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Exploring the potential of rosemary essential oil as an natural penetration enhancer for promoting transdermal absorption of crosslinked hyaluronic acid in-vitro

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ABSTRACT. ***Objectives:*** *This investigation aimed to study the effect of rosemary essential oil on the transdermal absorption of crosslinked hyaluronic acid in vitro.* ***Material and methods:*** *The most suitable concentration of crosslinked hyaluronic acid was chosen and enhancing effects of rosemary essential oil on the permeation of crosslinked hyaluronic acid were evaluated.* ***Results:*** *The optimization of carbazole sulfate method by the 96-well plate was used to establish the standard curve of hyaluronic acid successfully. Crosslinked hyaluronic acid containing 5% (v/v) rosemary essential oil showed better skin penetration than blank control group in transdermal experiments. The combination of 5% (v/v) rosemary essential oil and 2%(v/v) azone as a permeability aid can achieve more effect than 5%(v/v) rosemary essential oil as a permeability aid.* ***Conclusion:*** *This study showed the enhancing effect of rosemary essential oil on crosslinked hyaluronic acid percutaneous absorption.*

KEYWORDS: *Rosemary essential oil, Crosslinked hyaluronic acid, Transdermal absorption, Natural penetration enhancers*

1. Introduction

Crosslinked Hyaluronic Acid (CHA) is a polymer gel with three-dimensional structure obtained by crosslinking modification of HA with a crosslinking agent [1] (Fig.1A). Compared with natural HA, CHA has the advantages of good biocompatibility, non-antigenicity, moisturizing, viscoelasticity, anti-inflammatory and safety without toxicity. The obvious increase in molecular volume can make up for the shortcomings of poor stability of natural HA [2]. However, due to the relatively large molecular weight of CHA, it cannot penetrate the stratum corneum

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of the skin. So, the selection of proper penetration enhancers would be necessary to promote its transdermal absorption [3].

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 [4].

Rosemary essential oil is a colorless to pale yellow volatile liquid obtained from the leaves of rosemary (Fig.1B) by water vapor. The main ingredients include α-pinene, β-pinene, pinene, β-myrcene, limonene, 18-eucalyptol, p-cymene, camphor, ethylene bornyl ester, α-terpine ester, borneol, Dozens of ingredients such as verbalone [5].

2. Materials and methods

2.1 Chemicals and materials

Crosslinked Hyaluronic Acid (Changzhou Pharmaceutical Research Institute Co., Ltd.)[6]; 95% azone (Shenzhen Simeiquan Biological Technology Co., Ltd.); 99% Rosemary Essential Oil, (Changzhou Pharmaceutical Research Institute Co., Ltd.); Sodium chloride (analytical grade, Sino pharm Chemical Reagent Co., Ltd.); phosphate buffered saline (pH7.4)(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 ethanol (Shanghai McLean Biotechnology Co., Ltd.); 6-8 weeks 120-150g SD female Rat (Changzhou Cavins Experimental Animal Co., Ltd.); PM-996 sealing film (BEMIS).

2.2 Detection of HA and CHA

Establishment of Standard Curve: HA/CHA standard solutions with different concentrations (10.0, 5.0, 2.5, 0.625, 0.312, 0.156, 0.078, 0 mg/ml) were precisely prepared. The absorbance of CHA was measured by a 96-well plate carbazole sulfate method. Taking the CHA concentration (C) as the abscissa and the absorbance (A) as the ordinate.

Sulfuric acid-carbazole method was modified using 96-well plate in this study. CHA was hydrolyzed by sulfuric acid to uronic acid at high temperature. The uronic acid reacted with carbazole ethanol to produce a purple-red compound. The maximum absorption was detected at 530 nm with a microplate reader [7].

Adding 20 μL of the test solution to each well on ice and then adding 100 μL of sodium tetra-borate sulfuric acid solution. The 96-well plate was heated in 95 °C water for 15 min. After heated, it was quickly cooled to room temperature on ice.

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Then, adding 4 μL of carbazole ethanol solution to each well. As before, the 96-well plate was heated in 95°C water for 15 min and cooled to room temperature after heating. At last, measuring the OD value at 530 nm with a microplate reader. The standard curve equation of CHA was used to calculate the corresponding CHA concentration [8].

Optimization of CHA assay: The volume of sodium tetra-borate sulfuric acid solution was set as 50,100,200μL, and the volume of carbazole ethanol solution was set as 4, 8, 12μL.Then, the CHA standard curve was detected by orthogonal test respectively. Finally, the optimal combination is obtained by drawing the orthogonal test table (Table 1).

2.3 Transdermal experiment

YB-P6 type intelligent transdermal diffuser with an effective transdermal area of 1.62 cm^2 was used for in vitro 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 °C. The total transdermal time is 4 hours. 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%.

3. Statistical analysis

Graph-pad Prism version 7.0 software was used for statistical analysis and graph preparations. The Mann-Whitney non-parametric test was used to compare differences between groups. *$P<0.05$ was taken as significant.

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4. Results

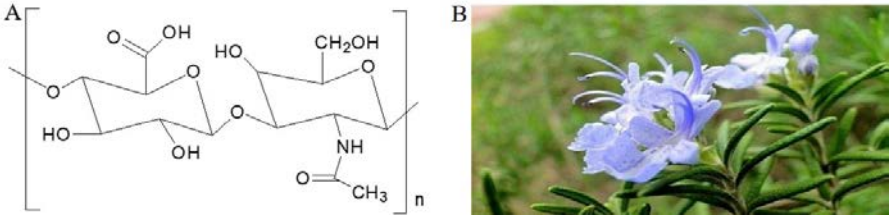

Figure. 1 (A) molecular structure of crosslinked hyaluronic acid; (B) the picture of rosemary.

Table 1 (A) Orthogonal test factor level table

Factors	Solution A(μL)	Solution B(μL)
1	50	4
2	100	8
3	200	12

Table 1 (B) L_9 (3^2) Orthogonal test results

Number	Solution A(μL)	Solution B(μL)	R^2
1	1	1	0.917
2	1	2	0.903
3	1	3	0.855
4	2	1	0.987
5	2	2	0.996
6	2	3	0.876
7	3	1	0.973
8	3	2	0.971
9	3	3	0.609
K1	2.675	2.877	
K2	2.859	2.870	
K3	2.554	2.340	
Range	0.305	0.537	
Best plan	K2	K1	

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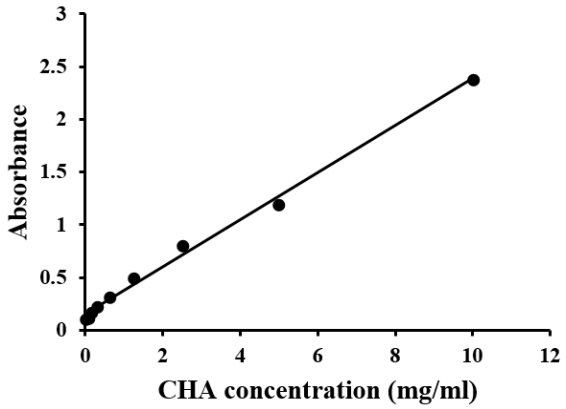

Figure. 2 Standard curve of crosslinked hyaluronic acid.

In this study, the linear relationships between the concentration (mg/ml) of CHA vs the absorbance (530nm) were well-established. The corresponding regression equations were $A = 0.2235C + 0.1494$ and $R^2 = 0.9943$ for CHA (Fig.2).

Table 2 Transdermal rate of CHA using penetration enhancers.

	1	2	3	4	5
Azone	-	2%	9%	-	-
Peppermint oil				2%	
Rosemary essential oil	-	-	-	-	5%
Transdermal rate (%)	0.48	0.24	3.10	1.65	2.86

Table 3 Transdermal rate of CHA using combination of penetration enhancers.

	1	2	3	4	5
Azone	-	2%	9%	-	-
Peppermint oil	-	-	-	2%	4%
Rosemary essential oil	5%	5%	5%	5%	5%
Transdermal rate (%)	2.86	8.46	2.07	1.34	3.03

Compared with the blank group, the 5% rosemary essential oil group was able to effectively enhance the transdermal rate of CHA, but lower than the positive control azone (9%) group. 5% rosemary essential oil increased the transdermal rate of CHA about 2.34%, compared with blank. Compared with the group of 2% peppermint oil, the 5% rosemary essential oil group was able to effectively enhance the transdermal rate of CHA. 5% rosemary essential oil increased the transdermal rate of CHA about 1.21%, compared with the group of 2% peppermint oil (Table 2). 5% rosemary essential oil mixed with or without 2% azone or 9% azone were used as the PEs for

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CHA transdermal experiments. Compared with the binary group of 2% azone with 5% rosemary essential oil, the CHA transdermal rate of binary group of 9% azone with 5% rosemary essential oil was significantly reduced. Compared with the group of 5% rosemary essential oil, the CHA transdermal rate of binary group of 4% peppermint oil with 5% rosemary essential oil was increased. The CHA transdermal rate of binary group of 4% peppermint oil with 5% rosemary essential oil increased the transdermal rate of CHA about 0.17%, compared with the group of peppermint oil. The CHA transdermal rate of binary group of 4% peppermint oil with 5% rosemary essential oil increased the transdermal rate of CHA about 1.69%, compared with the CHA transdermal rate of binary group of 2% peppermint oil with 5% rosemary essential oil (Table 3).

5. Discussion

In recent years, different forms of HA have been used in many ways, such as drug delivery, bio-material for medical use, beauty, cosmetics and food. It has been reported that after continuous administration of HA labeled with the isotope ^{14}C, approximately 8.8% of HA is absorbed by the body, 77% is eliminated with expiration, 3% is eliminated with urine, and approximately 9% is retained in the body, which proved that HA can be absorbed as a macromolecule [9]. Natural hyaluronic acid has a small molecular weight, which is beneficial for it to penetrate the skin. Considering the instability of HA, various forms of CHA have also been studied. The purpose of present study was to investigate whether CHA could be used as a topical material to achieve local tissue support through transdermal absorption.

Sulfuric acid-carbazole method is a common color reaction for detecting HA content [10]. There are some disadvantages to using UV spectrophotometer for measurement, for example that it is tedious steps when measuring large quantities of samples and the requirements of sample volume is high. These problems can be solved by using a 96-well plate. Orthogonal experiments can be used to optimize the conditions for detecting hyaluronic acid content in 96-well plates and reduce the volume of developer.

Although the effect of intra-articular injection of HA is significant [11], the required skills are very high, and patients need to endure more severe pain. This treatment is not conducive to long-term use. Long-term use of intra-articular injection of exogenous HA will inevitably bring trauma and infection to patients [12]. Oral administration is convenient and common but has some disadvantages such as slow absorption and the effect of the gastrointestinal tract on drug absorption and the "first pass effect" of the liver [13]. In contrast, TDDS can avoid the pain caused by injection of drugs to patients, and at the same time, it can improve the disadvantages of slow absorption and side effects of oral administration because it can treat specific sites [14].Compared to conventional synthetic PEs such as azone, dimethyl sulfoxide (DMSO) and ethanol, natural penetration enhancers have been shown to improve the permeation of both lipophilic and hydrophilic compounds [15]. One of the components of plants' essential oil is terpenes. Terpenes are promising, clinically acceptable enhancers because of their low systemic toxicity, high

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enhancement activity, and low cutaneous irritation at low concentration between 1 and 5% [16]. In the transdermal test of CHA, 5% concentration of rosemary essential oil showed better permeability than the control group without the permeability aid. In contrast, after administration of the transdermal liquid containing 2% azone, the concentration of HA in the receiving tank was higher in 4 h. It was found that 5% rosemary essential oil combined with 2% azone had better permeability to the same concentration of crosslinked hyaluronic acid. 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. Further studies are recommended to measure the penetration promoting effect to crosslinked hyaluronic acid of the main components of rosemary essential oil.

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Declaration of competing interest

All authors declare that they have no conflict of interest.

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