Iontophoresis-Targeted, Follicular Delivery of Minoxidil Sulfate for the Treatment of Alopecia

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ABSTRACT: Although minoxidil (MX) is a drug known to stimulate hair growth, the treatment of androgenic alopecia could be improved by delivery strategies that would favor drug accumulation into the hair follicles. This work investigated *in vitro* the potential of iontophoresis to achieve this objective using MX sulfate (MXS), a more water-soluble derivative of MX. Passive delivery of MXS was first determined from an ethanol–water solution and from a thermosensitive gel. The latter formulation resulted in greater accumulation of MXS in the stratum corneum (skin's outermost layer) and hair follicles and an overall decrease in absorption through the skin. Anodal iontophoresis of MXS from the same gel formulation was then investigated at pH 3.5 and pH 5.5. Compared with passive delivery, iontophoresis increased the amount of drug reaching the follicular infundibula from 120 to 600 ng per follicle. In addition, drug recovery from follicular casts was threefold higher following iontophoresis at pH 5.5 compared with that at pH 3.5. Preliminary *in vivo* experiments in rats confirmed that iontophoretic delivery of MXS facilitated drug accumulation in hair follicles. Overall, therefore, iontophoresis successfully and significantly enhanced follicular delivery of MX suggesting a useful opportunity for the improved treatment of alopecia. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:1488–1494, 2013

Keywords: iontophoresis; skin; controlled delivery; targeted drug delivery; drug transport; hair follicles; minoxidil sulfate; alopecia

INTRODUCTION

Androgenic alopecia, or male baldness, is a condition that affects a significant number of both men and women worldwide. The most frequent topical treatment is based on the drug minoxidil [MX; molecular weight (MW) = 209.2 Da] (Fig. 1a),[1,2] a potent antihypertensive agent, which promotes hair growth by various mechanisms including the induction of vasodilation and increasing blood flow in the dermal papillae,[1,3] as well as a direct effect via potassium channels (K_{ATP}) receptors in the follicle,[4] stimulating hair growth via a vellus-to-terminal hair follicle transformation and also via anagen induction and prolongation.[5] MX sulfate (MXS; MW = 289.3 Da) (Fig. 1b), an endogenous metabolite of MX, is a more potent vasodilator,[5] with greater aqueous solubility, and has been investigated as an alternative active moiety in formulations for the topical treatment of baldness.[6]

Current, commercially available formulations of MX are simple ethanol-based solutions of the drug[7] that require at least a twice-daily application to ensure pharmacological effect[8] and provide no specific "targeting" to hair follicles. The efficiency of these products in the treatment of alopecia, therefore, is at best modest.

The contribution of hair follicles to percutaneous drug delivery has been recently reviewed,[9–11] and attention has been drawn to the fact that follicular drug depots may be valuable assets for localized therapy, particularly the treatment of disorders such as acne,[10,11] some types of skin cancer (e.g., Bowen's

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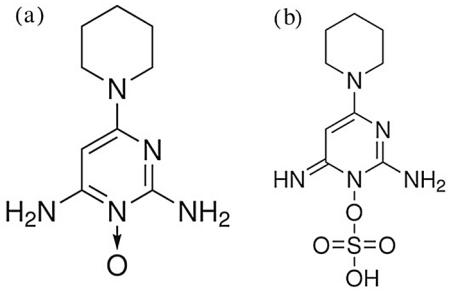

Figure 1. Chemical structure of (a) MX (MW = 209.2 Da; pK_a = 4.3), and (b) MXS (2,4-diamine-6-piperidine-pyrimidine-3-oxide) (MW = 289.3 Da; pK_a = 4.6).

disease),[12] and alopecia.[6,13,14] These perceived benefits, of course, can only be realized through the identification and optimization of drug delivery techniques that promote follicular transport.

Improved follicular delivery has been reported following drug formulation in, for example, liposomes, microspheres, and nanoparticles,[9–11] and specific enhancement of MX delivery incorporated into nanoparticles[14] and cosolvent systems[13] has been observed. In this paper, the use of iontophoresis, a technique that employs a small electric current (not higher than 0.5 mA/cm^2) to increase and control drug delivery through the skin,[15] is explored. The rationale for this approach is that the important contribution of follicular pathways to electrically enhanced topical/transdermal delivery has been clearly demonstrated, and attributed to the lower resistance of these routes across the skin [as compared with the inter- and intracellular pathways through the stratum corneum (SC)] under the influence of a potential gradient.[16–19] The working hypothesis, therefore, is that iontophoresis can "target" MXS to the follicular route and provide an approach to the improved treatment of alopecia.

The specific aim of this research, therefore, was to determine *in vitro* the extent to which iontophoresis is able to facilitate the follicular accumulation of MXS. As a control and benchmark, the passive delivery of MXS was first determined from an ethanol–water solution and from a thermosensitive gel of appropriate viscosity for a typical topical formulation.[20] MXS iontophoresis from the same gel was then undertaken at different pH conditions to assess the added benefit possible from the application of current. A preliminary study in rats was also carried out to investigate whether a short period of iontophoresis could improve the follicular penetration of MXS *in vivo*.

MATERIALS AND METHODS

Materials

Minoxidil sulfate (99%) was kindly provided by Galena Química e Farmacêutica Ltda. (Campinas, Brazil). Ag-wire (99.99%, ø = 1.5 mm), AgCl (99.99%) and Pt-wire, all used to prepare the electrodes, were purchased from Sigma–Aldrich (Steinheim, Germany). Poloxamer®407 (polyethylene–polypropylene–polyethylene triblock copolymer) was purchased from BASF (Lutol F127, Germany); HEPES and NaCl, used in buffer preparation, were obtained from Acros (Fair Lawn, New Jersey). Ethanol was purchased from Fisher Scientific (Leicestershire, UK). The solvents used for extraction and chromatographic analysis were all of HPLC grade: methanol and acetonitrile were purchased from Fisher Scientific (Leicestershire, UK), and acetic acid from Fluka (Steinheim, Germany). The water used in all preparations was of Milli Q grade (Millipore, France).

Skin

Porcine ear skin was used in all *in vitro* experiments. The ears were obtained less than 2 h post-sacrifice of the animal from a local abattoir. The whole skin was removed from the outer region of the ear, separated from its underlying layer, dermatomed to a nominal thickness of 500 μm, and stored frozen at −20°C for a maximum of 1 month before use.

MXS Physicochemical Characterization

Minoxidil sulfate solubility in pH 7.4 HEPES buffer containing 133 mM NaCl (i.e., the receptor solution used in the skin permeation experiments; see *Passive Permeation* section), was determined at room temperature. An excess of MXS (~5 g) was added to 10 mL of buffer and overnight continuously stirred for at least 12 h. The suspension was then filtered and analyzed for drug as described below.

The possible effect of electrical current on MXS stability was evaluated before the iontophoretic studies. A 0.4 mA direct current was passed through 10 mL of a 2% (w/w) MXS aqueous solution containing 89.5 mM of NaCl via Ag and AgCl anode and cathode, respectively, for 6 h. Samples were collected before and after current passage, and the MXS concentration and pH were measured.

The pK_a of MXS was determined via titration of 20 mL of 0.01 M MXS aqueous solutions with (i) 0.1 N NaOH, and (ii) 0.1 N HCl. The pH was measured (Digimed pH meter, model DM-20) after each 0.1 mL addition of the alkali or acid solutions. The pK_a of MXS was deduced from the inflection points of plots of measured pH versus the added volume of acid or base.

Preparation of MXS Formulations

MXS was incorporated at 2% (w/w) (69.1 mM) in (i) an ethanol–water solution (60:40), and (ii) a Poloxamer® gel. In both cases, the aqueous component contained 89.5 mM NaCl. The gel was prepared by cold dispersion of 16% (w/w) polymer in a mixture of

water–propylene glycol (95:5). The pH of both formulations after incorporation of the drug was 3.5. For the gel, the pH was also adjusted for some experiments to 5.5 using a sufficient volume of a 1 M NaOH solution.

Passive Permeation

The skin was thawed and mounted in a Franz diffusion cell (diffusional area = 0.8 cm^2). The donor compartment was filled with 1 g of either the ethanol–water vehicle (pH 3.5) or the gel formulation (both pH 3.5 and 5.5), and was closed with Parafilm® to minimize evaporation. The receptor chamber was perfused continuously at 1 mL/h (Pump Pro MPL580—Watson-Marlow Bredel Pumps, UK) with a physiological buffer containing 25 mM HEPES, pH 7.4 and 133 mM NaCl, stirred at 500 rpm and maintained at 37°C by a circulating water bath (Ecoline 003; E100, Lauda, Germany). Samples were collected automatically every hour (Fraction Collector PTFCII-Pharmatest, Germany) for 6 h. At the end of the experiment, the amount of the drug that permeated across the skin, that is, the amount of MXS recovered in the receptor solution, was determined analytically as described below. A minimum of four replicates were performed.

Iontophoresis

Iontophoretic experiments were conducted following essentially the same protocol for the passive experiments above except that a direct current of 0.4 mA (0.5 mA/cm^2) was applied to the skin, which, in this case, was mounted on flow-through iontophoretic cells (LG-1088-IC; Laboratory Glass Apparatus Inc., Berkeley, California).

The anodal, donor compartment contained the gel formulation at pH 3.5 or 5.5. Ag/AgCl electrodes were prepared as previously described,[21] and a constant current was passed between the electrodes from a power supply (Kepco APH 500DM Power Supply; Kepco Inc., Flushing, New York).

Differential Tape Stripping to Determine MXS Skin Distribution

After completion of the experiment, follicular and SC penetration of MXS was differentiated using a technique that combines tape stripping with cyanoacrylate skin surface biopsies.[22,23] The skin was removed from the diffusion cell and placed onto a flat surface with the SC facing up. That part of skin, which had been in contact with the MXS donor formulation, was cleaned with an isopropanol-soaked gauze pad and then tape stripped 15 times, using Scotch Book Tape No. 845 (3 M, St Paul, Minnesota). The glistening, exposed viable layers of the epidermis indicated that more or less complete removal of the SC was achieved after these 15 tape strips. MXS content in the tapes was determined as described below after exhaustive extraction of the drug with methanol over a 12 h period.

Subsequent to SC removal, a drop of cyanoacrylate superglue (Henkel Loctite, Dublin, Ireland) was applied to the stripped skin area and covered with a further tape strip using light pressure. After total polymerization of the glue (~5 min), this tape strip was then removed and the skin surface biopsy obtained in this way contained follicular casts,[11] from which MXS was extracted with methanol and quantified.

The efficiency of recovery of MXS from the SC and the follicular material was calculated by loading tapes containing either stripped SC, or cyanoacrylate biopsies, with known quantities of MXS in methanol. After solvent evaporation, the extraction process described above was performed and the amount of MXS recovered from each matrix was determined. It was found that the MXS recoveries from SC and follicular material were 93.9 ± 1.1% and 87.6 ± 1.7% ($n = 3$ in both cases), respectively.

Preliminary *In Vivo* Studies

Preliminary *in vivo* experiments were carried out in 4-week-old male Wistar rats ("Biotério Central", University of São Paulo, Brazil). The animals were housed at 24°C–28°C, exposed to daily 12:12 h light–dark cycles (lights on at 6 a.m.), and had free access to standard rat chow and tap water. The animal protocol was approved by the University of São Paulo Animal Care and Use Committee (Authorization number: 08.1.980.53.5).

MXS penetration into the skin was investigated after passive and iontophoretic administration of the Poloxamer gel formulation containing 2% (w/w) (69.1 mM) MXS, 89.5 mM NaCl, pH 5.5. Three animals were used for each condition investigated.

The hair on the abdominal skin of the animals was trimmed 48 h before the experiments were performed. A few minutes before the experiments, the rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), and were placed on their back. An open glass chamber (1 cm^2) attached to the skin with silicone grease was used to apply the formulation (2 mL). An Ag electrode was then introduced into the formulation and maintained at least 5 mm from the skin surface by means of a plastic lid that covered the chamber. An AgCl counter electrode was applied to another part of the animal through a chamber that contained a simple pH 7.4 buffer (25 mM HEPES, 133 mM NaCl) solution. A Phoresor II (model PM 850; Iomed, Inc., Salt Lake City, Utah) delivered a constant current density of 0.5 mA/cm^2 for 20 min. Passive experiments were performed in exactly the same way without application of current. At the end of the experiments, the rats were sacrificed by carbon dioxide asphyxiation. The drug-exposed skin areas were cleaned with cotton soaked in

water. The diffusion area of the treated skin was then dissected, pinned onto a piece of Parafilm, SC face up, and tape stripped eight times. Complete SC removal was indicated by the glistening of the exposed (viable epidermal) surface. Then, the follicles were removed from the skin with cyanoacrylate biopsies. MXS was extracted from the tapes and from the hair follicles with methanol. After extraction, MXS was quantified by HPLC, as described below.

Analytical Methods

Two analytical methods were used to determine MXS in aqueous and methanolic solutions, respectively. The amount of drug in receptor samples from the passive and iontophoretic experiments was quantified by reverse-phase HPLC. 10 μL aliquots of the samples were injected into a chromatograph (model LC-2010A HT; Shimadzu, Kyoto, Japan) equipped with an automatic sample injector and a reverse-phase column (Dionex 4.0 × 125.0 mm, 5 μm). The mobile phase was a mixture of water–acetic acid–acetonitrile (70:0.1:30, v/v), pH 3.3, at a flow rate of 1.0 mL/min. MXS was detected at 285 nm. The calibration curve was linear ($r = 0.999$) for the drug over the concentration range of 0.05–1.00 μg/mL.

MXS samples from methanol extractions of skin and follicular casts were analyzed using the same liquid chromatograph but, in this case, with mass spectrophotometric detection (model LCMS-2010 EV; Shimadzu). Five microliters of samples was eluted with a mobile phase of acetonitrile–water–acetic acid (70:0.1:30, v/v), pH 3.3, at a flow rate of 1.0 mL/min. Detection was accomplished in positive mode, 1.65 kV, $m/z = 210.05$. The calibration curve was linear ($r = 0.999$) and highly selective for MXS again over the concentration range of 0.05–1.00 μg/mL. The selectivity of the method was confirmed by analyzing blank SC and follicular casts and no interference was observed.

Data Analysis and Statistics

Three to six replicates of each transport experiment were performed. Results are presented as mean ± standard deviation (SD) and expressed in terms of the quantity of MXS per unit area of skin (μg/cm^2). The slopes of the linear portions of plots of the amount of drug permeated as a function of time yielded passive and iontophoretic fluxes of MXS across the skin.

Differences in the passive MXS penetration from the two formulations tested were assessed by an ANOVA followed by non-parametric Tukey's tests. Passive and iontophoretic penetration of drug into hair follicles and into the SC involved two 2-Factor ANOVAs (where the factors examined were the pH (3.5 versus 5.5) and current (iontophoresis versus passive). Statistical significance was fixed at $p < 0.05$.

RESULTS AND DISCUSSION

MXS Physicochemical Properties

The solubility of MXS in pH 7.4 HEPES buffer at room temperature was 93.4 ± 7.1 g/L, a value which ensured that all the permeation experiments were performed under sink conditions.

The concentration of MXS in the anodal donor compartment did not decrease significantly (less than 0.7%) from the initial value after 6 h of current passage; equally, the pH of the drug solution did not change appreciably either (less than 0.2% of the initial value). Furthermore, chromatographic evaluation of the MXS donor solutions revealed no additional peaks after current application, indicating that no detectable drug degradation occurred during iontophoresis.

The pK_a of MXS was found to be 4.6 ± 0.3, which is close to that reported for MX.[24] Being a weak base MXS will present a different charge depending on the pH of the formulation tested; that is, based on this value approximately 90% and 10% of MXS is positively charged at pH 3.5 and 5.5, respectively.

Passive Skin Uptake of MXS

Table 1 shows the cumulative skin penetration of MXS and its recovery from SC tape strips and follicular casts after 6 h of passive diffusion from the two tested formulations at pH 5.5. MXS flux through the skin was only slightly higher from the ethanol–water solution (1.0 ± 0.07 μg cm^{-2} h^{-1}) than from the Poloxamer® gel (0.5 ± 0.05 μg cm^{-2} h^{-1}). In contrast, MXS recovery from the SC and hair follicles was significantly (approximately threefold; $p < 0.05$) greater following application of the gel (Table 1).

The delivery of MX from different cosolvent mixtures has recently been investigated by Grice et al.,[13] who examined the passive uptake of MX into appendages and human skin SC, from different vehicles and as a function of time. Notably, the Poloxamer® gel used in the experiments reported in Table 1 led to substantially more skin retention of MXS than any of the previously studied formulations had been able to achieve.

Table 1. MXS Skin Uptake and Penetration After 6 h of Passive Diffusion from Ethanol–Water and Gel Vehicles at pH 5.5

	Vehicle	
MXS	Poloxamer® Gel	Ethanol–Water
Receptor phase (μg/cm^2)	1.5 (±0.1)[a]	3.0 (±0.1)
Stratum corneum (μg/cm^2)	52 (±16)[a]	17 (±0.5)
Follicles (μg/cm^2)	2.8 (±0.3)[a]	0.9 (±0.2)

Data shown are the mean (±SD) of at least four replicates.
[a]Result significantly different from that obtained with the ethanol–water formulation ($p < 0.05$).

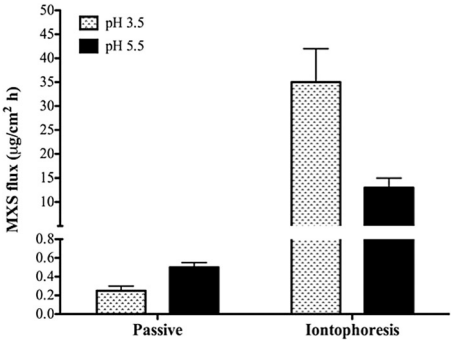

Figure 2. Passive and anodal iontophoretic fluxes of MXS through porcine skin from pH 3.5 and 5.5 Poloxamer® gels containing 2% (w/w) drug.

The passive skin transport of MXS from the gel formulation at pH 3.5 was 0.25 ± 0.05 μg cm^{-2} h^{-1}, approximately one-half of that measured at pH 5.5 (Fig. 2) and in line with the different degrees of ionization of the drug at these two pH values.

Iontophoresis of MXS

Iontophoresis significantly enhanced the penetration of MXS from the gel formulation through the skin at pH 3.5 and at pH 5.5 (Fig. 2). MXS delivery was greater at pH 3.5, consistent with the presence of a higher ionized fraction of the drug.

MXS recovery from the SC and follicles after 6 h of iontophoresis is compared with that following passive diffusion in Table 2. Although there was no significant change in the delivery to the SC, iontophoresis increased by two to four times (Table 2) the amount of MXS recovered from the follicles ($p < 0.05$). Further, from the gel at pH 5.5, a threefold increase in delivery to the follicles was observed relative to that from the pH 3.5 formulation ($p < 0.05$).

These experiments used porcine ear skin which, according to Lademann et al.,[25] represents a better *in vitro* model than excised human skin to investigate the penetration and uptake of topically applied substances into hair follicles. This is because pig skin contracts less after excision avoiding potential artifacts when the tissue is re-stretched to position it in a diffusion cell.[25] However, follicular density in human skin is generally higher than that in porcine skin although quite variable depending on body site. Specifically, porcine ear skin has approximately 20 follicles/cm^2,[26] whereas human occipital scalp has 123–354 follicles per cm^2 (mean = 239), and the chest from 36 to 85 follicles per cm^2 (mean = 66).[26,27] For the purpose of analyzing the data generated in this work, it was assumed that each cm^2 of porcine skin contained 20 follicles, thereby permitting an estimate of the amount of MXS delivered to each follicle to be calculated (Table 2). Iontophoresis improved uptake by two to four times at both pH 3.5 and 5.5 to 120 and 600 ng/hair follicle, respectively.

The pH of an iontophoretic donor formulation influences the drug ionization as well as its transport by electromigration and electroosmosis, the two key mechanisms underlying transport by iontophoresis.[15] Electromigration results from the direct interaction of the applied field with charged moieties in the system and its contribution to MXS transport is therefore greater at pH 3.5 (90% MXS ionized) than at pH 5.5 (10% ionization). However, the pH also modifies the permselective properties of the skin and the direction of the associated electroosmotic flow. The isoelectric point of porcine ear skin is ~4.4[28] meaning that, even with the pH gradient across the skin in the experiments described here (recall that the receptor is maintained at pH 7.4), the skin may be assumed to have had a net negative charge when the pH 5.5 gel was used, and to have manifested at least a partial reversal of this charge for the pH 3.5 formulation. Therefore, transport of MXS at pH 3.5 by electromigration would have been opposed (at least partially) by the cathode-to-anode electroosmotic flow, whereas that at pH 5.5 would have reflected both the anode-to-cathode electro-osmotic flow and the electromigration of the lower ionized (10%) fraction of the drug. It has been established[29–31] that electroosmosis is less efficient than electromigration and it makes sense, therefore, the delivery of MXS to the receptor was found to be lower at pH 5.5.

On the other hand, drug recovery from both the SC and follicles was higher for the pH 5.5 gel (Table 2), consistent with the higher lipophilicity of the unionized species, which predominates at this pH (an effect that has already been observed for the more lipophilic analogues of series of 5-ALA esters, NSAIDs and β–blockers[32–34]).

Table 2. MXS Recovery from SC Tape Strips and Follicular Casts After 6 h of Either Passive Diffusion or Anodal Iontophoresis from Poloxamer® Gels at pH 3.5 and 5.5

	Passive (μg/cm^2)		Iontophoresis (μg/cm^2)		Amount Per Hair Follicle (ng)	
MXS	SC	Follicles	SC	Follicles	Passive	Iontophoresis
Gel pH 3.5	25 (±6)	1.8 (±0.3)	35 (±9)	4.2 (±0.4)	90 (±15)	210 (±20)
Gel pH 5.5	52 (±16)	2.8 (±0.3)	73 (±23)	12 (±1.3)	140 (±15)	600 (±65)

Data shown are the mean (±SD) of at least three replicates. MXS penetration into hair follicles was enhanced significantly ($p < 0.05$) by iontophoresis and at pH 5.5 (two-factor ANOVA).

The situation with the follicles, however, may be more complicated as it has been demonstrated in earlier research that these "shunts" across the skin (i.e., follicles and sweat glands) are important—but not the only—contributors to the pathways of iontophoretic transport.[16,18,19,35,36] It also seems clear that the distribution of transport via, for example follicular versus nonfollicular routes, may be dependent upon the physicochemical properties of the permeant and that, for fully ionized species, the "shunt" pathways may be relatively significant participants (even though they represent only a small fraction of the total area of skin across which transport is possible[18]).

In terms of the practical significance of the results from this research, it is first important to establish whether the enhanced concentration of MXS in the follicular space achieved with iontophoresis is reproduced when the experiments are repeated *in vivo* (where the skin's microcirculation and natural, local clearance mechanisms are intact and functioning). A preliminary *in vivo* study was, therefore, conducted in rats to determine whether a short period of iontophoresis would improve the follicular penetration of MXS. Although rat skin is not the best model for the human counterpart, in terms of barrier function, as well as having a higher follicular density (289 follicules/cm^2),[37] this animal is accessible and convenient for a preliminary, proof-of-concept, *in vivo* experiment. Figure 3 shows that 20 min of anodal iontophoresis (0.5 mA/cm^2), similar to that used in the *in vitro* experiments, did not significantly increase MXS delivery to the SC as compared with passive delivery.

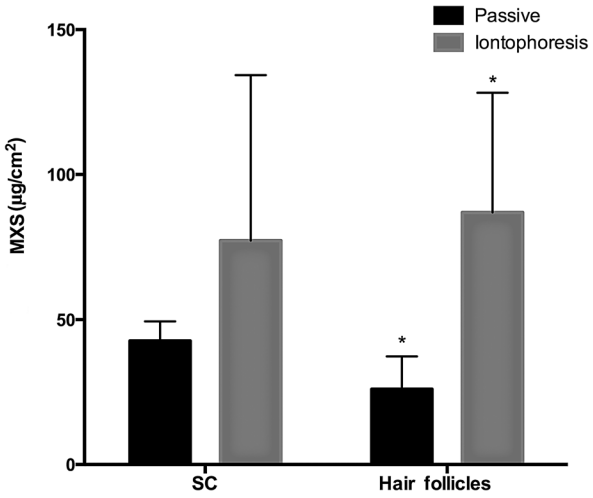

Figure 3. MXS recovery from SC tape strips and follicular casts of rat skin after 20 min of either passive diffusion or anodal iontophoresis from a Poloxamer® gel at pH 5.5. Data shown are the mean (±SD) of three replicates. MXS penetration into the rat hair follicles (*) was enhanced significantly ($p < 0.05$) by iontophoresis at pH 5.5 (ANOVA followed by nonparametric Tukey's tests).

In contrast, iontophoresis did enhance MXS penetration by 3.3-fold into the hair follicles ($p < 0.05$). It is important to point out that the quantities of MXS recovered from rat hair follicles after only 20 min of current passage were 10 times greater than those recovered from porcine skin after 6 h of iontophoresis. Differences in follicular density (follicular density in rats is 14 times higher than in porcine skin[38]) and skin thickness and composition may explain this difference. Interestingly, *in vivo* iontophoresis increased the amount of MXS delivered to hair follicles relative to passive delivery, in the same proportion (threefold to fourfold) as that found in the *in vitro* experiments.

These preliminary *in vivo* experiments suggest that iontophoresis should enhance the delivery of MXS into the follicular space in humans. However, this hypothesis requires confirmation in a skin model providing a closer representation of the follicular density[10,38,39] and properties found in sufferers of alopecia. In this respect, Eller et al.[39] reported some effects of the application site on the passive absorption of topical MX, which could be intensified during iontophoresis. Further, there is the opportunity to improve the formulation used for iontophoresis; the gels employed here have contained electrolytes in addition to the drug, providing competition to carry the applied current across the skin and decrease the efficiency of iontophoresis.[40,41] These are crucial factors to be considered before a prototypical device can be conceived.

CONCLUSIONS

Iontophoresis of MXS from simple gel formulations improved the delivery of the drug into hair follicles both *in vitro* and *in vivo*. Formulation of the gel at pH 5.5 produced the highest concentration of MXS in the follicular space. Further work is required to refine the approach and optimize the formulation properties for maximal drug delivery to the target site.

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