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Intestinal iontophoresis from mucoadhesive patches: a strategy for oral delivery

Amrita Banerjee[a], Renwei Chen[b], Shamsul Arafin[c], Samir Mitragotri[d,*]

[a] Department of Chemical Engineering, University of California, Santa Barbara, CA 93106, USA
[b] Center for Bioengineering, University of California, Santa Barbara, CA 93106, USA
[c] Department of Electrical and Computer Engineering, University of California, Santa Barbara, CA 93106, USA
[d] School of Engineering and Applied Sciences, Wyss Institute, Harvard University, Cambridge, MA 02138, USA

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ABSTRACT

Biologics have limited permeability across the intestine and are prone to degradation in the acidic-proteolytic milieu of the gastrointestinal tract, leading to poor oral bioavailability. Iontophoresis is a promising technology that can substantially improve transport of drugs across biological barriers and has been particularly explored for skin. In this study, we investigated whether iontophoresis across the intestine can be utilized to improve oral insulin transport. Application of electric current to intestinal cells resulted in opening of the tight junctions *in vitro* and a consequent about 3-fold improvement in paracellular transport of insulin. When evaluated *in vivo* using insulin-loaded mucoadhesive patches, iontophoresis produced profound hypoglycemia (63% blood glucose drop in 3 h) without damaging the intestinal tissue and the efficacy depended on insulin dose and current density. This study presents a proof of principle for intestinal iontophoresis as a novel method for oral protein delivery.

1. Introduction

Oral delivery is the favored route for drug administration by both patients and physicians due to its ease of administration. However, its use for biologics is limited because of their susceptibility to gastrointestinal degradation and limited permeability across the epithelium, leading to negligible oral bioavailability. This necessitates parenteral delivery of these therapeutic macromolecules, which lacks patient compliance and often results in non-adherence to dosing regimen, especially in the long-term. Various alternative approaches for oral delivery of biologics have been investigated including modification in protein/peptide structure to impart resistance to degradation by gastrointestinal acids and enzymes and/or improve permeability across intestinal wall, encapsulating them in enterically coated carriers or other novel formulations, using permeation enhancers and proteolytic inhibitors, amongst others [1–3]. We have earlier developed mucoadhesive intestinal patches for oral delivery of biologics such as insulin and salmon calcitonin and observed considerable improvement in oral bioavailability of the drugs [4,5]. These patches made using a specific combination of mucoadhesive polymers and coated with a water impermeable backing layer, improved intestinal permeability by strongly adhering to intestinal mucosa and providing a unidirectional transport of biologics from the drug depot in the patches. Additionally, the patches prevented enzymatic degradation of biologics by preventing access of intestinal enzymes to the loaded drugs. Furthermore, encapsulation of the patches in enterically coated capsules led to site specific delivery to the intestine. To further improve efficacy of the patches, in the current study we explored intestinal iontophoresis as a novel method to facilitate permeation of insulin across intestine both *in vitro* and *in vivo*.

Iontophoresis is a procedure that has been extensively used to enhance transport of drugs across biological barriers such as the skin [6–9]. The technique utilizes an electric current gradient to drive ionic or non-ionic drugs across the barriers and has been primarily utilized for treatment of hyperhidrosis, local inflammation and pain. The skin restricts passive transport of hydrophilic drugs or those with molecular weight $>$ 500 Da but with iontophoresis, controlled and painless delivery of many such medications is possible. Using small current gradient for short periods of time (generally ≤ 0.5 mA/cm^2 and 30 min for transdermal delivery), tissue trauma and risk of infections can be minimized using iontophoresis, unlike injectables [8]. In addition, a precise dose of medication can be delivered directly to the treatment site or systemically and the treatment can be terminated when desired by simply switching off the iontophoretic system [8,10]. Owing to its

* Corresponding author at: School of Engineering and Applied Sciences, Pierce 211, Harvard University, Cambridge, MA 02138, USA.
E-mail address: mitragotri@seas.harvard.edu (S. Mitragotri).

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non-invasive nature and high utility in drug delivery, several iontophoretic devices have been developed for delivery of various drugs including fentanyl (Ionsys®, *E*-Trans®), sumatriptan (Zecuity®; discontinued in 2016), lidocaine and epinephrine (Lidosite™), and fluoride (hyG ionic toothbrush), amongst others. Currently, drug delivery using iontophoresis is being investigated in several clinical trials with active recruitment of patients in about 15 such studies according to clinicaltrails.gov listing. However, the potential of this technology in enhancing oral drug delivery has not been explored yet. Here, we investigate if iontophoresis can be utilized to improve permeation of insulin across the intestinal cells, thus improve oral delivery of insulin.

2. Materials and methods

2.1. Materials

Human Insulin, fluorescein isothiocyanate (FITC)-insulin, pectin, sodium carboxy methylcellulose (SCMC), hematoxylin and eosin solutions were bought from Sigma-Aldrich (St. Louis, MO, USA). Eudragit® E PO was received as a gift from Evonik Industries (Parsipanny, NJ, USA). Caco-2 human colorectal adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, VA, USA). All cell culture solutions including Dulbecco modified eagle medium (DMEM) with or without phenol red, fetal bovine serum (FBS), penicillin/streptomycin (P/S) solution, Hank's balanced salt solution (HBSS) and 0.25% trypsin solution were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The transwells Millicell®-PCF cell culture inserts (3.0 μm pore size, 12 mm diameter) and trans epithelial electrical resistance (TEER) measuring device, Millicell®-ERS were bought from MilliporeSigma (Burlington, MA, USA). Electrodes for measuring TEER were purchased from World Precision Instruments, Inc. (Sarasota, FL, USA). Paraformaldehyde (16% *w/v*) was obtained from Alfa Aesar (Ward Hill, MA, USA) while Vectashield Hardset™ with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was bought from Vector laboratories Inc. (Burlingame, CA, USA). The patches were prepared using a bench top press (Carver, Inc., Wabash, IN, USA) and a pellet press (Pike Technologies, Fitchburg, WI, USA). Royovac heavy-duty 6 V lantern battery with screw tops was obtained from Fisher Scientific (Waltham, MA, USA) while analog panel current ammeters (0–200 μA DC) and 100 kΩ audio-taper potentiometers were bought from local stores. Male Wistar rats (200–300 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) while blood glucose measuring meter (Aimstrip plus) and strips were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other reagents were of analytical grade.

2.2. Caco-2 monolayer culture in transwells

Caco-2 human intestinal epithelial cells (passages # 5–10) were cultured in DMEM containing 10% *v*/v FBS and 1% v/v P/S and seeded in 24 well plate transwells at a density of 2×10^5 cells/ml. 200 μl DMEM with cells was placed in apical side while 600 μl cell free DMEM was placed in the basolateral side. The cells were allowed to grow for a period of 18–21 days and medium replaced with fresh DMEM every other day. TEER was measured on a regular basis and when it reached above 200 Ωcm^2, indicating sufficient tight junction integrity between the cells in the monolayer, iontophoretic transport study of insulin was performed [11,12].

2.3. Circuit setup and FITC-insulin transport assay

The circuit for *in vitro* transport study was set up as shown in Fig. 1. To maintain sterile conditions, all *in vitro* experiments including circuit set up were done under aseptic conditions in a biosafety cabinet. A 6 V battery was used to supply electrical current to transwells containing Caco-2 monolayer. The higher potential of the battery was connected to the basolateral side of the transwell while the lower potential of the battery was connected to the apical side while taking care not to touch the transwell membranes with Caco-2 cell monolayer. The current applied to the cell was monitored by an ammeter and its value was adjusted to 50 μA using a potentiometer. Three different branches, each attached to an ammeter, potentiometer and transwell were connected in parallel across the battery to enable three different measurements at a time.

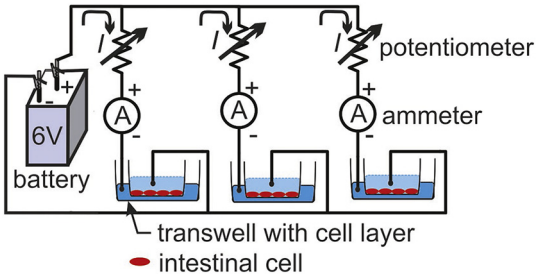

Fig. 1. Schematic of circuit set up for *in vitro* iontophoresis study.

Transport experiments were carried out in triplicates and at least 3 transwells were used per trial for each group. Before the start of the experiment, the existing medium in the transwell was replaced with phenol red, FBS and P/S free DMEM in both the apical (200 μl) and basolateral side (600 μl) and the cells were incubated for 30 min. At the start of study, the medium in the apical side was replaced with 200 μl of 500 μg/ml FITC-insulin prepared in phenol red, FBS and P/S free DMEM. Immediately after addition of FITC-insulin at the apical side, a 100 μl aliquot was withdrawn from the basolateral side and replaced with same volume fresh DMEM. This was performed at 0.25, 0.5, 1, 2, 3 and 5 h. A 50 μA electric current was applied to the cells during the first hour of the study for a period of 10 min, followed by a 10 min recovery period and this was repeated another two times. At the end of 1 h (total 30 min iontophoresis), the transwell plates were kept inside an incubator at 37 °C, 5% CO_2 and only taken out to remove 100 μl aliquots from the basolateral side followed by replacement with same volume fresh medium at the aforementioned time periods. After the end of study at 5 h, the FITC-insulin concentration in the aliquots were measured at 495/520 nm excitation/emission wavelengths using a Tecan Infinite M200 Pro multimode reader (Tecan US, Inc., Morrisville, NC, USA microplate reader). Results were plotted as % FITC-insulin transport *vs* time. TEER was measured at every time point when aliquots were withdrawn from the transwells. Apparent permeability coefficient (Papp) was calculated using the equation [13]:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A.\ C0.}$$

where dQ/dt is calculated from the slope of cumulative insulin transport across Caco-2 cells with respect to time, A is the area of the transwell surface and C0 is the insulin concentration in the apical side at time 0. The transport enhancement ratio (ER) was calculated using the equation [13]:

$$ER = \frac{P_{app}\, Iontophoresis}{P_{app}\, control}$$

2.4. Confocal microscopy

For qualitative analysis of FITC-insulin uptake by Caco-2 cells, the transwells from FITC-insulin transport study were washed two times with HBSS, followed by addition of 100 μl of 4% paraformaldehyde and kept at 4 °C overnight. On the next day, the transwells were washed with HBSS and the membrane cut out and placed in glass slides. Mounting media with DAPI was added to the membranes and covered with glass slides. Confocal imaging was performed using Olympus

Fluoview 1000 Spectral Confocal instrument at 60 X magnification.

2.5. Insulin mucoadhesive patch preparation

Insulin mucoadhesive patches were prepared as documented earlier [4]. Briefly, Eudragit E PO, pectin and SCMC were mixed in the ratio of 1:1:2 and known quantity of insulin was added to the mixture. About 110 mg of the mixture was pressed at 3-ton pressure using a hydraulic press into a patch. The patches were then cut into 5 × 3 mm (length x breadth) sized rectangular smaller patches. One side of the patch was completely covered with aluminum foil using minimal amount of super glue placed at one corner of the foil. Wires with a small section of their protective insulation removed were then stuck on the aluminum foil using super glue. Care was taken not to cover the entire wire placed over the foil with super glue. The exposed wire sticking on to the patches were then taped with an electrical insulating tape to prevent any loss of current *in vivo* through exposed wire. The insulin doses used in the patches were either 25 or 50 U/kg.

2.6. Circuit setup and in vivo efficacy study of intestinal iontophoresis using insulin mucoadhesive patches

All animal experiments were performed in accordance with the University of California Santa Barbara animal care committee guidelines and to the Guide for the Care and Use of Animals of the Institute of Laboratory Animal Resources, National Research Council.

The circuit for *in vivo* experiments was set up as illustrated in Fig. 2.

Intestinal iontophoresis was carried out using a 6 V battery connected to the intestine. A potentiometer and an ammeter were used in the electrical circuit to accurately control and measure the electric current. Given that the identified safe threshold for iontophoresis is 0.5 mA/cm^2, the electrical parameters were selected in order to perform the studies using a safe current density of 0.33 mA/cm^2 [8]. For the experiment, the rats were fasted overnight but given free access to water. Prior to the start of intestinal iontophoresis, the rats were anesthetized, a portion of abdomen prepped for surgery and an incision was made in the area to expose the intestine. Experiments were conducted using sterile instruments and following good surgical practices. Two small cuts about 5 cm afar were made in a small exposed section of the intestine and patches containing 50 U/kg insulin connected to wires and battery source were inserted. Thereafter current (45–50 μA) was allowed to flow for 1.5 min followed by 3.5 min of recovery time for 1 h, amounting to a total 18 min of iontophoresis. After 1 h, the patches were left in place but the wires were disconnected from the battery and cut close to the inserted patches. The intestinal incisions with the patches left in place were stitched, the intestine put back into the abdomen and the muscle and skin sutured. Blood glucose was determined at the start of the study and at every 0.5 h till the end of study at 3 h. The rats were kept anesthetized till the end of experiment. Afterwards, the animals were euthanized and the intestinal sections in and around the patches were dissected for further histological examination to determine morphological changes, if any, due to iontophoresis.

Control experiments with no electric current were conducted similarly. Additionally, subcutaneous (SQ) administration of 1 U/kg insulin solution was performed in 3 rats for comparison of efficacy. To determine whether decrease in current or insulin dose would bring about similar efficacy, studies were conducted using 50 μA current with patches containing 25 U/kg insulin or using 25 μA current with 50 U/kg insulin patches.

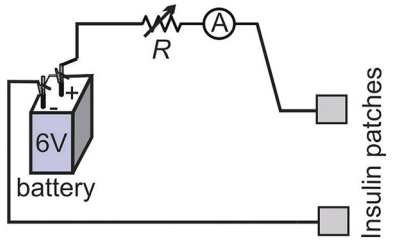

Fig. 2. Schematic of *in vivo* iontophoresis circuit set up.

2.7. Tissue histology

Tissues were fixed in 10% buffered formalin, dehydrated in ethanol, and embedded in paraffin. Five-micron cross-sections of intestine tissues were deparaffinzed, rehydrated, and stained with hematoxylin and eosin. Histological morphology was examined using a light microscope (Olympus BX60 Upright Compound Microscope).

2.8. Statistical analysis

All *in vitro* data are presented as mean ± standard deviation (SD) while *in vivo* data are presented as mean ± standard error (SE). The statistical analyses were performed using two-tailed student's *t*-test and p value < .05 was considered statistically significant. Graphs were plotted using Graphpad Prism 6.0, (GraphPad Software, La Jolla, CA).

3. Results

3.1. FITC-insulin transport

The transport of FITC-insulin across Caco-2 monolayers upon application of electric current was first assessed. Transport was higher with electric current right from the onset of study at 15 min (1.2 μg in electric current group compared to 0.2 μg in control group) but it improved significantly (p < .01–0.05) from 2 h onwards (Fig. 3). At the end of 5 h, approximately 14.1 μg FITC-insulin was transported across the cells to which electric current was applied compared to only about 5.5 μg transport in control group. The Papp of FITC-insulin across the cells in 5 h was 26.4 × 10^{-7} cm/s with electricity while that without electricity was 9.9 × 10^{-7} cm/s. The Papp value of insulin in control cells was very comparable to those obtained by other researchers using 21-day Caco-2 monolayer cultures [12,14,15]. The enhancement ratio calculated from the Papp was 2.7.

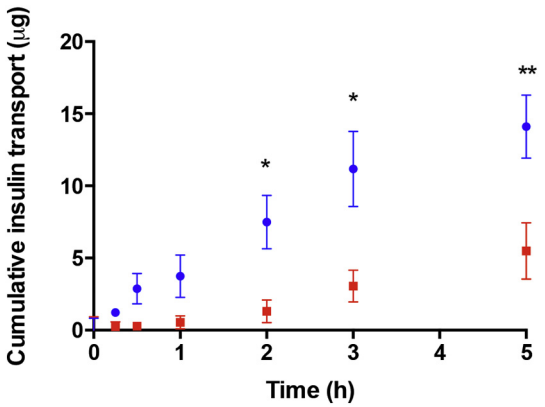

Fig. 3. Enhancement in FITC-insulin transport across Caco-2 monolayers upon iontophoresis. Blue circles represent insulin transport upon application of electric current while red squares denote insulin transport without electric current. Data represented as mean ± SD. A significantly higher insulin transport across the cells was observed from 2 h onwards upon application of electric current compared to control cells that were not subjected to treatment with electric current (* p < .05, ** p < .01), n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

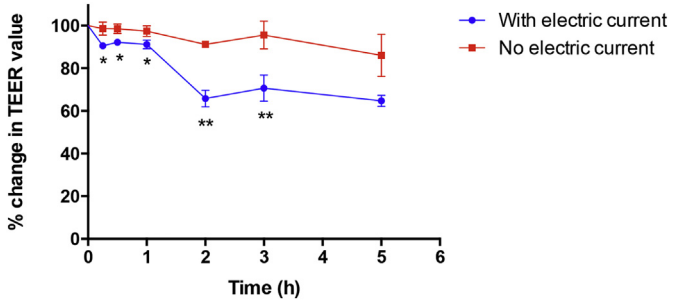

Fig. 4. Effect of electric current on tight junction integrity of Caco-2 cells. Data represented as mean ± SD. A significant reduction in TEER in cells was observed upon application of electric current from 15 min compared to no electric current control cells (* $p < .05$, ** $p < .01$), $n = 3$.

3.2. Measurement of tight junction integrity in Caco-2 monolayers upon application of electric current

TEER was measured to determine the tight junction integrity in Caco-2 cells upon application of electric current. TEER decreased significantly ($p < .05$) by 10% within 15 min of passage of current compared to no specific decrease in the control group (Fig. 4). After 2 h of the study, TEER dropped more significantly by 35% of initial levels (65.7 ± 3.8%) and was relatively constant during the remaining 3 h of the study. In comparison, in control (no current) wells, TEER reduced by around 9 and 4% of initial levels in 2 and 3 h respectively (91.2 ± 1.1% and 95.6 ± 6.5%).

3.3. Confocal micrograph images

Confocal laser scanning microscopy images of transwell membranes of wells with or without iontophoresis showed higher uptake of FITC-insulin by cells exposed to electric current compared to control wells (Fig. 5).

3.4. In vivo intestinal iontophoresis using mucoadhesive patches

After *in vitro* evaluation of intestinal iontophoresis of insulin, studies were conducted *in vivo*. Rats with 50 U/kg insulin patches inserted in their intestine and subjected to 45–50 μA current, demonstrated a significant ($p < .01$) drop in blood glucose levels from 2 h onwards to 35% of initial levels (65 ± 2.2%) that further decreased to 63% (36.6 ± 3%) in 3 h (Fig. 6). In contrast, rats with 50 U/kg insulin patches but without electric current treatment, showed only 3.4% drop in 2 h (96.4 ± 6.6%) followed by a maximum 12% drop in 3 h (88.1 ± 6.7%). Rats that were intra-intestinally injected with 50 U/kg insulin solution did not show any drop in blood glucose levels with time while those subcutaneously injected with 1 U/kg insulin solution showed a maximum drop of 42% in 1.5 h (57.9 ± 4.1%) that recovered to about 17% of initial levels (83.3 ± 2.6%) in 3 h.

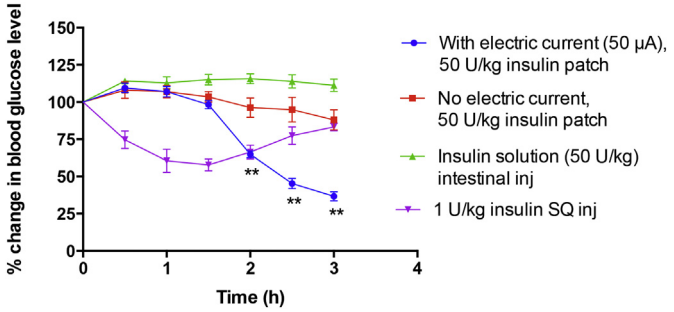

Fig. 6. Effect of intestinal iontophoresis through insulin mucoadhesive patches in lowering blood glucose levels in non-diabetic rats. A significantly higher reduction in blood glucose levels was observed using insulin patch based intestinal iontophoresis from 2 h onwards compared to control (no electric current) insulin patch. Data represented as mean ± SE. (** $p < .01$, electric current group compared to no electric current group), $n = 6$ for iontophoresis and controls and $n = 3$ for SQ injection.

3.5. Effect of varying electrical current and insulin dose on blood glucose reduction

The considerable drop in blood glucose levels upon application of electric current as observed in Fig. 6, prompted us to investigate whether similar efficacy could be achieved using lower current or insulin dose. To this end, we halved the current to 20–25 μA keeping the duration of current and insulin dose same (18 min and 50 U/kg respectively) and determined drop in blood glucose in 3 h. Lowering the current value to 20–25 μA did not bring about an extensive drop in blood glucose and a maximum 26% (74.04 ± 14.2% of initial level) decrease was noted in 3 h (Fig. 7). In comparison, same dose-no electric current control rats demonstrated only 12% (88.1 ± 6.7% of initial level) drop in blood glucose in 3 h. Next, blood glucose lowering efficacy by reducing insulin dose to 25 U/kg but keeping current and treatment duration same (45–50 μA current and 18 min respectively) was investigated. This modification also did not bring about a substantial drop in blood glucose levels and a maximum decrease of 23% (77.1 ± 8.8% of initial level) in 3 h was observed while its respective no electric current control resulted in a 15% drop (85 ± 12.2% of initial level). The study suggested that decreasing current or insulin dose would not be very effective in intestinal iontophoretic delivery of insulin.

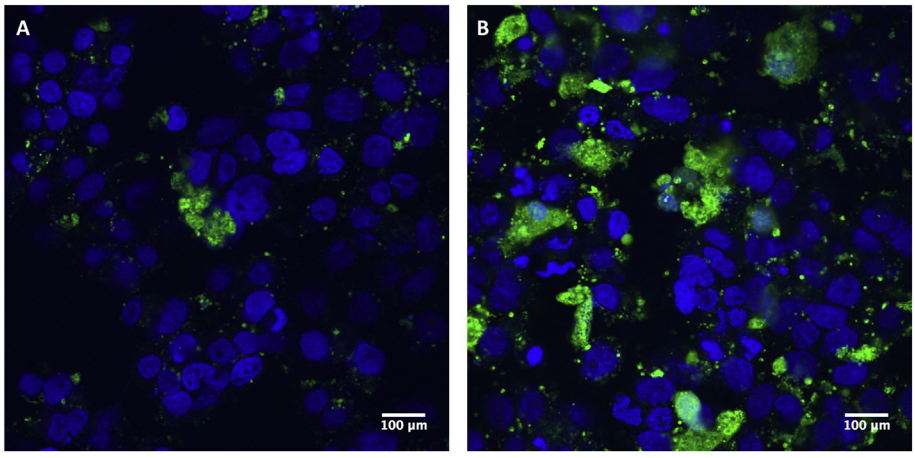

Fig. 5. Confocal micrograph images of transwell membranes containing Caco-2 cells upon treatment with FITC-insulin and (A): No electric current or (B) Electric current. The images present an overlay of blue colored nucleus staining from DAPI and green colored FITC-insulin staining. Images taken at 60 X magnification. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

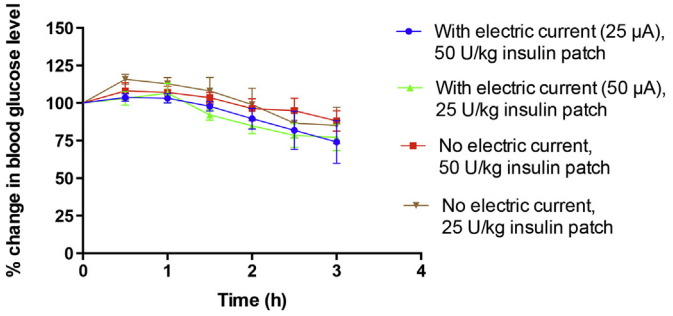

Fig. 7. Effect of low current/low insulin dose in lowering blood glucose levels in non-diabetic rats. No significant difference in blood glucose lowering efficacy of the patches was observed between iontophoresis group and its respective no current controls. Data represented as mean ± SE. ($n = 6$).

3.6. Assessment of toxicity to intestinal tissue upon application of electric current

Histological examination showed no difference with regard to morphology of intestinal tissue between the groups of rats with or without iontophoretic treatment (Fig. 8). In particular, sections where patches were placed or adjacent areas showed no structural damage upon the application of electric current. The lumen of the intestine (lamina propria and intestinal gland) was intact, and the thickness of smooth muscles was similar amongst all the tissues when measured with light microscopy.

4. Discussion

Iontophoresis is a technology to facilitate transport of drugs across biological membranes through the application of external current. It has been primarily utilized for transdermal delivery of small and large molecule therapeutics [16–18]. In the skin, the tightly packed corneocytes and lipid layers do not permit passive transport of hydrophilic molecules or those larger than 500 Da [19]. Various techniques have therefore been developed to enhance drug transport across the skin, which include chemical penetration enhancers, prodrugs, ultrasound, electroporation, thermal ablation, jet injectors and specially designed formulations to breach the skin barrier [20]. While passive transport techniques such as chemical penetration enhancers and prodrugs work well for small molecule drugs, invasive techniques using micro needles and jet injectors are usually required for macromolecule transport across the skin [21,22]. Iontophoresis has been largely studied as a non-invasive strategy for transdermal drug delivery and several formulations are commercially available, albeit for small molecule delivery mostly for the treatment of hyperhidrosis, inflammation and pain [20]. In addition, the technology has been tested for the treatment of myriad other disorders including musculoskeletal disorders, ulcers, cystic fibrosis, osteomyelitis and viral infections, amongst others [8,23]. For example, transdermal iontophoresis has been recently investigated for delivery of neostigmine/glycopyrrolate to induce bowel movement in patients with spinal cord injury and was found to be safe and effective in comparison to intravenous injection [24]. Similarly, cutaneous iontophoresis of vasodilator drug treprostinil, was recently found to improve skin blood flow at the treatment sites [25]. Apart from transdermal drug delivery, iontophoresis has also been investigated in ocular, buccal and vaginal drug delivery [26–30]. For example, Ocu-Phor™ iontophoretic system has been developed for drug delivery across the sclera in the lower eyelid. However, in oral drug delivery, only *in vitro* and *ex vivo* iontophoretic models have been explored [31,32].

The oral route although very convenient for drug administration cannot be used for delivery of biologics due to their propensity to degrade by gastric acids, proteolytic cleavage by intestinal enzymes and low permeability across the intestinal wall, that results in almost negligible oral bioavailability of such drugs [33]. To circumvent the issues of gastrointestinal degradation and low intestinal permeability, various techniques have been utilized including chemical modification of biologics, use of permeation enhancers, protease inhibitors, cell penetrating peptides, nanoparticles and encapsulation in enterically coated devices amongst others. For example, when 50 U/kg insulin conjugated to cell penetrating peptide, trans-activator of transcription (TAT), and placed inside elastic anionic niosomes was orally delivered to diabetic rats, it led to about 71% decrease in blood glucose levels in 12 h [34]. Chitosan-dextran sulfate nanoparticles loaded with 50 U/kg insulin brought about 35% decrease in blood glucose levels in diabetic rats at

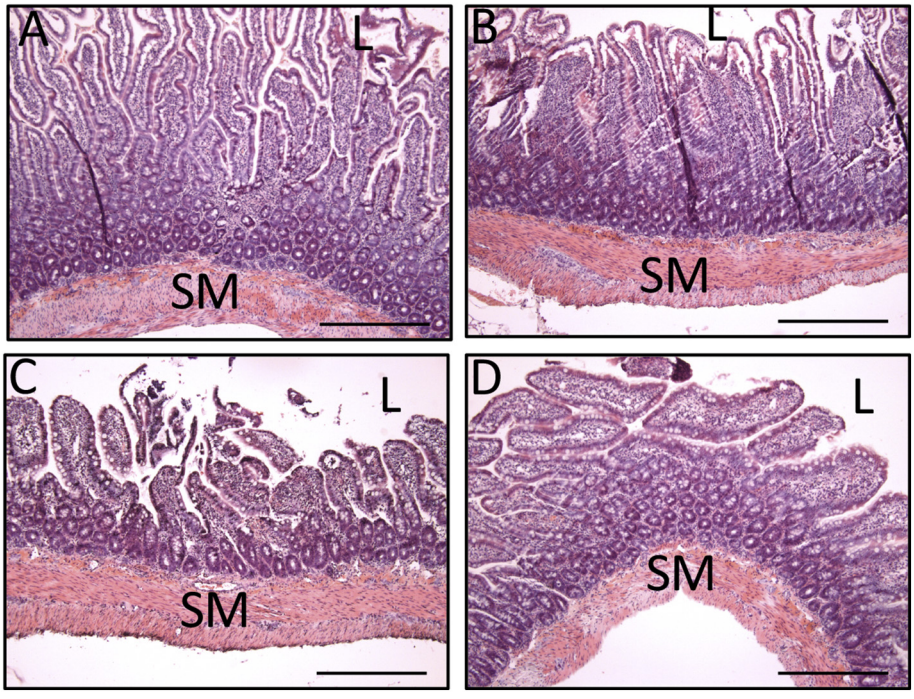

Fig. 8. Photomicrographs of hematoxylin and eosin staining of small intestine tissue sections. L: lumen of intestine (lamina propria and intestinal gland); SM: smooth muscle.
(A) Intestinal section where patch was placed in test rat (with electric current); (B) Intestinal section of test rat (with electric current) where patch was not present (section was taken from between the two areas where patches were placed); (C) Intestinal section of control rat (no electric current) where patch was placed; (D) Intestinal section of control rat where patch was not present (section was taken from between the two areas where patches were placed). Scale bar: 200 μm.

8 h [35]. Similarly, oral delivery of 50 U/kg insulin loaded in alginate-chitosan nanoparticles caused 40% drop in glucose levels in diabetic rats [36]. Radwan et al. screened the efficacy of various absorption enhancers in improving oral delivery of 60 U/kg insulin loaded in poly (ethylcyanoacrylate) nanospheres in diabetic rats and found significant blood glucose lowering efficacy using capric acid in about 10 h [37]. Earlier, 50 U/kg insulin micropatches co-administered with citric acid (a non-specific protease inhibitor) in enterically coated capsules, led to a 34% drop in blood glucose levels in 3 h in non-diabetic rats [38]. As evident, these techniques are effective to various extents, but there is still an unmet medical need to develop technologies that will substantially improve oral delivery of biologics. To this end, we explored intestinal iontophoresis as a novel oral protein delivery modality.

Transport of insulin across intestinal cells was enhanced close to about 3-fold with the application of intermittent electric current during first hour of the study compared to no current control. This can be attributed to significant ($p < .01$–0.05) decline in TEER from the onset of the experiment with a maximal 35% drop at 2 h. Interestingly, insulin transport across the cells was also significantly ($p < .01$–0.05) higher than control from 2 h onwards when TEER drop was maximum. The reduction in TEER suggests that insulin transport across Caco-2 monolayer subjected to electric current is mostly paracellular. Leonard and co-workers also noted about 40% drop in TEER in Caco-2 cells during application of electric current [31]. In the present study, at the end at 5 h, no significant difference in TEER between control and electric current group was observed, indicating that tight junction integrity may recover with time. This was earlier observed by Leonard et al. where cessation of electric current led to complete recovery of tight junction integrity [31]. In the same vein, Schaffnita et al. observed that iontophoresis across MDCK cell monolayers decreased electrical resistance across the cells but TEER recovered completely after 24 h of incubation [39]. In another study, ocular iontophoresis of various drugs including lidocaine, sodium benzoate and FITC-dextran improved transport across cornea and conjunctiva isolated from rabbits by 2–4 fold with an accompanying reduction in TEER that depending on applied current, recovered upon discontinuing iontophoresis [40]. In the present study, the higher transport of insulin across Caco-2 cells was corroborated by confocal images of transwell membranes that showed presence of higher FITC-insulin in cells subjected to iontophoresis.

To verify the results *in vivo*, we performed iontophoresis at an intestinal site and observed good correlation with *in vitro* data. Mucoadhesive patches loaded with 50 U/kg insulin were utilized for *in vivo* experiments because they had previously demonstrated to improve insulin transport across the intestine in non-diabetic rats [4,41]. These mucoadhesive patches were placed inside enterically coated capsules that enabled them to bypass the harsh acidic environment of the stomach and release drug loaded patches from the capsules specifically in the intestine. Upon release, the mucoadhesive polymers in the patches allow strong adhesion of the patches to the mucus layer of intestine. Additionally, the patches provide a concentrated depot of therapeutics at a single location that swell in an aqueous environment and facilitate concentration gradient mediated transport of biologics across the intestine while protecting them from proteolytic degradation by forming a physical barrier and preventing access of enzymes to loaded drugs. The objective of the present study was to investigate whether iontophoresis could further enhance the efficacy of intestinal mucoadhesive patches in oral insulin delivery. Keeping the current value similar for *in vitro* and *in vivo* experiments, we observed that iontophoresis greatly enhanced transport of insulin across the intestine leading to a remarkable 63% drop in blood glucose levels. Also, consistent with *in vitro* experiments, a very significant ($p < .01$) drop in blood glucose levels was observed at 2 h of the study that steadily declined till 3 h when animals became severely hypoglycemic and the study was terminated. However, modulation of electric current or insulin doses did not produce such remarkable efficacy, suggesting that 50 μA current and 50 U/kg insulin dose were critical for successful intestinal iontophoresis. Amongst other parameters, the efficacy of an iontophoretic therapy is greatly dependent on current density and drug concentration, indicating that any variation in these parameters may influence iontophoretic drug delivery [8]. A more thorough evaluation of iontophoresis parameters such as variation in treatment duration and electrode materials will need to be conducted to further optimize the process of intestinal iontophoresis.

Iontophoresis of insulin through the skin has been earlier attempted by other researchers but resulted in limited permeation [8,42]. Iontophoresis in combination with chemical penetration enhancers, microneedles and electroporation has been explored for transdermal delivery of biologics [8]. For instance, insulin iontophoresis through full thickness rat skin after pre-treatment with chemical penetration enhancers such as ethanol, propylene glycol, dimethyl acetamide, ethyl acetate or isopropyl myristate, led to enhanced transport of insulin across the skin in a few cases but many enhancers were reported to severely compromise skin barrier properties [43]. Iontophoresis in combination with electroporation led to high plasma insulin concentrations in rats [42,44]. Insulin encapsulated nanovesicles placed inside microneedles and iontophoretically delivered across the skin of diabetic rats resulted in about 65% drop in blood glucose levels in 6 h [45]. Apart from insulin, iontophoresis has been used to deliver therapeutic concentrations of salmon calcitonin across the skin of male hairless rats using wearable electronic disposable drug delivery (WEDD®) device [46]. Similarly, feasibility of electromigration of ribonuclease T1 and thyrotropin-releasing hormone across excised porcine and rabbit ear skin respectively has also been demonstrated [47,48].

In the present study, the intestinal samples obtained after *in vivo* experiments did not show signs of tissue damage which is consistent with observations of Leonard et al. who found that iontophoresis on colon tissue *ex vivo* did not change electrogenic ion response to forskolin compared to control tissues [32]. Similarly, ocular coulomb-controlled iontophoresis of methylprednisolone or acetyl salicylic acid in rabbits resulted in no toxicity or histological damage [49,50]. Iontophoresis works predominantly by establishing an electrical gradient for transport of molecules across pathways that already exist within a barrier, therefore any histological damage to tissues is not anticipated [51]. Also, given that the identified safe threshold for iontophoresis is 0.5 mA/cm^2, a low and safe current density of 0.33 mA/cm^2 or less were used in the study [8].

This study provides proof of concept that oral delivery of biologics can be enhanced through iontophoresis that modulates intestinal absorptive pathways to considerably improve permeability of such drugs across the intestine. Translation of the technology into clinics will require development of devices utilizing miniature electronic/electrical systems. Feasibility of this has been demonstrated by micro-electromechanical system (MEMS) based devices that have seen a recent upsurge in its application in drug delivery due to its capability to address myriad challenges in drug delivery [52]. For instance, Zhuang and co-workers have developed an electronic capsule that can deliver drugs in the gastrointestinal tract (GIT) in a controlled fashion [53]. The MEMS based device consisting of a timer, a microfluidic drug reservoir, power supply and a driving unit for drug release in the gut, was found to release mildronate in beagle dogs in the alimentary canal at a desired dose and time. Additionally, MEMS has been used to device a remote-controlled capsule for site specific delivery of drugs in the GIT [54]. Zhang et al. developed a MEMS based ocular iontophoretic implantable device that when placed under the eyelid of rabbits, enhanced transport of Mn^{2+} into the vitreous humor [55]. Along the same lines, microneedle based oral devices have also been developed for delivery of insulin [56]. When these devices were endoscopically placed in porcine stomach, they passed through the GIT without damaging the tissues and were excreted in a few days. For development of an intestinal iontophoretic device, insulin mucoadhesive patches with integrated circuits and on chip battery can be designed and placed in enterically coated capsules for site specific delivery of the device to intestine. Release of

iontophoretic device from the capsule in the intestine followed by adhesion of mucoadhesive patches to the intestinal mucosa and exposure to the aqueous environment may be used to trigger onset of iontophoresis. When drug load depletes leading to complete disintegration of patches and subsequent loss of mucosal adhesion, the device could be designed to safely pass through the GIT and get excreted. This study brings forth an unexplored approach for oral insulin delivery that can form the foundation for further development of this technology into clinical devices. This situation is akin to the field of transdermal drug delivery in the 90's when early stage results on various technologies including iontophoresis [57], electroporation [58], ultrasound [59], laser [60] and microneedles [61] were reported in the literature that inspired their development into clinically usable devices. We believe that a similar opportunity is on the horizon for oral delivery, and examples based on the use of patches [4,38], ultrasound [62] and microneedles [56] for oral delivery have already been reported. With this notion, we believe that this work will motivate further comprehensive investigation and development of novel iontophoretic devices for oral delivery that can potentially transform oral drug delivery, particularly of biologics. However, prior to this, iontophoretic parameters that can influence oral delivery will need to be determined systematically and use of permeation enhancers in the patches that can assist intestinal iontophoresis will need to be explored. In addition, comprehensive mechanistic studies to clearly understand the iontophoretic drug transport process as well as preclinical efficacy and toxicity studies in diabetic animals will need to be performed.

In summary, intestinal iontophoresis was conducted using insulin as a model drug and was found to remarkably improve insulin transport *in vitro* and its pharmacodynamics *in vivo*. A 35% drop in TEER was observed in Caco-2 monolayers subjected to iontophoresis that consequently led to almost three-fold enhancement in insulin transport across the cells. In rats, insulin loaded mucoadhesive patches integrated with iontophoretic circuit and surgically placed in the intestine, led to profound hypoglycemia that was dependent on insulin dose and current density. In addition, iontophoresis did not cause any structural damage to intestinal tissues, indicating that the technology is safe to use. We believe that development of MEMS based swallowable iontophoretic device is paramount to success of this novel technology and should be considered for its clinical translation.

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