

Potential treatment of atopic dermatitis: tolerability and safety of cream containing nanoparticles loaded with hydrocortisone and hydroxytyrosol in human subjects

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Abstract Hydrocortisone (HC), topical glucocorticoid along with hydroxytyrosol (HT), and anti-microbial- and anti-oxidant-loaded chitosan nanoparticles (CSNPs) were prepared in large scale and analyzed for their adverse effects on healthy human skin followed by repeated applications. Ten subjects were randomized to receive test (HC-HT CSNPs) and vehicle samples (aqueous (AQ) cream). They were applied on the arms for 28 days, and transepidermal water loss (TEWL), erythema intensity, and irritation score were measured. Blood samples were analyzed for blood hematology, blood biochemistry, and adrenal cortico-thyroid hormone (ACTH) levels. Skin biopsy was obtained to assess histopathological changes in the skin. HC-HT CSNP AQ cream was stored at 4, 25, and 45 °C for a period of 1 year, and its stability was assessed by monitoring their physical appearances, particle size, and pH. Spherical-shaped NPs were successfully upscaled using spinning-disc technology, with insignificant changes in particle size, zeta potential, and incorporation of drugs as compared to the well-established laboratory method. Particle size of HC-HT CSNPs was < 250 nm, and HC-HT CSNPs AQ cream remained stable when stored at 25 °C.

TEWL and erythema intensity for 28-day application did not indicate any signs of local irritation, redness, and toxicity, which were confirmed by normal Draize skin irritation scoring system and skin hematoxylin and eosin (H&E) staining results. Comparative results of blood hematology, blood biochemistry, and adrenal cortico-thyroid hormone level at day 0 and day 28 were not significant, indicating non-systemic toxicity. In conclusion, HC-HT CSNP AQ cream is safe, well-tolerated, and non-toxic, which may be useful in treating atopic dermatitis.

Keywords Atopic dermatitis · Topical drug delivery · Nanomedicine · Semi-solid · Skin

Introduction

The skin acts as an insulation sheath from the external environment and plays an important role in maintaining homeostasis [1]. This skin's protective function depends on several factors, including moisture content, cellular properties of corneocytes, interaction with the stratum corneum (SC), amount and composition of cellular lipids, and pH of the skin surface [2–4]. The defensive mechanism of the skin provides not only physical protection, but also metabolic, immunological, and UV protection [5]. Water levels in the SC maintain the flexibility and elasticity of the skin which also acts as a plasticizer in association with proteins and soluble matters [1]. The evaporation of water from the skin surface to maintain homeostasis is called transepidermal water loss (TEWL) [6]. Natural moisturizing factors (NMF) are breakdown products of filaggrin. NMF maintain the epidermal hydration, barrier function, and the skin pH. Reduction in the filaggrin breakdown results in decreased levels of NMF, decreased SC water

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level, and increased TEWL which lead to clinically dry skin. Increased pH of the skin may also result in the adhesion and proliferation of certain microbes such as *Staphylococcus aureus* [7].

Atopic dermatitis (AD) is a chronic skin disorder with intermittent flares, characterized by intense pruritis and disruption of skin barrier in both lesional and non-lesional skins. Dry skin is a consequence of compromised epidermal barrier, which further causes inflammation, scratching, lichenification, and increased TEWL, leading to the manifestation of AD [8]. There are many medications available for the treatment of AD, but topical glucocorticoids (TGs) are the main frontline and the most commonly prescribed drugs by dermatologists. The use of TGs for AD treatment is related to its anti-inflammatory, vasoconstriction, immunosuppressive, and anti-proliferative effects [9, 10]. However, AD treatment with TGs is associated with local side effects such as striae, skin atrophy, and rosacea. When absorbed systemically, it leads to systemic side effects such as hypertension, glaucoma, and suppression of hypothalamic pituitary adrenal axis [11]. The current challenges are the relapsing nature of the disease and the continuously increasing number of patients for whom the dosage form needs to be modified to minimize the side effects of TGs and to optimize TG-related beneficial effects. Many strategies have been used to minimize the side effects of TGs, but often, less consideration has been given to other factors that can control the delivery of drugs to the site of application such as iontophoresis [12], electroporation [13], and chemical penetration enhancer [14]. Among the other classes of drug delivery vehicles, polymeric nanoencapsulation is a holistic approach of targeting delivery [15].

Nanocarrier-based drug delivery and therapeutic efficacy mainly depend on their diffusion, affinity, and interaction between the polymer and membrane components. Several non-toxic, biodegradable, and naturally and synthetically occurring polymers such as PLGA (polylactic-co-glycolic acid), PLA (polylactic acid), and chitosan showed promising drug delivery and affinity to the site of action [15, 16]. Chitosan is a FDA-approved naturally occurring compound that has wound healing properties due to its excellent biocompatibility, biodegradability, and non-toxic nature [17, 18]. Hydrocortisone (HC) and hydroxytyrosol (HT) (naturally occurring antioxidant and anti-microbial) polymeric chitosan nanoparticles (HC-HT CSNPs) have been successfully developed and characterized in our laboratory [19]. Despite their efficacy in treating AD in animal models, their nanosize characteristic (250–300 nm) might cause side effects, due to deep penetration into the tissues and systemic absorption [20]. Therefore, various studies were carried out to determine the potential of acute dermal toxicity, acute dermal irritation, and repeated dose toxicity using different doses of CSNPs and to compare it with a standard treatment in animal models [21, 22].

However, to our knowledge, an investigation on the effect of CSNPs loaded with HC-HT on healthy human skin has not been performed. Therefore, this study was designed to determine potential HC-HT CSNP-related adverse effects on healthy human skin.

Generally, mass production of nanoparticles (NPs) is required to fulfill the demands of clinical applications. However, mass production could not be achieved by directly applying parameters used at the laboratory scale and involves transfer of procedures and technology to be able to manufacture the product at a rational cost. Although there are different methods of preparing polymeric NPs, ionic gelation method is the simplest and non-destructive method for scaling up [23]. In this respect, an overhead spinning-disc Silverson® mixer at various speeds (300–3000 rpm) was introduced for nanofabrication with an increased volume up to tenfold that of the laboratory scale. In the process of nanofabrication, the new method created interfering waves with high centrifugal force to provide intimate mixing as well as high surface-to-area interaction [24], which facilitates the cross-linking of protonated amine chitosan with anion tripolyphosphate. Additionally, the increased centrifugal force resulted in higher stirring speed, which produced finer NPs [25], as compared to magnetic stirring. In this study, HC-HT CSNP formulation was scaled up and characterized for their particle size, zeta potential, and morphology. Furthermore, successful incorporation of drugs in both NPs was confirmed by Fourier transform infrared spectroscopy (FTIR) analysis. In addition, the stability of this formulation was assessed on the basis of its physical appearance, pH, viscosity, and morphology of nanoparticles at different storage conditions (at 4 °C, 25 ± 2 °C/60 ± 5% RH, and 45 ± 2 °C/75 ± 5% RH) for a period of 1 year. HC-HT CSNPs were applied on the human skin for 28 days. Measurement of TEWL by Tewameter®, erythema intensity by Mexameter®, and irritation score by the standard Draize skin scoring method was performed at different time intervals for 28 days. Blood samples were drawn at the screening period and at the end of study for comparative analysis of blood hematology, blood biochemistry, and adrenal corticoid hormone (ACTH) levels. Skin biopsy was also obtained after patients provided an informed consent to assess histopathological changes in the skin.

Materials and methods

Materials

Analytical-grade HC (base), low-molecular-weight chitosan (CS) (specifications: MW, 70 kDa; deacetylation degree, 85%; viscosity 20–300 cP for 1% w/v solution in 1% v/v acetic acid at 25 °C), and HT were purchased from Sigma-Aldrich Chemicals Corp., Ltd., (St. Louis, MO, USA). The

pentasodium tripolyphosphate (TPP) was obtained from Merck KGaA Co., Ltd., (Darmstadt Germany). High-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, and ethanol were obtained from Fisher Scientific Korea Ltd. (Seoul, Korea). The aqueous cream (AQ cream) was a gift from Xorix Pharmaceuticals Pvt., Ltd. (Kuala Lumpur, Malaysia) and was used as the vehicle base for compounding the nanoparticle formulation.

Methods

Preparation of HC-HT CSNPs by overhead mixing method

NPs were prepared by ionic gelation method using overhead spinning-disc mixer L5M-A, Silverson® (UK) at room temperature. For scaling up, 500 mL of 0.2% w/v chitosan solution in 1% v/v of acetic acid was prepared and mixed overnight. Separately, 100 mL of HC (1% in 30% ethanol) and HT (1% in distilled water) was prepared. These three solutions were mixed in a large container with overhead mixer at a spinning speed of 500 rpm for 15–20 min. The solution was maintained at pH 5.0 with the addition of 1 M NaOH solution as the optimal pH for fabrication with high drug loading, and the nanosized particle was previously established at 5 [19]. HC-HT CSNPs were fabricated by adding 100 mL TPP solution (1% in distilled water) to the tank containing the HC, HT, and chitosan solutions. The TPP solution was added in a dropwise manner under spinning-disc rotation at 1000 rpm. HC-HT CSNPs were collected by ultracentrifugation at 35,000 rpm at 10 °C for 30 min. The resulting pellets were collected and resuspended in deionized water before they were centrifuged again. The procedure was repeated thrice to remove any non-encapsulated drugs. The pellets were sonicated to obtain a homogenous suspension of HC-HT CSNPs. HC-HT CSNP suspensions were freeze-dried later.

Preparation of HC-HT CSNPs by conventional method

Chitosan NPs were also prepared in a beaker by agitation with a magnetic stirrer. Briefly, 50 mL of 0.2% chitosan solution (w/v) was prepared in acetic acid 1% (v/v). It was then mixed with the 10 mL HC (1%v/v in 30% ethanol) and 10 mL HT (1%w/v in distilled water) at pH 5 by using the pH meter at room temperature. Co-loaded CSNPs were fabricated by adding 10 mL of TPP (1%w/v) solution in a dropwise manner under constant stirring at 900 rpm for ionic cross-linking of chitosan with TPP.

HC-HT CSNP physicochemical characterization

Average particle size, polydispersity index (PI), and zeta potential of both small- and large-scale NPs were measured by photon correlation spectroscopy (PCS) and laser Doppler

anemometry (LDA), respectively, using Nano-ZS (Malvern Instruments, Worcestershire, UK). Nanoparticle shape and surface properties were studied by field emission scanning electron microscope (FESEM; JEOL JSM-6700F, Tokyo, Japan) after vacuum-coating the NPs with platinum by an ion sputter (JEOL JFC-1100E). Experiments were conducted in triplicate, and results were expressed as mean of measurement for three different batches $\pm$ SD.

Entrapment efficiency and loading capacity

Entrapment efficiency (EE) and loading capacity (LC) of both drugs (HC and HT) were calculated according to Eqs. 1 and 2, respectively.

$$EE (\%) = W_t - \frac{W_f}{W_t} \times 100 \quad (1)$$

$$LC (\%) = W_t - \frac{W_f}{W_n} \times 100 \quad (2)$$

W_t is the total initial amount of HC or HT, and W_f is the amount of free drug in the supernatant after ultracentrifugation. W_n is the average weight of lyophilized drug-loaded NPs. The quantities of both HC and HT were determined by ultraviolet (UV) absorption of the supernatant [26]. Each sample was measured in triplicate. HC was measured at 248 nm (retention time at 9.58 min) where an intense characteristic peak was displayed, and the characteristic peak of HT was displayed at 280 nm (retention time at 3.34 min).

Fourier transform infrared spectroscopy analysis

The FTIR spectra of both small- and large-scale HC-HT CSNPs were recorded on FTIR spectrophotometer (spectrum 100; Perkin Elmer, Waltham, MA, USA). FTIR analysis was performed to certify the encapsulation as well as to understand the interaction between the drug and the excipients. Briefly, 2–3 mg of freeze dried HC-HT CSNPs from both small and large scales was blended with 200–300 mg potassium bromide (KBr) separately followed by compression to form a transparent pellet. Similarly, pellets of individual components (CS, HC, and HT) were prepared and analyzed over a scanning wavelength range of 4000–400/cm.

HC-HT CSNPAQ cream-based formulation

AQ was used for compounding formulations to make it appropriate topical drug delivery. The cream was liquefied in a water bath at 50 °C with vigorous blending. Freeze-dried HC-HT NPs were added to make a final concentration 0.5% of HC. Similarly, HC-HT solution was used to formulate non-NP systems (HC-HT AQ).

Table 1 Physicochemical characteristics of small- and large-scale HC-HT CSNPs

HC-HT CSNPs	Small-scale	Large-scale
Particle size (nm)	236.5 ± 15	221 ± 10
PdI	0.4 ± 0.06	0.4 ± 0.01
Zeta potential (mV)	+ 35.2 ± 0.32	+ 31.7 ± 2.65

All data are presented ± SD, $n = 3$

Stability of the formulation

Stability study was achieved as per standard International Council for Harmonization (ICH) guidelines at three different storage conditions (4 ± 2 , 25 ± 2 , and 40 ± 2 °C) over a period of 12 months in well-sealed glass vials. The samples were analyzed at the end of 0, 1, 3, 6, 9, and 12 months for the physical appearance, pH, viscosity, and morphology of nanoparticles.

Measurement of viscosity and viscoelasticity Samples were analyzed for their viscosity and viscoelasticity using a Bohlin Gemini HR nanorheometer (Malvern Instruments Ltd., UK) with cone rotor (2/20) and plate geometry at 25 °C. Data analyses were done with Bohlin R6.50.5.7 application software. Applied shear rate was increased in ascending order from 0 to 500 D (1/s) and then decreased in descending order from 500 to 0 D (1/s). The resulting shear stress (Pa) was measured. All measurements were made in triplicate at accurate control shear stress at the end of 0, 1, 3, 6, 9, and 12 months.

Measurement of sample pH pH was measured directly in each HC-HT CSNP and HC-HT AQ cream by using Mettler Toledo (the cream was thoroughly stirred and kept at room temperature before each test). Each measurement was made in triplicate at the end of 0, 1, 3, 6, 9, and 12 months.

Morphology of nanoparticles Morphology of HC-HT CSNP AQ cream at three different temperatures was analyzed using a transmission electron microscope (TEM,

Hitachi H-7000 TEM (Japan)). Each sample was placed on a 300-mesh copper grid coated with carbon and stained with phosphotungstic acid. Stained samples were allowed to evaporate and dry at room temperature (25 ± 2 °C). Analyses were made at the end of 0, 1, 3, 6, 9, and 12 months. Besides morphology of nanoparticles, particle size of HC/HT NPs in the AQ cream was measured to assess the stability of the system.

In vivo study

The main goals of this study were to evaluate the safety of HC-HT CSNP cream on healthy human skin and identify local and systemic adverse effects of NP formulation.

Study population Subjects enrolled in this study were ten 18–45-year-old healthy female Malaysian volunteers. All participants signed a written informed consent. All participants were receiving both nanoparticle and vehicle formulations, and the formulations were applied on the skin of a specific arm. The right arm anterolateral area was treated with the test sample (HC-HT CSNPs incorporated in AQ cream), while the left arm anterolateral area was treated with the vehicle sample (AQ cream) as a placebo. All of them had no history of skin disease, specifically AD or allergy to corticosteroids.

Study Design

Screening visit Healthy volunteers signed the written informed consent form during the pre-screening period on visit 2. The study was conducted according to the good clinical practice guidelines (GLP). Pre-study evaluation included skin observation, blood hematology, blood biochemistry, and ACTH levels in the blood.

Treatment period A 28-day, double-blind, vehicle-controlled study was designed to evaluate the safety of HC-HT CSNP cream in humans. The protocols of this study were approved by the research ethics committee of the National University of

Fig. 1 Comparable SEM micrographs of both small- (a) and large-scale (b) HC-HT CSNPs

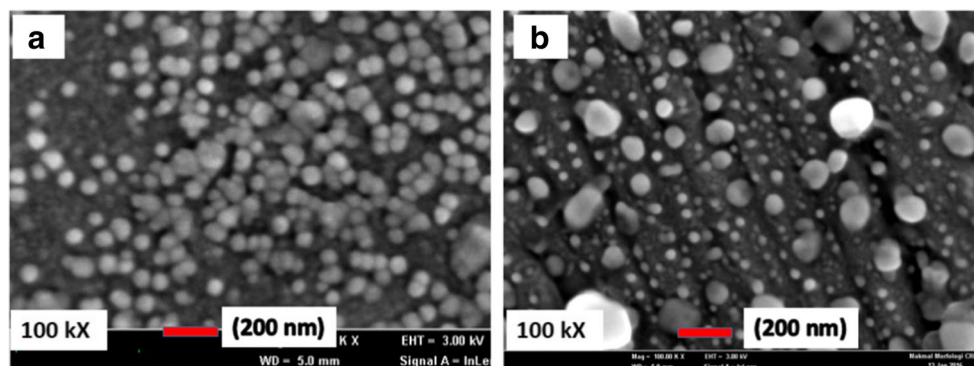
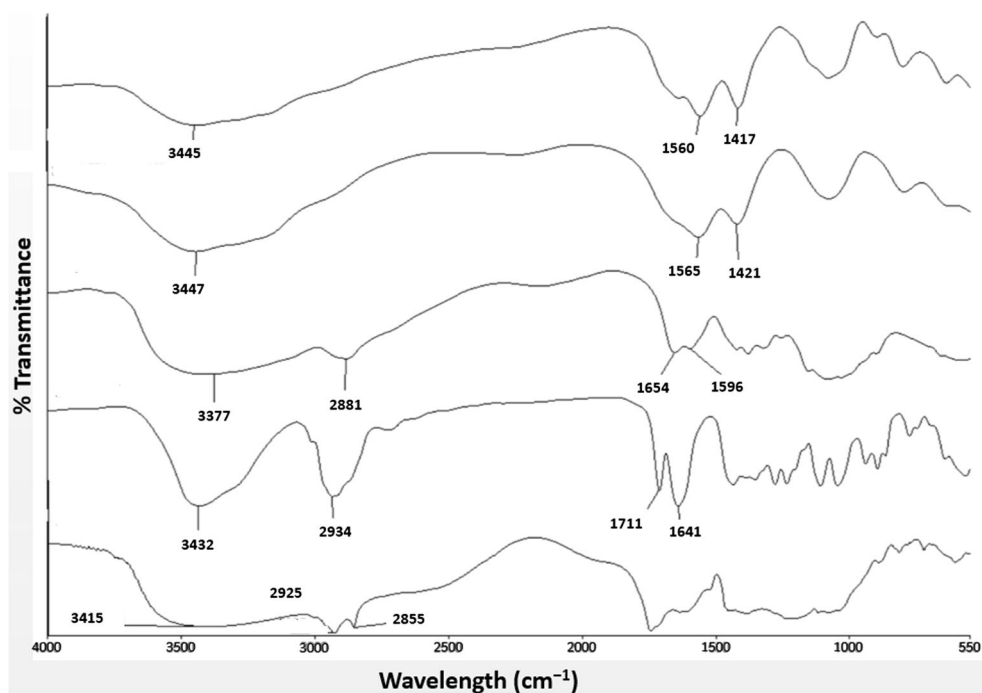


Fig. 2 FTIR spectra of large- (a) and small-scale (b) HC-HT CSNPs, chitosan (c), HC (d), and HT I (e)



Malaysia (UKM FPR.4/244/FF-2015-275). Healthy adult volunteers who were not taking any other medications and comply with the inclusion and exclusion criteria as listed below were randomized in the treatment group. Each subject was asked to apply a cream containing HC-HT CSNPs, equivalent to 250-mg HC (by applying the fingertip-unit method) and placebo aqueous (AQ cream (vehicle) on the right and left anterolateral aspects of the mid arm, respectively. Blood samples were collected from the subjects before the beginning (day 0) and at the end of the study (day 28) to determine any changes in their blood hematology, blood biochemistry, and serum cortisol levels. The subjects were requested to come to Universiti Kebangsaan Malaysia Medical Centre (UKMMC) for the first 3 days consecutively and then on their visits at days

7, 14, and 28 to measure TEWL and erythema intensity by Tewameter® and Mexameter®, respectively. The irritation level of the skin was also visually investigated by using the standard Draize skin irritation scoring method. A skin biopsy was performed only from subjects who provided an informed consent.

Inclusion criteria To be enrolled, the following criteria had to be fulfilled at visit 2:

1. Healthy adult of either sex between 18 and 45 years old
2. No significant diseases or clinically significant abnormal laboratory values during screening
3. On no regular medical treatment.

Exclusion criteria

1. Infected eczema or any other type of skin diseases, affecting the planned application site of the studied medication
2. Immunocompromised patients
3. Allergic contact dermatitis to topical HC or other topical medicaments
4. Patients with generalized skin atrophy
5. Patients requiring inhaled or intranasal corticosteroids
6. Patients who received oral or intramuscular corticosteroids in the past 8 weeks
7. Severe renal or liver impairment
8. Intake of any medications within 14 days before the beginning of the study
9. Diabetes mellitus, hypertension, and other chronic medical illnesses
10. Pregnant women and nursing mothers

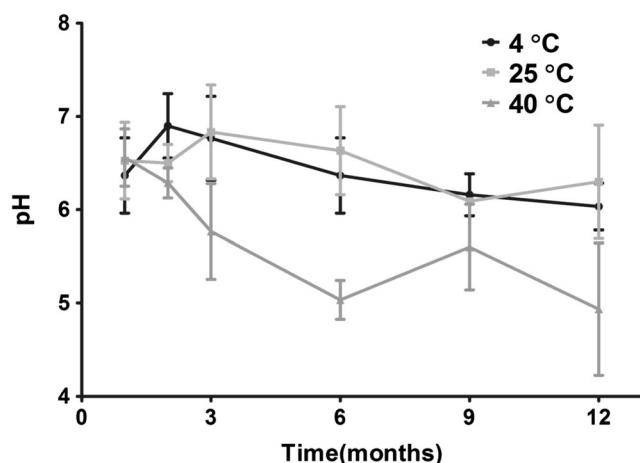


Fig. 3 pH values of HC-HT CSNPs in AQ cream at 4 °C (a), 25 °C (b), and 40 °C (c) over a period of 1 year

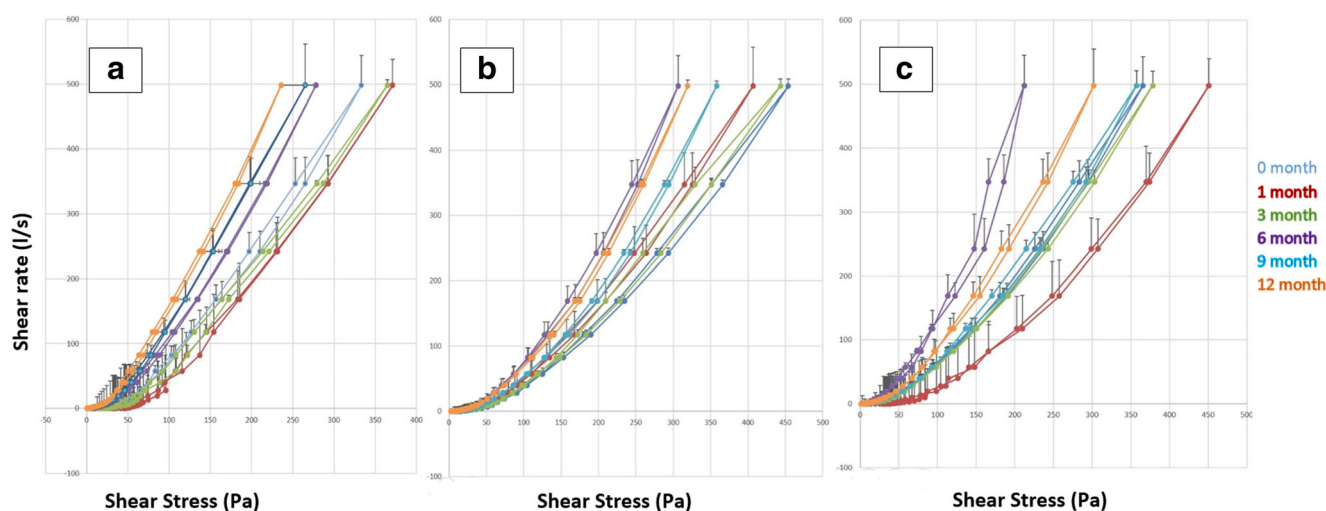


Fig. 4 Rheological behavior of HC-HT CSNPs in AQ cream at 4 °C (a), 25 °C (b), and 40 °C (c) over a period of 1 year

Treatment plan At visit 2, only eligible subjects received the study drug in a tube form, either the test product HC-HT CSNP cream or the placebo product. Study medications or the vehicle was applied twice a day on the specific area. Each subject was asked to apply one-half fingertip unit of cream containing HC-HT CSNPs and placebo AQ cream (vehicle) on the right and left anterolateral aspects of the arm, respectively, for 28 days.

Non-invasive biophysical assessments The standard Draize skin irritation scoring method was used to determine the irritation score on the basis of visual observation (Table 1). Adverse effects like burning or stinging sensations, itching,

redness, and rash were monitored throughout the study. TEWL was monitored by using Tewameter® as the loss of the skin barrier function, which results in increased water loss [6]. This increase in the TEWL is further associated with the change in the lipid content or NMF of the skin. As such, Sebumeter® was used to determine cutaneous biometry lipid levels and pigmentation of the skin. The following measurements were made at 0, 4, 24, 48, and 72 h and on the 7th, 14th, and 28th day. The treated area was examined for signs of skin atrophy, striae, telangiectasia, hypopigmentation, and folliculitis on days 1, 3, 7, 14, and 28.

Subjects were asked to report and record any signs or symptoms experienced throughout the study period in a diary.

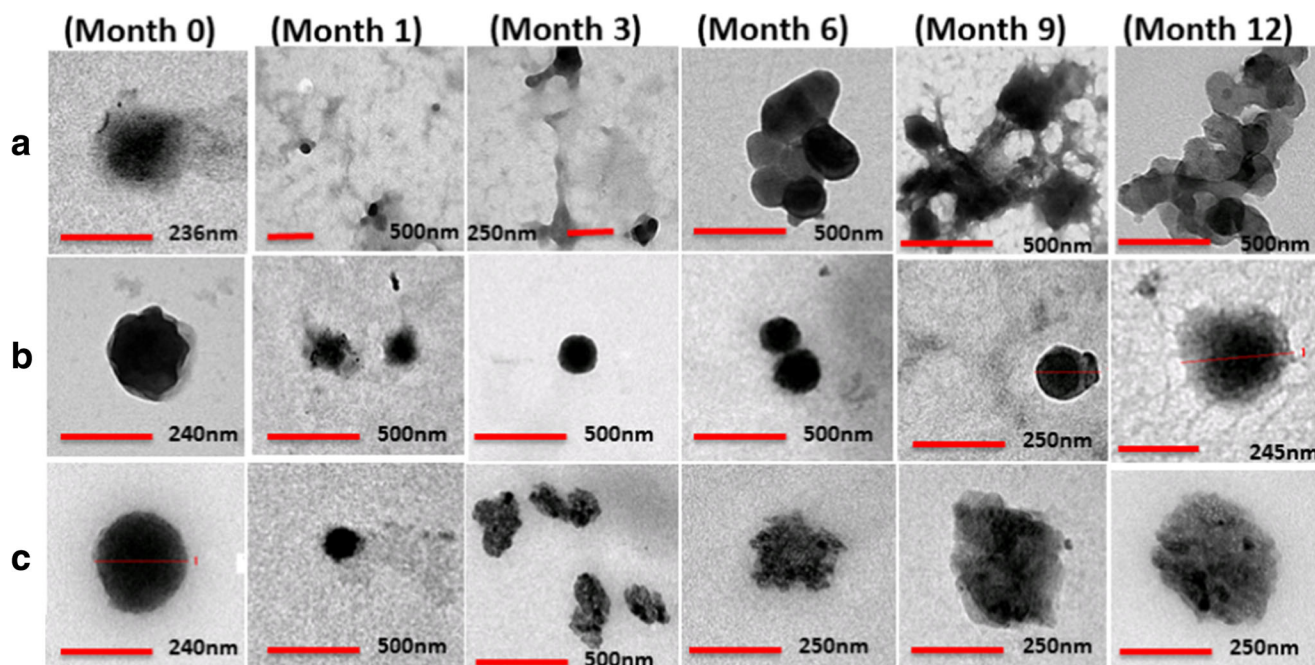


Fig. 5 Morphology of HC-HT CSNPs in AQ cream at pre-determined time points for 1 year. Morphology of NPs at storage temperatures of a 4 °C, b 25 °C, and c 40 °C

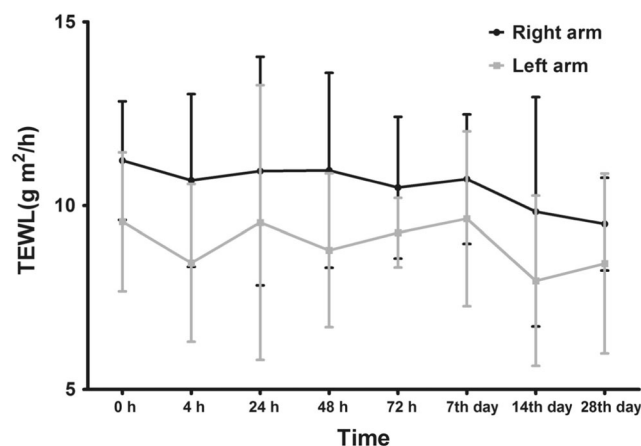


Fig. 6 TEWL during the 28 days of application of HC-HT CSNP AQ cream and AQ cream on the right and left arms, respectively ($n = 10$ /group)

Data analysis

Data were recorded as mean with standard deviations. Statistical analysis was carried out using t test. $p < 0.05$ and $p < 0.001$ are identified in tables and figures as significant (*) and highly significant (**), respectively. All graphs were drawn on GraphPad Prism version 6.04 for Windows (GraphPad software, La Jolla, CA, USA).

Results and discussion

Physicochemical characteristics

Particle size and zeta potential of HC-HT CSNPs prepared by using a magnetic stirrer (small-scale) and overhead rotating disc (large-scale) were measured as these characteristics determine the in vivo distribution, biological safety, toxicity, and the targeting ability of NPs. They also influence the drug

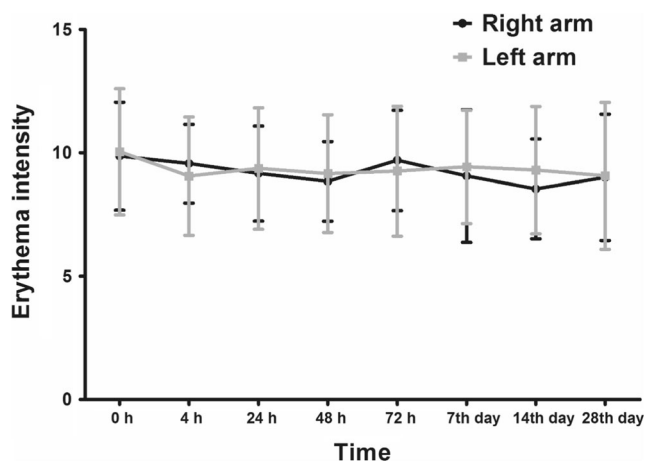


Fig. 7 Erythema intensity during the 28 days of application of HC-HT CSNP AQ cream and AQ cream on the right and left arms, respectively ($n = 10$ /group)

loading, drug release, and stability of NPs [27]. Polymeric CSNPs prepared by ionic gelation method have various advantages, including small-sized range of particles, use of aqueous solution, and better control of the particle size by varying TPP and chitosan concentrations [21]. According to the results obtained by laser Doppler anemometry, the mean particle sizes of both small- and large-scale CSNPs were not statistically significant. The stability of colloidal systems is usually determined by their zeta potential. Mean zeta potentials for small- and large-scale HC-HT CSNPs were $+35.2 \pm 0.32$ and $+31.7 \pm 2.65$ mV, respectively. Table 1 shows the mean particle size and zeta potential of both small- and large-scale resulting NPs. The EE percentages of HC and HT were found to be 73 and 56%, respectively whereas the LC percentages of both HC and HT were 38 and 23%, respectively. Analysis of variance (ANOVA) was used to compare the resultant values of physicochemical parameters of both production scales. The ANOVA results indicate that there was no significant difference between their particle size, PDI, and zeta potential values obtained from homogenous colloidal suspension of both small- and large-scale HC-HT CSNPs.

The SEM results demonstrated that NPs were uniformly distributed with a spherical and uniform shape (Fig. 1). Hence, it can be concluded that both techniques produced NPs with an acceptable limit of size.

FTIR analysis

Figure 2 shows the FTIR spectra of both small- and large-scale HC-HT CSNPs and their components (CS, HC, and HT). Each functional group in the compound has its own specific peak, which can be used for its identification by the FTIR instrument [28].

A chitosan spectrum shows two bands in the region of 1550–1650/cm. Both bands appear due to NH_2 bond stretching vibration of the amide I bonds. The splitting of the peak into two characteristic bands at 1654 and 1596/cm is due to the hydrogen bond in the polymeric structure. A broad absorption band in chitosan appears in the range of 3600–2800/cm due to the collective absorption of O–H and N–H groups in chitosan. This broad absorption shows the strong hydrogen-bonded structure. The characteristic peak of HT appears at 3415/cm due to O–H bending and at 2925 and 2855/cm due to C–H stretching [29]. Similarly, HC shows a characteristic peak at 3432/cm, corresponding to the O–H bending, at 2934/cm due to C–H stretching, and at 1711 and 1641/cm representing C=O bending. After ionic cross-linking, TPP and CS for both small- and large-scale CSNPs shifted from 1654 to 1565 and 1560/cm, respectively. Meanwhile, the peak of CS at 2881/cm disappeared and this may be due to the interaction between NH_3^+ of CS and PO_4^- of TPP. Moreover, intense peaks of HC and HT were not observed in the drug-loaded small- as well as large-scale NPs, indicating that the

Table 2 Erythema and edema scores as determined by the Draize skin irritation method ($n = 10$, $\pm$ SD).

Interpretation	Right arm (HC-HT CSNPs)								Left arm (vehicle AQ cream)							
	0 h	4 h	Day 2	Day 3	Day 7	Day 14	Day 28		0 h	4 h	Day 2	Day 3	Day 7	Day 14	Day 28	
Erythema score																
No erythema	0	0							0							
Very slight erythema	1	0							0							
Well-defined erythema	2	0	0	0	0	0	0		0	0	0	0	0	0	0	
Severe erythema	3	0							0							
Eschar formation	4	0							0							
Total score					0								0			
Edema score																
No edema	0	0							0							
Very slight edema	1	0							0							
Well-defined edema	2	0							0							
Severe edema (approximate raise 1 mm)	3	0	0	0	0	0	0		0	0	0	0	0	0	0	
Severe edema (raise more than 1 mm)	4	0							0							
Total score					0								0			

Dermal responses were scored according to the Draize skin irritation scoring system

drug was successfully encapsulated by intense interaction between CS and TPP.

Stability study

Alteration in physical properties during storage may influence clinical effectiveness. Accelerated testing of formulation kinetics is a common and important assessment for chemical stability [30]. pH of normal human skin ranges from 4.5–6.0, and therefore, pH of formulation should be within the range that is suitable for topical application as well as storage [31]. HC-HT CSNP AQ cream had an initial pH of 6.2 which was suitable for topical application on human skin. At 25 °C, pH of NP formulation insignificantly decreased to 5.8 at the

end of the 6th month (Fig. 3). On the other hand, at 40 °C, pH significantly decreased to 4.3, indicating destabilization of the cream, as temperature might induce hydrolysis and this would cause skin irritation. At 4 °C, pH of formulation was stable as pH was insignificantly different from the initial pH (month 0).

Corresponding shear stress (related to force applied) and shear rate were determined to describe the viscosity and flow

Table 3 Blood hematology and blood biochemistry on day 0 and at the end of study (day 28)

Blood hematology	Before	After
Hb (g/L)	129 $\pm$ 9	130 $\pm$ 10
RBC (10^{12} /L)	4.7 $\pm$ 0.2	4.8 $\pm$ 0.2
PCV (L/L)	0.4 $\pm$ 0.03	0.4 $\pm$ 0.03
MCV (fL)	87.7 $\pm$ 5	85.7 $\pm$ 5
MCH (pg)	27 $\pm$ 1.9	27 $\pm$ 2
MCHC (g/L)	310 $\pm$ 8	320 $\pm$ 8
RDW (%)	14 $\pm$ 0.6	14 $\pm$ 0.8
Neutrophils (10^9 /L)	3.8 $\pm$ 0.9	4.4 $\pm$ 1
Lymphocyte (10^9 /L)	2.7 $\pm$ 0.6	2.7 $\pm$ 0.6
Monocytes (10^9 /L)	0.4 $\pm$ 0.1	0.5 $\pm$ 0.2
Eosinophils (10^9 /L)	0.13 $\pm$ 0.04	0.13 $\pm$ 0.06
Platelets (10^9 /L)	287 $\pm$ 52	300 $\pm$ 49
Glucose (mmol/L)	4.8 $\pm$ 0.7	4.7 $\pm$ 0.4
ACTH (ng/L)	20.5 $\pm$ 6	21.8 $\pm$ 8

The values are presented as the mean $\pm$ standard deviation. $n = 10$ /group
RBC red blood cells, *Hb* hemoglobin, *PCV* pack cell volume, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *ACTH* adrenal cortico-thyroid hormone

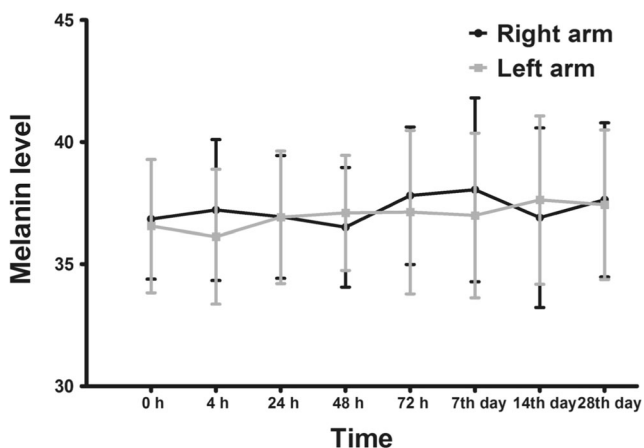
**Fig. 8** Melanin level during the 28 days of application of HC-HT CSNP AQ cream and AQ cream on the right and left arms, respectively ($n = 10$ /group)

Table 4 Blood biochemistry on day 0 and at the end of the study (day 28)

	Blood biochemistry	
	Before	After
Total protein (g/L)	76.9 ± 3.8	79.2 ± 2.9
Albumin (g/L)	43 ± 2	43.5 ± 2
Globulin (g/L)	33.8 ± 2.9	35.7 ± 1.9
Albumin/globulin	1.3 ± 0.1	1.2 ± 0.09
Alkaline phosphatase (U/L)	66 ± 15	66 ± 16
Total bilirubin (μmol/L)	8.6 ± 3.9	9.7 ± 6.5
GGT (U/L)	23.7 ± 15	24.7 ± 18
AST (U/L)	18.1 ± 6	18.9 ± 6
ALT (U/L)	17.7 ± 14	20.8 ± 14
Sodium (mmol/L)	140 ± 2	139 ± 2
Potassium (mmol/L)	3.9 ± 0.3	4.3 ± 0.2
Chlorine (mmol/L)	103 ± 1.7	106 ± 1.9
Urea (mmol/L)	4.3 ± 0.8	4.2 ± 0.8
Creatinine (μmol/L)	53 ± 9.8	51 ± 10.8
Uric acid (mmol/L)	0.3 ± 0.04	0.3 ± 0.05
Calcium (mmol/L)	2.2 ± 0.06	2.3 ± 0.04
Phosphate (mmol/L)	1.1 ± 0.1	1.3 ± 0.3
Renal albumin (g/L)	43 ± 2.1	43.5 ± 2.2

The values are presented as the mean ± standard deviation. $n = 10$

RBC red blood cells, Hb hemoglobin, PCV pack cell volume, MCV mean corpuscular volume, MCH mean corpuscular hemoglobin, MCHC mean corpuscular hemoglobin concentration, Reti reticulocytes, AST aspartate aminotransferase, ALT alanine aminotransferase, GGT gamma-glutamyl transferase

behavior of the formulation [32]. HC-HT CSNP AQ cream had shown non-Newtonian pseudoplastic shear thinning or flow as its curve in the graph of shear stress versus shear rate was convex towards the shear stress axis. This non-Newtonian flow and pseudoplastic behavior of the formulation at 25 °C showed that it will not flow on the skin at room temperature, when applied. Results of viscosity (η , Pa/s) are represented in Fig. 4. Furthermore, an increase in shear stresses was observed at 40 °C due to hydrolysis induced by high temperature. Drug analysis indicated that at high temperature, the drugs were not present in the nanoparticulate form but dispersed in the cream which further degraded them. Evaluation of the data was fitted in the Newtonian model by using Bohlin software.

The stability of a nanoparticle is important for its drug release and to produce the required therapeutic effects [26]. TEM analysis was carried out to determine the morphological behavior of NPs upon long-term storage at different temperatures (Fig. 5).

In general, NPs tend to adhere with one another and form aggregates to reduce surface energy and high temperature will aggravate the process. At high temperature, surface charge (charge repulsion) may not prevent these particles from forming aggregates [33]. HC-HT CSNPs showed the same morphological behavior as that of month 0 at 25 °C. Contrarily, particles were aggregated at 45 and 0 °C. Previously, the zeta potential of NPs had been reported to

remain stable at room temperature while it decreased at high temperature which led to the aggregation of NPs [34]. Particle size is an important parameter in evaluating NP stability as any change may indicate physical instability. It has been reported that indomethacin-loaded chitosan NPs were stable over a period of 6 months [35]. However, non-stability of chitosan NPs at extreme condition, over a longer period, has also been reported [34]. In the present study, storage of NPs at accelerated conditions (0, 45 °C) had demonstrated the instability of NPs for a long period of storage. On the other hand, NPs at 25 °C were quite stable and non-aggregated. Based on the results of shear stress, shear rate, and pH, HC/HT NPAQ cream-based formulations were relatively stable at lower temperatures (4 and 25 °C) but the particles were aggregated and formed large particles at 4 °C (which makes them unstable). It has also been reported that chitosan nanoparticles prepared by ionic gelation method showed excellent storage stability at room temperature and pH around 5.4 [36].

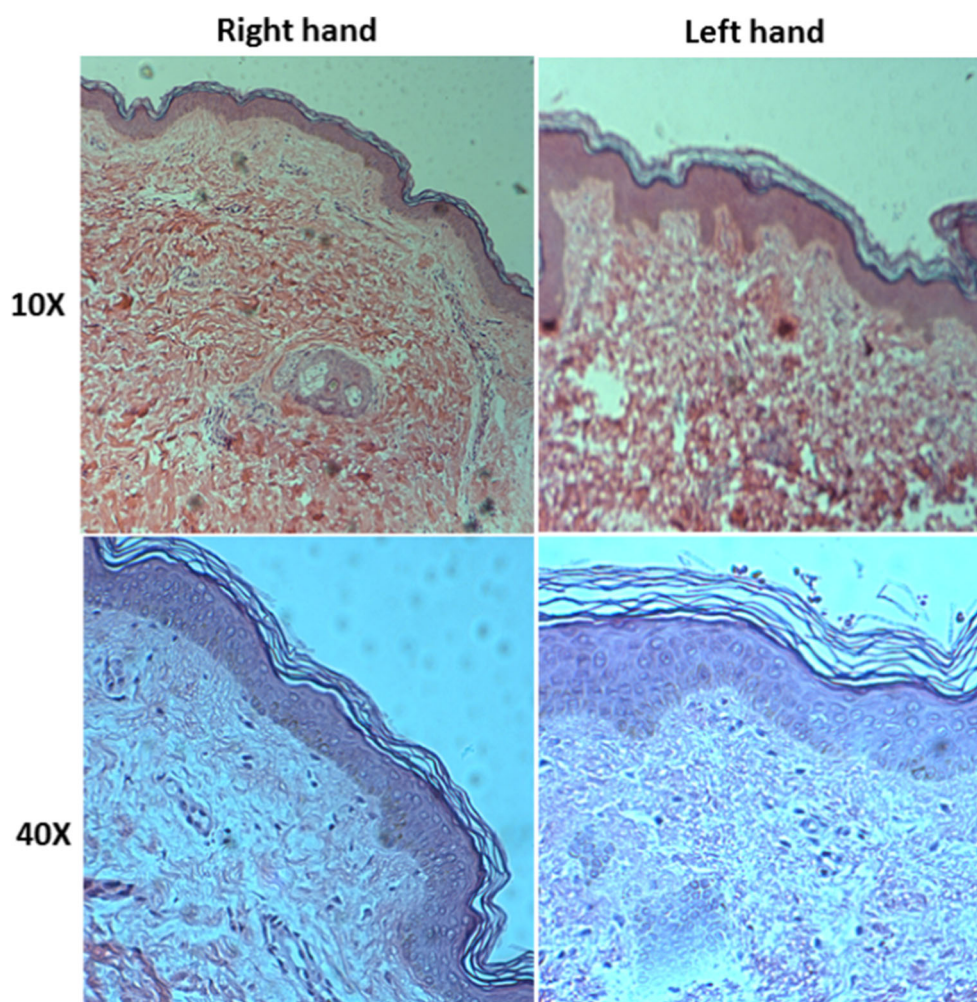
In-vivo study

Biophysical measurement with non-invasive techniques

Biophysical measurements of the application site were performed using different devices on various parameters at different times. The impaired barrier function of SC may play an important role in the development of AD. Restoration of barrier impairment is important to minimize the potential of AD. TG therapy has been used for decades as a standard treatment of AD. However, long-term use is associated with local adverse effects such as skin atrophy, telangiectasia, decreased barrier function, and decreased number of keratinocytes after 3–4 weeks of treatment, when evaluated by non-invasive methods or histologically [22]. In the present study, the application of NPs of TGs (HC) along with HT resulted in no distinct change in the skin as measured by non-invasive methods. All the biophysical measurements were conducted at the same room temperature and humidity (20 °C and 50 ± 10% humidity). Subjects were asked not to do any physical activities at least 15 min before the measurements. A change in TEWL represents a sensitive measure of the epidermal barrier function [37]. Application of an irritant on the skin may lead to delipidation and denaturation of proteins, which ultimately result in barrier dysfunction and, consequently, increased TEWL [37]. The difference in TEWL values between HC-HT CSNP AQ cream formulation and AQ cream (vehicle) was not statistically significant (Fig. 6). An increase in TEWL was observed on the 7th day in both groups. However, it was not significantly different from that at day 0. Moreover, the redness level of the skin by any irritants could be measured by the erythema level.

In this experiment, we measured the erythema intensity of the skin using a digital probe on pre-determined time intervals (Fig. 7).

Fig. 9 Comprehensive histopathological analysis of HC-HT CSNP and vehicle (right and left, respectively) treatment anterolateral areas of the human skin. Both were stained by H&E and observed at magnifications of $\times 10$ and $\times 40$



There were no significant changes between the formulation treatment group and the vehicle group. These results were further confirmed by the standard Draize skin irritation scoring system performed by a specialized dermatologist.

No erythema or irritation was observed based on the standard criteria of Draize skin irritation scoring system (Table 2).

A layer of lipid surrounding the comeocytes of SC prevents water loss from the skin as well as loss of NMF. Irritants cause a decrease in the lipid level, which may result in dry skin as well as susceptibility to certain skin diseases such as AD [37]. It has been reported that application of clobetasol propionate cream (TGs) on healthy skin for 3–4 weeks profoundly reduces the ceramide level [30, 31]. On the other hand, no change was detected in the melanin level, as determined by using the Sebumeter®, for 28 days (Fig. 8). In addition, no adverse reaction was reported during this study based on the patient's diaries.

Besides redness, skin irritation includes pain, pruritus, and burning sensation [32]. Skin irritation was determined by using the standard Draize skin irritation scoring method performed by a dermatologist as well as by using a digital sensory probe. It was important to correlate the irritation with the erythema level of the skin because it does not always describe the

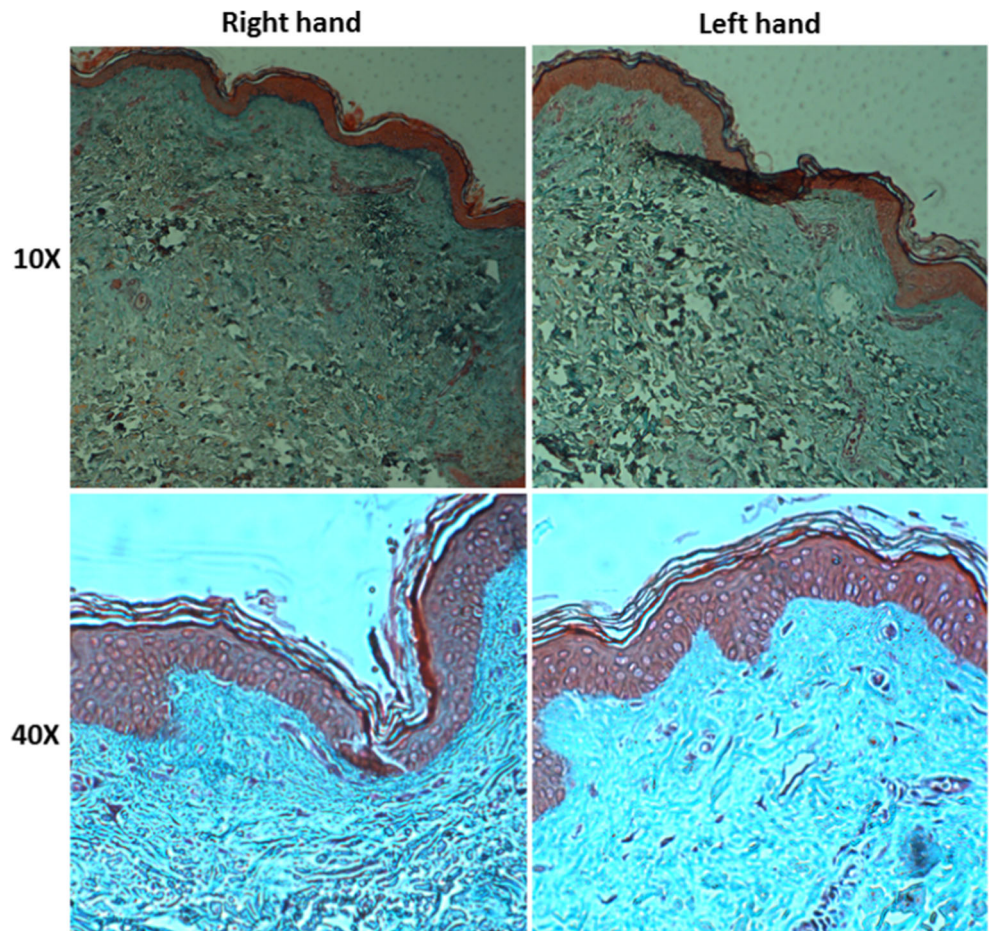
skin irritation well. The NP formulation did not cause any skin irritations as determined by Mexameter® as well as by using the standard Draize skin irritation scoring method. These results demonstrate that the NP formulation was well-tolerated and did not cause local adverse effects.

Systemic safety and tolerability

The safety of HC-HT CSNPs has been well-established through pre-clinical studies in rodents [19]. Furthermore, rodent toxicity data indicate that no adverse effect was observed (NOAEL) for 1000 mg/BSA/day in male and female rats [26]. Moreover, serum and plasma values of blood hematology and blood biochemistry of all healthy volunteers were normal. Besides, no significant difference was observed between values obtained before and at the end of the study. These present findings are consistent with the results obtained in healthy rats [26, 33].

The blood hematology results showed no significant change in Hb, RBC, and WBC (neutrophils, eosinophils, and monocytes) values before and at the end of the study (Table 3). It has been reported that application of TGs for 3–4 weeks causes an increase in the blood ACTH level. In

Fig. 10 Comprehensive histopathological analysis of HC-HT CSNP and vehicle (right and left, respectively) treatment anterolateral areas of the human skin. Both were stained by Mason's trichome and observed at magnifications of $\times 10$ and $\times 40$



contrast, the present study showed no change in ACTH level before and at the end of the study. These results suggested that positively charged polymeric CSNPs did not penetrate deep into the tissues and did not enter the systemic circulation (as in the rat skin) and that the small quantity of absorbed drugs was rapidly eliminated from the body [34]. As reported in the literature, long-term use of TGs causes hypertension by inducing Na^+ retention in the serum [35]. However, no increase in Na^+ level was observed after 28 days of application of HC-HT CSNPs (Table 3).

The liver enzyme level in the blood is usually measured to determine liver damage as well as overall liver health [36–38]. In the present study, no significant change was observed in ALT and AST levels before and at the end of study (after 28 days of application) (Table 4), indicating good liver health. It has been reported that application of HC cream for even a short period results in increased plasma cortisol level [39]. However, in the present study, 28 days of application of this HC-loaded NPs along with HT did not result in an increase in the cortisol level. The novel property of using the combination of drugs reported in this study provides better understanding of molecular interaction between the delivery system and drugs and the skin. This will provide a shift from current

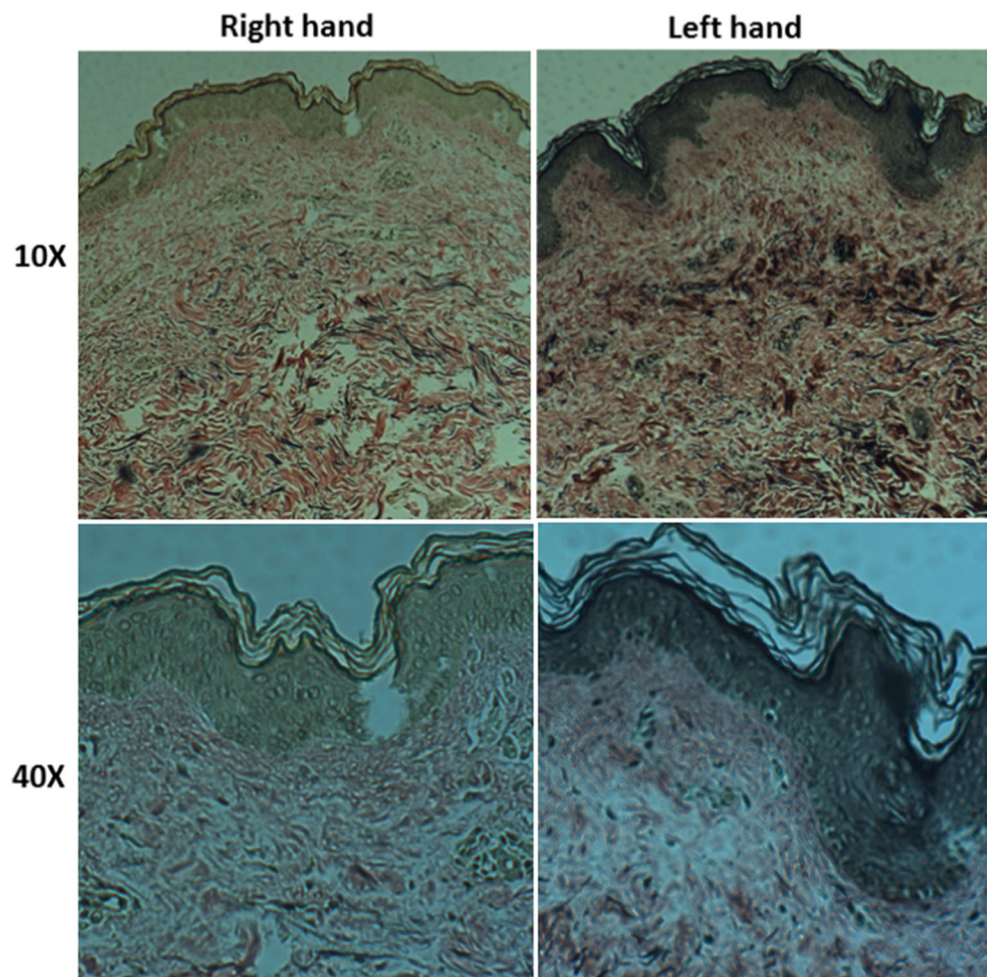
AD treatment concept by providing potential safety, clinical effectiveness, maintaining the normal anatomical infrastructure of the skin, and controlling pathological features.

Histopathology

The excised skin tissues from both the left and right application areas were histologically stained with H&E, Masson's trichome (MTC), and Verhoeff-Van Gieson (VVG) as shown in Figs 9, 10, and 11, respectively. Histopathological analysis of both right and left arms by H&E staining showed no sign of epidermal atrophy. Normal structure of the epidermal and dermal layers was observed as nuclei were detected in the cells (Fig. 9; right and left sections). Purple-colored nuclear chromatin material and pink-colored collagen fibers were observed in the tissue. Additionally, melanin pigment is produced and stored in the epidermis and acts as a barrier towards the sun's UV rays. As shown in Fig. 9 (left), at a high magnification of $\times 40$, melanocyte pigments were observed in both the right and left arm treatment groups.

MTC staining showed that nuclei were black, while the cytoplasm was stained in a reddish brown color (Fig. 10).

Fig. 11 Comprehensive histopathological analysis of HC-HT CSNP and vehicle (right and left, respectively) treatment anterolateral areas of the human skin. Both were stained by Verhoeff-Van Gieson and observed at magnifications of $\times 10$ and $\times 40$



Furthermore, both the right and left hands did not swell (edema) and did not show any degenerative changes. This finding was confirmed by MTC staining (Fig. 10) and VVG staining (Fig. 11), which indicated normal collagen, displaying no breakdown and fragmentation of reticular fibers in both HC-HT CSNP and vehicle treatment groups. Overall, there was no evidence of atrophy of the epidermal layer.

Conclusions

The results obtained in this study suggest that overhead mixing using spinning-disc technology by controlling the speed presents great potential for the preparation of NPs on a large scale with the same physicochemical characteristics as those obtained at the laboratory scale. HC-HT CSNPs were successfully prepared by the method with no significant change in their particle size, zeta potential, and morphology. The results of this study indicated that long-term use of HC-HT CSNP AQ cream was safe and well-tolerated as no sign of skin atrophy or irritation was observed. This study also confirmed that encapsulation of HC into CSNPs would not cause

systemic absorption and toxicity. Results of both preliminary quantitative and qualitative clinical data confirmed that nanodelivery was safe and well-tolerated, so further controlled studies are being conducted to authenticate the findings of this study and to determine more meticulous treatment regimen with HC-HT CSNPs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The study was approved by the Research Ethics Committee of the National University of Malaysia (UKM FPR.4/244/FF-2015-275). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional

and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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