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Transdermal Delivery of Adenosine and Eosin Y Using Microplasma Combined with $FeSO_4$ and DMSO Iontophoresis

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ABSTRACT: One of the main roles of transdermal drug delivery is to overcome stratum corneum; the first barrier of the skin. We combined several types of treatments to investigate their effect on skin permeability and drug absorption. Franz diffusion cell was used to investigate the permeation and retention of adenosine through Yucatan micropig's skin. The amount of the penetrated drug and the drug retained inside the skin was measured by HPLC. We compared adenosine permeation through the untreated skin, iontophoretically pre-treated skin with $FeSO_4 \cdot 7H_2O$ in DMSO followed by microplasma treatment, iontophoretically pre-treated skin with $FeSO_4 \cdot 7H_2O$ in water followed by microplasma treatment, and treated skin with DMSO. Eosin Y dye was used to analyze depth of the penetration after the treatment. Depth of penetration was displayed by microscopic observation and FE-SEM/EDS observation after skin sectioning. Absorption of eosin Y inside the skin measured by FE-SEM/EDS and optical microscope was consistent with absorption of adenosine measured by using Franz diffusion cell and HPLC. Iontophoretic pre-treatment followed by plasma treatment caused increased drug absorption in stratum corneum and plasma treatment itself increased drug penetration through the epidermal layer of the skin.

KEY WORDS: microplasma, $FeSO_4$, DMSO, iontophoresis, SEM, EDS, frozen section, Franz cell

I. INTRODUCTION

It is possible to observe growing number of publications studying plasma in drug delivery field from designing drug delivery systems[1–3] through drug delivery into cells[4] to drug delivery through tissues.[5–8] In transdermal drug delivery field, plasma has been studied to create microholes in stratum corneum to increase drug permeability by Gelker et al.[9,10] Creating microholes with plasma was combine with electroporation in study by Kim et al.[11] Similarly, microholes created by microneedles was combine with plasma discharge by Chen et al.[12] for improving the transdermal delivery of an immune checkpoint blockade. There was also a possibility of creating microholes in the work of Xin et al.,[13] when plasma discharge was used to enhance delivery of lidocaine to the skin before laser treatment to reduce pain caused by the laser.

However, creating microholes has not been observed in every study. Different mechanism was used in following studies. Small molecules such as Galantamine Hydrobromide (368 Da),[14] as well as medium (patent blue V – 1159.4 Da,[15] Cyclosporine A – 1203 Da[16]) or large molecules (DNA[17]) were successfully transdermally delivered. Enhancing effect of transdermal drug delivery by plasma treatment depends on the power of discharge.[18,19] Stratum corneum and the lipid bilayer between corneocytes are the first barrier of the skin. To permeabilize this barrier, it is necessary to influence the lipids by their extraction, oxidation or by changing their structure or composition. This is possible by using chemicals but plasma is capable of such action too.

The second barrier of the skin is composed of the tight junctions under the stratum corneum layer called stratum granulosum. Tight junctions create so-called kissing points between the skin cells, not allowing to pass chemicals inside or outside. Choi et al.[20] found out that plasma can also influence intercellular junctions of the skin keratinocyte cells and allow the penetration of a drug. In the study by Lee et al.,[21] production of nitric oxide in plasma treated keratinocytes was noticed. However, the effect of plasma induced nitric oxide on transdermal delivery needs to be investigate further.

In our recent research, Kristof et al.[22] focused on possible oxidation of stratum corneum lipids and influence on drug absorption or permeability through the skin. Based on this study, we speculated that the main reason of plasma induced skin permeability enhancement was not through lipid oxidation but other processes. We tried to induce lipid oxidation by introducing iron inside the skin to compare it with the purely plasma treated skin. Lipid oxidation of the lipids inside stratum corneum could increase the amount of drug absorbed by the skin.

The aim of the current research is to study how the supposed lipid oxidation influence drug distribution inside the skin. This distribution will be observed by optical microscope and FE-SEM/EDS (field emission scanning electron microscope) microscope/analysis of the frozen skin section. Model drugs used in this study were adenosine and eosin Y. We tried to induce lipid oxidation by the introduction of iron ($FeSO_4 \cdot 7H_2O$) into the skin by electroporation (iron served as a catalyst for oxidation) and plasma active species as oxidation agents. Permeability and drug retention of adenosine were investigated by Franz diffusion cells.

II. EXPERIMENT

A. Adenosine Penetration/Retention Study

1. Sample Preparation

The hairless Yucatan micropig's skin was ordered from Charles River Japan, Inc. (Yokohama, Japan). The skin samples were stored at –80°C. The fat layer was removed by knife prior to cutting the skin into pieces of dimensions 2 cm × 2 cm. The skin

samples were placed in 60°C distilled water for 1 min after 3 hours of soaking of the cut pieces at 4°C in phosphate buffered saline (PBS). Finally, the epidermal layer was peeled.

2. Franz Diffusion Cell

Franz diffusion cells were used to investigate the permeability of the skin. Figure 1 illustrates the procedure of the treatment. The skin samples were used as a membrane between the receptor and the donor compartment. The diffusion area was equal to 1.65 cm^2. The donor compartment was filled with 1 ml of adenosine solution dissolved in water (or DMSO) at a concentration of 3 mg/ml or 125 mg/ml in DMSO. The receptor compartment with a volume equal to 10 ml was filled with a solution of PBS and ethanol at a ratio of 70:30 (vol/vol). The Franz cell was kept in a water bath at 37°C with constant stirring.

a. Adenosine

Adenosine is an organic compound consisting of an adenine attached to a ribose. Adenosine has a molecular weight of 267 Da ($g \cdot mol^{-1}$) and a molecular formula of

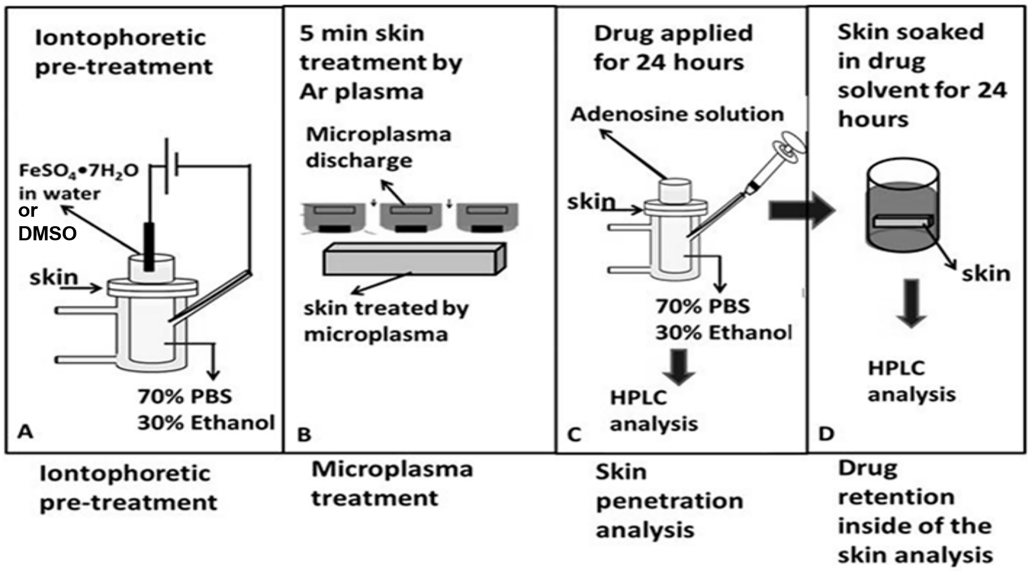

FIG. 1: Treatment procedure and analysis for the penetration of adenosine through the skin and the retention inside the skin with the following steps. (A) Iontophoretic pre-treatment by FeSO4·7H2O, (B) microplasma treatment, (C) drug application and penetration analysis, and (D) elution of the drug from the skin and the retention analysis.

$C_{10}H_{13}N_5O_4$. It is used to identify and convert heart rhythm. Adenosine was ordered from Fujifilm Wako Pure Chemical Corporation.

b. HPLC

The amount of the penetrated adenosine through the skin was measured using a High-Performance Liquid Chromatograph (HPLC LC-2010AHT, Shimadzu). The mobile phase consisted of acetonitrile and water with a ratio 10:90. The flow rate was set to 0.8 ml/min. An Inertsil ODS-SP 5 μm column with dimensions of 150 × 4.6 mm was used as a stationary phase. The column temperature was set to 25°C. Adenosine was detected at 260 nm with a retention time of 3.5 min.

3. Frozen Skin Section Study

a. Frozen Section

The full thickness of the skin sample without the fat layer were cut down to smaller pieces (approximately 20 mm × 20 mm). After the skin treatment, the skin was inserted into the Franz diffusion cell. Receptor compartment was filled by PBS and 1ml of 1% eosin Y solution was applied to donor compartment. After 24 hours (or 3 hours in some experiments), the skin was removed and washed under water flow. The skin was cut to pieces of 5 mm wide and frozen in water at –80°C for microscopic observation (skin sectioning). A frozen section of 10 μm thick were done by a microtome (REM-710, Yamato). Sectioned skin was placed on microslide glass for microscopic observation. Frozen section for FE-SEM and EDS observation were placed on copper sheet and deposited by 1 nm thick gold layer. Samples were observed FE-SEM (JSM-7001F, Jeol).

b. Eosin Y

Eosin Y is a pink dye commonly used in histology. Eosin Y has a molecular weight of 647.9 Da ($g \cdot mol^{-1}$) and a molecular formula of $C_{20}H_8Br_4O_5$. Pink color allows us to observe depth of penetration in the skin section. Eosin Y contains Bromine that is not naturally observed inside the skin and this allows us to also use FE-SEM/EDS observation to for estimation of depth of penetration. The 1% of eosin Y solution was ordered from Muto Pure Chemical Corporation.

4. Skin Treatment

Our procedure of the skin treatment composed of four steps graphically are described by Fig. 1. The procedures of the treatments were as follows:

A. The prepared skin samples were pre-treated by the iontophoresis with $FeSO_4 \cdot 7H_2O$ in water or DMSO solution to incorporate the iron into the skin

(details below in a. Iontophoresis) and after the pre-treatment, the content of the receptor and the donor parts of Franz diffusion cell were disposed.

B. Excess liquid on the skin was removed by tissue paper. The prepared skin samples were treated by argon microplasma discharge for 5 minutes (details below in b. Microplasma treatment).

C. A new receptor solution was prepared. The skin was inserted into Franz diffusion cell and 3 mg/ml of adenosine in water or in DMSO (125 mg/ml in the case of DMSO solution, or 1 ml of eosin Y solution in the case of the skin used for the skin sectioning) was applied for 24 hours (or 3 hours respectively) into the donor solution. After 24 hours, the samples from the receptor part of the diffusion cell were analyzed by HPLC to determine the amount of the penetrated drug through the skin. Or the skin was prepared for the skin sectioning according section 2.2.1. in the case of the full thickness skin.

D. The skin samples from Franz diffusion cells were removed and put into distilled water for 10 minutes to remove the surface drug solution. After 10 minutes, excess water on the surface of the skin was removed using a tissue paper and one layer of stratum corneum was peeled off by one tape strip to remove residual drug on the surface of the skin. Afterwards, the skin was inserted in a solution of a PBS and ethanol for the elution of adenosine from the skin. The elution was analyzed by HPLC to determine the retention of adenosine by the skin.

The samples and methods of the skin treatment are denoted as follows:

Control samples (C): The procedure is characterized by the steps C and D in Fig. 1 and using 3 mg/ml of adenosine for donor receptor part.

Microplasma-treated samples (MP): Procedure is characterized by the steps in B, C, and D in Fig. 1.

Iontophoretically pretreated samples in $FeSO_4 \cdot 7H_2O$ (in water) followed by microplasma treatment and applying drug in water solution (IWMW): Procedure is characterized by the steps in A, B, C, and D of Figs. 1 and 3 mg/ml in water is used in donor part. Iontophoretic pre-treatment is done in 30 mg/ml of $FeSO_4 \cdot 7H_2O$ dissolved in water.

Iontophoretically pre-treated samples in $FeSO_4 \cdot 7H_2O$ (in water) followed by microplasma treatment and applying drug in DMSO solution (IWMD): Procedure is characterized by the steps in A, B, C, and D of Figs. 1 and 3 mg/ml or 125 mg/ml of adenosine in DMSO is used in donor part. Iontophoretic pre-treatment is done in 30 mg/ml of $FeSO_4 \cdot 7H_2O$ dissolved in water.

Iontophoretically pre-treated samples in $FeSO_4 \cdot 7H_2O$ (DMSO) followed by microplasma treatment and applying drug in DMSO solution (IDMD): Procedure is characterized by the steps in A, B, C, and D of Figs. 1 and 3 mg/ml of adenosine in DMSO is used in donor part. Iontophoretic pre-treatment is done in 30 mg/ml of $FeSO_4 \cdot 7H_2O$ dissolved in DMSO.

DMSO-treated sample (DMSO): Procedure is characterized by the steps in C and D of Figs. 1 and 3 mg/ml or 125 mg/ml of adenosine in DMSO is used in donor part.

a. Iontophoresis

The skin was pre-treated by $FeSO_4 \cdot 7H_2O$ in order to incorporate the iron inside the skin. The skin was inserted into Franz diffusion cell. The donor compartment was filled with 1 ml of a solution of $FeSO_4 \cdot 7H_2O$ dissolved in water or DMSO at a concentration of 30 mg/ml. The receptor compartment with a volume equal to 10 ml was filled with a PBS and ethanol at a ratio of 70:30 (vol/vol). A pulsed current (at a frequency of 10 kHz), 5.55 mA/cm^2 was applied for 40 min. Franz diffusion cell was kept in a room temperature during the treatment according to Fig. 2 (left) and with the current waveform displayed in Fig. 2 (right).

b. Microplasma Treatment

Dielectric barrier discharge was generated by a thin-film electrode according to experimental set-up in Fig. 3. Atmospheric argon microplasma was maintained at a voltage of 1.6 kV and a frequency of 5 kHz. A saw-shape function of the voltage was set by a function generator (Tektronix, AFG3102) and amplified by high voltage amplifier (Trek, model 5/80). The voltage and the current waveforms are depicted in Fig. 4. The flow of argon was set at 5 L/min by a flow meter (Yamato). The skin treatment lasted for 5 minutes. The distance between the electrode and the skin was 0.5–1 mm.

III. RESULTS AND DISCUSSION

A. Penetration Study of Adenosine

Figure 5A shows adenosine permeability through the treated skin when concentration of 3 mg/ml is used, and Fig. 5B shows a comparison of 3 mg/ml with 125 mg/ml of adenosine in donor compartment. Non-treated sample (C) allowed the permeation of 4.5 μg of adenosine. When microplasma treatment (MT) was applied on the skin, almost 38 μg of adenosine permeated through the skin. 15 μg of adenosine permeated after

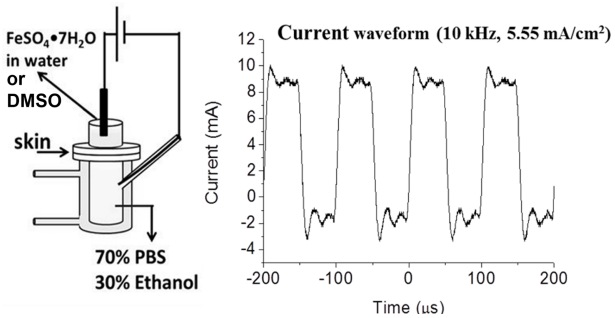

FIG. 2: (Left) Schematic description of iontophoretic pre-treatment of the skin. (Right) The current waveform of the iontophoresis at 10 kHz and the current density 5.55 mA/cm^2.

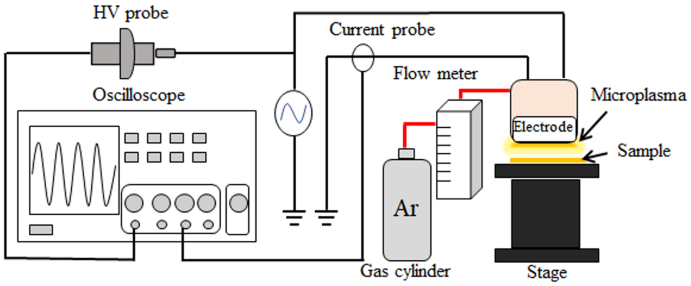

FIG. 3: Experimental setup of plasma treatment of the skin

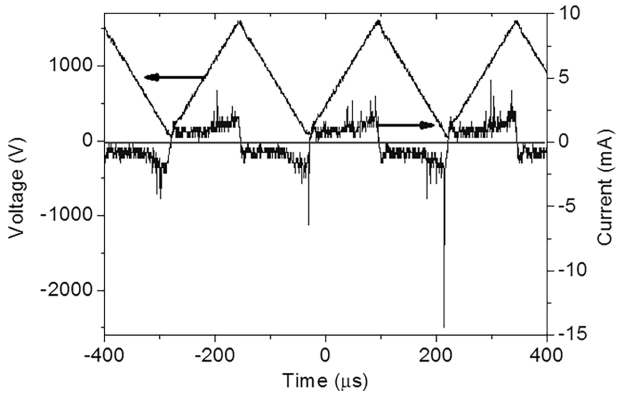

FIG. 4: Voltage and the current waveforms during the microplasma treatment

iontophoretic pre-treatment followed by microplasma treatment and applying adenosine water solution (IWMW). Permeability of the skin is possible to be improved by applying higher concentration of adenosine. However, solubility of adenosine in water is limited, so different solvent is needed. DMSO is one of such solvents. DMSO not only increase the solubility but moreover it can affect permeability in the skin. When 3 mg of adenosine in 1 ml of DMSO was applied on the skin, improvement of permeability was not observed. The same results were observed when skin was pre-treated by iontophoretic $FeSO_4 \cdot 7H_2O$ water solution followed by plasma treatment and applying adenosine in DMSO solution (IWMD) and when skin was pre-treated by iontophoresis $FeSO_4 \cdot 7H_2O$ DMSO solution followed by plasma treatment and application of adenosine in DMSO solution (IDMD).

However, when we increased the applied concentration to 125 mg/ml (approximately 40 times higher), amount of adenosine permeated through the skin increased by 4.25 times in the case of application with only DMSO (DMSO sample) and 12 times in the case of iontophoretic pre-treatment of the skin with $FeSO_4 \cdot 7H_2O$ water solution followed by plasma treatment and application of adenosine in DMSO solution – IWMD sample in Fig. 5B.

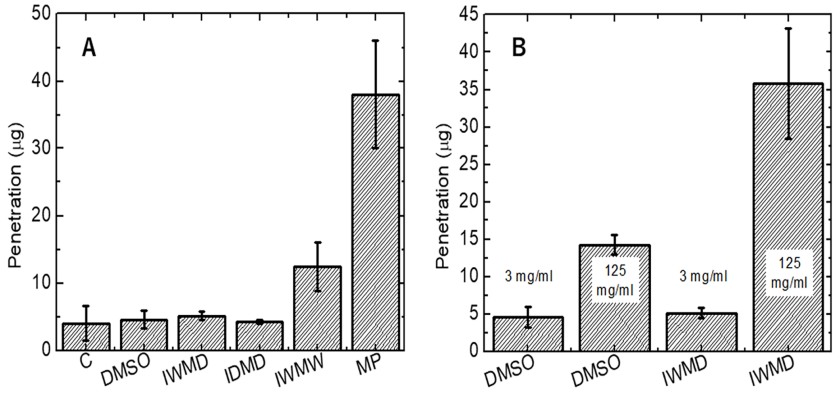

FIG. 5: (A) The amount of adenosine penetrated through skin of control sample (C), microplasma treated samples (MP), Iontophoretically pre-treated samples in FeSO4·7H2O (in water) followed by microplasma treatment and applying drug in water solution (IWMW), Iontophoretically pre-treated samples in FeSO4·7H2O (in water) followed by microplasma treatment and applying drug in DMSO solution (IWMD), Iontophoretically pre-treated samples in FeSO4·7H2O in DMSO followed by microplasma treatment and applying drug in DMSO solution (IDMD), DMSO treated sample (DMSO). (B) The amount of adenosine penetrated through skin when 3 mg/ml and 125 mg/ml of adenosine in DMSO was applied for chosen treatments.

A cell adhesion molecule called E-cadherin is essential for the appropriate intercellular junction formation[23] and it has crucial influence on the skin permeability.[19] Heartel et al.[24,25] observed the down-regulation of E-cadherin after air plasma treatment, suggesting that the products between the plasma and liquid inside the skin can play a key role in this down-regulation of E-cadherin. Difference between permeability of plasma treated skin and iontophoretically pre-treated skin indicates that plasma actively interacted with tight junctions (Fig. 5A, MP sample). This interaction is diminished in iontophoretically pre-treated sample (Fig. 5A, IWMW sample).

B. Retention Study of Adenosine

The amount of adenosine retained inside the treated skin is depicted in Fig. 6A when concentration of 3 mg/ml is used and comparison of 3 mg/ml with 125 mg/ml in Fig. 6B. Non-treated sample (C) retained 5 μg of adenosine inside of the skin and microplasma treated sample only 3 μg of adenosine. Based on previous study, we found out that the introduction of iron into the skin increase absorption of the drug. This finding was also confirmed in Fig. 6A. Iron from $FeSO_4 \cdot 7H_2O$ water solution was introduced into the skin by iontophoresis and followed by plasma treatment and the application of adenosine in water solution. The amount of adenosine retained after 24 hours was 20 μg. Drug applied with DMSO shows the retained amount of adenosine at 33 μg. Pre-treatment of the skin by $FeSO_4 \cdot 7H_2O$ iontophoresis in DMSO (sample IDMD) or in water (sample IWMD) retained less drug in comparison with DMSO treatment itself.

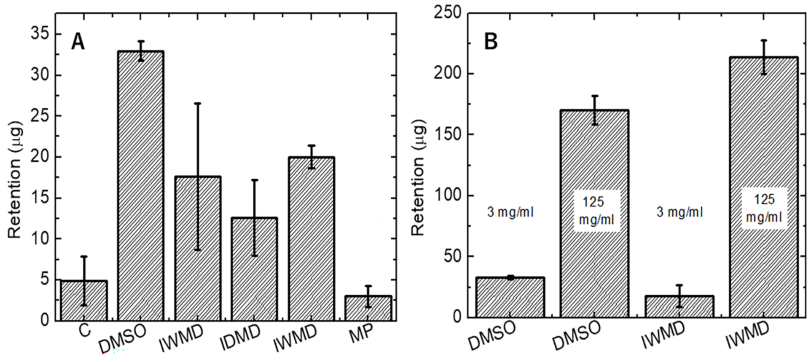

FIG. 6: (A) The amount of adenosine retained inside the skin of control sample (C), microplasma treated samples (MP), Iontophoretically pre-treated samples in FeSO4·7H2O (in water) followed by microplasma treatment and applying drug in water solution (IWMW), Iontophoretically pre-treated samples in FeSO4·7H2O (in water) followed by microplasma treatment and applying drug in DMSO solution (IWMD), Iontophoretically pre-treated samples in FeSO4·7H2O in DMSO followed by microplasma treatment and applying drug in DMSO solution (IDMD), DMSO treated sample (DMSO). (B) The amount of adenosine retained inside the skin when 3 mg/ml and 125 mg/ml of adenosine in DMSO was applied for chosen treatments.

Increased amount of applied adenosine to 125 mg/ml (approximately 40 times higher) caused the amount of adenosine retained inside the skin increasing 56 times in the case of application with only DMSO and 71 times in the case of iontophoretic pre-treatment of the skin with $FeSO_4 \cdot 7H_2O$ water solution followed by plasma treatment and the application of adenosine in DMSO solution (sample IWMD).

DMSO can change a gel phase structures of the skin ceramides inside stratum corneum to the more permeable liquid-crystalline phase. This behavior is induced by an accumulation of its molecules at the head group region of the ceramides. DMSO is competing with water molecules in creating hydrogen bonds with the ceramides.[26] At higher concentrations, DMSO can induce the formation of a "water pores" in the cell membrane,[27] and this phenomenon might not be reversible.[28] Using high concentration of DMSO in our study improved the adenosine retention inside the skin (Fig. 6), but not the permeation through the skin (Fig. 5). Oxidation of lipids in lipid membrane can cause degradation of ordering properties of lipid membrane.[29] Boonoy et al.[30] showed that a high formation of the aldehyde concentrations leads to unstable pores, evolving into micelles. Oxidized lipids can cause reduction of lipid bilayer,[31] and the oxidized skin lipids can make the permeation of another reactive oxygen species easier to permeate along the lipid bilayer.[32] It means that oxidation of lipids causes very similar effect as DMSO on lipid behavior (i.e. creating pores) but the combination of DMSO and supposed lipid oxidation caused by plasma after incorporation of iron inside the skin does not cause improvement of permeability or drug absorption by the skin but on the contrary diminishing effect (Fig. 6A). The reason can be these two processes competed to each other. However, the effect seemed to be a little different when a high concentration

of adenosine is used (125 mg/ml) (Figs. 5B and 6B). The experimental results show that DMSO and iontophoretic pre-treatment followed by plasma treatment make permeable the stratum corneum but they are not so effective in overcoming tight junctions in stratum granulosum.

C. Microscopic Observation

The amount of adenosine retained inside the skin from section 3.2 did not demonstrate any information about depth of penetration of the drug and did not allow us to analyze the reason of the effectivity or ineffectiveness of the skin treatment. Microscopic observation of dye in the skin section allowed us to analyze the treatments further. Eosin Y dye was used as a model drug after specific skin treatment. Figure 7A represents the skin section of non-treated sample, when only 1% eosin Y solution was applied for 24 hours. Microplasma-treated sample followed by application of eosin Y is depicted in Fig. 7B. Both figures demonstrate similar intensity of the dye presenting only in stratum corneum. Iontophoretic pre-treatment with followed by microplasma treatment and

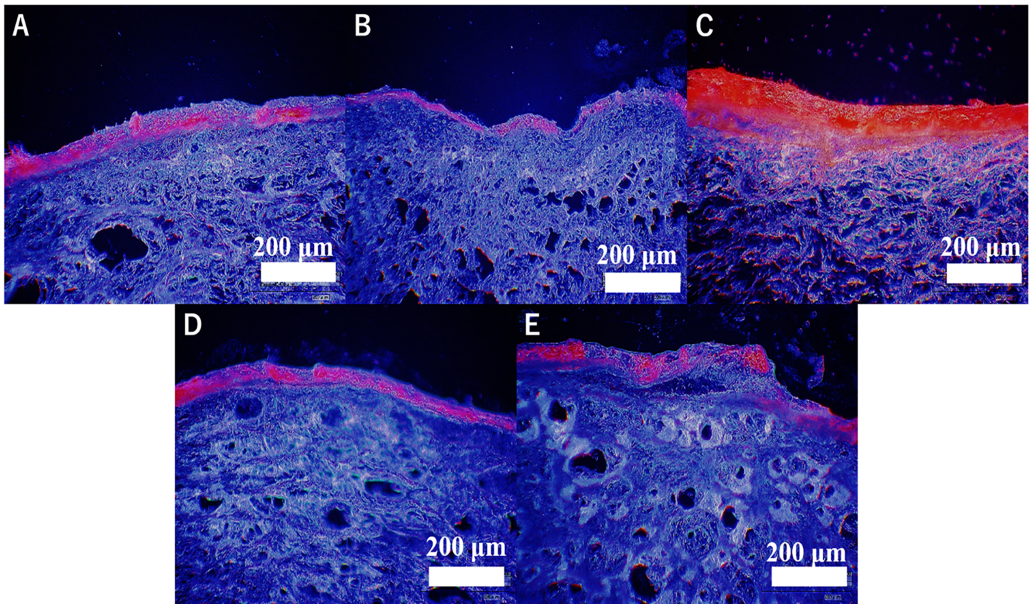

FIG. 7: (A) The skin section of non-treated sample, when only 1% eosin Y solution was applied for 24 hours. (B) Microplasma treated sample followed by the application of eosin Y for 24 hours. (C) Iontophoretically pre-treated samples in $FeSO_4 \cdot 7H_2O$ (in water) followed by microplasma treatment and the application of eosin Y for 24 hours. (D) Microplasma treated sample followed by the application of eosin Y for 3 hours. (E) Iontophoretically pre-treated samples in $FeSO_4 \cdot 7H_2O$ (in water) followed by microplasma treatment and the application of eosin Y for 3 hours. The natural color of samples was changed to increase the contrast of the photos.

the application of eosin Y solution in Fig. 7C shows stratum corneum was completely filled by eosin Y with strong red-orange color partially reaching areas under the stratum corneum. These results correspond with Fig. 6A where retention of adenosine was also highest after iontophoretic pre-treatment of those three types of treatments.

Figure 7D represents a skin section of the plasma treated skin, 3 hours after the application of eosin Y solution and, Fig. 7E shows iontophoretically pre-treated skin followed by microplasma treatment, 3 hours after the application of eosin Y. Intensity of the eosin Y was also present only in stratum corneum with comparable intensity in Fig. 7D and 7E. This observation indicates that high accumulation of the eosin Y in Fig. 7C occurred after 3 hours of application.

The skin is composed of two main barriers. There are stratum corneum and tight junctions present in stratum granulosum. To increase the skin permeability, both barriers have to be effectively overcome. Previous research demonstrated that tight junctions are "opened" temporally for approximately three hours.[16,20] This means that after 3 hours, when tight junctions are closed, the applied drug should be accumulated in stratum corneum. This was observed in the case of iontophoretically pre-treated sample in Fig. 7C. Non-treated skin (Fig. 7A) and plasma treated skin (Fig. 7B) displays lower amount of eosin Y indicating lower permeability of the stratum corneum in comparison with Fig. 7C (iontophoretically pre-treated skin). Plasma can cause disturbance of lipids in stratum corneum, indicated by FTIR measurements in Yahaya et al.[33] and Kristof et al.[34] This disturbance delayed up to 2 days but this did not play a significant role as it is seen in Fig. 7A and 7B. No presence of eosin Y under the stratum corneum can be explained by higher flow in this area and distribution in large volume. Stratum corneum is several micrometers thick, while layers under the stratum corneum can measure up to 1–2 mm.

D. SEM and EDS Observation

FE-SEM with EDS allowed us to verify measurements from optical microscope by observing concentration of Bromine (from eosin Y) and also distribution of iron to estimate possible depth of supposed lipid oxidation inside the skin.

The stratum corneum is shown as a bright part of the skin section in Fig. 8. The skin section was divided into 8 parts, 10 μm wide that were used for the detection of Br and Fe elements. The concentration of Fe and Br was normalized to concentration of deposed Au to minimize the effects of shadows or roughness of the sectioned surface.

Figure 8A depicts distribution of Fe of non-treated skin sample (blue or dark circles) and microplasma treated skin (white circles) with comparable and constant concertation of Fe up to 80 μm from the skin surface. In the case of iontophoretic pre-treatment followed by microplasma treatment (Fig. 8A, red or light circles), concentration of Fe is significantly higher and this concentration is decreasing from stratum corneum to deeper layers of the skin.

The concentration of Br, indicating presence of eosin Y (Fig. 8B) inside the skin, displays the highest concentration of Br (eosin Y) in iontophoretically pre-treated skin followed with plasma treatment. Br is accumulated with stratum corneum with partially

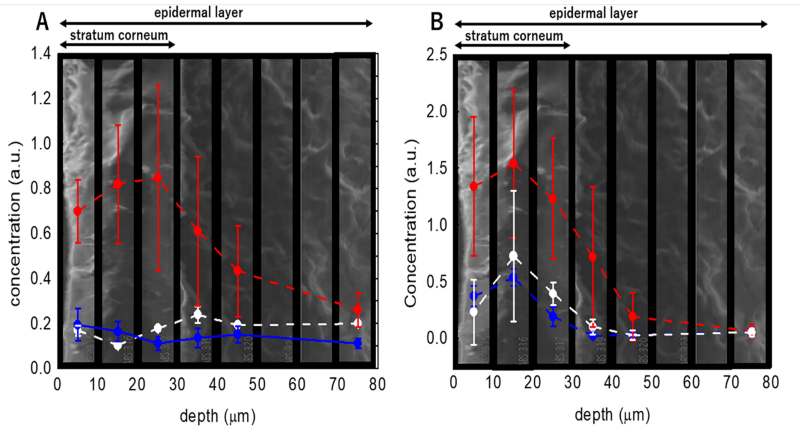

FIG. 8: (A) Distribution of Fe of non-treated sample (blue or dark circles), microplasma treated skin (white circles) and iontophoretic pre-treated skin by followed by microplasma treatment (red or light circles). Left side of the skin section shows top of the skin - stratum corneum 30 μm thick. The background is FE-SEM photograph of the skin section. (B) Distribution of Br of non-treated sample (blue or dark circles), microplasma treated skin (white circles) and iontophoretic pre-treated skin by followed by microplasma treatment (red or light circles). Left side of the skin section shows top of the skin - stratum corneum 30 μm thick. The background is FE-SEM photograph of the skin section, eosin Y containing Br was applied for 24 hours.

overlapping stratum granulosum under stratum corneum. Plasma treated skin and non-treated skin indicated accumulation of eosin Y in stratum corneum with similar concentration. These results agree with section of section C and section of B.

IV. CONCLUSION

The effect of plasma treatment of the skin with previously incorporated iron by iontophoresis was investigated. It was supposed that iron would mediate and enhance lipid oxidation in stratum corneum and it would increase the permeability of stratum corneum. DMSO as an alternative solvent to water was evaluated. The skin was the most permeable for adenosine after plasma treatment and its permeability was lower with iontophoretically pre-treatment by $FeSO_4$. When DMSO was used, permeability of the skin was as low as in non-treated skin. Using DMSO or iontophoretic pre-treatment of the skin by $FeSO_4$, adenosine is accumulating inside the skin. DMSO and iontophoretic pre-treatment of the skin by $FeSO_4$ can cause similar effect to the skin (water pores) but when they are used together, their effect is not superimposed but diminished at lower concentrations of applied drug. When higher concentrations of adenosine were used (125 mg/ml in DMSO), retention and penetration of iontophoretically pre-treatment by $FeSO_4$ (in water) following plasma treatment was superior to simple adenosine dissolved in DMSO. Using eosin Y demonstrated where the drugs can be accumulated after the skin treatment. Microscopic observations

were confirmed by FE-SEM/EDS measurements. Iontophoretic pre-treatment induced more accumulation of eosin Y in stratum corneum than the non-treated and the plasma treated skin. Iron concentration in the skin corresponded to concentration of eosin Y in this case. Accumulating effect of eosin Y can be explained by increasing permeability of the stratum corneum by iontophoretic pre-treatment followed by plasma treatment but with difficulties to permeate through tight junctions in stratum granulosum.

REFERENCES

1. Yamauchi Y, Kuzuya M, Sasai Y, Kondo S. A new drug delivery system using plasma-irradiated polysaccharide. J Photopolym Sci Technol. 2014;27:389–92.
2. Petlin DG, Anissimov YG, Tverdokhlebov SI. Plasma treatment as an efficient tool for controlled drug release from polymeric materials: A review. J Control Release. 2017;266:57–74.
3. Zahedi L, Beigi P G, Shafiee M, Zare F, Mahdikia H, Abdouss M, Abdollahifar MA, Shokri B. Development of plasma functionalized polypropylene wound dressing for betaine hydrochloride controlled drug delivery on diabetic wounds. Sci Rep. 2021;11:9641.
4. Pefani-Antimisiari K, Athanasopoulos DK, Marazioti A, Sklias K, Rodi M, de Lastic AL, Mouzaki A, Svarnas P, Antimisiaris SG. Synergistic effect of cold atmospheric pressure plasma and free or liposomal doxorubicin on melanoma cells. Sci Rep. 2021;11:14788.
5. Xin XW, Hamblin MR, Yue XY. Applications of cold atmospheric plasma for transdermal drug delivery: A review. Drug Deliv Transl Res. 2021;11:741–7
6. Vijayarangan V, Delalande A, Dozias S, Pouvesle JM, Robert E, Pichon C. New insights on molecular internalization and drug delivery following plasma jet exposures. Int J Pharm. 2020;589:119874.
7. Shimizu K, Kristof J. Microplasma drug delivery. In: Tutar Y, Tutar L, editors. Plasma medicine: Concepts and clinical applications. London: IntechOpen; 2018. p. 101–20.
8. Shimizu K, Kristof J. Enhancement of percutaneous absorption on skin by plasma drug delivery method. In: Maiti S, Sen KK, editors. Advanced technology for delivering therapeutics. Rijeka: InTech; 2017. p. 111–36.
9. Gelker M, Müller-Goymann ChC, Viöl W. Permeabilization of human stratum corneum and full-thickness skin samples by a direct dielectric barrier discharge. Clin Plasma Med. 2018;9:34–40.
10. Gelker M, Müller-Goymann Ch C, Viöl W. Plasma permeabilization of human excised full-thickness skin by µs- and ns-pulsed DBD. Skin Pharmacol Physiol. 2020;33:69–76.
11. Kim ES, Enkhzaya G, Hwang HS, Han J, Kim Ch S, Shin JW, Yoon YR, Kim NY. Highly Efficient transfection effect of transdermal drug delivery via skin by hybrid bipolar arc plasma stimulation and dual pulse electroporation technique. IEEE Access. 2021;9:24071–8.
12. Chen G, Wen Z, Wen D, Wang Z, Li H, Zeng Y, Dotti G, Wirz RE, Gu Z. Transdermal cold atmospheric plasma-mediated immune checkpoint blockade therapy. Proc Natl Acad Sci U S A. 2020;117:3687–92.
13. Xin Y, Wen X, Jiang X. Analgesic effect of topical lidocaine is enhanced by cold atmospheric plasma pretreatment in facial CO_2 laser treatments. J Cosmet Dermatol. 2021;20:2794–9.
14. Shimizu K, Tran AN, Kristof J, Blajan M. Investigation of atmospheric microplasma for improving skin permeability. Proceedings of the 2016 Electrostatics Joint Conference. 2016 Jun [cited 2021 Nov 17]. p. 9. Available from: www.electrostatics.org/images/I4.pdf.
15. Lv Y, Nie L, Duan J, Li Z, Lu X. Cold atmospheric plasma jet array for transdermal drug delivery. Plasma Process Polym. 2020;18:2000180.
16. Kristof J, Miyamoto H, Tran AN, Blajan M, Shimizu K. Feasibility of transdermal delivery of Cyclosporine A using plasma discharges. Biointerphases. 2017;12:02B40.
17. Connolly RJ, Lopez GA, Hoff AM, Jaroszeski MJ. Plasma facilitated delivery of DNA to skin. Biotechnol Bioeng. 2009;104:1034–40.

18. He T, Liu D, Xia W, Chen Z, Xu D, Rong M, Kong MG. Stimulation of lidocaine penetration into model tissues by an argon plasma jet. J Phys D Appl Phys. 2019;52:465202.
19. Lee HY, Choi JH, Hong JW, Kim GCh, Lee HJ. Comparative study of the Ar and He atmospheric pressure plasmas on E-cadherin protein regulation for plasma-mediated transdermal drug delivery. J Phys D Appl Phys. 2018;51:215401.
20. Choi JH, Nam SH, Song YS, Lee HW, Lee HJ, Song K, Hong JW, Kim GCh. Treatment with low-temperature atmospheric pressure plasma enhances cutaneous delivery of epidermal growth factor by regulating E-cadherin-mediated cell junctions. Arch Dermatol Res. 2014;306:635–43.
21. Lee S, Choi J, Kim J, Jang Y, Lim TH. Atmospheric pressure plasma irradiation facilitates transdermal permeability of aniline blue on porcine skin and the cellular permeability of keratinocytes with the production of nitric oxide. Appl Sci. 2021;11:2390.
22. Kristof J, Yokoyama R, Mustafa F, Yahaya AG, Blajan M, Shimizu K. Transdermal drug delivery of Adenosine by microplasma with iontophoresis and DMSO. J Biomed Syst Emerg Technol. 2021;S1:006.
23. Tunggal JA, Helfrich I, Schmitz A, Schwarz H, Günzel D, Fromm M, Kemler R, Krieg T, Niessen CM. E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. EMBO J. 2005;24:1146–56.
24. Haertel B, Hähnel M, Blackert S, Wende K, von Woedtke T, Lindequist U. Surface molecules on HaCaT keratinocytes after interaction with non-thermal atmospheric pressure plasma. Cell Biol Int. 2012;36:1217–22.
25. Haertel B, Wende K, von Woedtke T, Weltmann KD, Lindequist U. Non-thermal atmospheric-pressure plasma can influence cell adhesion molecules on HaCaT-keratinocytes. Exp Dermatol. 2011;20:282–4.
26. Notman R, den Otter W, Noro M, Briels WJ, Anwar J. The permeability enhancing mechanism of DMSO in ceramide bilayers simulated by molecular dynamics. Biophys J. 2007;93:2056–68.
27. Marren K. Dimethyl sulfoxide: An effective penetration enhancer for topical administration of NSAIDs. Phys Sportsmed. 2011;39(3):75–82.
28. Al-Saidan SMH, Selkirk AB, Winfield A J. Effect of dimethyl sulfoxide concentration on the permeability of neonatal rat stratum corneum to alkanols. J Invest Dermatol. 1987;89:426–9.
29. Neto JP, Cordeiro RM. Molecular simulations of the effects of phospholipid and cholesterol peroxidation on lipid membrane properties. Biochim Biophys Acta Biomembr. 2016;1858:2191–8.
30. Boonnoy P, Jarerattanachat V, Karttunen M, Wong-Ekkabut J. Bilayer deformation, pores, and micellation induced by oxidized lipids. J Phys Chem Lett. 2015;6:4884–8.
31. Mason RP, Walter MF, Mason PE. Effect of oxidative stress on membrane structure: Small-angle X-ray diffraction analysis. Free Radic Biol Med. 1997;23:419–25.
32. Yadav DK, Kumar S, Choi E-H, Chaudhary S, Kim M-H. Molecular dynamic simulations of oxidized skin lipid bilayer and permeability of reactive oxygen species. Sci Rep. 2019;9:4496.
33. Yahaya AG, Kristof J, Mustafa F, Blajan M, Shimizu K. Effect of plasma discharge on structure of epidermal layer of pig skin. Plasma Med. 2021;11:1–13.
34. Kristof J, Tran AN, Blajan MG, Shimizu K. A Study of the influence of plasma particles for transdermal drug delivery. Adv Intell Syst Comput. 2016;519:167–73.