In vitro study of transdermal penetration and iontophoresis of hepatitis B vaccines through rat skin

XU Ting, XU Yue-hong[*], WEI Min-yan, DENG Li-he, WU Chuan-bin

(*School of Pharmaceutical Science, Sun Yat-sen University, Guangzhou 510006, China*)

Abstract: *In vitro* percutaneous delivery of hepatitis B vaccines was investigated in order to assess the penetration of vaccine under passive diffusion and iontophoresis conditions. The study was carried out using Franz vertical diffusion cell through the hairless abdominal skin of Sprague-Dawley (SD) rats. Enzyme-linked immunosorbent assay (ELISA) was used to determine the cumulative amount of permeation and the retention amount of drug in skin. Passive diffusion alone resulted in less skin permeation and retention of hepatitis B vaccines, only (2.1 ± 0.1) ng·cm^{-2} and (2.3 ± 0.1) ng·cm^{-2} after 24 h when the initial concentration of vaccine in the donor compartment was 23 μg·mL^{-1} and 46 μg·mL^{-1}, respectively. After removing the stratum corneum, the permeation and retention amount of hepatitis B vaccines increased to (383.7 ± 86.2) ng·cm^{-2} and (16.8 ± 4.6) ng·cm^{-2}, respectively, 171.6-folds and 2.1-folds more than that from its intact skin with the drug loaded at 46 μg·mL^{-1}. Iontophoresis induced a significant increase of cumulative and retention amount of hepatitis B vaccines through the skin ($P<0.05$). Application of iontophoresis significantly enhanced the permeation of hepatitis B vaccines ($P<0.05$) by 2.7-folds and 6.6-folds for the intact skin, and by 1.6-folds and 1.8-folds for the tape-stripped skin with initial drug loading of 23 μg·mL^{-1} and 46 μg·mL^{-1}, respectively. Iontophoresis also significantly increased the amount of drug retained in the skin. After applying iontophoresis for 6 h, the amount of skin retention was nearly the same as passive diffusion for 24 h both from intact skin [(16.8 ± 4.6) ng·cm^{-2} *vs* (13.3 ± 5.4) ng·cm^{-2}] ($P>0.05$) and tape-stripped skin [(36.7 ± 14.1) ng·cm^{-2} *vs* (26.8 ± 11.2) ng·cm^{-2}] ($P>0.05$). Overall, these findings revealed that the transportation efficiency of bioactive substance like hepatitis B vaccines may be improved by iontophoresis, which can be potentially used in the field of transcutaneous immunization.

Key words: iontophoresis; hepatitis B vaccine; transdermal delivery

乙肝疫苗体外经大鼠皮肤渗透与离子导入给药特性研究

徐　婷, 徐月红[*], 韦敏燕, 邓礼荷, 吴传斌

(中山大学药学院, 广东 广州 510006)

摘要: 对乙肝疫苗进行体外经皮实验以评价疫苗在被动扩散和离子导入情况下的经皮渗透特性。体外透皮研究采用 Franz 扩散池, 以 SD 大鼠的腹部皮肤为渗透屏障, 以酶联免疫法测定药物累积渗透量和在皮肤中的滞留量。乙肝疫苗 (质量浓度为 23 μg·mL^{-1} 与 46 μg·mL^{-1}) 经完整皮肤被动扩散的经皮渗透量与皮肤滞留量均极少, 24 h 累积渗透量仅 (2.1 ± 0.1) ng·cm^{-2} 和 (2.3 ± 0.1) ng·cm^{-2}。去除角质层后, 经皮渗透量与皮肤滞留量分别提高至 (383.7 ± 86.2) ng·cm^{-2} 和 (16.8 ± 4.6) ng·cm^{-2}, 是完整皮肤的 171.6 倍与 2.1 倍 (46 μg·mL^{-1})。离子导入对于乙肝疫苗具有明显的经皮渗透促进作用: 完整皮肤经皮离子导入 6 h, 乙肝疫苗的累积渗透量是被动扩散 6 h 的

2.7 倍 (23 μg·mL^{-1}) 和 6.6 倍 (46 μg·mL^{-1}); 去角质层皮肤离子导入, 乙肝疫苗的累积渗透量是被动扩散 6 h 的 1.6 倍 (23 μg·mL^{-1}) 和 1.8 倍 (46 μg·mL^{-1})。离子导入也能显著增加疫苗在皮肤中的滞留量。离子导入 6 h 疫苗在完整皮肤中的滞留量和去角质层皮肤中的滞留量均与被动扩散 24 h 的皮肤滞留量接近 [完整皮肤: (16.8±4.6) ng·cm^{-2} vs (13.3±5.4) ng·cm^{-2}; 去角质层皮肤: (36.7±14.1) ng·cm^{-2} vs (26.8±11.2) ng·cm^{-2}] ($P>0.05$)。研究结果表明, 离子导入是促进乙肝疫苗等生物活性大分子经皮渗透的有效手段, 有希望应用于乙肝疫苗的经皮免疫领域。

关键词: 离子导入; 乙肝疫苗; 经皮转运

Hepatitis B is a serious worldwide public health problem with about 2 billion people having been infected by the virus. This results in 360 million people having chronic infection and 600 000 deaths occurring every year due to hepatitis B virus (HBV) related diseases or hepatocellular carcinoma[1]. Vaccination is an effective way to prevent HBV infection. Current available hepatitis B vaccines is mainly administered by intramuscular injection. Because three consecutive injections are needed at certain time intervals to achieve a desired immune effect, this treatment results in pain, the requirement for trained personnel, the risk of inducing needle-related diseases and low vaccination coverage due to failing to follow up. Thus, a needle free/non-invasive vaccination technique has become a global priority[2]. Transcutaneous immunization, a form of non-invasive vaccination, is an innovative immunization route which has become one of the rapidly developing areas of research[3]. Several reasons make skin an attractive route for vaccine delivery: highly accessible skin easy to monitor, presence of powerful antigen-presenting cells (APCs) in the epidermis, and no need to use needle or syringes for immunization[4]. Therefore, transcutaneous delivery can not only increase vaccine compliance but also improve vaccine safety, and immunization will become simple, practical and more economical in the future. Numerous studies have reported that humoral and cell-mediated protective immunity was successfully induced by this novel approach of immunization such as influenza virus[5], Tetanus toxoid[6] and diphtheria[7]. A number of clinical trials are already in progress[8, 9]. But the major challenge for topical administration of vaccine is how to effectively deliver sufficient amount of vaccine through the skin, in order to generate protective immune response. One of the possibilities to enhance the penetration of vaccine through the skin is the use of iontophoresis.

Transdermal iontophoresis (TI) is a physical enhancement technique used primarily to facilitate the delivery of charged molecules across the skin. It can be simply defined as the application of a small electric current to enhance the transport of both charged/polar and neutral compounds across the tissue barrier[10]. This technique is capable of expanding the range of compounds that can be delivered transdermally. Despite a great success in using transdermal delivery for topical or systemic therapy as reported in the literatures, it is generally considered that drug molecules more than 1 000 D may have difficulty in percutaneous absorption, especially for macromolecular drugs such as peptides and proteins. These drugs are often hydrophilic, with high molecular weight or charged, and make it difficult to reach or maintain effective therapeutic concentration by passive permeation. However, their transdermal absorption may be promoted by iontophoresis. Over the years, a wide range of proteins and peptides such as insulin[11, 12], luteinizing-hormone-releasing hormone (LHRH)[13], salmon calcitonin[14], and defibrase[15] have been studied for transdermal delivery via iontophoresis. It seems to be one of the most promising applications to employ iontophoresis to promote the percutaneous absorption of peptide and protein.

In this study, iontophoresis was used to promote the transdermal permeation of hepatitis B vaccines so as to study the possibility of transcutaneous immunization. The bioactive ingredient of hepatitis B vaccines is hepatitis B surface antigen which gives a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a molecular weight and an isoelectric point of 24 kD and 7.5, respectively. In the passive diffusion or iontophoresis experiments, the cumulative permeation amount of hepatitis B vaccines through the skin as well as the retained amount of the vaccine in the skin was quantitatively evaluated for comparison. The effect of the skin barrier on the transdermal delivery also studied by comparing the cumulative amount of hepatitis B vaccines through the stratum corneal (SC)-stripped skin

and the intact skin.

Materials and methods

The hepatitis B surface antigen (HBsAg, source-genetically modified yeast cells) was provided by Kangtai Biological Products Co. (Shenzhen, China). HBsAg enzyme-linked immunosorbent assay (ELISA) Kit was purchased from Kehua Bio-engineering Co. (Shanghai, China). Reference substance for HBsAg was provided by DaAn Gene Co. of Sun Yat-sen University (Guangzhou, China). Deionized water with a resistance of $\geqslant$ 18.2 MΩ·cm^{-1} was used to prepare all the test solutions. All other chemicals and solvents were of analytical reagent grade.

Skin membrane preparation All experiments were conducted according to the protocol approved by the Institutional Animal Ethics Committee (IAEC) of Sun Yat-sen University. Male Sprague-Dawley rats (200–220 g) obtained from the animal facility of the university was sacrificed by cervical dislocation. Hair was removed from the dorsal portion using an animal hair clipper and the skin was harvested with full thickness. Then the fat adhering on the dermis side was removed using a scalpel. Finally, the skin was rinsed with normal saline and then sealed in a plastic bag and stored at −20 ℃ for further study. The integrity of the rat skin was checked to exclude any damage prior to experiment. The SC-stripped skin was prepared by further adhering the intact skin with medical tape repeatedly to remove the stratum corneum (SC). This procedure was repeated 15 times[16].

Determination of hepatitis B vaccines Hepatitis B vaccines samples were analyzed by enzyme-linked immunosorbent assay (ELISA). A sample of 50 μL was added to each well and incubated for 30 min at 37 ℃. The plate was washed five times with phosphate buffered saline Tween-20 (PBS-T). Then 50 μL of substrate solution was added to each well and incubated for another 15 min. The reaction was stopped by adding 50 μL of 2 mol·L^{-1} H$_2$SO$_4$ solution. The absorbance was measured at 450 nm using a microlate ELISA reader (Multiskan Ascent, Thermo Labsystem, Helsinki, Finland). The method exhibited high reproducibility and accuracy in correlating the optical density with the concentration of hepatitis B vaccines (0.4−15 ng·mL^{-1}, r = 0.991 2). The assay provided a sensitivity of 0.2 ng·mL^{-1}, and the blank samples did not show any interference.

Transdermal passive diffusion experiment *in vitro* The *in vitro* skin permeation of hepatitis B vaccines was studied using Franz vertical diffusion cell with an effective permeation area and receptor cell volume of 2.27 cm^2 and 6.4 mL, respectively. The skin (intact skin or the SC-stripped skin) was mounted on a receptor compartment with the stratum corneum side upward facing the donor compartment, and the dermis facing the receptor compartment. A volume of 1 mL hepatitis B vaccines solution at different concentrations was applied on the skin in the donor compartment. The receptor compartment was filled with 6.4 mL of PBS (pH 6.5) containing 0.05% Tween 20 to prevent vaccine adsorption onto the surface of the electrodes or the wall of the chamber. During the experiment, the solution in the receptor side was maintained at 32 ± 0.5 ℃, and stirred by magnetic stirrer at 200 r·min^{-1}. At fixed time intervals, 1 mL of sample was withdrawn from receptor compartment through side tube and replaced with an equal volume of fresh pre-tempered buffer. The samples were tested for antigen content using a HBsAg ELISA Kit.

Transdermal iontophoresis experiment *in vitro* The iontophoresis experiments were conducted using the same set-up as for the passive diffusion study. Prior to the experiment, the anode was placed in the donor compartment and the cathode was placed in the receptor compartment, with the power supply from an iontophoresis apparatus (Guangzhou, China). During the experiment, the parameters of the iontophoretic device were fixed with current density set at 0.04 mA·cm^{-2} accompanying an on/off ratio of 9 : 1, and frequency of the pulse current at 99.8 Hz. The rest experimental and sampling conditions were similar to those described in the previous passive diffusion study.

Determination of hepatitis B vaccines retained in the skin At the end of the passive diffusion or iontophoretic absorption experiment, the skin was removed from the vertical cell and washed with distilled water. The treated skin area was weighed, placed in a tube of 5 mL phosphate buffer, and homogenized for 2 min using a tissue homogenizer (Euro Turrax, IKa Labortechink, Germany). Then the tube was vortex mixed for 1 min followed by 10 min of centrifugation at 12 000×g. After that 1 mL of the supernatant liquid was drawn and placed in a second test tube for assaying the amount of hepatitis B vaccines as previously described.

Data analysis The permeation data was analyzed

using the following formula[17]:

$$Q_n = \frac{C_n \times V_0 + \sum_{i=1}^{n-1} C_i \times V}{A}$$

where Q_n is the cumulative amount of drug transferred to the receptor compartment at time point $t = n$ (ng·cm^{-2}); V_0 is the volume of the receptor compartment (mL), V is the volume of sample (mL), C_n is the drug concentration in the receptor compartment measured at time point $t = n$ (ng·mL^{-1}), C_i is the drug concentration in the sample taken at time point $t = i$ (ng·mL^{-1}), and A is the effective penetration area (cm^2).

The cumulative amount of hepatitis B vaccines permeated through skin was plotted as a function of time. The permeation rate of hepatitis B vaccines at the steady state (J_{ss}, ng·cm^{-2}·h^{-1}) was calculated from the slope of the straight line obtained by plotting the amount of hepatitis B vaccines permeated *versus* time in steady state conditions.

$$P = J_{ss}/C_d$$

Where P ($\times 10^{-3}$ cm·h^{-1}) is the skin permeation coefficient, C_d is the donor concentration.

All experiments were done in triplicate and the results were reported as mean ± standard deviation. Comparisons were made between groups using *t*-test, and the statistical significance level was defined as $P < 0.05$.

Results and discussion

1　Transdermal passive diffusion of hepatitis B vaccines *in vitro*

Figure 1 shows the transport profiles of hepatitis B vaccines at different initial concentrations under passive diffusion for 24 h. As expected at the end of the experiment, the cumulative amount of hepatitis B vaccines permeated through the intact skin under passive diffusion was extremely low, only (2.07 ± 0.08) ng·cm^{-2} and (2.25 ± 0.10) ng·cm^{-2} when the initial concentration of vaccine in the donor compartment was 23 μg·mL^{-1} and 46 μg·mL^{-1}, respectively. The low permeation data suggests that stratum corneum is the rate-limiting factor for hepatitis B vaccines penetrating through the skin by passive diffusion. And there was no significant difference in transport profiles through the intact skin with different concentrations of drug loaded in the receptor compartment ($P > 0.05$). This is because, in the condition of intact skin with the strong barrier effect of the stratum corneum, the drug concen-

tration isn't the key factor affecting the percutaneous penetration of hepatitis B vaccines.

After the skin being pretreated with medical tape to remove the stratum corneum, the penetration of hepatitis B vaccines through the SC-stripped skin significantly increased as shown in Figure 1. For the stratum corneum removed skin, the cumulative amount of hepatitis B vaccines under passive diffusion increased to (276.7 ± 48.9) ng·cm^{-2} and (383.7 ± 86.2) ng·cm^{-2}, 132.5-folds and 171.6-folds more than that from intact skin with the initial drug load concentration of 23 μg·mL^{-1} and 46 μg·mL^{-1}, respectively. The determination of skin retention was only carried out with the drug loading concentration of 46 μg·mL^{-1}. With 46 μg·mL^{-1} of hepatitis B vaccines solution loaded, the amount of skin retention with tape-stripped skin was (16.8 ± 4.6) ng·cm^{-2}, 2.1-folds more than that from intact skin. According to Table 1, the comparison between the permeation rates of hepatitis B vaccines through intact skin and tape-stripped skin also approved the previous conclusion. The permeation rate of hepatitis B vaccines through intact skin was less than 0.1 ng·cm^{-2}·h^{-1} with the concentration of 23 μg·mL^{-1} and 46 μg·mL^{-1}, respectively. After the stratum conrneum was removed, the permeation rate of hepatitis B vaccines increased to (12.6 ± 1.9) ng·cm^{-2}·h^{-1}, and (15.4 ± 2.4) ng·cm^{-2}·h^{-1}, 197 times (23 μg·mL^{-1}) and 216 times (46 μg·mL^{-1}) as the intact skin respectively. Different from the intact skin, the permeation amounts of hepatitis B vaccines were greater at all the time points with a higher drug load in the donor compartment than those with a lower initial drug load ($P < 0.05$). This result could be attributed to the fact that the drug concentration, more specifically the concentration
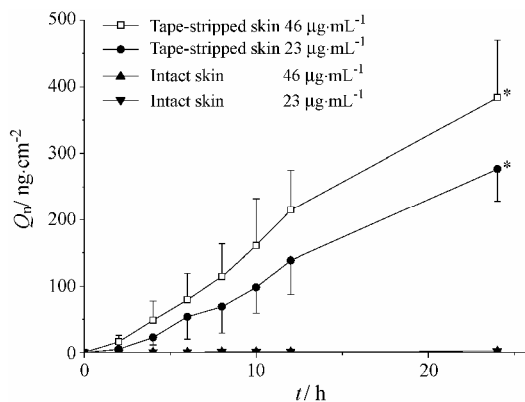

Figure 1　Cumulative permeation amount of hepatitis B vaccines under passive diffusion *in vitro* with different drug load concentrations ($n = 3$). $^*P < 0.05$ *vs* intact skin groups at the same concentration

Table 1 The permeation parameters of the hepatitis B vaccines under iontophoresis and passive diffusion condition ($n = 3$)

Group	Concerntrantion /$\mu g \cdot mL^{-1}$	Iontophoresis		Passive diffusion	
		J_{ss}/ng·cm^{-2}·h^{-1}	$P/\times 10^{-3}$ cm·h^{-1}	J_{ss}/ng·cm^{-2}·h^{-1}	$P/\times 10^{-3}$ cm·h^{-1}
Intact skin	23	0.277 ± 0.10	0.012 0 ± 0.004	0.064 ± 0.004	0.003 ± 0.000
	46	0.979 ± 0.152	0.021 2 ± 0.003	0.071 ± 0.005	0.002 ± 0.000
Tape-stripped skin	23	20.451 ± 8.582	0.889 ± 0.373	12.593 ± 1.924	0.335 ± 0.052
	46	39.422 ± 14.795	0.857 ± 0.322	15.422 ± 2.403	0.547 ± 0.084

gradient across the skin, became a major factor in percutaneous penetration under passive difussion after the diffusion barrier was removed.

2 Transdermal iontophoresis of hepatitis B vaccines *in vitro*

In order to investigate the effect of iontophoresis on skin permeation of hepatitis B vaccines, both intact skin and tape-stripped skin were used in the permeation experiment for 6 h under the conditions of anodal iontophoresis loaded with different concentrations of hepatitis B vaccines (23 $\mu g \cdot mL^{-1}$ and 46 $\mu g \cdot mL^{-1}$). The transport profiles were graphed in Figure 2, and a similar trend was observed as in passive diffusion study. It is obviously that the permeation of hepatitis B vaccines through the stratum corneum-stripped skin was significantly greater than that through the intact skin. Quantitatively, the cumulative permeation amount through tape-stripped skin was about 26 (23 $\mu g \cdot mL^{-1}$) and 19 (46 $\mu g \cdot mL^{-1}$) times as the permeation through the intact skin at the end of experiment. This suggested that the stratum corneum might also be a barrier for transdermal delivery of hepatitis B vaccines even under iontophoresis. It is also clearly shown that for both the intact and tape-stripped skin, a larger cumulative permeation amount was obtained when a higher concentration of drug was loaded in the donor compartment.

In order to clearly illustrate the improvement of transdermal delivery by iontophoresis, the accumulated permeation amount of hepatitis B vaccines after passive diffusion and iontophoresis applied for 6 h was compared in Figure 3. The results showed that the application of iontophoresis significantly enhanced the permeation of hepatitis B vaccines ($P < 0.05$) by 2.7-folds and 6.6-folds for the intact skin, and by 1.6-folds and 1.8-folds for the tape-stripped skin with initial drug load of 23 $\mu g \cdot mL^{-1}$ and 46 $\mu g \cdot mL^{-1}$, respectively. The permeation rates of hepatitis B vaccines across skins with or without the application of iontophoresis were also shown in Table 1. There was a more notable increase in permeation rate about 4.3-folds and 13.8-folds after iontophoresis applied on the intact skin with initial drug load of 23 $\mu g \cdot mL^{-1}$ and 46 $\mu g \cdot mL^{-1}$, respectively.

For the stratum corneum removed skin, the passive diffusion rate was relatively higher without the barrier, therefore, more enhancements in transdermal penetration by iontophoresis was observed for the intact skin with the permeation barrier.
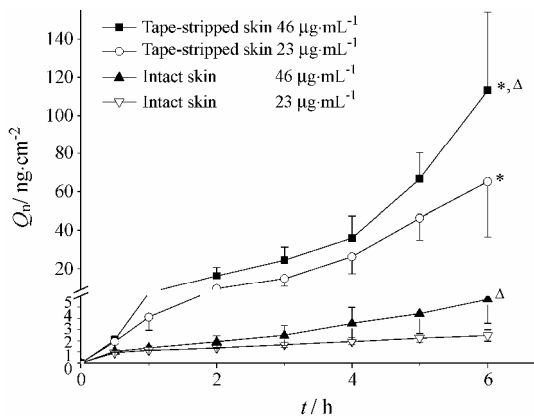

Figure 2 Cumulative permeation amount of hepatitis B vaccines under iontophoresis *in vitro* with different drug load concentrations ($n = 3$). $^*P < 0.05$ *vs* intact skin groups at the same concentration. $^\Delta P < 0.05$ *vs* the concentration group of 23 $\mu g \cdot mL^{-1}$ at the same condition of skin
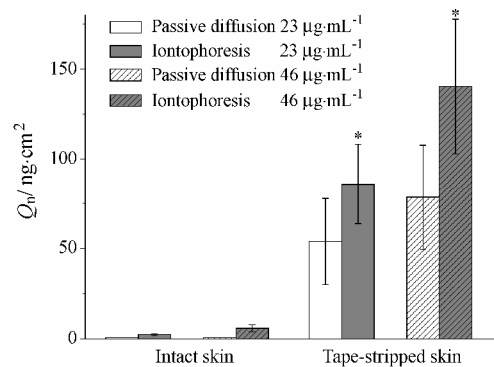

Figure 3 Cumulative amount of hepatitis B vaccines after passive diffusion or iontophoresis application for 6 h *in vitro* with different drug load concentrations ($n = 3$). $^*P < 0.05$ *vs* intact skin groups

Transdermal iontophoresis is available to increase the skin permeation of many types of molecules. Iontophoretic flux can be generated from not only

electrorepulsion but also electroosmotic solvent flow, which is produced by anodal iontophoresis from anode to cathode[17]. Thus, iontophoresis can be used to enhance transdermal delivery of ionic drugs as well as non-ionic compounds. In this study, as the hepatitis B vaccines carries a net positive charge at pH 6.2, its iontophoretic transport through skin will be from the anode chamber (anode iontophoresis), which is the same direction as the electroosmotic solvent flow. So the great enhancement of the permeability of hepatitis B vaccines can be attributed to the effects of electrorepulsion and electroosmosis together.

3 Hepatitis B vaccines retained in skin

Skin can be served as a temporary reservoir to retain the drug inside. Drug molecular weight is known as an important factor to affect the drug retention. Therefore, it is common for macromolecular drugs to retain in the skin during transdermal absorption[18, 19]. It is also well known that skin not only plays a role of barrier, but also is an important part of the immune system[20]. Therefore, it is important to determine the retained amount of hepatitis B vaccines in the skin after transdermal delivery studies.

As shown in Figure 4, the amount of hepatitis B vaccines retained in two types of skin was quantitatively evaluated at the end of the transdermal experiment. With 46 $\mu g \cdot mL^{-1}$ of hepatitis B vaccines solution loaded, the amount of skin retention after 6 h of iontophoresis was (16.8 ± 4.6) $ng \cdot cm^{-2}$ for intact skin and (36.7 ± 14.1) $ng \cdot cm^{-2}$ for tape-stripped skin, compared to the retention after 24 h of passive diffusion was (13.3 ± 5.4) $ng \cdot cm^{-2}$ for intact skin and (26.8 ± 11.2) $ng \cdot cm^{-2}$ for tape-stripped skin. The results suggest that iontophoresis not only promoted the permeation of hepatitis B vaccines through the skin, but also increased the retention amount of hepatitis B vaccines in the skin.
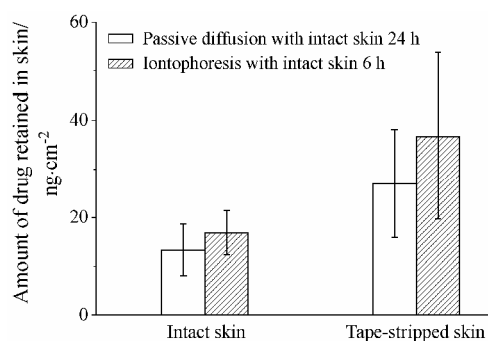

Figure 4　Retention of hepatitis B vaccines in rat skin after passive diffusion for 24 h or iontophoresis application for 6 h with drug load of 46 $\mu g \cdot mL^{-1}$ ($n = 3$)

Conclusion

The transport characteristics of hepatitis B vaccines across various types of skin under passive diffusion as well as iontophoresis were studied *in vitro*. As expected, the amount of hepatitis B vaccines permeation through the intact skin was very low and a greater permeability was observed with SC-stripped skin in the passive diffusion experiment. It suggests SC layer may be the major barrier for passive permeation of hepatitis B vaccines. The application of iontophoresis in transdermal experiment significantly improved the permeation rates J_{ss} ($ng \cdot cm^{-2} \cdot h^{-1}$) and coefficient P ($\times 10^{-3}$ $cm \cdot h^{-1}$) of hepatitis B vaccines for both intact and SC-stripped skin, and the vaccine cumulative permeation amount was increased with the increase of drug load. The increased retention of hepatitis B vaccines in the skin by iontophoresis might be helpful for better immune effect. The present study demonstrated the feasibility of transdermal iontophoretic delivery of hepatitis B vaccines *in vitro*. The permeation results obtained are encouraging and the work is further being extended on human cadaver skin and will evaluate its immunity effect *in vivo*. More research should be done before the successful application of iontophoresis for effective transcutaneous immunization.

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