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Effect of electroporation and iontophoresis on skin permeation of Defibrase® – a purified thrombin-like enzyme from the venom of *Agkistrodon halys ussuriensis* Emelianov

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The purpose of this study was to investigate electroporation and iontophoresis as a means for *in vitro* delivery of Defibrase – a thrombin-like enzyme (TLE) from *Agkistrodon halys ussuriensis* Emelianov snake venom – through human epidermis membrane (HEM). Electroporation was carried out using an exponential decay pulse generator (BioRad Genepulser, USA) for a period of 0.5 h, followed by a period of 5.5 h passive diffusion or iontophoresis. The results indicated that the combined use of electroporation and anodal iontophoresis in pH 6.4 permeation medium could effectively enhance the skin permeation of Defibrase, whose apparent permeability coefficient was $1.6 \pm 0.8 \times 10^{-4}$ cm · h^{-1}. The delivery of Defibrase by the combined use of electroporation and anodal iontophoresis was more effective than by electroporation alone ($P < 0.01$) or by the combined use of electroporation and cathodal iontophoresis ($P < 0.01$). Moreover, when the pH of the permeation medium was raised from 6.4 to 7.4 the permeation of Defibrase caused by a combined use of electroporation and anodal iontophoresis showed a tendency to increase. These results implied that electroosmotic flow effect might be important for the iontophoretic (following electroporation) skin permeation of Defibrase.

1. Introduction

Transdermal electroporation and iontophoresis are methods to enhance the transdermal transport of drugs. Electroporation of skin was first reported in 1993 [1], biomedical applications of iontophoresis have been around for more than a century [2]. Because of their programmable and controllable characteristics, both technologies have attracted considerable interest for the transdermal delivery of drugs including macromolecules [3–12].

In this report, electroporation and iontophoresis were applied separately and conjunctly for Defibrase transdermal transport *in vitro*. Defibrase is a thrombin-like enzyme (TLE) separated and purified from the venom of the northeast Chinese snake *Agkistrodon halys ussuriensis* Emelianov [13]. It gives a single band on SDS-PAGE, consists of 303 amino acids with a MW ca. 36,000 Da and has an isoelectric point of 4.3 [14]. Defibrase splits fibrinopeptide A from fibrinogen, in whole plasma or in purified form, producing a clot of modified fibrin. It does not activate factor XIII, and thus, in the absence of other activators, the Defibrase-produced fibrin clots are not cross-linked and are easily digested by plasmin. Defibrase can be used as a defibrinating agent for the treatment and prevention of thromboembolic diseases, but only a parenteral dosage form is available up to date. In this study, we investigated the skin permeation of Defibrase enhanced by electroporation and iontophoresis.

2. Investigations, results and discussion

2.1. Electroporation

The voltages applied to HEM were 300 V, 500 V and 1000 V with pulse time constant of 40, 8 and 6 ms, respectively. There was no obvious passive permeation of Defibrase during the experiment. The results of electroporation indicated that all the applied protocols could significantly enhance HEM permeation of Defibrase as compared to passive diffusion, but there were no significant differences for the permeation of Defibrase between the three protocols ($P > 0.05$, Fig. 1). We examined pH of donor and receptor solution, no evident changes could be found. The concentrations of Defibrase in donor compartments were also determined, more than 90% of its original amount were remained unchanged. According to Pliquett et al. [14], the applied voltages were quite different from the transdermal voltages that were about three-fold lower. There might be a range of effective voltages among which energy variation apparently has no effect on such a large molecule as Defibrase across HEM.

No significant difference of permeated Defibrase was found between the two electroporation protocols pulsed with electrodes of reversed polarity ($P > 0.05$, Fig. 2). The result indicated that neither electrophoresis nor electroosmosis contributed significantly to transport of Defibrase in this case, since both phenomena depend on electric field orientation.

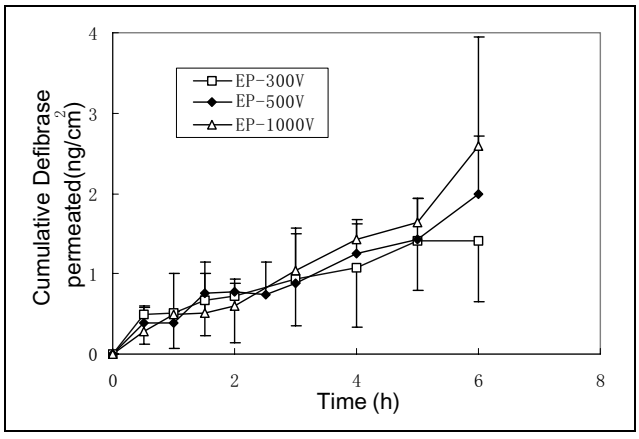

Fig. 1: Effect of pulse voltage on skin permeation of Defibrase (with cathode introduced in donor compartment). All protocols were applied for 0.5 h (6 pulses per min) followed by passive diffusion. The pulse time constant (τ) were 40 ms (300 V), 8 ms (500 V) and 6 ms (1000 V), respectively. Each point represents the mean (±SD) of three experiments.

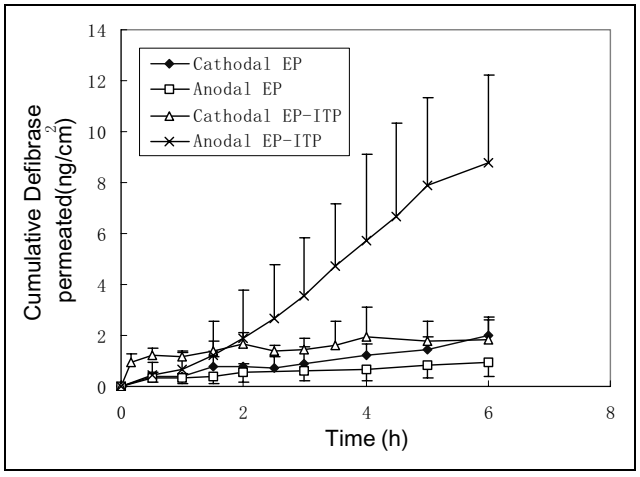

Fig. 2: Skin permeation of Defibrase enhanced by electroporation alone (500 V, 8 ms, 6 ppm, 0.5 h) or a combination of electroporation and iontophoresis (0.5 mA/cm^2, 5.5 h), with cathode or anode introduced in donor compartment. Each point represents the mean (±SD) of three experiments.

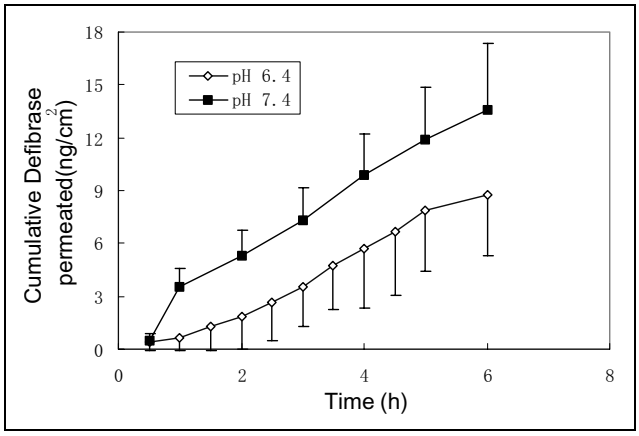

Fig. 3: Effect of pH on skin permeation of Defibrase by combined use of electroporation (500 V, 8 ms, 6 ppm, 0.5 h) and iontophoresis (0.5 mA/cm^2, 5.5 h), with anode introduced in donor compartment. Each point represents the mean (±SD) of three experiments.

2.2. Combined use of electroporation and cathodal iontophoresis

A combined use of electroporation and iontophoresis for Defibrase HEM permeation enhancement was investigated. Electroporation was applied for 30 min followed by 5.5 h iontophoresis with the cathode introduced in the donor compartment. There was no significant effect on the skin permeation of Defibrase (Fig. 2) compared with that of electroporation alone ($P > 0.05$). Defibrase is a very large anionic hydrophilic glycoprotein, with negligible ionic mobility [16], so it is extremely difficult to be transported through a negatively charged membrane (HEM).

2.3. Combined use of electroporation and anodal iontophoresis

During 30 min electroporation followed by iontophoresis with the anode introduced in the donor compartment, there was a significantly increased HEM permeation of Defibrase compared with that of electroporation alone ($P < 0.01$) or electroporation followed by cathodal iontophoresis ($P < 0.01$, Fig. 2). The apparent permeability coefficient of Defibrase was $1.6 \pm 0.8 \times 10^{-4}$ cm · h^{-1} by combined use of electroporation and anodal iontophoresis, which was 12-fold higher than that by electroporation alone ($1.3 \pm 0.7 \times 10^{-5}$ cm · h^{-1}). This could be due to Defibrase transport by convective solvent flow during anodal iontophoresis following electroporation. HEM here in pH 6.4 buffer was a cation selective membrane in which case there was a net osmotic water flux from anode to cathode. In order to verify the effect of electroosmosis, we changed the pH of the permeation medium from 6.4 to 7.4. As a result, there was an increased permeation of Defibrase in pH 7.4 medium (Fig. 3). This could be explained by the increased effect of convective solvent flow, which should be the result of the increased negative charge density of HEM surface in pH 7.4 medium.

The contribution of convective solvent flow was reported to have a great impact on the *in vitro* iontophoresis-facilitated skin permeation of drugs (especially the macromolecules). Burnette et al. [17] reported the iontophoretic transport of thyrotropin releasing hormone (TRH) across nude mouse skin. The flux of TRH in pH 8 buffer solution was greater than that in pH 4 buffer. They thought this was probably due to a greater extent of TRH transport by convective flow from the anode to the cathode at pH 8. Pikal [18] reported that the delivery of a large anion, carboxy insulin, during anodal iontophoresis was more effective than delivery during cathodal iontophoresis as a consequence of the electroosmotic flow effect. In their report of iontophoretic delivery of oligonucleotides across hairless mouse skin Oldenburg et al. [19] noticed an electroosmotic flow effect against drug flow from cathode to anode. As an unexpected result, the increased salt concentration had a slightly positive effect on mass flux. Singh et al. [20] calculated the enhancement ratios (iontophoresis/passive diffusion) for several solutes and found a higher flux enhancement of inulin (macromolecule) as compared to mannitol (small molecule). Their result was consistent with the observed molecular size dependency of flux enhancement due to electroosmosis [21, 22].

As for the electrically assisted delivery of Defibrase, the above results suggested that an electroosmotic flow effect might play an important role in iontophoretic (following electroporation) skin permeation of Defibrase, although there might be various other unknown reasons. This study also indicated that the combined use of electroporation and iontophoresis held some promise in transdermal delivery of larger molecules such as proteins.

3. Experimental

3.1. Materials

Defibrase was obtained from the Institute of Applied Ecology, Academia Sinica (Shenyang, China). Horseradish peroxidase was purchased from Dongfeng Biotechnology Co. (Shanghai, China). Polyclonal antibody against Defibrase was obtained and purified from Defibrase-immunized rabbits [23]. Horseradish peroxidase-labeled IgG was prepared by the periodate oxidation method. All other chemicals were of reagent grade from commercial sources. Excised human skin was obtained from a local hospital, and kept frozen at −20 °C for not more than three weeks. Human epidermis (HEM) was separated by an established heat-stripping method (60 °C, 45 s), and only skin samples with at least 20 kΩ cm^2 resistance were used.

3.2. Analytical techniques

Defibrase samples were analyzed by enzyme-linked immunosorbent assay (ELISA) [24]. Polystyrene 96-well microtiter plates (Beijing, China) were coated with 200 µl of 5 µg/ml polyclonal antibody in Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) at 4 °C overnight. At this or other steps, the wells were filled to a volume of 200 µl. The wells of the plate were washed four times with PBS-0.05% Tween-20 (pH 7.4) and incubated for 2 h at 37 °C with 5% low-fat milk in PBS-Tween-20 as a blocking step. After washing, a 1 h

incubation was performed with different preparations of Defibrase in 0.5% low-fat milk-PBS-0.01% Tween-20. The plates were washed again and incubated with horseradish peroxidase conjugated antibody diluted in 0.5% low-fat milk-PBS-0.01% Tween-20 for 1 h at 37 °C. Measurement of the peroxidase bound to the plates was made after the last washing cycle by incubation with peroxidase substrate solution (0.04% ortho-phenylenediamine, 0.012% H_2O_2 in pH 5.0 citrate-phosphate buffer) for 30 min at 37 °C, and the reaction was stopped by the addition of 50 µl per well of 2 M H_2SO_4. Spectrophotometric reading was taken at 490 nm on a microplate reader (Bio-Rad Model 3550 Microplate Reader, USA). The method exhibited high reproducibility and accuracy in correlating optical densities with Defibrase concentrations (0.2–30 ng/ml, $r^2 = 0.99$) [24].

3.3. Electroporation

Electroporation was carried out in custom-made side-by-side chambers (volume of 2.5 ml each). All the experiments were done under infinite dose conditions. HEM separated the donor and receptor compartments. The area of the skin available for drug permeation was 0.64 cm^2. The donor and receptor compartments were filled with saline and held at 4 °C overnight in order to allow the skin sample fully hydrated. The temperature was raised to 37 °C. The donor compartment was replaced with 2.5 ml of 10 µg/ml Defibrase (Defibrase is a glycoprotein which is well soluble in water. We used this low concentration of Defibrase because of the restricted sources. We thought that the diffusion experiments were carried out under sink conditions, since the molecules of Defibrase diffused very slowly and the concentration ratio of donor/receptor was more than 10-fold higher.) The receptor compartment was filled with 2.5 ml of 0.1 M, pH 6.4 phosphate buffer solution containing 0.3% BSA to prevent Defibrase adsorption onto the surface of the electrodes or the wall of the chamber. A pair of platinum electrodes was immersed in the solution and connected to an exponential decay pulse generator (BioRad Genepulser, USA). Three protocols of electroporation were applied: (i) voltage of 300 V, pulse time constant of 40 ms; (ii) voltage of 500 V, pulse time constant of 8 ms; (iii) applied voltage of 1000 V, pulse time constant of 6 ms. Each protocol was applied for 0.5 h (6 pulses per min), followed by a period of 5.5 h passive diffusion. Voltages were expressed as voltages applied across the electrodes. Samples of 0.5 ml each were taken at appropriate intervals from the receptor solution and immediately replaced with 0.5 ml of fresh buffer solution.

3.4. Combined use of electroporation and iontophoresis

Electroporation was applied with voltage of 500 V and pulse time constant of 8 ms for 0.5 h (6 pulses per min), followed by a period of 5.5 h iontophoresis with cathode or anode introduced in donor compartment. During iontophoresis, the platinum electrodes were connected to custom-made adjustable constant-current power source, the square-wave form pulse DC with a current strength of 0.5 mA/cm^2, a frequency of 2 kHz and on/off ratio of 1/1 was used.

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