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RESEARCH PAPER

Nanostructured lipid carriers (NLCs) composed of argan oil, the potential novel vehicle for caffeine delivery to stratum corneum and hair follicles

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ABSTRACT

Objective(s): The optimal formulation of Nanostructured lipid carriers (NLCs) that contains argan oil and caffeine was considered to be developed as a topical treatment for hair loss.

Materials and Methods: 26 batches were prepared according to the central composite design. Dynamic light scattering technique showed size, polydispersity index (PDI), and zeta potential, and electron microscopy depicted the morphology of NLCs. Caffeine encapsulation efficiency (EE) was measured by UV/Vis spectroscopy. Fourier-transform infrared spectroscopy (FTIR) was applied to show caffeine and NLCs interactions. The caffeine invivo morin model was designed to determine the caffeine penetration into follicles and stratum corneum.

Results: The optimal formulation consisted of 2% lipid, 2% surfactant, stearic acid/argan oil ratio of 3.5, and span/tween ratio of 1.25. Its spherical NLCs size, PDI, and zeta potential were 256.2 nm, 0.225, and -25.4 respectively. The caffeine EE% was 89% which was homogenously encapsulated in NLCs as shown in FTIR analysis. *In vivo* studies showed these nanoparticles have the ability to accumulate in the hair follicles by the time.

Conclusion: The NLC formulation optimized in this study is a potential formulation for intrafollicular delivery of caffeine.

Keywords: *Argan oil, caffeine, intrafollicular delivery, nanostructured lipid carriers*

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INTRODUCTION

The widespread prevalence of androgenetic hair loss and the limited number of drugs available to treat it has encouraged researchers to find new treatments. Caffeine is one of the most effective drugs that has been shown in many studies to prevent hair loss. Caffeine by the mechanism of phosphodiesterase inhibition, increasing the cAMP levels in cells and proliferation by stimulating cell metabolism show its effects, especially in the topical application (1-3).

Argan oil is one of the oils that has long been considered as a very high quality and efficient oil for skin and to prevent dryness and hair loss. Its various properties can be attributed to its compounds including sterols, carotenoids, xanthophyls, and potent antioxidants. Even now, in many cosmetic products for skin and hair, the valuable properties of this oil are included (4-6).

The pilosebaceous unit, which contains the hair follicle, has been one of the attractive targets for topical or systemic drug delivery. By targeting the active ingredients directly to this area, it is possible to increase the effectiveness of drugs in preventing hair loss. Various studies have shown that particle drug delivery using micro and nanoparticles has played an important role in increasing the concentration of drugs in this unit. Due to being size-related, the role of nanoparticles in this type of drug delivery is more prominent (7-9).

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Today, various microparticle and nanoparticle carriers, from polymers to lipids, have found their way into drug delivery researches for different routes of applications (10-12). One of the lipid nanoparticles that has been considered in drug delivery to hair follicles is nanostructured lipid carriers (NLCs). Their structural properties, which have liquid and solid lipid compartments, gave these nanoparticles high loading power and suitable drug release. Various studies have been performed on these nanoparticles to treat problems related to the pilosebaceous unit, including alopecia, acne, and other sebaceous gland disorders (13-15).

Therefore, in this study, the optimal formulation of NLCs that contain argan oil as the liquid lipid compartment and caffeine as the main pharmaceutical active ingredient will be developed. The physical properties of nanoparticles including size, zeta potential, carrier drug interaction, and drug encapsulation efficiency would be determined. Finally, the penetration of caffeine from the optimized formulation into the hair follicle and the stratum corneum will be evaluated in an *in vivo* morin model. To our knowledge, this is a novel formulation for the transfolicular delivery of caffeine and argan oil.

MATERIALS AND METHODS

Materials

Stearic acid, span 80 and tween20 purchased from Merck, Germany. Caffeine powder was obtained from Scharlab, Spain. Argan oil was purchased from Talaye Asia oil, Iran.

Methods

NLCa/c preparation process

Experimental design

To achieve the optimal NLCa/c spending the minimum amount of time and cost, 26 experiments based on Central Composite Design (CCD) using Design Expert® software (version: DX7Trial) were designed. The lipid%, caffeine%, surfactant%, span/tween, and stearic acid/argan oil were individually defined in 3 levels as independent variables. The dependent variables including nano particular main characteristics (particle size, polydispersity index (PDI), and zeta potential (ζ)) were analyzed as target characteristics of optimum formulation, Table1. The bias% was calculated using Equation1 for each target characteristic to have an insight about the level of correlation between experimentally obtained and predicted results. The optimum formulation proposed by the software was chosen for the further complementary analyses (16).

Bias%= estimated quantity-obtained quantity/ estimated quantity*100

Eqeuation1

NLCa/c method of fabrication

In this study, a combination of high shear homogenization (HSH) and ultrasonication (US) methods was used to make NLCa/c. For this purpose, the specified amount of lipophilic ingredients of the formulation, including stearic acid, argan oil, and span 80, were mixed and heated to 70 °C to melt. On the other side, the specified amount of hydrophilic compounds, including caffeine and tween20, dissolved in DW and reached a temperature of 70 °C to provide the aqueous phase. To create pre-emulsion, the aqueous phase was added to the lipid phase and homogenized by the high shear homogenizer (Heidolph, T25D, Germany) for 3 minutes. In the next step, by using a probe sonicator (Hielscher, UP100H, Germany) the product was subjected to ultrasound waves for 3 minutes. The prepared formulation rested in the closed container for 12 hours to form the final NLCa/c (17).

NLCa/c characterizations

Particle size distribution and ζ potential

The average particle size was estimated by photon correlation spectroscopy (PCS) based on the dynamic light scattering (DLS) technique. NLCa/c Formulations diluted 1: 100 with DW, were poured into square polystyrene cuvettes. The analysis was performed at a temperature of 25 °C, scattering angle of 173°, and λ = 633 nm using particle size analyzer instrument (Zetasizer Nano-ZS, Malvern Instruments). The effective diameters (intensity weighted mean diameter, Z-Ave) and polydispersity index (PDI) for each formulation were reported as the final responses. The ζ potential of each of the formulations (in the same dilution of the size determination process) was determined using a capillary zeta cell at 25 ◦C by the same instrument (on ζ potential determination mode). The average of 5 runs was reported as the final result (18).

NLCa/c surface morphology

The surface morphology of the NLCa/c formulation was visualized by scanning electron microscopy (Leo VP 1450, Carl-Zeiss, Oberkochen, Germany). To prepare the sample, the formulation was diluted 1/100 by DW and a drop of the aliquot

was deposited on the aluminum sample stub. The sample was air-dried and coated by the gold/ palladium atoms under an argon atmosphere. Microscopy was carried out at an accelerating voltage of 10 kV (19).

Analysis of caffeine content

Calibration curve and linear regression

From a freshly prepared 10 mg/ml aqueous stock of caffeine, a serial dilution was carried out to prepare standard samples in the concentration range of 100-1000µg/ml. The absorption of each sample was recorded against DW as a blank at λmax = 273 nm using a UV/Vis spectrophotometer (CE1021, CECIL, England) (20). The calibration curve of absorbance (dependent variable) was plotted against the concentration (independent variable) and its linear regression and coefficient of discrimination (R2) were obtained using Microsoft office excel 2007.

Encapsulation efficiency of caffeine in NLCa/c

To determine the encapsulation efficiency of caffeine in NLCa/c by an indirect spectrophotometry method, the unentrapped or "free" drug molecules were separated by the ultrafiltration centrifugation process. The ultrafiltration centrifuge tube (100 kD, Amicon Ultra-4, Millipore, USA) was used to separate the free drug from 0.5 ml of NLCa/c formulation (21). Following the centrifugation process (EBA 20, Hettich, Germany) at 6000rpm for 30 min, the filtrate was separated and analyzed spectrophotometrically as described previously. The caffeine content was calculated using linear regression of the calibration curve. Encapsulation efficiency was calculated using Equation2.

EE%= Added caffeine content – caffeine content in supernatant/ added caffeine*100 content

Equation 2

Fourier-transform infrared spectroscopic analyses

To investigate the chemical interactions of caffeine with the NLCs, the Fourier-transform infrared spectroscopy (FTIR) was performed. Sample preparation for the procedure was as follows: 10 mg of caffeine and the lyophilized powders without cryoprotectant (Alpha 1-4 LSCbasic freeze dryer, Christ, Germany) of blank NLC and NLCa/c were geometrically mixed with potassium bromide and pressed under a pressure of 10 tons for 2 min. The prepared pellets were individually placed in the FT-IR spectrophotometer (IRPrestige -21, Shimadzu, Japan) and scanned in the frequency range of 6000-400 cm-1. A KBr pellet was used to correct the background frequencies (22).

Penetration of caffeine molecules from the NLCa/c formulation to the stratum corneum of epidermis and hair follicles: *In vivo* morin model

Laboratory animal management and ethics

10 male BALB/c mice (6weeks old) were kept at 25 °C, 12 h/12 h darkness/light with free access to water and food in the animal research room of Zabol University of Medical Sciences. All the experiments were approved ethically by the Ethics Committee Acts (Ethical code: IR.ZBMU. REC.1399.140) of Zabol University of Medical Sciences.

Preparing the mouse skin and applying the topical treatment

Before the treatment, the back skin of all the mice was carefully depilated. Subsequently, they were divided into two groups of five. Each animal in group 1 received 2 mg/cm^2 of NLCa/c formulation, and in group 2 received caffeine aquase solution with an equivalent concentration of NLCa/c formulation, on the back while getting a superficial massage using a facial roller for 2 min for a facilitated absorption.

Harvesting the penetrated drug into stratum corneum of epidermis and hair follicles

After 1, 3, and 6 days of treatment, the differential stripping method was used to remove the collected drug in the stratum corneum, layer by layer. For this, 10 pieces of 2*3 cm^2 adhesive tapes were stuck on the treated area, rolled properly, and stripped respectively (23).

To obtain the drug molecules that reached the hair follicles, a drop of cyanoacrylate glue was placed on another part of the treated area, at the same time points of the previous experiment. It was pressed by a glass slide and then gently removed.

The drug content and residuals of adhesive tapes and glues were dissolved in chloroform-methanol (1:2) solvent, concentrated by vacuum evaporator (SPD1030A-115, Thermo Fisher Scientific, USA) at 5000 rpm, 37°C for 30 min, and the caffeine content was spectrophotometrically analyzed.

Table 1. Dependent variables (lipid%, caffeine%, surfactant%, span/tween ratio, and stearic acid/argan oil ratio) and outcomes (nanoparticles size, zeta potential, and PDI index) of 26 experiments designed by Central Composite Design (CCD) using Design Expert® software (version: DX7Trial)

No	Lipid w/w %	Surfactant w/w %	Ratio of lipid (Stearic acid/ Argan oil)	Ratio of surfactant (Tween/span)	Caffeine w/w %	Nanoparticle Size±SD	Zeta Potential±SD	PDI±SD
1	1	3	4	2	0.2	243.6±5.44	-20.8±4.31	0.341±0.014
2	2	1	3.5	1.25	1.35	353.3±2.37	-22.3±5.38	0.430±0.051
3	2	2	3.5	1.25	1.35	379.3±7.11	-22.8±6.12	0.322±0.07
4	3	3	3	0.50	2.50	461±5.31	-21.3±5.15	0.52±0.031
5	2	2	3.5	1.25	0.2	256.2±5.48	-25.4±4.18	0.225±0.042
6	3	1	4	2	0.2	421.9±4.71	-22.3±3.91	0.431±0.62
7	2	2	3.5	1.25	1.35	377.1±3.98	-21.8±6.11	0.336±0.083
8	2	2	3.5	1.25	1.35	365.5±5.93	-22.3±5.21	0.348±0.062
9	3	3	4	0.5	0.2	417.5±3.72	-22.1±5.92	0.641±0.052
10	2	2	3.5	0.5	1.35	381.8±4.51	-21.2±3.98	0.351±0.063
11	1	3	4	0.5	2.5	283.2±4.61	-22.3±6.20	0.287±0.68
12	3	2	3.5	1.25	1.35	451.3±6.39	-26.1±7.18	0.548±0.053
13	1	3	3	2	2.5	296.5±7.91	-23.2±6.75	0.285±0.080
14	2	2	3.5	1.25	1.35	379.3±4.38	-24.3±4.88	0.328±0.071
15	2	2	3.5	2	1.35	390.2±5.11	-23.2±3.90	0.335±0.032
16	2	2	3.5	1.25	2.5	395.2±5.38	-23.5±3.10	0.453±0.028
17	1	1	3	0.5	0.2	251.2±7.12	-22.5±7.91	0.242±0.030
18	1	2	3.5	1.25	1.35	287.3±3.25	-21.4±7.15	0.235.0.068
19	3	1	4	0.5	2.5	483.2±3.91	-23.2±6.90	0.538±0.075
20	3	3	3	2	0.2	416.3±4.32	-25.5±4.32	0.517±0.081
21	3	1	3	2	2.50	452.3±7.20	-24.3±6.71	0.539±0.067
22	2	2	3	1.25	1.35	376.3±6.52	-24.2±3.78	0.320±0.085
23	1	1	4	2	2.5	281.2±4.10	-22.2±5.17	0.220±0.065
24	2	2	4	1.25	1.35	385.3±4.95	-23.1±5.81	0.334±0.043
25	2	2	3.5	1.25	1.35	391.2±3.14	-22.4±5.32	0.358±0.068
26	2	3	3.5	1.25	1.35	362.3±4.22	-23.4±6.15	0.345±0.059

RESULTS AND DISCUSSION

NLCa/c characterizations

Particle size distribution and ζ potential

To optimize the NLCa/c preparation, 26 proposed formulation was prepared according to the lipid%, caffeine%, surfactant%, span/tween ratio, and stearic acid/argan oil ratio as presented in column 2-6 of Table 1. In columns 7-9 the resultant size, zeta potential and PDI index of all the NLC formulations are presented respectively. These results were compared with the results predicted by the software and the bias% was calculated. Bias% was ≤5% in the case of all the formulations. Formulation No. 5 was introduced as the optimal formulation. This formulation consisted of 2% lipid, 2% surfactant, stearic acid/ argan oil ratio of 3.5, and span/tween ratio of 1.25. Its size, PDI and zeta potential were 256.2 nm, 0.225 and -25.4 and the calculated bias% were 2.6%, 2.2% and 1.9% respectively.

The zeta potential is known as an indicator of the repulsion between particles. If it falls in the range of 20-40 for lipid-based nanoparticles, will lead to system stability and prevent it to create aggregations (24). As seen in Table 1, not only the formulation5 but all the formulations passed this criterion for stability. No significant correlation was shown between zeta potential and formulation content.

The PDI index is a value between 0-1, showing the uniformity of nanoparticle size, and the closer this number is to zero, the more uniform are the particle sizes. The values higher than 0.7 considered polydispers particles. This implied the homogeneity of particles sizes for all the batches and no.5 formulation as optimized formulation specifically (25).

Previous studies have shown that once the nanometer-sized particles were applied to the skin, they predominantly followed the transfolicullar pathway through the epidermal barrier (26). In this study, nanoparticles with particle sizes of 200-500 nm were synthesized that showed no aggregation potential and had good uniformity, therefore the

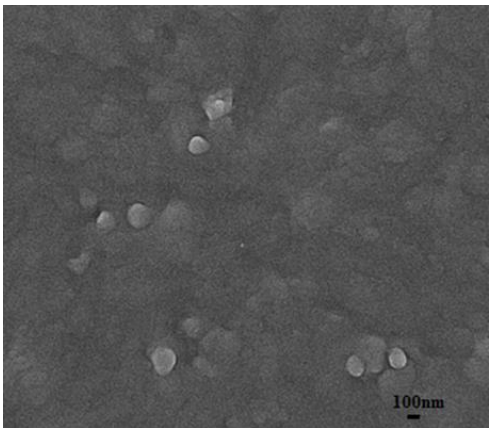

Fig 1. SEM image of NLCa/c optimized formulation (No.5)

follicular targeted drug delivery using these drug carriers will be efficacious.

NLCa/c surface morphology

In the SEM images obtained for formulation No. 5 (Fig. 1), spherical and uniform nanoparticles are observed which have nanometer dimensions and are in correlation with DLS results. However, in these images, the dimensions are somewhat smaller due to the usual difference in geometric and hydrodynamic dimensions recorded by SEM and DLS, respectively (27).

Encapsulation efficiency of caffeine in NLCa/c

Using the linear regression equation Y=0.0086x+0.7335 (R2=0.9982) of the caffeine calibration curve, caffeine encapsulation efficiency in NLCa/c optimized formulation was calculated to be 89.2%±3. Compared to other caffeine-containing NLC formulations in other studies, this parameter was significantly higher in this study (28).

Fourier-transform infrared spectroscopic analyses

One way to deduce drug loading in nanoparticles is to compare the spectrum of pure drugs with drug-containing nanoparticles. If the indicative

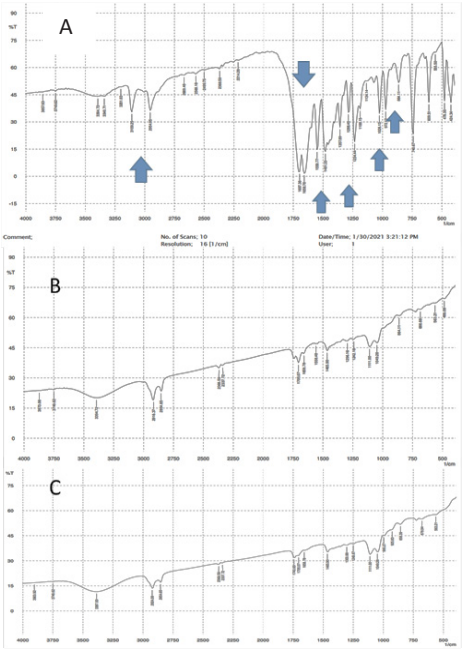

Fig 2. FTIR spectrum of A: pure caffeine, B: caffeine encapsulated NLC, C: blank NLC; (4000-400 cm^{-1})

peaks of the pure drug were undetectable in the drug containing nanoparticles spectrum and, the spectrum showed no significant difference with the blank nanoparticle spectrum, it indicates the complete encapsulation of the drug within the nanoparticles, which prevents the detection of the distinctive peaks (29). Comparing the spectra obtained from caffeine powder, blank NLC and NLCa/c (Table 2 and Fig. 2) confirmed the aforementioned fact that despite the quantifying the loaded caffeine in NLCa/c, its distinctive peaks are not observed in the FTIR spectrum of NLCa/c.

Penetration of caffeine molecules from the NLCa/c formulation to the stratum corneum of epidermis and hair follicles: *In vivo* morin model

In the final step of this study, to evaluate the

Table 2. Determinant groups and their region/vibration mode of caffeine in its FTIR spectrum (4000-400 cm^{-1})

Peak location	Bond	Mode of vibration
864,1026	C-C bond	Stretching
1218,1357	C-N	Stretching
1481-1550	C=C vibration	Stretching
	C=N bond	
1658,1697	C=O bond vibration	Stretching
2954,3109	Asymmetric vibration of C-H	Stretching
	bond of -CH3 group	

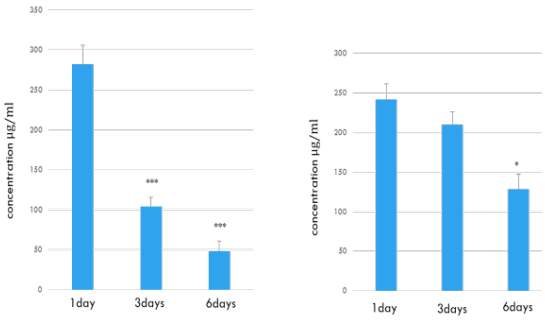

Fig 3. Caffeine concentration (µg/ml) in hair follicles in caffeine solution treated group (left) and NLCa/c treated group (right) in 24, 48 and 72 hours post-treatment in morin model, (mean±SD; ***=*P*≤0.001 and *= *P*≤0.1)

performance of NLCa/c on drug delivery via transfollicullar and transcellular pathways in an *in vivo* model, a combination of adhesive tape and cyanoacrylate slide methods was used. These methods were chosen because of their ease and speed of operation, non-invasiveness, differentiating the amount of drug in the stratum corneum and hair follicles, and their high level of accuracy (23, 30)

In this test, a morin model was considered. By topical application of NLCa/c formulation and caffeine solution along with a short massage for better absorption, in two different groups, the effect of the nanoparticle system on epidermal delivery was measured. By considering three different time points in both groups, the performance of the nanoparticle system in comparison with the drug solution in maintaining the drug in the stratum corneum and hair follicles was assessed.

As it was shown in the diagrams of Fig. 3, the elimination trend of caffeine in the hair follicle for NLCa /c formulation was slower than the caffeine solution on the third and sixth days after treatment compared to the first day.

The next point to consider about the difference in performance between the two treatment groups was the drug delivery to the hair follicles and the stratum corneum. Fig. 4 shows that on the

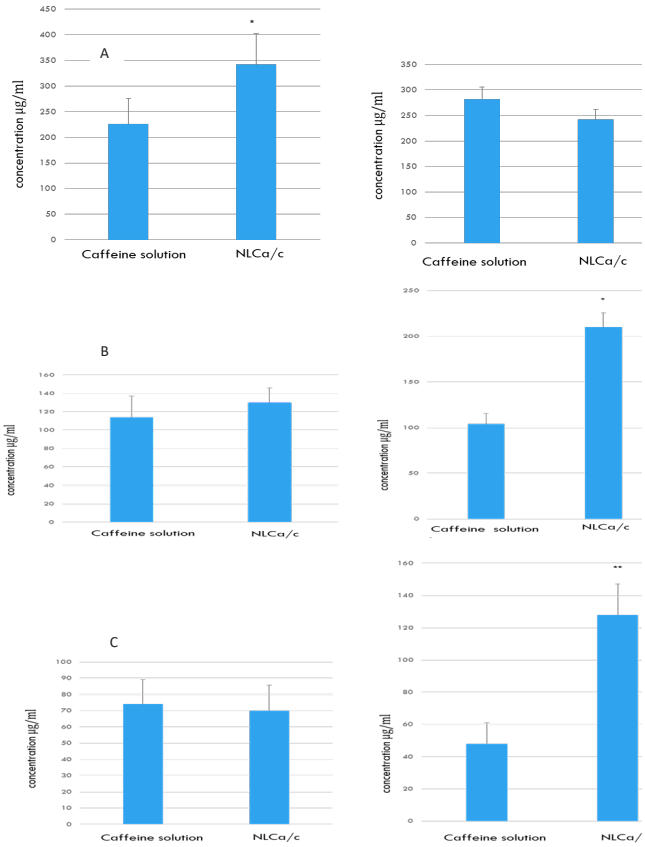

Fig 4. Caffeine concentration (µg/ml) in stratum corneum (left diagrams) and hair follicles (right diagrams) in caffeine solution and NLCa/c treated groups after 24 hr (A), 48 hours (B), and 7 hours (c) of topical application of treatment in morin model (mean±SD; ***=*P*≤0.001, **=*P*≤0.001 and *= *P*≤0.1)

first day after treatment, the amount of caffeine in the stratum corneum for the nanoparticle group was higher than the drug solution. But, this trend has changed in later days, and the nanoparticles have performed better in preserving the drug in the hair follicle than the stratum corneum. This effect has increased over time and on the sixth day, this difference has reached its maximum.

CONCLUSION

In this study, the NLC formulation containing argan oil and caffeine was optimized as a new formulation in drug delivery to hair follicles. The physical properties and drug loading showed that these nanoparticles have suitable nanoparticlular characteristics such as size, zeta potential and spherical morphology and can efficiently encapsulate caffeine. *In vivo* studies showed that these nanoparticles have the ability to accumulate in the hair follicles, which can lead to a stable and targeted drug delivery.

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The authors declare that there are no conflicts of interest in this study.

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