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Investigations on skin permeation of hyaluronic acid based nanoemulsion as transdermal carrier

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ABSTRACT

An alcohol-free oil/water hyaluronic acid nanoemulsion was developed to be applied as transdermal carrier for active lipophilic ingredient. In vitro hemolysis, skin penetration and histological examinations were carried out using α-tocopherol as model ingredient to assess skin permeability and bioavailability. Without any chemical enhancers, nanoemuslion performed desirable skin permeable capacity, being able to penetrate across stratum corneum and diffuse deeper into dermis compared with the control group (ethanol solution) via follicular and intercellular pathway. Penetration mechanism was preliminarily studied and suggested to be closely concerned with transmembrane concentration gradient, carrier characteristics and penetration enhancers. No irritation has been found in dermis and skin surface indicated hyaluronic acid nanoemulsions could be successfully used as purcutaneous delivery carrier of active lipophilic ingredient and favorable for drug and cosmetic applications.

1. Introduction

Skin is the largest organ in the body whose major function is to protect the body from dehydration and unwanted effects from the external environment (Schäfer & Redelmeier, 1996). Although the protective and impermeable qualities of the skin protect the organism from losing water, minerals and dissolved proteins, percutaneous delivery system has been taken advantage as a topical delivery system in pharmaceutical or cosmetic industries. In contrast to traditional drug administration pathway, transdermal administration is featured by its noninvasive procedure, which eliminates side effects, increases patient compliance and possibility for continuous and controlled drug absorption (Lee, Lee, Kim, Yoon & Choi, 2005). Besides, being a vital organ, the skin must be nourished as the other organs of the body by means of cosmetic formulations (Souto & Müller, 2008).

The stratum corneum (SC), the outermost nonviable layer of the epidermis, comprises flattened, stacked, hexagonal, and cornified cells (corneocytes or horny cells) anchored in a mortar of highly organized intercellular lipids, which is described as brick and mortar model (Contreras & Elizabeth, 2007). The corneocytes are surrounded by hydrophobic lipid bilayers which aligned approximately parallel to the surface of corneocytes, while intercellular spaces are abundant in SC lipids (Bouwstra, Pilgram, Gooris, Körten & Ponec, 2001). The particular structure is considered as the rate controlling barrier in the transdermal absorption of substances, which directly relate to lipophilicity of substances (Mahmoud, 2010). However, lipophilic molecule does not favorably partition out of the SC into the more aqueous viable epidermis (Potts, Bommannan & Guy, 1992), hindering deeper diffusion and further capillary uptake into circulation. In comparison, hydrophilic substances penetrate across the skin harder than lipophilic penetrants and as low as 10^{-4}-fold differences in flux can be noted (Sznitowska, Janicki & Williams, 1998).

In order to overcome the epidermal barrier as to increase transdermal transport, various transdermal carrier systems have been developed. Due to the permeability of a substance through the skin is inversely related to its size under certain conditions (Mahmoud, 2010), nanoparticulate systems investigations are promising, such as solid lipid nanoparticle (SLN), nanostructured lipid carrier (NLC), liposomes, microemulsions, and hexagonal phase nanodispersion (Küchler et al., 2009). In addition, as a good transdermal delivery system, beyond allowing desirable amounts of active ingredient to overcome the skin barrier, the material has to be biocompatible, preferentially biodegradable, or at least should be able to be excreted (Vauthier, Dubernet, Fattal, Pinto-Alphandary & Couvreur, 2003), ensuring non-irritancy to skin and sustain ingredients be active on the skin's surface or during the permeation process (Langer, 2004). Among those transdermal carrier systems, nanoemulsions appear to be attractive and competitive.

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Nano-sized emulsions are a class of stable emulsions composed of surfactant and oil suspended in water with a particle diameter ranging 50–200 nm (Kong & Park, 2011). Nanoemulsions seem to be ideal liquid vehicles for drug delivery since they provide all the possible requirements of a liquid system including easy formation, low viscosity with Newtonian behavior, high solubilization capacity for both lipophilic and hydrophilic ingredients, and very small droplet size (Kogan & Garti, 2006). The small droplets confer nanoemulsions large surface to volume ratio, favoring to close contact with the skin providing high concentration gradient and improved substance permeation. Moreover, low surface tension ensures better adherence to the skin. Also, the dispersed phase can act as a reservoir making it possible to transport bioactive molecules in a more controlled fashion (Elena, Paola & Maria, 2001).

Hyaluronan (hyaluronic acid, HA) is a sort of naturally occurring polymer, composed of unbranched repeating units of glucuronic acid and N-acetyl glucosamine linked by β1–3 and β1–4 glycosidic bonds (Ambrosio, Borzacchiello, Netti & Nicolais, 1999). Properties of HA including specific viscoelasticity, biocompatibility, hydration and lubrication (Garg & Hales, 2004) make this polysaccharide potentially very useful in the food, medical and cosmetic industries. By means of HA based nanoemulsion, a promising transdermal carrier was developed in previous study, whose transdermal capacity and bioavailability were investigated in this article. In-vitro percutaneous penetration was studied quantitatively and qualitatively using α-tocopherol as model lipophilic ingredient.

2. Materials and methods

2.1. Materials

Sodium forms of HA with a molecular weight (MW) of 110 kDa and 10 kDa were a gift of Kolon Life Science, Korea. EDC and monostearin (glycerol α-monostearate, GMS) were purchased from Tokyo Kasei Kogyo Co., Ltd (Japan). N-Hydroxy succinimide (NHS, 97%) was acquired from Aldrich Chemical. Methylene chloride, calcium chloride dehydrate, Sodium Bromide, Triton X-100 and acetonitrile (HPLC grade) were purchased from Duksan Pure Chemical (Korea). Phosphate buffered saline (pH 7.4), α-tocopherol (97%) (Vitamine E, VE), Rhodamine, Xylenes (histological grade) were purchased from Sigma–Aldrich. POE (20) Sorbitan monooleate (Tween 80) and Sorbitan Monolaurate (Span 20) were purchased from Samchun Pure Chemical (Korea). Tissue-Tek® O.C.T™ Compound was ordered from Sakura Finetek USA, Inc. Water, used for synthesis and characterization was purified by distillation, deionized and subjected to reverse osmosis using a Milli-Q Plus apparatus (Millipore, USA). All the chemicals were analytical grade and were used as received.

2.2. Preparation of HA-GMS nanoemulsions

Different batches of amphiphilic HA-GMS were synthesized by a previous method (Kong, Chen & Park, 2011), labeled as H6.5, H23 and L6 (capitals denote MW property of HA and the latter numbers are substituting degree of HA-GMS). Nanoemulsions were prepared through oil/water/surfactant (O/W/S) emulsifying system and solvent evaporation as previous paper (Kong & Park, 2011). Briefly, HA-GMS solution was the continuous phase, methylene chloride as oil phase, and Tween 80 and Span 20 (HLB = 12.5) as surfactants. The three components comprised in a mass ratio of 95/2/3. The disperse phase was added dropwise to a HA-GMS solution to form a coarse emulsion, which was further pulse-sonicated twice (pulse on, 10.0 s; pulse off, 2.0 s), using 0.05% $CaCl_2$ as crosslinking agent.

2.3. In vitro hemolysis assay

Hemolysis assay was performed using citrated human whole blood obtained from a healthy donor with permission. 10 mL blood sample was centrifuged (1500 g, 10 min). The supernatant was discarded and the erythrocytes obtained were resuspended in PBS (pH 7.4) and washed three times to remove debris and serum protein. The pellet was weighed and a 2% (w/w) erythrocytes stock dispersion was prepared in PBS (pH 7.4) that should be stored in 4 °C for a maximum of 24 h. The approximate amount of red blood cell was 3.25×10^8 cells/mL. A 0.5 mL aliquot of the erythrocyte stock dispersion was added per milliliter of nanoemulsion samples at different concentrations (0.50, 0.75 and 1.0 mg/mL). The mixtures were incubated up to 6 h at 37 °C to evaluate the influence of time on hemolysis in the presence of nanoemulsions. At predetermined intervals, oxyhemoglobin level was measured spectrophotometrically at 540 nm. Prior to measurements, the incubated mixtures were centrifuged (1500 g, 10 min) to remove unlysed erythrocytes and debris, while the supernatants were collected and incubated for another 30 min. The haemolysis given by the Triton X-100 solution (5%) was considered to be 100% while the haemolysis given by the PBS (pH 7.4) was taken as 0%.

2.4. In vitro skin penetration study

2.4.1. Rat skin preparation

Male Wistar Hannover rats (males, 200–250 g, Samtako Bio Korea) were sacrificed with prolonged ether anaesthesia and hairs on the skin were removed with electrical clipper. The full thickness dorsal skin of each rat was excised, subcutaneous tissues were carefully removed with forceps and surgical scissors. The skin was washed with PBS (pH 7.4) and blotted to dry, wrapped in aluminium foil and stored in polyethylene bag at −20 °C less than 4 weeks. All investigations were conducted in accordance with the guidelines of KOREA UNIVERSITY Institutional Animal Care & Use Committee.

2.4.2. Preparation of stratum corneum (SC)

The preparation of SC followed the reported method with certain modifications (Scott, Walker & Dugard, 1986; Scott, Dugard, Ramsey, & Rhodes, 1987). The skin was treated with 2 M sodium bromide solution in distilled water for 90 min at 25 °C. The SC from full thickness skin was separated using cotton swab moistened with water. SC sheet was cleaned by rinsing with PBS and blotted to dry and examined for cuts or holes if any. SC samples were spread carefully and identified the epidermal and dermal sides, packed with aluminium foil and stored at −4 °C till further use within 24 h.

2.4.3. Skin penetration

Skin permeation studies were performed on a group of modified static-type amber franz cells (diameter 17 mm, volume 15.88 mL, Daihan Labtech Co., Ltd, South Korea). The SC sheets were brought to room temperature and mounted between the donor and receiver compartments of the diffusion cells where the epidermal side faced upwards and the dermal side faced downwards. The residues were trimmed. Initially, the donor compartments were empty and the receiver chamber was filled with degassed PBS (pH 7.4). The acceptor fluid was stirred magnetically at a speed of 700 rpm and the assembled apparatuses were adjusted and maintained at 37.2 °C with the aid of digital thermo-controller. After equilibration for 30 min the receivers liquid was replaced with fresh one to the scale. 2 mL Nanoemulsion (1.0 mg/mL), loaded with VE (1.0 mg/mL) as modal active ingredient, were added to donors and screwed with caps to provide occlusive conditions. 1 mL samples were withdrawn at intervals (0.5, 1, 2, 4, 6, 8, 12 and 24 h) and replenished with 1.5 mL fresh degassed PBS. The whole process of manipulation should be done with great care to avoid formation of bubbles. The

samples were filtered through 0.45-mm membrane filter and analyzed for drug content by HPLC at 280 nm (Kong & Park, 2011). VE ethanol solution (1.0 mg/mL) was taken as control group for penetration contrast to nanoemulsion formulation. The experiments were repeated with the skin of at least another two donor rats.

2.4.4. Data analysis

Cumulative active ingredient penetration (Qt, μg/cm^2) through the skin was calculated from the following equation (Sintov & Shapiro, 2004):

$$Qt = VrCt + \sum_{i=0}^{t-1} VsCi \quad (1)$$

where Ct is the drug concentration of the receiver solution at each sampling time, Ci is the drug concentration of the ith sample, and Vr and Vs are the volumes of the receiver solution and the sample, respectively. Data were expressed as the cumulative drug permeation per unit of skin surface area, Qt/S. The steady-state fluxes (Jss, mg/cm^2 h) were calculated by linear regression interpolation of the experimental data at a steady state:

$$Jss = \frac{\Delta Qt}{(\Delta t \times S)} \quad (2)$$

Apparent permeability coefficients (Kp, $\times 10^{-3}$ cm/h) were calculated according to the equation:

$$Kp = \frac{Jss}{Cd} \quad (3)$$

where Cd is the active ingredient concentration in the donor compartment, and it assumed that under sink conditions the drug concentration in the receiver compartment is negligible compared to that in the donor compartment.

2.4.5. Fluorescence observation

To study the nanoemulsion localization within the transdermal penetration, nanosemulsion was labeled with rhodamine as previous paper (Kong et al., 2011) and subjected to fluorescence observation. Full thickness rats dorsal skin without subcutaneous tissue were applied to catch a whole view of penetration. 2 mL Rhodamine labeled nanoemulsion, loaded with VE, was added to donor apparatus and sealed with screw cap. After 10 h exposure, the skin was removed, cleaned remaining formulation and frozen at −80 °C. Cryotome sections of 30 μm thickness (LEICA CM 3050S, Germany) were subjected to normal and fluorescence light microscopy (Axioplan 2, ZEISS HBO100 equipped with AxioCam MHc Carl Zeiss microimaging GmbH, Germany).

2.5. Histological examination of skin

Full thickness rats dorsal skin without subcutaneous tissue were exposed to nanoemuslion using modified franz cells for 10 h. The exposed region was dehydrated using ethanol, replaced ethanol with xylene, embedded in paraffin for fixing, subjected to paraffin vertical sections (Thermo Scientific Microm HM 340 E Rotary Microtome, Germany), stained with haematoxylin and eosin. These samples were then observed under light microscope (Olympus CX40 equipped with DigiCAM II, Olympus, Japan) and compared with the control sample that was free of exposure to nanoemulsion.

2.6. Statistics

All statistical evaluations were performed by OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA). Statistical significance was found by One-Way ANOVA, Tukey test was used to analyze differences. The presented data (arithmetic mean value ± standard deviation) resulted from at least three independent experiments ($n \geq 3$), the value of $p \leq 0.05$ was considered as significant.

3. Results and discussions

3.1. In vitro hemolysis assay

Three different formulations of HA-GMS nanoemuslion free of VE were taken for hemolysis assays (Table 1). There was no pronounced size variation among samples regardless of VE loading. As seen in Fig. 1, concentration and time dependent behaviors were observed for either sample. After 1 h of incubation, there was no significant difference between control and treated groups ($p \leq 0.05$). The amounts of hemoglobin release (absorbance at 540 nm) increased as concentration ranged from 0.5 to 1.0 mg/mL, for H6.5, began with 1.0% (the absorbance of the samples/the absorbance of Triton-X100) to 3.9%, 1.6–6.4% for H23 and 1.6–2.8% for L6. This trend persisted till the end of 6 h treatment and led to significantly different level of hemolysis compared with the control ($p \leq 0.05$), where the lowest was 31.7% for L6 and the highest was even 56.6% occurred at concentration of 1.0 mg/mL, which were 11.3 and 8.8 folds individually higher than their counterparts after 1 h. The results suggested both long-time and high-dose exposures to nanoemulsion could render heavy hemolysis. Moreover, cross-

Table 1
Size variations and E.E. of HA-GMS nanoemulsions.

	VE free[b]		VE loading[c]		Encapsulation efficiency[d] (E.E.) (%)
	Size (nm)	PdI	Size (nm)	PdI	
H6.5[a]	48.2 ± 0.5	0.42	56.7 ± 0.3*	0.420	53.5 ± 3.2
H23	53.8 ± 0.3	0.38	59.1 ± 0.2*	0.416	70.3 ± 2.2
L6	52.3 ± 0.3	0.31	57.3 ± 0.2*	0.260	93.9 ± 1.4

* Statistically significant difference ($p \leq 0.05$, One-Way ANOVA) from the data obtained free of VE.
[a] Capitals denoted MW property of HA and numbers were DS of HA-GMS.
[b] Nanoemulsions were prepared at 1.0 mg/mL and crosslinked using 0.05% $CaCl_2$.
[c] Concentration of loaded VE (α-tocopherol) was 1.0 mg/mL.
[d] E.E. was calculated as the percentage of ratio of encapsulated VE mass to total VE mass.

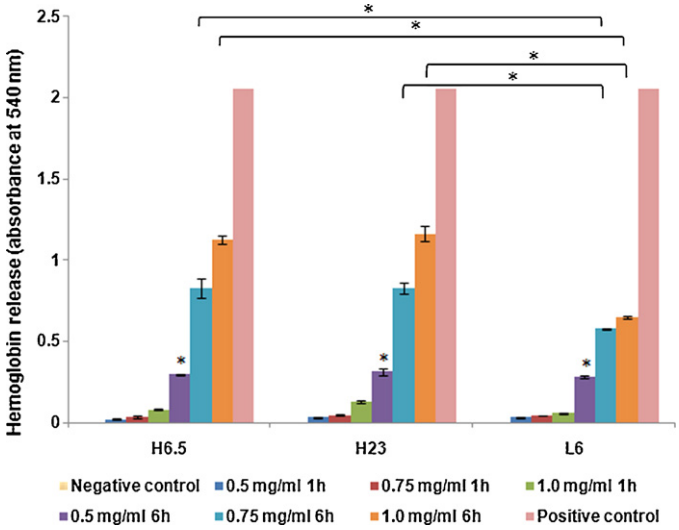

Fig. 1. The effects of different concentrations, time of the HA-GMS nanoemusions on hemoglobin release from the erythrocytes after 1 and 6 h of incubation. Mean ± S.D. ($n = 3$) of the data in each group are shown in each bar. The P values were obtained by One-Way ANOVA ($p \leq 0.05$). Triton X-100 (5%, v/v) was considered as the positive control and PBS (pH 7.4) as negative control.

comparison showed that H23 turned out to be the most hemolytic sample and L6 was relatively the most biocompatible one, whose hemolytic level was significantly lower than another two samples ($p \leq 0.05$).

3.2. Skin penetration

Owing to the least toxic performances based on the hemolysis assay and highest capacity in VE encapsulation efficiency (Table 1), L6 was taken as tested formulation in skin penetration experiment. VE ethanol solution (1.0 mg/mL) was taken as control group and PBS (pH 7.4) as acceptor liquid. As shown in Fig. 2, over 24 h's exposure to treatments, ever no presence of VE has been detected in acceptor liquid for the control group. Conversely, nanoemulsion displayed continuous permeation of VE 1 h after the beginning, and the cumulative permeation of VE in acceptor liquid turned out to be irregularly and approximately linear to testing time. The increment seemed to dramatically slow down from 8 h to 24 h, which varied from other reports that also used nanoemulsions as vehicles but displayed no platform stage within treatment period (Lee et al., 2005; Sintov & Shapiro, 2004; Zabka & Škoviera, 2003). Various factors, including test duration, sampling frequency, sink condition, stirring rate, diffusion membrane material and so on, may result in such differing phenomena. For in vitro skin penetration test via passive diffusion, sink condition regarding acceptor concentrations ≤10% of the donor concentrations is a prerequisite demand to be met to attain a desirable permeation profile (Li, Zhang, Zhu, Higuchi, & White, 2005). In this experiment, the VE concentration in acceptor liquid at 24 h was 8.29 ± 2.88 μg/mL, its being less than 1% of the donor concentration suggested the whole diffusion process was in perfect sink condition. Stirring rate was 700 rpm and did not create any vortex that was undesirable due to its potential to disrupt the static fluid layer adjacent to the membrane (Shiow-Fern, Jennifer, Francis, Victor & Gillian, 2010). Therefore, the real reason probably ascribed to test duration and sampling frequency. In other work, the test duration was not longer than 12 h typically shorter than ours, which might miss the possible platform stage that occurred (Lee et al., 2005; Sintov & Shapiro, 2004). Moreover, long time hydration of skin in donor samples may also bring changes to SC structure and affect the SC partition coefficient subsequently. It was reported that infrequent sampling did allow active ingredient concentrations in the receptor chamber to approach the sink condition limit (Shiow-Fern et al., 2010). In this test, sampling intervals were prolonged from 1 or 2 h in the pre-period to 4 h and even 12 h in the post-period. The mass accumulation of VE in acceptor liquid could also occur here and hinder continuous penetration afterwards, although the whole process fulfilled the sink condition.

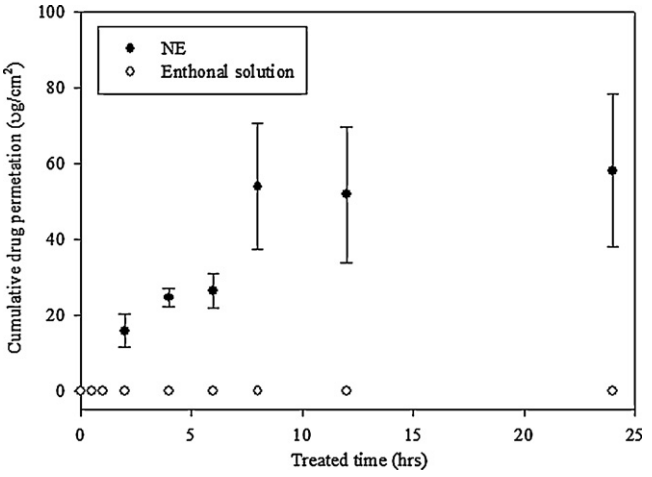

Fig. 2. Permeation profiles of vitamin E through the excised rat skin from HA-GMS nanoemulsion formulation (L6). The control sample was ethanol solution of VE (1.0 mg/mL) (Mean ± S.D., $n = 4$).

Table 2
In vitro percutaneous permeation parameters of VE in nanoemulsion and solution form through excised rat dorsal skin (Mean ± S.D., $n = 4$).

Formulation[a]	*Jss* (μg/cm² h)	*Kp* (×10⁻³ cm/h)	*Qt*/*S* (μg/cm²)
L6 nanoemulsion[b]	14.68 ± 4.13	14.68 ± 4.13	58.01 ± 20.15
Ethanol solution	–[c]	–	–

[a] Concentration of VE (α-tocopherol) was 1.0 mg/mL.
[b] Capitals denoted MW property of HA and numbers were DS of HA-GMS.
[c] No amount of VE in acceptor liquid has been detected.

The specific permeation parameters were listed in Table 2. The skin permeability appeared to be able or not for VE nanoemusion and solution. No detection of VE in acceptor liquid was able to offer evidence that VE in solution form was impermeable to SC layer. The lipid base of SC structure facilitated absorption of lipophilic substance like VE, rather than favored to partition out of the SC into the more aqueous viable epidermis, as aforementioned in introduction part. Failure of VE detection in acceptor liquid verified the inefficient partitioning capacity of SC layer. For nanoemulsion, the steady-state fluxes (*Jss*, mg/cm² h) was lower than similar study (Sintov & Shapiro, 2004) who took microemulsion as drug delivery vehicle on rat abdominal skin and showed higher than 60 mg/cm² h in their optimal formulations (MEb7A, MEb7B, and MEb7C) containing 2.5% lidocaine. It deserved to be clear that the notable *Jss* was obtained at cost of high content of surfactant and co-surfactants (60–70%, w/w), which were able to play roles of penetration enhancer and not desirable for biocompatible considerations (Kogan & Garti, 2006). In contrast, surfactant content was fixed and accounted merely for 3% (w/w) in our formulations. While in another study (Lee et al., 2005), whose *Jss* was 12.35 ± 1.14 for formulation G containing 1.5% (w/w) aceclofenac and absent of enhancers, which was parallel to our 14.68 ± 4.13 mg/cm² h. No additional enhancer was applied in this experiment either. In addition, the acceptor liquid used in this study was PBS (pH 7.4) without blending alcohol component to strengthen sink conditions. Better permeable efficiency of HA nanoemulsion could also be reflected from the apparent permeability coefficients (*Kp*, $\times 10^{-3}$ cm/h), $14.68 \pm 4.13 \times 10^{-3}$ cm/h, although the VE concentration was 1 mg/mL or 0.1% (w/w). It was able to be concluded that two principal factors govern the penetration of lipophilic ingredient from nanoemulsion—penetration enhancer and donor sample concentration. Increase of donor concentration or application of penetration enhancer was able to strengthen percutaneous penetration efficiency.

In order to clarify the preliminary percutaneous penetration mechanism, besides the quantitative measurement, qualitative studies were also conducted to catch the direct impression of the process. According to the skin penetration data, full thickness rat dorsal skin was treated with L6 formulation for 10 h to accomplish an adequate penetration. As shown in Fig. 3, typical native and the corresponding fluorescence images of the formulation, fluorescence emitted by rhodamine labeled nanoemulsion provided direct evidences of percutaneous penetration, verifying the successful permeation across SC layer and deeper diffusion (panel A, B, × 50). It was also observed that different exposed points on skin samples showed differing extent of penetration. Panel A, derived from the margin of the treated area, showed limited penetration that appeared to stop on the boundary of SC layer and the topmost layer of the epidermis. By contrast, panel B, derived from central treated area, showed deeper diffusion into dermis. This location dependent phenomena probably arose from hydrokinetic variations that, despite vortex was avoided in manipulation, agita-

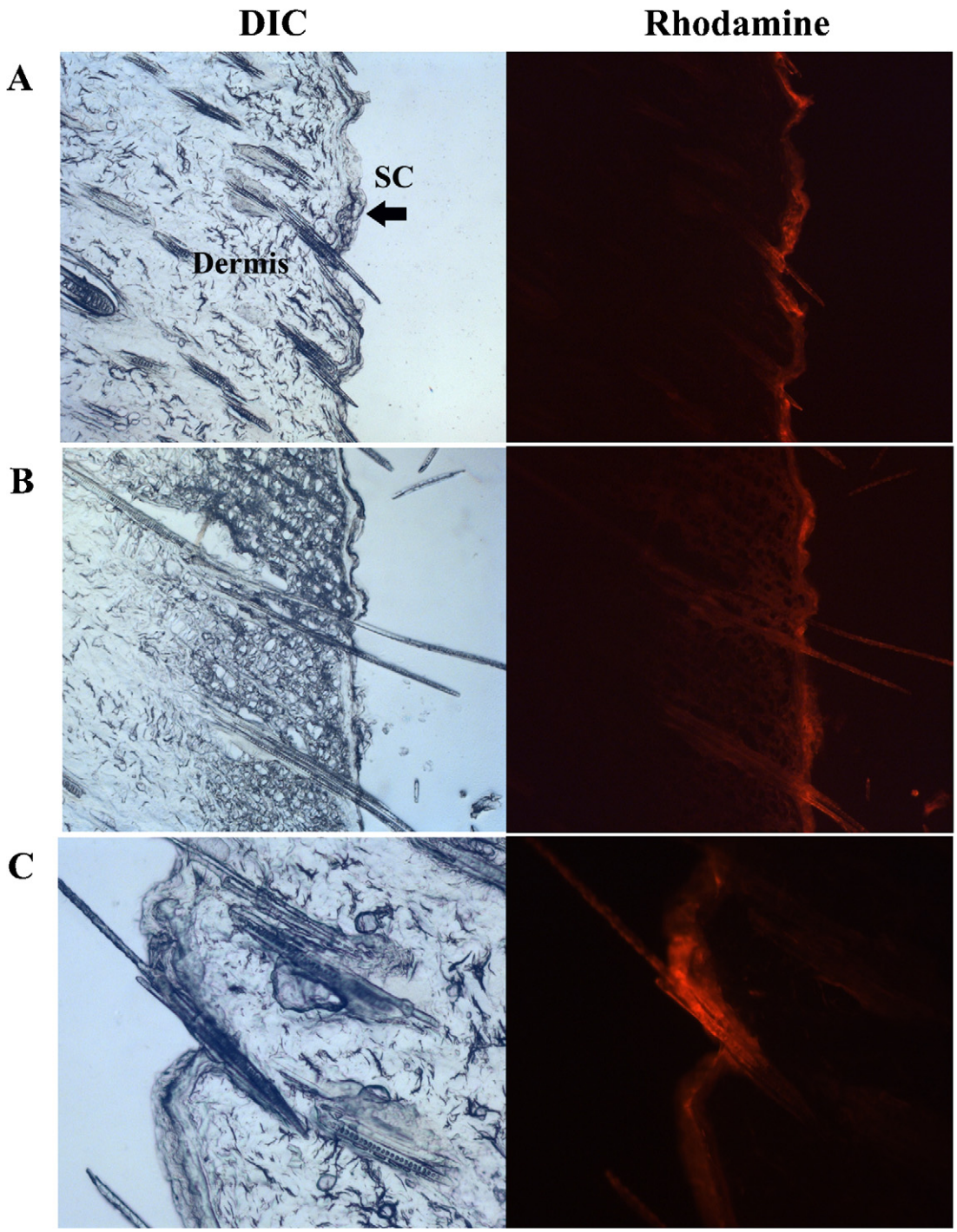

Fig. 3. Penetration of rhodamine labeled HA-GMS nanoemulsion (L6) into rat skin after 10 h exposure. (A) The margin area of treated skin sample (50×); (B) the central area of treated skin sample (50×); (C) magnification of follicle part (100×). Images are obtained in DIC and fluorescence mode of the same area.

tion still have brought heterogeneous and mild mass dispersion in acceptor liquid. Central area of treated skin sample offered better accessibility as well to donor formulation than the margin.

With regard to the pathway of skin penetration, there are four accessible pathways: intercellular, transcellular, pilosebaceic and the polar pores pathway (Kogan & Garti, 2006). Thereinto, the polar pores pathway could be regarded as sort of intercellular pathway. In SC's mortar and brick structure, the corneocytes are surrounded by hydrophobic lipid bilayers make them resistant to hydrophilic substances, like HA nanoemulsion in this study. Hence, transcellular pathway seemed to be impossible for nanoemulsion to penetrate. Pilosebaceic, known as follicular pathway, is surely proven to be effective in drug delivery especially for hydrophilic drugs (Jacobi, Toll, Sterry & Lademann, 2005) although their surface area covers only 0.1–1% of the total surface area of human skin (Kogan & Garti, 2006). Evidence was also provided by fluorescence image (panel C, × 100), where the site of follicle showed strong fluorescent intensity. Another important pathway for hydrophilic substances is intercellular, which is staggered and the polar pathway of penetration consists of the aqueous micropores localized intercellularly, between the laminar lipid structures (Sznitowska et al., 1998).

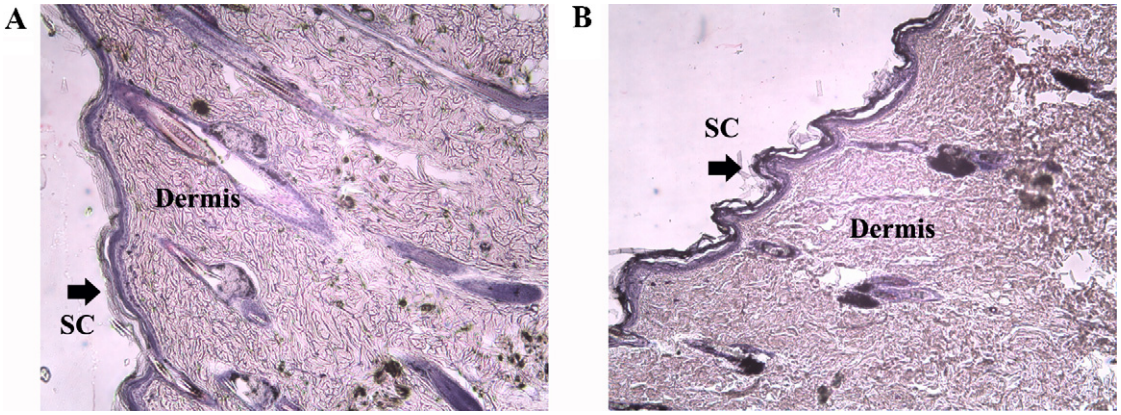

Fig. 4. Histological microimages (HE staining) of rat skin samples after 10 h exposure. (A) Control group showing normal epidermis, dermis and subcutaneous tissues (100×); (B) Skin sample from nanoemulsion treated skin (100×).

An effective purcutaneous drug delivery should be able to transport active ingredients not just across SC and perform good partitioning capacity into deeper tissue structures, while an effective cosmetic ingredient delivery emphasizes on skin retention rather than skin permeability. The transport of molecules across the skin through passive diffusion means that the solute flux was linearly dependent on the solute concentration gradient (Potts & Francoeur, 1991; Scheuplein, 1976), and the donor solvent concentration acted ignorable roles. In this skin penetration experiment, nanoemulsion was tried to be prepared with deionized water instead of PBS (pH 7.4), but no detection of VE has been identified in whatever kind of acceptor liquid even comprised PBS/isopropyl alcohol (50/50, v/v) and Tween 80 (10%, w/w) (data not shown). In addition, the transport are influenced by carriers characteristics and penetration enhancer, nevertheless, the latter typically induce skin irritation or sensitization, cause damage and reduce the barrier function for a rather long time. Concerning carrier characteristics, vesicle size and rigidity are primary to be considered. O/W nanoemulsion is sort of liquid vesicle composed of oil ingredient inside, surfactants interface and aqueous phase surrounded. Amphiphilic HA-GMS actually acted surfactant role and HA conferred superior water retention capacity on droplets surface. Being flexible, they were capable of adapting their shape and volume when passing through the SC. By contrast, classic rigid liposomes ruptured during transport through the SC (Dayan, 2005). Nanoemulsion also created a large surface-to-volume ratio for emulsion droplets that contacted skin, so more active ingredients got access to the skin in the surface-to-surface interaction between the emulsion and the skin. Moreover, curvature definitions of small emulsion surface components could affect emulsion efficacy and interaction characteristics with skin (Yechiel & Coste, 2005). In conclusion, nanoemulsion indicated desirable purcutaneous potency of lipophilic active ingredients for either drug delivery or cosmetic applications.

3.3. Histological observations

The image of untreated rat skin (control, Fig. 4A) showed normal status with well-defined epidermal and dermal layers. SC layer was intact and lied adjacent to the topmost layer of the epidermis. Dermis was free of any inflammatory cells and skin appendages were in normal status. When the skin was treated with nanoemulsion formulation (L6) for 10 h (Fig. 4B), the SC layer showed seemingly rigid that appeared to be fragile and lost its flexibility. Inter-lamellar gaps were extensively existed between SC and the topmost layer of the epidermis. These symptoms probably attribute to the lipid extraction from SC layers by nanoemulsion, which led to dehydration of SC with significant loss of moisture (Shakeel, Baboota, Ahuja, Ali & Shafiq, 2008), and might attribute to the hydration effect of HA. It worth noting there that certain degree of increasing intercellular spaces present in dermis, which also could be explained by HA's hydration effect. HA is a fundamental component of the extracellular matrix, in particular as intercellular space filler (Allison & Grande-Allen, 2006), which maintains the intercellular space within the dermis. The intercellular existence of HA in dermis in this study was confirmed by fluorescence image (Fig. 3B), where the diffusion of rhodamine fluorescence in dermis was clearly observed. This natural occurring moisture retention capacity of HA makes it a desirable moisturizing ingredient in cosmetics, such as Restylane® (USA), a commercially non-animal stabilized hyaluronic acid based dermal filler, which is used to replenish the dermal volume loss that allows wrinkles to form. Nevertheless, moisturizing capacity is just one of HA's various desirable properties (Collagen Nutraceuticals, 2006).

Dermis did not show any sign of inflammation (Fig. 4B) and no apparent signs of skin irritation (erythema and oedema) were observed through visual examination of the skin sample treated with nanoemulsion formulation. The results indicated this alcohol-free HA nanoemulsion containing no chemical enhancers, could improve drug penetration without causing skin irritation and bring benefit to skin care.

4. Conclusion

HA nanoemulsion targeting to be applied as transdermal carrier for active lipophilic ingredient has been developed in previous studies, whose stability and delivery potential have been verified. Concerning skin penetration capacity and bioavailability were assessed in this paper. In vitro hemolysis, skin penetration and histological examinations were carried out using α-tocopherol as model ingredient. Among the test formulations (H6.5, H23 and L6), L6 was the most biocompatible while H23 was the most hemolytic. It was noted that, in absence of any penetration enhancers, formulation L6 performed desirable SC permeability, efficient portioning capacity and available diffusion into deeper dermis compared with the control group (ethanol solution). Moreover, no irritation was found in dermis and skin surface. The penetration process was closely associated with transmembrane concentration gradient, carrier characteristics and penetration enhancers. The results suggested nanoemulsions could be successfully used as purcutaneous delivery vehicle of active lipophilic ingredient, and HA was favorable as a role in skin care for drug and cosmetic applications.

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