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Transdermal Delivery of Iron Using Soluble Microneedles: Dermal Kinetics and Safety

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1. Introduction

Transdermal delivery of iron with the help of various bio-physical enhancement techniques was reported earlier [1, 2]. Electrically mediated techniques such as iontophoresis and electroporation were able to enhance the transdermal delivery of Ferric pyrophosphate (a valuable transdermal iron source) significantly over the passive delivery. Microporation of the skin, *i.e.* pretreatment with microneedles was found to enhance the efficiency of iontophoresis even more in case of transdermal iron delivery [3]. Soluble microneedle system is an upcoming promising area of dermal/transdermal delivery of drugs. Soluble microneedle system consists of an array of microneedles prepared using water soluble polymers loaded with the therapeutic agent. The therapeutic agent would be released into the skin tissue fluids upon penetration of the microneedles. Development of a self-administrable dissolvable microneedle array system for delivery of iron can offer great advantages over oral and parenteral modes of administration. In the current study, soluble microneedles incorporated with Ferric pyrophosphate (FPP) was developed to deliver iron to meet higher iron demand in case of moderate to severe anemic conditions. Higher doses requirement could be met by increasing the area of patch, increasing the drug load in the microneedles and by increasing the frequency of administration. Ferric pyrophosphate (FPP) was incorporated into soluble microneedle array and the microneedle system was used to investigate the dermal kinetics of FPP in hairless rat model. The safety of administered iron compound was evaluated using human dermal fibroblast (HDF) cell lines. Cell viability studies and Reactive oxygen species assay was performed to assess the safety of FPP at different concentrations.

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2. Materials and Methods

2.1. Materials

Soluble Ferric pyrophosphate was obtained from Sigma-Aldrich Inc (St. Louis, MO). Phosphate buffered saline (PBS, pH 7.4) premixed powder was obtained from EMD Chemicals (Gibbstown, NJ). Ferrover[®] iron was purchased from Hach Company (Loveland, OH). Serum iron & TIBC kit was procured from Clinia Corporation (San Marcos, CA). All other chemicals and solvents used were of pure grade obtained from Fisher scientific (Fairway, NJ).

2.2. Methods

2.2.1. Preparation of Soluble Microneedles—Soluble microneedles containing FPP were prepared using water soluble polymers. Aqueous blends containing 15% w/w poly (methylvinylether/maleic acid (PMVE/MA) (Gantrez[®] AN-139, Ashland, Kidderminster, UK) and 143 mg of FPP/g were prepared and used to fabricate microneedle array using laser engineered silicone micromold templates. The array was composed of 121 (11x11) needles per 0.5 cm² perpendicular to the base. 300 mg of the drug and polymer mixture was added into the laser-engineered silicon microneedle molds and subjected to centrifugation at 550 x g for 15 min followed by drying at room temperature for 48 h. Upon removal from molds, the sidewalls of the microneedle arrays were removed using a heated scalpel blade. An accurate measurement of the final percentage content of active compound in the microneedles was determined based on mass loss calculations following water evaporation from the array.

2.2.2. Fate of microneedles in the skin—The fate of microneedles was assessed after insertion into the skin tissue by Scanning Electron Microscopy (SEM). Microneedle array was inserted into rat skin with mild force using thumb. The array was removed carefully after 1 h and 3 h and SEM pictures were obtained. For SEM, the microneedle array was fixed on aluminum stubs using glued carbon tapes and coated with gold using Hummer 6.2 sputter coater (Anatech USA, Union City, California). The sputter coating chamber was supplied with argon gas throughout the coating process. Photomicrographs of the microneedle array were acquired using a model JSM-5600 scanning electron microscopy (JEOL Ltd., Tokyo, Japan).

2.2.3. *In vivo* cutaneous microdialysis studies

a. Implantation of Microdialysis probe: All animal studies were approved by the Institutional Animal Care and Use committee (IACUC) at the University of Mississippi (Protocol # 11-016). Linear microdialysis probe (BASi, West Lafayette IN) with 5 mm length and 30 kDa cut-off molecular weight was used to perform dermal microdialysis studies. A 30G needle was inserted intradermally parallel to the *stratum corneum* surface, through a distance of 1 cm. The microdialysis probe was inserted through this needle and the needle was withdrawn leaving the probe implanted in the dermal tissue. The inlet tube was connected to an injection pump (BASi, West Lafayette, IN) and the outlet was placed in a sample collection vial.

b. Recovery of microdialysis probe: Microdialysis Probe recovery study was performed *in vivo* using retrodialysis method. A flow rate of 2 $\mu\text{L}/\text{min}$ was chosen for the entire study. Microdialysis probe was equilibrated with PBS (pH-5) for 30 minutes after implantation of probe and later, known concentration of drug was perfused and dialysate was collected at different time points [4]. The recovery was calculated using the following formula:

$$\text{Recovery}(\%) = 100 - \left(\frac{\text{concentration of dialysate}}{\text{concentration of Perfusate}} \times 100 \right)$$

c. Dermal kinetics of FPP: After implantation of the probe, the buffer was perfused for 30 minutes to equilibrate the probe with skin tissue fluid. The microneedle array was applied on the rat abdominal skin exactly at the site the probe was implanted, by firmly pressing it against the rat skin using thumb and the array was secured with the help of a surgical tape. Dialysate fluid was collected every hour and the array was removed after 3 hours and microdialysis sampling was continued up until 10 hours. The microdialysis samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS).

2.2.5. Evaluation of safety and toxicity of FPP in human skin cell lines

a. Cell culture: The safety studies were carried out using Human Skin Fibroblast (HDF) [CCD1093Sk (ATCC[®] CRL2115TM)] cell lines [ATCC, Manassas, VA]. HDF cells were grown in Eagle's Minimum Essential Medium (EMEM) (ATCC-302003) with 10% FBS in cell culture flasks (75 cm^2) to approximately 80% confluence in a 37°C, humidified 5% CO_2 incubator. The cell media was supplemented with penicillin (10000 units) and streptomycin (10 mg/mL) solution. The cells were seeded into clear/black wall 96-well microplates at a density of 200,000 cells/ml (200 μL) and cell proliferation/viability assay and reactive oxygen species (ROS) measurement assay were performed. Cell count was obtained with Bio-Rad automatic cell counter (Bio-Rad, Hercules, CA) after staining a 10 μL aliquot of cell suspension with 10 μL of Trypan blue stain (0.4 %) [Life Technologies, Grand Island, NY]. The passage number 6 was used for HDF cell lines in all the experiments.

b. Cell Viability Assay: Cell proliferation assay was carried out using the CellTiter 96[®] AQueous one solution reagent (Promega, Madison, WI) which can be used to measure the number of viable cells by colorimetric method. After reaching approximately 80% confluence of the cells in microplates, the media in all the wells were replaced with 100 μL of fresh medium (without serum and penicillin streptomycin solution) cell lines were exposed to 100 μL of either basal medium (control) or serial dilutions of FPP prepared at different concentrations (31.25, 15.62 and 7.81 mg/ml) or digitonin (positive control) [Promega, Madison, WI] prepared at different concentrations (60 $\mu\text{g}/\text{ml}$ –1.87 $\mu\text{g}/\text{ml}$) with serial dilutions for 24 hours. FPP solutions were prepared using basal medium and digitonin solutions were prepared from a stock of 20 mg/ml using cell culture grade dimethylsulfoxide (DMSO) [ATCC, Manassas, VA]. Untreated cells served as control in this study. The CellTiter 96[®] AQueous one solution reagent was completely thawed in water bath at 37°C for 10 minutes before use and 40 μL of the this reagent was added to each well in the 96 well plate containing the cells and the plate was incubated at 37 °C in a humidified, 5 % CO_2 atmosphere for 4 hours. After incubation the absorbance at 490 nm was recorded using

microplate Reader (SpectraMax[®] M5, Molecular Devices, LLC, Sunnyvale, CA). Testing at each concentration of the FPP and positive standard was performed in triplicates.

c. Measurement of Reactive Oxygen Species (ROS) activity/oxidative stress markers:

Possible generation of reactive oxygen species (ROS) and the intracellular ROS activity upon treating both HEK and HDF cell lines with FPP at different concentrations was measured using Oxiselect[™] intracellular ROS assay Kit (Cell Biolabs, San Diego, CA). After reaching approximately 80% confluence in 96-well microplates, both the cell lines was treated with cell permeable fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Cells loaded with DCFH-DA were washed gently with D-PBS (Sigma Aldrich, St. Louis, MO) 2–3 times and 100 μ L of fresh medium was added to all the wells and treated with 100 μ L of FPP prepared at different concentrations (31.25, 15.62 and 7.81 mg/ml) or H₂O₂ (positive control), prepared at different concentrations (250–62.5 μ M) for 24 h. Later cells were washed 2–3 times again with D-PBS and 100 μ L of fresh medium and 100 μ L of 2X cell lysis buffer was added. After 5 min incubation, 150 μ L of this medium was transferred to a black wall 96-well plate and fluorescence was measured. Untreated cells served as control in this study. All the studies were performed in triplicate.

3. Results and discussion

3.1. Morphology of soluble microneedles

Soluble microneedle array incorporating FPP (with 43 mg drug load) was prepared successfully using aqueous blends of 15% w/w poly (methylvinylether/maelic acid) (PMVE/MA), as described by Donnelly *et al* (2011) previously [5]. The polymer has a long history of safe use in denture adhesives, toothpastes and topical products. It is an excellent film-former and yields microneedles that are hard, but possess inherent flexibility. This means that the baseplate can conform to the surface contours of the skin, yet the microneedles efficiently penetrate the *stratum corneum*.

The final weight of the array was 36.2 ± 6.7 mg and the amount of FPP present in the actual needles of the 0.5 cm² array were determined to be ~600 μ g. Morphology of the soluble microneedle array was investigated using SEM and the prepared microneedles have an average height $\sim 540 \pm 50$ μ m with an average base radius of 250 ± 5 μ m and an average tip radius of 25 ± 5 μ m (Fig 1).

3.2. Fate of microneedles in the skin

The time required for disappearance of microneedles in the array was visualized with the help of SEM images (Fig 2). The arrays appear to start dissolving immediately after it was inserted into the skin and by the end of one hour, there was a significant deformity observed due to loss of content from the needles. The microneedles were found to completely dissolve and disappear in less than 3 hours delivering all the FPP loaded in the needles.

3.3. Dermal Kinetics of FPP

Microdialysis is a minimally invasive procedure useful for sampling water soluble small molecules from the biological tissues. Dermal microdialysis allows continuous sampling of

unbound therapeutic agent present in the dermal extracellular fluid. The recovery of contents by the probe depends on the nature of dialysis fluid, flow rate and dimensions of the probe. The retrodialysis method in the present work showed that the recovery of FPP by microdialysis probe in the cutaneous tissue was ~58%.

The dermal concentration-time profile of FPP followed a typical absorption elimination pattern like small drug molecules. The slow increase in the concentration of FPP in the dermal fluid indicates that the microneedle array are dissolving slowly over the duration of 3–4 hours, as observed in the previous study discussed in section 3.2. The time course of dermal concentration of unbound FPP would be a function of multiple biochemical and physiological process kinetics.

Previously several researchers have investigated the kinetics and mechanisms of elimination of iron from the dermal tissue [6–12]. Different iron salts were administered into the skin (intraepidermal/dermal compartments) and the disappearance of iron in the tissue was followed up. The amounts reported in most of the previous studies was the total iron content which includes the bound and unbound iron, whereas in the present study, figure 3 represents the kinetics of free or unbound iron only in the interstitial compartment where microneedles were administered.

Based on the previous reports by different investigators, the kinetics of intradermally administered iron could be schematically represented as in figure 3. Iron delivered intradermally binds to the available binding sites of transferrin molecules in the skin fluid (K_1). The binding could be instantaneous and absolutely depends on the amount of transferrin and its % saturation, leaving behind a pool of free iron. The free iron would be cleared by the systemic circulation (K_2) and the rate of clearance of free iron is predominantly a function of blood flow rate, the amount of free iron. The iron that enters the blood pool would be processed via a sequence of biochemical steps which are well investigated and reported in the literature. The Transferrin bound iron in the skin would generally undergo two major pathways of clearance. Most of the transferrin bound iron in the skin will be cleared into the lymphatic system (K_3) with a small fraction entering into the skin cells (K_5). A part of the transferrin bound iron could also enter the systemic pool directly (K_4). The iron that enters the skin cells, would serve as reserve with minute fractions being eventually released back into the extracellular compartment. It is likely that that the slowest phase of iron clearance from skin would be from the skin cells i.e. the rate of loss of intracellular iron to the interstitial fluid (K_6).

Beamish et al performed a systematic study to measure iron clearance from the skin [7]. ^{59}Fe -Ferrous citrate used as iron source was injected into sub-epidermal tissue of the volar aspect of the forearm to healthy volunteers and iron deficient subjects. In normal subjects, ^{59}Fe activity at the site of injection showed a rapid decline initially following a slow rate of disappearance thereafter. When the activity was plotted on a logarithmic scale, three exponential component were identified with an initial half-life about 30 minutes, second half-life of about one day (~19–29 hours) and a slow and third half-life about 60 (47–74) days. Cavill and Jacobs et al in 1971, reported that iron in the interstitial fluid of the skin was mainly cleared by lymphatic drainage. They also reported that a significant fraction

of iron bound to transferrin enters the skin cells and a relatively lesser amounts of intracellular iron was lost back to the interstitial fluid [11].

As mentioned earlier, in the present study, the time course of concentration free iron only was investigated in the dermal tissue. The C_{max} after administration of 0.6 mg of FPP was $10.60 \pm 1.77 \mu\text{g/ml}$. The AUC_{0-10} of FPP in the skin interstitial fluid was $70.21 \pm 7.92 \mu\text{g/ml.h}$. The downward time course of concentration followed a monoexponential trend with a dermal elimination constant K_d of $0.12 \pm 0.06 \text{ h}^{-1}$ which is essentially a function of K_1 and K_2 .

3.8. Safety and toxicity studies in cell lines

3.8.1. Cell viability Assay—The CellTiter 96® AQueous ‘one solution’ assay was improvised from previous CellTiter 96® AQueous Assay which contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. This MTS tetrazolium compound (Owen’s reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium [12]. Formazan is produced by an amount of NADPH or NADH dehydrogenase enzymes directly proportional to the number of living cells in culture as measured at 490 nm [13].

The decrease in the absorbance indicated a decrease in the mitochondrial activity due to cell death. FPP at 0.78 mg/ml level did not show a significantly different absorbance as compared to control (Fig 5). At higher concentrations of FPP, there was marginal decrease in the viability as compared to blank which could be due to increased osmotic function of the media. The levels of free FPP to which the HDF cells were exposed in this experiment and the duration of exposure was many fold higher in order than the levels and duration of exposure in vivo due to rapid binding of FPP with transferrin. Therefore, from the results, one can conclude that FPP as a safer source of iron for dermal delivery.

3.8.2. ROS Assay—Oxiselect™ intracellular ROS assay kit measures hydroxyl, peroxy, and other reactive oxygen species activity within a cell. Upon addition to cells, the non-fluorescent DCFH-DA permeates well into across the cell membrane and once inside the cell DCFH-DA was rapidly deacetylated by cellular esterases to 2', 7'-dichlorodihydrofluorescein (DCFH), which is also non-fluorescent in nature. DCFH will be rapidly oxidized to fluorescent 2', 7'-dichlorodihydrofluorescein (DCF) in presence of reactive oxygen species. The fluorescence intensity is proportional to the ROS levels within the cell cytosol. The amount of DCF generated is compared with the standard calibration curve obtained at different concentration of DCF, using relative fluorescence units (RFU). The DCF detection sensitivity limit of the kit is as low as 10 pM. Hydrogen peroxide was used as positive control at different concentrations as it generally crosses cell membranes readily, might be through the aquaporins in the cell [14]. Reactive oxygen species can cause oxidative stress at cellular level and oxidative stress can activate NF-3B signaling pathway, stress-activated kinases, and such activation could result in cell death by either apoptosis or necrosis [15]. From figure 6, the generation of reactive oxygen species as relative

fluorescence units after treating the cells with FPP was compared with standards (H₂O₂). Even at high concentrations of FPP, the amount of DCF generated as a measure of ROS was negligible ruling out any concern that the dermal administration of FPP would lead to free radical induced oxidative stress [16].

4. Conclusions

The ease of self-applicability and avoidance of gastrointestinal side effects are the greatest advantages with iron soluble microneedle system. In the present study, soluble microneedles for the delivery of FPP were successfully developed and evaluated. They were found to dissolve in the skin within a few hours (3–4 h). The safety and toxicity studies on cell lines proved that the amount of FPP loaded in microneedle array was safe and did not show any toxicity HDF cell lines. This study demonstrates the feasibility of transcutaneous iron replenishment therapy, a novel concept of treating iron deficiency and anemia.

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Figure 1. Microneedle array on the investigators finger. [Pictures were taken using Nikon Digital Camera (Nikon Inc., NY)] and SEM images of FPP soluble microneedle array containing 121 microneedles (18X magnification).

Figure 2. SEM Images of FPP soluble microneedle array (a) Microneedles before insertion into the skin (b) Microneedles inserted into rat skin and removed at 1 hour time point (c) Microneedles inserted into rat skin and removed after 3 hours (Dotted circles indicate the position of individual microneedles). All the mages were obtained at 75X magnification.

Figure 3.
The time course of free FPP concentration in the dermal interstitial fluid following the application of soluble microneedles ($n = 4 \pm \text{S.D.}$).

Figure 4.

Potential pathways of iron transport from the skin. K represents the rate process constant. Iron delivered intradermally binds to the available binding sites of transferrin molecules in the skin fluid (K_1). The free iron would be cleared by the systemic circulation (K_2). The Transferrin bound iron in the skin would generally undergo three major pathways of clearance. Most of the transferrin bound iron in the skin will be cleared into the lymphatic system (K_3) with a small fraction entering into the skin cells (K_5). A part of the transferrin bound iron could also enter the systemic pool directly (K_4). The transferrin bound iron that entered the intracellular compartment would reenter the interstitial fluid (K_6) at a relatively slower rate.

Figure 5. The mitochondrial activity (MTS activity) of HDF cells after 24 h exposure to 100 µl of FPP and digitonin. Results are combined from three independent exposures and expressed as mean ($n=3 \pm$ S.D.).

Figure 6. Induction of reactive oxygen species (ROS) by FPP or Hydrogen peroxide in HDF cells after 24 hours exposure. Results are combined from three independent exposures and expressed as mean ($n=3 \pm$ S.D.).