EFFECT OF TOPICAL MAGNESIUM APPLICATION ON EPIDERMAL INTEGRITY AND BARRIER FUNCTION

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Abstract

Dead Sea therapy is one of the oldest forms of treatment for skin disease and some chronic inflammatory diseases like arthritis and psoriasis. Much of the research to date has attributed the clinical effects of Dead Sea therapy to its mineral composition; mostly to magnesium salts. Magnesium salts, such as magnesium sulphate (Epsom salts), have long been used as a spa product and as a therapeutic to manage clinical conditions. The rationale of this PhD research was to understand the role of topically applied magnesium ions in epidermal integrity of human skin. We compartmentalized this research project into studying physiological, metabolic and proteolytic changes occurring at different layers of the skin. To begin with, we investigated the permeability of magnesium ions through stratum corneum. In Chapter 3, we developed a method to stain skin sections using mag-fura-2, a fluorescent dye that specifically binds to magnesium ions. When magnesium chloride (MgCl$_2$) solution was topically applied on normal and tape stripped skin, we observed that it is possible for magnesium ions to permeate through stratum corneum. We also varied the concentration and time of exposure of MgCl$_2$ solutions and found that at higher magnesium concentration, permeability is faster and penetration increases with progression of time. To further characterize permeability of the skin to magnesium, we blocked individual hair follicles using a novel method and subsequently treated skin with MgCl$_2$ solution. We found that hair follicles significantly contribute towards the permeation of magnesium ions. In Chapter 4, the hypothesis tested was that magnesium accelerates barrier recovery. Experiments were conducted on volunteers who did not have any history of skin conditions. We caused stratum corneum disruption on their volar forearm through tape stripping and subsequently treated that defined region with different salt solutions. The parameters we considered for understanding barrier function were transepidermal water loss, skin hydration and skin pH over 96 hours with repeated treatment at 24-hour intervals. We found that treatment with a variety of salt solutions, including magnesium chloride did not aid in lowering the TEWL to levels pre-tape stripping and no changes to skin pH were observed. However, we observed that treating the skin with 1.9 M magnesium solution nearly 2 fold (p < 0.05) increased hydration at 6 hours, relative to baseline at time zero, whereas water treatment increased hydration only 1.2 fold, and the hydration returned to normal levels within 24 hours in both cases. Skin barrier formation is a continuous process and occurs as a result of differentiating epidermal cells subsequently leading to terminal differentiation. In the case of disrupted barrier, the epidermal cells differentiate to replenish the lost layers of stratum corneum. Therefore as an extension to Chapter 4, experiments in Chapter 5 involved understanding the metabolic changes occurring in all three layers of the epidermis – granulosum, spinosum, basale. We used multiphoton tomography coupled with fluorescent lifetime imaging microscopy to observe
volunteers 0, 24 and 96 hours. Defined regions of volar forearm were treated with water or 1.9M MgCl₂ solution on normal and tape stripped skin at 24-hour intervals over 96 hours. In all the three layers, we observed that redox ratio significantly decreased (p<0.05) immediately after tape stripping and significantly increased (p<0.05) after 24 hours in the magnesium treated site in granulosum and spinosum layer, when compared to the normal skin site. This increase is indicative of changes in metabolic activity in the granular layer, suggestive of signs of differentiation and cell proliferation. However, we did not observe any significant change in NAD(P)H/FAD lifetime nor in the ratio of nuclear area to total cellular area (N/C).

In order to gain more understanding about the role of magnesium in terminal differentiation, as reported in Chapter 6 we studied the filaggrin (FLG) regulation under varying concentrations of salt solutions and due to stimulation by external factors. We found that treating the excised skin with high concentrations of magnesium and calcium chloride solutions resulted in increased levels of FLG after 30 minutes of treatment. The increase in FLG levels could be due to the interaction of divalent ions with the binding sites present in the profilaggrin, subsequently exposing the sites for cleavage by enzymes such as caspase-14. Also, inflicting damage to the epidermis in excised skin using 2,4-dinitrochlorobenzene (DNCB) and tape stripping increased FLG levels when incubated for 20 hours. Also, we found that treating the skin with these salt solutions prior to stimulating them with DNCB or TS did not allow FLG levels to peak above those observed with unstimulated skin. Increased levels of FLG as a result of salt treatment could have a possible role in preparing the epidermal cells for terminal differentiation eventually leading to barrier formation. We applied salt solution treatments on the volar forearm of human volunteers to measure physiological parameters and found a significant increase in hydration, but not skin pH when compared to the control site. This ensured that the changes observed in FLG levels are not due to pH changes.

From these results we conclude that magnesium ions can penetrate through healthy skin with intact stratum corneum, with significant contribution from hair follicles. At high magnesium concentrations the permeation of Mg²⁺ into the epidermis increases with time and is significantly higher than the baseline concentration of Mg²⁺ in the epidermis of untreated controls after 15 minutes of exposure. Even though magnesium treatment caused an increase in skin hydration at 6 hours, we did not find effects on TEWL or skin pH. Magnesium treatment also increased the redox ratio of cells in granulosum and spinosum layer indicating changed metabolic activity. We also found that magnesium ions could be involved in proteolytic cleavage of profilaggrin preparing the epidermal cells for terminal differentiation and providing a possible preventive effect against external stimulants.
Declaration by author

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Contributions by others to the thesis

Prof Ross T Barnard - Provided input on the conception and experimental design of the project. He also assisted in discussion and interpretation of the data, and with correcting the whole thesis.

Professor Michael S Roberts - Provided input on the design, interpretation of experiments and made available his knowledge of skin-biology, and provided access to his equipment and facilities.

Dr Jeff E Grice - Provided day-to-day guidance, input on the experimental designs and interpretation of data in all experiments.

Dr Yousuf H Mohammed - Helped develop method for topical application of salt solutions and hair follicle plugging.

Dr Washington Y Sanchez - Helped develop methods for experiments that needed multiphoton microscopy and image processing.

Dr David Liu - Helped with planning in vivo experiments on human volunteers.

Ms Eman Abd - Conducted an in vitro skin absorption study to validate hair follicle plugging method.

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ABBREVIATIONS

Ca$^{2+}$ Calcium ions
CaCl$_2$ Calcium chloride
DNCB 2, 4- dinitrochlorobenzene
FAD flavin adenine dinucleotide
FLG Filaggrin
FLIM fluorescent lifetime imaging microscopy
Mag-fura-2 magnesium- furaptura -2, tetrapotassium salt
Mg$^{2+}$ Magnesium ions
MgCl$_2$ Magnesium chloride
MPT multiphoton tomography
NaCl Sodium chloride
NAD(P)H nicotinamide adenine dinucleotide phosphate
OCT optimal cutting temperature
SB stratum basale
SC stratum corneum
SG stratum granulosum
SS stratum spinosum
TCSPC time-correlated single photon counting
TEWL transepidermal water-loss
TS tape stripping
CHAPTER 1: INTRODUCTION

1.1. LITERATURE REVIEW

Over the last two decades, several systematic studies have been conducted in an effort to understand the effect of magnesium ions (Mg\(^{2+}\)) in improving skin disorders. This literature review briefly elaborates the importance of magnesium in the biological system and its role in affecting inflammatory processes. We further describe the structure, barrier function and metabolism of human skin. We have collated literature about how magnesium have been widely used as for topical application. We also introduce the working principle of multiphoton microscopy, which has been extensively used in this PhD research.

1.1.1. Importance of magnesium in the biological system

Magnesium is a micronutrient required for normal growth and development. Magnesium ions bind to macromolecules and cellular membranes. Mg\(^{2+}\) is known to affect cellular functions, including the transport of potassium and Ca\(^{2+}\), modulation of signal transduction, cell proliferation and energy metabolism (Altura BM et al. 1995; Grubbs RD et al. 1987; Quamme GA et al. 1997; Shils ME et al. 1994). Numerous clinical disorders such as high blood pressure (Dickinson et al. 2006; Kass L et al. 2012), diabetes mellitus type 2 (Simmons et al. 2010), osteoporosis (Rude et al. 2009), and migraines (Sun-Edelstein C et al. 2009) have been associated with magnesium deficiency. Early stages of Mg\(^{2+}\) deficiency can be characterized by a wide range of symptoms such as anorexia, vomiting, weakness, paresthesia, muscular cramps, and irritability and impaired cognitive functioning reflected by a decreased attention span. Mg\(^{2+}\) deficiency is related to poor dietary Mg\(^{2+}\) intake, often as a result of lifestyle changes, leading to the aforementioned health disorders (Altura BM et al. 1991; Britton et al. 1994). The clinical disorders could be a consequence of stress response (Mazur et al. 2007; Petrault et al. 2002; Libby 2007).

1.1.2. Role of magnesium in affecting inflammatory process

Inflammation is a primary reaction brought about by magnesium deficiency, creating oxidative stress and subsequent immune stress. Mg\(^{2+}\) deficiency triggers inflammatory responses, including abnormal calcium homeostasis, activation of NMDA receptors, release of neurotransmitters, membrane oxidation and activation of NFκB (Mazur et al. 2007; Malpuech-Brugère et al. 1998).
Some of the important inflammation related clinical disorders such as asthma, arthritis, atherosclerosis and neuroinflammation, known to be caused or exacerbated by magnesium deficiency, are outlined below.

The pathogenesis of asthma, a chronic inflammatory disorder involves activation of NFκβ and, expression of pro-inflammatory cytokines, chemokines and inflammatory mediators (IFN-γ and ROS)(Bochner 1994). In this pathological condition, NFκβ activation leads to dysregulation of cytokines and infiltration of inflammatory cells such as mononuclear cells and fibroblasts in the lung(Bochner 1994; Yamamoto & Gaynor 2001). Indeed, increased NFκβ activity has been observed in the airways of asthmatic patients(Yamamoto & Gaynor 2001). Dietary Mg²⁺ intake has shown direct beneficial impact on lung function, airway reactivity and respiratory symptoms (Britton et al. 1994).

The synovial fluid from patients with rheumatoid arthritis contains elevated levels of TNFα (an activator of NFκβ), which is important in the pathogenesis(Yamamoto & Gaynor 2001; Feldmann & Maini 2008). Mg²⁺ deficiency can lead to lipid peroxidation and membrane oxidation, which in turn activates the NFκβ pathway(Mazur et al. 2007; Altura et al. 2003). Activation of inflammatory responses due to Mg²⁺ deficiency causes chronic inflammation leading to different types of arthritis, depending on the site of NFκβ activation.

Studies conducted in humans indicate that low Mg²⁺ intake and blood plasma concentration are linked with enhanced risk of atherosclerotic disease (Ma et al. 1995). Atherosclerosis is currently classified as an inflammatory disease, having interactions between modified lipoproteins, macrophages, T lymphocytes and the components of arterial walls (Ross R 1986), leading to the development of atherosclerotic lesions. Experimental results suggest regression of such lesions and suppression of atherogenesis in low-density lipid receptor deficient mice fed with Mg²⁺ supplement (Hellerstein et al. 1957; Whelton & Klag 1989). Infusion of Mg²⁺ at supraphysiological concentrations causes vasodilation of coronary arteries and systemic vasculature, antiarrhythmic effects and platelet inhibition (Altura BM et al. 1990). Studies also show dietary administration of Mg²⁺ attenuates atherosclerotic lesions by lowering serum cholesterol and triglycerides in cholesterol fed animals (Mazur et al. 2007). On the basis of these studies, it appears that Mg²⁺ concentration regulates lipid metabolism and reduces atherosclerosis in animal models (Mazur et al. 2007).
Even though the immune system and, in particular, the inflammatory response operates systemically, the inter-relationship between inflammation occurring in the nervous system and systemic inflammation needs to be better understood. It is widely accepted that several neurological disorders occur as a result of an inflammatory component (Akiyama et al.; McGeer & McGeer 2004). There are several drugs undergoing test that are posited to act by reducing neurodegeneration, at least in part through inhibition of the inflammatory response of glial cells (Ralay Ranaivo et al. 2006). However, these drugs exert their effect throughout the body, resulting in global immunosuppression (Giulian 1999). It would be ideal for such drugs to specifically target the glial cells and control inflammation in the brain without producing systemic immunosuppression(Ralay Ranaivo et al. 2006; Craft et al. 2005). Studies conducted on selective suppression of neuroinflammation, without producing extra-neural inflammation has yielded some positive outcomes (Ralay Ranaivo et al. 2006).

Mg\textsuperscript{2+} administration could be an effective way to treat neurodegenerative diseases by exploiting its Ca\textsuperscript{2+} channel antagonism to achieve selective suppression of neuroinflammation (Altura BM et al. 1983; Iseri L et al. 1984). If treatment for neurodegenerative diseases involved administration of Mg\textsuperscript{2+} locally to the brain, it could conceivably avoid the generalised stress on the immune system that is caused by non-targeted anti-inflammatory drugs. Experiments conducted on rat ischemic and excitotoxic brain injury models shows the activity of Mg\textsuperscript{2+} as a neuroprotective agent(Saris et al. 2000; Marinov et al. 1996; McDonald et al. 1990). This is achieved by Mg\textsuperscript{2+} blocking NMDA receptors to enhance regional cerebral blood flow to ischaemic areas of the brain, also Mg\textsuperscript{2+} inhibiting entry of Ca\textsuperscript{2+} into the cells through voltage-operated and receptor-operated channels (Huang QF et al. 1994).

The nervous and immune systems interact bi-directionally and Mg\textsuperscript{2+} deficiency is known to induce a systemic stress response by activating neuro-endocrine pathways, modifying production and activity of neuromediators such as acetylcholine, catecholamines, and substance P. Neuromediators, along with stress under conditions of magnesium deficiency, have well established effects in the progression of both local and systemic inflammatory responses(Weglicki et al. 1994). Administration of Mg\textsuperscript{2+} has been shown to block Ca\textsuperscript{2+} traffic through cell surface channels, acting as a broad inhibitor of neuroinflammation(Lee et al. 2011; Klegeris et al. 2005). Elevated systemic levels of Mg\textsuperscript{2+} have been shown to reduce damaging consequences of Ca\textsuperscript{2+} induced neuroinflammation in Parkinson’s disease and Alzheimer’s disease(Lee et al. 2011).
Taking into consideration that magnesium ions play such an important role in controlling inflammatory disorders, restoring magnesium levels in the biological system through topical application could be a viable preventive measure.

1.1.3. Topical magnesium application

Mineral based therapies such as Dead Sea therapy that have been in practise for several centuries point to numerous anecdotal health benefits. These are mostly skin conditions where the stratum corneum is compromised, such as psoriasis and dermatitis (Shani J, Eevn-paz Z, Avrach W.W., Rubinstein N., Livshin R. Justesen N.P.B. 1991).

Dead Sea therapy is one of the oldest forms of treatment for skin disease and some chronic inflammatory diseases like arthritis and psoriasis (Sukenik et al. 2006). Much of the research to date has attributed the clinical effects of Dead Sea therapy to its mineral composition; mostly to magnesium salts (Shani J, Eevn-paz Z, Avrach W.W., Rubinstein N., Livshin R. Justesen N.P.B. 1991; Proksch E, Nissen HP, Bremgartner M 2005). Magnesium salts, such as magnesium sulphate (Epsom salts), have long been used as a spa product and as a therapeutic to manage clinical conditions (Durlach et al. 2005).

In all these therapies, magnesium is believed to be the key component involved in ameliorating or subduing inflammatory response. There are several evidences that suggest increased levels of systemic magnesium through oral supplementation or through diet can prevent a range of inflammatory disorders (Malpuech-Brugère et al. 1999; Mazur et al. 2007). However, the effect of topical magnesium application on barrier function and epidermal integrity of human skin is less understood. In order for topically applied magnesium to be effective in treating inflammatory skin conditions, transport of its ions across the stratum corneum is a critical precondition.

A clinical study was conducted on 30 atopic dermatitis candidates, in which subjects were tested over six weeks for transepidermal water loss (TEWL), skin hydration, skin redness and skin roughness (Proksch E, Nissen HP, Bremgartner M 2005). Upon treating one of their arms with a 5% Dead Sea salt solution at 38°C - 42°C and the other arm with tap water (38°C - 42°C) as control, significant improvement, with reduction in atopic dermatitis symptoms in the Dead Seas salt group was shown (Proksch E, Nissen HP, Bremgartner M 2005).
1.1.4. Understanding human skin physiology

Skin is the largest organ in the human body accounting for 7% body weight, and is also the organ that is most exposed to the external stress and foreign particles (Baden & Goldsmith 1972). The skin not only acts as protective barrier but also plays a vital role in maintaining homeostasis through physiological and immunological processes (Marks 2004). The structure of skin is broadly classified into three main layers - the epidermis, the dermis, and the subcutaneous tissue (Masters 1997).

The outermost layer is stratum corneum that protects epidermis, is formed due to cornification of granular cells. In normal skin, the SC forms the outermost layer, formed by continuous replacement from the newly differentiated daughter cells of keratinocyte stem cells, displacing outwards (Nohynek et al. 2007). The stratum corneum forms the outermost layer of the epidermis and the skin as a whole, and is formed by the continuous replacement from the newly differentiated daughter cells of keratinocyte stem cells displacing outwards (Denda 2000). During terminal differentiation, exocytosis of lipid containing granules called lamellar bodies generates intercellular lipids (Denda 2000). In response to external stresses leading to damage, this continuous process leads to the maintenance of the stratum corneum (Denda 2000; Albert, M., Kligman 1983).

The epidermis is made of several layers of cells at different stages of differentiation and is about 120 μm thick containing 70% of the total water of the skin (Forslind et al. 1997; Egawa et al. 2007; Marks 2004). The major cell types that are found in the epidermis are keratinocytes (90-95%), along with melanocytes, Langerhans cells, and Merkel’s cells (Masters & So 2001). The epidermis is further arranged as stratum granulosum, stratum spinosum and stratum basale which is followed by the dermal layer. The epidermis also contains, nerve endings, hair follicles, and sweat glands thus integrating the skin along with the nervous and immune system, in order to achieve homeostasis (Masters & So 2001). The primary source of autofluorescence in epidermal cells are NAD(P)H, which can be harnessed to study metabolic changes occurring in this layer (Masters 1997).
Figure 1.1: Illustration of different layers of human skin.

Such a complex cellular arrangement is equally complemented by a complex circulatory system. Blood flow to the skin provides nutrition and regulates body heat through constriction and dilation of blood vessels. The circulatory system constitutes three major types of blood vessels – arteries, capillaries and veins. The subcutaneous venous plexus plays a major role in the conduction of heat and contains a major fraction of the cutaneous blood volume (Volmerhaus B et al. 2013).

1.1.5. Transdermal route for magnesium ions

Transdermal absorption is a potentially important route of transport for components that are involved in biological processes (Brisson 1974). Transport of magnesium ions (Mg$^{2+}$) through channels present in the skin is a critical precondition for the function of topical applications in treating skin or inflammatory disease. Even though much has been established in the area of cutaneous permeation and transdermal absorption in general (Winkelmann 1969; Albert, M., Kligman 1983; Brisson 1974), the transdermal absorption of Mg$^{2+}$ and subsequent interaction with cellular and subcellular mediators of the immune response remains to be clearly established.
Transdermal delivery is one of the important and well-characterised routes of administration for treatments that have local and systemic effects. Permeability of magnesium ions could be dependent on pathways associated with appendages, hydrated condition of skin and integrity (or lack thereof) of the stratum corneum (Chandrasekaran et al. 2014). The main pathways involved in transport of substances across the stratum corneum contributing to percutaneous absorption are, bulk diffusion, shunt diffusion and the intercellular route (Brisson 1974). Lipid-soluble substances penetrate through the lipid-rich membrane. Small, water-soluble molecules are able to enter through the 10Å pores created by protein subunits in the lipid membrane. This is known as bulk diffusion (Albert, M., Kligman 1983; Brisson 1974). Another means of diffusion is through the pilosebaceous units and sweat glands referred as shunt diffusion. Since the stratum corneum extends only superficially, it can be assumed that once a substance crosses this barrier, there is no substantial hindrance to its transport thereafter (Winkelmann 1969). The intercellular pathway is another route of transport enabling electrolyte movement through hydrophobic lipid membranes (Brisson 1974).

Stratum corneum in its normal structural conformation would be expected to repel Mg$^{2+}$. However, under hydrated and increased temperature conditions, the scenario changes, enabling permeation by such ions. Subsequently, the transport of the permeated Mg$^{2+}$ ions between cells is assisted by transmembrane proteins (Goytain & Quamme 2005; Sahni et al. 2007). For example, SLC41A2, a cell surface transmembrane protein with its N-terminus outside and C-terminus inside the cell membrane, is responsible for magnesium transport across the plasma membrane (Sahni et al. 2007).

Functional and topological studies conducted on epidermal cells using immunostaining have demonstrated a plasma membrane localisation of murine SLC41A2. The N-terminus of this protein, accessible to extracellular components, is involved in transcellular movement of Mg$^{2+}$, which is in turn required for homeostasis, cell growth and neuronal function (Sahni et al. 2007). The human SLC41A1 also functions as Mg$^{2+}$ transporter involved in magnesium homeostasis in epithelial cells (Sahni et al. 2007).

One important mechanism by which Mg$^{2+}$ intracellular homeostasis in humans is facilitated, is by the protein Transient Receptor Potential Melastatin 7 (TRPM7) (Chen et al. 2012). Knockout of TRPM7 in DT40 B cells (derived from an avian leukosis virus induced bursal lymphoma in a white leghorn chicken) resulted in lowered intracellular Mg$^{2+}$ and inhibition of cell proliferation (Sahni & Scharenberg 2008). Under stress (apoptic stimuli), TRPM7 is upregulated, releasing reactive oxygen species (ROS) from the cells to trigger local inflammation. Furthermore, increased intracellular ROS concentration results in increased Mg$^{2+}$ concentration in TRPM7-knockout cells. This increase in intracellular Mg$^{2+}$ in-turn results in increased expression of SLC41A2, resulting in
transport of Mg\textsuperscript{2+} into the cell (Chen et al. 2012). Meanwhile, another recent study suggests that sphingolipids may be homeostatic regulators of extracellular magnesium ion concentration influx and transport, and magnesium ion content in vascular muscle cells (Zheng T et al. 2011).

Based on transport mechanisms of human skin, and known sizes of magnesium ions, we could predict that the hydrated magnesium ion, of radius 4.76 Å, could potentially penetrate by bulk diffusion through the 10 Å pores created by protein subunits in the lipid membrane (Chandrasekaran et al. 2014; Elias et al. 2002). On the contrary, the barrier property of stratum corneum has been widely discussed, with the general view that the layer acts as a selective barrier to transport of ions (Elias & Friend 1975). 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 (Jahnen-Dechent & Ketteler 2012), thus leading to an assertion that it is almost impossible for magnesium ions to pass through biological membranes (Saris et al. 2000; Jahnen-Dechent & Ketteler 2012). However, we have re-examined this calculation and found that an error was made. Based on the ionic radii of dehydrated and hydrated magnesium ions, i.e., 0.65 Å and 4.76 Å respectively (Eigen 1963; Diebler et al. 1969; Maguire & Cowan 2002), we calculated that the radius of hydrated ion is only 5.47 fold higher than its dehydrated form. These conflicting arguments prompted us to conduct experiments to test and visualize the extent of magnesium penetration, localization and concentration in human epidermis (Chapter 3).

1.1.6. Mag-fura-2 as a suitable indicator dye for magnesium ions

Magnesium ions are important in mediating enzymatic reactions, DNA synthesis, muscular contraction and hormonal secretion. Using a suitable magnesium indicator enables us to understand the extent of magnesium permeation through skin. Some of the most commonly used magnesium indicators are magnesium green, mag-fura-2, mag-indo-1, and mag-fluo-4. These indicators are designed specifically to bind with magnesium ions at cellular level (Haughland 2002; Takahashi et al. 1999).
Figure 1.2: Structure of Mag-fura-2, tetrapotassium salt.
Mag-fura-2, tetrapotassium salt is a cell impermeant variant that specifically binds with magnesium ions.

Even though Mg$^{2+}$ indicators bind to Ca$^{2+}$, physiological concentrations of calcium do not interfere with Mg$^{2+}$ binding due to its low affinity. Even though dissociation constant for Mg$^{2+}$ of mag-indo-1 is 2.7mM, slightly higher than that of mag-fura-2, which is 1.9 mM, the affinities of mag-fura-2 and mag-indo-1 for Mg$^{2+}$ are reported to be essentially invariant at pH values between 5.5 and 7.4 and at temperatures between 22°C and 37°C (Lattanzio & Bartschat 1991).

Table 1.1: List of magnesium indicators

<table>
<thead>
<tr>
<th>Magnesium probe (AM ester)</th>
<th>Fluorophore</th>
<th>Dissociation constant $K_D$ (Mg$^{2+}$) (mM)</th>
<th>Affinity</th>
<th>Wavelength (nm)</th>
<th>pH sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Excitation</td>
<td>Emission</td>
</tr>
<tr>
<td>Magnesium green</td>
<td>-</td>
<td>1.0</td>
<td>High to Low</td>
<td>517</td>
<td>540</td>
</tr>
<tr>
<td>Mag-fura-2</td>
<td>Furan</td>
<td>1.9</td>
<td></td>
<td>340</td>
<td>510</td>
</tr>
<tr>
<td>Mag-indo-1</td>
<td>Indole</td>
<td>2.7</td>
<td></td>
<td>330</td>
<td>417</td>
</tr>
<tr>
<td>Mag-fluo-4</td>
<td>Fluorescein</td>
<td>4.7</td>
<td></td>
<td>493</td>
<td>517</td>
</tr>
</tbody>
</table>

The advantage of using mag-fura-2 is that it undergoes an appreciable shift in excitation wavelength upon Mg$^{2+}$ binding (Figure 1.2). The excitation-ratioable mag-fura-2 indicator is most useful for fluorescence microscopy, whereas the emission-ratioable mag-indo-1 indicator is preferred for flow cytometry. Many applications of mag-fura-2 involve estimation of the affinity and selectivity of Mg$^{2+}$ binding to proteins. Previously, researchers have used mag-fura-2 to measure intracellular Mg$^{2+}$ in a wide variety of cells, organelles and tissues, including cortical neurons (Brocard et al. 1993), isolated mitochondria (Jung et al. 1997). However, this is the first time we have used mag-fura-2 to stain human skin section to visualise permeation of topically applied magnesium solution.
Figure 1.3: Emission spectra of mag-fura-2.
a) Fluorescence excitation and b) fluorescence emission spectra of mag-fura-2 in solutions containing 0–35 mM Mg$^{2+}$.

1.1.7. Barrier function and epidermal homeostasis of human skin

The stratum corneum being the outermost layer functions as a physical barrier hindering, but not completely preventing, transdermal penetration through its cellular structure (Brisson 1974). It is also important for controlled transepidermal water loss (TEWL) through the skin. This exchange of water vapor helps the body to maintain homeostatic temperature regulation (Marks 2004). Defects in barrier function could lead to excess TEWL eventually leading to skin conditions such as atopic dermatitis (Addor & Aoki 2010). A substantial body of evidence points to magnesium and calcium ions being involved in restoring barrier function and epidermal homeostasis of impaired skin. However, the mechanism by which the repair process occurs, whether by increasing hydration conditions or redox ratio, is unknown.

1.1.8. Metabolism in human skin

The epidermal layers of skin are always in a continuous state of differentiation making them one of the most metabolically active organs in the human body (Menon 2002). Just like any other cells, they produce inflammatory mediators when stimulated and contain enzymes that regulate cellular processes.
In the epidermis, keratinocytes contain NAD(P)H (both NADH and NADPH) as the predominant fluorophores. NAD(P)H is an intrinsic cellular cofactor for enzymatic reactions involving oxidative metabolism (Ying 2008). The autofluorescence signals found in the dermis result primarily from collagen and elastin (Ying 2008). The coenzymes, NAD (i.e. NAD+ and NADH) and NADP (i.e. NADP+ and NADPH) are fundamental mediators of energy metabolism, mitochondrial function, calcium homeostasis, antioxidant/generation, gene expression, immunological function, aging and cell death. There are two major pools of NAD(P)H, cytosolic and mitochondrial (Ray & Shah 2005). In addition, NADH loses fluorescence upon oxidation to NAD+, and changes in the NADH fluorescence can be used to evaluate parameters that reflect metabolism (Niesner et al. 2004).

1.1.9. Multiphoton tomography

Multiphoton tomography (MPT) has emerged as an important non-invasive technique in medical research over the last two decades (Masters et al. 1997; Chia et al. 2008). This method not only enables understanding the cellular morphology but also detects specific components based on their intrinsic fluorescence or externally applied fluorescent dyes. MPT uses a two-photon laser for excitation as shown in the figure 1.3. Accordingly, the fluorophore simultaneously absorb two photons in a single pulse of excitation, thus making the rate of excitation proportional to average squared photon density. [30]. This significantly enhances background discrimination, reduces photodamage intrinsic to fluorophores in viable cells and minimizes photobleaching [30]. When multiphoton tomography is coupled with fluorescence lifetime imaging microscopy (MPT-FLIM), it enables the monitoring of changes in autofluorescence in NAD(P)H or FAD under varying conditions.
The potential benefits of MPT include enhanced background discrimination, low photodamage to viable tissue, reduced photobleaching and improved optical depth section acquisition. It is a non-invasive imaging assay that can be used for in vitro, in vivo and ex vivo applications. MPT is a two-photon excitation form of microscopy and the components are shown in Fig. 1.3. The rate of excitation is proportional to the average squared photon density (i.e. simultaneously absorb two photons per excitation process in the fluorophores).

FLIM is a sensitive, time-resolved technique that detects fluorescence lifetimes of (i.e. 1 ns) of molecules excited by the laser pulse [44]. DermalInspect® instrument comprises of a femtosecond near infrared laser source, a scanner module DI-SDM, and a control module DI-SM used in the MPT imaging [17]. The Ti:Sapphire tuneable laser system ranging between 710 nm and 920 nm generates a femtosecond pulse in the near infrared range with the repetition rate of 80/90 MHz. MPT-FLIM can be used to image samples up to of 200 µm depth and cross-sectional x, y imaging plane to detect endogenous autofluorescence from NAD(P)H, flavins, melanin and second harmonic generation (i.e. collagen). Emission signals pass through optical filters into photomultiplier

Figure 1.4: Schematic representation of multiphoton microscopy principle
detectors, including TCSPC 28 detectors. FLIM images thus generated were processed using SPCImage software. Factors such as ion intensity, molecular binding, and hydrophobicity may affect FLIM measurements. However, they are independent of dye concentration, photobleaching or excitation intensity [48].

1.1.10. Filaggrin – an important epidermal protein

Filaggrin or the filament-aggregating protein (Reference, PMC3805301) is one of the important epidermal proteins encoded by the gene FLG present on chromosome 1q21, formed in the stratum granulosum layer of the epidermis. It is formed by cleavage of profilaggrin and is found in the interface between the cornified and granular layer (Brown & McLean 2012).

Profilaggrin, the precursor of filaggrin is cleaved by the action of serine proteases, and its subsequent citrullination (deamination of arginine in a protein into the amino acid, citrulline) leads to the degradation of the protein to hygroscopic amino acids, which in turn helps in the retention of moisture. These amino acids including urocanic acid (UCA), and pyrrolidine carboxylic acid (PCA), collectively form the natural moisturising factor (NMF) of the skin. Thus, it closely interacts with the lipid envelope proteins and the keratin filaments to form the stratum corneum (SC), plays a significant role in various physicochemical properties such as retaining skin moisture and maintaining the selective permeability of the SC (Brown & McLean 2012).

A loss of function due to mutation in filaggrin is the main cause for genetic skin disorders such as ichthyosis vulgaris and atopic dermatitis (Bussmann et al. 2011). Absence of this protein can lead to a poorly formed stratum corneum causing excessive water loss leading to adverse skin conditions. These diseases are mainly characterised by the presence of symptoms such as dry, flaky skin and itchiness. However as established, a genetic dysfunction may not be the only cause for atopic dermatitis; it can also be caused by an up regulation of the serine protease activity, which could cause an imbalance in the filaggrin levels at the epidermis. Any irregularities in the production of filaggrin and its degradation at SC could result in a disfunction of the SC (Gerritsen et al. 1994).

Both FLG and profilaggrin have unique roles in maintaining epidermal integrity. As mentioned earlier, the epidermal cells undergo continuous differentiation leading to terminal differentiation where stratum granulosum cells are anucleated to form stratum corneum. The role of FLG is to prepare the cells to undergo terminal differentiation (Kuechle et al. 2000). In earlier studies FLG has been studied extensively for its loss- of-function related diseases characterised by malformation
of the barrier layer (Bussmann et al. 2011). However, the effect of external stimuli and divalent ions has not, to date, been studied in skin that does not have mutations in the FLG gene. Such a study could reveal the role of magnesium or calcium salts in affecting FLG levels in the skin under the influence of any stress.

1.1.11. Control of inflammation by Magnesium: possible mechanism

It is well established that Mg$^{2+}$ deficiency has a direct influence on inflammation. However, the molecular mechanism by which Mg$^{2+}$ restores inflammation to the normal range are unclear. A characteristic property of Mg$^{2+}$ is its antagonism of Ca$^{2+}$. It competes with Ca$^{2+}$ for entry into cells through voltage-gated channels and receptors and inhibits intracellular Ca$^{2+}$ release from the sarcoplasmic reticulum (Inesi G. et al. 1976; Weber A. et al. 1971).

The antagonism between Mg$^{2+}$ and Ca$^{2+}$, and competition for binding sites on receptors enables Mg$^{2+}$ to overcome the toxic effects produced by excessive Ca$^{2+}$ concentrations in cells of the immune system that are located in the brain (human microglial cells in tissue culture)(Lee et al. 2011). In the case of neuroinflammation, in vitro experimentation has shown that an influx of Ca$^{2+}$ into microglia (brain resident macrophages) and THP-1 cells activates their associated purinergic receptors and subsequently inflammation(Acuña-Castillo et al. 2007). Mg$^{2+}$ is effective in ameliorating the neurotoxic effect produced by over-activation of human microglial cells that occurs as a result of elevated levels of inflammatory cytokines in the cells such as TNF-α, IL-6, and nitrite ions(Lee et al. 2011; Zhao et al. 1998). These agents are released as a result of intracellular inflammatory pathway activation, via P38 MAPK and NFκβ (Lee et al. 2011).

NFκβ activity is regulated by various secondary messengers including intracellular calcium ions (Ca$^{2+}$). Low extracellular magnesium ions induce lipid peroxidation and activation of NFκβ in canine cerebral vascular smooth muscle (Altura BM et al. 2003) In rats fed a Mg$^{2+}$ deficient diet, where plasma Mg$^{2+}$ fell to 60% of control levels, a rise in Ca$^{2+}$ levels was observed. This rise in Ca$^{2+}$ secondary to a decrease in Mg$^{2+}$ is seen in a variety of systems including human patient studies, rodent models and cell culture, (including immune cells), all of which are mentioned in the 2010 review by Rayssiguier Y et al (Rayssiguier et al. 2010). The increased Ca$^{2+}$ can induce formation of reactive oxygen intermediates following an oxidative burst from cells such as neutrophils (Waddell et al. 1994), which in turn cause phosphorylation of IKβ (through an as yet unknown mechanism), which will release the active form of NFκβ for nuclear translocation.
The critical role of Ca\(^{2+}\) in the NFκβ pathway is supported by the observation that Ca\(^{2+}\) chelators prevent the induction of NFκβ activity \textit{in vivo} in murine models (Pahl & Baeuerle 1996). Other research has shown that when Mg\(^{2+}\) deficient rats were fed a Ca\(^{2+}\) deficient diet, the inflammatory effect was greatly reduced (as measured by reduced inflammation scores, prevention of leukocytosis and reduced splenomegaly) when compared to other hypomagnesaemic rats (Bussière et al. 2002). Once the active NFκβ crosses the nucleus it up-regulates the transcription of TNFα (Yamauchi et al. 2010; Drouet 1991). It should be mentioned, however, that the evidence for NFκβ-mediated TNFα expression is mostly limited to murine models, and studies relating to humans are limited (Baeuerle & Henkel 1994; Shea et al. 1996). However, in 2010 one study used mouse bone marrow-derived dendritic cells to show NFκβ-mediated positive expression of the TNFα gene (Yamauchi et al. 2010). It was also found that when vascular smooth muscle cells were exposed to low extracellular magnesium resulted in cytokines and chemokine synthesis concomitant with synthesis of ceramides. However, inhibition of ceramide synthesis markedly attenuated the release of cytokines and activation of NFκβ (Altura BM et al. 2012; Altura BM et al. 2013).

Additionally it would be of interest to study whether a high Ca\(^{2+}\), low Mg\(^{2+}\) state up-regulates NFκβ activity, and TNFα expression permitting TNFα synergism with STAT6 to switch B cells to IgE production (Geha et al. 2003). This is important to establish given the role of IgE in atopic and inflammatory conditions such as atopic dermatitis (Geha et al. 2003).

1.2. SCIENTIFIC QUESTIONS

1. How does topical magnesium salts penetrate normal or pathologic skins?

2. Which is the mechanism of magnesium-dependent healing process?

1.3. HYPOTHESIS

The overall hypotheses tested in this thesis were:

1. Magnesium salts are able to penetrate skin barrier.

2. Magnesium exerts protective effects on skin.

3. Magnesium affects skin metabolism and turnover.
4. Magnesium interacts with calcium to regulate skin homeostasis.

1.4. EXPERIMENTAL DESIGN

The aims of this thesis were to test hypotheses 1 through 4, by topically applying varying concentrations of magnesium chloride in solution. This was carried out in three ways.

1. Topically applying magnesium chloride solutions on excised human skin to investigate the permeability of the skin to magnesium ions and its concentration dependence (Chapter 3).

2. Investigating the effects of topical magnesium application to intact skin on TEWL, skin hydration, skin pH on barrier function (Chapter 4) and metabolic changes in the epidermis of healthy volunteers (Chapter 5).

3. Investigating the role of magnesium treatment in modulating skin regulatory proteins in excised human skin (Chapter 6).
CHAPTER 2: MATERIALS AND METHODS

Methods described in this chapter have been used in more than one chapter throughout this thesis.

2.1 PREPARATION OF EXCISED HUMAN SKIN

Human skin was donated from patients undergoing abdominoplasty, from various surgical facilities in Brisbane with the ethics approval of Princess Alexandra Hospital Research Ethics Committee (Approval no.1997/097). Donors of the excised human skin had previously read and signed informed consent forms. The donor skin was obtained on the day of removal from surgery and the experiment was performed within 24 hours to preserve the viability of the skin for ex vivo use of viable skin. The numbers of skin samples studied is described in each chapter. The subcutaneous fatty tissue was removed from the skin using a scalpel immediately after delivery. The surface of the skin was cleaned with deionised water and the excised skin was wrapped in aluminium foil and stored in polyethylene bags at 4°C for experiments conducted within 24 hours. Excised human skin were used in experiments mentioned in Chapter 3 and Chapter 6.

2.2. VOLUNTEER ENROLMENT FOR IN VIVO STUDY

All human subjects had previously signed informed consent forms before the experiment was performed. All experiments were done with approval of the Princess Alexandra Hospital and the University of Queensland Human Research Ethics Committees (Approval no.2008001342). Treatments conducted on volunteers vary according to experimental design and are explained in Chapters 4, 5 and 6 accordingly. All the studies were carried out on healthy subjects between ages 23 to 27, with undamaged skin and without a history of cutaneous disease. The volunteers were briefed about the details of the experimental protocol, following which their consent to participate was obtained. During the briefing, the volunteers were also instructed to abstain from the use of sunscreens or cosmetic skin products 24 hours prior to the experiment to avoid contamination. The area of skin on the proximal volar forearm was taken into consideration for carrying out the studies. Prior to starting the experiments, subjects were acclimatized for 30 minutes in laboratory conditions. Relative humidity and ambient temperature were maintained throughout the experiments.
2.3. TOPICAL APPLICATION OF SALT SOLUTIONS

Methods in all the chapters either involved pre-treatment on the epidermal side of excised skin or topical application on volar forearm of human volunteers with salt solution. The time of exposure ranged between 20 to 60 minutes depending on the protocol followed in each chapter. We developed a setup to achieve the above-mentioned treatment in a consistent manner (Figure 2.1). We used the donor component of the Franz cell, which is a cylindrical well with a circular frame surrounding it. By attaching this donor component to the skin surface with an O-ring double-sided adhesive tape to act as a leak proof seal. This set up now serves as a reservoir to hold the salt solution thus exposing the specific skin surface for the treatment.

![Franz cell – donor component](image1)

**Figure 2.1: Setup for topical application of salt solutions.**

All salts used were of molecular biology grade and were obtained from Sigma Aldrich, St Louis, USA. In Chapter 4, we used a commercially available Dead Sea salt, sold as Bokek® Premium (Dead Sea, Beer Sheva, Israel). The following table lists all the salt solutions used at various concentrations, at room temperature (23-25°C).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9 M Magnesium Chloride</td>
<td>Used 1.9 M magnesium chloride and 0.44 M calcium chloride as we calculated this to be the Dead Sea equivalent levels of magnesium and calcium (Ma’or et al. 2006).</td>
</tr>
<tr>
<td>0.44 M Calcium Chloride</td>
<td></td>
</tr>
<tr>
<td>5.64 M NaCl</td>
<td>We also used 5.64 M NaCl in Chapter 4 as this equivalent to ionic strength of 1.9 M MgCl₂.</td>
</tr>
<tr>
<td>1.9 M MgCl₂</td>
<td>Note that the solutions at high ionic strength had similar pH.</td>
</tr>
</tbody>
</table>
### Table 2.1: List of salt solutions used for topical application

<table>
<thead>
<tr>
<th>Salt dissolved in water</th>
<th>Concentration</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium chloride hexahydrate (MgCl$_2$. 6H$_2$O)</td>
<td>5 mM</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>52.8 mM</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>1.9 M</td>
<td>7.8</td>
</tr>
<tr>
<td>Calcium chloride (CaCl$_2$)</td>
<td>5 mM</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>0.44 M</td>
<td>7.2</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>5.64 M</td>
<td>7.9</td>
</tr>
</tbody>
</table>

For experiments in **Chapter 3**, we obtained excised human skin from patients undergoing abdominoplasty, as described in section 2.1. We then topically applied milliq water (vehicle control) and 5mM MgCl$_2$ solution for 30 minutes using the donor chamber of a Franz cell. We also applied 5mM MgCl$_2$ to skin that was tape stripped 30 times, in order to ease the permeation of magnesium through epidermis and represent as a positive control. We treated another set of excised skin in the same way as above, with 52mM and 1.9 M MgCl$_2$ solutions for 5, 15, and 60 minutes to study the effect of time and concentration in magnesium permeation.

For experiments in **Chapter 4**, we recruited 7 healthy volunteers as described in section 2.2. We marked 7 defined spots of 4 cm$^2$ area in their volar forearm as shown in figure 2.2. Parameters evaluating the skin conditions were measured at every stage of the experiment.

For experiments in **Chapter 5**, we recruited 3 healthy caucasian volunteers as described in section 2.2. We marked 6 defined spots of 4 cm$^2$ area in their volar and FLIM images were recorded for zero hour timepoint. After acquiring the images, three spots were tape stripped 20 times (section 2.4) to compare against corresponding normal skin sites. We acquired FLIM images immediately after tape stripping. We treated a normal and tape stripped skin site with 1.9M MgCl$_2$ solution for 60 minutes using the franz cell donor component as described in this section. We used the other four sites for milliq water treatment as vehicle control and no treatment control. This treatment was repeated after at 24-hour intervals for 4 consecutive days.
Figure 2.2: Representation of volunteer forearm.
Proximal volar forearm of volunteer were treated with various salt solutions within the 4cm² area using the setup described in figure 2.1.

For experiments in **Chapter 6**, excised skin was treated with 5mM & 1.9 M MgCl₂ and 5mM & 0.44 M CaCl₂ topically using similar setup.

### 2.4. STIMULATION OF HUMAN SKIN

In order to study the effect of salt solutions on impaired human skin, it was necessary to inflict damage to normal skin initially. We conducted tape stripping and 2,4-dinitrochlorobenzene (DNCB) as ways to physically and chemically stimulate the normal skin.

Tape stripping is an effective, non-invasive method that uses a flexible, adhesive tape to physically disrupt the stratum corneum (SC), outermost layer of skin. To remove the SC in the marked region of the skin, sequential tape strips were performed using D-SQUAME tapes applied with a pressure applicator (CuDerm, Dallas, USA) at constant pressure of 225 gm/cm² for 3 seconds per tape. For experiments in **Chapter 3** and **Chapter 6**, where excised skin has been used 30 tape strips were performed. In **Chapter 4** and **Chapter 5**, where the experiments involved human subjects 20 tape strips were performed on the marked region of volar forearm.

DNCB (Sigma-Aldrich, St Louis, USA) is skin sensitiser that is known to induce type IV hypersensitivity and contact dermatitis. In **Chapter 6**, we topically applied 50 μl of 0.1% DNCB in acetone in an attempt to stimulate filaggrin in the epidermis on untreated or pre-treated skin.
2.5. NOVEL METHOD FOR 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. 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 0.1μl acriflavine solution (dissolved in milliQ water) to form a globule around the follicle and subsequently adding 0.1μl of ethylcyanoacrylate (Loctite Super glue) (Henkel, Ohio, USA) on top of it. The polar monomers of cyanoacrylate 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 1.9M MgCl₂ solution for 15 minutes.

![Figure 2.3: Novel hair follicle plugging method.](image)

We developed a novel hair follicle plugging method to evaluate follicular contribution towards magnesium permeation. a.) Hair follicle, b.) Add 0.2μl acriflavine dissolved in water (1 mg/ml) around the hair. c.) Add equal amount of cyanoacrylate, d.) All hair follicles plugged.

2.5.1. Validation for hair follicle plugging method

Being a new method, its efficacy was tested by conducting in vitro skin absorption study in a Franz diffusion cell with an effective diffusion area of 1.33cm² and 3.4 ml receptor chamber capacity. The skin obtained from abdominoplasty 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 1ml 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, 200uL 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 1ml/min flow rate. The column used was Phenomenx Luna 5µm, c18 (150 mm ×4.6 mm); and caffeine was detected at 273 nm.

Figure 2.4: Validation for hair follicle plugging method.
We tested the effectiveness of hair follicle plugging method by topically applying 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 franz cell and evaluated using HPLC. We found that the plugging method was effective as there was no permeation in the first two hours.

2.6. MEASUREMENT OF SKIN PHYSIOLOGY PARAMETERS

2.6.1. Transepidermal water loss (TEWL)
Transepidermal water loss (TEWL) is the gold standard for measuring the barrier function of human epidermis (Pinnagoda et al. 1990). TEWL is the constant diffusion and evaporation of water from inside the body into the outer atmosphere through the epidermal layer of the skin (Pinnagoda et al. 1990). The measure of TEWL indicates the condition of the epidermis, whether normal or damaged. The stratum corneum, the outermost layer of the skin and the epidermis itself, governs TEWL primarily due to its structure. Therefore, if the SC is disrupted, the TEWL measure will be high owing to loss of excessive water. For our experiments, we used a commercial closed chamber TEWL instrument called Aquaflux (Biox, London, England). This design eliminates any air
movements and an internal condenser constantly removes the water vapour by converting it to ice. Before commencing of the experiment, the device was calibrated and baseline calibration was conducted prior to observing any readings.

Transepidermal water loss (TEWL) measurement is an established method to evaluate damage to stratum corneum. It is a biophysical method that quantifies the flux that occurs when water evaporates through the pores of the skin. Barrier function could be understood when a marked region of skin is constantly monitored for TEWL. Measuring skin hydration and skin pH would also provide useful information about disrupted barrier responds to these parameters (Blichmann & Serup 1988; Constantin et al. 2014).

Previous studies have demonstrated that magnesium concentrations are high in the SC layer and lower in other layers of the epidermis (Zglinicki et al). Similarly, the calcium ion gradient is higher in the upper layers of epidermis and gradually reduces towards basal layers. However, these studies used a concentration of magnesium and calcium salt different to the levels present in the Dead Sea. In this study we have used magnesium and calcium chloride salts in a concentration equivalent to Dead Sea levels (section 2.3), therefore reflecting the effect of these salt ions in improving barrier function.

Figure 2.5: Closed chamber TEWL.

2.6.2. Skin hydration and Skin pH

To measure skin hydration, we used a Corneometer CM825 (Courage+Khazaka electronic GmbH, Cologne, Germany) that works based on capacitance measurement of a dielectric medium. The
system measures variation in dielectric constant when skin surface hydration changes the capacitance of a precision capacitor (Constantin et al. 2014). We placed the probe on the defined area of the volar forearm of volunteers who participated in the study, for one second to record the hydration value. Before conducting the successive measurement, we waited 2 minutes in order to avoid any error. The same equipment housed a pH probe (Courage+Khazaka electronic GmbH, Cologne, Germany) specifically designed for measuring skin pH. The probe was placed on the site where pH had to be measured and the measure button was pressed until the reading was taken. A 10 second interval was provided between successive readings.

![Figure 2.6: Working principle of Corneometer.](image)

Corneometer measure the capacitance of skin surface thus indicating its hydration levels. Image obtained from Corneometer (Courage+Khazaka electronic GmbH, Cologne, Germany) product manual.

### 2.7. MICROSCOPY TECHNIQUES

#### 2.7.1. Multiphoton Microscopy

We used a LaVision Biotec Nikon multiphoton system with a tunable titanium Sapphire laser to visualize the magnesium skin sections. 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 of mag-fura
fluorescence in the epidermis (section 2.6). A 20× 0.95 NA water immersion objective (Olympus) was used.

2.7.2. Multiphoton Tomography coupled with fluorescence lifetime imaging (MPT-FLIM)

We performed MPT using the DermaInspect® system (JenLab GmbH, Jena, Germany) that used an ultra-short-pulsed, mode-locked, 80-MHz Titanium Sapphire laser (Mai Tai, Spectra Physics, Mountain View, California, USA) for excitation. It has a tunable laser within a range of 710-920 nm and has an 85 fs pulse width. We used a Plan-Neofluar high-NA oil-immersion 40X/1.30 objective lens (Carl Zeiss, Germany). An excitation wavelength of 740 nm (two-photon) was used to excite endogenous NAD(P)H/FAD fluorophores. The fluorescence emission was acquired through three spectral channels: 350-450, 450-515 and 515-620 nm.

Images were acquired for four layers of the epidermis for each spot on the volunteer forearm - the stratum corneum (SC; ~5-10 µm), stratum granulosum (SG; ~15-20 µm), stratum spinosum (SS; 25-30 µm) and stratum basale (SB; 35-40 µm). We ensured that the keratinocyte morphology matched with the characteristic of the specific layer and adjusted the depth accordingly.

2.7.3. Confocal Microscopy

We used Zeiss LSM 510 META confocal microscope for imaging FITC conjugated antibodies and propidium iodide stained skin sections with the following settings: argon laser with excitation and emission of 488 nm and 520 nm respectively for FITC and the Helium Neon laser (HeNe1) laser with excitation and emission of 543 nm and 555 nm respectively for propidium iodide. All images were recorded at 25X magnification with oil immersion objective. Each image was captured as z-stack at 60 µm thickness at 5 µm interval and the final images used were maximum intensity projection of the stack.
Figure 2.7: 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.

2.7.4. Image Analysis

Images obtained from multiphoton microscopy and confocal microscopy (section 2.13) were analysed using ImageJ (NIH, USA) software. The fluorescence intensity was quantified by gating the epidermis region and corresponding CTCF (corrected total cell fluorescence) was calculated using the formula below.

\[ \text{CTCF} = \text{Integrated density}_{\text{Viable epidermis}} - (\text{Area}_{\text{Viable epidermis}} \times \text{Mean fluorescence}_{\text{Background}}) \]

Mag-fura-2 (Chapter 3) and FITC (Chapter 6) fluorescence in the gated area were represented as CTCF values. The CTCF was then normalized to the mean area of viable epidermis.

2.7.5. FLIM analysis

To obtain FLIM measurements we used a time-correlated single-photon counting (TCSPC) SPC-830 detector (Becker and Hickl [B&H], Berlin, Germany), integrated into the MPT system. The
instrument response function (IRF) of the FLIM images was calibrated to a sucrose crystal standard (Ajax Finechem Pty Ltd, Sydney, NSW, Australia). Fluorescence lifetime parameters from the MPT-FLIM images were analysed using SPCIImage 4.8 software (Becker and Hickl GmbH, Berlin, Germany). FLIM data consists of several time channels distributed across the fluorescence decay curve. We used a double-exponential decay model that represents a sum of multiple exponentials, or components, as each pixel contains an overlay of fluorescence from several endogenous fluorophores at various conformations. The fitted decay curve establishes short (τ1) and long (τ2) fluorescence decay lifetimes (ps) and with corresponding relative amplitude coefficients α1 and α2 (%), respectively.

2.8. HISTOCHEMISTRY

2.8.1. General protocol

Histochemistry is the study of the chemical compounds distributed within biological structures. The most important and critical step in histochemistry is sectioning the tissue blocks as it defines the quality of the results. All the sample blocks used in experiments mentioned in Chapter 3 and Chapter 6 were embedded in Tissue-Tek® Optimal Cutting Temperature compound (Miles, Elkhart, Idaho) and stored at minus 80 °C. Skin sections were cut using the Leica CM1850 Cryostat (Leica Biosystems, Nussloch, Germany) and they were mounted on charged StarFrost® Hydrophilic slides (Light Labs, Dallas, Texas). The sections were stained immediately or stored at minus 80 °C until further use depending on the protocol. The sections were incubated in a lightproof humidity chamber. After the washing steps according to the protocol, BrightMount/Plus aqueous mounting medium (anti-fading) for fluorescent staining (Abcam, USA) was applied and the sections were covered with a coverslip. The edges of the coverslip were then lined with nail polish.

2.8.2. Mag-fura-2 staining

After treating the skin with the salt solutions described above (section 2.3), the skin were embedded in OCT and 60μm cryosections section were obtained. The sections were immediately stained with 30μl of mag-fura-2, tetrapotassium salt (10μg/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 as described in section 2.8.1. Staining human skin sections with mag-fura-2 dye has been done in this PhD for the first time.
2.8.3. Immunohistochemistry

All the skin samples after treatment were embedded in OCT medium and frozen into blocks. We obtained 10 μm thickness sections as described in section 2.8.1. The sections were stored at -80 °C until further immunostaining. Before commencing the wash steps, the slides were warmed to room temperature for 30 minutes. Slides were washed with PBS (pH 7.2) (Sigma Aldrich, St Louis, Missouri) for 5 min and milliQ water for 1 min. The sections were air dried for 1 min between washes to avoid sections being washed off. The sections were circled with a PAP pen (Enzo Life Sciences, Farmingdale, New York). We then incubated the sections with 30μL of anti-filaggrin primary antibody (Abcam, Cambridge, United Kingdom) with a 1:200 dilution in 12% BSA (GibcoBRL, Auckland, New Zealand) for 90 minutes at room temperature in a StainTray® humid chamber (ProSciTech, Turingowa, Australia). After incubation, the sections were washed with PBS for 5 minutes and milliQ H₂O for 1 min. Following the wash step, 30μL of the FITC conjugated goat anti-mouse IgG1 secondary antibody (Abcam, Cambridge, United Kingdom) at 1:50 dilution in 12% BSA was added to the section and incubated for 45 minutes at room temperature in the humid chamber. The secondary antibody incubation was terminated with a wash step with PBS (5 minutes) and distilled H₂O (1 min). The slides were then counterstained with 30μL of 4μM Propidium Iodide (Sigma Aldrich, St Louis, Missouri) for 5 minutes followed by a wash with PBS for 5 minutes and milliQ water for 1 min. The sections were mounted with anti-fade media and sealed as described in section 2.8.1.

2.9. BIOINFORMATICS

For developing further understanding about filaggrin interaction with divalent ions, in Chapter 6 we used basic bioinformatics techniques. We acquired filaggrin query sequence from the Uniprot PDB database. The motif search was performed using MOTIF, an online tool from GenomeNet. The motifs were visualised using PyMOL version 1.3r1 (Schroedinger) and SWISS MODEL (ExPASy). The predictive structure modelling was done using the multiple alignments threading method developed by University of Michigan (I-Tasser®).

2.10. STATISTICS

For all the experiments conducted, the measured parameters were expressed as mean ± SEM. A one way analysis of variance (ANOVA) was used for comparisons between more than two groups, and where relevant, a Tukey’s post-hoc comparison test. Where the term statistical “significance” is
used, it corresponds to a value for $p \leq 0.05$. All the statistical analysis and graphs were generated using the Graphpad Prism version 6.05.

In **Chapter 5**, we used one way ANOVA with sidak’s test to compare between control and tape stripped skin sites within each treatment. $P < 0.05$ was deemed statistically significant.

In **Chapter 6**, one-way ANOVA with a tukey’s test was performed to compare control group and other treatments. When comparing before and after measurement within one treatment group, in the case of skin pH and hydration results, we used one-way ANOVA with sidak’s test. $P < 0.05$ was deemed statistically significant.
CHAPTER 3: INVESTIGATING THE PERMEABILITY OF HUMAN SKIN TO MAGNESIUM IONS

3.1. INTRODUCTION

The objective of this chapter is to,

1. Demonstrate a method to visualize magnesium ions.
2. Observe magnesium ion absorption or localization in human skin.
3. Study the effect of magnesium chloride solutions with varying duration of treatment and concentrations.
4. To developed a method to plug hair follicles in an attempt to understand magnesium permeation through hair follicles.

Studies conducted in Chapter 4, Chapter 5, Chapter 6 of this thesis are based on topical magnesium application. It is therefore important to characterise the permeation of magnesium ions through stratum corneum, and its dependence on time and concentration to avoid ambiguity in this area.

3.2. RESULTS

3.2.1. Topically applied magnesium permeates through human stratum corneum

Cryosections of human skin pre-treated with 5 mM MgCl₂ solution showed increased fluorescence intensity relative to sections that were not pre-treated, when stained with Mag-fura-2 dye. This increase was observed in both stratum corneum intact, and tape stripped skin indicating the permeability of magnesium ions through stratum corneum. In skin sections not treated with MgCl₂, we observed that the Mag-Fura-2 dye emitted fluorescence upon binding with endogenous Mg²⁺ ions. We also found an increase in fluorescence intensity in tape stripped skin subsequently treated with 5 mM MgCl. We have also estimated the magnesium levels present in our sections by comparing its CTCF (section 2.6) to the CTCF of skin equilibrated with known concentration of MgCl₂ 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 resistance to the permeation of magnesium ions, as expected. However, where the stratum corneum is intact, permeability to magnesium ions still occurs, with the level of penetration a function of the thickness of the stratum corneum (which varies between donors).
Figure 3.1: Magnesium ions penetrate through skin but the extent depends on stratum corneum thickness.
(a) Images from three donors show increased fluorescence in tape stripped (TS) and SC intact skin treated with 5mM MgCl₂ solution for 30 min compared to skin untreated with MgCl₂. Magnesium ion penetration through SC intact skin appears to be less than in TS skin and dependent on the thickness of SC across donors. (b) Histograms showing normalized fluorescence intensity in viable epidermis in each donor. Scale bar = 50μm.

3.2.2. Magnesium permeability varies based on concentration and time of exposure

Figure 3.2 shows that the increase was similar after 60 minutes treatment with both 52 mM and 1.9M MgCl₂, even though increase becomes statistically significant after 15 minutes treatment with 1.9 M MgCl₂. This indicates that at higher Mg²⁺ concentration, permeability is faster and penetration increases with progression of time. However, there is no significant increase in magnesium penetration 5 minutes after exposure to either concentration of MgCl₂ and there was no significant increase after 15 minutes of exposure to 52 mM MgCl₂.
Figure 3.2: Magnesium permeation at varying time 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. We found that there was no significant penetration after 5 minutes for both the concentrations and no significant penetration after 15 minutes for 52 mM MgCl₂ solution. However, there was increased magnesium penetration at 15 minutes for 1.9 M MgCl₂ and at 60 minutes for both concentrations (* - p<0.05, ** - p<0.01).

3.2.3. 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 prevent any ion transport. We found reduced caffeine penetration through skin with hair follicles plugged when compared to unplugged skin. This validates our method of hair follicle plugging and enables us to use this method to understand magnesium penetration through hair follicles. Magnesium penetration was significantly increased in sections obtained from unplugged skin when compared to the sections of skin that were plugged. This indicates that hair follicles act as a major route of penetration through the skin for magnesium ions. We also observed fluorescence emission from the acriflavine-cyanoacrylate complex used to plug the hair follicle. This aids us with acquiring images at the plugged region, which could be difficult to do in other cases.
Figure 3.3: Hair follicles significantly contribute to magnesium permeation.
When 1.9 M MgCl₂ solution was topically applied over plugged and unplugged skin for 15 minutes, we found that mag-fura-2 fluorescence intensity is higher in unplugged skin section, indicating that follicles contribute significantly to magnesium permeation. a.) Plugged skin (plugged region shown in blue) prevents magnesium penetration and b.) Unplugged skin sections allow penetration of magnesium ions. c.) Increased penetration of magnesium ions is found in unplugged skin, indicating that hair follicles are a major route for penetration (* - p<0.05).

3.3. DISCUSSION

The treatment solution used in our experiments was magnesium chloride (MgCl₂6H₂O) that has been used for several years for therapeutic purposes, due to its higher effectiveness and reduced toxicity, compared to salts with other anions (Mittendorf et al. 2002). Once dissolved it dissociates into magnesium and chloride ions, which means that they do not form any ions pairs and exists as ions. This brings up the argument about the inability of ionic particles being able to traverse the stratum corneum layer of skin, which is negatively charged (Piemi et al. 1999; Baspinar & Borchert 2012; Rojanasakul et al. 1992).

Figure 3.1 demonstrates the ability of magnesium ions to penetrate through both intact skin and tape-stripped skin, although the penetration is more pronounced in the latter. This complies with our hypothesis that magnesium ions could permeate through skin based on their physical sizes further clarifying the existing ambiguity. Developing a method to stain magnesium ions in skin sections further led us to investigate the penetration profile under prolonged exposure and increased concentrations of magnesium chloride solution. We observed that magnesium ions in 1.9 M MgCl₂ solution penetrate rapidly 15 minutes after treatment when compared to 52mM MgCl₂ solution. According to Fick’s law of diffusion, flux goes from regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient (Brogioli & Vailati 2001; Brisson 1974). Thus the observed increase in fluorescence intensity is due to the
movement of magnesium ions from a region of high concentration to low concentration, with more rapid movement at higher external concentrations.

To further investigate the route of penetration of magnesium ions, we topically applied MgCl$_2$ solution on excised skin on exactly the same skin area with and without the plugging 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 possible drug 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 (Horita et al. 2014), 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 (Santappa & Sheriff n.d.). We also harnessed the fluorescent property 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 confirm that magnesium ions are able to penetrate through barrier-compromised SC and partially through SC intact skin. We also found that hair follicles contribute significantly (approximately 40% increase in mg-fura-2 CTCF (Figure. 3.3 c) the permeation of magnesium ions. However, further studies need to be conducted to quantitatively evaluate the magnesium permeation through skin (Denda et al. 2000).
CHAPTER 4: EFFECT OF TOPICALLY APPLIED SALT SOLUTIONS ON TEWL, SKIN HYDRATION AND SKIN pH ON BARRIER IMPAIRED HUMAN SKIN

4.1. INTRODUCTION

Skin is the largest organ in the human body, which is protected by a horny layer called stratum corneum. This outermost cutaneous permeability barrier is about 10-15 μm thick and it varies along different parts of the body and also from individual to individual (Menon GK, 2002).

Disruption to this barrier results in excessive loss of water from the epidermis further affecting their normal function. A homeostatic response is triggered when this layer is compromised with organic solvents or tape stripping, in order to restore the lost stratum corneum. Magnesium and calcium ions have been widely studied over the past few decades due to their role in epidermal homeostasis and accelerating barrier recovery. Several therapies for skin barrier integrity has been based on treatment with Dead Sea minerals that predominantly contain magnesium and calcium.

Measurement of transepidermal water loss, skin hydration and skin pH can be effective parameters to evaluate the integrity of barrier function.

The objective of this chapter is to,

1. Demonstrate the effectiveness of tape stripping.
2. Understand the effect of tape stripping that TEWL, skin hydration and skin pH.
3. Understand the effect of magnesium treatment on barrier impaired skin.

4.2. RESULTS

4.2.1. TEWL increases progressively with tape stripping

TEWL increased progressively as the number of tape strips increased (Figure 4.1,a). The number of tape strips required to completely remove the SC was estimated to be 35, by interpolating the y-intercept of 1/TEWL value (Fig 4.1,b). This indicates that by tape stripping 20 times substantial amount of SC could be removed.
Figure 4.1: TEWL increases with tape stripping, and it removes a substantial amount of SC.

a) Marked regions in the volar forearms of volunteers were tape stripped 20 times sequentially. TEWL values progressively increased with tape stripping (n=3). b) Number of tape strips required to completely remove stratum corneum has been estimated by interpolating the 1/TEWL values.

4.2.2. Tape stripping significantly increased TEWL and skin hydration, but not skin pH

Tape stripping significantly increased TEWL and skin hydration after tape stripping indicating the effectiveness of barrier disruption in marked regions of volunteer’s volar forearms. However, no change in pH was observed, consistent with previously reported studies (Figure 4.2).

Figure 4.2: Effect of tape stripping on TEWL, Hydration and pH.

c) TEWL and d) skin hydration in tape stripped sites showed significant increase when compared to baseline (BL). e) pH did not change upon tape stripping (n=7).
4.2.3. Effect of treatment on TEWL in tape stripped skin.
For all treatment spots, TEWL increased significantly after tape stripping at 24, 48, and 72 hour time points. There was no increase in TEWL for no treatment spot and 5.64 M NaCl treated spot, 6 hours after tape stripping. TEWL for no treatment spot was not significant to the baseline level at 96 hour timepoint.

Figure 4.3: Effect of salt solutions on TEWL over 96 hours.
Treatment with salt solutions for 20 minutes after tape stripping, significantly increased TEWL after 6 hours and remained high until 96 hours (after 4 repeated 20 minute treatments at 24 hour intervals) when compared to baseline TEWL, except in no treatment and 5.64M NaCl sites. TEWL at 5.64M NaCl and no treatment site returned towards baseline at 72 and 96 hours respectively, indicated by statistical no significance.

4.2.4. Effect of treatment on skin hydration in tape stripped skin.
Skin hydration increased significantly for no treatment (air), water, 1.88M MgCl₂, 0.44 M CaCl₂ treatment spots, 6 hours after tape stripping. There was no significant increase in hydration for other spots 6 hours after tape stripping.

4.2.5. Effect of treatment on skin pH in tape stripped skin.
Skin pH did not change for any treatment spot during the 96 hours of observation. This indicates that tape stripping or treatment with salt solution did not have any effects on skin pH.
Figure 4.4: Effect of salt solutions on skin hydration over 96 hours.
Skin hydration increased significantly 6 hours after salt treatment on tape stripped sites, except in sites treated with 5.64M NaCl and commercial Dead Sea salt. In all tape stripped sites, hydration drops towards baseline after 24 hours after treatment. Tape stripped site treated with 1.88M MgCl$_2$ has indicated significant decrease in hydration at 24, 48, 72 and 96 hours after treatment when compared with 6 hour time point. However, when mixed with 0.44M CaCl$_2$ the significant decrease of hydration occurs only at 72 and 96 hours after treatment.

Figure 4.5: Effect of salt solutions on skin pH over 96 hours.
Skin pH did not show significant change for any treatment condition.
4.3. DISCUSSION

Magnesium salts have been widely studied over the past few decades for their role in epidermal homeostasis and accelerating barrier recovery. Previous studies were conducted either on mice skin or human subjects with skin conditions using salt concentrations that do not correlate with Dead Sea levels (Denda et al. 1999; Proksch E, Nissen HP, Bremgartner M 2005). Also, the use of commercially available Dead Sea salts for such studies may not reflect the specific activity of each ion in barrier function. Evidence pointing to the beneficial effects of Dead Sea salt is also based on their richness in these ions. High levels of magnesium in horny layer of skin could be linked with improving several inflammatory conditions (von Zglinicki et al. 1993; Denda et al. 2000). However, the exact mechanism of how this happens still remains unanswered. The aim of this experiment was to evaluate the effect of magnesium and calcium ions on barrier function in case of a disrupted stratum corneum.

Tape stripping is an established method to cause barrier disruption, which is indicated by increase in TEWL and skin hydration. Several variables are involved in tape stripping such as surface area, pressure, ambient temperature and humidity in addition to inter and intra individual difference in SC thickness reported (Pinnagoda et al. 1990). In order to minimise such variability and to estimate that we are consistently removing the same amount of SC among all the subjects, we measured TEWL at regular intervals through the tape stripping process. These values were used to plot 1/TEWL versus number of tape strips, which was later extrapolated to estimate if substantial amount of SC was removed (Figure 4.1) (Breternitz et al. 2007; Gerritsen et al. 1994; Pinnagoda et al. 1990). We estimate that at least two-thirds of the stratum corneum was removed from each spot in the volar forearm of the donor.

Increase in TEWL and skin hydration immediately after tape stripping can be attributed to the loss of SC resulting in the efflux of water from the skin (Figure 4.2). However, this increase is not significant at 6 hours after tape stripping in the no treatment spot (Figure 4.3). On the other hand, skin hydration after 6 hours after tape stripping remained higher compared to baseline (Figure 4.4). As an effect of tape stripping, skin exudates such as lamellar body secretion and secretory granulocytes can temporarily form a mask at the tape stripped site inhibiting TEWL (de Koning et al. 2012). Increase in skin hydration at the same site could be due to the presence of these proteins and escaping water contained within that layer. An opposite effect was observed in site treated with high concentration of sodium chloride (5.64 M NaCl), where the hydration did not increase from the baseline, which could be due to its dehydrating property or interactions preventing skin exudates from being formed (Figure 4.4).
At a cellular level, tape stripping results in mitotic cell division at the basal layer that could occur for a week and cornification of granular layer in order to restore the lost SC (Menon 2002). It also results in dilation of skin blood vessels which makes the skin appear red in colour, increase in blood flow causes heat generation and chemical mediators causes rise in temperature for that skin spot. This epidermal injury leads to the development of temporary barrier by triggering an epidermal inflammation cascade that increase in cytokine secretion in stratum granulosum (Elias et al. 2008), to avoid water loss. The observed reduction in TEWL at 96 hours in the no treatment (air) spot could be indicative of this innate repair mechanism. While all the other treated spots after 96 hours did not show the same effect, which could be due the inhibition of repair mechanism by water, which is the vehicle (von Zglinicki et al. 1993). Previous studies reported improvement in barrier recovery 9 hours after treatment with magnesium and calcium salts in mice (Denda et al. 1999). Also, Denda et al. and Lee et al. (*vide infra*) have reported calcium ion delays the recovery of the stratum corneum in mice. On the other hand, our results do not indicate any significant improvement TEWL returning to the baseline level after treatment with different salt solutions. Difference in application of treatment, concentration of the solution, type of skin used (human volunteers in this case), could be the reason for contradiction in results (Denda et al. 1999; Lee et al. 1994).

We observed a peculiar pattern, with opposite effects on skin hydration in the 1.88 M MgCl$_2$ and 5.64 M NaCl treated spots, even though these concentrations are of equal ionic strength. This is consistent with the unique chemical natures of magnesium and sodium having hydrating and dehydrating properties, respectively. Increase in hydration levels could facilitate the expression of specific proteins by magnesium ions such as filaggrin, which in turn results in natural moisturising factors (Gerritsen et al. 1994). Twenty-four hours after tape stripping hydration levels of all treatment spots were similar to baseline. It has also been reported that the hydration of magnesium ions are higher than calcium, which is supported by the result in Fig 4.4 (Theophanides et al. 1990).

We found that tape stripping does not change the skin pH (Figure 4.5) neither did salt treatment over 96 hours have any effect on it. Acid mantle is the phenomenon that causes skin to maintain an acidic pH and is formed mainly in two ways. Exogenously, it is formed due to enzyme activity and membrane pump and endogenously, free fatty acids sweating from sweat glands, sebum secretion, microbial metabolite like lactic acid generation on the skin (Ali & Yosipovitch 2013). This acid mantle functions as antimicrobial in nature, non-pathogenic bacteria adhesion on the stratum
corneum and lowering the microbial growth. Hence, it is important that pH of the skin has to be always maintained at acidic levels.

Salt solutions used in this study were maintained at a pH closer to a neutral. Previous studies have reported increase in pH after 70-100 tape strips, whereas in our study skin pH before and after tape stripping were maintained at acidic levels (pH of 5.07 ± 0.62). Another possible reason why we did not observe improvement in barrier recovery with TEWL measurements could be due to subjecting the tape stripped skin to the solution with neutral pH, which may delay the recovery. We can correlate this with the case of dermatitis occurring mostly in children as their skin pH is neutral and stratum corneum layer is thin in children as compared to adult, so recovery of the barrier is delayed in children. Thus it implies that treatment with magnesium and calcium salts do not alter pH of treatment spots.

In conclusion we found that treatment with 1.9M MgCl$_2$ increased the skin hydration almost two fold within the first 6 hours after treatment. However, the TEWL values did not return to baseline levels even after 96 hours after tape stripping and subsequent treatment with salt solution. This could be due to the pH 7.8-7.9 (rather than the normal pH 5.5) affecting barrier recovery.
5.1. INTRODUCTION

In the previous chapter (Chapter 4), we investigated the effect of magnesium treatment on parameters that indicate barrier function. Since the stratum corneum formation is a result of continuous differentiation and anucleation of keratinocytes, it is important to understand the metabolic changes occurring in these cells. The skin is a metabolically active organ and contains enzymes, which catalyse hormones, steroids, inflammatory mediators, and xenobiotics such as drugs pesticides, and chemicals (Bronaugh et al. 1994). Subjecting the skin to treatment or physical disruption could affect the metabolic balances, which are mainly regulated by cellular cofactors and enzymes. These cofactors vary between different layers of the skin and have their unique intrinsic autofluorescence.

In the last two decades, MPT has developed into a powerful and important technique that enables the concurrent monitoring of both, morphology of the organ and solute transported into that organ including skin and is being used increasingly in clinical applications (Roberts et al. 2008) in biomedical research. This fluorescence technique allows detection of specific molecules based on fluorescence or autofluorescence signals. The result is the capacity to characterise cell physiology at subcellular resolution with limited toxic effects.

Previous studies have indicated the role of magnesium ions in cell proliferation and maintaining epidermal integrity (Lipkin et al. 1963; Denda et al. 1999).

The objective of this study is,

1. To test the hypothesis that treatment with magnesium solution could improve the metabolic effects implicated by tape stripping.
2. To study the changes in redox ratio, NAD(P)H/FAD lifetime and nucleus to cell ratio after tape stripping and subsequent magnesium treatment.

5.2. RESULTS

5.2.1. Magnesium treatment increases redox ratio
In the stratum granulosum and stratum spinosum layers of the epidermis, an effect of 30-tape strips difference between the redox ratio of control and tape stripped skin is significant at zero hours (p <0.05) (Figure 5.3). However, after 24 hours this difference was not observed in untreated and water treated site, but a significant increase in redox ratio of tape stripped site was observed when treated with 1.9M MgCl₂. We did not observe any significant change in NAD(P)H/FAD lifetime (Figure 5.2) and nucleus to cell ratio (Figure 5.4).

**Figure 5.1:** Representative images of epidermal layers acquired from MPT-FLIM. Representative images of a.) stratum granulosum, b.) stratum spinosum c.) stratum basale from three volunteers captured using DermaInspect® system (JenLab GmbH, Jena, Germany) that used an ultra-short-pulsed, mode-locked, 80-MHz Titanium Sapphire laser (Mai Tai, Spectra Physics, Mountain View, California, USA) for excitation. We acquired images before and after tape stripping at 0 hours and treated both normal and tape stripped skin with water and 1.9 M MgCl₂. We repeated the treatment over for 4 times with 24 hour interval and obtained images at 24 hours and 96 hour time point.
Figure 5.2: No significant changes in NAD(P)H/FAD (τm) Lifetime in epidermal layers after treatment.

Graphs showing comparison between normal and tape stripped skin under different conditions of treatment in all the layers of epidermis - a.) stratum granulosum, b.) stratum spinosum c.) stratum basale.
Figure 5.3: Magnesium treatment significantly increases the redox ratio in after 24 hours of magnesium treatment.
Tape stripping the skin 20 times significantly reduced the redox ratio in all three layers of epidermis - a.) stratum granulosum, b.) stratum spinosum, c.) stratum basale. We found that treatment with 1.9 M MgCl$_2$ significantly increased redox ratio in granulosum and spinosum layers.
Figure 5.4: No significant changes were observed in N/C ratio. Tape stripping increases N/C ratio in tape stripped skin when compared to normal skin in most treatment conditions, however the increase was not statistically significant.
5.3. DISCUSSION

Magnesium ions are important cofactors in regulating various cellular processes such as metabolism and proliferation (Swaminathan 2003). When the stratum corneum layer is removed physically through tape stripping, it not only exposes the granular layer but also triggers an inflammatory response (Gerritsen et al. 1994). In order to maintain the epidermal integrity in a healthy skin, the innate mechanism functions in such a way that the lost stratum corneum is replaced. In this chapter, we aimed to understand the metabolic changes that occur in the epidermal layers of skin when the barrier is disrupted by tape stripping and observe the subsequent effect of treating the tape stripped region with magnesium solution at 24 and 96 hours.

The use of MPT-FLIM has gained importance in recent times owing to its non-invasive nature of understanding cellular morphology and metabolic indicators that reflect the effect of external treatments. NAD(P)H lifetime is resolved as a two-component system with a short (~0.3–4 ns) and long (~2.3 ns) lifetime, that represents free and protein-bound conformations, respectively (Lakowicz et al. 1992; Niesner et al. 2004; Berezin & Achilefu 2010).

We measured the mean amplitude-weighted lifetime ($\tau_m$) and free:bound ratio ($\alpha_1/\alpha_2$) of NAD(P)H and FAD at different strata of the viable epidermis. The average lifetime ($\tau_m$) measurements in all the layers were lower in the tape stripped region when compared to normal skin site irrespective of the treatments at all measured time points. This was consistent with previous studies, where change in NAD(P)H and FAD lifetime reflected on metabolic perturbation. However the change observed in our study was not significant enough to make any assertions about the role of magnesium treatment in moderating metabolism.

On the other hand, we observed a significant reduction in the redox ratio immediately after tape stripping in all three layers of the epidermis, which reflect the onset of cell differentiation (Quinn et al. 2013). These cells will eventually undergo terminal differentiation to restore the lost stratum corneum layer. The free-to-bound ratio of NAD(P)H, represented by the ratio of the amplitude coefficients for the short and long lifetimes (i.e. $\alpha_1/\alpha_2$), is related to the NADH/NAD+ ratio and was used as an indicator for redox changes within the cell (Bird et al. 2005). Several T cell-mediated skin diseases such as psoriasis, atopic and contact dermatitis are associated with overproduction of reactive oxygen species and increased levels of lipid peroxidation, eventually leading to an imbalance in redox. When we observed skin sites 24 hours after treatment in granulosum and spinosum layers, we found that the untreated skin site and site treated with water had the same levels of redox ratios in both normal and tape stripped cases, whereas the tape stripped
site treated with 1.9M MgCl₂ had a significantly elevated redox ratio. The basale layer of tape stripped skin showed similar increase in redox ratio when compared to normal skin, 24 hours after treatment with magnesium, but was not significant. This phenomenon could be seen as a role of magnesium ions in improving the redox state of damage inflicted skin. However, after five subsequent treatments of water and 1.9M MgCl₂ at 24-hour intervals showed similar effects on redox ratio and the redox change caused by tape stripping seems to have returned to normal levels.

Therefore, we concluded that this study provides as model to suggest magnesium increases the redox ratio thereby contributing to improve metabolism, and promote differentiation to replenish the stratum corneum from time to time. However, extensive studies have to be conducted to demonstrate results with larger sample size.
CHAPTER 6: ROLE OF MAGNESIUM IN REGULATING FILAGGRIN LEVELS IN HYPERSENSITIVITY INDUCED HUMAN EPIDERMIS

6.1. INTRODUCTION

The importance of stratum corneum as an effective barrier has been discussed extensively in the previous chapters. The barrier formation is a continuous process occurring as a result of differentiating basal cells, and subsequent terminal differentiation. It is the process by which anucleation of keratinocytes is facilitated by epidermal proteins such as filaggrin.

We established in Chapter 3 that magnesium ions are able to permeate through skin, which leads us to hypothesise that topical magnesium application can modulate or regulate filaggrin levels in the epidermis due to the ions ability to bring conformational changes to profilaggrin and subdue inflammatory signals.

Even though the role of filaggrin has been well characterised and established that several skin conditions could be caused due a to loss function or mutation, the specific role of magnesium ions have not been studied. Profilaggrin could be a major target for magnesium ion due to the presence of calcium binding EF-hand motif (Markova et al. 1993). Anecdotal evidences of magnesium-based therapy for skin conditions are claimed to act by improving barrier function and hydrating the skin, complying with the characteristics of an epidermal system when filaggrin is well regulated (Proksch E, Nissen HP, Bremgartner M 2005; Denda et al. 1999).

The objective of this chapter is to,

1. To study the effect of tape stripping and DNCB on FLG levels.
2. To understand the effect of tape stripping and DNCB on skin treated with magnesium and calcium salts.

Hence studying the protein levels as a result of magnesium ion treatment could provide direct evidence to the therapeutic role of filaggrin in restoring epidermal integrity and its ion binding capabilities in normal, stimulated or inflamed human skin.
6.2. RESULTS

6.2.1. High concentration of magnesium and calcium salts increases FLG levels.

Topical application of highly concentrated salt solution, 1.9 M MgCl$_2$ (Figure 6.1, d) and 0.44 M CaCl$_2$ (Figure 6.1, f) for 30 minutes rapidly increased the FLG levels. This effect was not observed when the skin was not treated with any salt solution or when we topically applied 5mM concentration of magnesium (Figure 6.1, b) and calcium chloride (Figure 6.1, e). The two right hand columns of images in figure 6.1a and the two right hand columns in the histograms in fig 6.1 b to 6.1 f show the images and data for DNCB and TS skin immediately after application, subsequent to the 30 minutes pre-incubation with salt solutions.

6.2.2. Stimulation with DNCB and tape stripping increases FLG levels

We incubated excised skin at 4 °C for 20 hours after stimulating with 0.1% DNCB in acetone or tape stripping 30 times and observed increase in FLG levels when compared to FLG levels in normal untreated skin (Figure 6.2, b).

6.2.3. Magnesium and calcium pre-treated skin restricted rise in FLG levels when stimulated.

We pre-treated skin with magnesium and calcium chloride solutions for 30 minutes using the setup described in section 2.3. Excised pre-treated skin was then either stimulated by DNCB or TS, or left unstimulated, prior to incubation for a further 20 h at 4°C. After 20h at 4°C, unstimulated skin, that had been pre-treated for 30 minutes with 5mM CaCl$_2$, showed significantly higher levels of FLG compared to 5mM CaCl$_2$ treated skin that had subsequently been stimulated with DNCB or TS (p < 0.05) (Figure 6.2, e). As shown in figures 6.2 c, e and f, we found that skin pre-treated with 5mM MgCl$_2$, 5mM CaCl$_2$ or 0.44M CaCl$_2$, but unstimulated (white histograms) tended to have higher FLG levels than in skin stimulated with DNCB (light grey histograms) or TS (dark grey histograms) (p<.05 level in figure 6.2 e only). In the case of pre-treatment with 1.9M MgCl$_2$, there was no apparent decrease in filaggrin levels after DNCB stimulation (figure 6.2d). All of the salt solution treatments appear to have elevated the level of filaggrin in unstimulated skin, compared to unstimulated skin without salt solution treatment (p < 0.05, One way ANOVA, Tukey’s test).
Figure 6.1: Filaggrin expression is observed immediately after 30 minute treatment with high concentration salt solutions.

a.) Representative images showing FLG expression (green) and epidermal cells (red) on sections of skin that were stored in -80°C immediately after 30 minute pre-treatment or stimulation. Images were acquired with 20X oil immersion objective in Zeiss LSM 510 microscope. The intensity of fluorescence was measured using normalised CTCF values for b.) no salt pre-treatment, c.) 5 mM MgCl$_2$ treated, d.) 1.9 M MgCl$_2$ treated, e.) 5 mM CaCl$_2$ treated, and f.) 0.44 M CaCl$_2$ treated. Experiments were repeated in at least 3 donors with error bars representing mean ± SEM.
Figure 6.2: Effect DNCB and tape stripping after 20 hours on filaggrin expression in excised human skin pre-treated with magnesium and calcium salts.
a.) Representative images of showing FLG expression (green) and epidermal cells (red) on sections of skin that were incubated in PBS media at 4°C for 20 hours after pre-treatment or stimulation. Images were acquired with 20X oil immersion objective in Zeiss LSM 510 microscope. The intensity of fluorescence was measured using normalised CTCF values for b.) no salt pre-treatment, c.) 5 mM MgCl₂ treated, d.) 1.9 M MgCl₂ treated, e.) 5 mM CaCl₂ treated, and f.) 0.44 M CaCl₂ treated. Experiments were repeated in at least 3 donors with error bars representing mean ± SEM. Statistics was performed using one-way ANOVA Tukey’s test with * P<0.05.
6.2.4. Topical application of salt solutions increases skin hydration but does not change skin pH.
We measured the changes in skin pH and hydration on the volar forearm of human volunteers, before and after topical application salt solution. We did not observe any change in pH after treating defined regions of the skin as indicated in figure 6.3 a, with 5mM & 1.9 M MgCl₂ and 5mM & 0.44 M CaCl₂. At the same sites we found that treatment with these salt solutions significantly increased skin hydration (Figure 6.3, b).

Figure 6.3: MgCl₂ and CaCl₂ treatment does not change skin pH but increases skin hydration.
All a.) pH and b.) hydration measurements were taken on the inner forearm before (white columns) and after(grey columns) treatment with 5mM and 1.9 M MgCl₂ and 5 mM and 0.44 M CaCl₂. One way ANOVA using Sidak’s test was performed. * P<0.05 and *** P<0.001. n=6.

6.3. DISCUSSION
Profilaggrin has an important role in keratinization and maintaining epidermal integrity. It has been widely investigated to understand implications of its mutations in genetic skin disorders such as ichthyosis vulgaris and atopic dermatitis (Brown & McLean 2012; Bussmann et al. 2011). However, its relevance in normal skin particularly in response to haptens and mechanical stress has not been extensively studied. We hypothesized that the predominance of magnesium and calcium ions in epidermis and their role in metabolic and anti-inflammatory pathways could regulate FLG levels in normal skin, when subjected to stimuli (Figure 6.2,b). Profilaggrin has a domain that is known to have an affinity for binding magnesium ions, however it is four fold lower than that of calcium ions (Markova et al. 1993). There are several anecdotal observations and research data that attributes to the role of magnesium and calcium ions in skin barrier function(Proksch E, Nissen HP,
Bremgarter M 2005; Lipkin et al. 1963). Therefore we sought to investigate the effect of DNCB and tape stripping on human skin topically enriched with varying concentrations of magnesium and calcium solutions.

We observed enhanced levels of FLG in the epidermis, compared to normal skin (Fig 6.1,b), when magnesium chloride (Figure 6.1,d) and calcium chloride solutions (Figure 6.1,f) were topically applied for 30 minutes. This could be attributed to the binding affinity of N terminal domain EF-hand of pro-filaggrin to the divalent ions (Figure 6.4). The binding of these ions subsequently expose the sites for profilaggrin cleavage to filaggrin monomers by proteases, including Caspase-14 which is present in abundance in human epidermal cells (Eckhart et al. 2000). To obtain further understanding, we predicted the model of profilaggrin and specifically highlighted the EF-hand, calcium-binding domain found in its amino terminus. The filaggrin increase observed with magnesium salts could be due to the flexibility of residue 12 in the EF-hand of profilaggrin that can accommodate a magnesium ion in the place of calcium (Markova et al. 1993). This is possibly due to the binding of divalent ions present to the EF-hand motif of N-terminal in pro-filaggrin eventually leading to conformational changes exposing the cleavage sites. This suggests that both calcium and magnesium are capable of binding to profilaggrin and inducing its degradation to filaggrin. In addition, we established in Chapter 3 that magnesium ions were able to permeate through stratum corneum and localise in the upper granular region. This may further facilitate profilaggrin to FLG conversion.

We also demonstrated an increase (p<0.05) in FLG expression in both DNCB treated and tape stripped skin (Figure 6.2,b). Past studies have demonstrated that either epidermal LCs or dermal Ia+ APCs can induce contact hypersensitivity when stimulated with haptens or tape stripping (Sreilein 1989, Tsey 1990). Stimulation of DCs with DNCB and barrier disruption increases TNF-a production in a p38 MAPK or ERK dependent pathway and EGF dependant pathway, respectively (Fanny 2004). It is known that DNCB initiates cell-mediated immune response that increases TNF-a levels via p38 MAPK and ERK pathways. By the stimulation of TNF-a through these pathways, cytokines CD86, CD54, CD40 are expressed. These cytokines are also expressed through similar pathways under magnesium deficient conditions (Miyazawa M et al. 2008; Lee et al. 2011). This TNF-a increase acts as an apoptotic signal, in response to which the keratinocytes are primed by FLG to aid its terminal differentiation, leading to the formation of corneocytes (Raj et al. 2006; Kuechle et al. 2000).
Figure 6.4: Structure and position of the Calcium Binding Domain of Pro-filaggrin as predicted by threading using I-Tasser®.
The structure of Pro-Filaggrin (containing a single Filaggrin monomer) containing a calcium binding N terminal domain EF-hand (indicated in red). The residues at position 1, 3, 5, 7, 9 and 12 (indicated by multiple colours) of the 12 residue EF-hand form the buried Calcium binding pocket with limited solvent accessibility. The N terminal is indicated in green while the Filaggrin monomer is indicated in cyan. This structure was generated from 1-306 residues of a previously published sequence of Pro-filaggrin (PDB ID: P40930) using multiple alignment threading algorithm called I-Tasser® with a confidence score of -4.38 and estimated TM-score of 0.26±0.08.

We found that when excised skin was pre-treated with magnesium or calcium salts and subsequently stimulated with DNCB and TS, the FLG levels are always lower than that of the unstimulated salt treated skin (except in the case of 1.9M MgCl2 stimulated with DNCB, section 6.3.3). Of all the different concentrations used, we found that 5mM CaCl2 significantly increased FLG levels in excised skin when compared to 5mM CaCl2 treated skin subsequently stimulated with DNCB or TS. These findings suggest that magnesium and calcium chloride treatments exhibit a preventive action on the skin sections such that the repair/recovery required in response to the stimulations is contained and minimized in comparison to increased FLG levels observed when normal skin was stimulated with DNCB or TS (Figure 6.2,c,d,e,f).

Changes in pH also can cause profilaggrin to degrade into FLG monomers. As suggested by previous literature skin pH and hydration are important indicators of skin integrity (Egawa et al. 2007). As shown in figure 6.3, pH levels did not significantly change due to any magnesium and calcium salt treatment, irrespective of their concentrations. However, a significant increase in hydration levels were observed post treatment in most volunteers. Increase in hydration levels were
higher in skin treated with the magnesium salt when compared to the calcium salts. Increase in hydration indicated that the salt solution did not dehydrate the skin, but rather provided suitable conditions for profilaggrin cleavage. This could be directly related to the further degradation of filaggrin monomers to form natural moisturizing factors.

Previous studies have shown that magnesium and calcium antagonise each other, and when an environmental change occurs can compete with each other. Filaggrin expression in many cases has been previously linked to protective roles such as rehydration of damaged skin, cornification of new stratum corneum layers. Hence we conclude that both magnesium and calcium at high concentrations increase FLG levels within 30 minutes of treatment (section 6.3.1), raising the possibility of salts having a broader protective effect against haptens and mechanical stress by a preemptive elevation of filaggrin levels (section 6.3.3).
CHAPTER 7: CONCLUSION

7.1. OVERVIEW

Dead Sea therapy is one of the oldest forms of treatment for skin disease and some chronic inflammatory diseases like arthritis and psoriasis. Much of the research to date has attributed the clinical effects of Dead Sea therapy to its mineral composition; mostly to magnesium salts. Magnesium salts, such as magnesium sulphate (Epsom salts), have long been used as a spa product and as a therapeutic to manage clinical conditions. The rationale of this PhD research was to understand the role of topically applied magnesium ions in epidermal integrity of human skin. We compartmentalized this research project into studying physiological, metabolic and proteolytic changes occurring at different layers of the skin.

The overall hypotheses tested in this thesis were:

1) Magnesium ions are able to permeate through stratum corneum, and that this permeation is dependent on time and concentration

2) Magnesium ions accelerate barrier recovery and regulate metabolism in epidermal cells

3) Magnesium ions change levels of important epidermal proteins, such as filaggrin to maintain epidermal integrity.

We found that, contrary to the suggestion in several publications, magnesium permeates through the stratum corneum (section 3.3.1), in a concentration and time dependent manner (section 3.3.2), with significant contribution from hair follicles (section 3.3.3). Topical application of magnesium resulted in increased skin hydration levels (section 4.3.4) and increased FLG levels (section 6.3.1). Skin pre-treated with magnesium and calcium restricted rise in FLG levels when stimulated with DNCB or tape stripping (section 6.3.3). We did not observe signs of barrier recovery, as the TEWL measurements over 96 hours in vivo remained higher than baseline levels (section 4.3.3). However, redox ratio in tape stripped skin sites in vivo showed a significant increase over normal skin, 24 hours after treatment with magnesium chloride solution at 1.9M (section 5.3.1).

Future studies could be directed towards quantitative evaluation of magnesium permeated through the epidermal layers. In vivo experiments to investigate barrier recovery could be conducted over an elongated period of time to allow recovery of TEWL to normal or baseline levels. Even though the redox ratio seems to be significantly increased by topical application of magnesium, a similar study with larger sample size could yield more information about magnesium’s role in epidermal
metabolism. We have shown an increase in FLG levels when the skin is treated with salt solutions or when they are stimulated with external stress. Further research could focus on the mechanisms by which FLG is increased.

7.2. KEY FINDINGS

7.2.1. Magnesium permeates through stratum corneum, largely through hair follicles.

In Chapter 3, we topically applied 5mM magnesium chloride solution on excised human skin and found that magnesium ions permeate through stratum corneum. Significantly high fluorescence intensities relative to section untreated with MgCl₂, were observed at 15 and 60 minutes after the skin was exposed to 1.9 M MgCl₂ concentration and after 60 minutes of exposure to 52mM MgCl₂. This indicates that magnesium penetration increases with progression of time, and higher concentration accelerate permeation. According to Fick’s law of diffusion, flux goes from regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient (Brogioli & Vailati 2001; Brisson 1974). Thus the observed increase in fluorescence intensity is due the movement of magnesium ions from a region of high concentration to low concentration, with more rapid movement at higher external concentrations. We developed a novel method to plug the region surrounding the hair follicle based on polymerisation of cyanoacrylate. We topically applied MgCl₂ solution on excised skin on exactly the same skin area with and without the plugging 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 possible drug penetration routes. We also found that hair follicles contribute significantly to the permeation of magnesium ions.

7.2.2. Topical magnesium application increases skin hydration.

In Chapter 4, we found that treatment with 1.9M MgCl₂ increased the skin hydration almost two folds within the first 6 hours after treatment. Increase in hydration levels could facilitate the expression of specific proteins by magnesium ions such as filaggrin, which in turn results in natural moisturising factors (Gerritsen et al. 1994). Twenty-four hours after tape stripping hydration levels of all treatment spots were similar to baseline. It has also been reported that the hydration of magnesium ions are higher than calcium, which is supported by the result in Fig 4.4 (Theophanides et al. 1990). The TEWL values did not return to baseline levels even after 96 hours after tape stripping and subsequent treatment with salt solution. This could be due to the pH 7.8-7.9 of treatment solutions that we used (rather than the normal pH 5.5) affecting barrier recovery.
7.2.3. Redox ratio decreases upon tape stripping, but increases after topical magnesium application.

In Chapter 5, the stratum granulosum and stratum spinosum layers of the epidermis, as an effect of 30-tape strips difference between the redox ratio of control and tape stripped skin is significant at zero hours (p <0.05). When we observed skin sites 24 hours after treatment in granulostum and spinosum layers, we found that the untreated skin site and site treated with water had the same levels of redox ratios in both normal and tape stripped cases, whereas the tape stripped site treated with 1.9M MgCl₂ had a significantly elevated redox ratio. The basale layer of tape stripped skin showed similar increase in redox ratio when compared to normal skin, 24 hours after treatment with magnesium, but was not significant. This phenomenon could be seen as a role of magnesium ions in improving the redox state of damage inflicted skin. We observed significant reduction in redox ratio immediately after tape stripping in all three layers of the epidermis, which reflect the onset of cell differentiation (Quinn et al. 2013). These cells will eventually undergo terminal differentiation to restore the lost stratum corneum layer.

7.2.4. Magnesium and calcium treatment increased FLG levels in excised skin.

In Chapter 6, we found that both magnesium and calcium at high concentrations increase FLG levels within 30 minutes of treatment. It is likely that these salts have a protective effect against haptens and mechanical stress by pre-mediated increase in filaggrin levels.

Topical application of highly concentrated salt solution, 1.9 M MgCl₂ (Figure 6.1, d) and 0.44 M CaCl₂ (Figure 6.1, f) for 30 minutes rapidly increased the FLG levels. This effect was not observed when the skin was not treated with any salt solution or when we topically applied 5mM concentration of magnesium (Figure 6.1, b) and calcium chloride (Figure 6.1, e). This could be attributed to the binding affinity of N terminal domain EF-hand of pro-filaggrin to the divalent ions (Figure 6.4). The binding of these ions subsequently expose the sites for profilaggrin cleavage to filaggrin monomers by proteases, including Caspase-14 which is present in abundance in human epidermal cells (Eckhart et al. 2000).

We also demonstrated an increase (p<0.05) in FLG expression in both DNCB treated and tape stripped skin (Figure 6.2,b). Past studies have demonstrated that either epidermal LCs or dermal Ia⁺ APCs can induce contact hypersensitivity when stimulated with haptens or tape stripping (Sreilein 1989, Tse y 1990). Stimulation of DCs with DNCB and barrier disruption increases TNF-a production in a p38 MAPK or JNK dependant pathway and EGF dependant pathway, respectively (Fanny 2004). This TNF-a increase acts as an apoptotic signal, in response to which the
keratinocytes are primed by FLG to aid its terminal differentiation, leading to the formation of corneocytes (Raj et al. 2006; Kuechle et al. 2000; Eckhart et al. 2000).

### 7.3. FUTURE DIRECTIONS

We have successfully demonstrated that magnesium ions are permeable through stratum corneum, however, quantification of magnesium levels in the epidermal layers is still a challenge. Quantitative methods such as absorption spectroscopy could be used for analysing homogenates of dermatomed tissue to evaluate epidermal magnesium. Also, to further characterise the transdermal route of permeation for magnesium ions, a sandwich model of the excised skin could be used (Barry 2002).

*In vivo* experiments in Chapter 4, to investigate barrier recovery could be conducted over an elongated period of time to allow recovery of TEWL to normal or baseline levels, within a larger sample size. In Chapter 5, the changes observed in redox ratio after tape stripping and topical magnesium application serves as a good model for conducting similar experiments on a larger group of volunteers to obtain more confident results.

All the FLG immunohistochemistry experiments in Chapter 6 were conducted on excised human skin. We found that FLG levels increase upon treatment with high concentrations of magnesium or calcium and when stimulated with DNCB or TS, which are proteolytic and immunologic responses respectively. Since increase in FLG levels upon external stress could be due to expression of inflammatory cytokines facilitated by local immunological response in the skin, *in vivo* experiments could reflect changes in gene or protein expression. We have also progressed to a new study to show the involvement of epidermal dendritic cells or Langerhans cells (LCs) in excised human skin using the samples used for FLG experiment. These LCs could have a critical role in regulating inflammatory response in the skin at a local level, and in transmitting the signal systemically, either through cellular migration of release of signaling molecules. Figure 7.1 shows the presence of LCs in the epidermis when stained for langerin.
Figure 7.1: Preliminary result of langerin immunostaining on excised human skin section indicating the presence of Langerhans cells. Bright green spots indicate fluorescence of LCs.

7.4. SUMMARY

In summary, even though it is well established that magnesium is an important micronutrient involved in many biological processes, its penetration through the skin and its importance in epidermal homeostasis has been less studied. In this study, we have demonstrated that magnesium permeates through skin, predominantly through hair follicles, and at concentration equivalent to the concentration found in the Dead Sea, the magnesium ions permeate rapidly. We have also demonstrated that magnesium significantly increases skin hydration and the redox ratio. For the first time, we have also demonstrated that magnesium ions have a role in increasing FLG levels in the skin, suggestive of a protective effect against external stimuli. A significant amount of research is being done to gain a greater understanding about how topical magnesium application can impact inflammatory responses in the skin, which is the human body’s first line of defence. We believe that this study has built a foundation for new directions in this field, including investigation of the systemic neurological and immune effects of magnesium that has permeated through the skin.
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