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Effect of Rosmarinic Acid（RosA）on Enhancing Transdermal Absorption of Crosslinked Hyaluronic Acid（CHA）

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Abstract：To investigate the effects of Rosmarinic acid（RosA）on enhancing the transdermal absorption of cross-linked hyaluronic acid（CHA），RosA was obtained and CHA was prepared. The rat-skin transdermal in vitro model was established，the CHA micro-assay and cytotoxicity model were established. The stability of HA and CHA at different time-points and temperatures were investigated，the enhancing effects of RosA and combinations on CHA transdermal were evaluated，and the cytotoxicity of RosA were investigated. The results showed that 5% RosA enhanced skin penetration of CHA and was more effective than conventional azone. With the increase of RosA concentrations，the transdermal rate of CHA was increased（$^{***}P=0.001$）. RosA（10 mg/mL）used as the CHA transdermal penetration enhancer did not affect cell growth in vitro.

Key words：rosmarinic acid；cross-linked hyaluronic acid；transdermal absorption；natural penetration enhancer；cytotoxicity

迷迭香酸对增强交联透明质酸透皮吸收的作用

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摘要：研究迷迭香酸(RosA)对增强交联透明质酸(CHA)透皮吸收的影响，获取 RosA 并制备 CHA。建立体外大鼠透皮分析模型，通过微量 CHA 检测方法和细胞毒性测定模型，研究透明质酸和交联透明质酸在不同时间和温度下的稳定性，评估 RosA 对 CHA 透皮的增强作用，并与氮酮比较，同时考察 RosA 的细胞毒性。结果显示含体积分数 5% RosA 的 CHA 皮肤渗透增强，比常规氮酮有效。随着 RosA 质量浓度的增加，交联

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透明质酸的透皮率随之增加（*** $P=0.001$）。RosA 不显示体外细胞毒性，用于交联透明质酸渗透促进剂的 RosA（10 mg/mL）不影响细胞生长。

关键词：迷迭香酸；交联透明质酸；透皮吸收；天然渗透促进剂；细胞毒性

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Rosemary (Latin scientific name: *Rosmarinus officinalis*) is a dicotyledonous plant and lamiaceae, rosemary shrub. Rosemary essential oil is a colorless to pale yellow volatile liquid obtained from the leaves of rosemary by water vapor, and contains Rosmarinic acid, Pinene, Ursolic acid, etc. Rosmarinic acid (RosA) is a widespread compound with interesting biological activities, and a water-soluble antioxidant with a special herbal aromatic odor extracted from rosemary. It is also a polyphenolic hydroxyl-containing acid with effects of increasing skin elasticity and delaying aging[1]. Rosemary extract has been recognized as one of the natural products with high anti-inflammatory function and used for many traditional Chinese medicine[2]. These characteristics of RosA prompted us to design and use it as a permeability adjuvant in CHA transdermal experiment and to investigate the possibility for Rosemary extracts as the new NPE to promote CHA transdermal absorption.

Hyaluronic acid (HA) is an acidic mucopolysaccharide, widely distributed in the body, and has many important physiological functions[3], such as: regulating cell proliferation, migration and differentiation; natural moisturizing effect; lubricating joints to protect cartilage; regulating protein synthesis; regulating inflammatory response; regulating immune function; promoting wound healing. HA is widely used in medical biomaterials, skin support fillers, cosmetic and pharmaceutical excipients, but the natural HA is unstable. Crosslinked hyaluronic acid (CHA) is a polymer gel with three-dimensional structure obtained by crosslinking modification of HA with a crosslinking agent[4]. CHA also has the advantages of good biocompatibility, non-antigenicity, moisturizing, anti-inflammatory and safe without toxicity. Compared with natural HA, the obvious increase of CHA in molecular volume can make up for the shortcomings of poor stability of natural HA[5]. In addition, CHA is rich in viscoelasticity and enhanced mechanical strength, which facilitates the processing of materials[6-8]. However, due to the relatively large molecular weight of CHA, it cannot penetrate the stratum corneum of the skin, or in a lower rate with a small amount of permeation through the skin, resulting in the failure of its application purposes. So, the selection of proper penetration enhancers would be necessary to promote its transdermal absorption[9].

Transdermal drug delivery system (TDDS) has become a viable alternative to conventional routes of drug administration since it can avoid the hepatic first pass effect, improve the compliance of patients, decrease the administration frequency, and reduce the gastrointestinal side effects[10].

Penetration enhancers (PE) are substances that can temporarily adjust percutaneous penetration. Now commonly used PE are ethanol, azone, peppermint oil and so on. Natural penetration enhancers (NPE) usually have good permeability effect, mild, less irritation to the skin, low price and other advantages. The current applications of existing NPE in research and development are borneol, clove volatile oil, etc[11]. However, Rosemary extracts have not been well studied as the NPE.

1 Materials and methods

1.1 Chemicals and materials

Hyaluronic acid and crosslinked hyaluronic acid (Changzhou Pharmaceutical Research Institute Co., Ltd.)[12]; 95% azone (Shenzhen Simeiquan Biological Technology Co., Ltd.); 99% rosemary essential oil, rosmarinic acid (Changzhou Pharmaceutical Research Institute Co., Ltd.); sodium chloride (analytical grade, Sino pharm Chemical Reagent Co., Ltd.); phosphate buffered saline (pH=7.4) (PBS) (analytical grade, Beijing Regen Biotechnology Co., Ltd.); 99.5% sodium tetra-borate, decahydrate (Aladdin); sulfuric acid (analytical grade, Sino-pharm Group Chemical Reagent Co., Ltd.); ethanol (analytical grade, Sino-pharm Group Chemical Reagent Co., Ltd.); carbazole (Shanghai McLean Biotechnology Co., Ltd.); 6—8 weeks 120—150 g SD female Rat (Changzhou Cavins Experimental Animal Co., Ltd.); PM-996 sealing film (BEMIS); all cell culture materials were purchased from Gibco BRL (Grand Island, NY, USA).

1.2 Detection of HA and CHA

HA or CHA standard solutions with different concentrations (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0 mg/mL) were precisely prepared. The absorbance of HA/CHA was measured by a 96-well plate carbazole sulfate method. Taking the HA or CHA concentration (C) as the abscissa and the absorbance (A) as the ordinate.

In each well of 96 well plate, 20 μL of the test solution and 100 μL of sodium tetra-borate sulfuric acid solution were mixed well, and heated in 95 ℃ water bath for 15 min, followed by quickly cooling down on ice. Then, 4 μL of carbazole ethanol solution was added to each well, and then the 96-well plate was heated again in 95 ℃ water bath for 15 min and cooled to room temperature, the OD value was red at 530 nm with a microplate reader. The standard curve equation of HA was used to calculate the corresponding HA concentration[13].

1.3 Transdermal experiment

The back skin of SD rats was selected for experiment. Animal welfare and experimental procedures were carried out according to the National Guidelines for the Review of Experimental Animal Welfare Ethics. All works were undertaken with the approval of the Changzhou University Bio-Medicine Ethics Committee. SD rats (6—8 weeks) were sacrificed, the back skin was shaved and then peeled off. The fat layer of skin was removed with the saline solution. A concave para-film was placed on top of the receiving chamber and 400 μL of PBS was added to form a micro-transdermal receiving chamber. The rat skin was sandwiched between the supply chamber and the micro-transdermal receiving chamber, with the skin stratum corneum (outer layer) facing the supply chamber and the dermis (inner surface) facing the receiving pool. Adding 2 mL of different CHA sample solutions to each supply chamber. Putting the treated transdermal tester into the diffusion cell and waiting for the temperature to reach 35 ℃. The total transdermal time is 4 h. After the transdermal time is over, the liquid in the micro-receptor chamber in the diffusion cell was collected, and the volume of the permeate solution

and the concentration of CHA were measured. Transdermal rate of CHA was calculated: transdermal rate(%)=(CHA concentration×volume after penetration)/(CHA concentration×volume before penetration)×100%.

1.4 Cytotoxicity test

Fresh isolated rat synovial fibroblasts were used for cytotoxicity assays[14]. Cells were cultured in DMEM supplemented with 1% penicillin-streptomycin and 10% FBS and incubated at 37 ℃, 5% CO_2. When reaching the exponential growth phase, cells were seeded into 96-well plates at a density of 5 000 cells/well for attachment overnight.

RosA filtered by 0.22 μm sterile filter was diluted to different concentrations in serum free condition, and incubated with cells for 20 h. Thereafter 50 μL of 10% 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) solution was added to each wells, and incubated for another 4 h, at 37 ℃, 5% CO_2. Then the medium was carefully removed and 100 μL DMSO was added to each well. The plate was shacked for another 10 min at room temperature and then the absorbance was read at 570 nm with a microplate reader.

Determination of cytotoxicity: according to the United States Pharmacopoeia 29th edition, calculate the relative cell proliferation rate λ_{RGR} (λ_{RGR} = absorbance value of various types of hyaluronic acid intervention group/absorbance value of non-intervention control group × 100%), and judge the results according to the criteria[15].

1.5 Statistical analysis

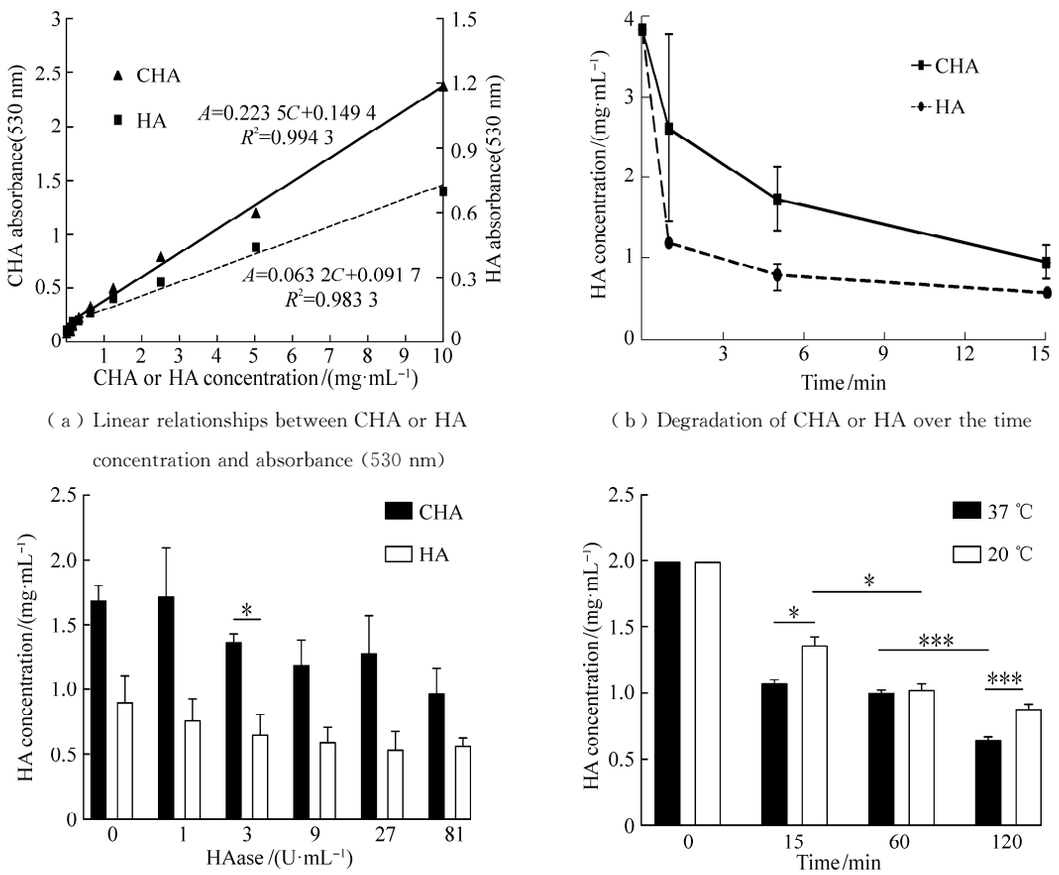
Graph-pad Prism version 7.0 software was used for statistical analysis and graph preparations. Unpaired student's test was used to compare differences between groups. $^{*}P<0.05$ was taken as significant.

2 Results

2.1 The degradation of CHA under different time and temperature conditions is slower than HA

The study was to establish the linear relationships between the concentration (mg/mL) of CHA or HA vs the OD value (530 nm) and examining the stabilities of crosslinked hyaluronic acid at different times, temperatures and concentration of HAase. The corresponding regression equations were $A=0.223\,5C+0.149\,4$, $R^2=0.994\,3$ for CHA; and $A=0.063\,2C+0.091\,7$, $R^2=0.983\,3$ for HA (Fig. 1 (a)). Fig. 1 (b) shows the degradation of CHA or HA over 0, 1, 5, 15 min under room temperature. The stability studies demonstrated that the degradations of CHA or HA were time dependent, as shown in Fig. 1 (b), both CHA and HA are quickly degraded at one min, but the degradation speed of HA was faster than CHA. In the first minute, the degradation rate of HA (58.5%) was 31.5% higher than that of CHA (27.0%); at 5 minutes, the degradation rate of HA (68.1%) was 21.6% higher than that of CHA (46.5%); at 15 minutes, the degradation rates of the two were similar. No matter what the concentration of HAase was, Fig. 1 (c) shows that the degradation of HA

was greater than that of CHA, and when the concentration of HAase was 3U/mL, the difference between the two was significant in degradation rate ($^{*}P<0.05$). The data also demonstrated that the time and temperature would affect CHA degradation rates, of which at 37 ℃ was faster than at room temperature (20 ℃) within the same time, e.g. At time-points 15 min ($^{*}P<0.05$) and 120 min ($^{***}P<0.001$), the difference between the two temperatures were significant. No matter what temperatures they were, both degradation speeds are time-dependents; at 37 ℃, CHA was degraded more in the process of 60 min to 120 min ($^{***}P<0.001$); at 20 ℃, CHA was more degraded in the process of 15 min to 60 min ($^{*}P<0.05$) (Fig. 1 (d)). All data shown are the mean ± SEM of three independent experiments.



(a) Linear relationships between CHA or HA concentration and absorbance (530 nm)

(b) Degradation of CHA or HA over the time

(c) Degradation of CHA and HA under different concentrations of HAase

(d) Effects of temperature and time on the degradations of CHA

Fig.1 Degradations of CHA or HA at difference conditions

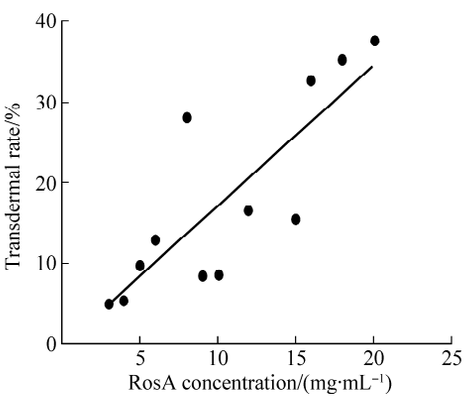
2.2 The transdermal rate of CHA was correlated with the increased RosA concentration

Therefore, CHA is more suitable for the study on transdermal absorption in vitro. This study was to evaluate the enhancing effects of RosA and combinations on CHA transdermal. The data from transdermal experiments shown that as the concentration of RosA increased (3, 4, 5, 6, 8, 9, 10, 12, 15, 16, 18, 20 mg/mL), the transdermal rate of CHA was also increased, the linear relationship between both has been well observed ($^{***}P=0.001$) (Fig. 2 (a)). Fig. 2 (b) shows the transdermal penetration of CHA at different time points with RosA (0, 5, 10 mg/mL) added. Data shown are the mean ± SEM of three independant experiments. Compared with 5 mg/mL, the concen-

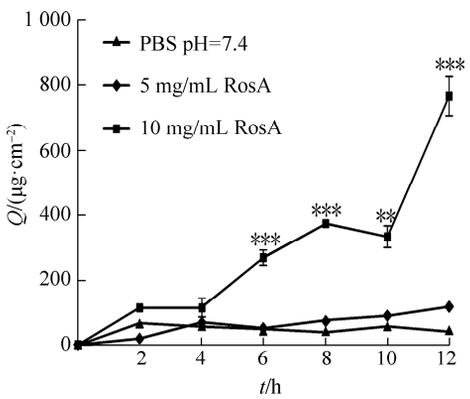
tration at 10 mg/mL of RosA was effectively increased the transdermal rate of CHA significantly, and especially after 6 h (** $P<0.01$, *** $P<0.001$). As shown in Table 1, Plot Q versus t at different times, and perform linear regression on Q versus T in the straight part after plotting. The slope obtained is the transdermal absorption rate (J) of CHA. In order to further compare the penetration-enhancing effects of penetration enhancers, calculate their penetration multiples according to the following formula: λ_{ER} (enhancement ratio) = J_i (including penetration enhancers) / J_e (excluding penetration enhancers) (Table 1). The λ_{ER} of RosA as permeability aid were 6.234 or 37.623 when the concentrations were 5 or 10 mg/mL, respectively.

5% rosemary essential oil mixed with or without azone (2% or 9%) were used as the PE for CHA transdermal experiments. Compared with the blank group and the 5% rosemary essential oil group, the 2% azone group and the binary group of 2% azone with 5% rosemary essential oil were able to effectively enhance thetransdermal rate of CHA (* $P<0.05$) (Fig. 2 (c)).

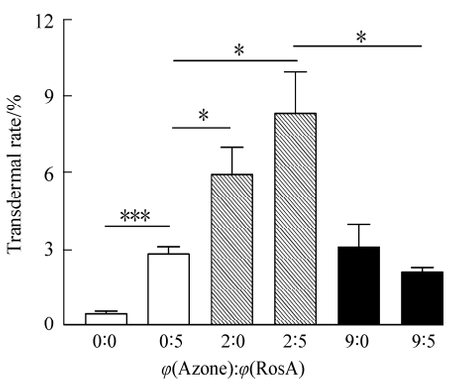
The transdermal rates of CHA in addition of RosA as the enhancers at different concentrations (3, 6, 9, 12 mg/mL) with or without 2% azone involved are shown in Fig. 2 (d), which demonstrated that when RosA concentration was 3, 6, 9, 12 mg/mL, 2% azone could effectively enhance the transdermal rate of CHA (*** $P<0.001$).



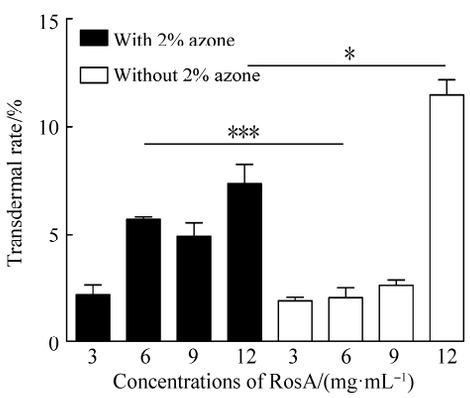
(a) Linear relationship of transdermal rate of CHA vs different concentrations of RosA



(b) Transdermal penetration of CHA at different time points with RosA (0, 5, 10 mg/mL) added



(c) Effects of 5% rosemary essential oil mixed with or without azone (2% or 9%) on the transdermal rate of CHA



(d) Effects of different concentrations of RosA in binary combinations of azone (2%) on the transdermal rate of CHA

Fig.2　Transdermal rate of CHA using NPE of rosemary extracts

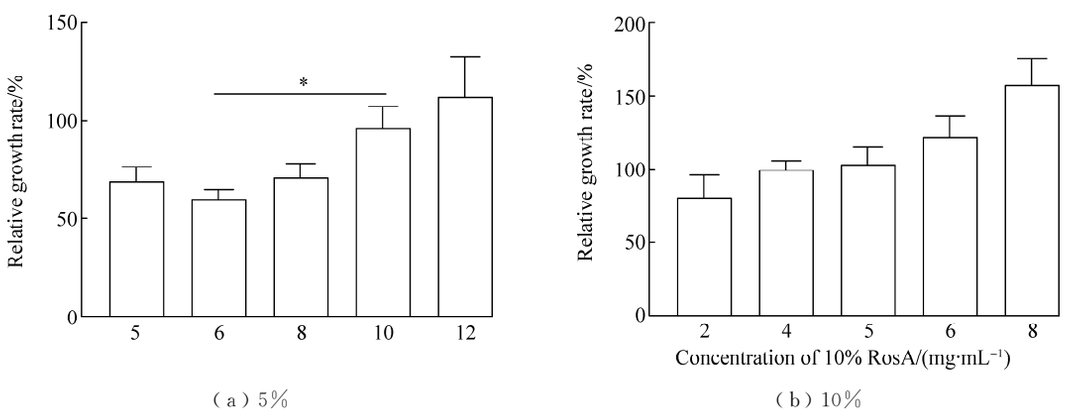
Table 1 Transdermal absorption parameters of CHA containing RosA

Rosmarinic acid/（mg·mL^{-1}）	Q/（μg·cm^{-2}）	J_i/（μg·cm^{-2}·h^{-1}）	λ_{ER}
0	42.249	1.426	
5	117.185	8.890	6.234
10	768.188	53.650	37.623

Note: Q—Skin penetration per unit area, 12 h; J_i—Skin penetration per unit time and per unit area; λ_{ER}—enhancement ratio.

2.3 RosA had no cytotoxic effects on cells

The results of cell cytotoxicity test have showed that both RosA (5%) had no effect on rat fibroblasts when its concentration around 10 mg/mL; RosA (10%) had no effect on rat fibroblasts when its concentration below 5 mg/mL. When the concentration of RosA (5%) was 5, 6, 8, 10, 12 mg/mL, the relative growth rates of rat synovial fibroblasts were 69.59%, 59.72%, 70.59%, 96.23%, 112.12%, respectively (Fig. 3 (a)). When the concentration of RosA (10%) was 2, 4, 5, 6, 8 mg/mL, the relative growth rates of rat synovial fibroblasts were 80.98%, 99.86%, 102.99%, 122.59%, 157.64% respectively (Fig. 3 (b)). According to the US Pharmacopoeia cytotoxicity test, the biosafety of the test materials (these concentrations of RosA) within the level 2 response ($\lambda_{RGR} \geqslant 50\%$) met the requirements. Data shown are the mean ± SEM of three independent experiments.



(a) 5% (b) 10%

Fig.3 Effects of different concentrations of RosA on rat synovial fibroblasts grow rates

3 Discussion

Rosemary has important application value in pharmaceutical, food, cosmetic and other fields due to its antioxidant, antibacterial and antiviral activities. It was reported that RosA has a function in inhibition of HAase activity, protecting nature HA from degradation caused by HAase[16-17]. This study investigated whether CHA could be used as a biomaterial for local tissue support through transdermal absorption. The results showed that CHA was more stable and suitable for high efficiency in cosmetic pharmaceutical and biomedical material applications. The aim of this study was also to investigate whether or not RosA can be a good natural penetrate enhancer for CHA application. Our present study demonstrated that it not only possess anti-infection and anti-inflammatory functions, but also helps CHA to penetrate cross rat skin.

Compared with intra-articular injection and oral administration, TDDS can avoid the pain caused by injection of drugs to patients, and at the same time, it can improve the disadvantage of slow absorption and side effects of oral administration because it can treat specific sites[18].

However, the delivery of most drug molecules via a transdermal route remains one of the major challenges in the development of TDDS. The principal barrier to TDD is located in the stratum corneum, the outermost layer of the skin, thereby limiting percutaneous absorption[19]. To achieve therapeutically effective drug levels at the proper site following TDDS, the barrier properties of the stratum corneum must be modified to enable sufficient drug permeation. A lot of approaches have been used to alter the stratum corneum barrier properties, and the most commonly applied approach is the application of PE[20-21]. Until now, efforts have been directed at identifying desirable PE which possess safe yet effective properties[22-24]. Compared to conventional synthetic PE such as azone, dimethyl sulfoxide (DMSO) and ethanol, NPE have been shown to own low systemic toxicity, high enhancement activity, and low cutaneous irritation at low concentration[25]. In the transdermal test of CHA, 5% concentration of rosemary essential oil showed better permeability than the control group without the permeability aid. It was also found that 5% rosemary essential oil combined with 2% azone had better permeability to the same concentration of crosslinked hyaluronic acid containing 2% azone. The results showed that rosemary essential oil had a certain permeability to CHA, but due to the poor solubility of rosemary essential oil in CHA, the permeability of rosemary essential oil with higher concentration to CHA could not be tested.

As an extract ofrosemary, RosA is a polyphenolic hydroxyl compound. Polyphenols are a promising and widely used enhancer because of their strong antioxidant, anti-inflammatory activity, lower skin irritation and high-water solubility[26-27]. These properties led us to use RosA as a permeability aid in CHA transdermal experiment. The results showed that RosA had a better permeability effect on CHA in vitro compared with rosemary essential oil, and the transdermal rate of CHA increased with the increase of RosA concentration. The enhancement ratios (λ_{ER}) of RosA (10 mg/mL) were determined to be 37.623, which was six folds higher that of RosA at 5 mg/mL at the same environment increasing the flux of CHA. Polyphenolic hydroxyl has ability to loose stratum corneum of the tight network skin[28], which might facilitate the diffusion of CHA through the stratum corneum; also its function of inhibiting HAases against CHA degradation may keep CHA integrity during penetration, as well as its oxygen-containing and hydrocarbon could form complexes to help in the stratum corneum partition of the CHA.

Despite most PEs performing fairly well in Transdermal drug delivery system, only a few of them have been approved for clinical application due to their skin toxicity or irritation. It is challenging to maintain the balance between safety and potency of PEs. RosA obtained from natural sources are generally considered to be less toxic comparedto synthetic PEs, such as azone[29]. Combined with the experimental results of toxicity of RosA at different concentrations on synovial fibroblasts in rats, when the concentration of RosA was 10 mg/mL, it met the requirements of good permeability and safety.

4 Conclusion

In conclusion, beyond its anti-inflammatory function, present study for the first time demonstra-

ted that RosA can act as a nature enhancer for transdermal percutaneous absorption of CHA with no cytotoxicity, which provides a new potential for pharmaceutical, surgical and cosmetic applications.

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