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マンニトールの *in vitro* 皮膚透過性に及ぼすエレクトロポレーションの効果

——イオントフォレシスとの比較——

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Effect of cathode and anode positions, frequency of applied pulse, and electrode materials at electroporation on the in vitro skin permeation of mannitol : Comparison with iontophoresis

非イオン性物質であるマンニトールのヘアレスラット摘出皮膚透過性に及ぼすエレクトロポレーションの効果を，陽陰の電極の位置，パルス頻度および電極材料をかえて測定した．まず，Ag 電極(陽極)を角質層側(donor)に，Ag/AgCl 電極(陰極)を真皮側(receiver)に配置したときと，この逆に電極を配置したときのマンニトールの皮膚透過性を比較した．いずれの電極配置でもエレクトロポレーション群では，非通電(コントロール)群にくらべてマンニトールの透過は著しく促進されたが，両エレクトロポレーション群のマンニトール透過に有意差はみられなかった．このことより，イオントフォレシスで報告されているような，convective flow による促進効果は生じないことが示唆された．また，パルス発生装置のコンデンサー容量を1から25 μF とすると透過速度が大きくなり，容量の増加に基づく通電時間の増加が透過促進に関係していることが示唆された．また，同様にパルス頻度を多くすると，通電時間の増加が原因と思われるマンニトールの透過促進が観察された．さらに，マンニトールの皮膚透過性に及ぼす電極位置の影響について試験した．皮膚を挟んで電極を配置した場合と陽極，陰極とも皮膚角質層側にセットした場合で，エレクトロポレーション効果に差はなかった．また，分極(Pt)電極と非分極(Ag/AgCl)電極を用いた場合を比較した結果，どちらの場合もマンニトールの透過はほぼ等しく，エレクトロポレーションでは電極の分極はほとんど引き起こらないか，もしくは促進効果に影響がないものと思われた．

以上より，エレクトロポレーションによる薬物透過促進では，電極の位置，材料に影響されない点でイオントフォレシスと大きく異なるが，電圧の適用時間に大きく左右されることではイオントフォレシスと同様であることが明らかとなった．

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Electroporation has been widely used for introducing DNA and RNA into cells, since its original model was developed by Neumann et al.[1] and Zimmermann et al.[2]. Based on the principle of electroporation, a high voltage applied to cells in a solution creates pores on the surface of the cell membrane, thus introducing genes into the cells through these pores[3].

Recently, this electroporation technology was utilized to increase transdermal drug delivery. Prausnitz et al.[4,5] reported the enhanced effects by electroporation on the skin permeation of calcein, by applying 50 to 500 V. Increased skin delivery of microcarriers was also reported by closely setting anode and cathode electrodes each other on the skin surface by Hoffman et al.[6,7]. In spite of such pilot studies on electroporation to increase skin delivery of drugs, details of its effects are not yet known.

We thus investigated the effect of electroporation on the *in vitro* skin permeation of a model compound, mannitol by changing cathode and anode positions, frequency of the applied pulse,

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and electrode materials. Mannitol was selected, because the current at iontophoresis due to non-ionic compound does not affect its skin permeation. Excised hairless rat skin was used in the study.

Materials and Methods

(1) Reagents and electrodes

^{14}C-D-mannitol (2.07 GBq/mmol with purity of more than 96.6%) was purchased from Amersham Life Science (Buckinghamshire, England), and cold D-mannitol was from Sigma Co., Ltd. (St. Louis, MO, USA). 0.9% NaCl for injection was supplied by Ohtsuka Pharmaceutical (Tokyo). Scintillation cocktail, Hionic-fluor, was obtained from Packard (Meriden, CT, USA).

Silver plate material was supplied by Murata Yohaku (Tokyo). Platinum and silver wire were obtained from Nirako (Tokyo). Needle type electrodes (1.0 mm in diameter) and ring type of electrodes (0.04 mm in thickness) were made in our laboratories. The tip of needle type electrode was bent not to cause any damage to the skin barrier. **Fig. 1** illustrates the ring type electrode. Ag/AgCl electrode was made by silver electrolysis in 0.9% NaCl.

(2) Skin permeation experiments

Male hairless rats (weight 150~180 g) were obtained from Saitama Experimental Animal Laboratory (Sugito, Saitama). The animals were housed in an air-conditioned room and quarantined for a week before use. Skin pieces were obtained from the abdomen in pentobarbital-anesthetized rats.

^{14}C-D-mannitol was diluted with cold D-mannitol in saline to adjust the concentration to 50 mg/ml and was used as a drug-donor solution.

A 2-chamber diffusion cell[8] with an effective diffusion area of 0.95 cm^2, or a Franz diffusion cell[9,10] with the area of 3.14 cm^2 were used.

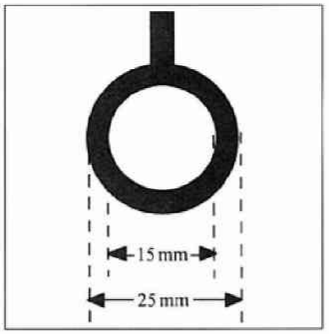

Fig. 1 Schematic representation of a ring type of electrode

When the 2-chamber diffusion cell was used, 2.7 ml of the donor solution was added to a cell facing the stratum corneum and the same volume of physiological saline was added to the receiver compartment (dermis side). On the other hand, when the Franz cell was used, 1 ml of the donor solution was added to the upper compartment (stratum corneum side) and the receiver compartment was filled with 17 ml of saline. These permeation cells were maintained at 37°C using an air bath. No significant difference was observed in the permeation rates of mannitol when using the 2-chamber cells or Franz cells. For the electroporation-treatment, electric pulse was applied by a Gene Pulser ® (Bio-Rad, Hercules, CA, USA). At predetermined times, an aliquot (0.1 ml) was sampled from the receiver compartment and replaced with fresh saline. Scintillation cocktail (10 ml) was added to the sample solution to measure the radioactivity by a liquid scintillation counter (TR-2200 Packard, Meriden, CT, USA).

Detail conditions were shown for each experiment as follows. A 2-chamber cell was firstly used to evaluate the effect of electroporation on the mannitol permeation. A 500 V-pulse was applied every minute, whereas the capacitance was set at 1 μF from the beginning of this

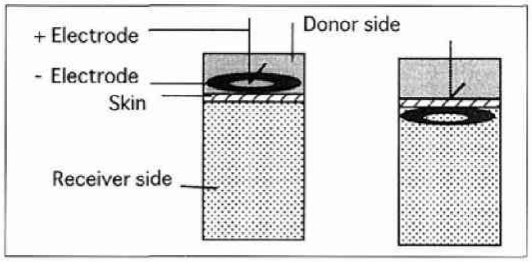

Fig. 2 Schematic representation of electrode position for Types 1 and 2 of experiments

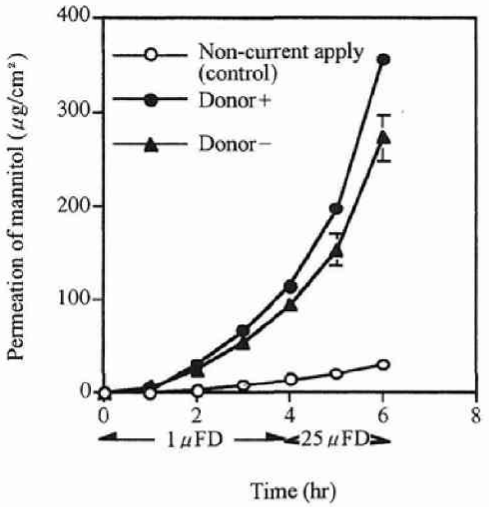

Fig. 3 Effect of electroporation on the permeation of mannitol through the excised hairless rat skin

permeation experiment till 4 hr, and changed to 25 μF from 4 to 6 hr. Needle type electrode was used both for anode and cathode, but the material was Ag for the anode and Ag/AgCl for the cathode. These anode and cathode electrodes were set in the drug-donor and receiver compartment, respectively and *vice versa*, and the distance between the electrode and the skin surface or the dermis layer-surface was set to be about 1.0 cm.

Franz diffusion cell was used for the following experiments to determine the effect of electrode position. Needle type-Ag and ring type-Ag/AgCl electrodes were used for anode and cathode, respectively. Two types (Types 1 and 2) of electrode positions were evaluated. **Fig. 2** illustrates the electrode positions. Both the electrodes were placed on the skin surface (Type 1) ; whereas anode was placed on skin and cathode was under the dermis layer (Type 2). A 500 V-pulse was applied every minute while the capacitance was set at 25 μF.

Needle typed Ag electrode was selected for anode and ring typed Ag/AgCl electrode was for cathode to evaluate to effect of the pulse frequency. The anode and cathode were placed on the skin surface. 500 V-pulse was applied every 30 or 60 minutes while capacitance was set to 25 μF.

Polarized electrodes made of Pt were used for the anode and cathode ; and Ag-anode and Ag/AgCl-cathode were selected as non-polarization ones, to check the effect of the electrode material on the mannitol permeation. In both the experiments, the anode and cathode were placed on skin. The anode was needle type and the cathode was ring type electrode. A 500 V-pulse was applied every minute while the capacitance was set to 25 μF.

Results and Discussion

(1) Effect of electroporation on the mannitol permeation

The effect of electroporation on the skin permeation of mannitol was measured in different positions of needle electrodes (anode or cathode in drug-donor or receiver compartment, respectively and *vice versa*). A control study was also carried out without any current application. **Fig. 3** shows the time course of the amount of mannitol permeated through the hairless rat skin. Higher permeation was observed by electroporation (500 V-application) compared to that of the control study,

Table 1 Comparison of mannitol flux

	Non-current apply (control)	Donor(+) Receiver(−)	Donor(−) Receiver(+)
0—4 hr (1 μFD)	—	29.31±1.86	23.43±6.9
4—6 hr (25 μFD)	—	120.1±8.3	89.90±13.44
0—6 hr	4.89±1.82	—	—

Mean±SE(n=5) ($\mu g/cm^2 \cdot hr$)

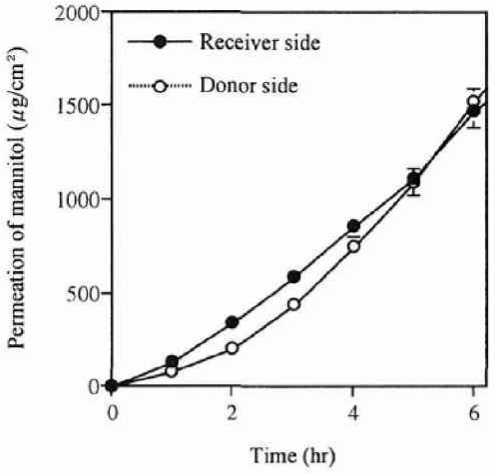

Fig. 4 Effect of electrode position on the permeation of mannitol through the excised hairless rat skin

suggesting that electroporation is useful to increase skin permeation of non-ionic compounds like mannitol. No significant difference was observed in the permeation whenever the anode or cathode was set in the drug donor side.

Early iontophoresis studies[11] reported that skin permeation of mannitol was higher when the anode was placed in the donor compartment than that of cathode being placed in the donor compartment. This was due to the effect of convective flow on the permeation of the non-ionic compounds. In this electroporation study, however, the permeation of mannitol was not dependent on the direction of the current flow. This suggested that very short term-current at electroporation did not bring a convective flow effect, and that the penetration-enhancing mechanism by electroporation was not due to electrical current-driving force, but was due to the pore production in the skin membrane as reported earlier[12].

This experimental data were also used to evaluate the capacitance effect on the mannitol flux. **Table 1** shows the flux for each study. When capacitance was set at 1 μF, the permeation enhancement ratio was about 5 times higher than the control, respectively. In contrast, when the capacitance was set at 25 μF, the enhancement ratio was about 20 times. Since the higher capacitance becomes the longer duration of the pulse application, the amount of mannitol permeation is dependent on the pulse time which is defined as the length of time between the beginning of the pulse (maximum voltage) and the time when the voltage reaches 37% of its initial value[13].

(2) Effect of anode and cathode position on the mannitol permeation

The effect of anode and cathode location on the mannitol permeation was evaluated. **Fig. 4** shows the results for Types 1 and 2 of experiments (**Fig. 2**). No significant difference in the permeation was observed when the electrodes were placed in different positions. Although a great enhancement ratio of 50 was observed for both Types 1 and 2 of studies (compared to control, **Fig. 3**), the skin permeation of mannitol was similar each other (cumulative amount ; about 1,500 $\mu g/cm^2$), suggesting that the two electrodes are not necessary to be set between the skin. Two electrodes can be set in the same drug reservoir in the electroporation, not like in iontophoresis.

(3) Effect of pulse frequency on the mannitol permeation

Application of pulse was reduced from 60

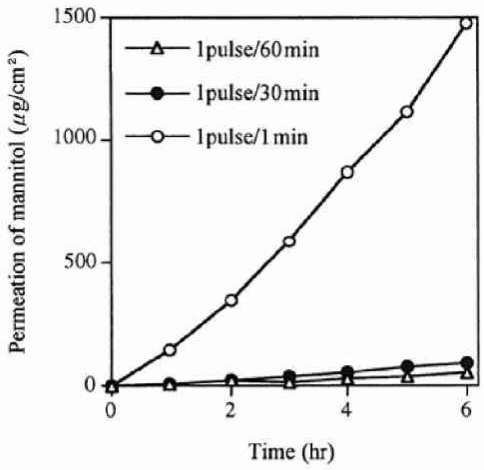

Fig. 5 Effect of application frequency of pulse on the permeation of mannitol through the excised hairless rat skin

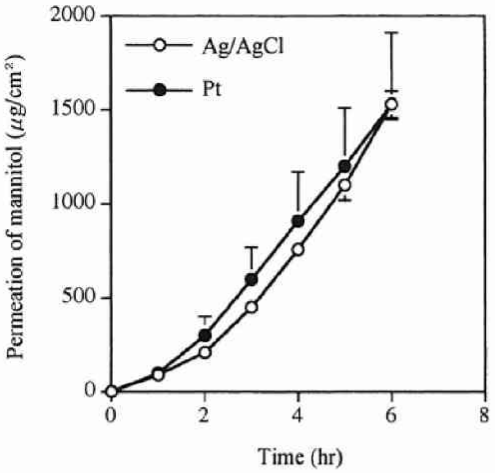

Fig. 6 Effect of electrode material on the permeation of mannitol through the excised hairless rat skin

pulses/hr to 1 or 2 pulses/hr so as to determine the effect of applied pulse frequency on the mannitol permeation. **Fig. 5** illustrates the results of the mannitol permeation. When electroporation was applied every minute(60 pulses/hr), the total permeation of mannitol was 1,500 $\mu g/cm^2$. On the other hand, when the application of the pulse was reduced to 2 pulses/hr, the total permeation was 86 $\mu g/cm^2$, and further reduction of pulse application to once every hour resulted in the permeation of 52 $\mu g/cm^2$. In the case of one pulse/hr, the permeation was only 1.7 times higher than the control.

(4) Effect of electrode material on the mannitol permeation

Fig. 6 shows the effect of polarized(Pt) or non-polarized(Ag/AgCl) electrodes on the mannitol permeation. No difference in the permeation till 6 hr was observed. The total amount of mannitol permeated was about 1,500 $\mu g/cm^2$. It was found from a previous patent for a constant voltage-iontophoretic drug delivery[14] that the drug permeation was lower in iontophoresis with the polarized electrodes than depolarized ones. Electrical double layer that was formed on the outer surface of the electrodes by iontophoresis, but not by electroporation could explain this phenomenon. This layer results in the consumption of electrical potential, and thus no enhancement by iontophoresis was observed. However, a similar permeation was found independent of the type of the electrode used in both the cases. The electroporation potential(i. e. 500 V) is much higher than polarization potential, so that the polarization potential does not have any decreasing effect on the drug permeation.

Conclusion

Material and position of electrodes did not influence the efficacy of electroporation. No effect by convective flow was observed. In addition, the present results suggest that electroporation does not increase electrochemical potentials, that it does affect directly skin barrier, and finally that skin-penetration enhancing mechanism of electroporation is much different from iontophoresis.

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