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ORIGINAL ARTICLE

Development and Evaluation of Methotrexate and Baicalin-Loaded Nanolipid Carriers for Psoriasis Treatment

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ABSTRACT

Objectives: Psoriasis is a chronic inflammatory, T-lymphocyte immune-mediated skin disease. In this study, skin-permeating nanolipid carriers (NLCs) of Methotrexate (MTX) and Baicalin (BL) were formulated. This further gave formulation of nano-lipid encapsulated carriers for dual-drug delivery of the hydrophilic and hydrophobic drugs through the liposomal gel.

Materials and Methods: Optimization of the formulation of NLCs was performed and characterized by determining their particle size, drug permeation, skin irritation, drug loading capacity, stability, *in vitro* drug release behavior, and *in vitro* cellular viability. *Ex vivo* skin permeation and *in vivo* psoriatic efficiency were also evaluated and compared.

Results: Results revealed that the amount of MTX permeating the skin was 2.4 to 4.4 fold greater for dual-drug s than for single NLCs. The optimized dual-drug loaded NLCs had an average particle size (150.20 ± 3.57 nm) and polydispersity index (0.301 ± 0.01) and high entrapment (86.32 ± 2.78% *w/w*). The MTX nanoparticles exhibit a positive Zeta potential of 38.6 mV. The psoriasis area and severity index scoring showed the lowest skin erythema, skin thickness and scaling. MTX-BL NLCs were inhibited the expression of inflammatory cytokines (tumor necrosis factor-alpha, and interleukin-17) .

Conclusion: It can be concluded that newer targeting strategies for NLCs for dual-drug delivery of nano-lipid carriers could be administered topically for the treatment of psoriasis.

Keywords: Psoriasis, Baicalin, Methotrexate, nano structured lipid carriers, topical delivery

INTRODUCTION

Psoriasis is a chronic inflammatory, T-lymphocyte immune-mediated skin disease characterized by the deregulated multiplication of skin cells, which increases skin cell thickness, causing the appearance of salmon-red plaques with a silver scaly surface. The etiology of psoriasis remains unknown. Several biochemical factors lead to the maturation and proliferation of epidermal cells.[1] Red and white/scaly patches are formed on the epidermis, which is caused by the immune system. The pathogens increase the epidermal growth and multiplication of epidermal cells.[2] Psoriasis is treated according to disease severity. Mild to moderate psoriasis symptoms are treated topically, whereas systemic therapy and phototherapy are used for severe disease. Systemic therapies have been of significant concern throughout history. They have been continuously developed and modified for the treatment of psoriasis.[3] The first-line treatment of psoriasis is Methotrexate (MTX) (cytotoxic drug), which is usually administered orally and parenterally. Therefore, transdermal and topical delivery of MTX with improved local and systemic delivery is

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preferred to reduce gastrointestinal side effects.[4] Different methods for topically delivering MTX to psoriasis lesions have been developed.[2] A traditional anti-psoriatic medicinal product, MTX is most effective when used as a single active ingredient or in combination with biologics.[5] MTX belongs to Dihydro-folate reductase enzyme inhibitor.[6] This drug shows good therapeutic activity in tumor necrosis factor (TNF), skin tumor, and rheumatoid arthritis. Due to its high molecular weight of MTX which is 454.56 D, water solubility, and ionized form, MTX will not diffuse passively through the Stratum Corneum.[7] Various types of MTX-based drug delivery systems, including nano-carriers solid lipid nanoparticles (SLNs), self-emulsifying nano-systems, transferosomes, liposomes, carbon nanotubes, polymeric nanoparticles, dendrimer, metallic nanoparticles, nanolipid carriers, and niosome formulated for the topical delivery of MTX.[7] Baicalin (BL) is extracted from the roots *Scutellaria baicalensis* is a traditional Chinese herb.[8] *S. baicalensis* has pharmacological activity against psoriasis.[9] Baicalin reduced the proliferation of keratinocytes and increased antitumor activity.[10] The liposomes were introduced to develop MTX-entrapped liposomes. The lipid carrier released the drug before permeation into the target area.[11] These deformable liposomes are composed of lipid content and a surfactant, an inner aqueous compartment surrounded by a lipid bilayer formulated to increase MTX skin penetration. The advantage of conventional liposomes over transfersomes is the characteristic of flexibility.[2] A topical formulation of MTX and etanercept for the treatment of psoriasis has previously been reported, and it provides a new pathway for combination formulations that noticeably increase bioavailability and better skin permeation compared with plain MTX gel. Dual-drug therapy is the most frequent approach to treat psoriasis, as it lowers the systemic toxic effects, improves patient compliance and increase the efficacy of the drug.[3] Topical preparation of co-loaded lipid nano-carrier lipid-soluble and water soluble drug formulated.[12] Dual-drug s with different polarities are formulated with the aid of an edge activator (EA) through the film hydration method (TFH).[13]

To achieve the treatment goal, MTX-entrapped transferomal formulations with improved permeability. Deformable transfersomes, elastic vesicles made of lipid materials and a surfactant with at least one inner aqueous compartment surrounded by a lipid bilayer, known as transfersomes were introduced previously.[2] Transfersomes are formulated from phosphatidylcholine (PC), EA sodium cholate (SC), and surfactant KG Dipotassium Glycyrrhizinate for the entrapment of MTX. By using KG as a surfactant, the amount of MTX permeated across the skin is 3-4 fold higher as compared to conventional liposomes.[14] Natural ingredient-based transfersomes are the choice because of the increased permeation of drugs into the skin.[2]

No previous study on MTX-BL transfersomes (TRs) gel co-loaded nano-lipid carriers has been reported. Both drugs have anti-psoriasis activity and have been used as single drug carriers. This study aimed to establish a nano-lipid carrier containing two drugs and evaluate their topical delivery for the topical treatment of psoriasis.

MATERIALS AND METHODS

Materials

MTX was gifted from Werrick Pharmaceutical (Islamabad, Pakistan). Tween 80 (Polysorbate 80) was purchased from Bio-Labs from the source of Hangzhou Zhongbao Imp. and Exp Corp. Ltd., China. Carboxy methyl cellulose purchased by bio-labs from the source of Qingdao Icd Biochemistry Co. Ltd. China. Sodium lauryl sulfate was purchased by bio-labs from the source of Emery Chemicals Malaysia. Soyabean PC was purchased from Bio-Labs from the source of Vigilant Tenent Laboratories. Carbopol 940 was purchased from Bio-Labs from the source of Lubrizol Advanced Materials INC. Brecksville USA. Carbopol 934 bio-labs were obtained from the source of Lubrizol Advanced Materials INC., Brecksville, USA. BL and SC were purchased from Sigma-Aldrich, USA. Phospholipon 90G was received as a gift sample from Lipoid AG, Switzerland. PBS (pH 7.4) and Alamar Blue reagent were obtained from Thermo Fisher Scientific, USA. Cytokine standards interleukin (IL)-17 and tumor necrosis factor-alpha (TNF-α) were purchased from BD Biosciences (Case, USA). All other used reagents were of pure analytical grade.

Preparation of MTX-BL-co-loaded TRs

Single MTX-TRs (Methotrexate and Transfersomes) and dual-drug-loaded MTX/BL TRs were prepared by the thin-TFH with some modifications.[15] Phospholipon 90G, tween 80, or SC as an EA, and MTX were dissolved in chloroform, methanol, and HCl 1:1:0 at pH 3 mixture and evaporated at 50 °C by using rotary evaporator under vacuum at 90 rpm for 20 minute. The thin film was evaporated under a vacuum to remove a few traces of the organic solvent. The dried film was hydrated with 100 mg of MTX solution in 20 mL PBS (pH 7.4) for 1 hour at 60 ± 1°. The transfersomes were extruded 5 times for 2 minutes through 450-and 200-nm filters. Dialysis was used to purify the formulation from the unbound drug. The vesicles were stored at 4 °C in glass vials.[16]

Encapsulation of BL

A mixture of Baicalin with cholesterol, chloroform, and Tween 80 or SC was formed. MTX loading was performed using Baicalin-loaded nano-lipid carriers in the MTX solution. Continue to stir in 1 mg/mL solution for 30 minutes. The excess drug present in the supernatant was removed by washing with water. The prepared MTX/BL TRs at 4 °C in a dark place. Dual-drug-loaded MTX/BL TRs were optimized in terms of entrapment efficiency (EE), vesicle size (VS), and elasticity by varying the percentage of Phospholipid or Sodum Cholate.[17]

Experimental design for the optimization of TRs

Nano-lipid carriers were prepared using the thin-TFH in a rotary vacuum evaporator. Phospholipon 90G, surfactant tween 80, sodium calcium, and cholesterol were added to a methanol: chloroform (1:9) mixture of 10 mL. In the rotary vacuum evaporator film, the film was allowed for 20 minutes at 50 °C temp and RPM 90. The quantity of phospholipon 90G and SC (PL:SC) ratio was varied as 90:10, 80:20, 70:30, 60:40, 80:20, and 80:20, and cholesterol was varied as 25 and 50 mg for preparation of trial batches of nano-lipid carriers formulations.

The formulations were kept in desiccators overnight for the removal of trace amounts of organic solvents by evaporation. The dried film was hydrated with 100 mg of the MTX solution in 20 mL PBS (pH 7.4) for 1 hour at 60 ± 1°. The batches were referred to as MTX/BL TRs 1, MTX/BL TRs 2, MTX/BL TRs 3, MTX/BL TRs 4, MTX-TRs, and blank TRs. Surfactants are selected according to the drug EE percentage and the VS of the nano-lipid carriers formed. SC lipid nanocarrier formulation has a higher flux value.[18]

Physicochemical characterization of co-loaded TRs

VS, polydispersity index (PDI), and zeta potential of the prepared co-loaded TRs were measured using a Zetasizer Nano ZS-90 instrument (Malvern instruments, Worcestershire, UK). All batches were diluted with millipore water at a 1:10 dilution and analyzed in triplicate using 90° scattering angle at 25 °C. Zeta potential was determined for drug-loaded nano-lipid carriers by Smoluchowski equation.[1] The EE of MTX was determined by direct method. Pellets were obtained by centrifugation of nano-lipid carriers for 15 mints at 15000 rpm. Then, it was treated with Triton X-100. Then 0.5 mL methanol was added to the disrupted nano-lipid carriers to make the drug more soluble. The samples were centrifuged at 10,000 rpm for 5 minutes. Prepared the dilution of 10 mL of TRs and diluted up to 5 mL with double-distilled water. Un-entrapped MTX was determined by direct method. The MTXs were unentrapped and separated from NLCs by exhaustive dialysis at 4 °C.[3] Then, MTX-TRs were added to the dialysis bag containing Molecular Weight cut off 12-24 kDa containing PBS (pH 7.4) and stirred with a magnetic stirrer. PBS was changed every 2 hours and its MTX content was determined using the atomic absorption spectrophotometer (AAS). The sample was dissolved with nitric acid, heated, and dried.[19]

Preparation of the gel

The optimized TRs were loaded with 100 mg of Carbopol 940 for topical preparation. Carbopol powder was added in 10 mL of distilled water and placed in a dark place for 24 hours. It swelled completely.[20] Drug-loaded MTX/BL TR gel was formulated by adding 50% *(w/w)* of MTX-TRs and BL/TRs slowly in carbopol gel. In contrast, constant stirring, while simple MTX-TR gel was prepared by adding 10% *(w/w)* of a single drug in the gel.[21] The formulation was adjusted by neutralizing with triethanolamine dropwise, resulting in a transparent gel was formed.[22]

Nano-lipid carriers stability

The optimized transfersomal preparations were stored at 4 °C for 3 months. The evaluation parameters were VS, PDI, EE, and zeta potential with different formulation's concentrations.[23]

Deformability index

To determine the deformability index, the developed TRs were formed using the extrusion technique. The VS of TRs were determined earlier and later of extrusion technique.[24]

Physicochemical and rheological evaluation of the MTX/BL TR gel

The MTX/BL TRs, MTX-TRs, and plain MTX gels were evaluated for pH, steady flow behavior, thixotropy, visco elastic behavior, and water-holding capacity.

Evaluation of pH

In 20 mL of distilled water add 1 gm of each gel; the pH of the gel was determined using a digital pH meter. A calibrated pH meter's electrode was dipped in the dispersion medium to determine the pH of the gel.

Homogeneity

To improve patient compliance, it is necessary to evaluate the homogeneity of topically applied transfersomal gels. The consistency of the gel was measured by applying a small amount of gel to the thumb and index finger and rubbing them over each other. The homogeneity was measured by its consistency.

Spreadability

A 0.5 g gel sample was placed between two transparent circular glass slides. Rest the gel over the glass for 5 minutes. The diameter was the indicator of spreadability. Measured the diameter of the gel circle.

Drug content determination

The MTX content was determined using an analytical method of MTX content (equivalent to 10 mg) in a 100 mL volumetric flask. Stirred the dilution and remained for 24 hours. The samples were filtered and analyzed using AAS.[19]

Rheological studies

The gel's viscosity was assessed using a Brookfield viscometer. Spindle no. 96 was used in the viscometer to measure the gel flow behavior. The sample was placed in the holder, a spindle was attached to it, and it was allowed to rotate at a speed of 5 rpm for a 10 s run time at 37 °C to attain the minimum turning force of 10%. Various rotational speeds were used to determine the viscosity of gels.

In vitro drug release and release kinetic study

Franz diffusion cell and dialysis membrane were used for *in vitro* drug release for transferomal dispersion. For activation of the dialysis membrane, soak it for 1 hour. PBS (pH 7.4) with sodium lauryl sulfate solution was used as the release medium. Filled the dialysis bag with 1 mL of transpersonal formulation while the the release medium was added to a separate vial of 10 mL. Place in a shaker bath with a shaking speed of 100 rpm at 37 °C. A sample of 5 mL was collected at a time interval of 0.5, 1, 3, 6, 24, 48, 96, and 120 hours and replaced with 5 mL fresh PBS medium. The MTX contents were analyzed using an AAS until no MTX appeared. Nitric acid was added after dialysis, followed by heat for complete drying. HCl:water (1:1) was added. Then it was boiled. Then, the best-fit model was used for regression co-efficient.[25] The permeation flux study was conducted for optimized transferomal and plain gels. The slope of the percentage of drug release vs. time is expressed for permeation flux.[10]

In vivo screening model, CFA, and formaldehyde

To induce psoriasis, a mixture of CFA and formaldehyde (1:10 ratio) was prepared. [26] Removed hair from the dorsal side of rats approximately 2 x 2 cm. A volume of 0.1 mL of the prepared

mixture was applied topically to the shaved area (n= 5 animals per group) on days 1, 2, and 3. The psoriatic lesions were observed daily for 7 days.[27]

Anti-psoriatic activity of the MTX/BL TR gel

Psoriasis was induced using the CFA and formaldehyde method. Animals were divided into 5 groups: 1) disease untreated, 2) plain drug MTX (water soluble), 3) single-loading MT 4. dual-drug-loaded MTX/BL TRs. The all groups treated every 24 hours for 21 days with MTX/BL TR gel (20 mg/kg) and MTX/BL TR gel (20 mg/kg) while the control group was left untreated. Drug efficacy was measured using the PASI. The intensity of psoriasis was found by stain smears through microscopic examination.[28]

Ex vivo permeation and drug deposition studies

Ex vivo skin penetration studies were performed for all trial batches using dialysis membrane and franz diffusion cell. The permeation fluxes of trial batches of transferomal gel and plain drug were determined MTX 20 mg/kg[2] and BL 5 mg/kg.[15] The outcomes of applying MTX 20 mg/kg and Baicalin 5 mg/kg formulation on normal mice skin showed no MTX and BL in the acceptor compartment within 24 hours, but the same dosage of MTX and BL applied on psoriatic skin, and 50% penetration was detected in the acceptor compartment. First, the abdominal hair of BALB/c mice was removed using an animal hair clipper. Mice were then sacrificed. The skin samples and abdominal fat tissues were excised. The excised skin was organized on the donor and receptor compartment with the SC side in the direction of the donor and dermis layers toward the receptor of the Franz diffusion cell apparatus. 7 mL of PBS (pH 7.4) was filled into the receptor compartment with a constant stirring rate of 300 rpm at 32 °C. 1 gm of the simple MTX gel, single MT/TRs, or MTX/BL TRs gel (equivalent to 20 mg/kg and 5 mg/kg of both drugs (MTX-BL) was placed on the skin surface. The cumulative amounts of MTX and BL permeated were assessed by the AAS method per unit area plotted against time.[16] Skin samples from the *ex vivo* permeation study were saved and blot-dried. Using the tape stripping method, the skin pieces were stripped into 20 parts. The entire tape was collected and placed in a beaker. MTX was extracted when the tape was added to a mixture of HCL:water (1:1). The remaining skin was chopped, meshed and homogenized.[22,29]

Evaluation of skin structure after MTX/BL TR gel treatment

In vivo skin irritation and histopathological study

In the histopathological study, epidermal changes and irritation potential in psoriatic mice were examined. The animals were divided into 5 groups with 5 animals in each group: group I had normal mice with epidermis psoriasis not induced to them, group II acted as an untreated control group, group III received plain drug MTX, group IV received single-loaded MTX, and group V received dual-drug-loaded MTX/BL TRs. Respectively, which were applied topically for 1 week. Histopathological examination was performed to determine pathological changes during topical application of gels. Striped skin samples were prepared from sacrificed mice in different treatment groups. Stained the skin samples with Hematoxylin and Eosin and a cryostat microtome on slide and observe under electric light microscope.[4]

Macrophage cytotoxic assay

Several cytokines are involved in the regulation of immunity against psoriasis. IL-17 is mainly produced by Th-17 cells. IL-17 plays an important role in the production of chemokines and secretions of neutrophils and antimicrobial proteins at the site of inflammation. In psoriatic skin samples, the cytokine levels (TNF-α and IL-17) were determined by enzyme-linked immunosorbent assay (ELISA).[30] Skin tissues of induced psoriasis were treated with PBS, and then the mixture was properly homogenized in a tissue homogenizer at 3000 rpm for 5 minutes. After centrifugation at 10,000 rpm for 15 minutes at 4 °C, the levels of TNF-α and IL-17 were determined by ELISA according to manufacturer's protocol.[14]

In vivo efficacy of the formulation in a BALB/c infection model of psoriasis

Clinical severity was expressed by the psoriasis-affected area and severity index (PASI). It was developed on the basis of the PASI. Redness, scales, and erythema were scored independently on a scale from 0 to 4: 0: none, 1: slight, 2: moderate, 3: marked, and 4: marked. PASI was calculated based on redness, erythema, and the scale scores. Anesthesia was administered at the end, and skin samples were collected. Preserved in 10% formalin solution for histological examination. Stained the rat skin specimen in hematoxylin and eosin dye for histological examination.[10,31]

Statistical analysis

The analysis of trial batches of MTX/BL TRs gel was performed using the response surface methodology. Assessment responses were analyzed using surface and contour plots to observe the design space and determine suitable quantities of excipients for maximum responses. The optimization plots explain the formulation factors and levels that produced the desired target responses. One-way analysis of variance (ANOVA) was applied for comparisons between groups. For significant *p* values, multiple Tukey's tests were used to compare the means of different groups. The significance level of this study was set at 0.05. SPSS V23 software was used. Kruskal-Wallis test for non-parametric statistical differences was used.[19]

RESULTS

The optimized dual-drug formulations were characterized on the basis of physiological parameters, including the varying concentrations of surfactant and EA, VS, polydispersity index, EE, and *in vitro* and *ex vivo* drug permeation study.

Selection of the surfactant and EA

For the flexibility of MTX/BL TRs, various EAs, such as SC, tween 80, phospholipon 90G, sorbitan monolaurate, sorbitan monopalmitate, sorbitan stearate, and sorbitan monolaurate.[2] SC was selected on the basis of observations seen in the dispersion; it increased the flexibility of the vesicles while with other surfactants, frothing was seen in the dispersion.

Formulation of nano-lipid carriers

Six formulations were prepared with different concentrations of phospholipon 90G and SC. The Transfersomal preparations were prepared using thin-TFH.[16] After the formulation of dual-drug-loaded carbopol gel TRs, they were evaluated on the basis of key parameters, *i.e.* VS, PDI, deformability Index and EE. Optimized formulation codes with MTX/BL TRs 4 were selected for further study.

Physicochemical characterization of NLCs

MTX/BL TRs were prepared by thin-TFH, SC is used as an EA. Co-delivery of MTX/BL TRs allows targeted delivery of nanoparticles to the immune system involved in the pathology of psoriasis. The main results of the physicochemical properties are shown in Table 1. These results show that mean VS decreases with increasing SC, with a quick reduction in VS as SC reaches 10%. It was established that the VS of single MTTRs was considerably higher ($p < 0.01$) at 10% SC than that of MTX/BL TRs. In Table 1 the PDI value is < 0.3 of formulations containing 5% or 10%. This results in homogenous dispersion. When SC% increases from 10% to 20%, PDI exhibits an increase in value. MTX/BL TRs incorporation did not affect the average TR size. TRs had a PDI in the range of 0.116 (blank TRs) to 0.359 (MTX/BL TRs). The PDI values suggest that the transferredomal preparation was homogenous with a low tendency toward aggregation. The average VS of the MTX/BL TRs was 170.1 ± 3.7 nm, with a PDI value of 0.138 and a ZP value of -38.6 Figure 1. The PDI values of MT/TRs and MTX/BL TRs ranged from 0.15 to 0.359, indicating better uniformity and homogeneity of the formulations.

The deformability index is the major parameter of NLCs for topical drug delivery because it allows drug molecules to easily permeate into the skin with the help of EA.[24]

Moreover, the effect of SC on the deformability index increased with each other to the extent of 10% ($p < 0.01$), and the deformability index decreased. The values of the deformability index of blank TRs, single MTX TRs, and MTX/BL TRs were 59.7 ± 3.7, 56.1 ± 3.3 and 52.8 ± 2.4, respectively. This explains that the addition Baicalin created a detrimental influence on the deformability of elastic vesicles, whereas the addition of MTX had an optimistic effect. It was observed that lipid content increased the particle size of nano-lipid carriers.

Effect of independent variables on vessel size

The VS of the optimized co-loaded nano-lipid carriers were evaluated using a Zetasizer nano ZS-90 instrument (Malvern Instruments, Worcestershire, UK). Various concentrations of PL:SC had significant effects on dual-drug-loaded transferomal preparations. The VS of formulations is shown in Table 1. There was no considerable difference in VS at 70:30 or above concentrations, but at 60:40 the rapid reduction of dual-drug-

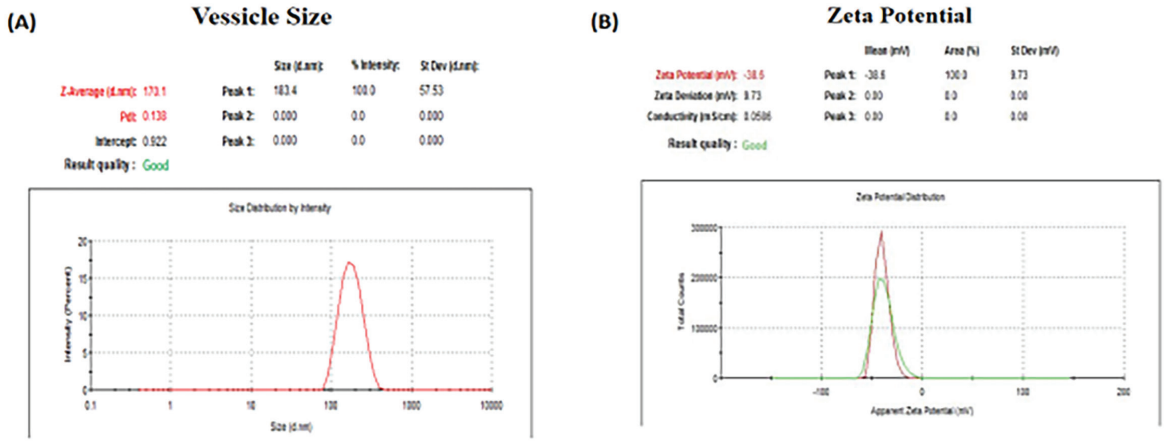

Figure 1. Independent variables, (A) vesicle size, (B) zeta potential

Table 1. Physicochemical characterization

Formulation code	PL:SC	Vesicle size (nm)	PDI	Deformability index	EE% ± SD (PT/CR)
MTX/BL TRs 1	90:10	241.5 ± 7.4	0.154	45.3 ± 2.7	71.9 ± 5.2/86.8 ± 5.7
MTX/BL TRs 2	80:20	170.1 ± 3.7	0.138	52.8 ± 2.4	69.5 ± 4.7/81.9 ± 4.4
MTX/BL TRs 3	70:30	111.3 ± 4.1	0.272	49.7 ± 2.9	47.0 ± 4.1/59.7 ± 4.8
MTX/BL TRs 4	60:40	56.4 ± 3.5	0.359	33.5 ± 2.6	37.8 ± 5.0/44.1 ± 4.9
MTX TRs	80:20	152.5 ± 5.0	0.131	56.1 ± 3.3	33.7 ± 4.1
Blank TRs	80:20	141.2 ± 3.5	0.116	59.5 ± 3.7	-

PL: Phospholipon 90G, SC: Sodium cholate, PDI: Polydispersity index, MTX/BL TRs: Methotrexate-baicalin dual loaded transfersomes, MTX TRs: Single Methotrexate loaded transfersomes, EE: Entrapment efficiency, SD: Standard deviation

loaded MTX/BL TRs was observed. The preferred VS was obtained by sonication for 10 minutes.

Effect of the independent variable on zeta potential

Vesicle's charge is evaluated by the Zeta potential. The zeta potentials of single MTX-TRs and MTX/BL TRs are described in Table 1. The zeta potential was approximately 38.6 mV, which is considered as a stable colloidal dispersion. MTX/BL TR incorporation did not have significant interference in the zeta potential values and consequently in the stability of the formulations. Neutral charged or slightly negative nano-lipid carriers with a zeta potential ranging from 10 to and 10 mV are acceptable.

Effect of the independent variable on the percentage EE

The EE of TRs were evaluated. It was observed that EE gradually decreased with increasing PL:SC until 10%. Increased PL:SC resulted in an immediate decrease in EE. Single MTX-TRs have lower EEs than dual-drug-loaded MTX/BL TRs. After consideration of all the important factors, MTX-co-loaded nano-lipid carriers with a PL:SC of 60:40 and VS 56.4 ± 3.5 nm were selected for the rest of the studies because they showed high EE. Dual-drug-loaded TRs showed an increase in EE compared to single-loaded hydrophilic drugs.

The EE percentage of MTX TRs was approximately 37.8% and that of blanks was 33.7%, which indicates that the further incorporation of the drug was not affected by the functionalization process. The EE% values of the results were relatively high, which shows that the transferomal preparation has better stability and good drug entrapment.

Physicochemical and rheological evaluation of TR gels

Spreadabilty

MTX/BL TRs were formulated with a carbopol 940 gel base, which retained the concentration of the drug for the prolong period of time into the stratum corneum. The spreadability factor of the TR gel was evaluated for the characteristics of topical gel formulations of the MTX/BL TR gel, MTX-TRs, and blank TRs. No significant difference in the physicochemical properties of the transferredomal carbopol gels was observed. At the initial stages, the spreadability profiles of all transfersomal gels were similar.

The MTX/BL TRs gel was evaluated for rheological behavior. The rheological properties of the transferredomal gel were analyzed for topical application. The results were compared with those of the blank drug gel. The viscosities of the gels were analyzed using a Brookfield viscometer spindle no 96.

The dual-loaded drug-incorporated gel showed shear thinning characteristics after the application of the slightest shear stress, which explained the pseudoplastic behavior. This assumption assumes physical stability of the formulations under several conditions during manufacturing and transportation.

Ex vivo skin penetration

The total amount permeated per unit area from simple MTX gel, single MTX-TR gel 31.42 µg/mL was released with standard deviation (SD) ± 9.4 µg/cm^2, single BLTRs gel 73.2 µg/mL was released with SD ± 12.4 µg/cm^2. In combination drug delivery, the MTX/BL TRs the MTX was 218.6 µg/mL permeated with SD ±19.5 µg/cm^2 and the BL was 237.61 µg/mL permeated with SD ± 25.5 µg/cm^2, respectively. When drugs were applied with co-loaded MTX/BL TRs, the skin permeation of the drugs was significantly improved ($p < 0.01$). Co-loaded MTX/BL TRs were more efficiently deposited in the skin than simple MTX and single MTX gels.

VS and lipid content of nano-lipid carriers (liposomal formulations) affected the release pattern of TRs. Additionally, the deposition of MTX/BL TRs in the skin was much higher than that of the single drug-loaded MTX gel. It has a longer retained period of time at the site of psoriasis due to more skin deposition than less skin permeation (Figure 2).[21]

Evaluation of skin structure after MTX/BL TRs treatment

Histopathological examinations compared the normal skin of healthy mice with the typical epidermis and dermis with that of psoriatic-induced treated or untreated mice. The results obtained from group I (normal mice epidermis) Figure 3A, group II (untreated control) Figure 3B, group III (plain drug MTX) Figure 3C, group IV (single loaded MTX) Figure 3D and group V (dual-drug loaded MTX/BL TRs) Figure 3E, respectively.

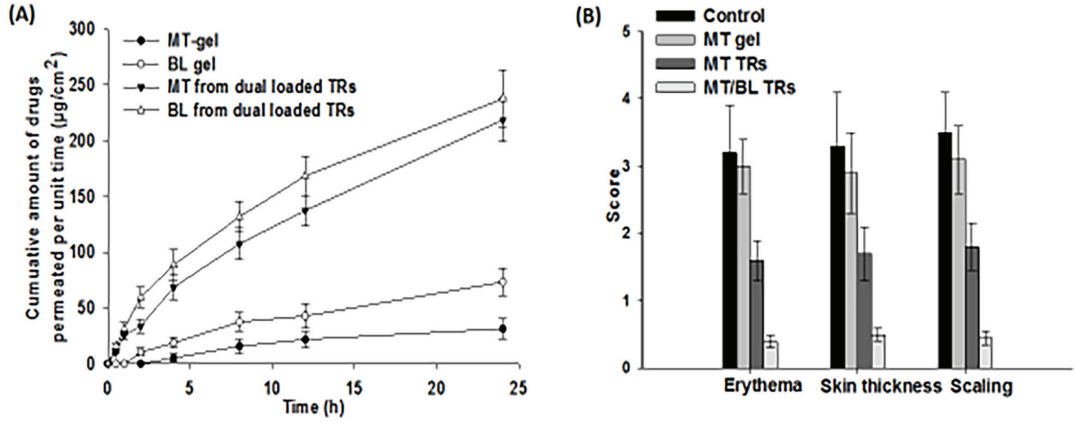

Figure 2. A, B) Drug's permeation and skin penetration studies

MT: Methotrexate, BL: Baicalin, TRs: Transfersomes

Group I included normal skin with well-defined epidermis, dermis, subcutaneous tissue, and muscles. The epidermis shows stratified squamous keratinized epithelium supported by a dermis layer of dense fibro elastic connective tissue that is devoid of inflammatory cells. Histopathological changes in the treatment group were highly dependent on formulation type. The thickness index varies among the groups. During the treatment the thickness was reduced. After treatment with MTX gel, psoriatic skin showed no significant reduction in epidermal thickness. This result indicated that the anti-psoriatic activity of the single MTX gel was relatively partial. Single MTX/TRs showed better anti-psoriatic activity by reducing epidermal thickness, which indicates that nano-lipid carriers of MTX within nano-lipid carriers have better anti-psoriatic activity, but MTX/BL TRs, a dual-drug delivery gel, showed similar histopathological characteristics as compared to the normal epidermis of mice, where the epidermis of the skin was almost normalized. This confirms that single MTX/TRs and MTX-BL nano-lipid carriers have significant ($p < 0.05$) reduced thickness in all groups. Relatively acceptable safety profile and does not cause irritation in clinical trials when applied topically. This was based on the absence of apparent signs of skin irritation in the *in vivo* study (Figure 2).

Scoring of skin inflammation

For 30 consecutive days, the PASI scores for skin erythema and skin thickening in the psoriasis-affected area were observed. All formulations of simple MTX drug, single MTX TRs, and MTX/BL TRs exhibited clear PASI scores on day 10, which were later more improved on day 15. Compared with these formulations, the dual-drug-loaded MTX-BL/TR gel showed reduction in both skin erythema and skin thickening and had the best anti-psoriatic activity. The PASI score ranged from 0 to 6, and the scoring parameters were 1. Erythema, 2. skin thickness, 3. scaling. The formulations for PASI scoring were the control, simple MTX, MTX-TR, and MTX/BL TRs gels. The control group had a score of 3 in erythema, skin thickness, and scaling. The control group had the highest score in all the parameters, which indicates severe erythema, skin thickness, and scaling. Simple MTX gel: showed less score of 3 compared to the control group for all the parameters of erythema, skin thickness, and scaling. Simple MTX gel showed a score of 2.5 for all the parameters, which indicates that the severity of erythema, skin thickness, and scaling decreased. MTX-TRs gel: The PASI scoring scale decreased by up to 1.5. The TR gel preparation has more efficacy and permeation into the skin than the plain MTX gel. MTX/BL TR gel: The combination dual-drug delivery has shown the scaling of zero or above for all parameters, which shows that the dual-drug combination of MTX/BL TRs gel has the lowest erythema, skin thickness, and scaling, which leads to improving the improve the condition of psoriasis.

Cytotoxicity assay

Increased levels of cytokines characterize psoriatic skin is characterized. TNF-α and IL-17.[32] ELISA was performed to find the level of TNF-α and IL-17, as shown in Figure 4A and Figure 4B. The TNF-α and IL-17 levels were analyzed in 5 groups. Each group consisted of 5 members. The optimized topical transferomal gel for psoriasis decreased the level of cytokines TNF-α, IL-22 and IL-17.[22]

TNF-α

Figure 4A shows the results of the relative % of TNF-α at a scale of 0-120 at the Y-axis and different group formulations of control, untreated, MTX solution, MTX TRs, and MTX/BL TRs. The results show major differences between the control and untreated groups. The percentage of TNF-α increased up to 90%, simple MT sol:showed 65% TNF-α. The relative percentage of TNF-α decreased as compared to the untreated

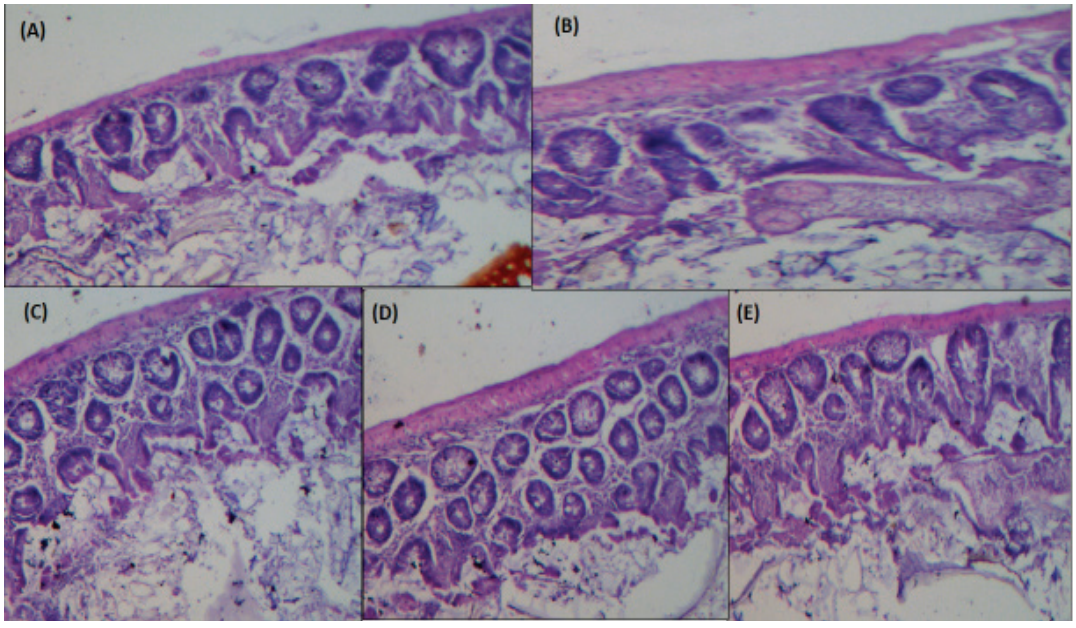

Figure 3. Histopathological studies. A) Normal, B) Psoriasis, C) Single Methotrexate drug, D) Single Methotrexate transfersomes, E) Dual-loaded Methotrexate-Baicalin transfersomes

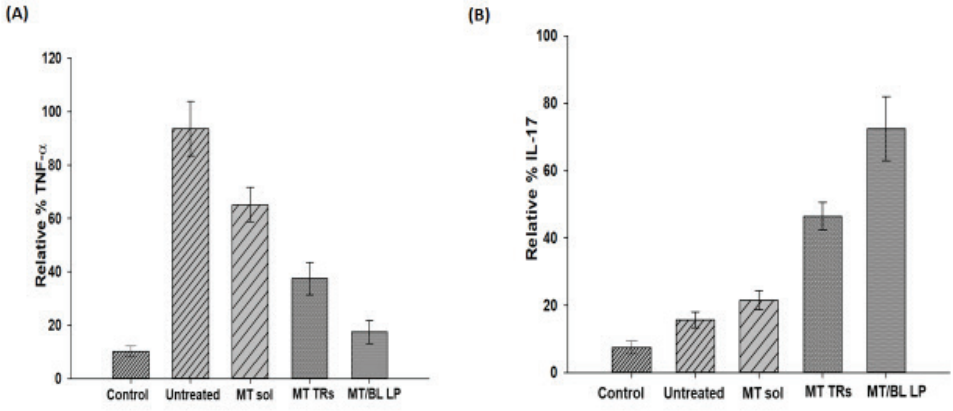

Figure 4. Cytotoxicity sssay A: TNF- α, B: IL-17

MT: Methotrexate, BL: Baicalin, TRs: Transfersomes, LP: Liposomes, TNF- α: Tumor necrosis factor-alpha, IL-17: Interleukin-17

group. MTX is effective in psoriasis as the results showed a decrease value. The MTX TR gel was incorporated with MTX. The gel showed more penetration permeation than the plain MTX solutionl. The TNF-α was decreased up to 35%. The MTX/BL TR gel percentage relative of TNF-α has been 10%, which was near 5% of the control group. The results were interpreted as indicating that nano-lipid carriers have more permeation and penetration than plain gel. Transfersomal gels have greater efficacy than plain single-dose drug delivery. The results are similar to those of the control group, indicating a better choice of nano-lipid carriers for topical drug delivery. The results of the control group are much more concise than those of the other groups. The control group was used as a standard, and the relative percentage of TNF-α was 5%. Other groups had high relative percentage of TNF-α, the untreated group had the highest percentage of TNF-α.

IL-17

The relative percentage of IL-17 was significantly higher in patients with psoriasis than in the control group. Because it was treated with different formulations, the relative percentage of IL-17 was twice that of other formulations. Figure 4B shows the results of the relative % of TNF-α at a scale of 0-120 at Y-axis and different group formulations of control, untreated, MTX solution, MTX-TRs, and MTX/BL TRs. The results show major differences between the control and untreated groups. The percentage of IL-17 increased up to 65%, and the Simple MTX solution produced 20% IL-17. The relative percentage of IL-17 was increased compared with the untreated group. MTX is effective against psoriasis as evidenced by the increased value. The MTX-TR gel was incorporated with MTX. The gel showed more penetration permeation than the plain MTX sol. The IL-17 was increased up to 40%. The MTX/BL TR gel percentage relative of IL-17 was 65%, which is significantly different from the control group. The results were interpreted as indicating that nano-lipid carriers have more permeation and penetration than plain gel. Transfersomal gels have greater efficacy than plain single-dose drug delivery. The results are similar to those of the control group, indicating a better choice of nano-lipid carriers for topical drug delivery. The results of the control group are quite more concise than those of the other groups. The control group was used as a standard, and the relative percentage of IL-17 was 20%. Other groups had a high relative percentage of IL-17, whereas the untreated group had the lowest percent of IL-17. The comparison between TNF-α and IL-17. In comparison with the control group the TNF-α and IL-17 levels were relative of 20. In untreated group the level of TNF-α were increased up to 90% in psoriatic-induced untreated skin, respectively. After treatment with MTX- solution, MTX-TRs, and MTX/BL TRs the level of TNF-α was decreased by 65%, 25%, and 20%, respectively. Similarly, in the untreated group, the level of IL-17 was raised compared with the control group, indicating the induction of psoriasis (Figure 4). After the application of MTX- solution, MTX-TRs, and MTX/BL LP, the level of IL-17 was increased by 20%, 40% and 65%. The present data showed that an increased serum concentration of IL-17 was present. The role of IL-17 in neutrophil production at the site of inflammation and chemokine production. The TNF-α cytokine assay data shows that in the psoriasis-affected group (untreated) the serum level was increased, which was later on decreasing when treated with MTX nano-lipid carriers. The IL-17 cytokine assay data showed that in the psoriasis-affected group (un treated) the serum level was decreased, which was later on increasing when treated with MTX nano-lipid carriers. Both cytokines assays showed the activity of nano-lipid carriers of MTX solution, MTX-TRs, and MTX/BL TRs. TNF-α interacts with inflammatory cells to trigger cytolysis.

DISCUSSION

In this study, dual-drug nanocarrier physiological and *in vitro* and *in vivo* characteristics were examined. The topical treatment of psoriasis is preferred over systemic drug delivery because of fewer adverse effects.[33] Topical formulations have greater bioavailability with fewer side effects. Dual-drug delivery shows a significant therapeutic approach at a very low dose for complicated and single therapy resistant diseases.[16] Adverse drug reactions were reported with systemic MTX

therapy in patients with psoriasis, but there was no significant effect on liver and serum enzyme level.[8] To improve the efficacy of treatment of various pathological diseases, dual-drug therapy is required. The limitations of the dual-drug delivery method are the entrapment of different charged molecules, physicochemical incompatibility, solubility, selection of surfactants, stability, and various drug concentrations.[11] Co-loaded nano-lipid carriers penetrated and permeated into the deeper layer of dermis, slowly releasing a dual-drug into the subcutaneous.[34] Previously, many studies have described the MTX nano-carrier skin penetration results for the topical application for the treatment of psoriasis.[35] It was concluded that the VS of single MTX-TRs was considerably higher ($p < 0.01$) at 10% SC as an EA compared to MTX/BL TRs.

EAs act as stabilizers to increase the drug permeation of lipid-nano carriers.[7] The VS decreases with increasing SC concentration from 10% to 20%, and the PDI shows an increase in its value. The PDI value of MTX-TRs and MTX/BL TRs ranges from 0.15 to 0.359, which shows better uniformity and homogeneity of formulations.[36] The hydrophobic drugs have a higher membrane flux and have direct interaction with the lipid bilayer of the skin. Phospholipon 90G increased the permeation flux ($p < 0.05$) of the MTX formulation because of its high lipid content. The effect of SC on the deformability index increased with each other at the extent of 10% ($p < 0.01$). The amount of EE significantly decreased when the amount of surfactant increased.[37] The nano-lipid carriers prepared by SC have a concise particle size and an EE% higher than that of tween 80. SC provides better EE compared to other surfactants. The formulation of MTX, phospholipon 90G, sodium calcium, and cholesterol has the most concise particle size and better EE. In TR vesicles, the greater the lipid content, the greater is the EE. The effect of cholesterol in formulations is the least effective, but its effect on EE is greater due to its high lipid content. Phospholipon 90G had a greater influence on EE%. This explained that the addition of Baicalin created a detrimental influence on the deformability of elastic vesicles, whereas the addition of MTX had an optimistic effect. To increase the deformability of ultra-deformable liposomes, the EA is of vital importance. The effect of SC as an EA is concentration-dependent.[38] Advanced rigid molecular structures have more skin permeation, rigid VS and more bioavailability.[39] In the present study, single MTX-TRs had lower EEs than dual-drug loaded MTX/BL TRs. Decreased concentrations of EA and Phospholipon 90G decrease the rapid reduction of dual-drug loaded TRs vs. more increase in phospholipon 90G and SC resulted in immediate decrease in EE. Single MTX-TRs had lower EE compared with dual-drug loaded MTX/BL TRs. Dual-drug loaded TRs showed an increase in EE compared to single-loaded hydrophilic drugs. Transfersomal preparations have better stability and good entrapment of a drug. VS of nano-lipid carriers plays an important role in topical delivery. To achieve advanced targeted drug delivery and deeper penetration of the drug, the formulation should be optimum and characterized on the basis of particle size. These formulations varying from different (phospholipon 90G and SC) concentrations were prepared by thin-film hydration. Polar and high-molecular-weight molecules diffuse through the stratum corneum by encapsulation with a non-ionic surfactant of particle size.[29] To increase the stability of the formulation, the vesicle should have a highly negative zeta potential charge due to electrostatic repulsion. Highly positively charged nanoparticles are more cytotoxic because they cause protein aggregation in the blood. To determine the stability, cellular uptake, and cytotoxicity of transferomal preparation, the Zeta potential is required.[1] The stable colloidal dispersion exhibited a Zeta potential of approximately 38.6 mV. Highly positively charged nanoparticles are more cytotoxic because they cause protein aggregation in the blood. The surface charge of nano-lipid carriers maintains its stability.[22] The Ideal pH for carbopol gel is 5.0-8.0 which does not affect its rheological properties, so it can be used as a topical formulations.[40] The formulations of carbopol gel having different concentrations showed a non-Newtonian, higher shear-thinning, which increased the drug's retention time, bioavailability, and therapeutic efficacy.[16] Nano-carrier emulsion gels are more beneficial in topical preparations hence they covered the maximum coverage area.[41] The dual-drug delivery of MTX and Baicalin into the nano-carrier molecule improves the therapeutic activity as compared to single drug delivery. The previous studies has been reported.[34] The thin film was formed at 50 °C temperature and 90 RPM at 20 minutes in a rotary vacuum evaporateator. Nano-lipid carriers have 5 fold more penetration of drug into the Stratum Corneum.[22] Lipid nano-carrier is the advanced Drug Delivery System in Cosmeceuticals. The Novel Drug Delivery system termed as nano-safe carriers due to their safety profile.[42] The components were selected based on the characteristics of skin permeation, molecular compatibility, and GRAS condition.[41] In a previous study, the dual-drug delivery of MTX lipid ultra-deformable liposomes formed by a carbopol gel showed increased skin drug bioavailability.[3] For the Topical route of drug administration, spreadability is an important characteristic for the development and formulation of appropriate drugs in the target area. The PSRAL gel and PSRCL gel showed that the rheological properties of gels result in a reduction of viscosity due to shear stress.[43] Drug loaded incorporated gel showed shear thinning characteristics by applying the slightest shear stress, which explained the pseudoplastic behavior. This shows the stability of the formulations. *In vitro* and *ex vivo* permeation studies of all the transferomal batches were conducted to determine drug release and permeation studies. The MTX/BL TRs improved the skin permeation of drugs was much improved ($p < 0.01$) However co-loaded MTX/BL TRs were more efficiently deposited when applied into the skin compared to a simple MTX and single MTX gel. The VS and lipid content behavior of TRs affected the release pattern of TRs. It has a longer retained period of time at the site of psoriasis due to more skin deposition. *In vitro* results of MTX SLNs showed a 8 hour sustained release.[3] The dual-drug MTX-loaded TRs decreased the PASI score, and the formulation was developed for treating psoriasis topically.[1] *In vitro* results explained the lipid nano-carrier anti-psoriatic activity, decreased the IL-17

and TNF-α. Cell lines explain the decreased levels of NO, IL-2, IL-6 and IL 1β.[31]

MTX with Baicalin transferomal gel showed significant penetration and permeation parameters in psoriasis affected skin. Psoriasis is a chronic inflammatory, T-lymphocyte immune-mediated skin disease. The etiology of psoriasis is unknown, but the risk factors are drugs, IBD, lifestyle, environmental, and genetic factors, which lead to the proliferation of keratinocytes.[13] As a result, silver scales, papules, and plaques are formed due to epidermal thickening. Scaly skin lesions are usually observed at the elbows, knee joints, palms, soles, and extensor surfaces, and erythrodermic psoriasis diffuses in areas covering > 90% of the body surface. Psoriasis treatment focuses on relieving symptoms and improving skin function. Depending upon the type and severity of psoriasis, the treatment should be planned, which may include phototherapy, systemic treatments, Monoclonal antibodies, or topical treatments.[44] Application of Drug Directly at Topical Affected Psoriasis Sites with a narrow Therapeutic Window Reduced Systemic Absorption.[45] MTX (orally as well as systematically) is the gold standard drug for the treatment of psoriasis. MTX is a dihydro-folate reductase enzyme inhibitor. This drug shows good therapeutic activity in TNF, skin tumor, and rheumatoid arthritis. Due to the high molecular weight of MTX (454.56 D, water solubility, and its ionized form, it will not diffuse passively through the Stratum Corneum.[46] Various types of MTX-based drug delivery systems, including nano-carriers, SLNs (solid lipid nanoparticles), self-emulsifying nano-systems, nano-lipid carriers, liposomes, carbon nanotubes, polymeric nanoparticles, dendrimer, metallic nanoparticles, nanolipid carriers, and niosome, have been formulated for the Topical Delivery of MTX.[47]

MTX-entrapped nano-lipid carriers are elastic vesicles made of lipid materials and a surfactant with at least one inner aqueous compartment surrounded by a lipid bilayer.[48] By using KG as a surfactant, the amount of MTX permeated across the skin is 3-4 fold higher as compared to conventional liposomes. Natural ingredient-based nano-lipid carriers are the better choice because of increased permeation of drugs into the skin. Formulations were analyzed and optimized by thin-film rehydration using phospholipon 90G, tween 80, and cholesterol. Optimization and characterization of drug carriers based on particle size, zeta potential, and drug EE. Evaluation of pH, homogeneity, spreadability, rheological studies, and drug content determination for all formulations showed that drug-loaded TRs (MTX/BL TRs) have better physicochemical properties than plain drugs.

The result of VS at 70:30 or higher concentrations but at 60:40 the rapid reduction of dual-drug-loaded MTX/BL TRs was observed. The zeta potential was approximately 38.6 mV, which is considered stable colloidal dispersion. The incorporation of MTX/BL TR did not have significant interference in the zeta potential values and consequently in the stability of the formulations. Neutral charged or slightly negative nano-lipid carriers with zeta potentials ranging from 10 mV to and 10 mV are acceptable. Highly positively charged nanoparticles are more cytotoxic because they cause protein aggregation in the blood. The EE percentage of MTX-TRs was approximately 37.8% and that of blanks was 33.7%, which indicates that the further incorporation of drug was not affected by the functionalization process. The EE% values of the results were relatively high, which shows that the transferomal preparation has better stability and good drug entrapment. The dual-loaded drug-incorporated gel showed shear thinning characteristics after the application of the slightest shear stress, which explained the pseudoplastic behavior. This assumption assumes physical stability of the formulations under several conditions during manufacturing and transportation.

Characterization by *in vitro* release and membrane diffusion studies of transferomal gel formulations revealed that the VS and lipid content of nano-lipid carriers (liposomal formulations) affected TR release patterns.[49] Additionally, the deposition of MTX/BL TRs in the skin was much higher than that of the single drug-loaded MTX gel. It has a longer retained period of time at the site of psoriasis due to more skin deposition than less skin permeation.

The effects of the formulations on anti-psoriatic efficacy were evaluated by PASI scoring of skin severity and thickening. The scoring parameters were set to 1. erythema, 2. skin thickness, 3. scaling. The combination dual-drug delivery has shown the scaling of zero or above for all parameters, which shows that the dual-drug combination of MTX/BL TRs gel has the lowest erythema, skin thickness, and scaling, which leads to improving the improve the condition of psoriasis.[50]

Histopathological examinations compared the normal skin of healthy mice with the typical epidermis and dermis with that of psoriatic-induced treated or untreated mice. The epidermis is characterized by stratified squamous keratinized epithelium supported by a dermis layer of dense fibroelastic connective tissue that is devoid of inflammatory cells. Histopathological changes in the treatment group were highly dependent on formulation type. The thickness index varies among the groups. During the treatment the thickness was reduced. After treatment with MTX gel, psoriatic skin showed no significant reduction in epidermal thickness. This result indicated that the anti-psoriatic activity of the single MTX gel was relatively partial. In contrast, single MTX-TRs showed better anti-psoriatic activity by reducing epidermal thickness, which indicates that nano-lipid carriers of MTX within nano-lipid carriers have better anti-psoriatic activity, but MTX/BL TRs, a dual-drug delivery gel, showed similar histopathological characteristics as compared to the normal epidermis of mice; the epidermis of the skin was almost normalized.[51]

Increased levels of cytokines characterize psoriatic skin is characterized. TNF-α and IL-17.[49] ELISA assay was performed to find the level of TNF-α and IL-17. The increased levels of the pro-inflammatory cytokines IL-17, IL-23, TNF-α and IL-27 due to the activation of Th1 and Th 17 cells (CD4+T cells and CD8+T cells) enhance the inflammatory response.

Stable MTX/TR-based transferomal gel with advanced efficiency for the psoriasis model in BALB/c mice was investigated for the liposomal targeted drug delivery of MTX/BL TRs. The results

establish that MTX/BL TRs were more potent and exhibited better penetration and permeation than single-loaded drugs.

Study limitations

The sample size in this study was small, 25 animals in 5 groups because of limited Research and Development facilities. To study more effectively ELISAs and more cytokines assays should be applied, More variables of surfactants and EAs should be studied.

Future perspective

This study will create new opportunities for the release of the profile of topical dual delivery of anti-psoriasis drugs. Further evaluation of the synergistic mechanism and cytotoxicity of novel co-loaded nano-lipid carriers for the treatment of psoriasis.

CONCLUSION

It can be concluded that newer targeting strategies for NLCs for dual-drug delivery of nanolipid carriers MTX/BL TRs gel that could be administered topically for the treatment of psoriasis. Furthermore, this approach opens new avenues for continued and sustained research in pharmaceuticals with much more effective outcomes.

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Ethics

Ethics Committee Approval: *In vitro* and *ex vivo* permeation studies were conducted as per the experimental protocol approved by the IBADAT International University, Islamabad Faculty of Allied Health Sciences Bioethics Committee (approval number: #BEC/0525, date: 23.02.2023).

Informed Consent: Not required.

Authorship Contributions

Surgical and Medical Practices: S.S., S.A., Concept: M.J.D., Design: K.I., Data Collection or Processing: S.S., S.K., Analysis or Interpretation: M.J.D., K.I., Literature Search: S.S., H.S., Writing: M.J.D., S.A.

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