



Research paper

Improved transdermal delivery of morin efficiently inhibits allergic contact dermatitis

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ABSTRACT

The skin is an important site for local or systemic application of drugs. However, most of the drugs have poor permeability through the skin's outermost layer, stratum corneum. The aim of this study was to develop a method to enable transdermal delivery of morin (3, 5, 7, 2, 4-pentahydroxyflavone), which is a poorly water-soluble drug with anti-inflammatory properties obtained from natural products. Morin phospholipid complex (MPC) was prepared and then loaded in Carbopol 940 hydrogel (MPC-gel), which can significantly increase the transdermal flux of morin based on the *in vitro* skin penetration data presented in this paper. To further enhance permeation, different compositions of penetration enhancers were dispersed in the gel and screened. After applied onto the mouse skin, MPC-gel showed apparent reduction of ear swelling in 2, 4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD). Further determination of cytokines levels, histopathological analysis and T lymphocytes proliferation indicates that the MPC-gel is potent enough to reduce the inflammatory response mediated by the DNFB in ACD mice model. Collectively, we anticipate that such an approach may provide a new treatment for topical ACD.

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1. Introduction

Stratum corneum (SC), the uppermost layer of the skin, is a challenge for transdermal drug delivery and is the main physical barrier to drug permeation (Vitorino et al., 2013). Over the past decade, successful attempts to solve this problem have been made through chemical permeation enhancers, microneedles, electroporation, ultrasound and low-frequency ionophoresis, but success has been limited (Han and Das, 2013; Lane, 2013; Tuan-Mahmood et al., 2013). The potential for skin irritation associated with chemical permeation enhancers and the invasiveness of microneedles, and ease of application of some electrical devices all have their disadvantages with respect to transdermal delivery. The complexity and expensive cost of production of many transdermal drug delivery systems may also deter clinical applications of these systems. The use of chemical penetration enhancers (CPEs) may be considered as a relatively more cost-effective approach. These CPEs

have been used in transdermal research since the 1960s (Aqil et al., 2007; Barry, 2004). Terpenes, such as menthol, cineole and limonene, obtained from natural sources are generally considered as safe and effective penetration enhancers, where the Food and Drug Administration classifies terpenes as generally regarded as safe (GRAS) (Vaddi et al., 2002).

Allergic contact dermatitis (ACD) is a common chronic skin inflammatory disorder caused by T cell-mediated delayed hypersensitivity of type IV, which has a significant impact on patients' quality of life (Fonacier et al., 2015; Kaplan et al., 2012). Approximately, 15–20% of the world's population suffer from this condition (Keegel et al., 2009; Shi et al., 2015). Haptens, such as 2, 4-dinitrofluorobenzene (DNFB) could easily penetrate into the skin. Dendritic cell (DC) maturation is induced after the haptens entry into the epithelia and covalently bind to carrier proteins to cause a full immunogenic response (Kadowaki, 2007). Therefore, *in vivo* models of ACD are often used to investigate Ag-specific T cell-mediated immune responses (Saary et al., 2005). Apart from ACD, T cells also play a crucial role in many other autoimmune-related diseases such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (Petta et al., 2014). Glucocorticoid

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is one of the most commonly used anti-inflammatory drugs clinically, but its systemic use often results in severe side-effects particularly after prolonged use and/or high doses, due to the ubiquitous glucocorticoid receptor distributed throughout the body (Baschant et al., 2012). Thus, alternative treatments that avoid the need for glucocorticoid whereby the toxicity and side-effects are minimized are desirable.

Recently, several effective flavonoid compounds such as morin hydrate (3, 5, 7, 2, 4-pentahydroxyflavone) and quercetin have been found in fruits, wine, tea, and other family members of herbs (Das, 1994). These low molecular weight substances have shown to possess anti-inflammatory, anti-oxidative, anti-cancer, and anti-hypertensive properties, where they have helped to improve eyesight and strengthen joints (Fang et al., 2003; Francis et al., 1989; Iwase et al., 2001; Kang et al., 2004; Kitagawa et al., 2004). The main advantage of flavonoids over synthetic immunomodulatory compounds is that they are relatively non-toxic and can be used as a dietary supplement (Jakhar et al., 2014; Penalva et al., 2016). In some countries, quercetin is accepted as a dietary supplement with the recommended daily oral doses of 200–1200 mg (Penalva et al., 2017). However, only a few studies have focused on the applicable formulation of morin. Most investigations employ simple aqueous formulations of morin and administering high doses via the intraperitoneal injection route to achieve the effective pharmacological effect (Sinha et al., 2015; Zhang et al., 2010). Injection administration provides effective systemic treatment but need for frequent application, skilled administration and potential for abuse warrant other means of delivery (Luby et al., 2005). Thus, the potential of developing a transdermal form of morin to help improve patient compliance-by providing a sustained delivery of low therapeutic doses of the drug transdermally was investigated. As a poorly water-soluble drug, the use of phospholipid complex has been studied in the literature to help improve the solubility of morin for oral application, and the researchers found that morin phospholipid complex (MPC) can significantly improve both the lipophilicity and hydrophilicity of morin (Zhang et al., 2015, 2011). However, to date, the therapeutic effect of morin via transdermal delivery has not been fully established.

Carbopols are hydrophilic polymers of acrylic acid cross-linked with polyalkenyl ethers or divinyl glycol, which make them as potential candidates for topical use gel type formulation. They are considered to be non-toxic and non-irritant materials with no evidence of their hypersensitivity on human when used topically (Chawla and Saraf, 2012). Carbopol 940 was selected as a gelling agent because it can produce an acceptable physical appearance, with suitable spreadability due to its water absorption capacity, hydration and swelling ability. In addition, Carbopol 940 has high molecular weight (104,400 g/mol), so they cannot penetrate into the skin and offer good alternative to oil based vehicles (Chawla and Saraf, 2012). In this study, a Carbopol 940 gel based on the use of MPC technique and CPEs to enhance the transdermal delivery of morin and reduce the inflammatory response mediated by the DNFB has been developed.

2. Materials and methods

2.1. Materials

Morin hydrate, ConA, and 2, 4-dinitrofluorobenzene (DNFB) were purchased from Sigma-Aldrich Co. (Shanghai, China). Carbopol 940 was obtained from Yuan Cheng Technology Co. (Wuhan, China). Lipoid E80 (Purified Ovocleithin) was purchased from Shanghai Tai-wei Pharmaceutical Co., Ltd. (Shanghai, China). Menthol, olive oil, isopropyl myristate, and 1, 2-propanediol were obtained from Aladdin (Shanghai, China). Compound dexamethasone acetate cream was purchased from Baiyunshan

Pharmaceutical Co., Ltd. (Guangzhou, China). The Mouse ELISA Ready-SET-Go! Kits were purchased from eBioscience (Affymetrix Inc, US). All other chemical reagents were of analytical or high performance liquid chromatography grade.

2.2. Animals

Female BALB/c mice 6–8 weeks old were purchased from the Dashuo Experimental Animal Co., Ltd (Chengdu, P. R. China). All mice experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University. Mice were acclimated to the environment 2–7 days, with water and standard diet available ad libitum.

2.3. Preparation of morin-phospholipid complex gel (MPC-gel)

MPC was prepared and characterized according to previous work (Zhang et al., 2011). In order to obtain suitable viscosity values for transdermal application, MPC was loaded into Carbopol 940 hydrogel. Briefly, 160 mg of MPC and prescription amount of chemical penetration enhancers were dispersed in 2.4 ml of ethanol at room temperature by gentle agitation. Then distilled water was added to the ethanol dispersion with a final ethanol concentration of 30% (v/v), and a clear mixture was obtained. Next, 0.5% (w/v) of Carbopol 940 was sub-divided into the mixture under gentle stirring. After Carbopol 940 was fully swollen overnight at room temperature, it was subsequently neutralized using triethanolamine to promote gelation. As a control, Morin-gel was prepared using the process described for the MPC-gel.

2.4. Evaluation of MPC-gel

The prepared MPC-gel was evaluated for drug uniformity, drug content, viscosity, pH, and centrifugation stability studies.

The drug content was determined by taking 1 g of accurately weighed MPC-gel which was diluted with methanol to 100 ml and analyzed by HPLC. The HPLC method we used to determine morin was according to a published method with some modifications (Zhang et al., 2011). The analysis was performed on an Agilent 1260 infinity HPLC system equipped with a G1314C UV detector. A Kromasil C18 (Sciencelab, China) analytical column (150 mm × 4.6 mm, 5 μm) was used with a mobile phase consisting of acetonitrile and 0.5% phosphoric acid (35:65, v/v) by a flow rate of 1.0 ml/min. Detection wavelength was 252 nm, and the injection volume was 10 μl. Studies showed that the precision, accuracy, and recovery of this HPLC method all met the measurement requirements.

Viscosity studies were carried out using a DV-C digital viscometer (Brookfield engineering, MA, USA). Briefly, Take the appropriate volume of MPC-gel in a 25 ml beaker with spindle S64 at 4 rpm and at temperature 25 ± 0.5 °C.

The pH of the dispersion was measured using a pH meter (METTLER TOLEDO). Briefly, 1 g of the MPC-gel was weighed in 25 ml volumetric flask and then added distilled water to 25 ml.

As for centrifugation stability studies, MPC-gel was weighed 5 g in 10 ml EP tube, and then centrifuged at 4000 rpm/min for 30 min. Changes in preparation before and after centrifugation were observed. All measurements were done in triplicate.

2.5. In vitro release studies

To identify the characteristics of morin release from Carbopol 940 gel, release studies were carried out based on a dialysis technique. Briefly, 1 g of Morin-gel or MPC-gel was transferred into dialysis bags (MWCO ~ 14,000) and the bags were ligated with surgical suture lines. Then, the sealed bags were dialyzed against 50 ml of release medium (PBS, pH 7.4) and shaken in a horizontal

shaker under controlled conditions (100 rpm/min, $37 \pm 1^\circ\text{C}$). At predetermined intervals (0.25, 0.5, 1, 2, 4, 6, 8 and 12 h) aliquot of dialysate (1.0 ml) was withdrawn and replaced with 1 ml of fresh medium. Morin concentration in dialysate was analyzed by established HPLC method. Each experiment was run in triplicate.

2.6. In vitro skin penetration studies

We used BALB/c mouse dorsal skin as skin model. To prepare the skin, the mice were euthanized, hairs on the backs were removed using a razor over an area of about $3 \times 3\text{ cm}^2$, and subcutaneous fat was removed only using scissors and tweezers. Full thickness mouse skin was used for in vitro skin penetration studies.

In vitro skin penetration studies were performed in a vertical Franz diffusion cell with an effective diffusion area of 1.13 cm^2 and the receiver fluid volume was 15 ml. The receiver compartment was filled with 30% v/v methanol in PBS (pH 7.4) and stirred at 300 rpm/min. Each gel formulation (1 g) was placed in the donor compartment. The system was kept at $37 \pm 0.5^\circ\text{C}$ via a circulating water bath to keep the epidermis surface temperature at $32 \pm 0.5^\circ\text{C}$ throughout the experiment. At predetermined time intervals (0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 30 h), samples (1 ml) were collected from the receptor chamber and immediately replaced with an equivalent volume of fresh receptor medium to maintain the sink condition. The amount of drug (morin) that crossed the skin was quantified by HPLC methods. All values represent the averages of parallel experiments performed in triplicate.

The cumulative amount of morin (Q_n , $\mu\text{g}/\text{cm}^2$) was plotted as a function of time (t , h), and the cumulative amount of morin diffused per unit area of the excised skin was calculated on the following Eq. (Lv et al., 2009)

$$Q_n = (C_n \times V_0 + \sum_{i=1}^{n-1} C_i \times V_i) / A$$

2.6.1. Effect of MPC on transdermal flux

In order to verify the MPC could improve the transdermal flux of morin. Gel formulations (Morin-gel and MPC-gel) were prepared for in vitro skin penetration studies. The permeation studies were conducted according to the procedure described in above.

2.6.2. Screening of chemical permeation enhancers

Menthol, azone, oleic acid, isopropyl myristate and medium chain triglycerides were considered as chemical permeation enhancers for further screening. Menthol and azone were tested at 2% (w/v). Oleic acid, isopropyl myristate and medium chain triglycerides were tested at 5% (w/v). The concentration of 1, 2-propylene glycol (PG) was kept at 5% (v/v). The procedure for preparing MPC-gel is described in Section 2.3. All formulations were uniform upon visual inspection. Then we further screened the concentration of selected permeation enhancers in order to obtain the best transdermal flux. The permeation studies were conducted according to the procedure described in above.

2.7. Induction of allergic contact dermatitis (ACD) by 2, 4-dinitrofluorobenzene (DNFB)

ACD was induced in BALB/c mice according to a published method with minor modifications (Kim et al., 2010). Briefly, the back skin of the mice was sensitized by applying $20\text{ }\mu\text{l}$ of 0.5% DNFB in 4:1 (v/v) acetone-olive oil mixture on days 1 and 2. On day 6, the elicitation phase was treated with $20\text{ }\mu\text{l}$ of 0.25% DNFB to the right ear of each mouse. 24 h after elicitation (on day 7), the left and right ears were cut using a sharp clamp, and the weight of the

pieces was calculated using an electronic balance. The ear swelling (mg) was evaluated by measuring the weight difference between the right and left ears. The thickness of right ear (μm) was measured using Axiovision image analysis 4.7 software (Carl Zeiss Vision GmbH, Munich, Germany). H&E stained tissue images were captured with the digital microscope camera to measure their thicknesses. Their blood samples, spleens, thymuses and lymph nodes were harvested for further measurements. As a control, the normal mice were painted with an identical volume of menstruum (4:1 v/v acetone-olive oil).

The mice were randomly divided into five groups ($n=5$ per group): acetone-olive oil treated normal group, DNFB treated ACD model group, the mice treated with DNFB were transdermal administered MPC-gel (5 mg/kg), Morin-gel (5 mg/kg) and Compound dexamethasone acetate cream (dexamethasone 1 mg/kg) from day 1 to day 6 on their sensitized back.

2.8. Assay of spleen and thymus indices

24 h after challenge, the weight of the mice were measured, and then sacrificed. The spleen and thymus were removed and weighed. Spleen and thymus indices were defined as the spleen or thymus wet weight, divided by the body weight.

2.9. Measurement of cytokine levels in the serum and white blood cell in the blood

The blood samples were collected from the mice on day 7 after elicitation by 0.25% DNFB. The serum was separated. ELISAs were used to measure the IFN- γ , TNF- α and IL-4 production in the sera, with according to the manufacturer's instructions. Absorbance was measured at 450–570 nm and the sample concentrations were determined from a standard curve. In order to counting the number of white blood cells in the blood, blood samples were collected through the mouse orbital puncture in a tube containing EDTA-2K anticoagulant. Then, the blood was measured by automatic blood analyzer MEK-6318 (NIHON KOHDEN, Japan).

2.10. Histopathological analysis

The histopathological examination was carried out according to published methods (Yuan et al., 2010). Briefly, earlobes and spleens were harvested 24 h after DNFB challenge, fixed in 4% paraformaldehyde, and embedded with paraffin. The sections ($4\text{ }\mu\text{m}$) were stained with hematoxylin and eosin (H&E) for pathological analysis. The thickening of the skin and infiltrated inflammatory cell in the dermis were observed by a microscope.

2.11. Cell culture and measurement of cell proliferation

The draining lymph nodes in groin of the ACD mice treated with or without MPC-gel were removed, ground, and filtered through a $70\text{ }\mu\text{m}$ cell strainer under aseptic conditions. Then a single-cell suspension was prepared, cells were washed, collected, and rinsed using RPMI 1640 (Gibco, UK) medium supplemented with 10% (w/v) fetal bovine serum (FCS, Life Technologies, Inc., UK), penicillin (100 IU/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). Next, they were seeded in 96-well plates (5×10^5 cell/well), and stimulated with ConA (5 $\mu\text{g}/\text{ml}$) for 48 h at 37°C in 5% CO_2 , and the cell proliferation was measured with an CCK-8 assay. Absorbance was measured at 450 nm. The proliferation of lymphocytes was expressed as a proliferation index, which was defined as the mean of stimulated experimental data divided by the mean of the unstimulated control.

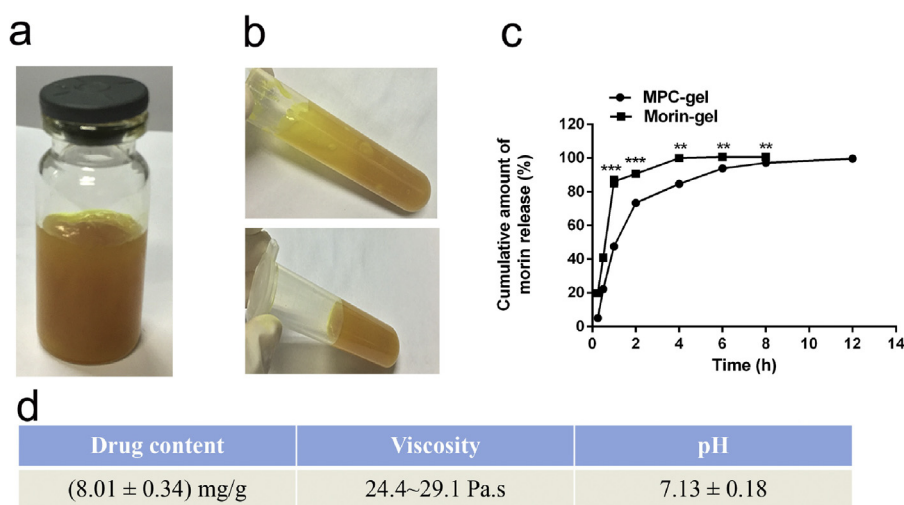


Fig. 1. Physicochemical properties of MPC-gel. (a) Photograph of physical appearance of MPC-gel. (b) Centrifugation stability of MPC-gel: 1) before centrifugation and 2) after centrifugation. (c) Release curves of morin from Carbopol 940 gel based on the dialysis method. Data expressed as mean ± SD (n = 3), **p < 0.01, ***p < 0.001 versus MPC-gel. (d) Drug content, viscosity and pH of MPC-gel. Data expressed as mean ± SD (n = 3).

2.12. Statistical analysis

All statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). And treatment effects were analyzed using one-way ANOVA, followed by Dunnett's two-tailed *t*-test. The criterion for significance was *P* < 0.05.

3. Results

3.1. Evaluation of MPC-gel

The appearance of the formulation is shown in Fig. 1a. It is a yellow semi-solid gel with fine texture and color uniformity. The drug content was 8.01 ± 0.34 mg/g. The values for viscosity were found to be in the range of 24.4 ~ 29.1 Pa.s and all the formulations were easily spreadable in nature. In addition, after diluted with

water, the pH of the MPC-gel was 7.13 ± 0.18, which is acceptable for topical application (Fig. 1d). After 30 min centrifugation at 4000 rpm/min, the bubbles in the preparation have been eliminated and no stratification was observed, which confirmed the stability of the MPC-gel (Fig. 1b).

3.2. In vitro release studies

The release of morin from Morin-gel was relatively fast (Fig. 1c). More than 80% of morin was released from the Morin-gel by 1 h, and the release was almost completed after 4 h. On the other hand, the MPC-gel slowed down the release of morin, where >80% drug was released only after 4 h.

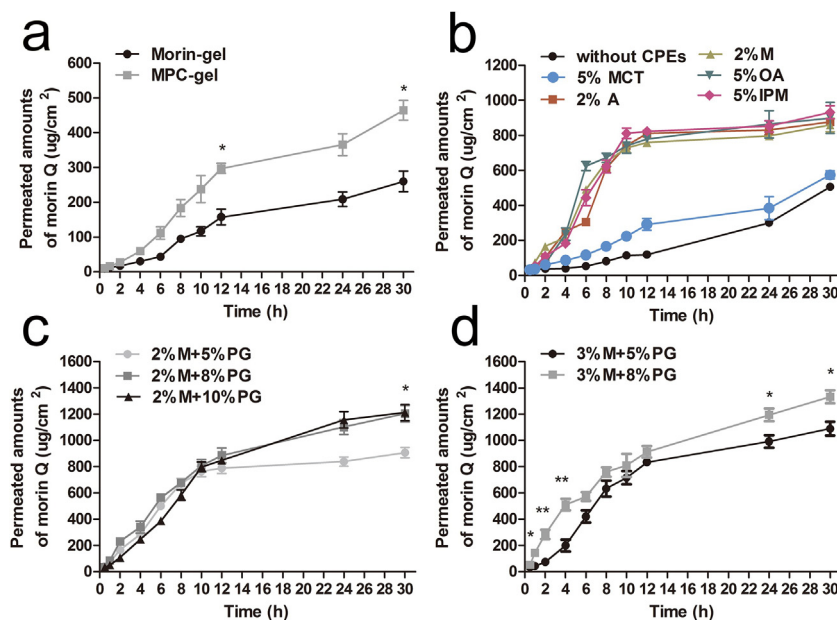


Fig. 2. In vitro mouse skin penetration studies. (a) The permeation kinetic curves of Morin-gel and MPC-gel. (b) Cumulative amount of morin permeated through mouse skin with different penetration enhancers in MPC-gel. (c, d) Effects of different concentration of menthol and 1, 2-propanediol on transdermal delivery of MPC-gel. All results are presented as the means ± S.D (n = 3), *p < 0.05, **p < 0.01. Key: M: menthol; PG: 1, 2-propanediol; A: azone; OA: oleic acid; IPM: isopropyl myristate; MCT: medium chain triglycerides.

Table 1

Formulations and respective permeation parameters for morin. Key: M: menthol; PG: 1, 2-propanediol; J_{ss} = Flux at steady-state; Q_{12} and Q_{30} = Cumulative amount of morin permeated after 12 h and 30 h, respectively; Data are expressed as mean $\pm$ SD. (n = 3), ** $p < 0.01$, *** $p < 0.001$ compared to MPC-gel without penetration enhancer as reference.

Formulations	CPEs	J_{ss} ($\mu\text{g}/\text{cm}^2/\text{h}$)	Q_{12} ($\mu\text{g}/\text{cm}^2$)	Q_{30} ($\mu\text{g}/\text{cm}^2$)
Morin-gel	without	8.66 ± 1.38	157.89 ± 32.15	259.82 ± 41.42
MPC-gel	without	15.47 ± 1.34	297.32 ± 20.13	464.24 ± 40.07
	2%M + 5%PG	$30.24 \pm 2.30^{**}$	$790.01 \pm 70.58^{**}$	$907.33 \pm 69.05^{**}$
	2%M + 8%PG	$40.12 \pm 3.62^{**}$	$885.39 \pm 97.03^{**}$	$1203.47 \pm 108.44^{**}$
	2%M + 10%PG	$40.43 \pm 3.57^{**}$	$847.07 \pm 36.76^{***}$	$1212.76 \pm 107.06^{**}$
	3%M + 5%PG	$36.32 \pm 3.06^{**}$	$835.10 \pm 36.62^{***}$	$1089.68 \pm 91.80^{**}$
	3%M + 8%PG	$44.46 \pm 2.85^{***}$	$913.43 \pm 77.23^{**}$	$1335.85 \pm 85.48^{***}$

3.3. In vitro skin penetration studies

3.3.1. Effect of MPC on transdermal flux

The cumulative permeated amounts of morin from Morin –gel and MPC-gel were $259.82 \pm 41.42 \mu\text{g}/\text{cm}^2$ and $464.24 \pm 40.07 \mu\text{g}/\text{cm}^2$ after 30 h, respectively (Fig. 2a). Treatment of the skin with MPC-gel enhanced the cumulative amount of morin that crossed the skin after 30 h (Q_{30}) by ~ 2 times compared to morin treatment alone.

3.3.2. Screening of chemical permeation enhancers

To further enhance permeation, 2% (w/v) of menthol and azone, 5% (w/v) oleic acid, isopropyl myristate and medium chain triglycerides were incorporated into the MPC ethanolic dispersion. When fixing the concentration of 1, 2- propanediol (PG) at 5% (v/v), these chemical enhancers resulted in higher flux enhancement, with the exception of medium chain triglycerides (MCT) (Fig. 2b). However, there were no statistical differences ($p > 0.05$) between the extent of permeation resulted from the addition of either menthol, azone, oleic acid or isopropyl myristate after 30 h of treatment. As it is acknowledged that the local application of

menthol in the skin can play a cooling and analgesic effect (Craighead and Alexander, 2016), menthol was chosen as the permeation enhancer for subsequent formulation optimization.

When fixing the menthol concentration at 2% (w/v), and increasing the concentration of PG to 8% (v/v), a significant increase (20%) in permeation was evident after 30 h. Nevertheless, a further increase of PG concentration to 10% (v/v) did not result in a further increase in permeation (Fig. 2c). Similarly, when a higher concentration of menthol was applied (3% w/v), the increase of PG concentration from 5% (v/v) to 8% (v/v) also augmented the extent of permeation (Fig. 2d and Table 1). Since the MPC-gel formulation that contain the permeation enhancers increased the steady-state flux (J_{ss}) and cumulative amount (Q_{12} and Q_{30}) of morin after 30 h (Table 1), the MPC-gel formulations containing 30% ethanol (v/v), 3% (w/v) menthol and 8% PG (v/v) were taken forward for further development.

3.4. MPC-gel inhibited DNFB induced ear swelling in mice

The mouse model of 2, 4-dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis (ACD) was used to explore the

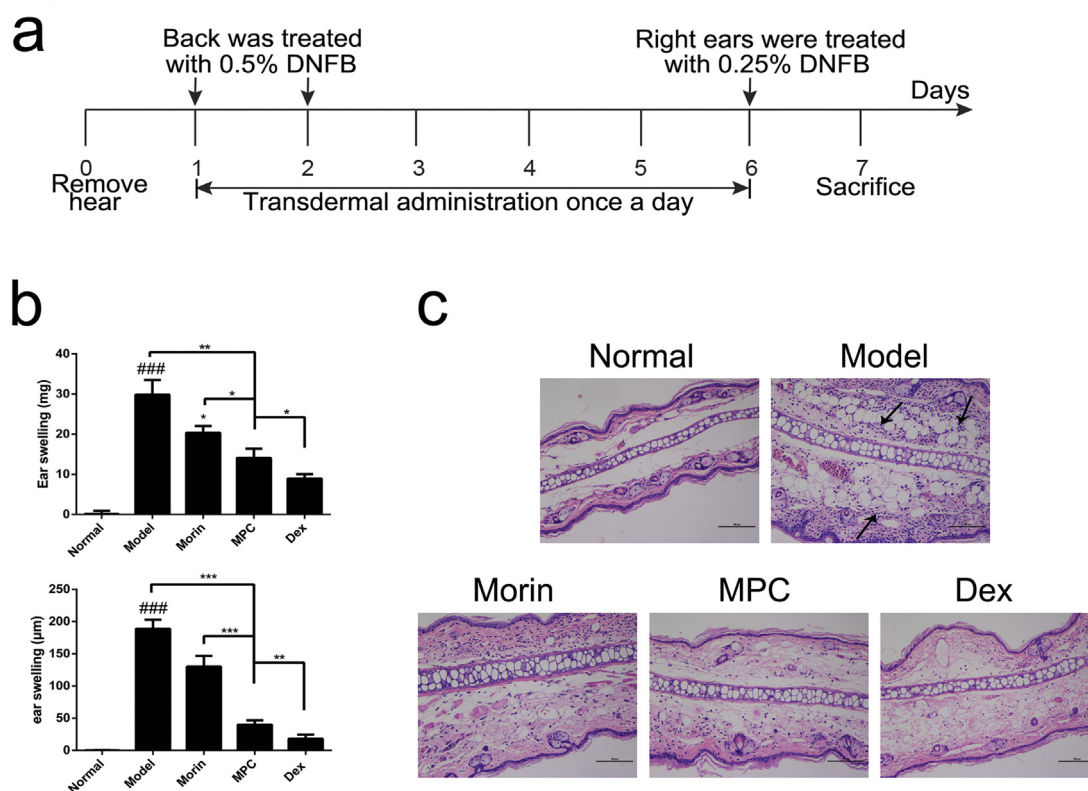


Fig. 3. Effect of MPC-gel on ear tissues in DNFB-induced ACD mice. (a) Experimental design. (b) Effect of MPC-gel on ear weight and ear thickness of the mice model of ACD (means $\pm$ S.D, n = 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the MPC-gel group; ### $p < 0.001$ versus normal group). (c) Effects of MPC-gel on the histopathology of H&E-stained ear skin sections. (inflammatory cell infiltration: black arrows; magnification: $\times 200$, scale bar: 100 μm).

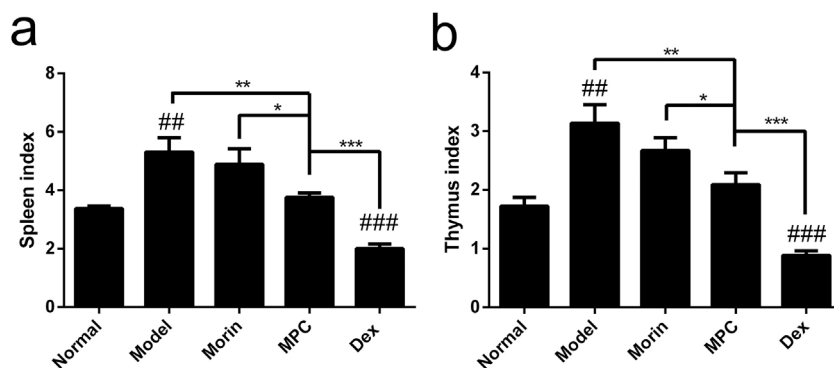


Fig. 4. Effect of MPC-gel on spleen and thymus indices in mice treated with DNFB. All results are presented as the means $\pm$ S.D (n=5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the MPC-gel group; ## $p < 0.01$, ### $p < 0.001$ versus normal group.

effectiveness of MPC-gel in skin inflammatory diseases *in vivo*. The normal mice were painted with an identical volume of menstruum (4:1 v/v acetone-olive oil mixture) and model mice were sensitized and elicited by applying DNFB. MPC-gel (5 mg/kg) was applied transdermally to the mice back in the sensitization phase and elicitation phase of ACD (days 1–6). Dexamethasone cream (1 mg/kg, transdermally) was used as the positive control, while Morin-gel (5 mg/kg) as another control. The establishment of the DNFB-induced ACD model and the dosage regimen for the mice are shown in Fig. 3a. Ear swelling of the DNFB-induced ACD model mice increased when compared with acetone-olive oil treated normal mice, but was remarkably reduced in the MPC-gel treatment groups (Fig. 3b). Histological analysis of the skin of the mice ear indicated that the MPC-gel and dexamethasone cream effectively alleviated DNFB-induced inflammatory cell infiltration and edema (Fig. 3c).

3.5. MPC-gel reduced spleen and thymus indices in mice treated with DNFB

As shown in Fig. 4, the spleen and thymus indices of the DNFB-induced ACD model group were significantly higher when compared with the acetone-olive oil treated normal mice ($p < 0.01$), and splenomegaly was observed in model mice when treated with DNFB alone (Fig. 6a). In contrast, treatment with the MPC-gel (5 mg/kg) markedly reduced the spleen and thymus indices, and splenomegaly in DNFB-treated mice. The spleen and thymus indices were also significantly reduced following dexamethasone cream (1 mg/kg) treatment even when compared with the acetone-olive oil treated normal group ($p < 0.001$). This reduction was also greater than observed from the MPC-gel treated group.

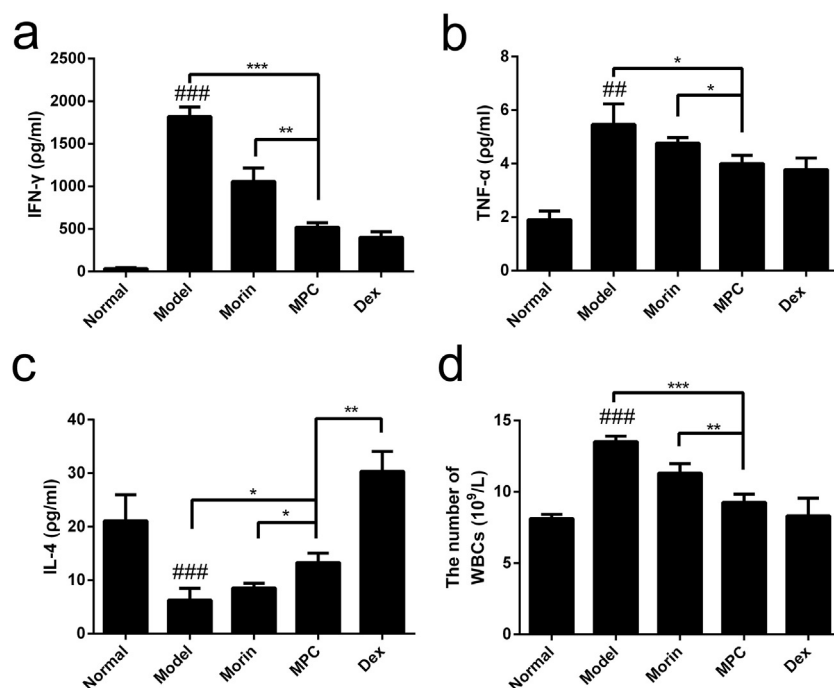


Fig. 5. Effects of MPC-gel on the production of cytokines in the sera and white blood cells (WBCs) in the blood of the mice model of ACD. (a–c) The IFN-γ, TNF-α, and IL-4 levels in sera were analyzed with ELISAs. (d) The number of WBCs in the blood was measured. (All results are presented as the means $\pm$ S.D, n=5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the MPC-gel group; ## $p < 0.01$, ### $p < 0.001$ versus normal group).

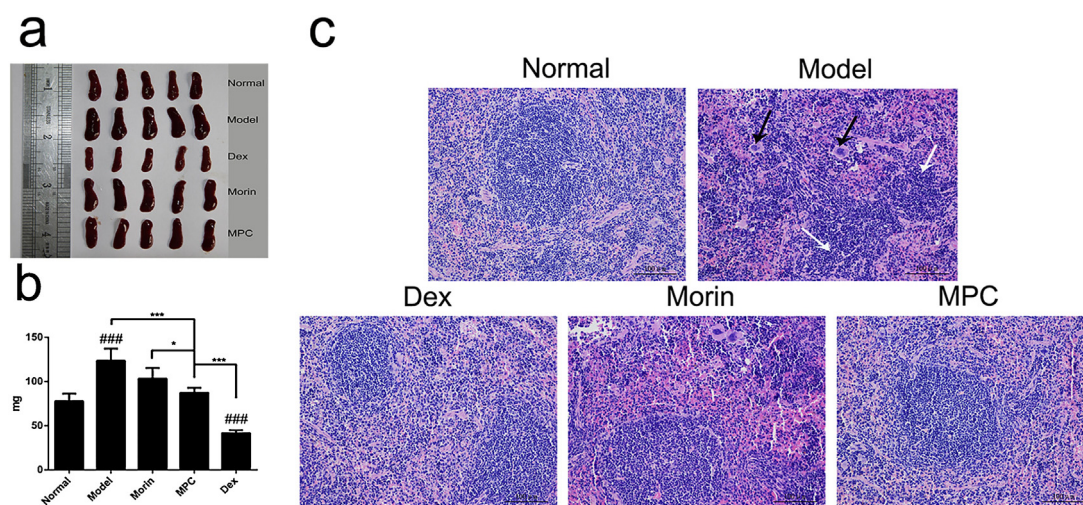


Fig. 6. Effects of MPC-gel on spleen histopathology in mice treated with DNFB. (a) Representative photographs of spleen after DNFB challenge 24 h. (b) The weight of spleen was measured. All results are expressed as means $\pm$ S.D, $n = 5$, * $p < 0.05$, *** $p < 0.001$ compared with the MPC-gel group; ### $p < 0.001$ versus normal group. (c) Effects of MPC-gel on the histopathology of H&E-stained spleen sections. DNFB treated ACD model group: the histopathology displayed obvious multinucleated macrophage hyperplasia (black arrows) and lymph node hyperplasia (white arrows). Original magnification 200 $\times$, scale bar = 100 μ m.

3.6. MPC-gel regulated the cytokine levels in serum and white blood cell in the blood

As shown in Fig. 5, the data presented here confirm that the immunological and inflammatory processes induced by ACD are mainly regulated by cytokines. DNFB challenge to the mice resulted in an increase in interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) levels in serum. Compared with the model group treated only with DNFB, treatment with MPC-gel (5 mg/kg) significantly reduced cytokines levels of IFN- γ and TNF- α (all $p < 0.05$). Since a strong contact sensitizer such as DNFB has been shown to induce a mixed Th1/Th2 response (Adenuga et al., 2012), the typical Th2 cytokine IL-4 was also measured. It is noteworthy that DNFB-induced ACD mice showed apparent reduction in IL-4 level (Fig. 5c), whilst the mice treated with MPC-gel (5 mg/kg) displayed a significant increase in levels of IL-4 ($p < 0.05$). Thus, confirming that the topical application of MPC-gel modulates Th1 and Th2 cells secretion of cytokines in DNFB-induced ACD.

The number of white blood cells (WBCs) in the blood is an important indicator of inflammation. Typically, the number of WBCs in the untreated normal mice ranges from 4 to 12 $\times 10^9$ /L. As shown in Fig. 5d, the quantity of WBCs recorded for the DNFB treated model group were significantly higher ($p < 0.001$), and exceeded the normal range, whereas the number of WBCs were remarkably reduced in the MPC-gel (5 mg/kg) treated group.

3.7. MPC-gel ameliorated spleen histopathology in mice treated with DNFB

The spleen is a very unique and important adaptive immune organ for the body. Representative photographs of the spleen after DNFB challenge 24 h are shown in Fig. 6a. In addition, the weight of spleen was measured (Fig. 6b). In comparison with DNFB-induced ACD model group, treatment with MPC-gel (5 mg/kg) significantly reduced splenomegaly. However, after continuous treatment with dexamethasone cream (1 mg/kg) for six days, the spleen was found to be significantly smaller than the acetone-olive oil treated normal group. From the spleen histopathology study, lymphoid hyperplasia and multinucleated macrophage proliferation were evident in the model group (Fig. 6c). The degree of spleen injury was also significantly alleviated in the hyperplasia of lymph node

and multinucleated macrophage proliferation of mice treated with MPC-gel.

3.8. MPC-gel suppressed t lymphocytes proliferation

Considering the key role of T lymphocytes during the induction of ACD, evaluation of the effects of MPC-gel on T lymphocytes proliferation in the groin lymph node was made and ConA was used to stimulate T-lymphocyte proliferation. Lymphocytes from DNFB challenged and acetone-olive treated normal oil mice were isolated, and stimulated with or without 5 μ g/ml of ConA at 37 $^{\circ}$ C for 48 h. As shown in Fig. 7c, the T cell proliferation index in MPC-gel (5 mg/kg) treated mice were lower than the acetone-olive treated normal group ($p < 0.05$). The data demonstrate that the MPC-gel has an inhibitory effect on DNFB induced T lymphocyte proliferation, as confirmed in the representative photographs and the weight of lymph nodes after DNFB challenge 24 h shown in Fig. 7a and b.

4. Discussions

Drugs such as corticosteroids, non-steroidal anti-inflammatory drugs and biologics are available for the treatment of inflammatory diseases (Gautam and Jachak, 2009). These drugs may cause unwanted side-effects or be expensive to manufacture as in the case of biologics. There are a large number of alternative compounds with anti-inflammatory properties from natural products, which also offer great promise as potential drugs to treat inflammatory disorders. Unlike corticosteroids, morin hydrate polyphenols have been studied in a variety of human diseases with relatively mild side-effects. However, morin is difficult to dissolve in water and oil, resulting the poor permeation across the skin. To date, the therapeutic effect of morin via transdermal delivery has not been fully established. On the other hand, morin hydrate, a yellowish pigment has low molecular weight (302.2357), so it has the potential to penetrate into the skin with the appropriate pharmaceutical technique.

In this study, for the first time, a MPC-gel combined with penetration enhancers to enhance the penetration of the poorly water-soluble drug, morin, across the mouse skin for transdermal drug delivery has been investigated. It has been found that drug-phospholipid complex has a stronger lipophilicity than the original

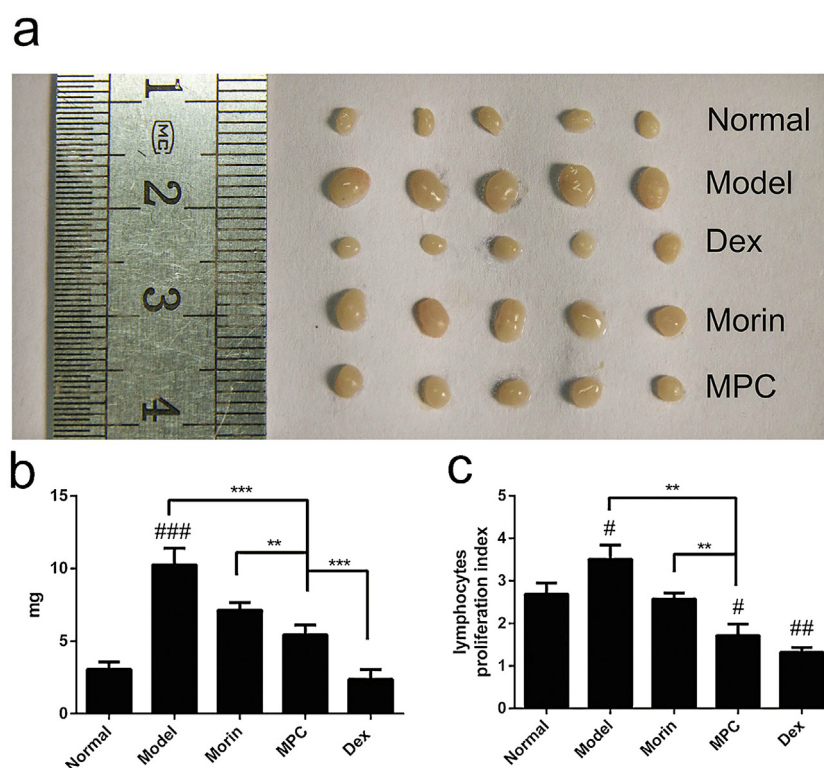


Fig. 7. Effect of MPC-gel on lymph nodes in mice treated with DNFB. (a) Representative photographs of lymph nodes after DNFB challenge 24 h. (b) The weight of lymph nodes was measured. (c) Lymphocytes proliferation index was measured with the CCK-8 assay after activated with ConA for 48 h. All results are expressed as means $\pm$ S.D, $n = 5$, ** $p < 0.05$, *** $p < 0.001$ compared with the MPC-gel group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with the normal group.

drug in many studies. As shown in Fig. 2, the transdermal delivery of morin increased significantly after the formation of MPC ($p < 0.05$). Morin and phospholipids are likely to be non-covalently linked, such as hydrogen bonds and van der Waals forces, and the morin molecules are “entrapped” in the polar head of phospholipids molecules, the drug and phospholipids form a whole (Zhang et al., 2011). Thus, MPC can significantly improve the lipophilicity of morin. Phospholipids are a class of lipid molecule, which are a key component of all cell membranes (Singh et al., 2017). Owing to their similarity to the biomembrane, phospholipids are recognized as non-allergic, bio-friendly permeation enhancers (Elnaggar et al., 2014). This makes MPC is more easily penetrated through the stratum corneum to reach the dermis than morin. The incorporation of chemical permeation enhancers, including menthol (3% w/v) and 1, 2-propanediol (8% v/v), to MPC-gel resulted in significant increase in transdermal delivery of morin than other combinations. In agreement with the literature, menthol has been reported as an effective enhancer for transdermal delivery of a variety of drugs such as imipramine hydrochloride, caffeine, hydrocortisone, triamcinolone, propranolol hydrochloride, and zidovudine (Amnuakiet et al., 2005; Godwin and Michniak, 1999; Jain et al., 2002; Narishetty and Panchagnula, 2005a, 2005b; Pandit et al., 2015). It is believed that menthol disrupts the hydrogen bond network of the ceramides head in the lipid bilayer, and increase electrical conductivity of tissues by opening the polar pathways in the stratum corneum (Narishetty and Panchagnula, 2005a; Narishetty and Panchagnula, 2005a, 2015b; Sznitowska et al., 1998). The analgesic and anti-septic properties of menthol are also desirable for the application of this study (Cal et al., 2001).

Repeated exposure to allergens to damaged skin can cause allergic contact dermatitis, which is a common chronic inflammatory skin disorder. In hapten-induced ACD, T cells secrete cytokines such as IFN- γ and TNF- α (Hirasaki et al., 2011; Zhang et al., 2013). They are considered to be key factors in the pathogenesis of a

variety of inflammatory diseases. Thus, an attractive approach to the treatment of Th1-mediated immune disorders is the selective modulation of IFN- γ and TNF- α signaling. In this study, according to the results of serum cytokines levels, morin relieves DNFB-induced inflammation mainly by down-regulating expression of pro-inflammatory cytokines IFN- γ and TNF- α level, and up-regulating expression of IL-4 (Fig. 5). After transdermal application of morin onto DNFB-induced ACD mouse back, the effect of the regulation on cytokines secretion was increased significantly after the formation of MPC.

In addition, the developed MPC-gel could remarkably ameliorate DNFB-induced ear swelling when compared to the DNFB-treated ACD model group and Morin-gel group (Fig. 3). In this study, MPC-gel (5 mg/kg, transdermally), was superior to Morin-gel (5 mg/kg, transdermally) for decreasing DNFB induced spleen and thymus indices, and alleviating lymphoid hyperplasia and multinucleated macrophage proliferation in the spleen. Besides, MPC-gel also produced a significant suppression on ConA-stimulated T lymphocyte proliferation in the lymph node.

On the other hand, these results also demonstrated that MPC-gel (5 mg/kg, transdermally) is not as effective as dexamethasone cream (1 mg/kg, transdermally). The data presented in this study confirmed that dexamethasone had potent anti-inflammatory property (Bao et al., 2016; Yu et al., 2015). Although the anti-inflammatory effect of dexamethasone was better than MPC-gel, after continuous treated with dexamethasone (1 mg/kg, transdermally) only for six days, the mice had smaller spleens, thymuses and lymph nodes than the acetone-olive oil treated mice. In contrast, MPC-gel showed good anti-inflammatory efficacy and potent immune-suppressive, but milder effect on immune organs. These results showed that MPC-gel was capable of inhibiting T cell-mediated delayed hypersensitivity of type IV immune reactions in a more safe way.

5. Conclusion

In conclusion, the proposed MPC-gel can provide transdermal delivery of morin and suppress allergic contact dermatitis symptoms. The MPC-gel combination with penetration enhancers significantly increased the transdermal flux of morin. In the ACD mice model, MPC-gel regulates the T cell-mediated allergic responses mainly by suppressing the systemic production of IFN- γ and TNF- α , which results in reduced ear swelling, inflammatory cell infiltration in the ear tissue, and decreased T lymphocyte proliferation after re-application of DNFB. Therefore, the topical administration of MPC-gel with chemical penetration enhancers represent an efficient way on inhibiting ACD induced by DNFB.

Authors' contributions

Xun Sun, Kawai Wan and Jiao Yu designed the research; Jiao Yu performed the research; Xun Sun contributed new reagents or analytic tools; Jiao Yu generated laboratory data, performed preliminary data analysis; Jiao Yu wrote the draft manuscript; Kawai Wan and Xun Sun contributed to manuscript writing and proofreading.

All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpharm.2017.07.062>.

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1. Differential scanning calorimetry (DSC)

Differential scanning calorimetry was performed on an EXSTAR 6000 thermogravimetric analyzer (Seiko Instruments, Tokyo, Japan). morin, MPC, blank gel with chemical penetration enhancers and MPC-gel were placed in conventional aluminum pans (about 10 mg), and a scan speed of 10 °C/min was employed. A scanning temperature range was 10-320°C by nitrogen flow protection, and the flow rate: was 20 ml/min. The result was shown below.

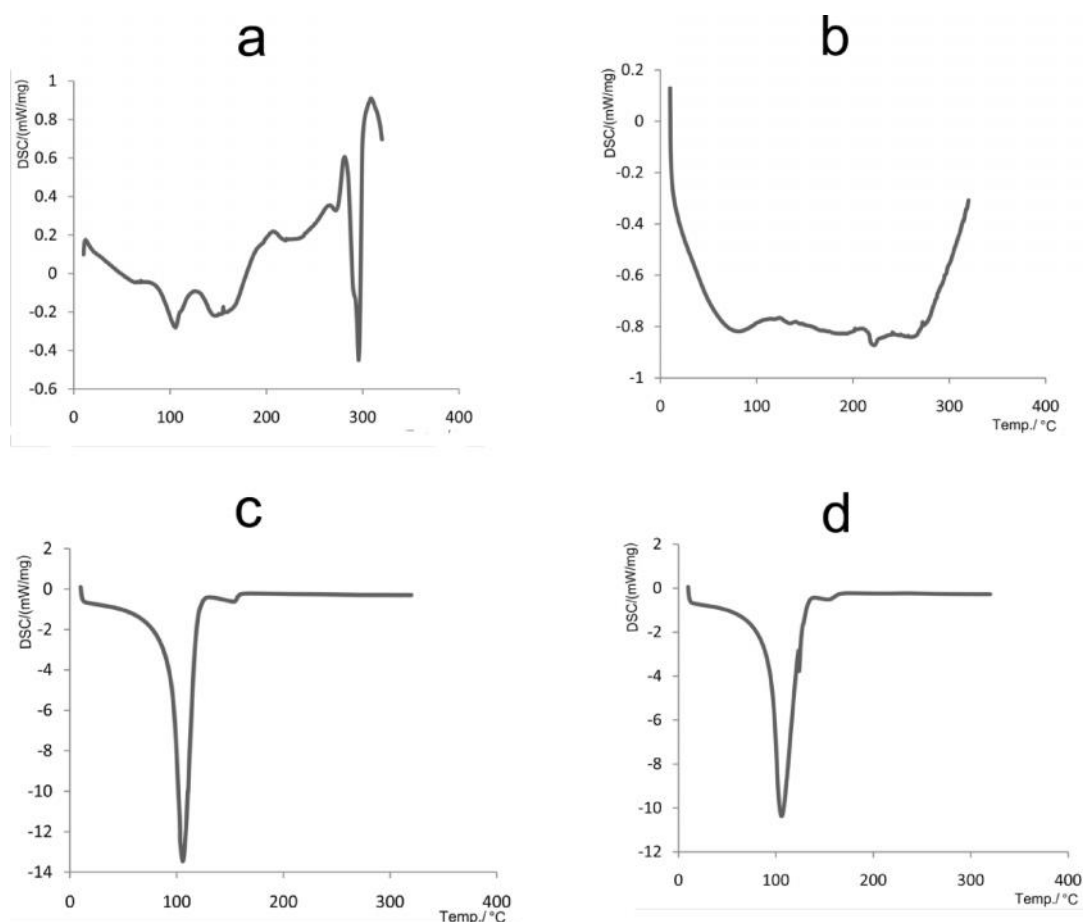


Fig. S1. DSC thermograms of morin, MPC, blank gel with chemical penetration enhancers, MPC-gel. (a) DSC of Morin. (b) DSC of MPC. (c) DSC of blank gel with chemical penetration enhancers. (d) DSC of MPC-gel.

2. *In vitro* porcine skin penetration studies

Methods for the study of porcine skin penetration *in vitro* refer to the Section 2.6. The result was shown below.

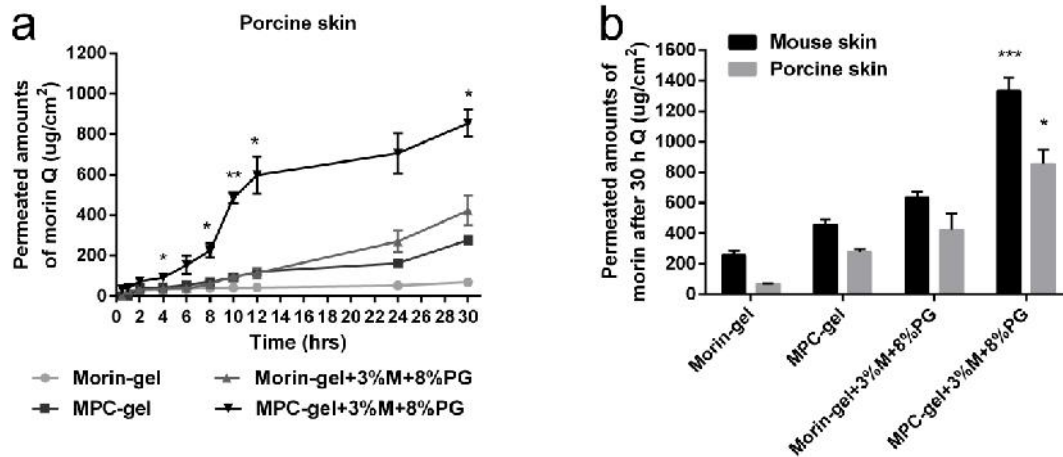


Fig. S2. *In vitro* porcine skin penetration studies. (a) The permeation kinetic curves of different formulations on porcine skin. (b) Cumulative amount of morin permeated after 30h on mouse skin and porcine skin. All results are presented as the means $\pm$ S.D (n=3), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$.