Iontophoresis Enhances Transdermal Delivery of Methylene Blue in Rat Skin (I): The Effect of Current Application Duration

■Jae-Hyoung Lee, PT, PhD; Eun-Young Choi, PT, PhD[1]

■Department of Physical Therapy, Electrotherapy Research Laboratory for Tissue Growth & Repair, Wonkwang Health Science University; [1]Department of Physical Therapy, Kwangyang College

Purpose: The objectives of this study were to determine the enhancing effect of iontophoresis method as it transdermally deliver methylene blue (MB) using visual examination, in terms of penetration depth and tissue distribution in the skin, and to determine the effect of application duration on the efficacy of iontophoresis.

Methods: Twenty-four male Sprague-Dawley rats were randomly divided into 5-, 10-, 20-, and 40-minute groups. These rats were exposed to either topical or anodic iontophoresis of 1% MB using a direct current of 0.5 mA/cm^2 for 5, 10, 20, and 40 minutes. Using cryosections of rat tissues, the penetration depth of MB was measured using light microscopy.

Results: Significant differences in the penetration depth (F=54.20, p<0.001) were detected among the four groups. *Post hoc* comparisons of the penetration depth of MB data pooled across groups showed no significant difference between all topical application groups and 5-minute iontophoresis group, but did reveal a significant difference in the penetration depth between all topical application groups and 5-minute iontophoresis group versus 10-minute group, between the 10-minute and 20-minute group, and between the 20-minute and 40-minute iontophoresis group (p<0.05).

Conclusion: The results demonstrate that iontophoresis enhances transdermal delivery of MB across stratum corneum of skin barrier by visual examination. Furthermore, the penetration depth of iontophoretic transdermal delivery of MB was dependent on the application duration. The duration of iontophoresis is one of the important factor in the efficacy of iontophoresis application.

Keywords: Iontophoresis, Penetration depth, Tissue concentration, Transdermal transport, Application duration, Methylene blue

I. Introduction

Transdermal drug delivery systems have been widely used for non-invasive administration of therapeutic agents due to several advantages that include avoidance of gastrointestinal disturbances, bypassing of hepatic first-pass metabolism and patient acceptability. In iontophoresis, the appendages provide pores to ions and allow large polar molecules permeation. The drugs penetrate through hair follicles, sebaceous glands, sweat ducts, across corneocytes and intercellular spaces. The epithelial cells of the pilosebaceous gland are more permeable than corneocytes and the molecules can reach the dermis by entering the follicle and the sebaceous gland.[1] However, the pore route usually contributes poorly to drug transportation because of the small size of available appendageal area for transport which is measured about 0.1% of whole skin surface.[2,3] Thus, most of the drugs introduce transdermally permeate mainly through the intercellular lipid lamellar region. But the intact stratum corneum of the epidermis pose a barrier to the permeation of many therapeutic substances. This barrier is effective and selective since it has a brick and mortar structure that behaves like a wall. The corneocytes of hydrated keratin comprise the 'bricks', embedded in a 'mortar', which are composed of multiple lipid bilayers of ceramides, fatty acids, cholesterol and

cholesterol esters.[4] The composition of this barrier makes it difficult to deliver a therapeutic dose because it prevents the transportation of drugs to the epidermis at a sufficient rate even if the drug is potent.[5]

As a consequence, to achieve the therapeutic effects of drugs, many enhancing techniques have been used to overcome the barrier, including chemical modification, electrical and mechanical disruption of the barrier function.[3] Recently, there has been a great emphasis on the use of physical enhancing methods such as iontophoresis, electroporation, phonophoresis, thermophoresis and magnetophoresis.[6,7] Among these physical methods, one of the most efficient and popular methods to enhance transdermal drug delivery is iontophoresis.[8] Iontophoresis is the administration of drugs across the skin by the application of low intensity direct current for the management of a variety of medical conditions.[9]

To determine the penetration depth of the drug in the tissues, the concentration of tissue layers using n high-performance liquid chromatography (HPLC) from tissue extracts,[10-12] radiolabeled specimens from tissue biopsies[13,14] were employed. Insertion of hypodermic needle is also an option since it is used to determine the penetration depth of local anesthetic, but many patients experience discomfort during the application. Worth noting is that all these methods cannot directly and accurately determine the penetration depth of the drug in tissues. Since these methods only provide some estimates which make it difficult to establish an accurate and safe therapeutic dosage, effective alternatives are used in several studies using visual determinations of the penetration depths and distribution of ions in the tissue such as confocal laser scanning microscopy,[15] cryo-scanning electron microscopy and transmission electron microscopy,[16] nuclear magnetic resonance imaging,[17] but all these methods are costly and are impractical to use.

The effect of iontophoresis is largely based on the penetration, distribution and concentration of ionic drugs in the target tissues and there are few published studies that investigate the exact penetration depth of iontophoresis by visual examination under a microscope. The purposes of this study, therefore, were to determine whether the microscopic visual examination could be useful method to observe the penetration depth and ion distribution by iontophoresis and to determine the enhancing effect of iontophoresis and the efficacy of application duration of iontophoresis as it transdermally deliver

methylene blue (MB) into the tissue.

II. Materials and Methods

1. Preparation of the Solution

One percent (1%) MB solution was formulated by mixing methylene blue dihydrate (MW. 319.85, Junsei Chemical Co., Ltd., Japan) and ultrapure water. A solvent, which is a mixture of 0.1 N sodium hydroxide and absolute methanol with a 1:1 ratio, was prepared for the procedure. The sodium hydroxide (Sigma Chemical Co., USA) and the methanol absolute (Sigma Chemical Co., USA) were of analytical grade.

2. Iontophoresis of MB

Twenty-four male Sprague-Dawley rats (270.0±2.3 g were used, and utmost adherence to the principles of laboratory animal care was observed in this study. The rats were randomly divided into 5-, 10-, 20- and 40- minute groups (n=6 for each group). These rats were anesthetized by intraperitoneal injection of ketamine HCl (75 mg/kg) and xylazine (10 mg/kg). The hair in the upper and low back area of the rats was electrically clipped and cleaned to reduce skin resistance. The dispersive electrode, which is a soft cotton pad (4 × 4 cm) soaked in sterile saline solution, was held on the upper back and attached to the cathode terminal of the direct current generator. The active electrode, which is a soft cotton pad (2.5 × 2.5 cm) soaked in 600 μl of 1% MB solution, was placed on the left and right side of the low back. The active electrode was placed on the left side in the iontophoresis of MB, and an anodal current of 3.2 mA (0.5 mA/cm^2) was applied for 5, 10, 20 and 40 minutes to the four groups using a direct current generator, respectively (Endomed 982, Enraf-Nonius, Netherlands). In the topical application of MB, the active electrode was placed on the right side of low back and maintained for 5, 10, 20 and 40 minutes groups without electricity. The distance between the electrodes was maintained at 5 cm apart.

3. Measurement of Penetration Depth

Immediately after treatment, the skin was rinsed to remove superficial MB and excised a 0.4 × 0.4 cm full-thickness skin samples. The samples were embedded on a metal disk using embedding matrix (Tissue Tek, Sakura Finetechnical Co., Ltd.,

Japan) and an instant freezing aerosol (Lip Freeze, L.I.P. Ltd., UK), then cut into 7 μm thickness serial sections at -20°C using a cryotome (Shandon, Thermo Electron Corporation, USA). Images of serial sections were captured under 100x light microscope (Olympus BX 50, Olympus Optical Co., Ltd., Japan) with a color CCD camera (IK-642K, Toshiba CCD color camera, Toshiba Co., Japan). Images at 768×512 pixels on a 1.2 mm × 0.8mm size of the dermis were made in 5 areas. One pixel covered a surface of 1.6 μm x 1.6 μm in the section. From these digitally captured images of sections, penetration depth and surface area were measured in five sites using the computerized image analysis system (Image-Pro® Plus, version 3.01, Media Cybernetics, Inc., USA).

4. Measurement of Tissue Concentration and Transdermal Transport

A 0.4 x 0.4 cm Full-thickness skin samples (mean wet weight of 30.5mg) were immersed in a 1 ml of 0.1 N NaOH in absolute methanol (1:1) in a 1.7ml tube and placed into the incubator at 37°C for 12 hours. The samples were then centrifuged in the same tube. Prior to sample measurements, calibration of the spectrophotometer and normalization of absorbance was performed daily. The supernatant fractions of the samples were diluted with 3 ml distilled water. The absorbance of the dissolved supernatant was measured in a 10 mm path length quartz cuvette at a wavelength of 600 nm using a spectropho-tometer (Spectronic 20+, Spectronic Instruments, USA). Total concentration of MB in solvent was determined from an

absorbance versus standard concentration plot. Tissue concentration of MB (mg MB/g tissue) was calculated as the total concentration of MB in mg divided by the wet weight of skin sample. Transdermal transport was calculated as the amount of transported MB (μg) across the skin surface area (cm^2).

5. Data Analysis

To compare the differences among the four groups in terms of penetration depth, amount of tissue concentration and trans-ported MB in tissues, a one-way analysis of variance was used. To determine intergroup differences, Duncan multiple range test was used. Statistical differences were considered to be significant at 0.05. All statistical analyses were performed using SPSSWIN 10.0 (SPSS Inc., USA).

III. RESULTS

1. Penetration depth and distribution

MB penetration into the skin was observed using the prepared cryosections. After 5 minutes of topical application, no MB was observed (Figure 1a). The topical application of 10 and 20 minutes showed minimal staining of the skin sections (Figure 1b, c), and in the 40 minute topical application group, a weak blue spotty stained pattern in hair follicles and around hair shafts was observed (Figure 1d). With iontophoresis, the 5 minute group revealed limited presence of MB in the stratum corneum (Figure 1e), whereas in the 10- and 20-minute group, a dark blue
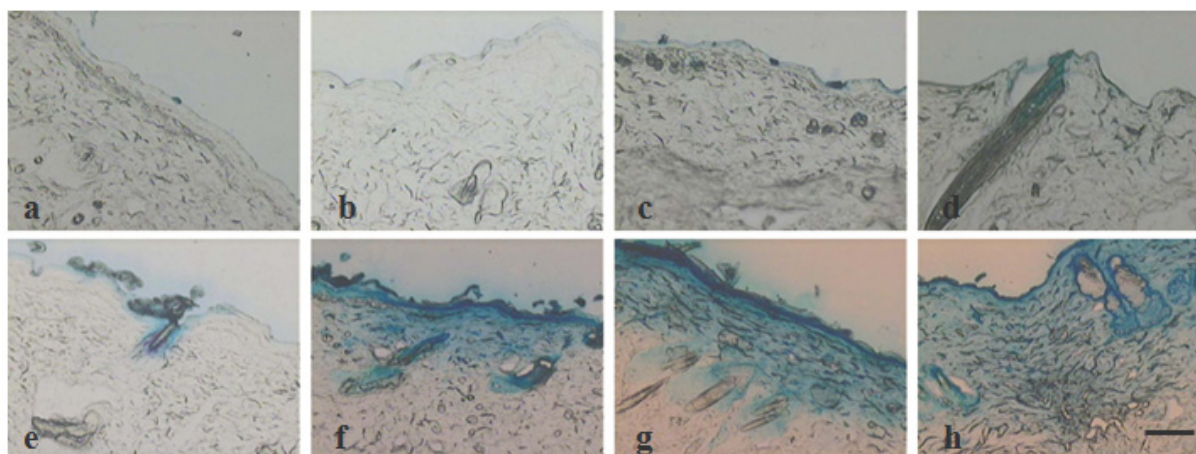

Figure 1. Photomicrographs (light microscopy, 100x) of rat skin after MB application. Dark bluish-stained MB can be seen in the cryosections. Topical application of MB for 5 minutes (a), 10 minutes (b), 20 minutes (c) and 40 minutes (d). Iontophoresis of MB for 5 minutes (e), 10 minutes (f), 20 minutes (g) and 40 minutes (h). Bar: 100 μm.

stained was observed in the epidermis like a band indicating MB migration through the epidermis and into the superficial dermis (Figure 1f, g). In the 40 minute ontophoresis group, MB presence in the deep dermis was seen (Figure 1h).

Mean values and standard deviations of penetration depth are shown in Table 1. The result of the one-way analysis of variance of penetration depth are shown in Table 2. The significant differences in the penetration depth was detected among the groups (F=54.20, p<0.001). *Post hoc* comparisons of the penetration depth data pooled across groups showed no significant difference between all topical application groups and the 5 minute iontophoresis group. There are significant difference, however, between all topical application groups and 5 minutes iontophoresis group versus the 10 minute group (p<0.05), between the 10 minute and 20 minute group (p<0.05), and between the 20 minute and 40 minute iontophoresis group (p<0.05). The penetration depth increased significantly in the 10 minute iontophoresis group compared to the 5-, 10-, 20- and 40 minute topical application groups and to the 5 minute iontophoresis group. The penetration depth in the 20 minute iontophoresis group was deeper than in the 10 minute iontophoresis group. The penetration depth in the 40 minute iontophoresis group was the deepest among the groups (Figure 2).

Table 1. Means and standard deviations of penetration depth, tissue concentration and transdermal transport of MB in topical application and iontophoresis groups

	Penetration Depth (μm)	Concentration (mg/ 1 g tissue)	Transport (μg/cm^2)
	Mean±S.D.	Mean±S.D.	Mean±S.D.
TA 5 min	0.00±0.00	0.003±0.001	0.341±0.04
TA 10 min	3.22±2.93	0.005±0.003	0.97±0.75
TA 20 min	8.32±10.24	0.01±0.009	1.07±0.68
TA 40 min	20.62±28.48	0.04±0.030	3.98±2.99
IP 5 min	46.19±43.83	0.05±0.035	6.73±4.04
IP 10 min	208.92±72.50	0.24±0.046	26.15±7.71
IP 20 min	281.59±65.76	0.47±0.168	50.28±13.26
IP 40 min	349.22±73.96	0.70±0.165	93.25±19.14

TA: topical application, IP: iontophoresis, S.D. : standard deviation.

Table 2. Summary of one way analysis of variance

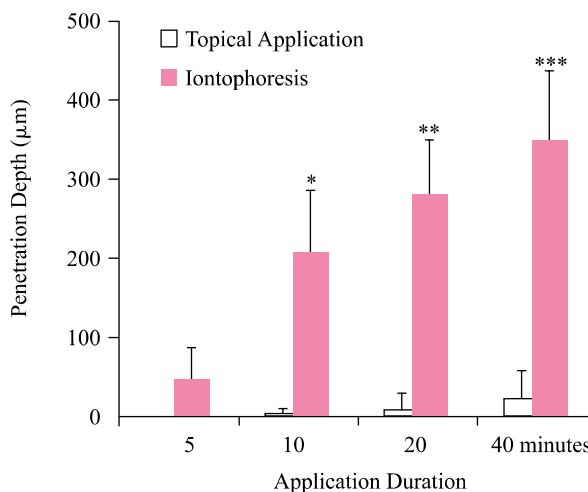| Variables	df	SS	MS	F	p
Penetration Depth	7	872871.70	124695.64	54.20	0.000
Concentration	7	2.92	0.42	56.01	0.000
Transport	7	46765.55	6680.79	85.10	0.000

Figure 2. Comparisons of penetration depth of MB in the cryosections of rat skin. There were significant differences in the penetration depth among the groups (p<0.001). Post hoc comparisons revealed that 40-minute group has the deepest penetration comparing all other groups(***), 20 minute groups has deeper penetration than 10- and 5 minute groups(**), 10-minute group is deeper than 5 minute groups(*) (p<0.05).

Values are mean±standard deviation (n=6).

2. Tissue concentration of MB

Mean values and standard deviations of tissue concentration are shown in Table 1. The result of the one-way analysis of variance of tissue concentration are shown in Table 2. The significant differences in the tissue concentration was detected among the groups (F=56.01, p<0.001). *Post hoc* analysis revealed that tissue concentration increased significantly in the 10 minute iontophoresis group compared to all topical application groups and the 5 minute iontophoresis group (p<0.05). The tissue concentration in the 20 minute iontophoresis group was higher than the 10 minute iontophoresis group (p<0.05). The mean tissue concentration in the 40 minute iontophoresis group was the highest (p<0.05) (Figure 3).

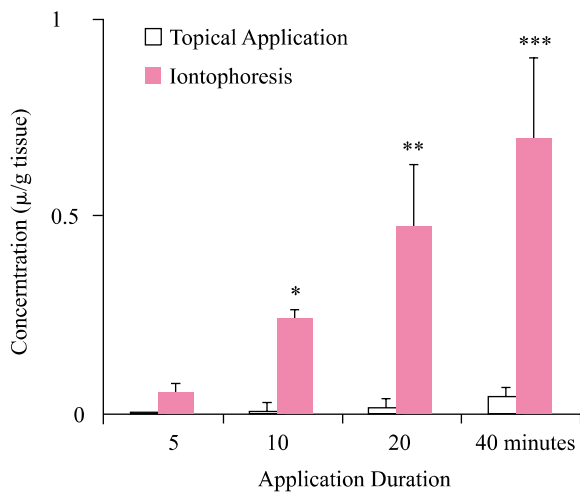**Figure 3.** Comparisons of tissue concentration in the rat skin. There were significant differences in the tissue concentration among groups (p<0.001). *Post hoc* comparisons of the tissue concentration showed significant differences between all topical application groups and 5 minute iontophoresis group and the 10 minute group(*), between the 10 minute and 20 minute groups(**), and between the 20 minute group and the 40 minute iontophoresis group (***) (p<0.05).

Values are expressed in mean±standard deviation (n=6).
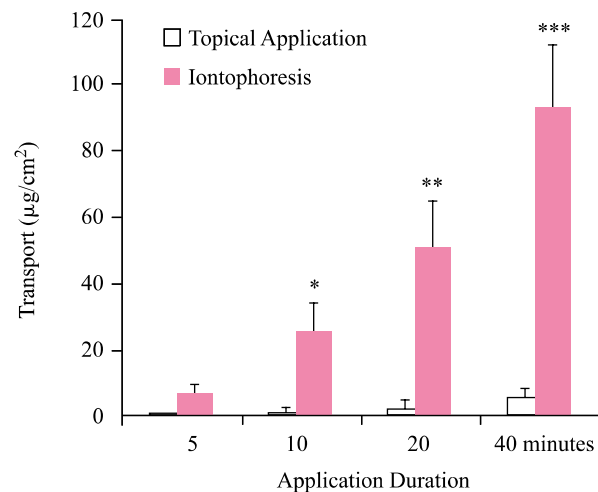

Figure 4. Comparisons of transdermal transport across the skin. There were significant differences in the transdermal transport amount among the groups (p<0.001). *Post hoc* comparisons of the transdermal transport amount showed significant differences between all topical application groups and 5 minute iontophoresis group versus 10 minute(*), and between 10 minute versus 20 minute(**),and between 20 minutes versus 40 minute iontophoresis group (***)(p<0.05).

Values are expressed in mean±standard deviation (n=6).

3. Transdermal transport of MB

Mean values and standard deviations of transdermal transport are shown in Table 1. The results of the one-way analysis of variance of transdermal transport are shown in Table 2. The amount of transdermal transport into the skin revealed significant differences among the groups (F=85.10, p<0.001). *Post hoc* comparisons showed significant differences between all topical application groups, the 5 minute iontophoresis group and the 10 minute group, between the 10 minute and 20 minute groups, and between the 20 minute and 40 minute iontophoresis group (p<0.05). Figure 4 shows the comparisons of transdermal transport across the skin. It reveals significant differences in the transdermal transport amount among the groups (p<0.001). *Post hoc* comparisons of the transdermal transport amount showed significant differences between all topical application groups, 5 minute iontophoresis group and the 10 minute group, between the 10 minute and 20 minute groups, and between the 20 minute and the 40 minute iontophoresis group (p<0.05). Values are expressed in mean± standard deviation (n=6).

IV. Discussion

In our study, the light microscopic examination was a useful technique to determine visually the penetration depth and distribution of MB in the non-fixed fresh cryosection of rat skin biopsy following iontophoresis. Under microscopy, the penetration depth and distribution of MB can be compared and differences revealed. MB was evenly distributed throughout the epidermis and the dermis after iontophoresis. It appeared to be darker in the epidermis, in the surrounding hair follicles and fibroblasts due to its affinity to the nucleic acid present in keratinocytes, follicular epithelial cells and fibroblasts during its migration by iontophoresis. The results indicate that MB migrated across corneocyte and intercelluar space of stratum corneum, and into the dermis by entering the more permeable pilocebaceous epithelial cells.

In this study, we have observed that MB ions penetrated the stratum corneum and traveled a distance of 0.2~0.35 mm in the dermis after 10 to 40 minutes of iontophoresis, and there

was no indication of MB penetration to the stratum corneum after topical application. However, our results differ from other researchers where their penetration depth studies of tissue extracts reported deeper penetration. Their values range from 1 to 50 mm. While Costello and Jeske[14] reported that radiolabeled lidocaine penetration into the tissue of a rabbit following iontophoresis of 5 mA intensity applied for 20 minutes was at least 1 cm into the gluteal muscles. Riviere et al.[18] have detected presence of [^{14}C] lidocaine at a depth of 1.35 mm in a pig skin after iontophoresis using a 0.9 mA (0.2 mA/cm^2) intensity for 1 hour. In another study conducted by Glass et al.,[13] a radiolabeled dexamethasone phosphate was seen in a tissue of a rhesus monkey following iontophoresis of 5 mA for 20 minutes and penetrated the deeper tissue to a depth of 17 mm reaching joint capsules. Singh and Roberts[10] have observed the salicylic acid in tissue at approximately 3~4 mm below the application site after iontophoresis in rats. Panus et al.[19] have reported that ketoprofen was introduce into porcine medial thigh by iontophoresis of 4 mA (0.28 mA/cm^2) intensity for 40 minutes (160 mA·min) and penetrated the tissue to a depth of 10~50 mm. They estimated the penetration depth using HPLC by measuring the concentration of drugs from extracted skin tissue, fascia and muscle. Moreover, Bjerring and Arendt-Nielsen[20] described that local anesthetics (EMLA) provide anesthesia to a maximal depth of 5 mm, whereas Ashburn[21] reported that a 20 mA·min dose of iontophoretic lidocaine provides anesthesia to a depth of 7.6 mm and a 40 mA·min dose provides anesthesia to a depth of 8.6 mm.

The difference in the penetration depth between our study and those of the previous studies is probably explained by the differences in the molecular weight, subject, dosage and assay method. They estimated the penetration depth of lidocaine (234.34), dexamethasone (392.47), salicylic acid (138.12) and ketoprofen (254.29) by radiolabel assays and HPLC by measuring the concentration of drugs from extracted tissue at skin, fascia and muscle. In our study, the penetration depth of MB (319.85) was measured by visual microscopic observation of the tissue. It is well known that smaller ions are transported at a faster rate than larger ions.[9] We believe that this value, which we determined by direct microscopic technique, is accurate because observation and measurement were done in situ.

We used MB in this study because it allows accurate visualization once it penetrates into the tissues. Methylene blue

(3,7-bis (dimethylamino)-phenazathionium chloride tetrame-thylthionine chloride,) is a water soluble cation. The color of MB permits visual examination in the tissue under the microscope, its absorbance property (668 nm) allows spectrophotometric detection, and its cationic property serves as a model for a number of cations for iontophoresis.[22]

We confirmed that iontophoresis enhances transdermal delivery of MB in a rat skin in vivo by colorimetric examination. In this study, negligible amount of MB was found in the skin tissue following topical application, whereas a substantial amount of this same substance was seen in the skin tissue following iontophoresis. The amount of MB in the skin tissue and the amount of MB transported per unit area were greater after iontophoresis than after topical application.

In this study, iontophoresis for 10, 20 and 40 minutes has transported transdermally MB as far as 22.49%, 30.32% and 37.60% of skin thickness. All of these are observed and documented in a rat skin with an average thickness of 928.88 μm, epidermis 54.60 μm, dermis 874.28 μm, respectively. In addition, tissue concentration of MB following iontophoresis for 10, 20 and 40 minutes was greater by 4.6, 8.9, and 13.2 times than after iontophoresis of 5 minutes. While transdermal transport of MB after iontophoresis of 10, 20 and 40 minutes was greater by 3.9, 7.5, and 13.8 times than iontophoresis of 5 minutes. We also observed that trandermal delivery of ion, penetration depth, and MB tissue concentration increase as the iontophoresis duration increases. Following a 10 minute iontophoresis, transdermal transport of MB was greater than topical application of any duration and the 5 minute iontophoresis. It is reasonable to suspect then that topical application and a short duration iontophoresis did not exert a significant influence in enhancing transdermal delivery of ion, in this case, MB. Thus, a direct relationship between the concentration of MB in tissues and intophoresis duration exists. This study clearly showed that transdermal delivery of ions depends on the duration of iontophoresis and its result is consistent to the research findings of other investigators where deeper penetration of ion was observed following a longer application duration iontophoresis.[23,24]

The present results demonstrate that the enhancing effect of iontophoresis can be established by microscopic visual examin-ation and colorimetric examination. The light microscopic visual examination may serve as an accurate technique to

determine the penetration depth and ion distribution following iontophoresis. The penetration depth, MB tissue concentration and the amount of transported ion were enhanced by iontophoresis and not by topical application and were dependent on the application duration of iontophoresis. Thus, the duration parameter of iontophoresis is one of the important factors that should be considered in improving the efficacy of transdermal delivery of ions and in determining the depth of penetration of ions in target tissues.

Author Contributions

Research design: Lee JH, Choi EY

Acquisition of data: Lee JH, Choi EY

Analysis and interpretation of data: Lee JH, Choi EY

Drafting of the manuscript: Lee JH

Research supervision: Lee JH

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