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Research Article

Enhanced Permeation of Methotrexate *via* Loading into Ultra-permeable Niosomal Vesicles: Fabrication, Statistical Optimization, *Ex Vivo* Studies, and *In Vivo* Skin Deposition and Tolerability

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Abstract. The aim of this study was to incorporate methotrexate (MTX) into ultra-permeable niosomal vesicles, containing cremophor RH40 as an edge activator (EA) and polyvinyl alcohol (PVA) as a stabilizer to enhance the drug permeation. Formulae were prepared by ethanol injection method following a Box-Behnken design in order to optimize the formulation variables (EA%, stabilizer %, and sonication time). To investigate the role of both cremophor RH40 and PVA, conventional MTX niosomes and MTX niosomes containing PVA only were fabricated. Drug entrapment efficiency percent (EE%), particle size (PS) analysis, zeta potential (ZP) measurements, and transmission electron microscopy (TEM) were conducted to characterize the vesicles. Cell viability studies and *ex vivo* permeation experiments of the optimized formula were conducted. Lastly, *in vivo* skin deposition of MTX from both the optimized formula and MTX solution was performed in rats. Besides, histopathological changes in rat skin were assessed. The optimized MTX ultra-permeable niosomal formula demonstrated spherical morphology, with an EE% of 65.16% and a PS of 453.6 nm. The optimized formula showed better physical stability in comparison with that of the same composition but lacking PVA. The cell viability studies verified the superior cytotoxicity of the optimized formula, and the *ex vivo* permeation studies revealed its ability to improve the drug permeation. The optimized formula demonstrated a significant deposition of MTX in rat dorsal skin, and histopathological evaluation confirmed the tolerability of the optimized formula in rats upon topical application. Accordingly, ultra-permeable noisomes, as a stable nanosystem, could be promising for effective delivery of MTX.

KEY WORDS: methotrexate; niosomes; edge activator; Box-Behnken design; IC_{50}; histopathology.

INTRODUCTION

Methotrexate (MTX) is a dihydrofolate reductase enzyme inhibitor, an enzyme playing a vital role in the synthesis of deoxyribonucleic acid (DNA), and therefore, the drug has shown good activity to control skin cancer, tumor necrosis factors, and rheumatoid arthritis (1). MTX is usually administered in large doses combined with other antineoplastic drugs in the management of solid tumors, hematologic malignancies (2), and non-melanoma skin cancer (3). On the other hand, MTX is used in low doses as an immunosuppressant and anti-inflammatory and therefore shows great potentialities in psoriasis and rheumatoid arthritis treatment (4,5). MTX is frequently administered per oral and parenteral to accomplish satisfactory results in the treatment of skin diseases. Unfortunately, the use of MTX by these conventional routes is accompanied by serious adverse effects, such as liver fibrosis and cirrhosis, anemia, bone marrow suppression, leucopenia, oligospermia, menstrual alteration, gastrointestinal disorder, mucosal ulceration, stomatitis, loss of appetite, depression, and other psychic disorders (6). This demands a formulation possessing enhanced penetration characters that could be used topically, so that it would reach the site of action in doses effective to achieve successful outcomes. The main shortcoming with dermal application of MTX is its diminished aptitude for diffusing passively through the stratum corneum (SC). This is owing to its large molecular weight (454.56 Da), extensive water solubility, and its presence mainly in the ionized form at the physiological pH (7).

The utilization of nanosized drug carriers, such as self-emulsifying nanosytems, liposomes, transferosomes, carbon nanotubes, polymeric nanoparticles, dendrimers, metallic

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nanoparticles, nanolipid carrier, and niosomes, has been attempted to augment the topical delivery of MTX (8,9). Niosomes are non-ionic surfactant vesicles with the ability to encapsulate both hydrophobic and hydrophilic drugs. The main goals of nisomes are exploiting the drug penetration and sustaining the release of the drug and hence increase patient compliance (10). Up to date, the incorporation of an edge activator (EA) and a stabilizer in niosomes and studying their effects on the characteristics of the vesicles have been rarely explored.

In this study, a new strategy was conducted to improve MTX dermal delivery through its entrapment into ultra-permeable niosomes. The present work aimed to achieve two targets. The first one was the fabrication of ultra-permeable niosomes *via* the incorporation of an EA and a stabilizer to maximize the percutaneous penetration and therapeutic effectiveness of MTX upon topical administration. The optimized parameters for an ultra-permeable stable niosomal MTX formulation were customized using Box-Behnken statistical design (BBD). The characteristics of MTX-loaded ultra-permeable niosomes, including entrapment efficiency percent (EE%), particle size (PS), polydispersity index (PDI), and zeta potential (ZP) were measured. Transmission electron microscopy (TEM) was used to visualize the inherent morphology of the ultra-permeable niosomes. The second target was to verify the hypothesis of the enhanced permeability and deposition of MTX ultra-permeable niosomes across rat skin through *ex vivo* and *in vivo* studies. Besides, MCF-7 cancer cell line was used to assess the cytotoxicity behavior of MTX ultra-permeable niosomes. Finally, the possibility of histopathological changes that may result from the topical application of the optimal formulation to the dorsal skin of rats was also assessed.

MATERIALS AND METHODS

Materials

MTX was kindly gifted by EIMC United Pharma Co. (Cairo, Egypt). Sorbitan monostearate (Span 60), polyvinyl alcohol (PVA, crystalline powder, 99% hydrolyzed, viscosity of 4% aqueous solution = 55–65 cP, molecular weight = 146,000–186,000), cremophor RH 40 (polyoxyl 40 hydrogenated castor oil, acid value ≤ 0.8 mg KOH/g, iodine value ≤ 1 g I_2/100 g), Cholesterol (assay ≥ 99%, molecular weight = 386.65) and acetonitrile (HPLC grade) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). Glacial acetic acid and sodium acetate anhydrous were purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, disodium hydrogen phosphate, and sodium chloride were purchased from Merck (Darmstadt, Germany). Ethanol (95%) was obtained from El-Nasr pharmaceutical chemicals Co. (Cairo, Egypt). The marketed MTX solution, Unitrexate1®, was manufactured by EIMC United Pharma Company, Cairo, Egypt.

Preparation of MTX Ultra-permeable Niosomes Using Ethanol Injection Method

In our study, ethanol injection method was utilized to prepare MTX ultra-permeable niosomal vesicles (11). In brief, span 60 and cholesterol at 2:1 weight ratio were dissolved in a predetermined volume of ethyl alcohol at 60°C. The alcoholic solution was gradually injected into a 2.5-fold larger volume of phosphate buffer (pH 8) containing MTX with continuous stirring at 60°C. Cremophor RH 40 (EA) and PVA (stabilizer) were previously dissolved in the aqueous phase. Stirring was continued till the complete evaporation of ethyl alcohol followed by cooling the obtained dispersions to room temperature. Some of the prepared formulations were subjected to bath sonication (Crest Ultrasonics Corp., NJ, USA) to study the influence of sonication time on the properties of the obtained formulae. The vesicular dispersions were kept overnight at 4°C to mature and then were used for characterization.

Optimization of MTX Ultra-permeable Niosomes

A 3^3 Box-Behnken design (BBD) was used to statistically optimize the formulation variables for MTX ultra-permeable niosomes preparation. Design Expert® software (Stat-Ease, Inc., Minneapolis, Minnesota, USA) was used to generate and evaluate the experimental design. Fifteen experiments were conducted; the mid-point of each edge of the multidimensional cube is represented by 12 experiments, while the remaining three trials represent the replicates of the cube's center point. In this study, EA (Cremophor RH 40) percentage (A), stabilizer percentage, PVA (B) (both percentages are with respect to the total weight of Span 60 and cholesterol), and sonication time (C) were evaluated as independent variables, whereas the dependent variables included encapsulation efficiency percent (Y_1: EE%), particle size (Y_2: PS), and polydispersity index (Y_3: PDI) (Table I). In addition, Table II shows the composition of the prepared MTX ultra-permeable niosomes.

Optimized formulation was selected for further investigations based on the desirability criterion. To check that the calculated formulation variables and predicted responses are valid, the suggested optimized MTX ultra-permeable niosomal formula was prepared and evaluated in triplicate. The observed responses should lie within the 95% prediction interval represented in Table I to be considered acceptable.

In Vitro Characterization of MTX Ultra-permeable Niosomes

MTX Entrapment Efficiency Percent (EE %) Determination

To determine the EE%, the MTX-loaded vesicles and the un-entrapped drug were separated by centrifugation at 20,000 rpm for 1 h at 4°C using cooling ultracentrifuge (Sigma 3-30 KS, Sigma Laborzentrifugen GmbH, Germany). Un-entrapped MTX concentration was determined spectrophotometrically at λ_{max} 307 nm (Shimadzu, model UV-1601 PC, Kyoto, Japan). MTX EE% was calculated using the following equation:

$$\mathrm{EE\%} = \frac{\text{Total amount MTX} - \text{Unentrapped MTX}}{\text{Total amount of MTX}} \times 100 \quad (1)$$

Table I. BBD for the Optimization of the Ultra-permeable MTX Niosomal Formulae, Model Summary Statistics of Quadratic Model, Constrains for Optimization, and Factor Levels for Optimized Ultra-permeable MTX Niosomal Formula and Their Predicted and Observed Values

Factors (independent variables)		Levels of variables				Optimized level	
		Low (−1)	Medium (0)	High (+1)			
A: EA (cremophor RH 40) percentage (%)		5	10	15		14.68	
B: stabilizer (PVA) percentage (%)		1	3	5		4.5	
C: sonication time (min)		0	1	2		0	
Responses (dependent variables)	r^2	Adjusted r^2	Prediction r^2	Constrains	Predicted	Observed	95% prediction interval
Y_1: entrapment efficiency (%)	0.914	0.900	0.872	Maximize	68.27	65.16	58.36–78.18
Y_2: particle size (nm)	0.941	0.915	0.850	Minimize	440.82	453.67	263.27–618.33
Y_3: PDI	0.931	0.900	0.824	Minimize	0.537	0.492	0.461–0.612

Particle Size (PS), Polydispersity Index (PDI), and Zeta Potential (ZP) Determination

Dynamic light scattering technique was used to measure the mean PS and PDI of the appropriately diluted MTX-loaded vesicles utilizing Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). ZP was determined for the prepared dispersions using the same instrument.

Stability Studies

The storage stability of the optimized formula *versus* the same formulation but lacking the stabilizer, PVA, was assessed through the storage in refrigerator (6 ± 2°C) for 3 months. The Formulation instability was checked by the inspection of the changes in the physical properties of the dispersion (*e.g.*, visual appearance and PS) and drug EE% (12).

Transmission Electron Microscopy (TEM)

The inherent morphological features of the optimized formula were inspected using TEM (Joel JEM 1230, Tokyo, Japan). One drop of the freshly prepared sample was appropriately diluted and settled onto the surface of copper grid and left to dry in the air. The grid was negatively stained with phosphotungstic acid dye (2%, *w/v*) for 5 min and then air dried for 1–2 min. Subsequently; the grid was probed and visualized *via* TEM (13,14). *Cellular Cytotoxicity Assessment.* The cytotoxicity of MTX ultra-permeable niosomes was assessed by Sulfo-Rhodamine B assay (15). The MCF-7 cells at density of 10,000 cells/cm^2 were seeded into 96-well plates containing 10% fetal bovine serum (FBS), phosphate-buffered saline of pH 7.4 (PBS), and incubated at 37°C for 24 h to permit the adherence of the cells to the plates prior to use. At the beginning, the assays were carried out for the evaluation of free MTX toxicity. This was done through the incubation of cultured MCF-7 cells with increasing MTX

Table II. Composition, EE%, PS, and PDI of the Prepared Ultra-permeable MTX Niosomal Formulae in the BBD (ZP Are Also Presented in the Table)

Formula	Factors levels in actual values			Y_1: EE% ± SD (%)[a]	Y_2: PS ± SD (nm)[a]	Y_3: PDI ± SD[a]	ZP ± SD (mV)[a]
	EA percentage (%)	Stabilizer percentage (%)	Sonication time (min)				
N1	5	1	1	56.36 ± 2.81	713.70 ± 23.30	0.66 ± 0.03	−50.4 ± 4.93
N2	15	1	1	32.03 ± 4.26	618.40 ± 18.90	0.54 ± 0.06	−50.5 ± 2.17
N3	5	5	1	52.97 ± 5.62	965.80 ± 35.10	0.73 ± 0.04	−48.4 ± 2.62
N4	15	5	1	39.95 ± 1.07	508.90 ± 14.90	0.51 ± 0.02	−53.5 ± 3.16
N5	5	3	0	66.23 ± 2.97	1406.0 ± 67.20	0.70 ± 0.04	−50.3 ± 2.48
N6	15	3	0	68.27 ± 3.52	427.50 ± 12.40	0.53 ± 0.03	−47.9 ± 3.77
N7	5	3	2	40.52 ± 3.07	535.20 ± 20.60	0.65 ± 0.07	−47.2 ± 2.51
N8	15	3	2	33.73 ± 1.88	533.10 ± 27.10	0.57 ± 0.03	−48.2 ± 1.44
N9	10	1	0	58.91 ± 5.91	559.40 ± 13.70	0.59 ± 0.04	−43.3 ± 1.28
N10	10	5	0	63.43 ± 3.22	765.10 ± 36.70	0.55 ± 0.02	−51.7 ± 3.94
N11	10	1	2	32.6 ± 2.74	551.40 ± 22.20	0.54 ± 0.05	−54.3 ± 2.76
N12	10	5	2	28.92 ± 1.26	464.30 ± 16.30	0.43 ± 0.01	−43.2 ± 2.18
N13	10	3	1	35.43 ± 2.21	604.00 ± 11.70	0.45 ± 0.03	−51.4 ± 1.94
N14	10	3	1	39.67 ± 3.58	595.90 ± 14.30	0.44 ± 0.06	−50.1 ± 2.49
N15	10	3	1	33.73 ± 2.46	613.70 ± 12.80	0.45 ± 0.07	−52.3 ± 3.29

[a] All measurements are performed in triplicates

concentration (125–500 μg/mL). To ensure the safety of the ultra-permeable niosomes, the cellular cytotoxicity of plain ultra-permeable niosomes (unloaded with drug) and that of the optimized MTX ultra-permeable niosomal formula was assessed at the same concentrations. The cells monolayer was incubated after drug treatment in the incubator in 5% CO_2 for 24 h at 37°C. After washing the plates with PBS, 50 mL of sulfo-rhodamine B stain solution was poured in each well. The attached stain was subjected to lysis by shaking for 30 min with 250 mL of lysis buffer (10-mM Tris-EDTA buffer). The optical density was determined using ELISA micro-plate reader (Meter Tech. S960, Warminster, PA, USA) at 564 nm. MTX dose response curve was constructed, and the half maximal cell growth inhibitory concentration (IC50%) was calculated to signify the concentration that provides 50% cell viability. The cytotoxicity was assessed by determining the absorbance for the treated cells relative to that of control (untreated) cells. All measurements were established using three identical experiments replicates.

Ex Vivo Permeation Study

Skin Preparation

All the animal study protocols were approved by the Research Ethics Committee, Faculty of Pharmacy, Cairo University, Egypt. Dorsal skin of rats (newly born) weighing 70 ± 20 g was excised after anesthesia and scarification humanely. Subcutaneous tissues and adhering fats were removed, to clean the dermal surface, without damaging the epidermal surface. The permeation experiment was conducted using the cleaned skin within 30 min of the sacrifice of the animals.

Ex Vivo Permeation Study

MTX permeation through rat skin from the optimized ultra-permeable MTX niosomal formula (containing both PVA and cremophor RH 40) in comparison with conventional MTX niosomes, MTX niosomes containing PVA only, and MTX solution in phosphate buffer (pH 8) was evaluated. The skin was kept in phosphate buffer saline (PBS, pH 7.4) solution for 2 h for equilibration. The skin was placed on a diffusion cell in which the donor compartment was faced by the SC, while the receptor compartment was fronted by the dermis. The surface area of skin membrane through which diffusion takes place was 0.636 cm^2. Either 0.5 mL of MTX-loaded vesicles or MTX solution (10 mg/mL) was added to the donor compartment, while 20 mL of PBS (pH 7.4) was added to the receiver compartment and stirred at 50 rpm at 32 ± 0.5°C. Samples from the receptor fluid (0.5 mL) were withdrawn at different time points up to 8 h and replaced by fresh buffer solution to keep the volume constant and maintain sink conditions. The validated isocratic HPLC method reported by Abdelbary and AbouGhaly was used to determine MTX concentration in the withdrawn samples Abdelbary and AbouGhaly (16). The cumulative amount of MTX permeated per unit area (μg/cm^2) was plotted against time (h). The flux (J_{max}) at 8 h, the total amount of MTX permeated in 8 h, and the enhancement ratio (ER) were calculated from the following equations membrane (17):

$$J_{max} = \frac{\text{Amount of drug permeated}}{\text{Time} \times \text{Area of membrane}} \quad (2)$$

$$\text{ER} = \frac{J_{max} \text{ of the nanovesicles}}{J_{max} \text{ of the drug solution (control)}} \quad (3)$$

Statistical analysis of the obtained results was performed by one-way ANOVA using SPSS software 19.0 (SPSS Inc., Chicago, IL, USA). Tukey's HSD (honest significant difference) test was used for post hoc analysis. P value ≤ 0.05 implies statistical significance.

In Vivo Skin Deposition Studies

The *in vivo* studies were performed using 30 male Wistar rats (150–200 g). The rats were placed in individual cages and provided with tap water and standard diet *ad libitum*. One day before sample application, rat dorsal skin hair was shaved with an electric clipper. On the experiment day, the rats were randomly assigned into two groups, each containing 18 rats. Bottle caps (4.91 cm^2), serving as drug pools, were fixed to the shaved zone, and 0.5 ml of the tested formulations (the optimized formula and MTX solution) was charged non-occlusively into the drug pool. Three rats from each group were humanely sacrificed applying an overdose of anesthetic ether (18) at predetermined time points (1, 2, 4, 6, and 8 h). Following, the dorsal rat skin in contact with the formulation was cut out and washed twice with 5-mL normal saline. Skin samples were shredded into pieces and placed in a beaker containing 5-mL dimethyl sulfoxide (DMSO). Afterwards, the beakers were sonicated for 30 min. The samples were filtered through a 0.45-mm membrane filter, and MTX concentration was measured using the same HPLC method used for the *ex vivo* permeation study. MTX deposition in skin from the tested formulations was then calculated.

In Vivo Histopathological Study

The aim of performing *in vivo* histopathological experiment was to evaluate the possibility of any irritation that may result from the application of the optimized formula through the microscopical examination of the ultrastructural alterations in the skin. Twelve rats were randomly placed into two groups of equal sizes. Rats in group I were kept untreated (control), while the rats in group II received topical treatment with the MTX optimized formula onto the skin surface, three times a day for 1 week. Rats from both groups were sacrificed, and the skin was removed for histopathological examination according to the method stated earlier by Bancroft *et al.* (19).

RESULTS

Statistical Analysis of BBD

The Effect of Formulation Variables on EE%

EE% of MTX ultra-permeable niosomes ranged from 32.03 ± 4.26 to 68.27 ± 3.52% as demonstrated in Table II. Quadratic model was the best model to describe the EE% due to the higher adjusted and prediction r^2 values compared with the linear and two factor interaction (2FI) models as shown in Table I. ANOVA was adopted to identify the significant quadratic model terms on EE%. Model terms with P value < 0.05 are statistically significant. Significant factors and interactions for EE% are related in the following equation:

$$EE\% = 37.15 - 5.26A - 15.13C + 7.53A^2 + 8.17C^2 \quad (4)$$

ANOVA showed that both EA percentage (A) and sonication time (C) had significant effects on EE%. The response surface plot for the effect of EA percentage and sonication time on EE% is displayed in Fig. 1a.

The Effect of Formulation Variables on PS and PDI

PS of MTX ultra-permeable niosomes ranged from 427.50 ± 12.40 to 1406.00 ± 67.20 nm, while their PDI was in the range of 0.43 ± 0.01 to 0.73 ± 0.04 as shown in Table II. According to the adjusted and predicted r^2 values shown in Table I, the quadratic model was selected to describe the PS and PDI. Significant factors and interactions for PS and PDI are related in the following equations:

$$PS = 593.40 - 191.60A - 134.25C + 244.10AC + 120.18A^2 \quad (5)$$

$$PDI = 0.47 - 0.074A - 0.021C + 0.024AC + 0.12A^2 + 0.04C^2 \quad (6)$$

Statistical analysis revealed that both EA percentage and sonication time demonstrated a significant quadratic effect on PS and PDI. Figure 1b, c shows the response surface plots for the effect of EA percentage and sonication time on PS and PDI, respectively.

Optimization of MTX Ultra-permeable Niosomes

By applying the constrains showed in Table I on EE%, PS, and PDI, the optimized MTX ultra-permeable niosomal formula was suggested by the Design Expert® software with an overall desirability of 0.873. The suggested formula (containing 14.68% of cremophor RH40 as an EA, 4.5% of PVA as a stabilizer, and was not subjected to sonication) was prepared and assessed. The observed responses lie within the 95% prediction interval as represented in Table I, showing that the optimization and prediction processes are valid.

Stability Studies

The optimized MTX ultra-permeable niosomes formula and the MTX ultra-permeable niosomes formula of the same composition but containing no PVA were subjected to stability study. The obtained results revealed that there was no significant change in the EE% ($P > 0.05$) and the physical appearance of both formulations. On the other hand, the optimized formula showed no significant change in the PS during the storage period at 4–8°C for 3 months, while the PS of the formulation lacking PVA was significantly increased at the end of the storage period ($P < 0.05$).

Transmission Electron Microscopy (TEM)

TEM image of optimum MTX ultra-permeable niosomes is displayed in Fig. 2. TEM micrographs showed spherical and homogenously distributed niosomal vesicles with uniform PS.

Cellular Cytotoxicity Assessment

The cytotoxicity of MTX solution, drug-free ultra-permeable niosomes, and the optimized MTX ultra-permeable niosomes was assessed using MCF-7 cell lines. MCF-7 cells were subjected to equally increasing MTX concentrations in both MTX solution and the optimized MTX ultra-permeable niosomes. Regarding the unloaded niosomes, increasing niosomal dispersion amounts corresponding to that added in case of the optimized MTX ultra-permeable niosomes were used. The cell viability percentage was determined after 24 h.

The plain niosomes showed better viability results after 24 h reaching up to 89.4% at 500 μg/mL compared with both MTX solution and the optimized MTX ultra-permeable niosomal formula, which manifested cell viability of 26.8 and 14.3%, respectively, at the same concentration as shown in Fig. 3. These findings confirm the safety of the vehicle used for this study. On the other hand, the optimized MTX ultra-permeable niosomes achieved better cytotoxicity results compared with MTX solution. These results were in accordance with the IC_{50} results, where the IC_{50} of the plain formula was undetectable at the end of the study. However, IC_{50} of the optimized MTX ultra-permeable niosomes was 98.3 μg/mL, which is significantly lower than that of MTX solution having an IC_{50} of 118 μg/mL.

Ex Vivo Permeation Study

MTX therapeutic effect on skin disorders would be enhanced when MTX penetrates to deeper skin layers, so it is a necessity to improve its permeation across the SC barrier. Figure 4 shows the cumulative amount of MTX permeated per unit area of rat skin relative to time from optimized MTX ultra-permeable niosomes compared with conventional MTX niosomes and MTX niosomes containing PVA and MTX solution. Permeation parameters, including the flux (J_{max}), total amount of MTX permeated in 8 h, and ER, are shown in Table III. Based on the MTX permeation studies from different formulae, the optimized ultra-permeable MTX niosomes showed significantly higher flux and total amount of MTX permeated per unit area compared with the other

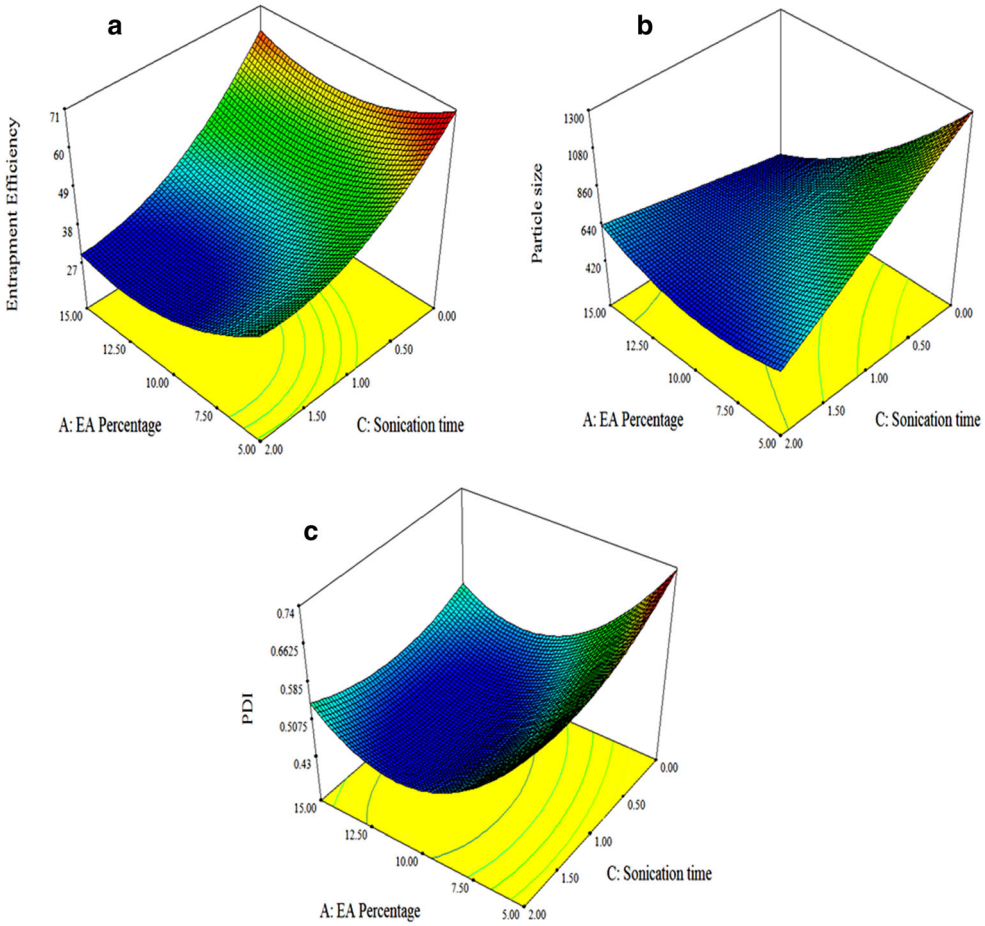

Fig. 1. Response surface plots demonstrating the effect of factors, A: EA % and C: sonication time, on different responses studied. **a** EE%, **b** PS, and **c** PDI

tested niosomes and MTX solution ($P < 0.05$). MTX percutaneous permeation to deep skin layers can be displayed in the following sequence: ultra-permeable MTX niosomes > MTX niosomes containing PVA ≈ conventional MTX niosomes > MTX solution. According to the calculated ER values, the ultra-permeable MTX niosomes enhanced MTX permeation by a factor of 2.67 compared with MTX solution. This is markedly higher than the ER of MTX niosomes containing PVA and conventional MTX niosomes, which were 1.55 and 1.52, respectively.

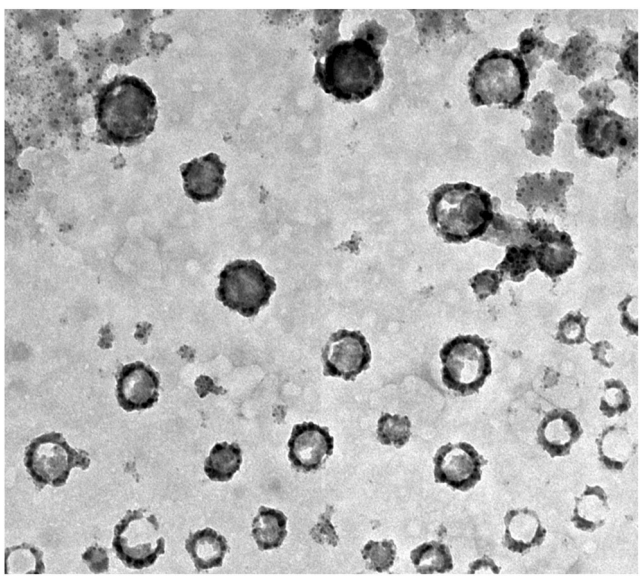

Fig. 2. Transmission electron micrograph of the optimized MTX ultra-permeable niosomes

In Vivo Skin Deposition

Skin deposition is considered as an estimate for the potentiality of the nanosystem to travel through different layers of the skin and target specific action site. The outcomes of the *in vivo* skin deposition of MTX from ultra-permeable MTX niosomes *versus* that of MTX solution are presented in Fig. 5.

Histopathological Examination

The stained rat skin layers were inspected under light microscope. The histopathological micrographs of the control group and the second group treated three times daily with the application of the MTX optimized formula are shown in Fig. 6a, b.

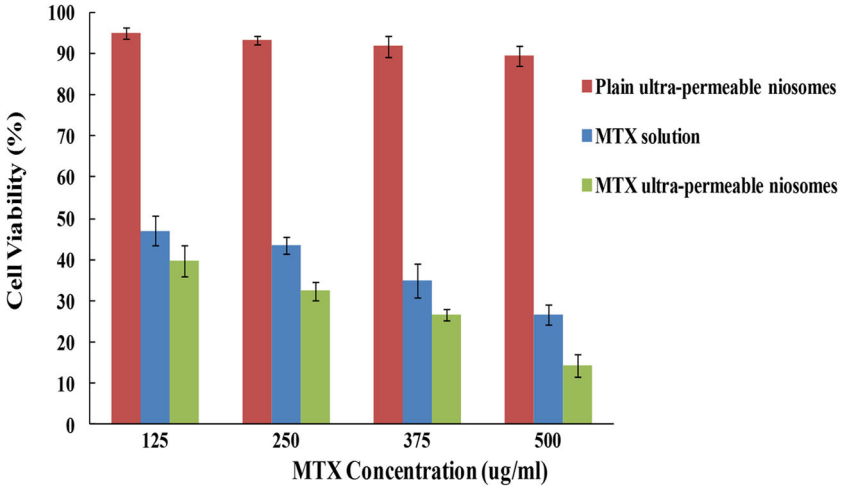

Fig. 3. Cell viability of the optimized MTX ultra-permeable niosomes compared with plain ultra-permeable niosomes and MTX solution in MCF-7 cell line

DISCUSSION

BBD is a quadratic independent design, which entails less trials and time. Three independent variables were assessed, namely, EA percentage (A), stabilizer percentage, PVA (B), and sonication time (C). In this design, the dependent variables were EE% (Y_1), PS (Y_2), and PDI (Y_3). The ZP values of the prepared formulae are given in Table II. It is obvious that all the prepared formulae showed acceptable ZP values (-43.3 ± 1.28 to -54.3 ± 2.76 mV), indicating their good stability (20). The high negativity of ZP values can be attributed to the fact that MTX is a dicarboxylic acid, which is highly ionized exhibiting two negatively charged groups in phosphate buffer (pH = 8) (21). It is worthy to mention that ZP was not included as a response in the model analysis as all the independent variables showed a non-significant effect on ZP.

Regarding the EE%, increasing both the EA percentage and sonication time was accompanied with a significant reduction in the EE% ($P < 0.0001$). The EA, cremophor RH 40, is a hydrophilic non-ionic polyethoxylated surfactant (22), which was incorporated in the niosomal formulations in order to increase their permeability characters. Increasing the EA concentration led to pore formation and disruption in the membrane of the prepared vesicles, leading to drug leakage and decreased entrapment. Similar results were obtained by Abdelbary *et al.*, who found that increasing the amount of the EA led to a significant decrease in EE% in terconazole loaded ultra-deformable bilosomes (23). In addition, it was clear that increasing the sonication time leads to a decrease in the PS of MTX-loaded vesicles, limiting the internal space available for drug charging and thus decreasing the EE% (24).

With respect to PS analysis, results revealed that increasing the EA percentage significantly decreased the PS ($P < 0.0001$). The polyethylene oxide (PEO) units of cremophor RH 40 act as steric stabilizer, preventing the aggregation of the niosomal vesicles. So, higher EA percentage would result in adequate coverage of the MTX ultra-permeable niosomes surface, leading to higher steric

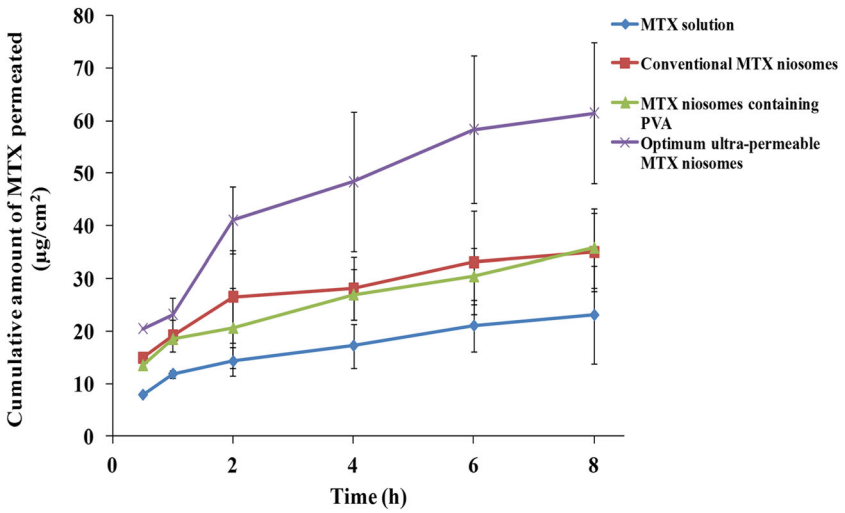

Fig. 4. Cumulative amount of MTX permeated per unit area across skin *via* the optimized MTX ultra-permeable niosomes compared with conventional MTX niosomes and MTX niosomes containing PVA and MTX solution

Table III. Permeability Parameters Obtained from *Ex Vivo* Permeation Studies of the Optimum Ultra-permeable MTX Niosomes, Conventional MTX Niosomes, and MTX Niosomes Containing PVA and MTX Solution

Measured parameters	Formulae			
	Ultra-permeable MTX niosomes	Conventional MTX niosomes	MTX niosomes containing PVA	MTX solution
J_{max} (μg/cm^2/h)[a]	7.68 ± 1.11	4.37 ± 0.81	4.47 ± 0.43	2.88 ± 0.78
Total amount permeated per unit area in 8 h (μg/cm^2)[a]	61.47 ± 8.85	34.95 ± 6.51	35.77 ± 3.44	23.05 ± 6.25
ER	2.67	1.52	1.55	1

[a] All measurements are done in triplicates

stabilization and lower interfacial tension forming niosomes of smaller PS (23,25). This is in agreement with Al-Mahallawi *et al.* who stated that increasing the EA% resulted in a significant decrease in the PS of ciprofloxacin-loaded nanospanlastics (26). Moreover, increasing the sonication time significantly decreased the PS ($P < 0.0001$). Increasing the sonication time led to the release of higher sonication energy in the dispersion medium resulting in smaller PS (24). Regarding the PDI, increasing both factors led to a significant decrease in PDI and consequently more homogenous PS distribution.

The better physical stability of the optimized MTX ultra-permeable niosomes formula (containing 14.68% of cremophor RH40 as an EA, 4.5% of PVA as a stabilizer, and was not subjected to sonication) compared with that lacking PVA can be interpreted in terms of steric stabilization provided by PVA. It is well known that PVA is widely used in the manufacture of different nanosystems due to its ability to coat the nanoparticles surface, thus providing a shield which prevents particle aggregation (27).

Concerning the *ex vivo* permeation experiment and cell viability studies, the superiority of the optimized MTX ultra-permeable niosomes relative to the other tested formulations were evident. According to Tukey's HSD post hoc analysis, the permeation parameters of the conventional MTX niosomes and MTX niosomes containing PVA were non-significant from each other, but both showed significantly higher permeation compared with MTX solution. This may be attributed to the ability of the niosomes to bind with the SC lipids due to the presence of cholesterol in the cell membrane as well as the niosomal structure. This may lead to higher MTX concentration gradient at the skin surface promoting its permeation to the deep dermal layers (28). In addition, the presence of non-ionic surfactants in niosomes may act as mild penetration enhancers. On the other hand, permeation parameters of ultra-permeable MTX niosomes were significantly higher than both tested niosomes formulae and MTX solution. The higher permeability of MTX ultra-permeable niosomes compared with the other tested niosomal formulae may be due to the existence of the EA, Cremophor RH40, in their structure. It is well known that EA disturb the cell membrane causing an increase in the epithelial permeability. It may also open the tight junctions in the epithelial wall reversibly, facilitating the drug transport *via* the membrane (17). Similarly, the enhanced cytotoxicity of the optimized formula can be attributed to the incorporated EA. Cremophor RH 40 significantly enhanced the permeation of the applied MTX ultra-permeable niosomes facilitating their penetration into the cell membrane and internalization into the cellular compartments, resulting in improved cytotoxicity and a significantly lower IC_{50}.

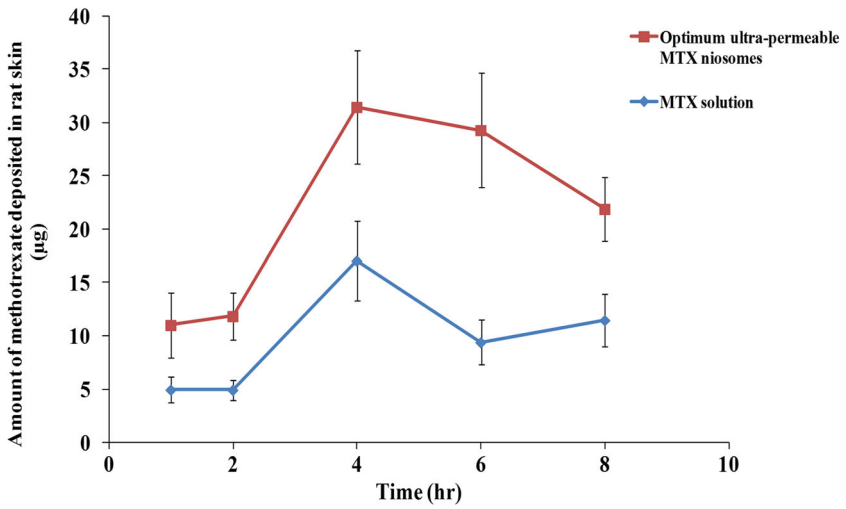

Fig. 5. Cumulative amount of MTX deposited in rat skin *via* the optimized MTX ultra-permeable niosomes compared with MTX solution

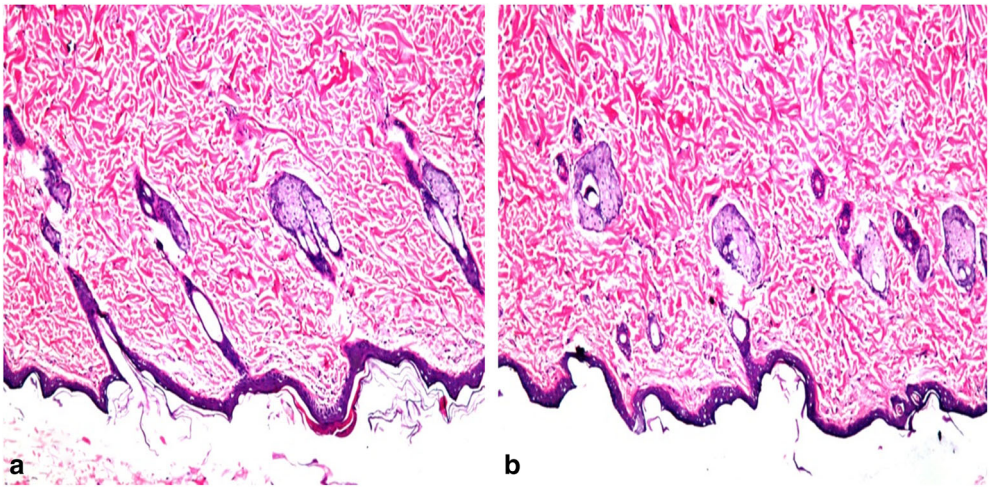

Fig. 6. Photomicrographs viewing histopathological sections (hematoxylin and eosin stained) of **a** normal untreated rat skin and **b** rat skin treated with the optimized MTX ultra-permeable niosomes

Moreover, the optimized formula was capable to deposit larger MTX amounts in skin relative to MTX solution. The calculated AUC for the formula was markedly higher than that of the drug solution (172.110 and 76.588 μg h, respectively), where the optimized formula showed 2.25-fold increase in the AUC than that of the drug solution. These findings validate the potentiality of the optimized formula to shuttle the drug molecules through the skin layers and to enhance its skin deposition, circumventing the SC barrier upon topical application. Finally, the histopathological studies revealed that the control group exhibited normal skin structure, with no substantial change in the structure of both the epidermis and dermis (Fig. 6a). For the second group, treated with the application of the MTX optimized formula, the micrograph displayed normal skin structure with no signs of skin inflammation or skin irritation. The epidermis, as well as the underlying dermis, was totally intact (Fig. 6b). As a conclusion, ultra-permeable MTX niosomes are safe to be applied topically without any irritation or inflammation possibilities.

CONCLUSIONS

Ethanol injection technique was successfully applied for the fabrication of MTX-loaded ultra-permeable niosomes with acceptable EE% and small uniform PS. BBD statistical analysis showed that the quadratic model is the best to describe EE%, PS, and PDI. Analysis of different models showed that the EA% and the sonication time exhibited a significant effect on EE%, PS, and PDI. The optimized MTX ultra-permeable niosomes with a desirability value of 0.873 were selected and evaluated. It had an EE% of 65.16%, PS of 453.67, and PDI of 0.492. The optimized formula showed good stability upon storage with no significant changes in EE% and PS. The performed cell viability studies demonstrated that the MTX ultra-permeable niosomes exhibited superior cytotoxicity and a significantly lower IC_{50} compared with MTX solution. The *ex vivo* permeation study of optimum MTX ultra-permeable niosomes showed a 2.67 ER and significantly higher flux and total amount of MTX permeated per unit area compared with the other tested niosomes and MTX solution, which can be attributed to the presence of the EA, Cremophor RH40, in their structure. The *in vivo* studies revealed that the optimized formula displayed a greater extent of MTX deposition in the rat dorsal skin relative to MTX solution. Moreover, the *in vivo* histopathological experiments demonstrated the non-irritant nature of the applied formula. These findings suggested that the MTX ultra-permeable noisomes are a promising tool to enhance the MTX penetration to reach the site of actions in effective doses, so decreasing the side effects associated with MTX oral administration.

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