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Passive and Iontophoretic Transport Enhancement of Insulin Through Porcine Epidermis by Depilatories: Permeability and Fourier Transform Infrared Spectroscopy Studies

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ABSTRACT

The effect of thioglycolate-based depilatory lotions was studied on the in vitro passive and iontophoretic permeability of insulin through porcine epidermis and biophysical changes in the stratum corneum (SC) lipids and proteins. The porcine epidermis and Franz diffusion cells modified for iontophoresis were used for the in vitro transport studies. Cathodal iontophoresis was performed at 0.2 mA/cm^2 current density. Resistance of the control- and depilatory-lotion–treated epidermis was determined according to Ohm's law. Biophysical changes were studied on porcine SC before (control) and after treatment with the depilatory lotions using Fourier transform infrared (FT-IR) spectroscopy. Asymmetric (~2915 cm^{-1}) and symmetric (~2848 cm^{-1}) Carbon-Hydrogen (C-H) stretching absorbances were studied to estimate the extent of lipid extraction. Fourier self-deconvolution and second derivative procedures were applied to amide I band (1700-1600 cm^{-1}) in order to estimate quantitatively the changes in the secondary structure of the SC protein. The passive permeability of insulin was significantly ($P < .05$) increased through depilatory-lotion–treated (ie, Better Off, Marzena, and Sally Hansen) epidermis in comparison to control. Iontophoresis significantly enhanced ($P < .05$) the permeability of insulin through depilatory-pretreated epidermis in comparison with the control epidermis. Further, we were able to achieve the desired flux of insulin (5.25 U/cm^2/d) through Better Off–treated epidermis using 0.2 mA/cm^2 current density and 100 U/mL donor concentration of insulin. The SC treated with depilatory lotions showed a decrease in peak areas of C-H stretching absorbances in comparison with untreated SC. Depilatory lotion treatment also decreased ($P < .05$) the epidermal resistance in comparison with the control epidermis. The decrease in the α-helix conformation and the increase in the random and turn structures were observed in the SC proteins due to depilatory lotion treatment. The changes in the secondary structure of proteins and lipid extraction from the SC are suggested as the cause of the decrease in the epidermal resistance and the increase in the passive and iontophoretic permeability of insulin through depilatory-pretreated epidermis in comparison with the control epidermis.

KEYWORDS: transdermal, insulin, depilatory lotion, iontophoresis, epidermal resistance

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INTRODUCTION

Different techniques have been used to increase the transdermal transport of insulin. Stripping of the stratum corneum (SC)[1] and use of penetration enhancers[2] have been reported to increase the transdermal transport of insulin. Delipidization of mouse skin by gentle wiping with absolute alcohol increased the iontophoretic flux of insulin.[3] Studies have suggested that depilatory lotions alter skin permeability by changing the skin barrier function.[4,5] A depilatory cream significantly increased the skin absorption of testosterone.[6] Calcium thioglycolate, when used as a penetration enhancer, led to a 40-fold increase in the plasma concentration of theophylline.[7]

Dry SC is composed of (by weight) approximately 75% protein, 25% lipid, and a small percentage of low molecular weight materials. Skin penetration enhancers can either alter the lipid structure or interact with intercellular SC lipid, or simultaneously exert both actions, resulting in improved drug permeation. The role of the

lipids in the barrier function of the SC is well established, and techniques to reduce their role are often used in transport studies. Proteins, being the major component of the SC, draw attention for investigations. Denaturation of keratin in the SC has been suggested as a mechanism for permeability enhancement of SC.[8] SC contains 2 fibrous proteins, 1 with an α-helical conformation and the other with a cross-β-structure, the latter being the minor component.[9] Protein conformational changes were noticed when porcine SC was pretreated with skin penetration enhancer–azone/propylene glycol.[10] Five percent azone in propylene glycol as a penetration enhancer increased the iontophoretic flux of insulin by a factor of 2.75 as compared with iontophoresis alone.[11] Therefore, the SC proteins might play an important role in the transdermal transport enhancement of insulin.

Electrical resistance of the skin is an average measure of the difficulty that the charge carriers have in traversing the skin. Changes in electrical properties also indicate the changes in membrane properties of the skin.[12] Fourier transform infrared (FT-IR) spectroscopy allows the simultaneous investigation of the lipid and protein components of a biological membrane. This technique can be employed to study the extent of lipid extraction from porcine SC. Decreases in the absorbances of C-H stretching peaks have been linked to the SC lipids extraction.[14] FT-IR spectroscopy is an appropriate method to study the conformation of membrane proteins.[14] The widely used application of FT-IR spectroscopy to proteins is based on the assessment of the amide I band that arises from the in-plane Carbon=Oxygen (C=O) stretching vibration of the peptide backbone. Since deconvolution and derivative methods preserve the band position, they have been used extensively to resolve and identify the position of overlapped protein amide I bands. Second derivative analysis of the amide I band is a reliable method to quantitatively determine the secondary structures of proteins.[15]

The application of a depilatory lotion prior to the transport study was found to enhance the passive and iontophoretic transdermal delivery of insulin.[16,17] However, these studies did not elaborate the mechanism of transport enhancement. It is important to recognize the changes in the SC by the depilatory lotion treatment in order to understand the mechanism of transport enhancement of insulin.

The overall objective of our research is to achieve the transdermal transport of therapeutic doses of insulin required for the treatment of diabetes. This study, for the first time, would provide the biophysical basis of understanding the mechanism of transport enhancement of insulin through depilatory-lotion-treated porcine epidermis. This study investigated the effects of pretreatment of the epidermis with depilatory lotions on the epidermal resistance and in vitro passive and iontophoretic transepidermal transport of insulin. Changes in protein secondary structures and the extent of lipid extraction of the SC by depilatory lotions were studied by FT-IR spectroscopy.

MATERIALS AND METHODS

Materials

Thioglycolate-based depilatory lotions used were Better Off (Personal Care Group Inc, Montvale, NJ), Marzena (Marzena Bodycare Products Inc, Badger, CA), and Sally Hansen (Del Laboratories Inc, Farmingdale, NY). [^{125}I] Human recombinant insulin (specific activity; 2000 Ci/mmol) was purchased from Amersham Pharmacia Biotech Inc (Piscataway, NJ). Lispro insulin (Humalog) was obtained from Eli Lilly and Company (Indianapolis, IN). All chemicals and reagents used were of analytical grade. Deionized water (Resistivity ≥ 18 MΩ-cm) was used to prepare all solutions and buffers.

Preparation of Epidermis and SC

The epidermis was prepared from porcine ears by heat separation technique.[18] The whole skin was soaked in water at 60°C for 45 seconds, followed by careful removal of the epidermis. The epidermis was washed with water and used in the in vitro percutaneous absorption studies. SC samples from the epidermis were prepared using the trypsin digestion method.[18]

In Vitro Transport

Depilatory lotion (100 mg/cm^2), sufficient to spread over the sample, was used to treat the epidermis for 10 minutes and then washed with deionized water. Franz diffusion cells, modified for iontophoresis, were used in the in vitro transport studies. The treated or untreated (control) epidermis was sandwiched between the cells with the SC facing the donor compartment. The maximum capacity of the donor and receiver compartment was 1 mL and 5 mL, respectively. The effective diffusional area was 0.785 cm^2. The donor compartment contained 1 mL of insulin solution (0.2 μCi of insulin in 0.9% wt/vol sodium chloride [normal saline]), and the receiver compartment was filled with 5 mL of normal saline. The donor concentration of insulin used

was 0.1 μmol/mL (0.6124 ng/mL). The cells were maintained at 37 ± 0.5°C by a PMC Dataplate stirring digital dry block heater (Crown Bioscientific Inc, Somerville, NJ).

Scepter iontophoretic power source (Keltronics Corporation, OK) and Ag/AgCl electrodes were used in iontophoresis. Cathodal iontophoresis was performed at 0.2 mA/cm^2 current density. The contents of the receiver compartment were stirred with the help of a magnetic bar at 100 rpm. At appropriate times, 0.5-mL samples were withdrawn from the receiver compartment and normal saline (0.5 mL) was added to the receiver compartment to maintain a constant volume. The samples were counted in a gamma counter (Beckman Coulter, Gamma 5500B, Fullerton, CA) for quantification of I^{125}-labeled insulin in disintegrations per minute (dpm). Net dpm for the samples was obtained by subtracting background dpm. The results were expressed as the mean ± SD of 3 experiments.

Epidermal Resistance

The electric resistance of the epidermis treated with the depilatory lotion and the control (no treatment) was investigated using modified Franz diffusion cells and Scepter iontophoretic power source. The treated or control epidermis was sandwiched between the cells with the SC facing the donor compartment. The donor and receiver compartment contained 1 mL and 5 mL of normal saline, respectively. Direct current (DC) 0.2 mA/cm^2 (I) was applied by placing the anode in the donor and the cathode in the receiver solution. The potential difference (V) across the epidermal sample was noted. The resistance (R) was calculated according to Ohm's law ($V = I \times R$).

FT-IR Spectroscopy

After depilatory lotion treatment of the SC for 10 minutes, the samples were washed with deionized water, vacuum dried (650 mmHg) at 21 ± 1°C for 3 days, and stored in a desiccator. The same amount of depilatory lotion (100 mg/cm^2) was used for treatment of SC samples for FT-IR and epidermal samples for in vitro transport and epidermal resistance studies. The SC samples were squeezed between the potassium bromide plates, and clamped and mounted in the enclosed sample chamber, away from moisture to get their spectra. The samples were then subjected to FT-IR (Nicolet 210, Nicolet Instrument Corporation, Madison, WI) spectroscopic study. Spectra were obtained in the frequency range 4000 to 1000 cm^{-1} in absorbance mode. All spectra analyzed represent an average of 64 scans with resolution of 4 cm^{-1}, Happ-Genzel apodization, and zero filling factor of none. For each SC sample, the spectra were taken before and after the treatment, which allowed each sample to serve as its own control. OMNIC FT-IR software (Nicolet Instrument Corporation) was used to analyze the spectra. FT-IR experiments with each treatment were performed in triplicate with samples coming from different subjects.

To estimate the lipid extraction caused by depilatory lotion treatment, attention was focused on the decrease in absorbance (measured in terms of peak area) of asymmetric and symmetric C-H stretching bands (near 2850 and 2920 cm^{-1}, respectively) sensitive to alkyl chains of the lipids. Second derivative, fourth derivative, and self-deconvoluted spectra were generated for amide I peak. Fourth derivative spectra were generated from the second-derivative spectra by repeating the latter procedure. Fourier self-deconvolution was performed with a bandwidth at half-height of 13 cm^{-1} and enhancement factor of 3. In order to save space, only the second derivative spectra are shown, although the fourth-derivative and self-deconvoluted spectra gave similar results. The fraction of a component was computed to be the area of the component peak divided by the sum of areas of all the component peaks of the amide I band.

Data Treatment

The receiver compartment concentration of insulin was corrected for sample removal.[19] The cumulative amount of insulin permeated per unit epidermal surface area was plotted against time, and the slope of the linear portion of the plot was estimated as the steady state flux (J_{ss}). The permeability coefficient, K_p was calculated as

$$K_p = \frac{J_{ss}}{C_v} \tag{1}$$

where C_v is the donor concentration of insulin. Statistical comparisons were made using the Student t test. The level of significance was taken as $P < .05$.

RESULTS AND DISCUSSION

Figures 1 and **2** show the transport profiles of insulin during passive and iontophoresis, respectively. **Table 1** shows the permeability coefficient and enhancement ratios in the permeability coefficient of insulin through epidermis. Depilatory lotions (ie, Better Off, Marzena,

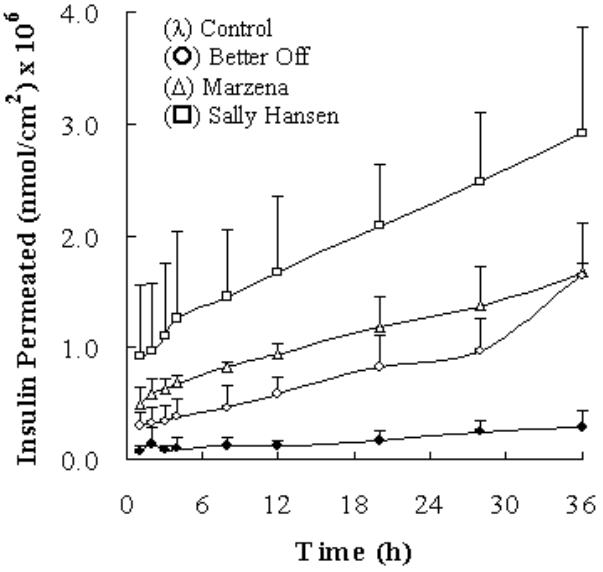

Figure 1. Effect of depilatory lotion treatment on the in vitro passive transport of insulin through porcine epidermis. Each data point is the mean ± SD of 3 determinations.

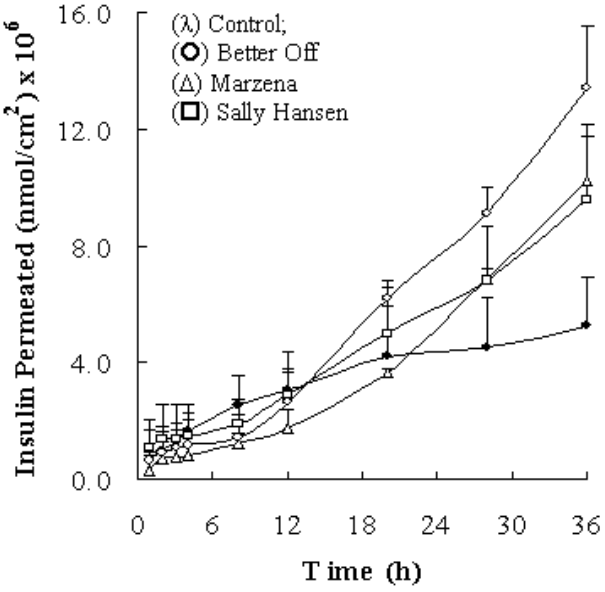

Figure 2. Effect of depilatory lotion treatment on the in vitro iontophoretic transport of insulin through porcine epidermis. Each data point is the mean ± SD of 3 determinations.

Table 1. Permeability Coefficient of Insulin Through the Porcine Epidermis and Enhancement Ratio Due to Depilatory Lotion Treatment for Passive and Iontophoretic Transport*

Treatment	Permeability Coefficient (cm/h) x 10^4 (mean ± SD, n = 3)		Enhancement Ratio	
	Passive	Iontophoresis	ER_1	ER_2
Control	1.17 ± 0.42	8.50 ± 3.00†	--	7.26
Better Off	4.18 ± 0.20†	43.97 ± 13.70†,‡,§	5.17	37.58
Marzena	3.03 ± 1.44†	35.72 ± 9.73†,‡,§	4.20	30.53
Sally Hansen	5.15 ± 1.84†	27.37 ± 5.37†,‡,§	3.22	23.39

*Control indicates no treatment; ER, enhancement ration; ER_1, iontophoretic permeability coefficient with treatment/iontophoretic permeability coefficient with control; and ER_2, iontophoretic permeability coefficient with treatment/passive permeability coefficient with control.

†Significantly ($P < .05$) greater than control (passive).

‡Significantly ($P < .05$) greater than control (iontophoresis).

§Significantly ($P < .05$) greater than the corresponding passive flux.

and Sally Hansen) significantly ($P < .05$) increased the passive permeability coefficient compared with the passive control. Iontophoresis significantly ($P < .05$) enhanced the permeability of insulin through depilatory-pretreated epidermis in comparison with the control epidermis. We observed 24- to 38-fold enhancement in iontophoretic permeability of insulin through different depilatory-lotion–treated epidermis in comparison with passive permeability through the control. Kari[1] and Siddiqui et al[20] had used depilatory lotion to remove the hair from rabbits and rats, respectively, before the transdermal transport of insulin. The hypoglycemic effects observed in their studies may partially be due to the use of depilatory.

Taking body fat, blood glucose and insulin sensitivity into consideration, insulin dose may vary from 5 to 10 United States Pharmacopeia (USP) insulin human units per day to 49 to 175 USP insulin human units per day in divided doses.[21] The transport studies were performed with a very low donor concentration (0.2

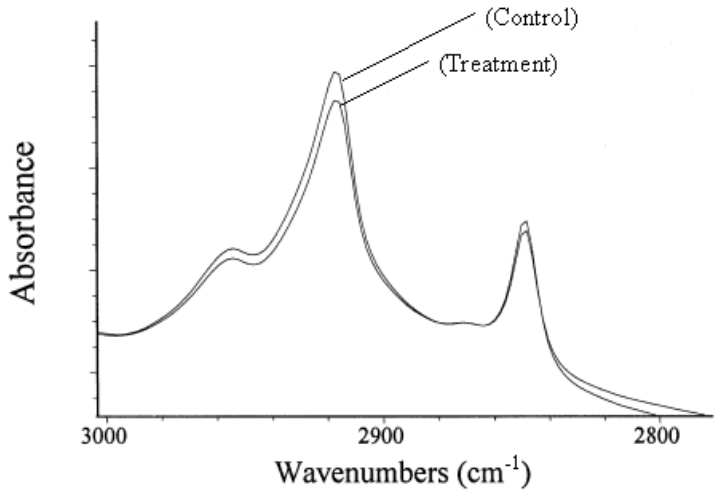

Figure 3. The representative FT-IR spectra (3000-2800 cm^{-1}) of porcine SC treated with a depilatory lotion (Better Off) for 10 minutes, and the control.

Table 2. Changes in Symmetric and Asymmetric C-H Stretching Absorbance Peak Areas After Treatment With Depilatory Lotions*

Treatment	Peak Area					
	Asymmetric			Symmetric		
	Control	Treatment	% Decrease†	Treatment	Control	% Decrease†
Better Off	41.99 ± 3.66	34.78 ± 3.32	17.77	8.31 ± 1.17	7.55 ± 2.43	10.52
Marzena	52.58 ± 2.77	43.46 ± 2.43	17.17	11.26 ± 0.44	9.35 ± 0.31	16.87
Sally Hansen	37.53 ± 2.52	34.57 ± 7.87	7.25	7.75 ± 0.94	7.03 ± 1.04	7.79

*Peak area is expressed as mean ± SD, n = 3.
†% Decrease = 100 −[(absorbance peak area due to treatment/absorbance peak area due to control) × 100]

μCi/mL, ie, 1.68 $\times 10^{-5}$ U/mL) of insulin. In order to determine the amount of insulin transported with a usual donor concentration of 100 U/mL, in vitro iontophoretic transport study was performed through Better Off–treated epidermis. The current density was 0.2 mA/cm^2, and the donor compartment carried 1 mL of a 50 mM NaCl solution containing 100 U of Lispro insulin spiked with 0.1 μCi [^{125}I] insulin. The Lispro insulin flux was 5.25 (± 1.05) $U/cm^2/d$. A patch as small as 20 cm^2 should be sufficient to achieve a flux of 100 U/d. This flux could further be enhanced by using higher current density and larger patch area. Iontophoretic current up to 0.5 mA/cm^2 has been found to be safe.[22]

Figure 3 shows the representative FT-IR spectra from 3000 to 2800 cm^{-1} of SC treated with a depilatory lotion (Better Off). Decreases in peak areas of the asymmetric and symmetric C-H stretching absorbances were observed, revealing the extent of lipid extraction. Lipid extraction caused by different depilatory lotions was little (as indicated by 7% to 18% decrease in C-H stretching absorbance peak areas) and varied marginally from one another (**Table 2**).

Derivative spectral analysis and Fourier self-deconvolution have been applied to obtain the position of the overlapping components of the amide I band and assign them to different secondary structures. The representative original and second derivative spectra of the amide I band of the control and the SC treated with a depilatory lotion (Better Off) are shown in **Figure 4**. The frequency regions for different structures were assigned as follows: 1661 to 1647.5 cm^{-1} to α-helix; 1689 to 1682 cm^{-1} to β-sheet 1; 1637.5 to 1627.5 cm^{-1} to β-sheet 2; 1627.5 to 1615 cm^{-1} to low frequency β-sheet; 1644.5 to 1637.5 to random; 1682 to 1661 cm^{-1}

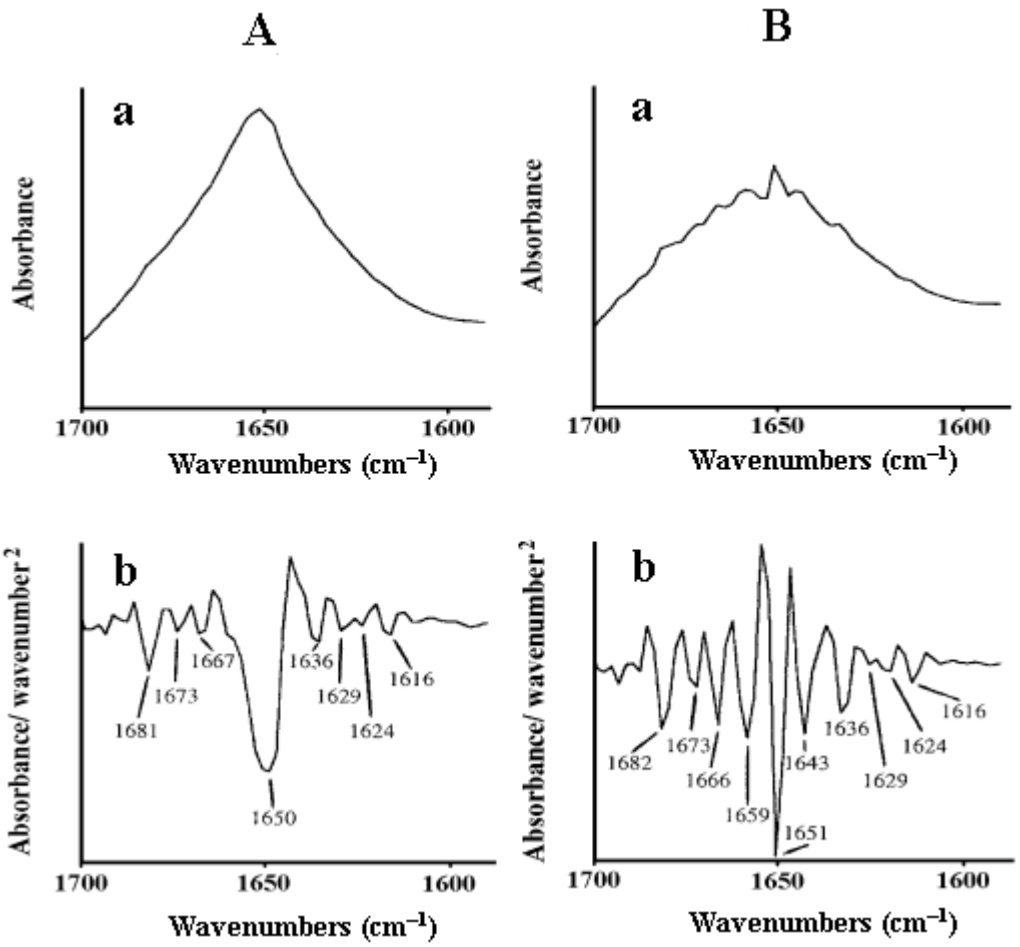

Figure 4. The representative (a) original, and (b) second derivative, FT-IR spectra (1700-1600 cm^{-1}) of the amide I band of porcine SC proteins. (A) Control (no treatment); (B) Depilatory lotion (Better Off) Treated.

to turns.[23,24] Random and turn structures are added and referred to as "unordered" structures. In this study, the possible contribution caused by vibrational modes of membrane lipids overlapping with amide I band was not taken into account.

The area under the peaks of second derivative spectra can be used to determine the percentage composition of the various structures.[24,25] **Tables 3** and **4** compare the percentage composition of protein secondary structures before and after treatment with depilatory lotions. Second derivative quantitation of untreated SC revealed α-helix (~67%), β-sheet 1 (~10%), β-sheet 2 (~9%), low frequency β-sheet (~3%), and turn structures (~9%).

There was a remarkable decrease in α-helix conformation, accompanied by equivalent increase in unordered structures in the depilatory-lotion-treated SC. A new peak at 1642 cm^{-1} emerged in the treated SC, which was assigned to random conformation. This contributed to the increase in unordered structures. Random peak is indicated by Fourier self-deconvolution and fourth derivative analysis in all the treated SC, though the peak was too small to appear in the second derivative spectra for quantitation in some SC samples. A distinct peak at 1659 cm^{-1} separated out and appeared as a shoulder to α-helix peak in the untreated SC. This peak was assigned as another turn conformation. Major increase in unordered structure came from increase in turn conformation. So the observations indicate that depilatory lotion treatment is affecting the α-helix conformations of the SC proteins resulting in a conformational transition to random and turn structures, β-sheet structures mostly remaining unchanged. The helix to random coil transition is a thoroughly studied phenomenon. This transition is very abrupt and can occur upon change of temperature, pH, solvent etc.[26]

The depilatory lotion treatment led to decrease in the epidermal resistance. The epidermal resistance of the

Table 3. Estimation of α-Helix and β-Sheet (1, 2, Low Frequency) of SC Proteins Before (Control) and After Treatment With Depilatory Lotions, Using Second Derivative Methods*

Treatment	% α-Helix		% β-Sheet 1		% β-Sheet 2		% β-Sheet low frequency	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
Better Off	70.90 ± 2.96	22.42 ± 3.13	9.77 ± 1.30	9.37 ± 1.04	7.74 ± 2.74	7.54 ± 1.61	3.92 ± 0.40	4.37 ± 2.03
Marzena	67.53 ± 0.51	46.50 ± 2.38	10.25 ± 1.21	12.85 ± 1.43	10.17 ± 0.67	9.81 ± 0.91	2.91 ± 1.45	6.17 ± 0.33
Sally Hansen	66.44 ± 0.96	35.06 ± 10.10	12.17 ± 1.98	15.22 ± 3.54	7.76 ± 2.43	6.29 ± 1.47	3.41 ± 1.01	4.59 ± 2.72

*Values are expressed as mean ± SD; n = 3.

Table 4. Estimation of Unordered Structure (Turns and Random) of SC Proteins, Before (Control) and After Treatment With Depilatory Lotions, Using Second Derivative Methods*

Treatment	% Turns		% Random		% Total Unordered	
	Control	Treatment	Control	Treatment	Control	Treatment
Better Off	7.68 ± 0.99	41.41 ± 1.90	0.00 ± 0.00	14.89 ± 2.54	7.68 ± 0.99	56.29 ± 2.46
Marzena	9.15 ± 0.83	24.66 ± 2.28	0.00 ± 0.00	0.00 ± 0.00	9.15 ± 0.83	24.66 ± 2.28
Sally Hansen	10.23 ± 2.42	34.72 ± 9.02	0.00 ± 0.00	4.12 ± 3.77	10.23 ± 2.42	38.85 ± 11.75

*SC indicates the stratum corneum. Values are expressed as mean ± SD; n = 3.

control was 8.12 KΩ.cm^2. Depilatory-lotion–treated epidermis showed the resistance of 3.93-5.72 KΩ.cm^2 (**Table 5**). Changes in the secondary structures of the protein and lipid extraction of the SC due to depilatory lotion treatment are suggested for the decrease in the epidermal resistance.

Proteins, 90% of which are water-insoluble, represent a major component of the SC.[8] It has been reported that protein in keratinized tissue is rich in cysteine residues, and the disulfide bridges they form might attribute to the proteins' insoluble nature.[27] A strong reducing agent, 2-mercaptoethanol, which can cleave disulfide bonds, resulted in hydration and solubilization of proteins in keratinized tissue.[28] This mechanism was suggested for the reducing agents dithiothreitol and ascorbate for increasing the penetration of diclofenac across rat skin.[29] This is in good agreement with the observation of Baden et al[9] that the stability of α-helix and conformational changes in SC proteins are related to the number and nature of disulfide bonds. The mechanism of action of thioglycolates for depilatory effect involves reduction of cystine linkages. With the decrease in disulfide bridges, the hydration capability of the protein would increase resulting in the increase in hydration of the SC.[29] It is likely that transport of ionic hydrophilic molecules occurs via an aqueous route, and since insulin is present in ionized form, increased hydration of the SC would result in an increased permeability of insulin. Therefore an increase in the permeability of insulin through the thioglycolate-based depilatory-lotion–treated epidermis may have resulted because of the solubilization of the SC proteins causing α-helix structures to lose shape and increase the amount of turns structure and random coil conformations. The contribution of the appendageal pathways to the permeability of insulin has not been studied in the current investigation. The concentration of thioglycolic acid among different depilatory lotions usually varies between 2.5% and 4.0% by weight in the form of its alkali salt. Different thioglycolate content, alkalinity of the formulation, and presence of other ingredients in the depilatory lotions studied might have resulted in enhancement of insulin permeation to different extent.

CONCLUSION

The depilatory lotions (ie, Better Off, Marzena, and Sally Hansen) significantly ($P < .05$) increased the passive flux of insulin in comparison with the control (epidermis not treated with depilatory lotion). Iontophoresis further increased ($P < .05$) the flux of insulin through depilatory-lotion–treated epidermis. The decrease in α-helical conformations and the increase in the unordered conformations of protein in combination with lipid extraction of the SC appear to be the main reasons for increase in the passive and iontophoretic

Table 5. Changes in Epidermal Resistance After Treatment With Depilatory Lotions*

Treatment	Resistance ($K\Omega.cm^2$)	% Decrease in Resistance†
Control	8.12 ± 1.55	--
Better Off	5.12 ± 0.15	37.01
Marzena	5.72 ± 1.76	29.52
Sally Hansen	5.22 ± 1.42	35.76

*Control indicates no treatment; n = 3.

†Percentage decrease in resistance = 100 - [(resistance of treated epidermis/resistance of control epidermis) × 100]

permeabilities of insulin through the depilatory-treated epidermis. Finally, this study indicates that depilatory lotions or their active components can be considered as penetration enhancers in developing commercial transdermal iontophoretic delivery systems for insulin.

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