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# Effect of electroporation and pH on the iontophoretic transdermal delivery of human insulin

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**Abstract**

The synergistic effect of electroporation (EP) and iontophoresis (IP) on the in vivo percutaneous absorption of human insulin was evaluated in rats. Passive diffusion and IP alone (0.4 mA/cm$^2$) resulted in almost no skin permeation of insulin at pH 7, whereas EP treatment (150 or 300 V, 10 ms, and 10 pulses) resulted in a high plasma level of insulin and the combined use of EP and IP led to a further increase of the plasma level of insulin compared with that measured after EP alone. Interestingly, a much higher plasma level was observed when the pH of the insulin solution at 7 was increased to 10. One of the reasons was the different aggregation properties of insulin at pH 7 and pH 10. The nonassociation ratio of insulin was significantly higher at pH 10 than at pH 7. Insulin monomers and dimers were observed in addition to the normal form of insulin, hexamer, albeit in low percentages, at pH 10, whereas most of the insulin was in the hexamer form at pH 7. To confirm the influence of the aggregation properties of insulin, the commercially available human insulin analogue insulin lispro was then evaluated. Its skin permeation was found to be extremely high compared to that of conventional human insulin without increasing the solution pH. Marked decreases in blood glucose levels reflecting the increases in the plasma concentration of insulin were also observed after EP/IP treatment.

The present study suggests that percutaneous absorption of insulin is synergistically enhanced by a combined use of EP and IP and that altering the aggregation properties of insulin is important to enhance the percutaneous absorption of insulin by IP and/or EP.


*Keywords:* Insulin; Percutaneous absorption; Electroporation; Iontophoresis; Insulin lispro

## 1. Introduction

Insulin has been administered to type I diabetic patients by parenteral routes because it is easily degraded or metabolized in the gastrointestinal tract. Subcutaneous administration by self-injection is the commonest route for insulin delivery. Pain associated with the injection and risk for infection at the injection site cannot be completely avoided, and a much simpler, non-invasive method has long been desired. Transdermal delivery of insulin can avoid its metabolization and the first-pass effect and may thus be the optimum route of administration.

Electroporation (EP) is used in gene transfer into bacterial, animal and human cells, since it produces reversible tiny pores on the cell membrane under conditions of high electric field (Neumann and Rosenheck, 1972; Zimmermann et al., 1973). Recently, EP has drawn attention as a method to increase transdermal delivery of several compounds, namely benzoic acid (Yoshida et al., 2000; Sugibayashi et al., 2001), calcein ($M_W$: 684) (Prausnitz et al., 1993; Tokudome and Sugibayashi, 2003a,b), oligonucleotide ($M_W < 5000$) (Regnier et al., 2000) and FITC dextran ($M_W$: 4.4–38 kDa) (Lombry et al., 2000; Tokudome and Sugibayashi, 2003a,b). These studies suggested that EP might enhance skin permeability of macromolecules with a molecular weight of at least 40 kDa as well as small molecules.

Iontophoresis (IP) using a low voltage for a few hours has been commonly used to enhance transdermal delivery of water-soluble macromolecular drugs, such as insulin ($M_W$: 36 kDa) (Sage, 1997), salmon calcitonin (sCT) ($M_W$: 3.6 kDa) (Chang et al., 2000a) and human parathyroid hormone (PTH) ($M_W$: 4.1 kDa) (Singh et al., 1998). However, transdermal delivery enhanced by IP is limited to compounds having a molecular weight of 20 kDa or less, since the main permeable pathway created by IP is the skin appendages such as hair pouches and

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sweat glands (Turner et al., 1997). Since the stratum corneum is the main barrier against skin permeability for insulin hexamers ($M_W$: 36 kDa), no effect of IP alone on percutaneous absorption has been observed (Kanikkannan et al., 1999).

The skin permeation of monomeric insulin synthesized by gene transfer techniques has already been evaluated (Kanikkannan et al., 1999). A combined use of IP and a keratolytic agent (Kari, 1986), depilatory cream (Siddiqui et al., 1987) or chemical enhancer (Choi et al., 1999), was found to decrease blood glucose levels. There have been several in vitro studies on the effect of EP combined with IP in the transdermal delivery of several compounds; namely luteinizing hormone releasing hormone (LHRH) ($M_W$: 1.2 kDa), sCT (3.6 kDa), PTH (4.1 kDa), buprenorphine, calcein, sodium nonivamide acetate, timolol and atenolol (Chang et al., 2000b; Guy et al., 2000; Pliquett et al., 1995; Vanbever et al., 1997; Denet et al., 2003; Fang et al., 2002). Some of these studies demonstrated markedly high skin permeability by the combined use of EP/IP, but other reports showed almost no synergistic effect, suggesting that the efficacy would widely vary depending on the molecular weight and electric charge of drugs. High synergistic effects of EP/IP were observed in transdermal delivery of LHRH (Bommannan et al., 1994), sCT and PTH (Chang et al., 2000a), suggesting that the combined use of EP/IP may be useful for drugs with a relatively high molecular weight. No reports, however, were found on the in vivo evaluation of the combined effect of EP/IP.

In the present study, the effect of EP or IP alone and in combination on the percutaneous absorption of human insulin was evaluated in solutions with different pHs using various electric parameters in rats. The percutaneous absorption rates of insulin in rats were evaluated based on the plasma concentration of insulin.

## 2. Materials and methods

### 2.1. Materials

Human insulin (humulinR U-100) and insulin lispro (genetic recombination) (Humalog) were purchased from Eli Lilly (Kobe, Japan). Glucose reagent CII-Test kits were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and commercially available. Silver plate material was supplied by Murata Yohaku (Tokyo, Japan). Parallel plate-plate electrodes (0.04 mm in thickness) for electroporation were prepared using silver plates (Murata Yohaku) in our laboratory (Fig. 1).

### 2.2. Animals

Male Sprague–Dawley rats (250–300 g) were obtained from Saitama Experimental Animal Laboratory (Fukaya, Saitama, Japan). The animals were housed in an air-conditioning room and quarantined for a week before use. These animal experiments were performed in accordance with the guidelines of the Life Science Research Center, Josai University.

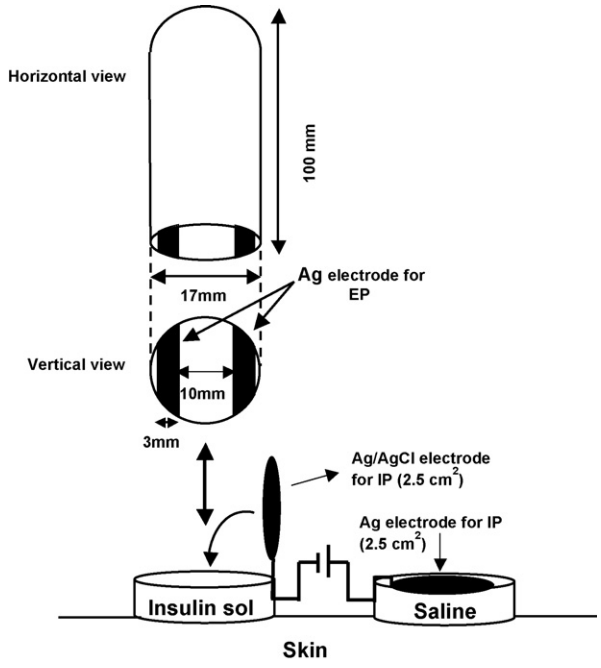


Fig. 1. Schematic representation of electrodes for EP and IP.

### 2.3. Determination of plasma Insulin and blood glucose

Blood (ca. 0.2 mL) was collected from the jugular vein in rats at 0.5, 1, 2, and 4 h after starting the experiments. Blood glucose levels were measured using Glucose CII-Test Wako and reported as % of control values. Plasma was obtained by centrifugation (12,000 rpm for 5 min) and then the plasma insulin level was determined using enzyme immunoassay kits (Dinabot Co., Ltd., Tokyo) and presented as microunits per milliliter.

### 2.4. In vivo experiments

Rats were anesthetized with an i.p. injection of urethane (25% aqueous solution, 1.5 mg/kg) before an experimental procedure. The anesthetized rats were kept warm during all experiments by placing them on a heating pad. The hair of the abdominal area was carefully shaved with electrical clippers, and the skin surface was washed using cotton soaked in 70% ethanol. Two acrylic cells were attached to the skin (each application area: 2.5 $cm^2$). One cell was filled with 2.0 mL insulin solution (50 U/mL) containing 0.2% (w/v) bovine serum albumin to avoid adsorption of insulin onto the surface of electrodes and the acrylic diffusion cell. The other cell was filled with physiological saline for IP receptor. The pH of the insulin solution was adjusted to 7 or 10 with/without 0.2N NaOH (or pH 2 or 3 for comparison). Insulin is predominantly negatively-charged at pH 7 or 10. EP (150 or 300 V and 1, 5 and 10 ms) was applied using a square pulse generator (Electro Square Porator T820, BTX, San Diego, CA), followed by 240 min-passive or 60 min-IP delivery. During EP treatment (150 or 300 V, 10 ms, and 10 pulses), the plate type Ag-electrode was kept in the insulin solution while contacting the skin surface. Then IP was applied by setting the AgCl-electrode at about 50 mm above the skin sur-

face. DC-cathodal iontophoresis was applied at 0.4 mA/cm$^2$ for 60 min using ADIS-HP (Hisamitsu Pharmaceutical Co., Ltd., Tsukuba, Ibaraki, Japan). The electrodes positions for EP and IP are schematically shown in Fig. 1.

### 2.5. Self-association of insulin

The effect of solvent pH on the conformation or self-association of insulin was determined by extracorporeal ultrafiltration using centrifugal filter devices (Microcon® Millipore, Billerica, MA). Each insulin solution was applied onto the upper side of a filter device. After centrifugation (14,000 × $g$, 30 min), the filtrate was injected into an HPLC apparatus composed of a pump (LC-10AS, Shimadzu, Kyoto, Japan), UV detector (SPD-10A, Shimadzu), integrator (C-R5A, Shimadzu), system controller (SCL-10A, Shimadzu), auto injector (SIL-10AXL, Shimadzu) and a reverse phase column (Inertsil® ODS-3, 4.6 mm × 150 mm, GL Science Inc., Tokyo). The flow rate was 1.0 mL/min, the mobile phase was acetonitrile: 0.015 M phosphate buffer (pH 6.8) (1:1) and insulin was detected by UV at 205 nm. The % filtrate recovery was calculated by

$$\%\ \text{filtrate recovery} = 100 \times \frac{W_f \times C_f}{W_0 \times C_0} \quad (1)$$

where $W_f$ and $W_0$ are the weight of the filtrate and starting material, and $C_f$ and $C_0$ are the insulin concentration in the filtrate and starting material, respectively.

### 2.6. Data analysis

The percent plasma glucose against the initial plasma glucose level was plotted against time. The area above the plasma glucose level curve was determined using a trapezoidal rule.

### 2.7. Statistical analysis

Analysis of variance (ANOVA) was used to analyze data from each experimental group. Tukey's test was performed to determine the level of significance. The data were considered to be significant at $p < 0.05$ or 0.01.

## 3. Results

### 3.1. Effect of iontophoresis or electroporation alone on the skin permeation of human insulin

To evaluate the effect of IP or EP alone on the in vivo percutaneous absorption of insulin, drug solutions (50 U/mL) with a pH 7 or 10 were used. In the study of IP alone, a donor solution was placed on the cathode, and a constant current (0.4 mA/cm$^2$) was applied for 60 min, considering the isoelectric point (p$I$ = 5.3) of insulin. In the study of EP alone, on the other hand, 300 V, 10 ms square pulse (10 pulses) was applied every second for 10 s at the time point of 0 min. Fig. 2 and Table 1 show the effect of IP or EP on the time course of plasma insulin concentrations and the changes in blood glucose levels, respectively. When the insulin solution of pH 7 was applied on the skin, no percutaneous absorption of insulin nor a significant change in blood glucose levels was observed by either IP alone (0.4 mA/cm$^2$, 60 min) or EP alone (300 V). When the insulin solution of pH 10 was applied, the plasma concentration of insulin was increased to, approximately 100 μU/mL, at 60 min by IP alone, and rapidly decreased when IP was off. The plasma insulin concentration was also increased by EP alone and it reached approximately 50 μU/mL at 240 min after the start of the experiment. However, the blood glucose levels showed almost no tendency to a decrease.

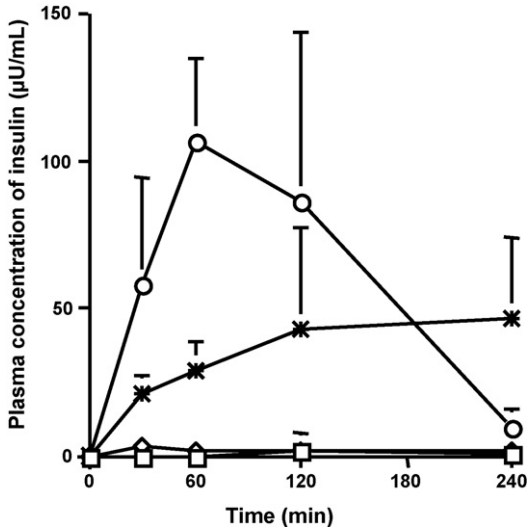


Fig. 2. Effect of cathodal iontophoresis or electroporation on the time course of plasma level of human insulin in rats and applied pH (7 or 10). Symbols: □, iontophoresis (pH 7); ◊, electroporation (pH 7); ○, iontophoresis (pH 10); x, electroporation (pH 10). Each point represents the mean ± S.D. of three to five experiments.

### 3.2. Synergistic effects of iontophoresis and electroporation on the percutaneous absorption of human insulin

To evaluate the synergistic effect of EP/IP, IP (0.4 mA/cm$^2$) was conducted for one hour immediately after EP (150 or 300 V, 10 ms, 10 pulses) using a solution of pH 7 (Fig. 3). The percutaneous absorption of insulin by IP was significantly enhanced after pretreatment with EP, and its effect depended on the voltage

Table 1
Synergistic effect of electroporation and iontophoresis on blood glucose levels and their % change in rats ($n$ = 3–5, mean ± S.D.)

| $n$ = 3–9 | Electrical condition | %Average | %Change |
|---|---|---|---|
| Passive | – | 105 ± 2.0 | 5.0 ± 4.9 |
| IP (pH 7) | 0.4 mA/cm$^2$ | 98.6 ± 1.8 | 12.2 ± 1.3 |
| IP (pH 10) | | 90.6 ± 3.2 | 17.4 ± 9.4 |
| EP (pH 7) | 300 V | 102 ± 2.9 | 3.0 ± 2.8 |
| EP (pH 10) | | 99.6 ± 3.0 | 0.0 ± 8.1 |
| EP/IP (pH 7) | 150 V/0.4 mA/cm$^2$ | 80.7 ± 2.6 | 22.7 ± 6.3 |
| EP/IP (pH 10) | | 73.7 ± 6.3 | 39.7 ± 9.5 |
| EP/IP (pH 7) | 300 V/0.4 mA/cm$^2$ | 69.7 ± 3.1 | 52.5 ± 7.9 |
| EP/IP (pH 10) | | 69.3 ± 1.4 | 61.7 ± 0.3 |

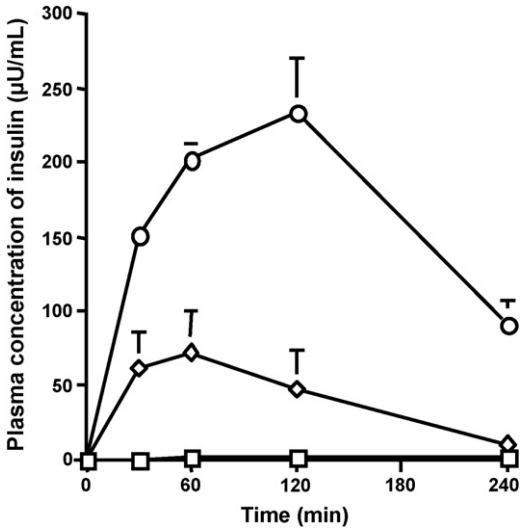


Fig. 3. Effect of electroporation voltage on the time course of plasma insulin level under iontophoretic delivery in rats. The pH of the insulin solution was adjusted to 7. Symbols: □, iontophoresis without electroporation; ◊, electroporation at 150 V with iontophoresis; ◯, electroporation at 300 V with iontophoresis. Each point represents the mean ± S.D. of three to five experiments.

applied (10 pulses of 150 or 300 V). Furthermore, blood glucose levels were significantly changed by the combined use of EP/IP, reflecting the increases in plasma insulin concentrations, to approximately 23% in the 150-V group and to approximately 53% in the 300-V group 120 min after administration against the initial level (Table 1).

To evaluate the effect of pH of the drug solutions on the percutaneous absorption of insulin enhanced by the combined use of EP/IP, an experiment using an insulin donor solution with pH 10 was conducted in the same manner as mentioned above (Fig. 4). As a result, enhanced absorption was also observed depending

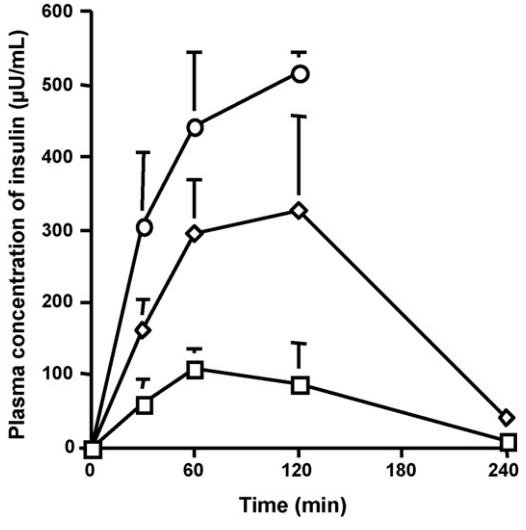


Fig. 4. Effect of electroporation voltage on the time course of plasma levels of insulin under iontophoretic delivery in rats. The pH of the insulin solution was adjusted to 10. Symbols: □, iontophoresis without electroporation; ◊, electroporation at 150 V with iontophoresis; ◯, electroporation at 300 V with iontophoresis. Each point represents the mean ± S.D. of three to five experiments.

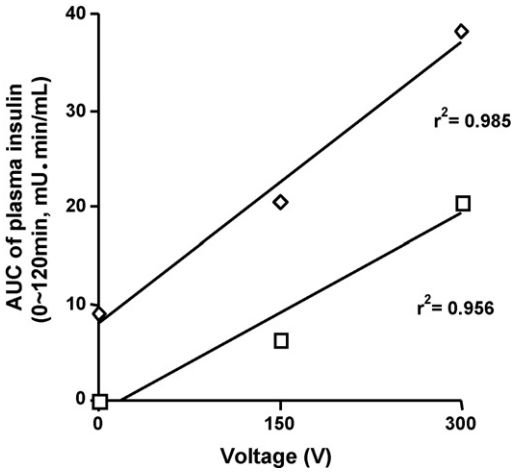


Fig. 5. Relationship between electroporation voltage and AUC of plasma concentration of insulin from 0 to 120 min. Symbols: □, iontophoresis with/without electroporation at pH 7; ◊, iontophoresis with/without electroporation at pH 10.

on the EP voltages (150 and 300 V), and higher plasma insulin concentrations were obtained compared with those obtained with the solution with pH 7. The blood glucose levels at 300 V decreased to approximately 30% of the initial value (Table 1), and many rats died due to hypoglycemia. Therefore, the plasma drug concentrations measured until 120 min after the dosing were adopted. Furthermore, the effects of the number of pulses (10 and 5 pulses) and pulse duration (10, 5 and 1 ms) of EP on IP insulin absorption were evaluated, and the obtained results showed that insulin absorption depended on the strength of each EP parameter (data not shown).

The area under the plasma concentration versus time curve of insulin from 0 to 120 min ($AUC_{0–120}$) was calculated and plotted to demonstrate the relationship between the percutaneous absorption of insulin at pH 7 and pH 10 and the voltage applied for EP (Fig. 5). A good linear relation was observed between voltage and the AUC for experiments at pH 7 and pH 10. Higher absorption was obtained with the solution of pH 10.

### *3.3. Dependence of self-aggregation of insulin on pH*

The insulin conformation in solutions at pH 7 and pH 10 with or without EP was analyzed by HPLC using centrifugal filter devices to demonstrate the effect of alkaline pH and high electric fields (150 and 300 V) on the self-aggregation of insulin (Table 2). Insulin solution of pH 7 was subjected to centrifugal filtration using a filter membrane with a molecular weight cut off of 30 kDa, and the filtrate was analyzed by HPLC. No peak of insulin was detected. Then the insulin solution of pH 10 was analyzed using filter membranes with a molecular weight cut off of 10 and 30 kDa in the same manner as above. As a result, a single peak of insulin was detected in the filtrates obtained in both filtration experiments using different filter membranes. Therefore, the self-aggregation property of insulin may slightly change when the pH value shifts to the alkaline side, and monomers and dimers must be presented in spite of a few percentages. In addition, the influence of a high electric field on insulin self-aggregation was investigated in the same manner. No significant difference was

Table 2
Dependence of insulin aggregation on pH with and without electroporation ($n = 3$–5, mean ± S.D.)

| Association | % of total insulin | | | | | |
|---|---|---|---|---|---|---|
| | Without EP | | EP-150 V | | EP-300 V | |
| | pH 10 | pH 7 | pH 10 | pH 7 | pH 10 | pH 7 |
| Monomer | 0.83 | ND | 0.68 | ND | 0.36 | ND |
| Dimer, tetramer | 7.78 | ND | 7.3 | ND | 9.14 | ND |
| Hexamer | 91.4 | ≒100 | 92 | ≒100 | 90.7 | ≒100 |
| Total insulin | 100 | 100 | 100 | 100 | 100 | 100 |

found in the filtration ratio of insulin between those with and without a high electric field. Thus, no peak of decomposed matters was produced: insulin did not decompose and was stable even under a high electric field.

### 3.4. Synergistic effects of iontophoresis and electroporation of insulin lispro

Insulin lispro is a human insulin analogue created by reversing the position of proline at the 28th position and lysine at the 29th position on the B chain. Fig. 6 shows the combined effect of EP/IP on the time course of the plasma concentration of insulin lispro. In the IP alone group, almost no absorption was observed when using insulin lispro solution pH 7 (data not shown), similar to the results obtained with human insulin (Fig. 3). However, the absorption tended to be significantly increased by the combined use of EP/IP, and the plasma concentration of insulin was approximately twice that obtained with human insulin. In addition, the plasma concentration of insulin increased immediately after the initiation of power distribution (for EP and IP) and decreased immediately after the completion of power distribution (for IP). Blood glucose levels also decreased significantly under EP/IP treatment in the insulin lispro group as compared with the human insulin group (data not shown).

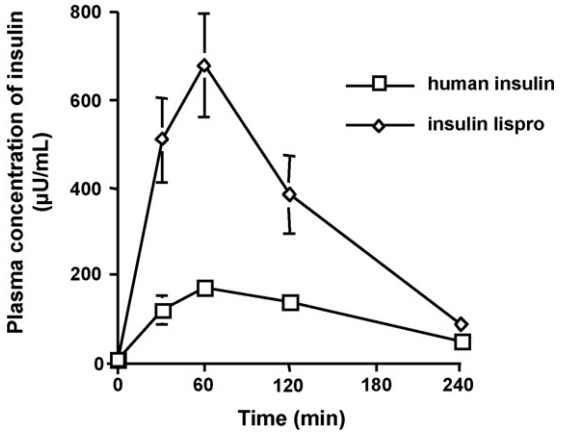


Fig. 6. Effect of combined electroporation and iontophoresis on the time course of plasma concentration of insulin after application of human insulin and insulin lispro at pH 7 (EP condition: 10 pulses of 150 V–10 ms and IP condition: 0.4 mA/cm$^2$). Each point represents the mean ± S.D. of three to five experiments.

## 4. Discussion

We have previously designed electrodes whose anode and cathode are set on the stratum corneum for the in vivo application of EP. These electrodes include needle-needle electrodes, needle-ring electrodes and parallel plate-plate electrodes. We then demonstrated that the electric fields produced on the skin and current density distribution in the skin barrier markedly varied depending on the shape and position of electrodes and greatly influenced the skin permeability (Yoshida et al., 2000; Sugibayashi et al., 2001). Analysis by computer simulation revealed that the most uniform electric field and current density distribution are obtained with parallel plate-plate electrodes, suggesting that this electrode system was optimum for EP treatment. Thus, we investigated the pretreatment effect of EP on IP delivery of insulin using the parallel plate-plate electrode system. The influence of animal species differences on the transdermal iontophoretic delivery of hPTH(1–34) ($M_W$; ca. 3.0 kDa) was reported by Suzuki et al. (2001): a good correlation was observed between its skin permeation rate and the density of hair follicles in the experimental species. On the other hand, macromolecules such as poly-L-lysin (>20 kDa) and hexameric insulin (ca. 3.6 kDa) hardly penetrated through the intact skin of furry and hairless animals by iontophoresis alone (Turner et al., 1997). We thus investigated the synergistic effect of EP and IP on the skin permeation of human insulin in furry rats.

Since the isoelectric point of insulin is 5.3, the peptide is negatively charged at the physiological pH in skin tissues. Then, the insulin solution was applied at the cathode side in the present in vivo IP delivery, and most experiments were conducted at pH 7 or 10. Percutaneous absorption of insulin by IP or EP alone varied depending on the pH of drug solutions, and significant absorption of insulin was observed only when the insulin solution of pH 10 was applied (Fig. 2). A similar experiment was conducted using insulin solutions in which the pH was shifted to acidic (pH 2–3). However, no absorption of insulin was observed (data not shown).

The effect of IP on the skin permeation of charged drugs is closely related to two primary mechanisms of IP, electrorepulsion and electroosmosis. Since insulin is positively charged at pH 2, the direction of electroosmosis is from the skin reservoir to the pH 2 insulin donor solution topically applied (Marro et al., 2001). Thus, the observed absorption of insulin was not increased by IP.

It has been reported that pores formed by EP are generally reversible and disappear within several hundred milliseconds, and the drug flux a few minutes after application of EP completely returns to that before the application of EP (Pliquett et al., 1995). However, it has also been reported that the persistence of tiny pores was prolonged and the effect of EP was maintained for a long time when polymers were delivered using EP (Vanbever et al., 1997). In addition, a synergistic effect of EP and IP was obtained when sCT (3.6 kDa) and PTH (4.1 kDa) (Chang et al., 2000b) were administered after pretreatment with EP under IP delivery. In our study, the enhancing effect of EP alone and of EP combined with IP on insulin absorption was observed. The results were attributable to the formation of an irreversible

route on the stratum corneum, through which insulin could be absorbed.

When EP alone was applied using insulin solution at pH 10, the blood glucose level did not decrease in spite of increased skin permeation. The following two reasons may be considered: one is the low absorption rate of insulin after only EP treatment, and the other may be a slight increase in the blood glucose level by urethane injection (see Table 1). Aisaka et al. (1989) have already studied the effect of urethane on the blood glucose level and found that urethane increased the blood adrenaline level leading to hyperglycema. A slight increase in blood glucose level was also observed in the present experimental group with no insulin absorption. Therefore we used %Average and %Change for variations of the blood glucose level. It was confirmed that the effect of IP on the blood glucose level was much higher than that of urethane.

The synergistic effect of EP and IP on insulin absorption can be expected when the driving force of IP is effectively used. The drug delivery by IP is generally expressed by the following formula, which indicates the balance of electrorepulsion and electroosmosis (Guy et al., 2000):

$$J_{\text{total}} = J_{\text{electrorepulsion}} + J_{\text{electroosmosis}} \quad (2)$$

However, $J_{\text{electroosmosis}}$ cannot be expected in this experiment, since cathodal iontophoresis was adopted considering the isoelectric point of insulin. Thus, $J_{\text{total}}$ was expressed by the following formula:

$$J_{\text{total}} = J_{\text{electrorepulsion}} = \frac{z_{\text{ins}} u_{\text{ins}} c_{\text{ins}}}{F \sum z_i u_i c_i} I \quad (3)$$

where $z_{\text{ins}}$, $u_{\text{ins}}$ and $c_{\text{ins}}$ and $z_i$, $u_i$ and $c_i$ represent the number of charges, mobility and concentration of insulin and ion $i$, respectively, and $I$ and $F$ represent current density and Faraday constant, respectively. These formulas revealed that the changes in transference number by the combined use of EP/IP led to the enhancement of insulin absorption, since the same current of IP was used with and without EP in this experiment. Insulin was absorbed through the stratum corneum under the IP delivery system alone, suggesting that this peptide may show very low mobility.

We have already reported that EP combined with IP had a synergistic effect on the skin permeation of high molecular compounds, and analyzed the effect based on changes in electroosmosis and transference number (Tokumoto et al., 2005). The present permeation data can also be analyzed using the transference number of insulin. The migration rate of insulin changed due to production of a new permeation pathway and the associated change in the transference number of insulin.

Percutaneous absorption of insulin from a pH 10 solution was greater than that from a pH 7 solution. Using the alkaline solution, skin may be damaged, insulin would be negatively charged and the self-aggregation property of insulin would be altered. First, a similar experiment was conducted 120 min after applying the solution of pH 10 on the skin to examine the effect of skin damage caused by the alkaline solution on the percutaneous absorption of insulin. However, no significant difference in insulin absorption was observed (data not shown). These

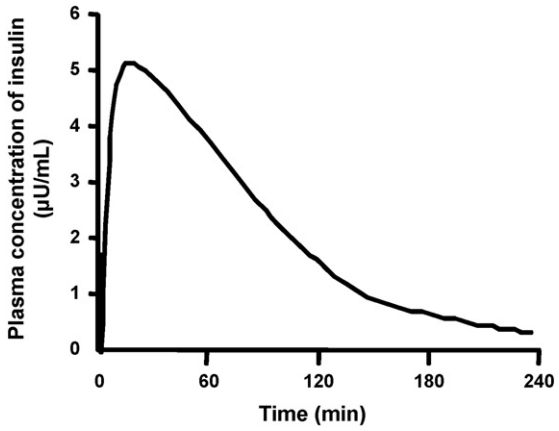


Fig. 7. Effect of electroporation voltage on blood glucose levels under iontophoretic delivery of human insulin in rats. The pH of the insulin solution was adjusted to 10. (EP condition: 10 pulses of 150 V–10 ms and IP condition: 0.4 mA/cm$^2$). Each point represents the mean ± S.D. of three to five experiments.

results may closely related to pH change in the insulin solution during the absorption process. In the present experiment, the pH of the insulin solution decreased from 10 before skin application to 8.5 240 min after the application, suggesting low irritation of the skin membrane. The MTT assay using three-dimensional human cultured skin model (LSE-high, Toyobo, Osaka, Japan) showed no membrane damage caused by the insulin donor solution (Watanabe et al., 2002).

In general, insulin exists as hexamers in a relatively concentrated solution. The aggregation property of insulin was found to be influenced by its concentration, pH, metal ions and additives in the solution, and monomeric insulin formed in solutions with pH 2 or lower, and with pH 9 or higher (Denet et al., 2003). Our results were consistent with those of that previous report, and showed that monomeric insulin formed when the pH of the solution was shifted to the alkaline side (Table 2). Then, we demonstrated the synergistic effect of EP and IP on the absorption of hexameric insulin ($M_W$: 36 kDa) as well as human insulin using a pH 7 solution, and that the higher enhancing effect of EP and IP in the solution of pH 10 was due to the formation of a low-molecular weight compound by disruption of the conformation of insulin hexamer as well as changes in the charged condition of insulin.

Furthermore, the percutaneous absorption of insulin lispro was markedly enhanced at pH 7 with a short $T_{\max}$ value. The effect of the structural modification of insulin by reversing the position of aminoacids Lys (B28) and Pro (B29) in insulin lispro is considered the reason for its high and rapid skin permeation. However, the direct mechanism of the enhanced effect of EP/IP on the absorption of insulin lispro could not been demonstrated, since insulin lispro generally exists as a hexamer in solutions. Using these data on percutaneous absorption, the absorption rate was calculated and the bioavailability was calculated by deconvolution technique. The blood concentrations of insulin were then simulated based on the disappearance parameters in healthy subjects under an assumption that a 10 cm$^2$ preparation would be used (Fig. 7). As a result, the bioavailability was approximately 0.4% in the insulin lispro group that showed the highest

absorption, and the simulation of blood concentrations revealed a $C_{max}$ of approximately 5 μU/mL. Therefore, it was suggested that this method for drug delivery might be effective for basal insulin replacement therapy. An optimization of the dose and the design of electrodes with lower electric stimulus are required to increase the bioavailability of the drug and to provide a safer procedure for drug delivery. Since the obtained simulation was based on the absorption rate of insulin in furry rats, the predicted value may be overestimated for humans. Nevertheless, the present results provide useful information on the feasibility assessment of transdermal delivery of insulin using EP and IP technologies.

## 5. Conclusion

1. No adequate absorption of insulin was obtained by IP or EP alone, whereas EP and IP had a synergistic effect on the percutaneous absorption of insulin.
2. The synergistic effect of EP and IP was significantly influenced by the conformation of insulin in the solution and was enhanced by inhibition of insulin aggregation.
3. The human insulin analog insulin lispro showed low self-aggregation and can be used to enhance the percutaneous absorption of insulin even if the pH is not alkaline.

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