Skin pretreatment with microneedles prior to pilocarpine iontophoresis increases sweat production

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Summary
Collection of sweat via pilocarpine iontophoresis is commonly used to diagnose cystic fibrosis (CF), with thousands of tests performed each day. The main source of resistance to the passage of pilocarpine ions to the sweat glands is the electrical resistance of the stratum corneum. It was hypothesized that pretreating the skin with 0.5 mm-long microneedles would significantly decrease this resistance, thus increasing pilocarpine’s permeation into the skin. Improved permeation should result in significantly reduced time to sweat initiation, time to collection of a clinically meaningful amount of sweat, and increased total amount of sweat produced in 15 min. Subjects (n = 12) had two 5 cm² areas on the forearm measured, marked and randomized to experimental (microneedles + iontophoresis) or control (iontophoresis alone). Microneedle pretreatment was conducted using a 35-needle microneedle stamp in a manner that 20 applications completely covered the 5 cm² treatment area. This was repeated five times for a total of 100 applications. Both experimental and control sites were placed under iontophoresis (~15 mA) for 5 min. Microneedle pretreatment significantly decreased mean skin resistance (260 ± 27 kΩ versus 160 ± 19 kΩ, P = 0.006), while significantly increasing mean sweat rate (0.76 ± 0.35 versus 0.54 ± 0.19 µl cm⁻² min⁻¹, P = 0.007). No significant difference was found concerning pain (P = 0.059), number of active sweat glands (P = 0.627) or the osmolality of the collected sweat (P = 0.636). The results of this study suggest that microneedle pretreatment prior to pilocarpine iontophoresis significantly increases sweat production. Such results have the potential to improve the methodology currently used to diagnose cystic fibrosis and, more broadly, to administer drugs via the skin.

Introduction
Iontophoresis is a process in which the transport of ionic solutes into the skin is enhanced by applying an electrical field across the skin (Khan et al., 2011). Iontophoresis is used as a delivery method for dozens of drugs treating a multitude of diseases ranging from gouty arthritis to calcified tendonitis (Watts, 2005). One of the drugs, pilocarpine, is a cholinergic agonist which promotes region-specific sweating (Colman & Atwell, 1962). Collection of sweat following iontophoresis of pilocarpine is currently the most commonly used test for the diagnoses of cystic fibrosis (CF) (LeGrys & Retsch-Bogart, 1997; Chernick, 1998) with thousands of tests performed worldwide each day using the procedure (LeGrys, 2001). At the majority of CF testing locations, 10–15 µl of sweat is the minimum amount that must be collected to have a usable sample to determine osmolality (LeGrys, 2001). The major problems associated with the procedure, from a clinical point of view, are as follows: (i) the procedure requires 30 min, which is time-consuming and (ii) that approximately 15% of subjects do not sweat enough to provide an adequate sample volume for subsequent analysis (LeGrys, 2001). As such, these patients then require retesting. Anything that decreases the amount of time needed to collect an adequate sweat sample and/or increases the percentage of testable subjects has potential for clinical value.

The current standard of care regarding the diagnosis of CF uses iontophoresis to drive pilocarpine ions into the skin using a small electrical current (~1.5 mA). The main source of resistance to the passage of pilocarpine is the skin’s outer layer, namely the stratum corneum (Delgado-Charro & Guy, 2001). Pilocarpine is not unique in this regard as the physical and electrical barrier presented by the stratum corneum impedes the absorption of many drugs (Rawat et al., 2008), while stopping others altogether (Sloan & Saltani, 1986; You et al., 2010).
In the last decade, microneedles have been investigated as an independent method of transdermal drug delivery (Kim et al., 2012). Solid microneedles, as used in the current study, measure hundreds of microns in length, taper to a sharp tip often of just a few microns in diameter and are arranged in multi-needle arrays. Because of their small size, microneedles are generally reported as painless by human subjects (Gill et al., 2008). Pretreatment with microneedles creates micron-scale punctures in the stratum corneum, which leads to increased drug absorption (McAllister et al., 2003; Wermeling et al., 2008; You et al., 2010) and reduced skin electrical resistance (Gupta et al., 2011). Micropores made in this way resel within a few hours in uncovered skin, but can remain open for days if the skin is occluded (Gupta et al., 2011). Most studies have allowed drugs to diffuse passively into the skin after microneedle pretreatment, but iontophoresis has been shown to increase rates of delivery after microneedle pretreatment (Lin et al., 2001). Alternatively, drugs have been coated onto solid microneedles (Daddona et al., 2011) or encapsulated within solid microneedles that dissolve in the skin (Sullivan et al., 2010) for direct deposition of drugs within the skin. Hollow microneedles have also been used for injection of liquid formulations into the skin, including an approved product for intradermal influenza vaccination (Atmar et al., 2010). Finally, solid microneedles often assembled on rollers are widely used cosmetically to improve skin appearance (Aust et al., 2008).

In this study, we hypothesized that microneedle pretreatment would significantly decrease the skin’s electrical resistance, thus increasing the amount of pilocarpine that permeated the skin during iontophoresis. Improved ion permeation should, in turn, result in significant decreases in terms of time to sweat initiation and time to collection of a clinically meaningful amount of sweat (15 μl) and increased overall sweat rate. It was further hypothesized that microneedle pretreatment combined with iontophoresis would not be significantly more painful than iontophoresis alone.

Methods

Subjects were recruited when they responded to fliers that were posted throughout the San Diego State University (SDSU) campus and surrounding community. The study was approved by the SDSU IRB, and prior to testing and data collection signed informed consent was obtained. The subjects consisted of seven men and five women with a mean ± SD age of 32.7 ± 9.2 years, height of 172.5 ± 11.9 cm, and weight of 73.92 ± 13.46 kg. Subjects were instructed to report to the laboratory well rested and were asked not to have exercised or eaten at least 3 h prior to testing. Participants sat quietly with their dominant arm resting on a table top at approximately heart height, while two 5 cm² circular areas on the flexor surface of the proximal half of the forearm were measured and marked with a permanent pen. These two positions were then randomized to experimental (microneedles + iontophoresis) or control (iontophoresis alone).

Prior to beginning iontophoresis in either area, microneedle pretreatment of the treatment area was conducted using a microneedle derma stamp (DRT, Beijing, China) with an array of 35 microneedles, each of which was 0.5 mm in length spread across five rows. The skin area was first cleaned with isopropyl alcohol, and then, the microneedle array was firmly pressed into the treatment area in a manner that 20 applications completely covered the 5 cm² treatment area. This was repeated five times for a total of 100 stamp applications. This process was completed in approximately 30 s. A measurement of pain was taken at this time using the visual analogue scale, which has been shown to have good reliability as a tool to measure low levels of acute pain (Bijur et al., 2001). Next, a disposable Ag/AgCl electrode (3M, St. Paul, MN, USA) was applied, and skin resistance was measured using a digital multimeter (Radio Shack, Fort Worth, TX, USA). The digital multimeter provided measurement data updated every second. Measurements of skin resistance were allowed to come to a steady state, defined as a change in skin resistance of <5 kΩ for ten (10) consecutive data readings. The mean of the ten data points was then considered for statistical analysis. Skin resistance of the control site was gathered by cleaning the area with alcohol, waiting 30 s before applying the electrode and measuring skin resistance in the manner discussed above.

The order of iontophoresis for the treatment and control sites was randomized. Each spot (5 cm²) was placed under iontophoresis (1.5 mA) for 5 min using 2 ml of 0.01 g ml⁻¹ pilocarpine solution (Spectrum Chemical Company, Gardena, CA, USA) at the positive electrode and 2 ml of saline at the negative electrode. Immediately following iontophoresis, the visual analogue pain scale was again employed to measure subject’s perceptions of pain.

Following iontophoresis, the area was thoroughly cleaned and dried, and then, a Wescor macroduct coil (Logan, UT, USA) was affixed with a velcro strap to prevent leakage. Time to sweat initiation and time to collection of 5, 10 and 15 μl of total sweat were recorded. Collection was allowed to continue for a total of 15 min before the final sweat position was marked, and the macroduct was removed. The final position was measured, and the sweat was collected and frozen for later analysis of osmolality using a Wescor 5500 Vapor Pressure Osmometer (Wescor Inc., Logan, UT, USA).

The number of sweat glands at both the experimental and control locations was quantified in four 0.25 cm² areas using a method that has been previously described in the literature (Sato & Dobson, 1970). In brief, iodine impregnated paper was held against the skin of the treated (e.g. iontophoresed) area for approximately 5 s to allow individual sweat glands to be effectively visualized. A transparent plastic sheet with a 0.25 cm² marked box was then placed over the sweat marked iodine paper. Each sweat ‘spot’ within the marked area was counted using a magnifying glass. This process was repeated four times in each treatment area and summed to give a gland per cm² value. A single investigator conducted all of the sweat gland visualization and counting.

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Control and treatment data for all sweat rate variables were analysed using paired t-tests. Pain analogue data were compared using a repeated measure ANOVA. Significance was set at the 0.05 level for all tests.

Results

Mean ± SD skin resistance was 260 ± 27 kΩ at the control site and 160 ± 19 kΩ at the microneedle pretreatment site (P = 0.006). In all but one subject, prior microneedle treatment yielded a reduction in measured skin resistance at the treated area.

Figure 1 shows the differences in time to sweat initiation and time to complete the collection of three clinically meaningful sweat volumes. In all cases, time differences were significant, with sweating at the site pretreated with microneedles taking approximately 65% as long to initiate (P = 0.006) and approximately 80% as long to collect clinically meaningful volumes (P = 0.030, 0.049, and 0.010 at 5 μL, 10 μL, and 15 μL of sweat, respectively).

Total sweat collected and the derived mean sweat collection rate were also significantly increased with microneedle pretreatment. Mean sweat volume was 40.2 μL at the control site and 56.6 μL at the treatment site (P = 0.004). This translates to a 143% greater overall sweat rate (P = 0.007) following microneedling pretreatment. These data are visualized in Fig. 2. The greater overall sweat collection rate was the product of both an earlier initiation of sweating (Fig. 1), and a faster sweat rate as evidenced by a 39% greater slope (4.3 versus 3.1) in the sweat volume versus collection time relationship presented in Fig. 3.

The visual analogue pain scale assessment yielded a mean value of 1.48 ± 0.61 during the microneedle pretreatment. In comparison, iontophoresis of the control and microneedle treatment areas yielded mean pain values of 0.99 ± 0.73 and 0.90 ± 0.74, respectively. There was no significant difference (P = 0.059) in the amount of pain experienced during microneedle pretreatment of the treatment area, iontophoresis of the pretreated area and iontophoresis of the control site.

Number of active sweat glands and sweat osmolality were also compared to determine whether either of those parameters was affected by microneedle pretreatment. The mean number of sweat glands per cm² was 105 ± 20 at the control site and 103 ± 19 at the microneedle treatment site. Sweat osmolality was 109 ± 44 and 111 ± 42 mmol kg⁻¹ at the control and treatment sites, respectively. Neither the number of sweat glands nor sweat osmolality was significantly different between the treatment and control sites (P = 0.627 and 0.636, respectively).

Discussion

Direct and indirect measurements indicate that there are more available transport pathways through the stratum corneum following microneedle treatment (McAllister et al., 2003; Wermeling et al., 2008; You et al., 2010; Duan et al., 2011). This, in turn, increases the passive absorption of a variety of topically applied drugs including naltrexone (Wermeling et al., 2008), L-ascorbic acid (Shah et al., 2011) and lidocaine hydrochloride (Duan et al., 2011) among others. The results of the current study suggest that there may be a profound effect when microneedle pretreatment is combined with iontophoresis. It appears that microneedles may create new, micron-scale, transport pathways into the skin and iontophoresis provides an added driving force to deliver drugs through those pathways by electrophoresis. This effect was suggested by previous research showing that both microneedle pretreatment and long-duration iontophoresis increased permeation of 5-aminolevulinic acid through the stratum corneum of swine nearly...
15-fold (Fang et al., 2004). However, when combined, there was an additional 5- to 15-fold increase over either process alone. Similar synergistic effects have been reported for delivery of oligonucleotides and high molecular weight dextrans (Lin et al., 2001; Wu et al., 2007).

The present study builds on previous literature as the first study to assess pharmacodynamics of a drug’s biological effect in vivo and the first study to examine the combined effects of microneedle pretreatment and iontophoresis for transdermal delivery in human subjects. Although further research is required, proof of concept has been established that the combination of microneedle pretreatment and iontophoresis may be a viable delivery mechanism for a variety of drugs that were previously too large or too hydrophilic to cross the stratum corneum.

Of potentially immediate clinical interest is the fact that the increased permeation of pilocarpine significantly decreased the amount of time necessary to collect 15 μl of sweat; a usable sample volume for cystic fibrosis testing (LeGrys, 2001). Reducing collection time could have an immediate impact on clinical practice and staffing and patient inconvenience; yielding substantial time and cost savings for both practitioners and patients. Additionally, by increasing the total sweat collected, microneedle pretreatment in combination with iontophoresis has the potential to ensure that less than the currently estimated 15% of patients (LeGrys, 2001) are forced to repeat the test due to insufficient sweating.

All of the visual analogue pain scale ratings were within the minimal range, and there was no significant (P = 0.059) increase in pain with microneedle pretreatment compared with iontophoresis alone. These data are consistent with prior observations (Gill et al., 2008) in suggesting that microneedles of 0.5 mm in length can be used without increasing perception of pain. However, the near statistical significance of the differences in pain following treatment in adults does raise real concerns about the applicability of this treatment in a paediatric setting. Indeed, what is considered ‘minimal’ pain to an adult may be substantially more painful for an infant. Future testing should focus on determining optimal needle length, needle arrangement and number of applications to minimize pain, while maximizing the efficiency of pilocarpine permeation and associated sweat induction.

In conclusion, the current study is the first to demonstrate the effects that microneedle pretreatment has on the iontophoresis of pilocarpine into skin. Specifically, microneedle pretreatment significantly increases sweat production following pilocarpine iontophoresis, which could potentially be used to expedite and increase efficacy of clinical testing for cystic fibrosis. As the first study in human subjects, this finding more broadly suggests clinical use of skin pretreatment with microneedles followed by iontophoresis for delivery of a wide variety of molecules into the skin for local or systemic therapies.

Conflict of interest

M.R.P. is an inventor of patents that have been licensed to companies developing microneedle-based products, is a paid advisor to companies developing microneedle-based products, and is the founder/share holder of companies developing microneedle-based products. The resulting potential conflict of interest has been disclosed and is managed by Georgia Tech and Emory University.

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