

RESEARCH ARTICLE

Antidermatitic Perspective of Hydrocortisone as Chitosan Nanocarriers: An *Ex Vivo* and *In Vivo* Assessment Using an NC/Nga Mouse Model

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ABSTRACT: The aim of this study to administer hydrocortisone (HC) percutaneously in the form of polymeric nanoparticles (NPs) to alleviate its transcutaneous absorption, and to derive additional wound-healing benefits of chitosan. HC-loaded NPs had varied particle sizes, zeta potentials, and entrapment efficiencies, when drug-to-polymer mass ratios increased from 1:1 to 1:8. *Ex vivo* permeation analysis showed that the nanoparticulate formulation of HC significantly reduced corresponding flux [$\sim 24 \mu\text{g}/(\text{cm}^2 \text{ h})$] and permeation coefficient ($\sim 4.8 \times 10^{-3} \text{ cm/h}$) of HC across the full thickness NC/Nga mouse skin. The nanoparticulate formulation also exhibited a higher epidermal ($1610 \pm 42 \mu\text{g/g}$ of skin) and dermal ($910 \pm 46 \mu\text{g/g}$ of skin) accumulation of HC than those associated with control groups. An *in vivo* assessment using an NC/Nga mouse model further revealed that mice treated with the nanoparticulate system efficiently controlled transepidermal water loss [$15 \pm 2 \text{ g}/(\text{m}^2 \text{ h})$], erythema intensity (232 ± 12), dermatitis index (mild), and thickness of skin ($456 \pm 27 \mu\text{m}$). Taken together, histopathological examination predicted that the nanoparticulate system showed a proficient anti-inflammatory and antifibrotic activity against atopic dermatitic (AD) lesions. Our results strongly suggest that HC-loaded NPs have promising potential for topical/transdermal delivery of glucocorticoids in the treatment of AD. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: Corticosteroids; allergic contact dermatitis; percutaneous; biodegradable polymers; nanoparticles; *in vitro* model; *ex vivo* permeation; *in vivo* efficacy

INTRODUCTION

Glucocorticoids (GCs) are highly effective drugs that are widely used in dermatology for the management of various inflammatory complications. However, several local and systemic adverse effects often accompany their long-term use,^{1,2} limiting the clinical significance, and excluding their applicability in chronic maintenance therapies. In recent times, hydrocortisone (HC), a mild-potency agent of the GC series, has been administered percutaneously to minimize the development of unwanted systemic effects. Compared with other high-potency GC compounds, HC possesses mild vasoconstrictive activity³; therefore, the incidence of adverse effects is significantly lower than that associated with the related anti-inflammatory

compounds.⁴ To further limit the adverse effects associated with GCs, special approaches have been attempted to mitigate the transcutaneous absorption of GCs and to increase therapeutic feasibility and patient compliance.

The development of successful topical/transdermal drug delivery systems has been limited⁵ because of the significant penetration barrier provided by the stratum corneum (SC), the topmost skin layer. The SC is composed of a multilayered “brick- and mortar-” like structure, in which the bricks are composed of keratin-rich corneocytes, and the mortar is an intercellular matrix with a unique composition of lipids.⁶ To overcome these penetration problems, various active and passive penetration-enhancing approaches, including chemical enhancers,⁷ electroporation,⁸ iontophoresis,⁹ sonophoresis,¹⁰ microneedles,¹¹ and laser ablation,¹² have been tested. However, each of these techniques has limitations in terms of toxicity to SC and therapeutic practicability.

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Nanotechnology is one of the more advanced and noninvasive techniques adopted for improving skin permeation of various drugs.¹³ Among the various nanocarriers, micelles, liposomes, and polymeric nanoparticles (NPs) have been proposed to facilitate percutaneous delivery of therapeutic agents while mitigating damage to the skin barrier function of SC.^{14,15} These colloidal nanocarrier systems could target GCs to the epidermis, where inflammatory reactions take place.¹⁶ Among these colloidal nanocarriers, NPs have been reported to be more appropriate for topical/transdermal delivery because of their exceptional biopharmaceutical properties, such as high entrapment efficiency (EE), controlled release rates, and low enzymatic degradation.¹⁷ Among the various biodegradable and biocompatible polymers used for preparing NPs, chitosan (CS) has generated enthusiasm because of its mucoadhesive and transepidermal penetrative properties, which are marked by regulation of intercellular tight junctions.¹⁸

Subsequent to optimization of colloidal characteristics (in terms of particle size, zeta potential, entrapment, and loading efficiencies) of unloaded and HC-loaded NPs, the study was aimed at mitigating the systemic absorption of HC and its accumulation in the epidermal and dermal layers. *Ex vivo* drug permeation across dermatomed NC/Nga mouse skin was investigated using Franz diffusion cells. To evaluate the *in vivo* clinical proficiency of the nanoparticulate system, transepidermal water loss (TEWL), erythema intensity, dermatitis index, and skin thickness were also assessed using the NC/Nga mouse model of atopic dermatitis (AD). To harmonize our results, histopathological examinations of NC/Nga mouse skin specimens were also performed by using hematoxylin–eosin and Masson's trichrome staining techniques.

MATERIALS AND METHODS

Materials

Eight-week-old NC/Nga mice were purchased from Experimental Animal Devision, RIKEN BioResource Center, Tsukuba Ibaraki, Japan. Isoflurane (inhalation anesthetic) was obtained from Pacific Pharmaceuticals (Lahore, Pakistan). CS (MW, 70 kDa; deacetylation degree, 85%), HC (base form), phosphate-buffered saline (PBS), hematoxylin–eosin, and Masson's trichrome stains were purchased from Sigma–Aldrich Chemicals Company Ltd. (Kuala Lumpur, Malaysia). Pentasodium tripolyphosphate (TPP) was sourced from Merck KGaA Company Ltd. (Darmstadt, Germany). High-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, and ethanol were obtained from Fisher Scientific Korea Ltd. (Seoul, Korea). QV cream (Ego

Pharmaceuticals Pty. Ltd., Kuala Lumpur, Malaysia) was used as a vehicle base to formulate the nanoparticulate formulation.

Preparation of CS/TPP NPs

CS/TPP NPs were prepared via ionic gelation of CS with TPP.¹⁹ CS solution (0.2%, w/v) was prepared in 1% (v/v) acetic acid at room temperature, and CS/TPP NPs were tested at various CS/TPP mass ratios (1:2 to 1:8) and different pH values of TPP solution (2, 3, 4, 5, 6, 7, and 8). CS/TPP NPs were spontaneously developed by adding 10 mL of TPP solution dropwise into 25 mL of CS solution with constant stirring at 700 rpm. Thereafter, CS/TPP NPs were centrifuged ($72,000 \times g$) using Optima L-100 XP Ultracentrifuge (Beckman–Coulter, Brea, California, USA) with NV 70.1 Ti rotor (Beckman–Coulter) at 10°C for 30 min.

Preparation of HC-Loaded NPs

HC-loaded NPs were prepared by the incorporation method. CS solution (0.2%, w/v) was mixed and incubated with HC solution (1 mg/mL in a 30:70 ratio of ethanol and water solvent mixture) for 30 min at various drug-to-polymer mass ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, and 1:8) before addition of TPP solution (0.1%, w/v). Thereafter, 10 mL of TPP solution was added dropwise to each reaction mixture with constant stirring (700 rpm). Finally, the HC-loaded NPs were harvested by centrifugation ($72,000 \times g$) for 30 min and lyophilized at -40°C for 24 h.

Particle Size and Zeta Potential

To determine the mean particle size and zeta potential, the resulting pellets of unloaded and HC-loaded NPs obtained after centrifugation ($72,000 \times g$) were resuspended in distilled water. Then, the mean particle size and zeta potential of NPs were measured using a ZS-90 Zetasizer[®] (Malvern Instruments, Worcestershire, UK) based on the photon correlation spectroscopy technique. All of the measurements were performed in triplicate at 25°C with a detection angle of 90°. Results were reported as mean $\pm$ standard deviation.

Entrapment Efficiency and Loading Capacity

To measure drug EE and loading capacity (LC) of HC, the standard corresponding calibration curve was made by analyzing various standard HC solutions (1–1000 $\mu\text{g/mL}$) by using reversed-phase HPLC (RP-HPLC) (Waters 600 controller, in-line degasser AF, 2707 Autosampler, and 2998 Photodiode Array Detector) with Waters Symmetry C₁₈ column (250 $\times$ 4.5 mm; 5 μm) at 248 nm (λ_{max}). EE and LC of HC were calculated using Eqs. 1 and 2,

respectively²⁰:

$$\text{Entrapment efficiency (\%)} = W_t - W_f / W_t \times 100 \quad (1)$$

$$\text{Loading capacity (\%)} = W_t - W_f / W_n \times 100 \quad (2)$$

where W_t is the total initial amount of HC and W_f is the amount of free HC in the supernatants. W_n is the weight of lyophilized HC-loaded NPs. All of the measurements were performed in triplicate, and results were reported as mean $\pm$ standard deviation ($n = 3$).

QV Cream-Based Topical Formulation

For compounding the formulation with semisolid consistency appropriate for topical/transdermal delivery, QV cream was used as a vehicle base. For adequate uniformity of contents and homogenous dispersion, QV cream was liquefied in a water bath at 50°C with subsequent vigorous blending of HC-loaded NPs to formulate the nanoparticulate system (HC-NPs) and was then allowed to cool prior to further analysis.

Ex Vivo Drug Permeation Studies

Ex vivo permeation studies were carried out using a jacketed Franz diffusion cell comprising six cells with a diffusional surface area of 0.654 cm² and receptor cell volume of 5 mL; the cell was operated in continuous stirring mode (600 rpm) at 37°C (Perme-gear, Hellertown Pennsylvania, USA). The receptor compartments were filled with PBS (pH 7.4) and acetonitrile in the ratio 70:30. In current experiment, the solvent mixture consists of PBS and acetonitrile was used to improve solubility of HC (because of its hydrophobic nature) and its permeation across the dermatomed NC/Nga mouse skin. The dermatomed full-thickness mouse skin was immediately mounted on diffusional cells with the SC facing the donor compartments and equilibrated using a receiver compartment solution for 1 h to facilitate skin hydration. For all of the permeation experiments, the cumulative permeating flux of HC at different time intervals was estimated using RP-HPLC. The HC permeation flux $\mu\text{g}/(\text{cm}^2 \text{ h})$ was calculated from the slope of the plot of the cumulative amount permeated from the dermatomed NC/Nga mouse skin versus time by using a linear regression analysis. The permeability coefficient (k_p) of HC across the full-thickness mouse skin was estimated using Eq. 3 (Ref. 21):

$$\text{Permeability coefficient} = K_p = J/C \quad (3)$$

where J is the flux and C is the concentration of HC in the donor compartment.

Analysis of the Amount of HC Retained in Various Skin Layers

To analyze the amount of drug retained in the epidermis and dermis, the cleaned dermatomed mouse skin mounted on Franz diffusion cells was subjected to a heat separation technique²² to separate the epidermis from the dermis. Subsequently, the epidermis and/or dermis was weighed and extracted with 10 mL of methanol and acetonitrile (3:1) by mechanical shaking in a water bath maintained at 37°C overnight. Thereafter, all of the samples were harvested by centrifugation ($13,200 \times g$) at 10°C for 20 min. HC in the examined skin layers was quantified using RP-HPLC, and expressed in terms of micrograms of HC per gram of skin tissue.

In Vivo NC/Nga Mouse Model for Allergic Contact Dermatitis

Experimental Animals

Eight-week-old NC/Nga mice were bred with the aim of obtaining a single line of animals from the same parents to reduce genomic diversity. They were housed in an individual ventilated cage assembly with inlet air filters in an air-conditioned animal room with a 12-h light/dark cycle at a temperature of $22^\circ\text{C} \pm 1^\circ\text{C}$ and humidity of $60\% \pm 5\%$. The mice were provided with a laboratory diet and water *ad libitum*. The experimental protocol for animal handling was conducted in accordance with the National Institute of Health (NIH) guidelines and approved by the Animal Ethics Committee of Universiti Kebangsaan Malaysia (UKMAEC) (FF/2011/HALIZA/30-NOVEMBER/408-NOVEMBER-2011-DECEMBER-2012).

Induction of AD-Like Skin Lesions

Induction of AD-like skin lesions was accomplished by applying 100 μL of a 0.15% solution of 2,4-dinitrofluorobenzene (DNFB) in acetone/olive oil (3:1) onto the shaved dorsal skin of anesthetized mice, once per day on days 1 and 5. Then, on the ninth, eleventh, and thirteenth days, the mice were sensitized again by applying 100 μL of 0.2% DNFB on the dorsal skin as described previously.²³ The animals were randomly divided into six groups ($n = 6$). The baseline group comprised normal mice and second group comprised negative control (NG-CON) that received repeated DNFB applications without any treatment. The vehicle group (VGR) was the AD-induced (through DNFB application) group and was treated with vehicle cream (QV cream). The fourth and fifth groups were the positive control groups (POS-CON-0.5 and POS-CON-1) consisting of AD-induced mice treated with 0.5% HC and 1% HC commercial creams, respectively. The sixth group (HC-NP) comprised

AD-induced mice treated with HC-NPs incorporated in QV cream (nanoparticulate system).

In Vivo Clinical Efficacy

Transepidermal Water Loss and Erythema Intensity

Transepidermal water loss [TEWL; g/(m² h)] and intensities of erythema were determined using a Tewameter[®] TM 300 and a Maxameter[®] 18, respectively (Courage + Khazaka Electronic GmbH, Cologne, Germany). Prior to each testing session, all of the mice were isolated in an air-conditioned room (20 ± 3°C) with relative humidity of 50%–60%.

Dermatitis Severity and Thickness of Dorsal Body Skin

Efficacy of HC-NPs was further assessed by determining the dermatitic severity and skin thickness of all of the NC/Nga mouse groups. Dermatitic severity was assessed by two persons blinded to the treatment group according to the criteria described by Park et al.²⁴ The severity of AD was evaluated as dermatitis index, established on the basis of the following criteria: (1) erythema/hemorrhage, (2) dryness/scaling, (3) edema/swelling, and (4) erosion/excoriation, each of which was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores was then taken as the dermatitis index.²⁵ At the end of the experiment, all of the mice were euthanized with a lethal dose of inhalation anesthetics (isoflurane). The severity of AD was further evaluated by determining the skin thickness of all of the mouse groups by using a dial thickness gage.

Microscopic Analysis by Advanced Digital Light Microscope (Dinolite[®])

Dinolite[®] is an advance digital light microscope (Courage and Khazaka, Germany) used to examine the microscopic signs and symptoms of AD-like skin lesions. To perform this analysis, NC/Nga mice were anesthetized at the end of experimental period (sixth week of treatment), and the Dinolite[®] probe was placed on the dorsal skin surface by adjusting focus and magnification (10–50×) using digital zooming capability. The Dinolite[®] micrographs clearly highlighted the microscopic signs and symptoms of AD-like skin lesions.

Histopathological Examination

To examine the histopathological changes, NC/Nga mice were euthanized at the end of the treatment period (6 weeks), and the dorsal skin specimens (5 mm) were punched by skin biopsy punch and fixed in 10% buffered formalin to embed in paraffin wax. The embedded skin specimens were then serially sectioned (5 µm) with microtome and stained with either hematoxylin–eosin to observe histopathological features of skin or by Masson's trichrome stain to examine

the variable deposition of collagen fibers (acquired green color) and skin fibrosis at lesional skin under light microscope using an image analysis software (VideoTest-Master Morphology: Video Test; St Petersburg, Russia).

Statistical Analysis

All data are presented as mean ± standard deviation. Data were analyzed with either paired *t*-tests or independent *t*-test and ANOVA, followed by a Tukey's post-hoc analysis. For the analysis of particle size, zeta potential, EE, and LC of NPs, a *p* value of less than 0.05 was considered to indicate a significant difference. For the TEWL, erythema intensities, and dermatitis index, a *p* value of less than 0.01** was considered to indicate a significant difference. For the data obtained from *ex vivo* permeation, drug skin retention, and skin thickness, a *p* value of less than 0.001*** was considered to indicate a significant difference.

RESULTS AND DISCUSSION

Optimization of Unloaded CS NPs

Figures 1a and 1b indicate that the zeta potential and particle size of unloaded NPs varied from +20 ± 3 to +53 ± 3 mV, and from 102 ± 17 to 676 ± 22 nm, respectively, by increasing the CS/TPP mass ratios from 1:2 to 1:8. The increasing trend in zeta potential and particle size of NPs might be caused by variable cross-linking extent between the individual opposite charges donated by CS (–NH₃⁺) and TPP (–P₃O₁₀^{–5}) at various CS/TPP mass ratios. Similarly, the number of individual charges (–NH₃⁺ and –P₃O₁₀^{–5}) and the corresponding charge density of both CS and TPP ions also imparted a great impact on the ionic interaction and thus on the zeta potential of NPs. For example, at a lower CS/TPP mass ratio (1:2), the TPP solution might donate a larger number of negative ions (–P₃O₁₀^{–5}), which could sufficiently neutralize the positive charges (–NH₃⁺) on the contour of CS, and thus result in a lower zeta potential of NPs and vice versa, as shown in Figure 1a. On the contrary, the increment in the particle size of unloaded NPs might be caused by the possible electrostatic interaction (promoting aggregate formation) between the NPs because of a decrease in zeta potential of NPs that occurred as the CS/TPP mass ratios increased (Fig. 1b).

Despite that, Figures 1c and 1d show that the zeta potential of NPs decreased from +55 ± 4 to +14 ± 2 mV and particle size from 323 ± 22 to 124 ± 13 nm, respectively, as the TPP solution pH increased from 2.0 to 8.0. The decrease in zeta potential and particle size of NPs could be explained by the variable extent of dissociation/ionization of TPP into the

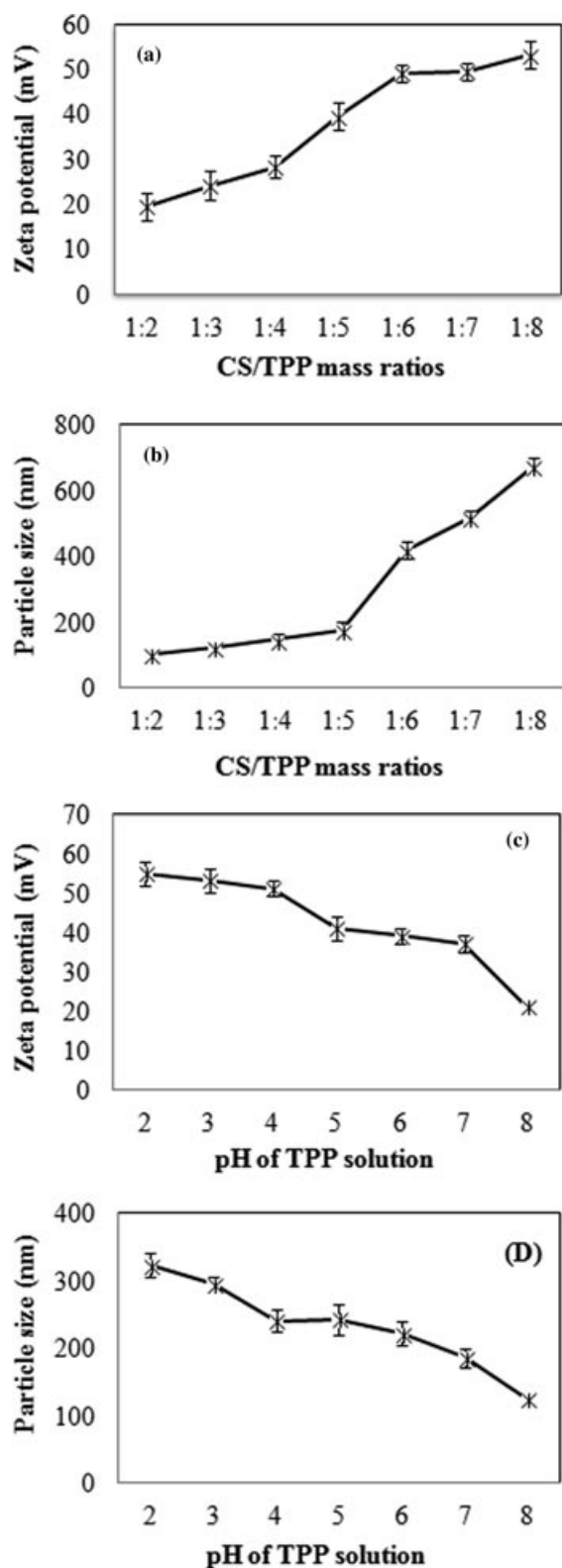


Figure 1. Effects of CS/TPP mass ratios on zeta potential (a), particle size (b), and effect of TPP solution pH on zeta potential (c), and particle size (d) of unloaded NPs (Mean $\pm$ S.D., $n = 3$).

reaction mixture, and by different extents of deprotonation of $-\text{NH}_3^+$ groups on CS at various pH values of the TPP solution. For example, at a high pH value (8.0), a greater number of TPP ions ($-\text{P}_3\text{O}_{10}^{5-}$) were produced in solution because of the higher ionization extent of TPP, which might sufficiently be gelled with the $-\text{NH}_3^+$ groups of CS (to form NPs), thus reducing the surface charge of NPs and vice versa. Similarly, the $-\text{OH}$ ions produced at a high pH value might also cause the deprotonation of $-\text{NH}_3^+$ groups of CS to the neutral amine ($-\text{NH}_2$) group, further reducing the zeta potential of NPs. On the contrary, in a TPP solution with a low pH value (2.0), TPP ions were not completely dissociated in the solution and may exist as $-\text{P}_3\text{O}_{10}^-$, $-\text{P}_3\text{O}_{10}^{2-}$, $-\text{P}_3\text{O}_{10}^{3-}$, and $-\text{P}_3\text{O}_{10}^{4-}$. These incompletely dissociated TPP ions were unable to completely neutralize the $-\text{NH}_3^+$ groups within CS and thus resulted in a high positive charge (zeta potential) on the surface of NPs (Fig. 1c). Similarly, the incremental increase in the particle size of unloaded NPs could be explained by formation of aggregates of the suspended NPs when their surface charge decreased with the increase in the pH value of the TPP solution (Fig. 1d).

Optimization of HC-Loaded NPs

The zeta potential of the HC-loaded NPs varied from $+14 \pm 2$ to $+45 \pm 4$ mV, and the mean particle size ranged from 382 ± 14 to 187 ± 12 nm, respectively, as the drug-to-polymer mass ratios linearly increased from 1:1 to 1:8, as shown in Figures 2a and 2b. The incremental increase in the surface charge of HC-loaded NPs (from 1:3 to 1:5) was predicted to result from the increase in the number of free residual amine groups ($-\text{NH}_3^+$) on the contour of CS (Fig. 2a). This could be explained by the linear increase in the amount of polymer (CS) when the drug-to-polymer mass ratio increased from 1:1 to 1:8, which may have caused a linear increase in residual $-\text{NH}_3^+$ groups on CS while in solution. Alternatively, the trend toward a decrease in the particle size of HC-loaded NPs could be explained by a greater surface charge density of HC-loaded NPs obtained at higher drug-to-polymer mass ratios (1:8), preventing the particles from interacting with each other to form aggregates,²⁶ and thus maintaining the size of NPs.

Further, the experimental data also suggested that the EE of HC increased significantly ($p < 0.05$, paired t -test) from $42\% \pm 4\%$ to $86\% \pm 3\%$ as the drug-to-polymer mass ratio increased from 1:1 to 1:8 (Fig. 2c). An increase in the EE of HC may indicate that the reaction mixture becomes more viscous at higher drug concentration (1:1 ratio), which would tend to increase the forces opposing the extensive incorporation of HC inside the polymer matrices and hence reducing the EE of HC.¹⁹ However, the viscosity of the reaction medium decreased as the drug-to-polymer mass

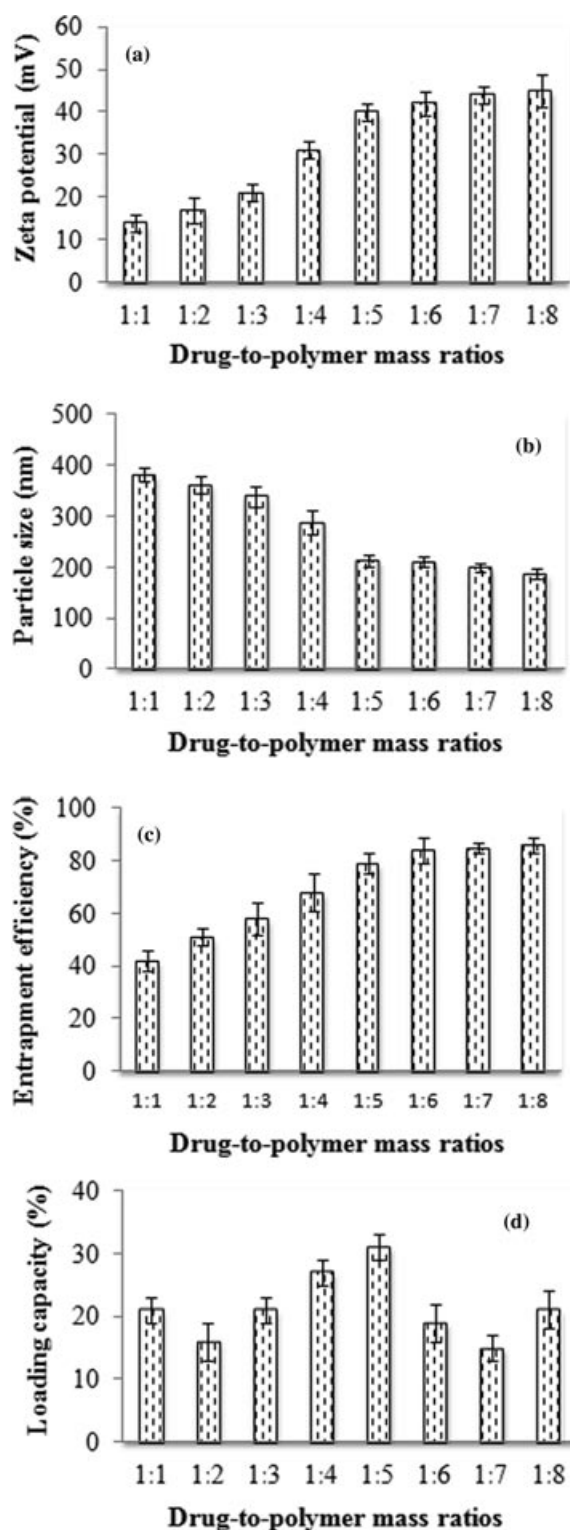


Figure 2. Effects of drugs-to-polymer mass ratios on zeta potential (a), particle size (b), entrapment efficiency (c), and loading capacity (d) of HC-loaded NPs (Mean $\pm$ S.D, $n = 3$).

ratio increased from 1:1 to 1:8, which resulted in an increase in the tendency of HC molecules to be incorporated into polymer matrices. Moreover, the surface charges of HC-loaded NPs, which play a very impor-

tant role in improving the EE and LC of the drug, were also found to increase as the drug-to-polymer mass ratio increased. In spite of this, the LC of HC fluctuated and reached the highest percentage of 31 ± 4 at a 1:5 drug-to-polymer mass ratio (Fig. 2d).

Ex Vivo Drug Permeation Studies

To assess the ability of HC-NPs to mitigate the transcutaneous permeation of HC across the full-thickness NC/Nga mouse skin, the permeation data from positive controls (to which commercially available 0.5% and 1% HC creams were applied) were also compared with that of the HC-NP group. Previously, we reported that QV cream was observed to be superior in terms of release characteristics of HC from the nanoparticulate system using an *in vitro* model.²⁷ Hence, in the current research, we had selected QV cream-based nanoparticulate system to assess its ability to mitigate bioavailability of HC and its adverse effects. To maintain sink condition, it was assessed that total initial concentration of HC in the donor compartment was $4941 \pm 37 \mu\text{g/g}$ for POS-CON-0.5 formulation; however, for POS-CON-1, the total concentration of HC was $9880 \pm 69 \text{ mg/g}$. On the contrary, for the nanoparticulate formulation, total initial concentration of HC estimated in the donor compartment was $4912 \pm 51 \mu\text{g/g}$. At the end of permeation experiment, it was revealed that $2179 \pm 42 \mu\text{g}$ and $6139 \pm 38 \mu\text{g}$ of HC were present in the donor compartment for POS-CON-0.5 and POS-CON-1 formulation, respectively. In contrast to that, the nanoparticulate formulation, a concentration of $1806 \pm 27 \mu\text{g}$ of HC/1 g of cream was determined in the donor compartment at 24 h or $\sim 65\%$ of HC had permeated out from the donor compartment, which is the highest for HC permeation. Moreover, the permeation coefficient (K_p ; cm/h) for all of the formulations in the donor compartment was 18.3×10^{-3} , 25.8×10^{-3} , and 15.3×10^{-3} cm/h for POS-CON-0.5, POS-CON-1, and HC-NPs formulation, respectively, at 24 h.

The concentration, corresponding flux, and K_p of HC were also investigated in the receiver compartment. Data revealed that only $\sim 10\%$ of the amount of HC initially applied using the nanoparticulate system was recovered in the receiver compartment at the end of diffusional experiment (Fig. 3). In contrast, greater percentages of HC ($\sim 24\%$ and $\sim 32\%$) had permeated across the full-thickness dermatomed mouse skin from POS-CON-1 and POS-CON-0.5 groups, respectively ($***p < 0.001$). After 24 h, the total estimated amount of HC that had permeated across the mouse skin on using HC-NPs was $581 \mu\text{g/cm}^2$ (Fig. 3). The corresponding permeation flux (J/h) and the K_p of HC were $\sim 24 \mu\text{g}/(\text{cm}^2 \text{ h})$ and 4.8×10^{-3} cm/h, respectively, on using HC-NPs. However, the total amounts of HC transported across the mouse skin in POS-CON-1 and POS-CON-0.5 groups were 2390

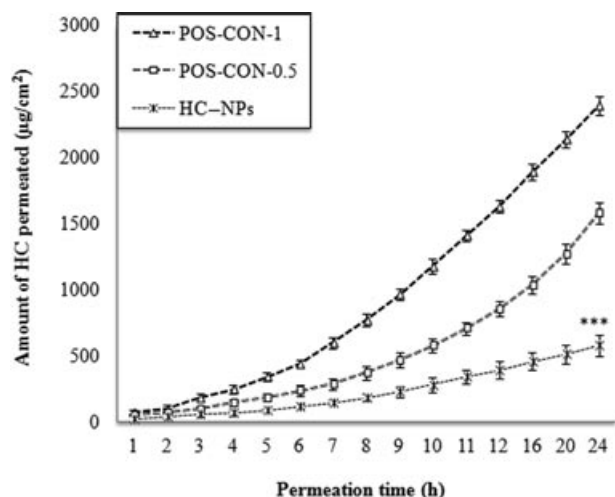


Figure 3. *Ex vivo* Franz diffusional permeation of HC across dermatomed NC/Nga mouse skin from HC-NPs in comparison with POS-CON-0.5 and POS-CON-1 formulations. Data were presented as mean $\pm$ S.D, $n = 3$; significance (***) $p < 0.001$ of HC-NPs against positive control groups.

and $1579 \mu\text{g}/\text{cm}^2$, respectively. The K_p of HC for the POS-CON-1 and POS-CON-0.5 groups was 10×10^{-3} and $13 \times 10^{-3} \text{ cm}/\text{h}$, respectively, which was ~ 2.0 and ~ 2.7 times higher than that of the HC-NP group, respectively. Similarly, the corresponding flux of HC in the POS-CON-1 and POS-CON-0.5 groups was 4.1 and 2.7 times higher than the corresponding flux in the HC-NP group. Conclusively, the results indicate that the HC-NPs may afford a well-controlled reduction in transcutaneous penetration of HC, which is predicted to alleviate the systemic adverse effects.

Estimation of HC Retained in the Epidermis and Dermis

For successful management of topical/transdermal inflammatory diseases (such as AD), it is necessary to retain significant GC contents in skin layers. In current research, the amount of HC retained in the epidermis and dermis was estimated using RP-HPLC with results expressed as micrograms of HC retained per gram of skin tissue. We found that the total amount of HC retained in the epidermis ($1610 \mu\text{g}/\text{g}$ of skin) with HC-NPs was significantly greater (***) $p < 0.001$) than that retained in POS-CON-0.5 and POS-CON-1, which was 690 and $820 \mu\text{g}/\text{g}$ of skin tissue, respectively (Fig. 4). Data also revealed that the total amount of HC retained in the dermis in the HC-NP group was estimated to be ~ 2 and ~ 1.8 times greater than that in the POS-CON-0.5 and POS-CON-1 groups, respectively (Fig. 4). These results were also in accordance with previously published data, which explored whether HC-NPs exhibited greater efficiency for topical/transdermal delivery of drug contents with minimal systemic absorption.²⁸

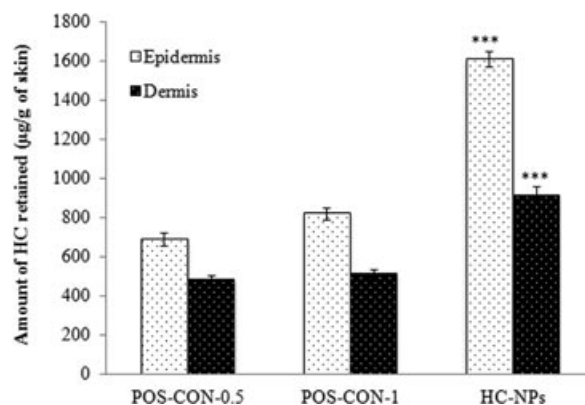


Figure 4. Amount of HC accumulated in the epidermis (lighter bars) and dermis (darker bars) at the end of permeation experiment. Data were presented as mean $\pm$ S.D, $n = 3$; significance (***) $p < 0.001$ of HC-NPs against positive control groups.

In Vivo Clinical Efficacy

TEWL and Erythema Intensity

Interpretation of the results from the TEWL studies indicated that, by the end of the sixth week of treatment, both the NG-CON group and VGR had shown a progressive increase in TEWL (Fig. 5a). Similarly, mice in the positive control groups POS-CON-0.5 and POS-CON-1 also showed an increase in TEWL during the course of treatment but with less severity than the NG-CON group and VGR. Interestingly, despite having received a higher dose of HC, the POS-CON-1 group showed a greater degree of TEWL [$41 \text{ g}/(\text{m}^2 \text{ h})$] after the fourth week of treatment than that shown by the POS-CON-0.5 group [$35 \text{ g}/(\text{m}^2 \text{ h})$]. This may have been a response due to predisposition to skin atrophy or skin thinning in mice, reflecting a dose-dependent adverse effect of GCs. In contrast, the HC-NP group showed better control [$15 \text{ g}/(\text{m}^2 \text{ h})$] in maintaining TEWL during the course of AD than the other tested groups.

Further, all of the tested groups also experienced a progressive increase in erythema intensity through the end of the sixth week of treatment, except for the HC-NP group (Fig. 5b). This group showed a slight reduction in erythema intensity after the fourth week of treatment. This might have been because of a synergistic or additive effect of HC and CS NPs, promoting SC regeneration as well as diminishing the underlying inflammatory cascades. This ultimately resulted in reduced TEWL and erythema intensity. To understand the mechanism underlying AD, it was addressed that DNFB application would promote involuntary scratching in mice, leading to a physical disruption of SC with exacerbation of TEWL²⁹ and a reduction of the water binding efficiency of SC.³⁰ Interestingly, the findings of current research

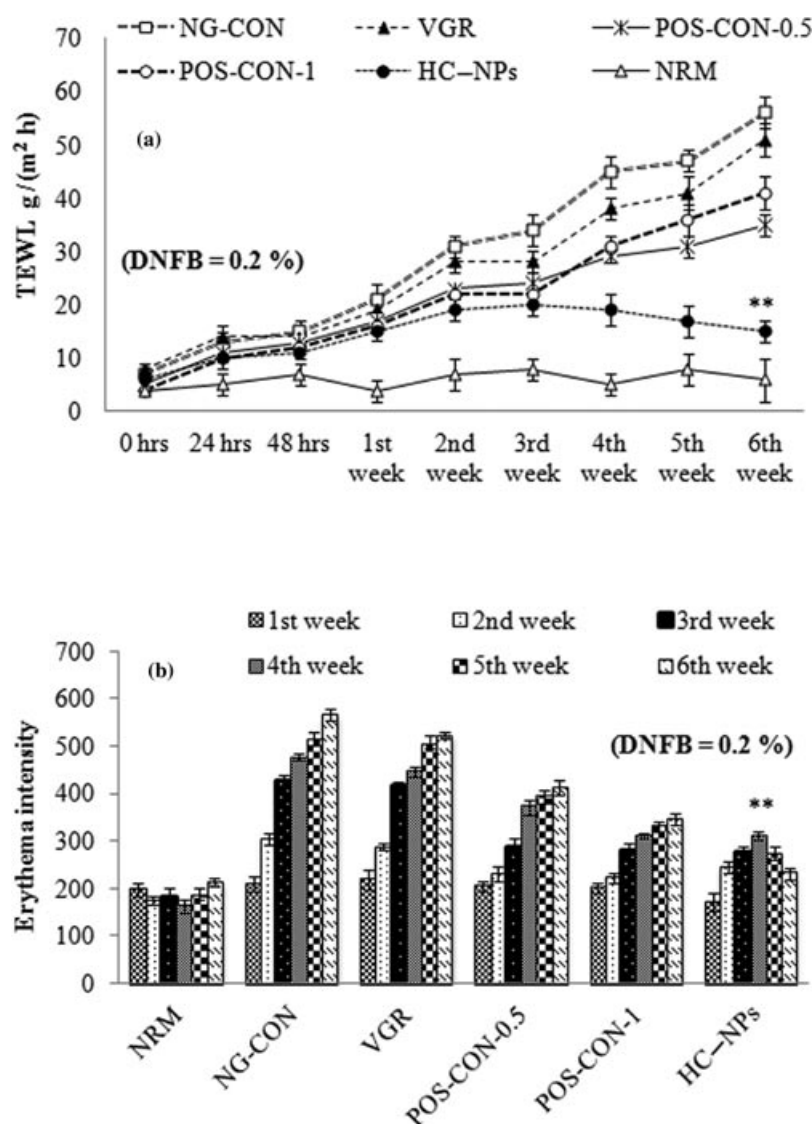


Figure 5. Clinical effectiveness of HC-NPs in comparison with all of the other tested groups in terms of TEWL (a), and erythema intensity (b). Data were presented as mean $\pm$ S.D, $n = 3$; significance (** $p < 0.01$) of HC-NPs against NG-CON and VGR.

suggested that the HC-NP system held promise in reducing TEWL and the erythema intensity (Figs. 5a and 5b; ** $p < 0.01$).

Dermatitis Severity and Dorsal Skin Thickness

The inhibitory efficacy of the nanoparticulate formulation was further assessed by evaluating the dermatitis index and dorsal skin thickness by using the NC/Nga mouse model. The NG-CON group showed the highest grades with regard to pathological features, including severe erythema, hemorrhage, edema, deep excoriation, and intense dryness (Fig. 6). Nevertheless, the VGR also experienced clinical symptoms similar to those shown by the NG-CON group. However, the dermatitic index of this group was lesser than that of the NG-CON group during

the course of treatment, which might have been because of the lower individual scores of dryness and erythema than those of the NG-CON group. In spite of this, the positive control groups, especially POS-CON-1, showed suppressed AD symptoms than those shown by the NG-CON group and VGR, particularly at the fifth week of treatment. On the contrary, treatment with HC-NPs maintained the skin integrity more efficiently throughout the course of treatment (** $p < 0.01$) as compared with other tested groups, with the milder symptoms of dryness and erythema.

Moreover, the obtained results further revealed that the skin thickness of the NG-CON group following euthanasia was the highest (Fig. 6b; *** $p < 0.001$) compared with all of the other tested groups. This might have resulted from underlying inflammatory

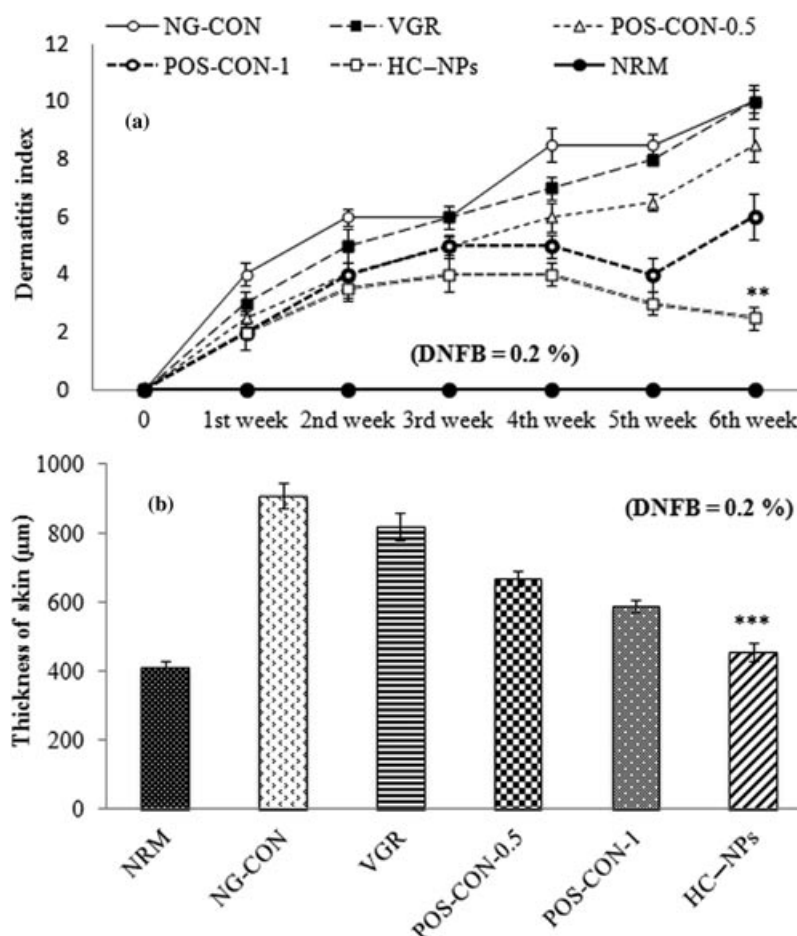


Figure 6. Clinical effectiveness of HC-NPs in comparison with all of the other NC/Nga mice groups in terms of dermatitis index (A), and thickness of skin (B). Data were presented as mean $\pm$ S.D, $n = 3$; significance (** $p < 0.01$) of HC-NPs against NG-CON and VGR for dermatitis index and significance (** $p < 0.001$) of HC-NPs against NC-CON and VGR for skin thickness.

cascades associated with AD pathogenesis that are induced as inflammatory mediators accumulates, initiating neo-vascularization, keratinization, and epithelization, and resulting in increased skin thickness. The signs and symptoms of increasing skin thickness were observed to be decrease down from the NG-CON group ($910 \pm 37 \mu\text{m}$) to the HC-NP group ($456 \pm 27 \mu\text{m}$) (Fig. 6b). Interestingly, despite low HC concentration (0.5% HC, w/w incorporated into NPs), the HC-NP group showed a better control over skin thickness than that shown by the POS-CON-1 group. This may have resulted from an additive or synergistic effect of HC and CS NPs, inhibiting the inflammatory cascades underlying AD pathogenesis.

Advanced Digital Light Microscopic Analysis of Mouse Skin

Moreover, to harmonize our findings, the severity of AD was further assessed using an advanced digital light microscope (Fig. 7). The obtained results

demonstrated that the nanoparticulate system efficiently mitigated the progression of AD. The HC-NP group showed only mild AD-like complications. In contrast, the NG-CON group clearly had presented with the highest degree of erythema/hemorrhage, dryness/scaling, edema, and epidermal excoriation. The severe AD-like skin symptoms in the NG-CON group were probably caused by severe disruption of physical barriers (SC) associated with severe itching, as well as by the underlying inflammatory reactions initiated in response to DNFB application. The Dinolite[®] micrograph of the VGR presented with less severe symptoms of AD than those in NG-CON, including exudates/transudates, hemorrhage, and erosion/excoriation. In contrast, the Dinolite[®] micrographs of the positive control groups (POS-CON-0.5 and POS-CON-1) showed some anti-AD effects that mitigated the progression of dermatosis that might have been caused by their inhibitory action against underlying inflammatory cascades. The microscopic data shown

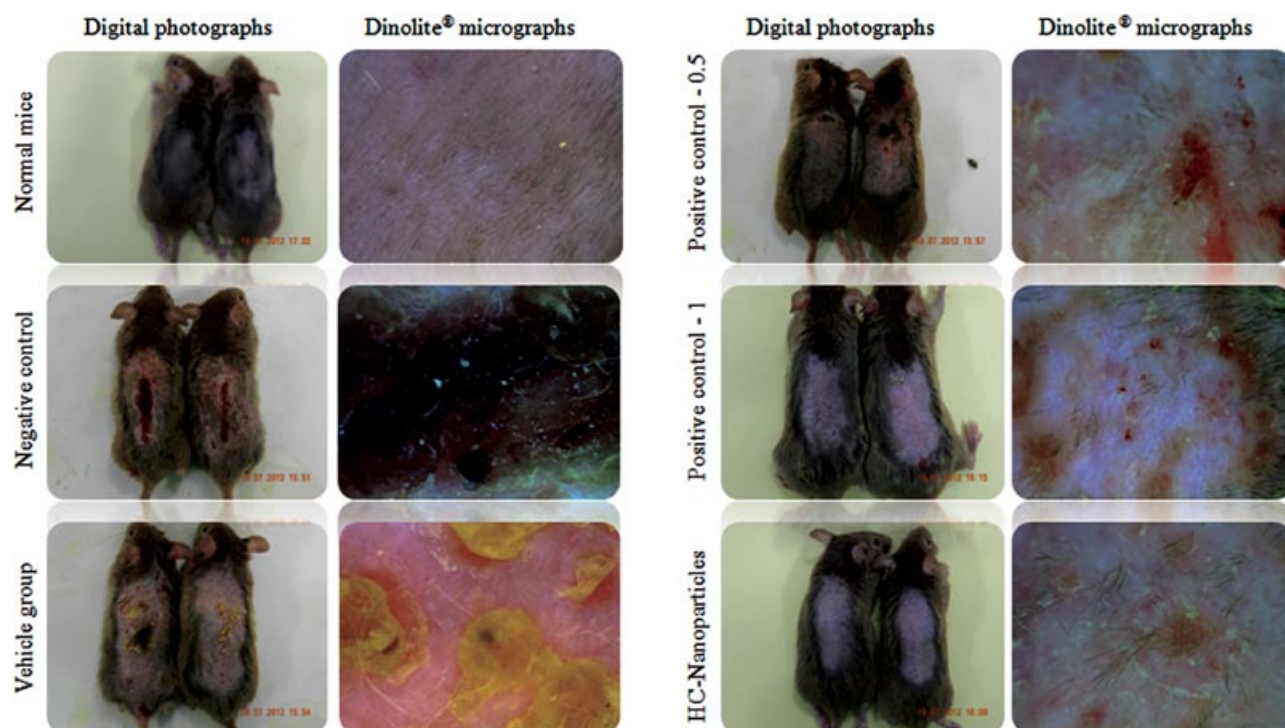


Figure 7. Digital camera and Dinolite® micrographs of various NC/Nga mice groups indicating severity of AD in terms of microscopic signs and symptoms of AD.

in Figure 7 lent further support to the idea that the use of HC-NPs helped efficiently control the signs and symptoms of AD.

Histopathological Examination

Figure 8a shows the histopathological features of integumentary system of all of the NC/Nga mice groups. Resulting monographs revealed that the normal mice group presents with normal anatomical features of skin in terms of epidermal and dermal thickness, keratinized stratified epithelium, and intact hair follicles. Comparatively, the NG-CON group indicated pronounced epidermal hyperplasia, parakeratosis, acanthosis, hyperkeratosis, and fragmented stratified epithelium with large number of infiltrated inflammatory cells. Together, the VGR shows almost similar characteristic features as that of NG-CON group but the hyperkeratosis and acanthosis were observed to be slightly less severe than the NG-CON group. Interestingly, POS-CON-1 mice group was presented with a significant control on the infiltration of inflammatory cells with hardly visible hair follicles; however, it showed marked epidermal hyperplasia compared with POS-CON-0.5. Surprisingly, the mice group treated with the HC-loaded nanoparticulate formulation (HC-NPs) distinctly alleviates the signs and symptoms associated with pathogenesis of AD with mere developed hair follicles indicating the restoration of skin integrity.

Taken together, Figure 8b identified an appropriate NC/Nga mouse model of AD for an intensive deposition of collagen scaffolds, especially in the papillary dermis via mason's trichrome staining. The extent of collagen deposition in various NC/Nga mice groups was of paramount importance because this collagen deposition directly reflects the severity of developing skin fibrosis at the site of AD-like skin lesions. The obtained results revealed that the repeated topical applications of DNFB in NG-CON group results in the overexpression of fibrogenesis and deposition of collagen fibers (acquired green color by Masson's trichrome stains) in the dermis, especially in papillary dermis at original magnification $\times 50\mu\text{m}$. However, in case of positive control groups (POS-CON-0.5 and POS-CON-1), comparatively a lower integer of collagen fibers were deposited. In contrast, the topical application of tested nanoparticulate (HC-NPs) formulation remarkably diminished DNFB-induced fibrogenesis and thus may rationalize to alleviate skin fibrotic remodeling. Moreover, the numbers of fibrocytes (playing a key role in tissue remodeling and skin fibrosis) migrated in the inflamed tissues were also observed to be downregulated by the HC-NPs mice group, as shown in Figure 8b.

CONCLUSIONS

In the current research, optimized NPs were successfully formulated for percutaneous delivery of HC

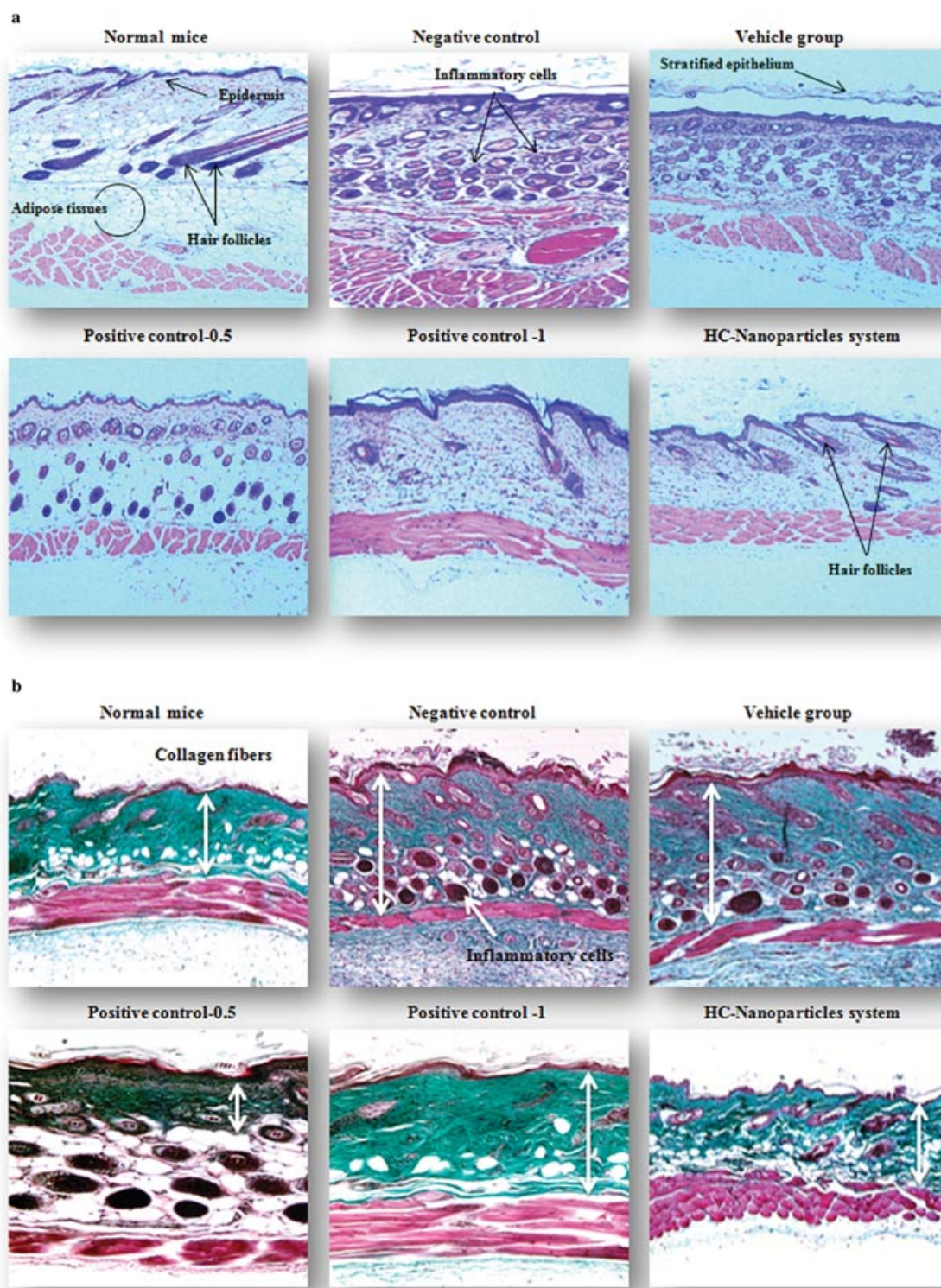


Figure 8. Histopathological features of AD-like skin lesions in all of the NC/Nga mice groups by using hematoxylin–eosin staining (a) and Masson's trichrome staining (b). White arrows indicating the magnitude of collagen deposition (collagen fibers turn green by Masson's trichrome stains) to provoke skin fibrosis. All of the monographs were observed under original magnification of $\times 50 \mu\text{m}$.

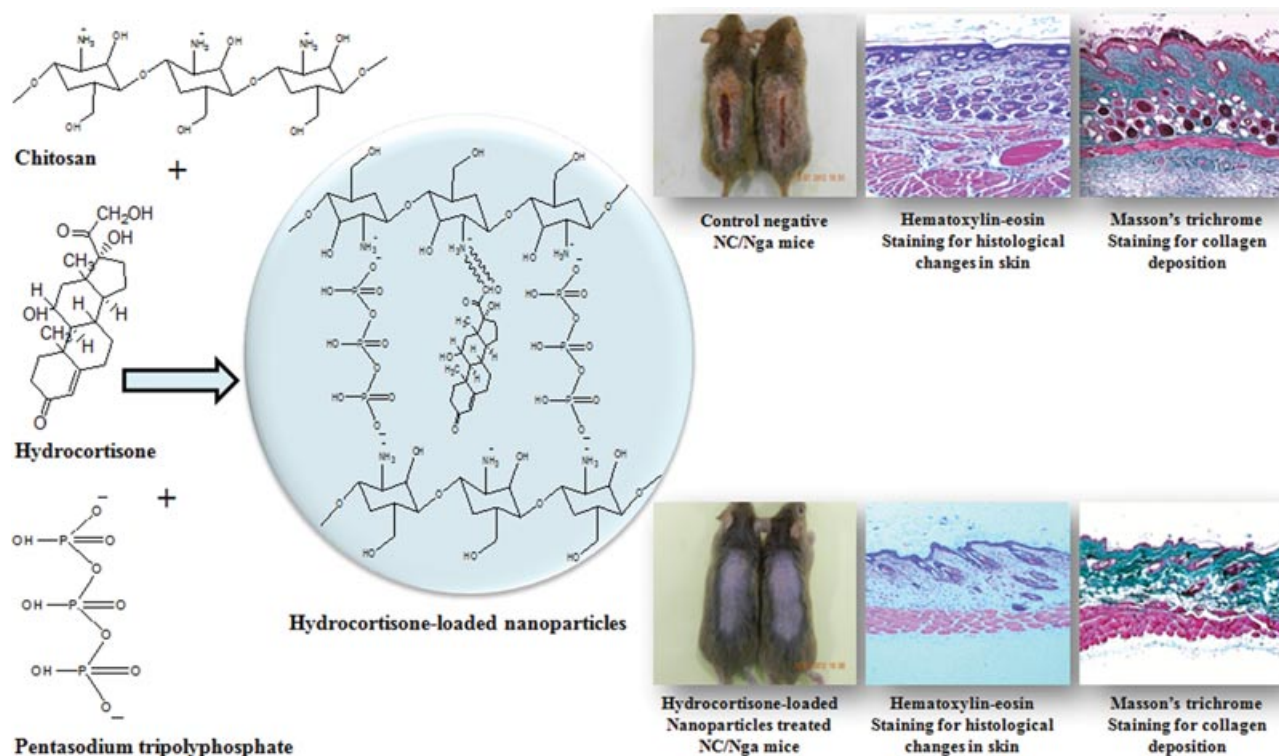


Figure 9. Schematic illustration of research work.

with the aim to alleviate transcutaneous absorption of GCs. *Ex vivo* permeation study revealed that the use of HC-NPs efficiently reduced the permeation of HC across the full-thickness NC/Nga mouse skin. The analysis of drug retention in the skin further showed that HC-NPs significantly accumulated HC in the epidermis and dermis and therefore could help to reduce the risk-benefit ratios of GCs. The *in vivo* clinical studies suggested that the HC-NPs had the ability to efficiently alleviate the signs and symptoms of dermatosis. Taken together, these findings were further supported by histopathological assessment in which HC-NPs could efficiently mitigate the pathological changes in skin integrity and prohibit the collagen deposition at the skin lesion. We propose that HC-NPs have potential for percutaneous delivery of anti-inflammatory drugs in the treatment of AD.

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REFERENCES

1. Zöller N, Kippenberger S, Thaçi D, Mewes K, Spiegel M, Sattler A, Schultz M, Bereiter-Hahn J, Kaufmann R, Bernd A. 2008. Evaluation of beneficial and adverse effects of glucocorticoids on a newly developed full-thickness skin model. *Toxicol In Vitro* 22:747–759.
2. Schoepe S, Schacke H, May E, Asadullah K. 2006. Glucocorticoid therapy-induced skin atrophy. *Exp Dermatol* 15:406–420.
3. Fang J, Shen K, Huang Y, Wu P, Tsai Y. 1999. Evaluation of topical application of clobetasol 17-propionate from various cream bases. *Drug Dev Ind Pharm* 25:7–14.
4. Fang J, Fang C, Sung K, Chen H. 1999. Effect of low frequency ultrasound on the *in vitro* percutaneous absorption of clobetasol 17-propionate. *Int J Pharm* 191:33–42.
5. Brown M, Martin G, Jones S, Akomeah F. 2006. Dermal and transdermal drug delivery systems: Current and future prospects. *Drug Deliv* 13:175–187.
6. Moser K, Kriwet K, Naik A, Kalia Y, Guy R. 2001. Passive skin penetration enhancement and its quantification *in vitro*. *Eur J Pharm Biopharm* 52:103–112.
7. Karande P, Jain A, Mitragotri S. 2004. Discovery of transdermal penetration enhancers by high-throughput screening. *Nat Biotechnol* 22:192–197.
8. Sammeta S, Vaka S, Murthy S. 2010. Transcutaneous electroporation mediated delivery of doxepin-HPCD complex: A sustained release approach for treatment of postherpetic neuralgia. *J Control Release* 142:361–367.
9. Kalia Y, Naik A, Garrison J, Guy R. 2004. Iontophoretic drug delivery. *Adv Drug Deliv Rev* 56:619–658.
10. Mitragotri S, Blankshtein D, Langer R. 1995. Ultrasound-mediated transdermal protein delivery. *Science* 269:850–853.
11. Prausnitz M. 2004. Microneedles for transdermal drug delivery. *Adv Drug Deliv Rev* 56:581–587.

12. Lee W, Shen S, Wang K, Hu C, Fang J. 2002. The effect of laser treatment on skin to enhance and control transdermal delivery of 5-fluorouracil. *J Pharm Sci* 91:1613–1626.
13. Cevc G, Vierl U. 2010. Nanotechnology and the transdermal route: A state of the art review and critical appraisal. *J Control Release* 141:277–299.
14. Shim J, Kang H, Park W, Han S, Kim J, Chang I. 2004. Transdermal delivery of minoxidil with block copolymer nanoparticles. *J Control Release* 97:477–484.
15. Alvarez-Roman R, Naik A, Kalia Y, Guy R, Fessi H. 2004. Skin penetration and distribution of polymeric nanoparticles. *J Control Release* 99:53–62.
16. Maia CS, Mehnert W, Schaller M, Korting H, Gysler A, Haberland A, Schafer-Korting M. 2002. Drug targeting by solid lipid nanoparticles for dermal use. *J Drug Target* 10:489–495.
17. Wu X, Price G, Guy RH. 2009. Disposition of nanoparticles and an associated lipophilic permeant following topical application to the skin. *Mol Pharmacol* 6:1441–1448.
18. He W, Guo X, Zhang M. 2008. Transdermal permeation enhancement of N-trimethyl chitosan for testosterone. *Int J Pharm* 356:82–87.
19. Wu W, Yang W, Wang C, Hu J, Fu S. 2005. Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate. *Int J Pharm* 295:235–245.
20. Papadimitriou S, Bikiaris D, Avgoustakis K. 2008. Chitosan nanoparticles loaded with dorzolamide and pramipexole. *Carbohydr Polym* 73:44–54.
21. Yamane M, Williams A, Barry B. 1995. Terpene penetration enhancers in propylene glycol-water co-solvents systems: Effectiveness and mechanism of action. *J Pharm Pharmacol* 47:978–989.
22. Padula C, Sartori F, Marra F, Santi P. 2005. The influence of iontophoresis on acyclovir transport and accumulation in rabbit ear skin. *Pharm Res* 22:1519–1524.
23. Kim T, Jung J, Kim G, Jang A, Ahn H, Park Y, Park C. 2009. Melatonin inhibits the development of 2,4-dinitrofluorobenzene-induced atopic dermatitis-like skin lesions in NC/Nga mice. *J Pineal Res* 47:324–329.
24. Park E, Park K, Eo H, Seo J, Son M, Kim K, Chang Y, Cho S, Min K, Jin M, Kim S. 2007. Suppression of spontaneous dermatitis in NC/Nga murine model by PG102 isolated from *Actinidia arguta*. *J Invest Dermatol* 127:1154–1160.
25. Matsuda H, Watanabe N, Geba G, Sperl J, Tsudzuki M, Hiroi J, Matsumoto M, Ushio H, Saito S, Askenase P, Ra C. 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 9:461–466.
26. Avadi MR, Sadeghi AM, Mohammadpour N, Abedin S, Atyabi F, Dinarvand R, Murtaza RT. 2010. Preparation and characterization of insulin nanoparticles using chitosan and Arabic gum with ionic gelation method. *Nanomedicine* 6:58–63.
27. Katas H, Hussain Z, Ling TC. 2012. Chitosan nanoparticles as a percutaneous drug delivery system for hydrocortisone. *J Nanomater* 2012:11 pages, doi: 10.1155/2012/327725.
28. Şenyiğit T, Padula C, Özer Ö, Santi P. 2009. Different approaches for improving skin accumulation of topical corticosteroids. *Int J Pharm* 380:155–160.
29. Werner Y, Lindberg M. 1985. Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. *Acta Derm Venereol* 65:102–105.
30. Gioia F, Leonardo C. 2002. The dynamics of TEWL from hydrated skin. *Skin Res Technol* 8:178–186.