Permeation of topically applied Magnesium ions through human skin is facilitated by hair follicles

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Abstract. Magnesium is an important micronutrient essential for various biological processes and its deficiency has been linked to several inflammatory disorders in humans. Topical magnesium delivery is one of the oldest forms of therapy for skin diseases, for example Dead Sea therapy and Epsom salt baths. Some anecdotal evidence and a few published reports have attributed amelioration of inflammatory skin conditions to the topical application of magnesium. On the other hand, transport of magnesium ions across the protective barrier of skin, the *stratum corneum*, is contentious. Our primary aim in this study was to estimate the extent of magnesium ion permeation through human skin and the role of hair follicles in facilitating the permeation. Upon topical application of magnesium solution, we found that magnesium penetrates through human *stratum corneum* and it depends on concentration and time of exposure. We also found that hair follicles make a significant contribution to magnesium penetration.

Key words: magnesium, hair follicle, multiphoton microscopy, mag-fura-2

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Mineral based therapies such as Dead Sea therapy have been used for several centuries and have been associated anecdotally with a range of health benefits. These are mostly skin conditions where the *stratum corneum* (SC) is compromised, such as psoriasis and dermatitis [1]. In all these therapies, magnesium is believed to be the key component involved in ameliorating or subduing inflammatory response. Much evidence exists demonstrating that increased levels of magnesium through oral supplementation, or dietary intake can ameliorate inflammatory disorders [2-4]. However, in order for topically applied magnesium to be effective in treating inflammatory skin conditions, transport of its ions across the SC is a critical precondition.

Transdermal delivery is a commonly used route of administration for treatments that are intended to have both local and systemic effects. Permeability of the skin to magnesium ions

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could be dependent on pathways associated with appendages, glands, hair follicles, hydration state or integrity of the SC [5]. The barrier property of SC has been widely discussed, with the general view promulgated in the literature that the layer acts as a selective barrier to transport of ions [6, 7]. It has also been conjectured that the negatively charged SC would not allow permeation of any charged molecules such as magnesium ions. The radius of the hydrated magnesium ion has been reported to be 400 times higher than its dehydrated form [8], leading to the assertion that it is almost impossible for magnesium ions to pass through biological membranes [8, 9]. However, when we re-examined this calculation, it was found that the *volume* of the magnesium ion is 451.76 $Å^3$ [$\frac{4}{3}\pi(4.76)^3$] in the hydrated state, but based on the ionic radii of dehydrated and hydrated magnesium ions, i.e., 0.87 Å and 4.76 Å respectively [10-13], we calculated that the hydrated *radius* of the ion is only 5.47 fold ($\frac{4.76\,Å}{0.87\,Å}$) greater than its dehydrated radius. Based on our recalculation, and on published data showing systemic effects of topical magnesium [1, 5], we postulated that the hydrated magnesium ion could potentially penetrate by bulk diffusion through the 10 Å pores formed by protein subunits in the lipid membrane [5, 14], or by other means, such as hair follicles. To test this postulate, we conducted experiments to test and visualize magnesium penetration, localization and concentration in human epidermis.

In this article, we have demonstrated a method to visualise magnesium ions in human skin sections using mag-fura-2, tetrapotassium salt, a fluorescent dye that specifically binds to magnesium ions. This enabled us to identify the influx or localisation of magnesium ions in human skin. Subsequently, we varied the duration of treatment and concentrations of magnesium chloride solutions applied to the skin. We used 5 mM $MgCl_2$ solution, as it was closer to the physiological levels found in human tissues. The concentrations 52 mM and 1.9 M, even though far from physiological levels, are equivalents of $MgCl_2$ in ocean water and Dead Sea water, respectively, were used to understand the relevance of Dead Sea therapy. Moreover, we developed a novel method to plug hair follicles in an attempt to investigate possible magnesium permeation through hair follicles.

Methods

Topical magnesium solution application

We obtained excised human skin from patients undergoing abdominoplasty. We then topically applied deionised water (milliQ) (vehicle control) and 5 mM $MgCl_2$ solution (pH 7.2) for 30 minutes, at 23-25 °C using the "donor" (upper) chamber of a Franz cell. We also applied 5 mM $MgCl_2$ to skin that was tape stripped 30 times, in order to facilitate the permeation of magnesium through epidermis and represent a positive control. We treated another set of excised skin in the same way as above, with 52 mM and 1.9 M $MgCl_2$ solutions (pH 7.4 and 7.8 respectively) for 5, 15, and 60 minutes to study the effect of time and concentration in magnesium permeation.

Hair follicle plugging

We developed a novel method to plug the hair follicles on excised skin to evaluate their contribution in magnesium permeability across the skin (*figure 1*). According to this method we closely identified all the hair follicles in a marked region using a Zeiss Primostar microscope and image captured using Zeiss AxioCam Erc 5s connected to Axiocam software (Zeiss, Oberkochen, Germany). The plugging procedure involved applying 0.1 μL acriflavine solution (dissolved in milliQ water) to form a globule around the follicle and subsequently adding 0.1 μL of ethylcyanoacrylate (Loctite Super glue) (Henkel, Ohio, USA) on top of it. The polar monomers of ethylcyanoacrylate polymerize when they come in contact with the water molecules in the presence of acriflavine dye. This dye-sensitised polymerisation forms long and strong chains, thus plugging the skin and hair follicle. We then treated the plugged and unplugged skin with 1.9 M $MgCl_2$ solution for 15 minutes under the conditions described above.

Validation for hair follicle plugging method

The efficacy of this new method was tested by conducting an *in vitro* skin absorption study in a Franz diffusion cell with an effective diffusion area of 1.33 cm^2 and a 3.4 mL receptor chamber capacity. The skin obtained from abdominoplasty

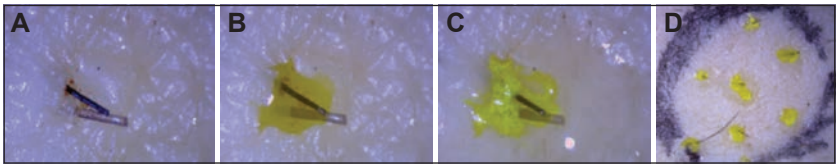

Figure 1. Novel hair follicle plugging method. Novel hair follicle plugging method to evaluate follicular contribution towards magnesium permeation. **A)** Hair follicle. **B)** Add 0.2 μL acriflavine dissolved in water (1 mg/mL) around the hair. **C)** Add equal amount of cyanoacrylate. **D)** All hair follicles plugged.

was cut into disc pieces and mounted in the Franz diffusion cell between donor and receptor compartments, with the *stratum corneum* side facing the donor chamber and the dermal side facing the receptor chamber. We used 1 mL of 3% (w/w) caffeine totally solubilized in aqueous solution as donor solution, as it is known to permeate through hair follicles and PBS buffer as receptor solution at pH 7.4 and 35 °C (physiological skin temperature). The donor chamber was then wrapped with Parafilm after addition of the solution to prevent evaporation. At different point times over 12 hours, 200 μL of the receptor solution was withdrawn and replaced with the same amount of fresh PBS buffer. The sample thus withdrawn was analysed for caffeine levels by sensitive and rapid high performance liquid chromatography (HPLC), consisting of Shimadzu SIL-20 a HT, CBM-20A system controller, a SPD-20A detector, LC-20AD a pump and an auto injector. The mobile phase of caffeine (95% water, 2% acetonitrile, 2% tetrahydrofuran and 0.5% acetic acid) was pumped across the system at 1 mL/min flow rate. The column used was Phenomenx Luna 5 μm, c18 (150 mm × 4.6 mm); and caffeine was detected at 273 nm (*figure 2*).

Histochemistry staining

After treating the skin with the magnesium solutions, the pieces were embedded in OCT and 60 μm cryosections section were obtained. The sections were immediately stained with 30 μL of Mag-fura-2, tetrapotassium salt (Biotium, Fremont, CA, USA) (10 μg/mL) and incubated for 5 minutes in a light-proof chamber. The dye was washed off with milliQ water for 10 seconds. The section was then mounted on the slide.

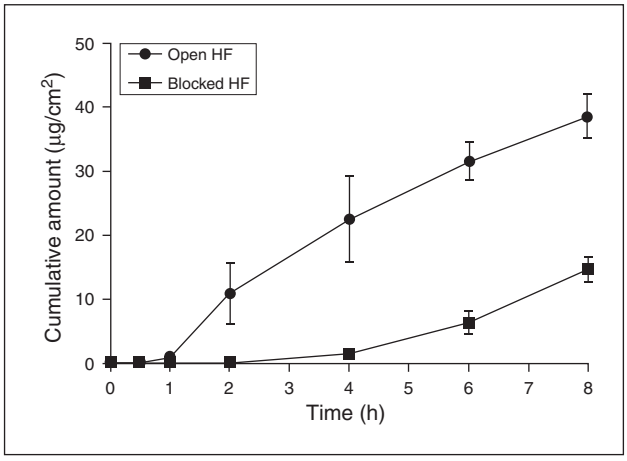

Figure 2. Validation for hair follicle plugging method. Test of the effectiveness of hair follicle plugging method by topical application of 3% caffeine solution on both normal skin (open hair follicle) and plugged hair follicle over 8 hours using a Franz cell setup. The resulting transdermal permeation of caffeine was sampled every hour from the receptor chamber of the Franz cell and evaluated using HPLC.

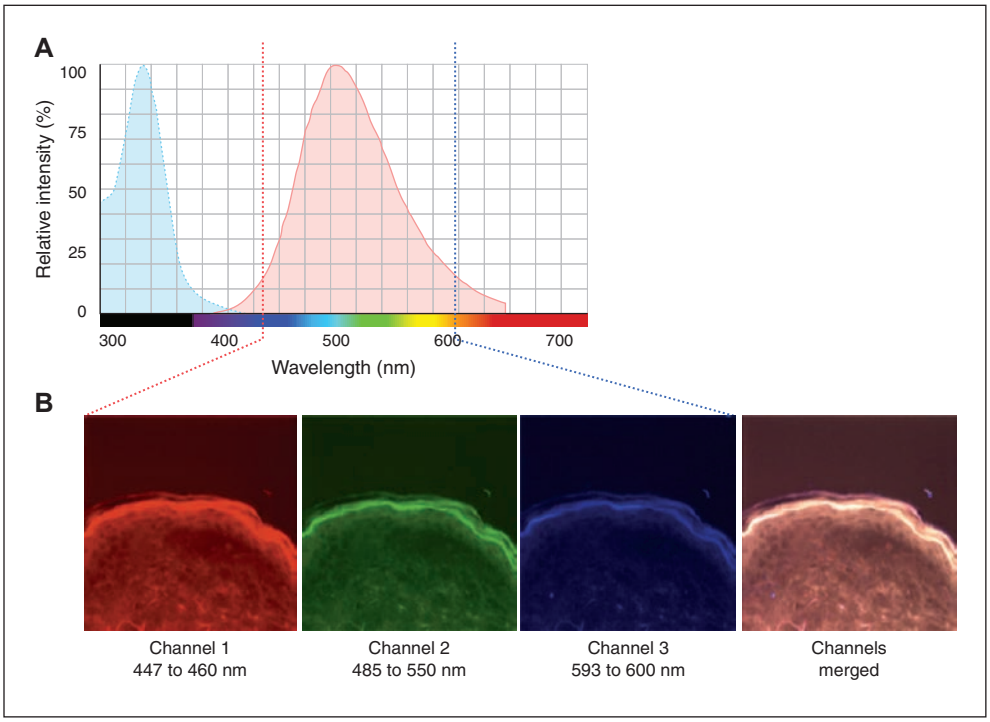

Figure 3. Imaging Mag-fura-2 fluorescence using multiphoton microscopy. **A)** Excitation (320 nm) and emission spectra (520 nm) of mag-fura-2, tetra potassium salt. **B)** We used a LaVision Biotec Nikon multiphoton system with a tunable titanium Sapphire laser to visualize the skin sections. Excitation wavelength was 740 nm and emission was detected using three non-descanned filters: 447 to 460 nm, 485 to 550 nm, and 593 to 600 nm.

Multiphoton Microscopy

We used a LaVision Biotec Nikon multiphoton system with a tunable titanium Sapphire laser to visualize the magnesium skin sections (*figure 3*). The slides were imaged with excitation wavelength was 740 nm and emission was detected using three non-descanned filters: 447 to 460 nm, 485 to 550 nm, and 593 to 600 nm, and resulting images were analysed using ImageJ to measure CTCF (corrected total cell fluorescence) of Mag-fura fluorescence in the epidermis. A 20 × 0.95 NA water immersion objective (Olympus) was used. CTCF was calculated using the formula:

$$\mathrm{CTCF} = \mathrm{Integrated\ density}_{\mathrm{Viable\ epidermis}} - \left(\mathrm{Area}_{\mathrm{Viable\ epidermis}} \times \mathrm{Mean\ fluorescence}_{\mathrm{Background}}\right)$$

Results

Topically applied magnesium permeates through human stratum corneum

Cryosections of human skin pretreated with 5 mM $MgCl_2$ solution for 30minutes showed increased fluorescence intensity relative to sections that were not pretreated, when stained with Mag-fura-2 dye. This increase was observed in both *stratum corneum* intact, and tape-stripped skin indicating the permeability of stratum corneum to magnesium ions. We also found a greater increase in fluorescence intensity in tape-stripped skin that was subsequently treated with 5 mM $MgCl_2$. In control skin sections not treated with $MgCl_2$, we observed that the Mag-fura-2 dye emitted some background fluorescence upon binding

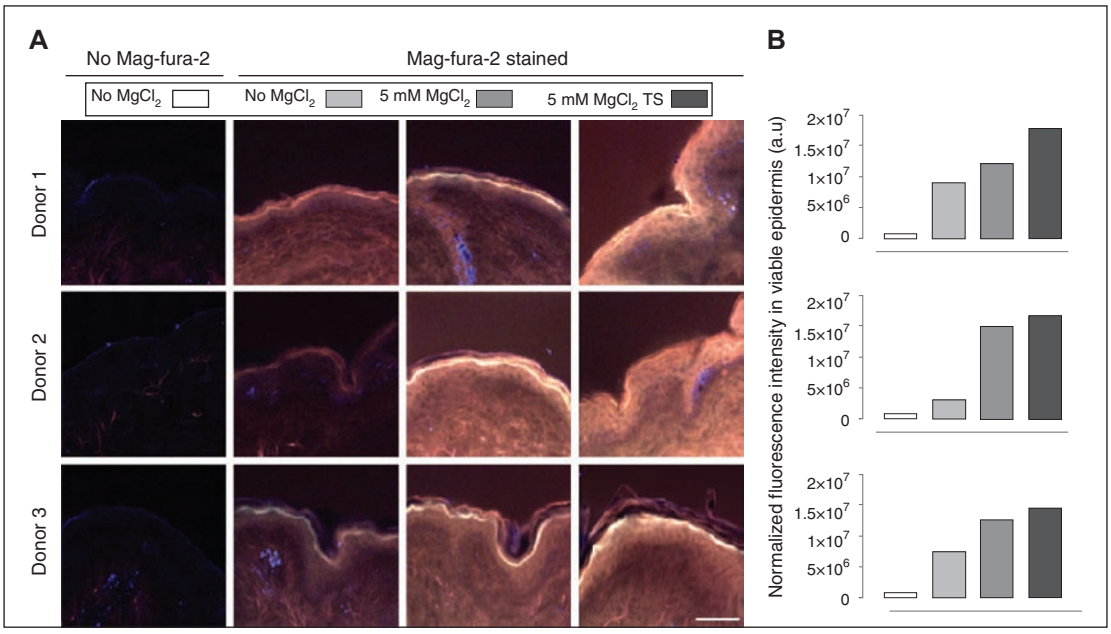

Figure 4. Magnesium ions penetrate through skin but the extent depends on stratum corneum thickness. **A)** Images from three donors showed increased fluorescence in tape stripped (TS) and SC intact skin treated with 5 mM $MgCl_2$ solution for 30 min compared to skin untreated with $MgCl_2$. **B)** Histograms showing normalized fluorescence intensity in viable epidermis in each donor. Scale bar = 50 μm.

with endogenous ions. We have also estimated the magnesium levels present in our sections by comparing the CTCF to the CTCF of skin equilibrated with known concentrations of $MgCl_2$ solution. The greater fluorescence intensity and its progressive distribution through the viable epidermis in the tape-stripped skin, compared to unstripped skin, indicates that the *stratum corneum* offers some resistance to the permeation of magnesium ions, as expected. However, where the *stratum corneum* is intact, permeability to magnesium ions still takes place, with the level of penetration a function of the thickness of the *stratum corneum* (which varies between donors) (*figure 4*).

Magnesium permeability varies based on concentration and time of exposure

Higher fluorescence intensities relative to sections untreated with $MgCl_2$, were observed at 15 and 60 minutes after the skin was exposed to 1.9 M $MgCl_2$ concentration ($p<0.05$, one way ANOVA, Tukey's test) and after 60 minutes of exposure to 52 mM $MgCl_2$ ($p<0.01$). This indicates that magnesium penetration increases with time, and higher concentration enhances permeation. However, although there is a trend towards an increase in magnesium penetration 5 minutes after exposure to either 52 mM or 1.9 M $MgCl_2$ it was not significant ($p>0.05$). After 15 minutes treatment with 1.9 M $MgCl_2$ there was a significant increase in measured magnesium penetration ($p<0.05$) and a further increase at 60 minutes (*figure 5B*). After treatment with 52mM $MgCl_2$, a trend to increase was apparent after 15 minutes of exposure, however, a significant increase was only observed at 60 minutes at this lower concentration ($p<0.05$) (*figure 5*).

Hair follicles have significant contribution towards magnesium penetration through skin

We developed a novel method to plug the region surrounding the hair follicle in order to test the role of hair follicles in ion transport. To validate

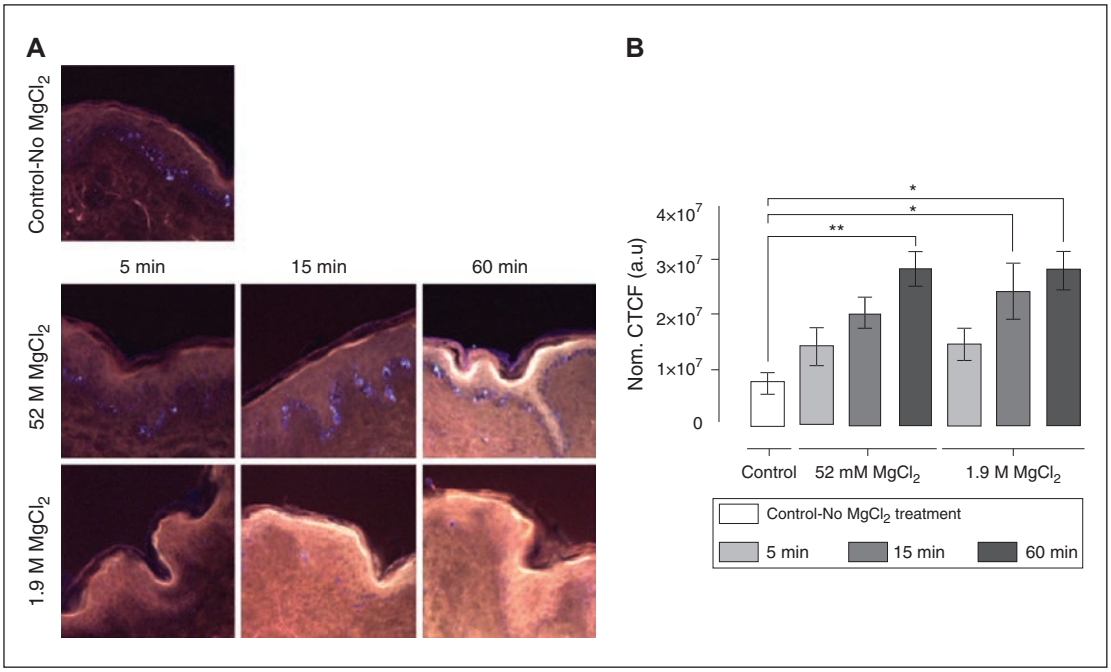

Figure 5. Magnesium ion permeation at varying times and concentrations. **A)** Representative images indicating penetration of magnesium ions at varying times and concentrations. **B)** The skin was treated with 52 mM and 1.9 M MgCl2 solutions for 5, 15 and 60 minutes. (* $p<0.05$, ** $p<0.01$).

the plugging method, we tested caffeine penetration through plugged and unplugged skin. We found reduced caffeine penetration through skin when hair follicles were plugged, compared to unplugged skin. The plugging method was effective, as there was no permeation in the first two hours, and very limited permeation even after four hours (*figure 2*). We then proceeded to use this method to test magnesium penetration through hair follicles. Magnesium penetration

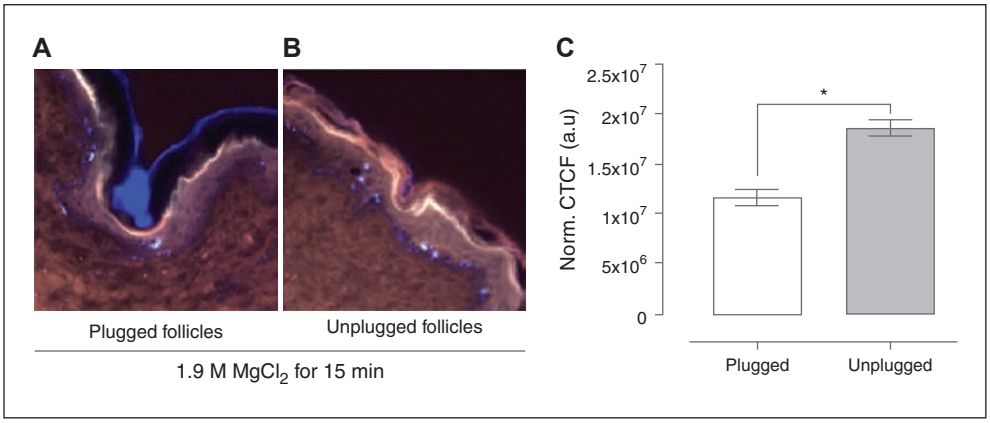

Figure 6. Hair follicles significantly contribute to magnesium permeation. **A)** Plugged skin (plugged region shown in blue). **B)** Unplugged skin sections allow penetration of magnesium ions. **C)** Increased penetration of magnesium ions in unplugged skin, (* $p<0.05$).

was significantly decreased in the sections of skin that were plugged, compared to penetration through unplugged skin (*figure 6*). This indicates that hair follicles act as a major route of penetration of magnesium ions through the skin. We also observed fluorescence emission from the acriflavine-cyanoacrylate complex used to plug the hair follicle. This aided with acquisition of images at the plugged region, which is difficult to do without this local labelling.

When 1.9 M $MgCl_2$ solution was topically applied over plugged and unplugged skin for 15 minutes, we found that Mag-fura-2 fluorescence intensity was higher in unplugged skin sections, demonstrating that hair follicles contribute significantly to magnesium permeation (*figure 6*).

Discussion

The treatment solution used in our experiments was magnesium chloride ($MgCl_26H_2O$) which has been used for several years for therapeutic purposes. It has been reported to have greater effectiveness and reduced toxicity, compared to salts with other anions [15]. Once dissolved it dissociates into magnesium and chloride ions. The question of fundamental importance is whether ions are able to traverse, by any mechanism, the *stratum corneum* layer of skin, which is negatively charged [16-18].

Figure 4 illustrates the ability of magnesium ions to penetrate through both intact skin and tape-stripped skin, although the penetration is more rapid in the latter. This confirms our hypothesis that magnesium ions could permeate through skin. Utilization of a method to indirectly stain magnesium ions in the various layers of skin sections further allowed us to investigate the penetration profile under prolonged exposure and increased concentrations of magnesium chloride solution. We observed that with the application of the 1.9 M $MgCl_2$ solution Mg^{2+} ions penetrated rapidly, that is, within 15 minutes of treatment. In the case of the 52 mM $MgCl_2$ solution, permeated Mg^{2+} was visible only after 60 minutes application to the skin. According to Fick's law of diffusion, ion flux moves from regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient [19, 20]. Thus the observed increase in fluorescence intensity results from the movement of magnesium ions from a region of high concentration to low concentration, with more rapid movement at higher external concentrations. However, mechanisms other than simple diffusion could be operating.

To further investigate the route of penetration of magnesium ions, we topically applied $MgCl_2$ solution onto equally marked out areas in excised skin, with and without plugging of the hair follicles. We observed a significantly higher fluorescence intensity indicating magnesium penetration when the hair follicle orifices were open. The artificial blocking of the hair follicle orifices ensured they were excluded as ion penetration routes. Consequently, Mg^{2+} should only pass through the interfollicular epidermis and its lipid domains, and possibly through the sweat glands. Unlike the previous methods of hair follicle plugging with a microdrop of special varnish-wax-mixture [21], we used a combination of acriflavine dye and cyanoacrylate. The resulting polymerization reaction of cyanoacrylate molecules when they contacted acriflavine solution formed a rigid bond around the hair follicles blocking the orifice [22]. We also harnessed the fluorescence of acriflavine to visualize the plugged region of the follicle, along with Mag-fura-2 fluorescence when observed under the multiphoton microscope. In conclusion, we demonstrate that magnesium ions are able to penetrate through barrier-compromised SC, and, at a slower rate, through SC intact skin. We also found that hair follicles contribute significantly to the permeation of magnesium ions (an approximately 40% increase in Mag-fura-2 CTCF over 15 minutes (*figure 6C*). Our work establishes that magnesium ions are able to traverse the *stratum corneum* in a time dependent manner, and that this process is facilitated by hair follicles.

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Disclosure

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Human Research Ethics Clearance: This research was approved by the University of Queensland and QPAH Ethics Committees: HREC/16/QPAH/064, UQ HREC number 2008001342.

Statement of Author Contributions. All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; NCC, WYS and YHM conducted the experiments. NCC, RTB, JEG and MSR wrote the manuscript.

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