment.

Keywords: Melatonin, Microemulsion, Transdermal, Androgenic alopecia, Topical

Background

Hair is a significant factor in maintaining the way of positively perceiving oneself and is linked with youthful vitality. Androgenetic alopecia (AGA) is one of the utmost familiar chronic disorders which affects a wide range of genetically predisposed persons. Around 30% to 40% of women and 65% to 85% of males suffer from this disorder [32].

Melatonin (MEL), or N-acetyl-5-methoxytryptamine, is a renowned neuroendocrine mediator which controls a wide variety of body functions and plays a vital role in primary circadian pacemaker, to synchronize the body functions with environmental light/dark conditions [54]. Due to its lipophilic nature (Log $P = 1.2$ at 28.0 °C) [26], MEL can cross all biological barriers and exhibits an effect on all subcellular compartments including nuclei and mitochondria, acting directly or through receptors [41]. Numerous studies revealed that the mammalian organisms skin owns melatonergic receptors type 1 (MT1) and type 2 (MT2) receptors of high binding affinity to MEL [25, 56]. The genes encoding the MT1 receptors have been located in epidermal sites such as dermal papilla fibroblasts, keratinocytes and hair follicle [49], while MT2 receptors are principally found in adnexal structures like the inner root sheath, the eccrine glands and the blood vessels of human skin [48, 50]. Thus, the pleiotropic biochemical action of MEL at skin compartments allowed transdermal and topical application of MEL for effective treatment of androgenetic alopecia [19].

Microemulsions own various advantages compared to other drug delivery systems, like simplicity in ways of preparation and excellent lipophilic drugs loading

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capacity which leads to high drug concentration gradients on the way through the skin allowing free and fast drug diffusion [52]. There are three possible potential ports of skin penetration which are intercellular penetration pathway, transcellular penetration pathway and the follicular penetration pathway [45]. These pathways are stated to be of relevance for substances applied topically, which have got their own limitations and must not be considered selectively, as most of the substances possibly use more than one penetration pathway parallelly [37]. Several studies proved that O/W microemulsion delivery system showed effective skin permeation [5, 29, 52].

This study was constructed to elucidate the underlying mechanism of percutaneous permeation of MEL employed in optimized ME formulae and inspect a concept for the possible MEL transdermal delivery. The effect of the presence of large water content, that aids skin hydration along with the oil phase and nonionic chemical permeation enhancers, will be investigated.

Materials and methods

Materials

MEL was purchased from Skin actives company, Gilbert, AZ, USA. Tween 20 (polyethylene glycol sorbitan monolaurate), Tween 80 (polyoxyethylene (80) sorbitan monooleate), PEG 400 (polyethylene glycol 400), oleic acid, isopropyl myristate, coconut oil, olive oil and jojoba oil, methanol (HPLC grade) and acetonitrile (HPLC grade), potassium persulfate were supplied from Sigma-Aldrich (St. Louis, Missouri, USA). Disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride (for preparation of phosphate-buffered saline pH 7.4) were purchased from El-Nasr Pharmaceutical chemicals (Cairo, Egypt). Xyla-ject (Xylazine Hydrochloride 23.3 mg/ml) and formol saline were bought from Adwia Pharmaceuticals (Cairo, Egypt). Other solvents and chemicals were of analytical grade.

Methods

Determination of solubility of MEL in different oils and surfactants

All experiments involving MEL were conducted in amber colored containers to achieve protection of MEL from light due to its photoreactivity [2, 4, 33]. Solubility studies were done based on the method reported by Bhusnure et al. [6] for appropriate selection of oils (coconut oil, olive oil, jojoba oil, oleic acid), surfactants (Tween80, Tween20) that have good MEL solubilizing capacity. Excess quantity of MEL was being dissolved in 2 ml of different oils and surfactants and co-surfactant in 5 ml capacity stoppered vials. All vials were tightly sealed and then continuously stirred at 32.0 ± 0.5 °C inside a shaking incubator (KS 4000 from IKA, Beijing, China) at 250 strokes/min for 72 h. After reaching equilibrium, samples were withdrawn from each vial and then were centrifuged using a cooling centrifuge (Sigma Laborzentrifugen GmbH, Osterode, Germany) for 15 min at 5000 rpm. The supernatant was drawn and filtered with 0.45 µm membrane filter (Thomas Scientific Philadelphia, USA), to be dissolved in ethanol, and the amount of MEL dissolved was quantified by UV–Vis spectroscopy (Shimadzu UV–Vis spectrophotometer 240j/PC, Tokyo, Japan) at λ_{max} 278 nm subsequent to appropriate dilution with ethanol. Molar absorption coefficient of MEL was detected at its λ_{max} in different media: ethanol (for solubility studies), methanol and phosphate-buffered saline (PBS) pH 7.4 (for ex vivo permeation studies).

Preparation of melatonin-loaded microemulsion

Pseudo-ternary phase diagram was constructed via water titration method at 25.0 °C, in order to investigate the performance of systems containing oil, surfactant/co-surfactant mixture (Smix) and water. Surfactants and co-surfactant (Smix) were vortexed (Heidolph vortex, Germany), in various ratios (1:1, 2:1, 3:1 and 4:1). The ratio of oil to the Smix was varied as per weight-to-weight ratios 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Under vortex mixing, double distilled water was titrated dropwise to substantiate the efficacious formation of ME. The titrated water quantity was recorded and phase diagram was designed using Triplot 4.1.2 software (Todd A. Thompson USA) software and all experiments were repeated in triplicate [17].

Preparation of melatonin-loaded MEs

Drug-loaded ME formulae were prepared by dissolving MEL gradually in coconut oil to be stirred for 24 h, to reach dissolution equilibrium. The Smix was added, and then, double distilled water was titrated to the mixture using a vortex mixer. The un-dissolved drug was precipitated by centrifugation, and the amount of MEL in the supernatant was quantified by UV–Vis spectrophotometer. The MEs were set aside for 24 h to reach equilibrium before further investigation. MEL (0.5% w/w) dissolved in coconut oil was used as a control for ex vivo permeation studies [30].

Characterization of melatonin-loaded MEs

Droplet size, polydispersity index (PDI) and zeta potential (ZP) determination

The droplet size, ZP and PDI of one gram of each ME formula containing (0.5% w/w) of MEL were measured by dynamic light scattering (DLS) without dilution, by Malvern Zetasizer Nano-ZS instrument (Malvern instruments Ltd., Worcestershire, UK). Scattering of light was observed at 25 °C at 90° angle [42]. ZP was being

measured in the form of the potential difference of charge between the continuous phase and the particles [16].

Visual and optical inspection

Visual inspection of ME formulae was determined for 3 months, through monitoring non-medicated ME samples in clean and transparent containers in the presence of good lightening and medicated ME samples wrapped in aluminum foil due to MEL photosensitivity [4, 33]. Samples were observed against black and white illuminated background for the following characteristics homogeneity: color, transition of clear system to opaque, phase separation and the presence of any precipitates [13].

Ex vivo melatonin skin permeation study

MEL permeation study through rat skin was performed for the optimized ME formulae based on the guidelines implemented by Research Ethics Committee of Faculty of Pharmacy, Future University in Egypt (FUE) No (REC-FPSPI-11/78). Firstly, the rats were subjected to euthanasia through administration of sodium thiopental overdose, and then, abdominal hair removal took place through shaving using an electric shaver. After removing all abdominal hair, full-thickness rat skin was removed and examined for any bites, scratches or anomalies prior carefully removing the subcutaneous fat without causing any damage to the epidermis and then the skin was immersed in PBS solution for 10 min prior to execution of the permeation study [44].

Onto the receptor compartment, the full thickness skin was mounted with the stratum corneum side opposite to the donor chamber and then the ME formulae were applied into each cell in the Franz diffusion cell (Hanson research, Vision Microelle autosampler, 6-Cell drive system, autofill collector system, California, USA). Each cell was sealed by parafilm to prevent any water evaporation and ensure occlusive conditions. The automatic sampler was adjusted to sample and replace the PBS solution volume in the receptor chamber at time intervals of 1, 2, 3, 4, 5, 6, 8, 12, 24 h, and then, the withdrawn sample was added in a rack coded with numbers related to each cell [44]. Finally, samples were then collected for measurement of drug concentration via UPLC system (ultra-performance liquid chromatography) (Agilent 1290 Infinity LC system equipped with G4220 degassing unit, G4204A Quat pump, G1316C column compartment, G4226A thermostatted autosampler and G4212A photo Diode Array Detector). MEL concentrations were detected quantitatively via an analytical assay developed and validated in our laboratory. The samples were injected with a 10-µl autosampler injector onto C8 reversed-phase analytical column. The mobile phase consisted of acetonitrile and acetic acid (1%, v/v) and (80:20, v/v) in an isocratic elution mode at a flow rate of 0.2 mL/min. The UV detector wavelength was established at 256 nm. There was interference detected from any formulae constituent during MEL elution. The cumulative quantity of MEL permeating across rat skin was plotted against time. This experimental work was executed in triplicates.

The permeability coefficient (P) of MEL across rat skin was calculated based on Eq. (1) [43].

$$P = \frac{1}{AC_{\mathrm{D}}} \cdot \frac{\mathrm{d}Q}{\mathrm{d}t} \tag{1}$$

where A is the diffusional area of diffusion cell, C_{D} is the concentration at the donor chamber, and $\mathrm{d}Q/\mathrm{d}t$ is the slope in the steady-state region of the amount of the drug permeated. Experiments were executed for 24 h so that the steady-state regions were almost three to five times longer than the lag time.

MEL steady-state flux (J_{ss}) was derived from the following equation:

$$J_{\mathrm{ss}} = PC_{\mathrm{D}} \tag{2}$$

Tape stripping of skin for stratum corneum (SC) retention study

After Franz permeation study, the skin in each cell was rinsed well to remove any excess formulation followed by drying with clean filter paper. Skin samples were attached and secured properly on a flat clean surface, and then, the SC layer was taken off using 20 adhesive tape strips (D-Squame tapes, CuDerm Corp., Dallas, TX, USA). For MEL extraction, the tape strips (excluding the first one) were immersed in separate containers (groups of five) and being soaked in 5 mL methanol overnight prior to being analyzed via UPLC. The experiments were performed in triplicate [1, 8]. The quantity of MEL found in the SC was calculated by the equation adopted from Subongkot [52].

$$\text{Drug content in SC}\left(\mu\mathrm{g/cm}^2\right) = Qs/P \tag{3}$$

where Q_{s} is MEL quantity in SC (tape strips) (µg) and P is skin penetration area (cm^2).

Transmission electron microscopy (TEM)

The optimized ME formula was visualized for morphology and structure using TEM (JEM-2100, Jeol, Tokyo, Japan). A blend of diffraction modes and bright-field imaging at increasing magnification was applied to reveal the size and form of the ME. A thin layer of the prepared ME sample was negatively stained with 1% of phosphotungstic acid and left till dry for 10 min. Right after placing the copper grid in the electron chamber of the TEM,

an image was shaped due to the transmitted electrons' interaction through the ME sample. On an imaging device, the image was focused and magnified, to be seen on a computer screen [12, 38].

Statistical analysis

Statistical analysis designed for all experiments was performed through applying one-way analysis of variance (ANOVA) trailed by post hoc test, by means of SPSS® software (IBM 20), USA. The significant level in all statistical tests was set at p value ≤ 0.05. All the results were expressed as mean values ± SD.

Results

Determination of solubility of melatonin in different oils and surfactants

MEL solubility in different oils was measured and is arranged in Fig. 1 as follows: coconut oil [12.82 ± 0.5 mg/mL] > oleic acid [10.22 ± 0.4 mg/mL] > olive oil [9.5 ± 0.6 mg/mL] > Jojoba oil [7.3 ± 0.8 mg/mL].

Drug solubility of MEL in Tween 80, Tween 20 and PEG 400 was found to be [10.7 mg/mL], [10 mg/mL] and [8.1 mg/mL], respectively. Tween 80 and Tween 20 showed no significant difference ($p < 0.05$) in MEL solubilizing capacity.

Preparation of melatonin-loaded microemulsion

In various ratios, surfactant and co-surfactant were blended; hence, pseudo-ternary phase diagram was developed (data not shown), and the composition of stable ME formulae is presented in Table 1. It should be clarified that all formulae established with the oil/Smix ratio (1:9) to (7:3) could incorporate unlimited water content without showing any signs of turbidity. On the other hand, those incorporating the oil/Smix ratio (8:2) and (9:1) clearly displayed that turbidity and ME formation did not occur.

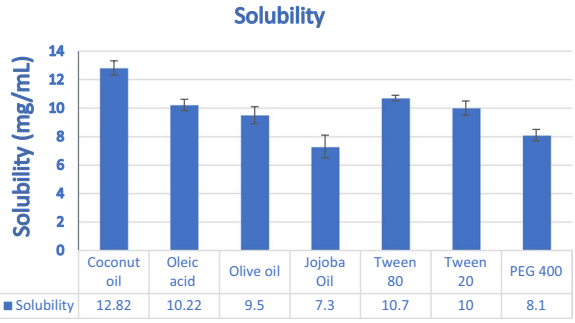

Fig. 1 Solubility of MEL in oils, surfactants and co-surfactants, data expressed as mean ± SD ($n = 3$)

Characterization of melatonin-loaded MEs

Droplet size, polydispersity index (PDI) and zeta potential (ZP) determination

For the stable MEL-loaded ME, formulae were determined, and results are displayed in Table 1. Basically, droplet size of MEs with Tween 80 and PEG 400 ranged between 31 ± 0.2 and 98 ± 0.6 nm. The PDI values ranged between 0.1 and 0.3, and the ZP values were found to be near zero.

Visual and optical inspection

The visual inspection experiment was carried out for 3 months by withdrawing ME sample at weekly interval for the first month and monthly interval for the subsequent months. The visual observation showed no evidence of phase separation or any precipitation or flocculation.

Ex vivo melatonin skin permeation study

Eight selected 0.5% MEL-loaded ME formulae were subjected to ex vivo full thickness skin permeation study and were compared to controls composed of 0.5% MEL PBS dispersion as well as 0.5% MEL in coconut oil. Skin permeation parameters and profiles at various time intervals are presented in Table 2 and Fig. 2. Results revealed that all ME formulae exhibited controlled release of MEL, compared to MEL in coconut oil and MEL PBS dispersion.

MEL PBS dispersion exhibited the lowest permeation parameters. On the other hand, MEL in coconut oil showed an obvious burst effect after 8 h with the highest cumulative amount permeated that reached 4500 µg/cm^2 after 24 h (Fig. 2).

Owing to the data shown, formulae with high oil content, TPA7, TPC7, TPB7, TPD7 have been shown by pronounced elevation in permeation parameters. On the contrary, formulae with higher Smix content showed low permeation parameters despite the skin permeation enhancement capacity of Tween80 and PEG400 [45].

Melatonin retention in the stratum corneum

Based on the data presented in Table 3, TPC5 scored the highest total amount of MEL in SC after 24 h with a value 336.083 µg/cm^2. The same formula showed the lowest flux value releasing 52% of cumulative amount of MEL after 24 h in Table 2.

According to the previous findings, TPC5 was selected for further experimental investigations and in vivo study. The main objective is to prove that MEL ME would provide high follicular accumulation along with the sustained permeation characteristics that is evaluated in Fig. 2.

Table 1 Composition and physicochemical properties of different MEL-loaded ME

Formula code	Percentage ratio (%w/w)					Droplet size (nm) ± SD	PDI ± SD	ZP (mV) ± SD
	Smix ratio	Oil wt%	Tween 80	PEG400	Water %			
TPA1	1:1	3.3	15	15	66.6	55 ± 0.30	0.3 ± 0.01	− 1.0 ± 0.01
TPA2	1:1	6.6	13.3	13.3	66.6	66 ± 0.10	0.2 ± 0.03	0.1 ± 0.01
TPA3	1:1	10	11.6	11.6	66.6	68 ± 0.50	0.3 ± 0.05	0.2 ± 0.01
TPA4	1:1	13.3	10	10	66.6	98 ± 0.60	0.3 ± 0.03	− 1.0 ± 0.01
TPA5	1:1	16.6	8.3	8.3	66.6	88 ± 0.20	0.1 ± 0.06	− 0.1 ± 0.01
TPA6	1:1	20	6.6	6.6	66.6	67 ± 0.40	0.3 ± 0.05	− 1.0 ± 0.01
TPA7	1:1	23.3	5	5	66.6	61 ± 0.10	0.1 ± 0.01	− 1.0 ± 0.01
TPB1	2:1	3.3	20	10	66.6	44 ± 0.10	0.3 ± 0.01	0.5 ± 0.01
TPB2	2:1	6.6	17.7	8.8	66.6	45 ± 0.20	0.3 ± 0.03	0.1 ± 0.01
TPB3	2:1	10	15.5	7.7	66.6	54 ± 0.70	0.3 ± 0.04	0.1 ± 0.01
TPB4	2:1	13.3	13.3	6.6	66.6	57 ± 0.60	0.2 ± 0.03	0.5 ± 0.01
TPB5	2:1	16.6	11.0	5.5	66.6	69 ± 0.20	0.1 ± 0.04	− 0.1 ± 0.01
TPB6	2:1	20	8.8	4.4	66.6	60 ± 0.60	0.3 ± 0.05	1.0 ± 0.01
TPB7	2:1	23.3	6.6	3.3	66.6	56 ± 0.10	0.1 ± 0.01	− 0.1 ± 0.01
TPC1	3:1	3.3	22.5	7.5	66.6	30 ± 0.20	0.3 ± 0.01	− 1.0 ± 0.01
TPC2	3:1	6.6	19.9	6.6	66.6	39 ± 0.01	0.3 ± 0.03	− 1.0 ± 0.04
TPC3	3:1	10	17.5	5.8	66.6	44 ± 0.50	0.3 ± 0.04	− 1.0 ± 0.01
TPC4	3:1	13.3	15	5	66.6	49 ± 0.30	0.2 ± 0.03	− 0.9 ± 0.05
TPC5	3:1	16.6	12.4	4.1	66.6	84 ± 0.20	0.1 ± 0.04	− 1.0 ± 0.01
TPC6	3:1	20	9.9	3.3	66.6	69 ± 0.20	0.2 ± 0.05	− 1.0 ± 0.02
TPC7	3:1	23.3	7.5	2.5	66.6	63 ± 0.10	0.1 ± 0.01	− 1.0 ± 0.01
TPD1	4:1	3.3	24	6	66.6	31 ± 0.20	0.2 ± 0.01	− 0.6 ± 0.01
TPD2	4:1	6.6	21.3	5.3	66.6	32 ± 0.01	0.2 ± 0.03	− 0.8 ± 0.04
TPD3	4:1	10	18.6	4.6	66.6	38 ± 0.50	0.3 ± 0.02	− 0.1 ± 0.01
TPD4	4:1	13.3	16	4	66.6	39 ± 0.30	0.3 ± 0.03	− 0.5 ± 0.05
TPD5	4:1	16.6	13.2	3.3	66.6	41 ± 0.20	0.1 ± 0.01	− 0.9 ± 0.01
TPD6	4:1	20	10.6	2.6	66.6	37 ± 0.20	0.2 ± 0.05	− 0.5 ± 0.02
TPD7	4:1	23.3	8	2	66.6	30 ± 0.10	0.1 ± 0.02	− 0.5 ± 0.01

Table 2 Composition and permeation parameters of selected MEL-loaded ME formulae (mean ± SD, $n = 3$)

Formulation code	Composition % (w/w)				Permeability coefficient P (cm/h) × 10^{-5}	Steady-state flux J_{ss} (μg/cm^2/h)
	Coconut oil	Tween80	PEG400	Water		
MEL in PBS	–	–	–	–	0.0029 ± 1.2	14.337 ± 1.4
MEL in coconut oil	100	–	–	–	0.0136 ± 0.7	67.903 ± 0.08
TPA5	16.6	8.3	8.3	66.6	0.005 ± 1.1	24.801 ± 1.2
TPA7	23.3	5	5	66.6	0.0118 ± 0.15	58.938 ± 0.19
TPB5	16.6	11.0	5.5	66.6	0.0046 ± 0.5	23.148 ± 0.4
TPB7	23.3	6.6	3.3	66.6	0.0067 ± 0.7	33.386 ± 0.9
TPC5	16.6	12.45	4.15	66.6	0.0035 ± 0.9	17.474 ± 0.86
TPC7	23.3	7.5	2.5	66.6	0.0107 ± 2.1	53.524 ± 2.3
TPD5	16.6	13.28	3.32	66.6	0.0035 ± 2.3	17.737 ± 2.1
TPD7	23.3	8	2	66.6	0.0065 ± 0.4	32.504 ± 0.5

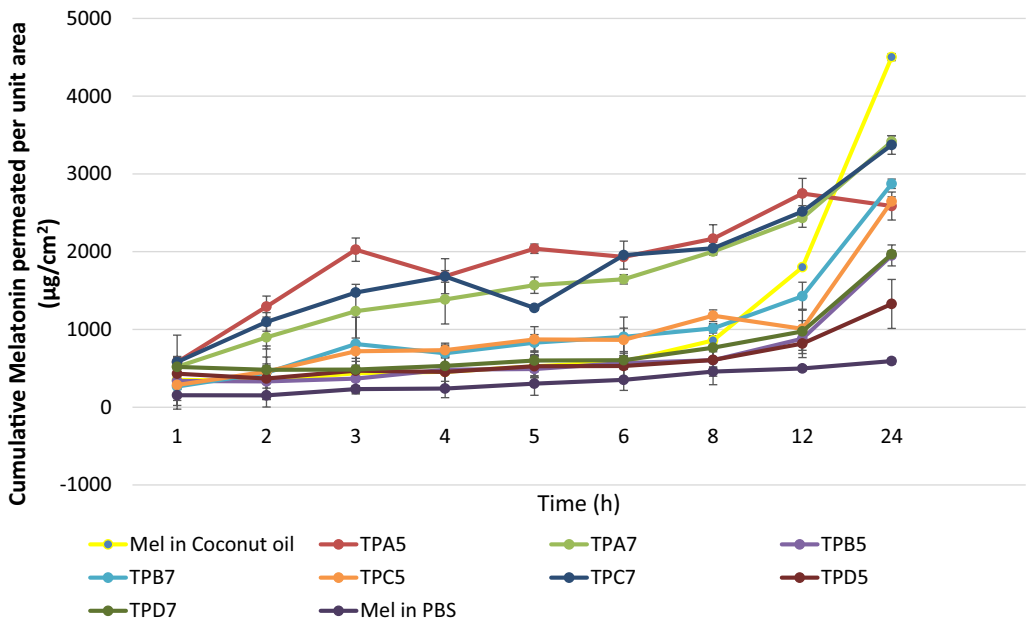

Fig. 2 Melatonin permeation profiles from different optimized ME formulae in PBS (pH 7.4, 37 °C ± 0.5) through excised rat skin (mean ± SD, $n = 4$)

Table 3 MEL deposition in stratum corneum (SC) from different formulae

Formulation code	MEL content in SC (µg/cm²)
MEL dispersion	51.330 ± 3.0
Coconut oil	55.765 ± 3.0
TPA5	68.391 ± 8.0
TPA7	227.693 ± 1.3
TPB5	210.831 ± 1.6
TPB7	192.314 ± 2.1
TPC5	336.083 ± 0.4
TPC7	230.926 ± 0.7
TPD5	74.819 ± 9.1
TPD7	73.54 ± 4.2

Transmission electron microscopy (TEM)

Morphology and droplet shape of the optimized ME (TPC5) are illustrated in Fig. 3A, B. The ME droplets appeared as dark spheres with bright surroundings. The droplets were visualized as nanometer-sized (75–100 nm) spherical and uniform particles, with no observable drug crystals which consequently indicates ME of good condition. The result was almost consistent with the DLS particle size distribution analysis.

Discussion

Drug solubility in excipients plays a significant part in determining the stability of formulae [45]. The system should have convenient solubilizing capacity to be able to add single drug dose in minimum formula volume. The structural and physical composition of coconut oil was found to be responsible for increased solubility of MEL [55], as it is mainly composed of high levels of short chained saturated fatty acids, C8:0 (caprylic), C10:0 (capric), C12:0 (lauric), C14:0 (myristic), palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2) and linolenic acids (C18:3) [27]. Coconut oil was selected as the oil phase due to the significant resistance to oxidation [31] and high drug solubilizing capacity which leads to engagement of low amount of surfactant and co-surfactants, accordingly reducing the toxic effects of surfactants [34].

Despite the similarity of the solubilizing effect of Tween 20 and Tween 80, Tween 80 was selected as the surfactant. A study by Ja'afar et al. [22] reported that systems having Tween 20 revealed poor stability without microemulsion being formed. As a result for the similarity between the hydrophobic tail of Tween 20 and lauric acid which is a major chemical component in coconut oil, thus, Tween 20 was incapable of reducing the interfacial tension of water–oil system, which may affect the ME stability [22].

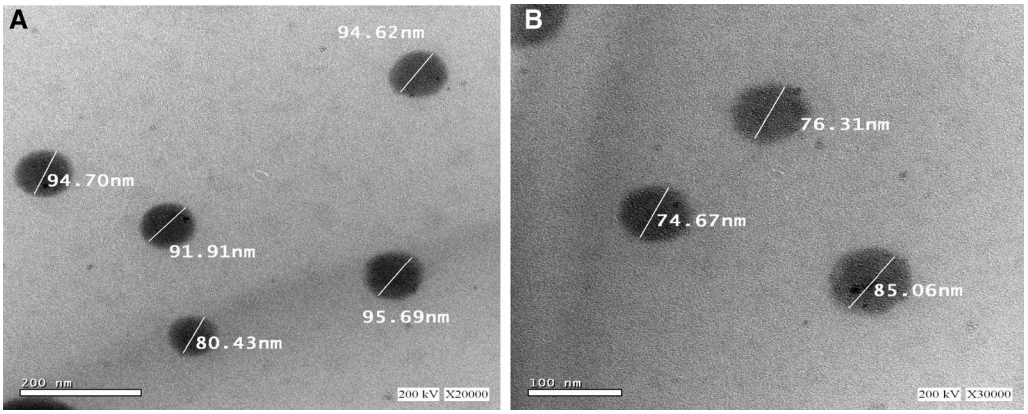

Fig. 3 Representative TEM images of TPC5 (**A**) at magnification 20,000 and (**B**) at magnification 3000

Generally, surfactants with HLB values in the range of 8–20 are used to form O/W emulsions [23]. Therefore, Tween 80 surfactant having HLB value 15.0 was selected in this study to elevate the possibility for ternary mixtures for O/W ME formation. Moreover, Tween 80 can produce droplets smaller in size than those attained by means of other surfactants (Span 80, poloxamer 407) [40]. Moreover, adding a co-surfactant is compulsory because it lessens the interface's bending stress and offers appropriate flexibility to the interfacial film so as to implement various curvatures required to produce ME with small droplet sizes. To summarize, coconut oil was the selected oil phase and Tween 80/PEG 400 was the Smix used for ME formulae.

Chiappisi et al. [11] demonstrated that after further dilution with water, ME droplets swell till a bicontinuous ME is formed or/and the emulsification boundary is reached. Accordingly, the unique behavior of high-water content incorporation assisted in adopting the idea of incorporating fixed water percentage with all ME formulae in our work while studying the effect of different oil-to-Smix ratios. This novel idea came in compliance with the study done by Rahdar et al. [39] who examined the effect of the chain length of oil on rhodamine B in the AOT nanodroplet microemulsions at fixed water content. The results attained indicated that there were other factors along with HLB value, such as the long hydrophobic chains of unsaturated fatty acid in Tween 80 which imparted flexibility to the ME structure and hence better emulsification potential [40]. The stability of all the formed MEs could be due to the high HLB value of Tween 80, and the high solubilizing capacity of PEG 400 [23].

Regarding the droplet size, PDI and ZP measurements our results from Table 1 showed that the relative proportion of Smix broadly affected the droplet size, as increasing the Smix ratio led to a significant decrease in the mean droplet size. Such results might be accredited to the reduction in surface free energy of the formed droplets and condensing the interfacial film, upon the addition of Tween 80 to the ME; meanwhile, the co-surfactant resulted in film expansion [35].

The average droplet size in each formula was found to increase with elevation of oil content along with lowering Smix ratio. Expansion of oil droplet of ME hindered the Smix effect in decreasing the interfacial tension between the oil phase and water phase, which eventually elevated the amount of free energy required to disrupt the droplets, causing droplet diameter increase [57]. Such findings were in accordance with a former report by Chen et al. [10] mentioning that the average droplet size of triptolide ME containing 1.5% and 6% oil was 12.7 and 59.8 nm, respectively.

The PDI values demonstrated the size distribution uniformity, leading to increased stability against sedimentation, flocculation and coalescence due to reduced Ostwald ripening [9]. The ZP values were found to be near zero, which is ascribed to the nonionic nature of the surfactant and co-surfactant. However, such components are reported to provide better stability to the formula against any ionic interactions which comes in accordance with the results reported by Anarakdim et al. [3] and Sharma et al. [46]. On basis of all the previous data, a wide range of stable, homogenous formulae were formed in the ME size range (1–100 nm) exhibiting equivalent characteristics. Accordingly, 8 formulae were selected for ex vivo study through full thickness rat skin; selection was based on internal system components. Four formulae containing the largest amount of coconut oil incorporated along with least amount of Smix (7:3 oil to Smix) were selected (TPA7, TPC7, TPB7 and TPD7). For broader evaluation, 4 formulae containing equal amounts of coconut oil and

Smix (1:1 oil to Smix) were selected (TPA5, TPB5, TPD5 and TPC5), as it was postulated that higher surfactant concentration increases the affinity of drug to the ME vehicle besides decreasing the thermodynamic activity that helps with providing a slow drug release from the vehicle to the skin leading to a sustained release manner along with better drug deposition in skin compartments [21].

The noticeable low permeation value of MEL PBS might be attributed to the insoluble large-sized drug particles. On the other hand, the burst effect and high cumulative amount of MEL in coconut oil could be credited to the presence of high levels of saturated fatty acids in coconut oil, particularly lauric acid (C12:0) and myristic acid (C14:0) [15]. Throughout a previous study by Kandimalla et al. [24] where the effect of fatty acids on MEL permeation across porcine skin and rat skin was investigated, it was concluded that MEL skin permeation was extremely enhanced by saturated fatty acids through disruption of the tightly packed lipids filling the extracellular spaces found in the stratum corneum. Additionally, it has been stated that C-10 to C-12 fatty acids own optimal balance between solubility parameters, partition coefficient and drug affinity to skin [47]. Moreover, the low molecular weight and very low viscosity of coconut oil might be another reason as reported by Therese et al. [53].

On the contrary to formulae with high oil content, the formulae with higher Smix content showed low permeation parameters despite the skin permeation enhancement capacity of Tween80 and PEG400 [45]. Increasing the ratio of surfactants, probably, formed a strong oil/water interface, preventing MEL fast permeation from the nanosized droplets. This observation comes in accordance with that reported by Cardoso and Barradas [7] who found that increasing the proportion of surfactant stabilized the nanoemulsion interface and prevented minoxidil rapid release from nanosized droplets. Moreover, it was reported by Kumar and Sarkar that the interfacial film formed by the surfactant can regulate the permeation properties of ME and surfactant adsorption to the interface could hinder mass transfer of the encapsulated drug molecules [28]. The effect of surfactant on membrane permeability showed an apparent concentration-dependent biphasic action, revealing an increase in membrane permeability that occurred at low surfactant concentrations. However, regarding the concentration of surfactant, more is not considered better at all times, and one of the reasons for this phenomenon was the possible changes in the thermodynamic activity of the drug [10].

It can be concluded that the effect of the surfactant at the interface played the major role in controlling MEL permeation, in spite of the oil permeation enhancing effect, the high-water content and the nanosized droplets.

One last additional factor is the influence of water content on ME internal structure. It is well known that O/W than W/O systems have pronounced effect on penetrating lipophilic drugs through skin, as 15% to 20% of SC weight is made up of water, so when hydration of skin occurs, it disrupts the SC structure which leads to its swelling and the opening of its tightly packed brick structures subsequently elevating permeability [51]. As some lipid chains in the stratum corneum are covalently attached to the corneocytes, hydration of these proteins will also lead to the disorder of the lipid bilayers [57]. It has been stated that prolonged hydration of skin distends the intercellular spaces, expanding the lacunar networks which join the water pool system's connections in SC interstices, that are normally disconnected in ordinary circumstances [14].

Microemulsions have been known to elevate skin hydration. As water is regarded as an enhancer, this effect might have contributed to the formulae penetration-enhancing characteristics. In a study by Hathout et al. [20], the ratio between the absorption of peak amide I and amide II expanded (as determined by means of attenuated total reflectance Fourier-transform infrared spectroscopy) which resulted in increased water percentage in the ME, signifying a progressive rise in the hydration of SC. Same observations were stated by Gupta et al. [18]. Additionally, bearing in mind that a few compounds in the oil phase have got occlusive characteristics such as vegetable oils [36], there is a possibility that such components alter the gradient of water in upper skin layers through avoiding evaporation.

The amount of MEL was determined in stratum corneum via tape stripping technique with TPC5 showing highest SC retention value after 24 h (Table 3).

According to the previous findings, TPC5 was selected for TEM showing spherical and uniform particles, with no observable drug crystals which consequently indicates good condition. Further experimental investigations and in vivo study would be performed. The main objective is to prove that MEL ME would provide sustained permeation characteristics that is evaluated in Fig. 2.

Conclusions

In the present study, we developed spherical and uniform particles of MEL-loaded microemulsion of good condition and high stability. In ex vivo permeation study, MEL ME showed decrease in steady-state skin flux along with high SC deposition that suggested controlled release profile compared to MEL in coconut oil and MEL PBS dispersion. This would make it a promising candidate for future in vivo studies for androgenic alopecia treatment.

Abbreviations
MEL: Melatonin; ME: Microemulsion; AGA: Androgenic alopecia; SC: Stratum corneum; Tween 20: Polyethylene glycol sorbitan monolaurate; Tween 80: Polyoxyethylene sorbitan monooleate; PEG 400: Polyethylene glycol 400; PBS: Phosphate-buffered saline; Smix: Surfactant/co-surfactant mixture; PDI: Polydispersity index; ZP: Zeta potential; UPLC: Ultra-performance liquid chromatography; TEM: Transmission electron microscopy.

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Declarations

Ethics approval and consent to participate
Animal study was performed according to the guidelines implemented by Research Ethics Committee of Faculty of Pharmacy, Future University in Egypt (FUE) No (REC-FPSPI-11/78).

Consent for publication
All authors have approved the final article and consented to publication.

Competing interests
All authors declare that they have no competing interest.

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