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Formulation and Toxicity Evaluation of Nanostructured Lipid Carriers for the Treatment of Acne

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Acne vulgarise is an inflammatory disease involving the pathological alteration of the sebaceous glands of the body. It is not a life-threatening disease but has a great influence on lifestyle. Topical combination therapy of vitamin A and antibacterial drugs is an effective treatment for acne.
Materials and Methods: The current work investigates the nanostructure lipid colloidal carrier system of Tretinoin and Clindamycin phosphate. Nanostructured lipid carriers (NLCs) were prepared by highspeed homogenization-sonication technique and characterized for physicochemical properties, permeation, *in vivo* anti-acne and toxicity (acute 2000 mg/Kg, repeat 1000 mg/kg) in Wistar rats.
Results: The prepared system was found to be stable, homogenous with more site retention of drugs having non-irritation and toxicity potential. The formulation showed a size of 283 nm, polydispersity index (PDI) 0.43 and Zeta potential (ZP) -37.9 mV with drug entrapment 92.0% and 66.15% for tretinoin and clindamycin respectively. Observed permeation was 18 % and 45% for Tretinoin and Clindamycin less than marketed formulation which is more focused on dermal retention of drug. No significant abnormalities and toxicological symptoms were observed for acute

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and repeat dose toxicity study for histopathology and haematological examinations of organs.
Conclusion: Prepared NLC formulation was aimed at epidermal targeting. Based on obtained results it is concluded that developed lipid-based nanocarrier system of selected drugs showed the targeting potential for effective acne treatment.

Keywords: Nanostructured lipid carriers; acute toxicity; repeat dose toxicity.

1. INTRODUCTION

Acne vulgarise is the most common, prevalent skin disease. A significant number of the population is affected by acne. It is not life-threatening but influences quality of life [1-3]. Acne patients exhibit low self-esteem owing to the diverse lesions on their face, chest and back. A common type of bacteria *Propionibacterium (P) acnes* contributes to acne by causing inflammation. It is an inflammation of sebaceous follicles, characterized by seborrhoea, papules, comedones, nodules, pimples and pustules. Factors responsible for acne are sebum, hormones, follicular differentiation, bacteria *Propionibacterium acnes*, inflammation and nutrition [4, 5].

Many treatments exist such as retinoids, antibiotics, keratolytic, anti-seborrheic, anti-androgen medications and hormones-based treatment. Anti-bacterial such as clindamycin, erythromycin, minocycline, tetracycline, hydroxyquinoline and vitamin A derivatives such as tretinoin, isotretinoin, adapalene can be used [6-11]. Topical therapy of vitamin A which exhibits anti-comedolytic activity and antibacterial drug clindamycin phosphate is a better acne treatment [12-17]. Novel drug deliveries providing alternatives for enhanced impact as a drug is loaded in lipid carrier systems like nanocrystals [18,19], liposomes [20,21], niosomes [22], micro-emulsion [23], lipid nanoparticles [24, 25]. The first generation of solid lipid nanoparticles (SLN) were prepared from only solid lipid. The second-generation nanostructured lipid carriers (NLCs) are prepared by a mixture of solid, liquid lipid [26-33]. NLCs are also used as delivery carriers for hydrophobic and hydrophilic drugs, expected to have low toxicity and biodegradability.

Clindamycin phosphate (2mg/10 ml) is more water-soluble than tretinoin (< 0.1g/100mL). This work focuses on the incorporation of Clindamycin phosphate and Tretinoin into colloidal nanostructured lipid carrier formulation followed by characterization for morphology, entrapment efficiency, permeation, *in vivo activity* and toxicity potential. *In vivo* activities tested on Wistar rats. Dermal toxicity studies outcome is outlined in this article to characterize the skin irritation, local and systemic toxicity potential after dermal administration and evaluated as a better potential for acne treatment [34-36].

2. MATERIALS

Tretinoin was offered as a gift sample from Ranbaxy Pharmaceuticals, Delhi, India whereas Clindamycin phosphate was provided by SRS Pharma aqLTD. Mumbai, India. Cremophore El was gifted by BASF, Mumbai. Shea butter was supplied by Anshul Pharmaceuticals, Mumbai. Emulcier 61 was gifted by Gattefossae Ltd, Mumbai, India. Olive oil, Jojoba oil was gifted by Aromax Pharma LTD. Mumbai, India. Stearic acid, Cetyl palmitate, Span 20, Span 80, PEG 200, Oleic acid, Medium chain tryglycerides and other solvents used for analysis were purchase from S. D. Fine Chem. Mumbai, India. The remaining materials used were of analytical grade.

3. METHODS

3.1 Screening of Drug in Liquid Lipids and Surfactants

Drug solubility in selected liquid lipids (jojoba oil, oleic acid, olive oil, medium chain triglycerides) and surfactants (Cremophore EL, Span 20, Span 80, PEG 200) was determined. The vials were filled with an excess quantity of drug and 2 ml of liquid lipid and surfactant. It was stirred to achieve equilibrium for 24 h at 25^0 C. Vials were then centrifuged using ultracentrifuge at 10,000 rpm, 30 min. The clear supernatant was collected and drugs were quantitated by U.V. Spectrophotometer. Tretinoin was determined using solvent methanol with suitable dilutions and analyzed at 339 nm whereas Clindamycin was evaluated using methanolic sodium hydroxide 0.1 N (1:1) at 215 nm after suitable dilutions [37,38].

3.2 Screening of Solid Lipids

Solid lipid solubility (stearic acid, Cetyl palmitate, Emulcier 61, Shea butter) was done by adding

both drugs (5 mg) in a wide-mouth, amber coloured, screw-capped bottle. The lipids were heated till melting. This molten lipid was added in a small portion. The addition of lipid is continued until no drug particle is seen visually. Lipid melt was quantified for Tretinoin by dissolving in methanol and for clindamycin phosphate in methanolic sodium hydroxide 0.1 N by using UV Spectrophotometer [39-41].

3.3 Preparation and Evaluation of NLCs

High- speed homogenization-sonication technique: Based on solubility screening of both the drugs in liquid, solid lipids and surfactants oleic acid, shea butter, Emulcier 61, Cremophor EL and Span 20 were selected for formulation preparation. Various batches with different proportions of both the lipids, surfactant and co-surfactant were prepared to select a suitable combination to get a stable batch.

Selected lipids were weighed and melted. Tretinoin was added to the hot, liquified lipid phase. Clindamycin was dissolved in water (aqueous phase) and heated to emulsification temperature same to the oil phase. Both aqueous and lipid phase were mixed at a similar temperature around 60-70 ^{0} C using a magnetic stirrer, 2000 rpm, 10 min to prepare a stable primary emulsion and then with a high-speed homogenizer at 60,000 rpm, 20 min. This mixture was further processed by probe sonicator, 20 min to get NLCs. Obtained NLC formulation was of thick, creamy consistency. The prepared formulation was stored in an amber colour bottle for further evaluation [42-43].

Morphological evaluation: Particle size, zeta potential, polydispersity were evaluated using a Horiba particle size analyzer zeta sizer. Before size determination, suitable dilutions were with double distilled water. The diluted sample was evaluated at a fixed scattered angle of 173^0 and 25^0 C temperature [44-46].

% Entrapment Efficiency (EE): % EE of prepared NLC was calculated by centrifugation. NLC formulation was diluted with equal parts of water and centrifuged at high-speed at 15,000 rpm, 30 min with a refrigerated centrifuge. The clear supernatant was separated and quantified for tretinoin by dissolving supernatant in methanol by U.V. Spectrophotometer at 339 nm and in methanolic sodium hydroxide 0.1 N at 215 nm for clindamycin [47].

% EE =(Drug added in the formulation – Unentrapped drug/ Drug added in the formulation) X 100

Drug permeation: A drug permeation was estimated using Franz diffusion assembly (12 ml capacity, 3.14 cm^2 area). Rat skin (Wistar rats, about 150-200 g) obtained from the college animal house. Hairs were removed using a hair trimmer and epilator cream and used as a diffusion membrane for study. The doner compartment consisted of 0.5 g of prepared NLCs formulation. The receptor chamber was filled with pH 7.4 phosphate buffer saline (PBS 7.4). The experiments were performed at temperature 37±0.5^0 C, 50 rpm. At pre-decided time interval of 0.5 h, 1 h, 2 h, 4 h, and 6 h, 0.5 ml aliquot from the receptor chamber was collected and replenished with an equal volume of media. Samples were analysed by spectrophotometer and the drug permeation profile was plotted to compared with marketed formulation [48].

Experimental animals: Wistar rat (200-250 g) species were used for *in vivo* anti-acne model and toxicity evaluation. The animals were acclimatized to laboratory conditions (25^0 C, 35-60% humidity).

3.4 *In vivo* Anti-acne Activity

Acne induction and treatment: A rat model was used to reflect the inflammation and acne genesis by injecting a culture of *P. acnes.* The lyophilized culture of bacteria *P. acne* was procured from the Indian Institute of Microbial Technology (IMTECH), Chandigarh, India. Induction of acne was done by injecting heat-killed bacterial culture (60 ^{0} C for 30 min) of concentration 10^{-2} CFU / ml, 20µl was injected to the right ear pinna subcutaneously for 4 days, once a day. Left ear pinna was without induction and considered as a control for comparison. Acne induction was identified by the presence of redness, inflammation and acne.

Acne on the right ear pinna. After induction animal ears were treated with the test formulation, marketed formulation and placebo formulation. Once a day 0.5 g of the formulation was applied to the affected area and observed for healing and any other changes [49].

3.5 *In vivo* Acute and Repeat Dose Toxicity Study

Acute dermal toxicity: The dermal toxicity was performed as per the OECD guidelines no.402

for the chemical testing. Wistar rats of 150-200 g were acclimatized to the laboratory condition for a week. Animals were assigned to the treatment and placebo groups. Each group contains 5 female and 5 male rats. A limit dose, 2000 mg/kg body weight was selected. Animals were shaved at the dorsal area before 24 hr. About 10% of the body surface area was shaved for the test. Formulations were applied locally for 24 hrs on the first day of the study. Test substances were held in touch with the skin with a porous cotton dressing throughout the testing time. The test site of the animals was observed after 30 min, 2 h, 6 h, 24 h, 48 h, 72 h and 14 days. Animals were visually observed for mortality, morbidity, behaviour (salivation, convulsions, tremors, sleep, diarrheal, lethargy and coma, injury, changes in physical appearance, pain, signs of illness were conducted once daily during the study, as well as any changes in fur, mucous membrane, eyes) respiratory, circulatory, autonomic, nervous system were noted. Body weights were checked during the study [50].

Repeat dose toxicity study: Repeat dose toxicity test was performed according to the OECD guideline 410. Animals were divided into two groups treatment and placebo control consisting of 10 animals (5 females, 5 males). Animals were shaved before testing at the dorsal area. About 10 % of the body surface area was shaved for the application of the test substance. The test was performed at a limit dose of 1000 mg/kg body weight. The test substance was applied daily to the skin to the test and placebo control group for 6 h for a day for 28 days. The formulation was retained at a place by a non-adhesive bandage. The rat's weight were noted and visually observed for mortality, morbidity, general behaviour same as the acute study. For histopathological evaluation animals were necropsied. Skin, liver, kidneyand heart were isolated and stored in 10% formalin and processed for histopathology [51].

4. RESULTS AND DISCUSSION

4.1 Screening of Drug in Lipids and Surfactants

Both drug solubility in selected solid, liquid lipids and surfactants is predicted in Fig. 1 and Fig. 2. As per solubility studies, tretinoin and clindamycin exhibited maximum solubility in Emulcier 61(4.1 mg/g for tretinoin, 19.12 mg/ g for clindamycin phosphate) ,oleic acid (18.63 mg/ml for tretinoin, 10 mg/ml for clindamycin phosphate), Span 20 (10.5 mg/ml for tretinoin, 4.5 mg/ml for clindamycin phosphate) and Cremophor EL (10.6 mg/ml for Tretinoin, 3 mg/ml for Clindamycin phosphate).

Selection criteria for lipids and surfactants for the development of tretinoin-clindamycin NLCs include pharmaceutical acceptability, non-irritation and sensitization potential and GRAS (generally regarded as safe) category. As per solubility studies, NLC was prepared using Emulcier, shea butter, as a combination of solid lipids, Oleic acid as liquid lipid and Span 20, Cremophor EL as a combination of surfactant and co-surfactant. Trial batches were prepared with selected solid, liquid lipid and surfactant co-surfactant combination to evaluate stability and suitability of selected ingredients for batch preparation [52-53].

4.2 Preparation of NLC

The solubility of tretinoin was observed more in Emulcier 61, shea butter, oleic acids and trial batches were observed to be more stable in presence of Span 20 and Cremophor. Table 1 represents the composition of a finalized batch obtained after various trials. The lipids were selected depending upon drug solubility and miscibility with each other. Prepared trial batches were evaluated for room temperature stability and particle size. The selected batch showed maximum stability and less particle size as compared to other prepared batches prepared by high speed homogenisation [54,55].

Table 1. Batch composition

Ingredients	% w/w
Tretinoin	0.05
Clindamycin phosphate	0.1
Oleic acid	4
Emulcier 61	2
Shea butter	2
Span 20	7
Cremophor EL	3
Water q. s.	100

4.3 Characterization of NLCs

Morphological measurements: Particle size was found around 283 nm and zeta potential was -37.9 mv, polydispersity index 0.43 indicating a nano-size formulation with a stable behaviour. Fig. 3 represents the SEM image of NLC formulation. The SEM size was observed less than 100 nm size and of uniform spherical shape. From this data it is depicted prepared batch is of particle size (up to 200 nm) with good homogeneity, stable range zeta potential, thus represents a stable NLC system [56].

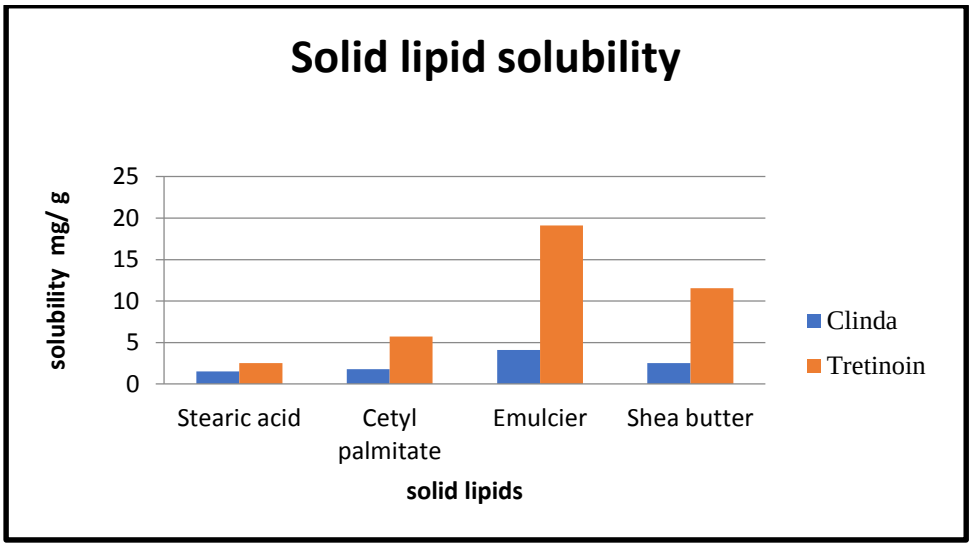

Fig. 1. Solubility of drugs in solid lipids

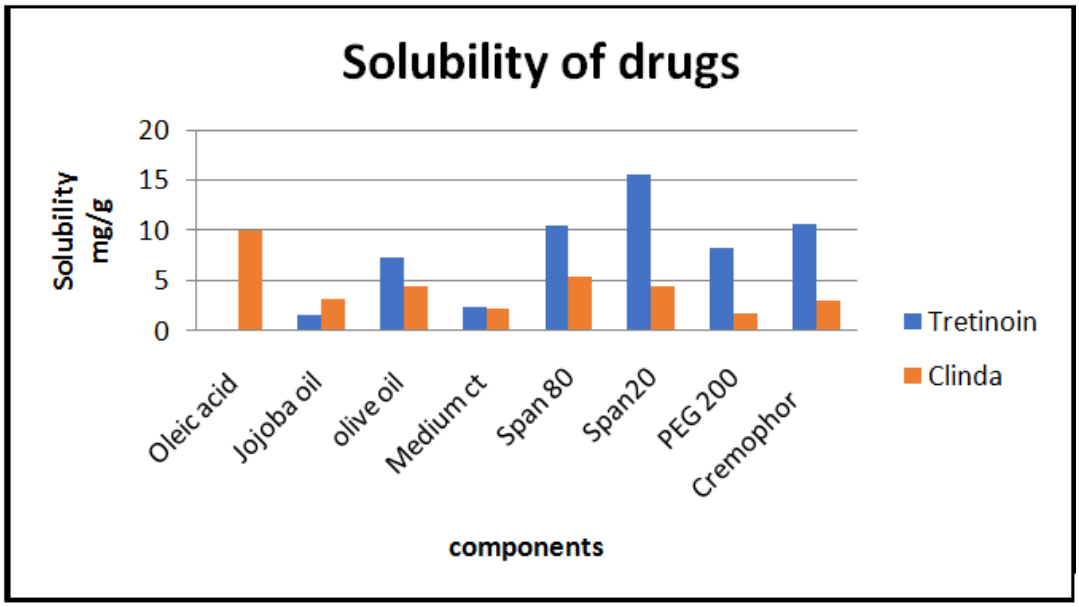

Fig. 2. Solubility of drugs in liquid lipids and surfactants

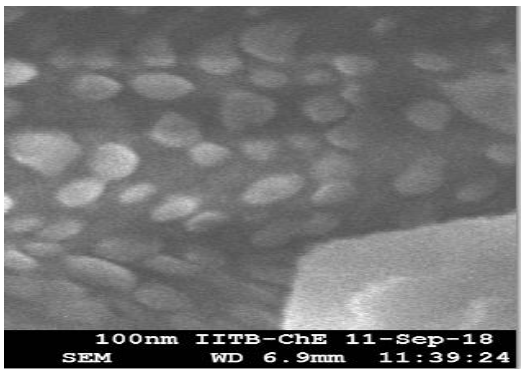

Fig. 3. SEM images of prepared NLCs

Entrapment efficiency: Observed entrapment for Tretinoin was 92.5% and for clindamycin phosphate was 66 %. The entrapment efficiency (EE), is drug encapsulated inside or adsorbed over the surface of nano lipid core, was determined by estimating free Tretinoin and clindamycin in the supernatant. The lipids used for formulation solubilize the added Tretinoin. The encapsulation efficiency was found less for clindamycin as it is a hydrophilic nature. More drug is present in aqueous phase but surfactant co-surfactants help to entrapped it in colloidal lipid micelle by emulsification. Tretinoin is a fat soluble vitamin which is having more

entrapment due to the lipoid nature and presence of surfactant co-surfactant combination [57].

Drug permeation: The present study is focused on the evaluation of topical target potential of the prepared system using Franz diffusion cell in PBS 7.4 at 37 ^{0} C, 50 rpm using cellophane membrane. The amount of both drugs permeated from prepared NLC formulation was determined and compared with marketed formulation for 6 hr is shown in Fig. 4. A higher release is observed for both the drugs of the hydrogel-based marketed formulation. The initial slow release may be due to un-entrapped drug of aqueous phase and some amount of drug adsorb on the lipid carrier surface. Tretinoin release is less as entrapment efficiency in lipid core is more as compared to Clindamycin which also suggests that release prolongation is due to entrapment in lipid core. The drug permeation for NLC is found less than marketed hydroalcoholic gel formulation. Less permeation of the drug through the skin for lipid-based formulation indicated more drug retention into the skin layer and less permeated across the skin. Retention of NLCs by the skin is due occlusive property and less diffusion through the skin which is desirable for site effective treatment. NLC based drug formulation is design for controlling the drug release as well as skin targeting [58,59].

***In vivo* anti-acne rat model**

An inflammatory acne mouse model was used to evaluate an anti-acne activity of the prepared NLC formulation. Ear pinnae were observed daily for sign of acne and inflammation. After two to three days of induction micro-comedones and inflammation at injected left ear pinna were seen and compared to the uninfected right ear. Infection was treated with the marketed formulation and prepared NLC formulation once a day 0.5 g. Inflammation and acne signs disappeared after treatment with prepared NLCs formulation in two days where it required 3 days for the standard treatment group and required five days for the control group. Antiacne *in vivo* rat model images of rat ear pinna of a group A formulation test group, B standard treated group are shown in Fig. 5. Animals were infected by injecting heat-killed *P.acnes* culture into the animal ear pinna. Injected *P.acnes* induced the granulomatous inflammation followed by the formation of micro-comedones which is about mild to moderate type of acne. Smooth healing was observed for the test group as some scars were visible on the standard treatment group. The prepared formulation showed faster healing compared to the standard group. *In vivo* results suggested that prepared NLCs effectively treated bacterial infection and healed ear pinna better than marketed preparation [60].

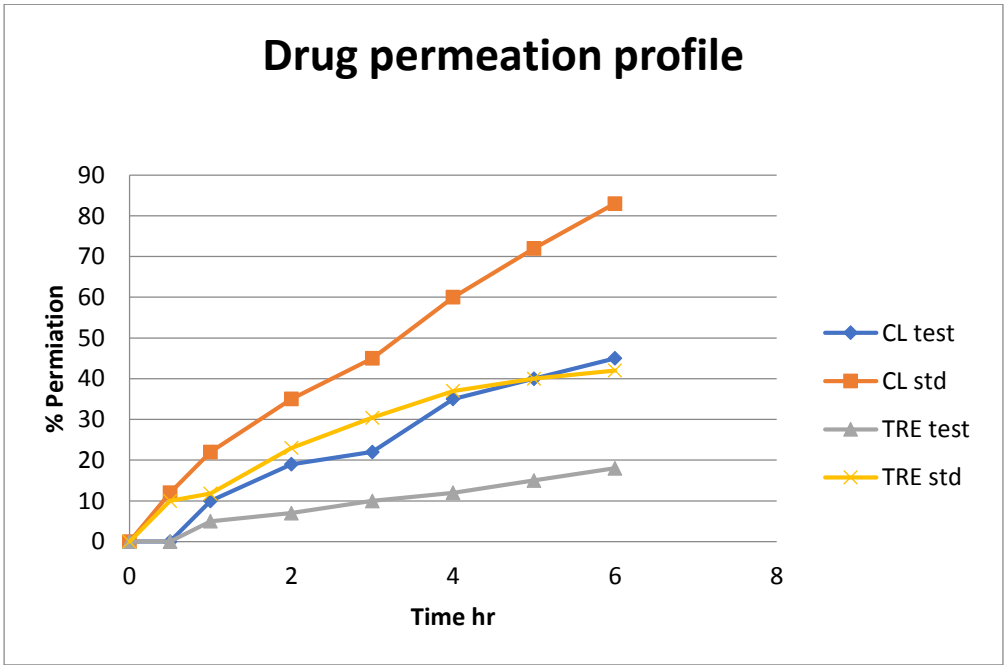

Fig. 4. Permeation profile of prepared NLC and marketed formulation

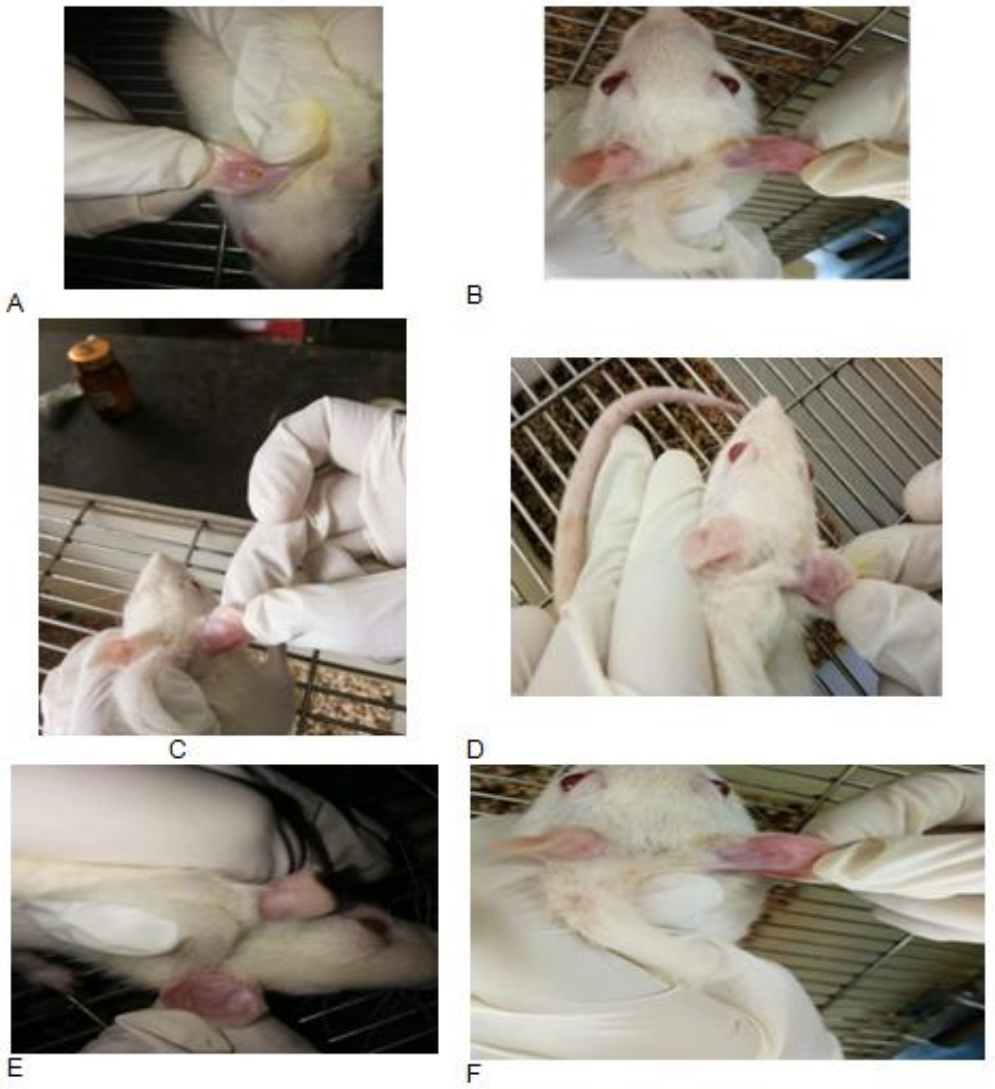

Fig. 5. Images of antiacne activity evaluation of prepared NLCs. Infected formulation test group a) day one c) day three, e) day five. Infected standard treated group b) day one, d) day three, f) day five

Acute toxicity: In acute dermal toxicity dose, 2000 mg/kg dose was found to be toxicologically insignificant. The treatment group did not show any toxicity sign on the surface of the skin at 24 and 48 h, 72 h after patch removal till 14 day, and the reaction was graded as "0" as per the Draize test score for erythema and oedema. No clinical signs of toxicity, mortality and changes in body weights were observed in treated animals. The treatment and placebo group did not show any changes in conduct, skin impacts, breathing, food, water consumption. No considerable weight loss was observed for all the animals [61].

Subchronic toxicity: Observed parameters for general signs are summarised in Table 2. Observed changes in toxicity study are noted in Fig. 6.

Haematological and histopathological evaluation: All the erythro and leuko parameters are shown in Table 3. Microscopic structural changes of the organ liver, kidneys and skin is shown in Fig. 6.

General signs: No harmful signs or mortality were seen in test animals. Animals did not show any critical change of conduct, skin impacts, breathing, food and water consumption, postural irregularities. The physical appearance of skin, fur and eyes was normal. The bodyweight of the rats

was found to be more. This indicated that applying the formulation on the skin had no effect on the growth. A proper intake of nutrients and water was observed.

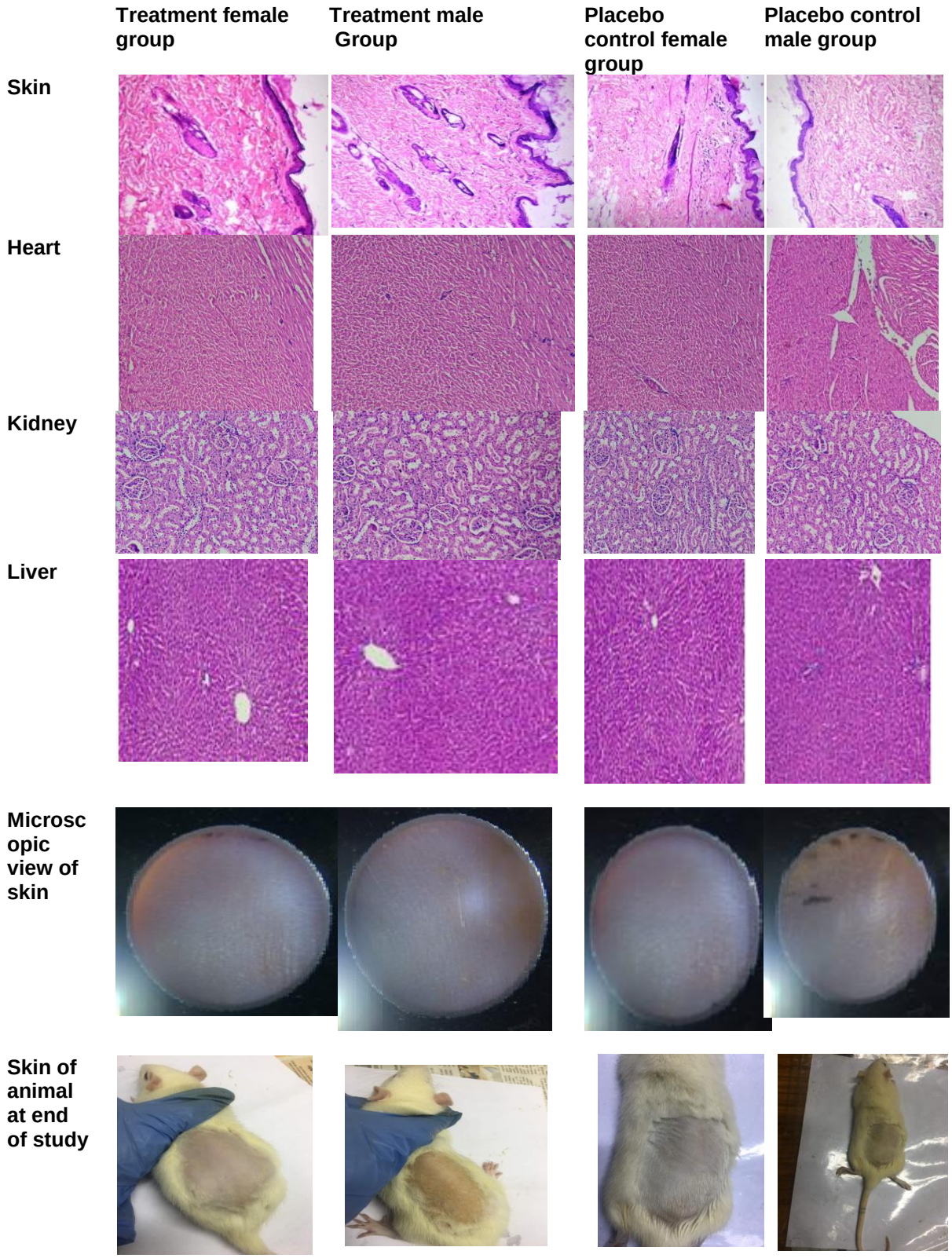

Fig. 6. Organ histopathology for the repeat dose study

Table 2. General signs for acute and repeat dose toxicity

Test parameter	Acute toxicity		Repeat dose toxicity	
	Treatment group	Control group	Treatment group	Control group
Skin, fur	No alteration	No alteration	No alteration	No alteration
Eyes	No alteration	No alteration	No alteration	No alteration
Mucosa membrane	No alteration	No alteration	No alteration	No alteration
Salivation	No alteration	No alteration	No alteration	No alteration
Behavioural pattern	No alteration	No alteration	No alteration	No alteration
Lethargy	No alteration	No alteration	No alteration	No alteration
Sleep	No alteration	No alteration	No alteration	No alteration
Diarrhoea	No	No	No	No
Tremors	No	No	No	No
Body weight	Weight gain	Weight gain	Weight gain	Weight gain

Table 3. Evaluation of various haematological parameters for the repeat dose toxicity

Group	Hb gm %	RBC x 10^6 / cmm	WBC X 10^3 /cmm	PLT X 10^3/ cmm	PCV %	MCV fl	MCH pg	MCHC gm/dl	N %	E %	L %	M %
Test group Female	16.0	9.13	23.0	762	49.6	54.4	17.5	32.2	57	4	38	1
Test group Male	16.1	8.84	12.3	623	49.2	55.7	18.2	32.7	66	2	31	1
Placebo control female	15.4	8.48	12.9	655	40.1	55.0	18.4	33.5	59	2	39	0
Placebo control male	15.0	8.35	13.6	869	43.0	51.5	17.9	34.8	68	0	32	0

Hb: Hemoglobin, RBC: Red Blood Cell, WBC: White Blood Cell, PLT: Platelets, PCV: Packed Cell Volume, MCV: Mean Corpuscular Volume, Retic: Reticulocyte, MCHC: Mean Corpuscular Hemoglobin Concentration, MCH: Mean Corpuscular Hemoglobin, N: Neutrophils, E: Eosinophils, L: Lymphocytes, M, Monocytes

Haematological evaluation: After 28 days of the treatment no significant changes in haematological and serum biochemistry parameters level were observed in control and treatment groups.

Histopathology examination: It shows in considerable variations in control and treatment groups. Hence, it is suggested that the formulation is non-toxic. Histo-pathological evaluation of organs indicated no structural damage to the selected organs.

Liver histology revealed no alteration in portal and central vein, bile duct and hepatic artery in the control and treated rats. There was no fibrosis, necrosis, inflammation, or local fatty degeneration in the liver hepatocytes. The kidney displayed no adverse effects. No morphological alteration was observed in cardiac muscles, arteries and veins. The microscopic examination of the skin of rats did not indicate any changes in the layers of the skin compared to the control group [62].

5. CONCLUSION

Both hydrophilic and lipophilic drug-loaded NLCs were prepared with Span 20, Oleic acid, Emuicier 61, Shea butter and Cremophor EL using homogenization-sonication method. The NLCs have more target potential to the topical part of the skin. NLCs were found to reduce the irritation potential and more efficacious in treatment for *in vivo* anti-acne study results. The results of the toxicity study suggest that formulation is with no death or no signs of toxicity at 2000 mg/kg (acute study) and 1000 mg/kg (repeat dose toxicity study), thus proving safety for use. The organ histology revealed no changes in both the groups and was considered safe since they did not induce dermal toxicity, irritation and sensitization. The results of the presented study suggest NLCs as improved nanocarriers for Tretinoin and clindamycin formulation as they offer many advantages over marketed gel.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) of the institute (Protocol no: KMKMP/IAE/081718).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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