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*Research Article*

# Magnesium Compounds Increase Aquaporin-3 in Human Epidermal Keratinocyte HaCaT Cells

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Several studies have shown that magnesium can be a useful tool in preventing various skin disorders. However, the mechanism remains unclear. In this study, we analyzed the mechanism by which magnesium improves skin function, focusing on the water channel aquaporin-3 (AQP3), which is a cutaneous functional molecule. Magnesium compounds (magnesium acetate, magnesium chloride, magnesium sulfate, and magnesium lactate) were added to human epidermal keratinocyte HaCaT cells, and the mRNA and protein expression levels of AQP3 were analyzed. We also investigated the mechanism by which magnesium acetate regulates AQP3 expression. Several magnesium compounds were individually added to HaCaT cells, and 6 hours later, the AQP3 mRNA expression level in the treated cells was significantly increased compared to that in the control cells. Among the magnesium compounds, magnesium acetate had a strong effect and markedly increased the AQP3 mRNA expression level by approximately 3.5 times and the protein expression level by approximately 3 times. Magnesium acetate also enhanced the phosphorylation of cAMP response element-binding protein (CREB), which is involved in AQP3 transcription. Furthermore, the increase in AQP3 expression levels induced by magnesium acetate was suppressed by treatment with the protein kinase A (PKA) inhibitor H-89. Magnesium compounds increased the expression level of AQP3 in epidermal keratinocytes and may have a skin-moisturizing effect. The magnesium-induced phosphorylation of CREB may be associated with the activation of the cAMP/PKA pathway. Overall, magnesium compounds may be useful for the prevention and treatment of age-associated xeroderma.

## 1. Introduction

The skin consists of the epidermis and dermis, and its water content is maintained by moisturizing factors such as ceramide, collagen, and hyaluronic acid. The levels of these moisturizing factors decrease with age, resulting in a decrease in the dermal water content and dryness [1–3]. Aging-related skin dryness causes wrinkles and itching. Substances that increase the levels of these moisturizing factors are being investigated from the viewpoint of antiaging. For example, 3-O-laurylglyceryl ascorbate, which promotes the synthesis of ceramide [4]; vitamin C-squalene bioconjugate, which promotes the production of collagen [5]; and epigallocatechin gallate, which increases the activity of hyaluronic acid synthase [6] have been identified. Although promising results in regard to age-associated xeroderma are expected, almost no cosmetics have shown dramatic effects, and investigations of materials with new mechanisms of action are now taking place.

We recently investigated the possible involvement of aquaporins (AQPs) in the development of age-associated xeroderma [7]. AQPs are proteins that transport water, and 13 subfamilies (AQP0-12) have been identified in humans [8]. In the skin, AQP3, which is classified as aquaglyceroporin, is highly expressed in epidermal keratinocytes [9]. When we investigated the relationship between skin conditions and AQP3 expression in young and old mice, we found that the aged mice had decreased dermal water content and markedly downregulated the expression of

AQP3 in the skin [7]. Moreover, AQP3-knockout mice have been reported to have reduced dermal water content [10]. Therefore, it is possible that a decrease in cutaneous AQP3 expression levels is involved as one of the causes of xeroderma, and substances that increase AQP3 expression levels may be useful for the prevention and treatment of age-associated xeroderma.

Magnesium is a mineral and plays an important role in maintaining the homeostasis of living organisms. Several studies have shown that magnesium can be a useful tool against various skin disorders. For example, it has been reported that the topical application of a magnesium-rich Dead Sea salt solution increased the water content in the skin of patients with atopic dry skin [11] and that the topical application of magnesium salt improved skin barrier function in mice [12]. Recently, we have revealed that administration of magnesium sulfate, which is commonly used as a laxative, increases AQP3 expression levels in the colon and that this increase plays an important role in laxative activity [13, 14]. However, it was unclear what effect magnesium treatment would have on AQP3 expression levels in the skin. Therefore, in this study, we aimed to clarify the mechanism by which magnesium improves skin function, focusing on AQP3.

## 2. Materials and Methods

*2.1. Cell Culture.* Human epidermal keratinocyte HaCaT cells (Cell line service, Eppelheim, Germany) were cultured in Dulbecco's modified Eagle medium containing potassium penicillin G, streptomycin, and 10% fetal bovine serum.

Magnesium acetate, magnesium chloride, magnesium sulfate, magnesium lactate (final concentrations: 10 mM and 25 mM), or sodium chloride (final concentrations: 15 mM and 37.5 mM) were added to HaCaT cells. After culture for 3 hours, 6 hours, 9 hours, 12 hours, or 24 hours, real-time RT-PCR or Western blotting was performed. In addition, magnesium acetate (final concentration: 25 mM) was added alone or simultaneously with H-89 (final concentration: 30 $\mu$M), and the cells were cultured for 3 hours or 6 hours and analyzed in the same manner.

*2.2. Real-Time RT-PCR.* Total RNA was extracted using TRI reagent (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). synthesis of cDNA from total RNA was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, Calif., USA). SsoAdvance Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), target gene forward and reverse primer solutions, and the cDNA solution were added to a PCR plate, and PCR was performed. The following primer pairs were used. AQP3; forward: AGACAGCCCCTTCAGGATTT, reverse: TCC CTTTGCCCTGAATATCTG, ribosomal protein L30 (RPL30); forward: GAAGACGAAAAAGTCGCTGG, reverse: GACCAATTTCGCTTTGCCTT. RPL30 was used as a reference gene for gene expression normalization.

*2.3. Western Blotting.* HaCaT cells were suspended in RIPA buffer (Nacalai tesque Inc., Kyoto, Japan) containing protease inhibitor (Nacalai tesque Inc.) and phosphatase inhibitor (PhosSTOP; Roche Diagnostics GmbH, Mannheim, Germany). After centrifugation (15,000 ×g, 15 minutes, 4°C), the total protein concentration of the obtained supernatant was measured by the bicinchoninic acid method. Electrophoresis was performed on a polyacrylamide gel, and the proteins were transferred to a polyvinylidene difluoride membrane. After blocking, the membranes were incubated with primary antibodies (rabbit anti-rat AQP3 antibody (Alomone Labs, Jerusalem, Israel); rabbit anti-cAMP response element-binding protein (CREB) antibody (Cell Signaling Technology, Danvers, MA, USA); rabbit anti-phospho-CREB antibody (Cell Signaling Technology); mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (EMD Millipore Corp, Burlington, MA, USA)) and secondary antibodies (donkey anti-rabbit IgG-HRP antibody (GE Healthcare, Fairfield, CT, USA); sheep anti-mouse IgG-HRP antibody (GE Healthcare)). The membranes were then reacted with ECL prime Western blotting detection reagents (GE Healthcare).

*2.4. Statistical Analysis.* Experimental values are shown as the mean ± standard deviation (S.D.). Dunnett's test or Tukey's test was used to test for statistical significance, and differences were considered significant when $p < 0.05$.

## 3. Results

*3.1. Effects of Magnesium Compounds on AQP3 mRNA Expression Levels in HaCaT Cells.* Magnesium compounds (magnesium acetate, magnesium chloride, magnesium sulfate, and magnesium lactate) were added to HaCaT cells, and the AQP3 mRNA expression level was analyzed 6 hours later.

It has been reported that the addition of 10 mM magnesium compounds to HaCaT cells attenuates ultraviolet-B-induced damage [15]. It has also been reported that bathing containing magnesium compounds at a concentration of about 50 mM improves skin barrier function and dry skin in patients with atopic dermatitis [11]. Based on the above, in this study, the concentrations of magnesium compound added to HaCaT cells were set at 10 mM and 25 mM. No morphological changes in cells were observed at a concentration of 25 mM for any magnesium compound.

All magnesium compounds significantly increased the mRNA expression level of AQP3 in HaCaT cells compared to control cells. Among them, the rate of increase in AQP3 levels induced by magnesium acetate was the highest, significantly higher than the control at low concentration (10 mM) by approximately 2-fold and at high concentration (25 mM) by approximately 3.5-fold (Figure 1).

The above mentioned results revealed that magnesium compounds increase the expression level of AQP3 in HaCaT cells.

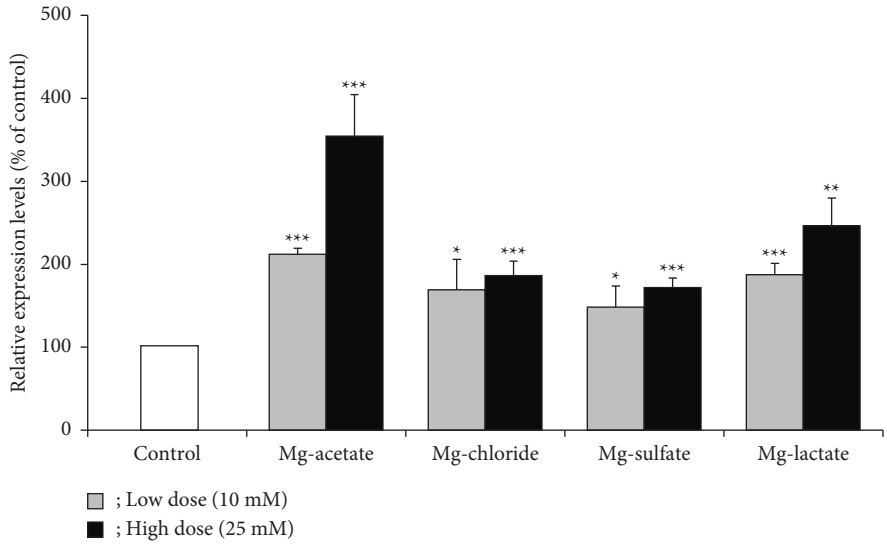


Figure 1: Effects of magnesium compounds on AQP3 mRNA expression levels in HaCaT cells. HaCaT cells were treated with magnesium acetate, magnesium chloride, magnesium sulfate, or magnesium lactate (10 mM or 25 mM). After 6 hours, the mRNA expression level of AQP3 was analyzed by real-time RT-PCR. The mRNA expression levels were normalized to RPL30, and the mean value of the control was expressed as 100% (mean ± S.D., $n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control).

*3.2. Effects of Magnesium Acetate on AQP3 Expression in HaCaT Cells.* When magnesium acetate was added to HaCaT cells at a concentration of 10 mM, the mRNA expression level of AQP3 peaked 9 hours after the treatment. However, it then began to decrease and reached almost the same level as the control cells 24 hours after treatment. A similar change was observed when magnesium acetate was added at a concentration of 25 mM but the AQP3 expression level remained twice as high as that in control cells 24 hours after treatment (Figure 2(a)).

The protein expression level of AQP3 was significantly increased by approximately 2.5-fold and 4-fold after magnesium acetate treatment at a concentration of 10 mM and 25 mM after 24 hours, respectively (Figure 2(b)).

The above mentioned results clarified that magnesium acetate increases the expression of AQP3 at both the mRNA level and the protein level.

*3.3. Effects of Osmotic Pressure on AQP3 Expression.* It has been reported that the expression level of AQP3 increases with increasing osmotic pressure [16]. Therefore, we examined the possibility that the increase in AQP3 expression by magnesium acetate was caused by the increase in osmotic pressure. Specifically, a medium containing sodium chloride was prepared to obtain the same osmotic pressure as that observed when magnesium acetate was added, and the mRNA expression levels of AQP3 were compared.

The mRNA expression levels of AQP3 were markedly increased in the magnesium acetate-treated cells compared to the control cells. However, when sodium chloride was added to account for the osmotic pressure differences, the expression level of AQP3 of the sodium chloride-treated cells increased compared to the control cells but was lower than that when magnesium acetate was added (Figure 3).

The above mentioned results showed that changes in osmotic pressure were not responsible for the magnesium acetate-induced increase in AQP3 expression.

*3.4. Effects of Magnesium Acetate on the Expression Level of Phosphorylated CREB.* It has been reported that phosphorylation of CREB is involved in the transcription of AQP [17, 18]. Magnesium acetate was found to significantly increase the mRNA expression level of AQP3 at 3 to 6 hours (Figure 2(a)). Therefore, it was considered that phosphorylation of CREB was already enhanced 3 hours after magnesium acetate addition. In this study, we analyzed the phosphorylation of CREB 3 hours after addition.

Treatment of HaCaT cells with magnesium acetate at a concentration of 10 mM significantly increased CREB phosphorylation by approximately 2-fold compared to that of control cells 3 hours after treatment. Moreover, when magnesium acetate was added at a concentration of 25 mM, phosphorylation of CREB was significantly increased by approximately 3.5-fold (Figure 4).

The above mentioned results suggested that CREB phosphorylation may be involved in the upregulation of AQP3 expression induced by magnesium acetate treatment.

*3.5. Effects of PKA Inhibitors on the Magnesium Acetate-Induced Upregulation of AQP3 Expression.* CREB is phosphorylated by PKA activation [19, 20]. Therefore, the PKA inhibitor H-89 was used to investigate whether PKA activation is involved in the upregulation of AQP3 expression by magnesium acetate treatment.

Six hours after treatment with magnesium acetate, the mRNA expression level of AQP3 was significantly increased by approximately 4-fold compared to that in control cells. However, simultaneous addition of magnesium acetate and

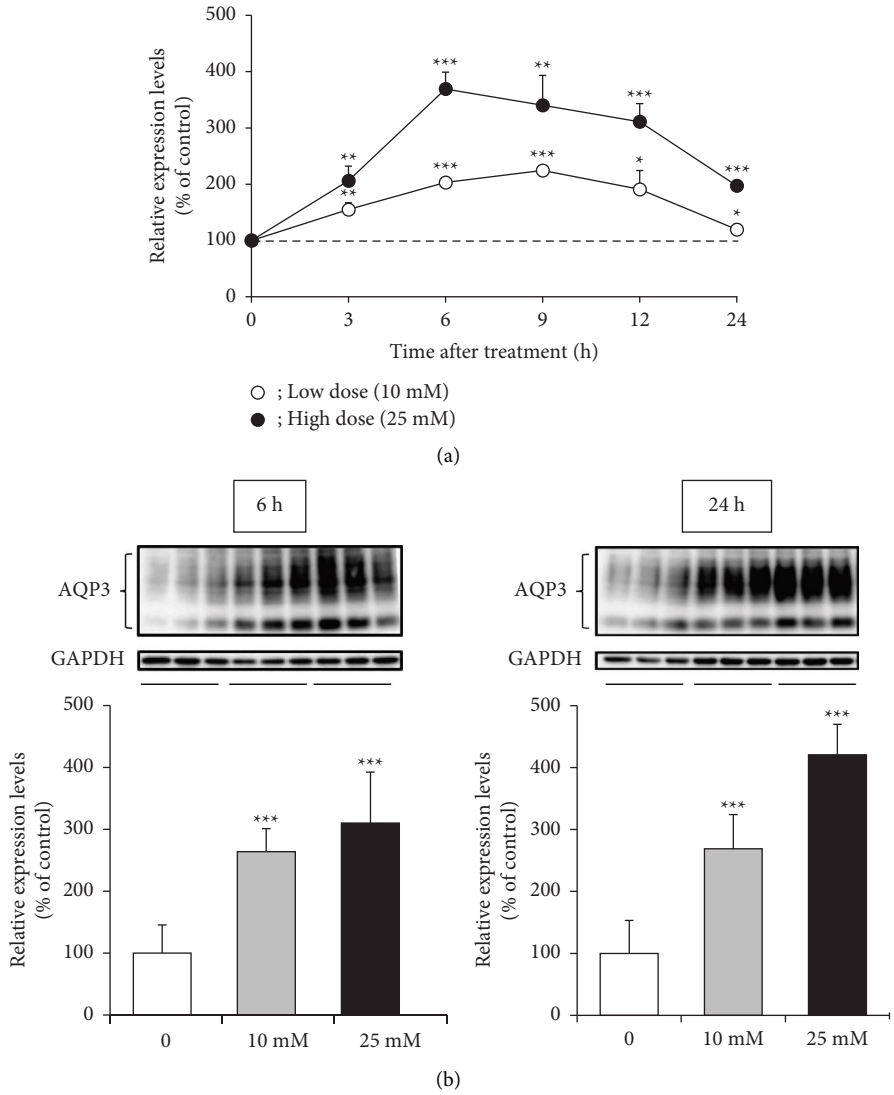


Figure 2: Effects of magnesium acetate on AQP3 expression in HaCaT cells: (a) HaCaT cells were treated with magnesium acetate (10 mM or 25 mM). After 3, 6, 9, 12, or 24 hours, the mRNA expression level of AQP3 was analyzed by real-time RT-PCR. The mRNA expression levels were normalized to RPL30, and the mean value of the control was expressed as 100% (mean ± S.D., $n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control). (b) HaCaT cells were treated with magnesium acetate (10 mM or 25 mM). After 6 or 24 hours, the protein expression level of AQP3 was analyzed by Western blotting. The protein expression levels were normalized to GAPDH, and the mean value of the control was expressed as 100% (mean ± S.D., $n = 3$, $^{***}p < 0.001$ vs. control).

H-89 suppressed the increase in AQP3 expression levels (Figure 5).

The above mentioned results suggested that magnesium acetate increases the expression level of AQP3 by activating PKA.

## 4. Discussion

Magnesium compounds have been reported to increase skin hydration and improve skin barrier function [11, 12]. In this study, we analyzed the effect of magnesium on improving skin function, focusing on AQP3 expression in the skin.

We investigated the effects of various magnesium compounds on AQP3 expression in the skin using HaCaT cells. HaCaT cells are immortalized keratinocytes that are cultured under $Ca^{2+}$ concentration and temperature conditions different from normal culture conditions. HaCaT cells are known to express AQP3 [21] and are widely used in investigations searching for substances that increase AQP3 levels [22, 23]. HaCaT cells were treated with magnesium acetate, magnesium chloride, magnesium sulfate, or magnesium lactate and cultured for 6 hours. All magnesium compounds significantly increased the mRNA expression level of AQP3 in HaCaT cells (Figure 1). Among them, magnesium acetate remarkably increased the expression level of AQP3. Therefore, in subsequent experiments, we focused on magnesium acetate and performed detailed analyses.

Treatment with magnesium acetate caused the mRNA expression level of AQP3 to increase from 6 hours to

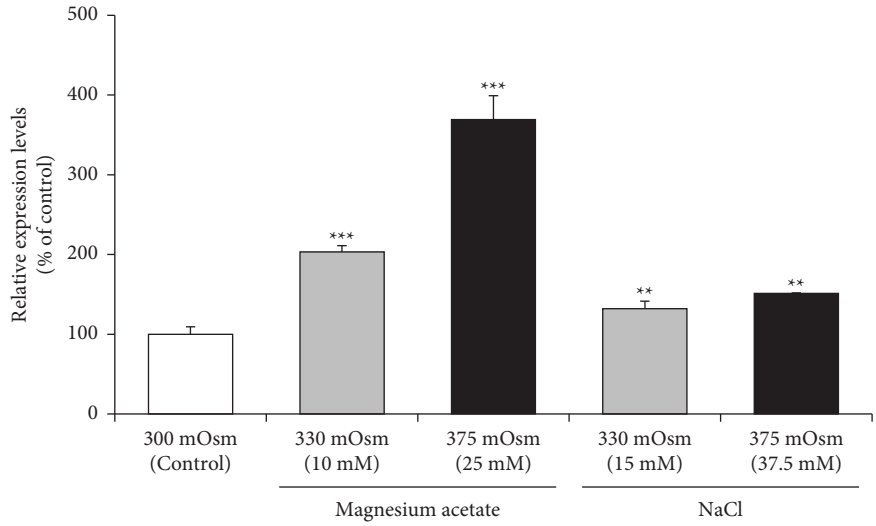


Figure 3: Effects of osmotic pressure on AQP3 expression. HaCaT cells were treated with magnesium acetate (10 mM; 330 mOsm or 25 mM; 375 mOsm) or sodium chloride (15 mM; 330 mOsm or 37.5 mM; 375 mOsm). After 6 hours, the mRNA expression level of AQP3 was analyzed by real-time RT-PCR. The mRNA expression levels were normalized to RPL30, and the mean value of the control was expressed as 100% (mean ± S.D., $n = 4$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control).

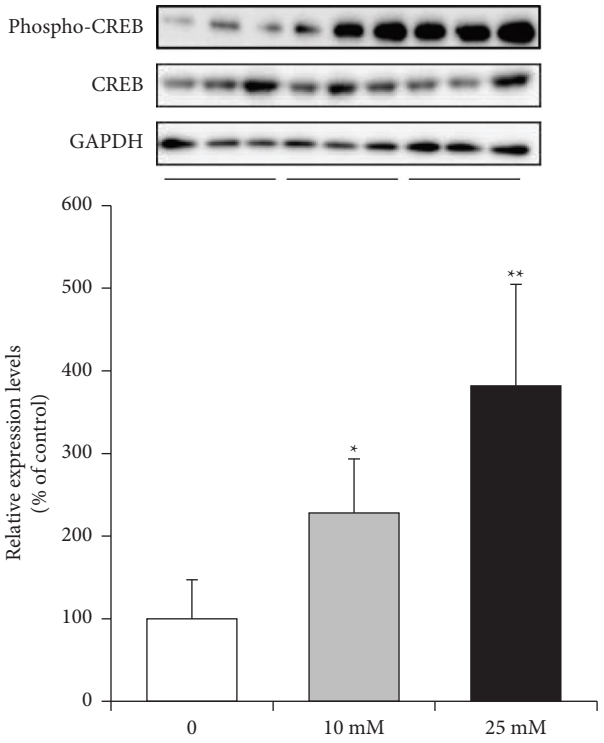


Figure 4: Effects of magnesium acetate on the expression level of phosphorylated CREB. HaCaT cells were treated with magnesium acetate (10 mM or 25 mM). After 3 hours, the protein expression level of CREB or phospho-CREB was analyzed by Western blotting. The protein expression levels were normalized to CREB, and the mean value of the control was expressed as 100% (mean ± S.D., $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control).

9 hours and then decrease (Figure 2(a)). We also found that magnesium acetate significantly increased the protein expression level of AQP3, and this increase was concentration-dependent (Figure 2(b)). These results demonstrated that magnesium acetate enhances the transcription of AQP3 in keratinocytes and increases AQP3 protein expression.

How does magnesium acetate increase the AQP3 expression level? It has been reported that the expression level of AQP3 increases with increasing osmotic pressure [16]. However, results from an experiment using sodium chloride suggested that the increase in AQP3 expression levels by magnesium acetate was unlikely to be due to increased osmotic pressure (Figure 3).

Then, we analyzed the increase in AQP3 expression levels by magnesium acetate at the molecular level. Extracellular magnesium ions are taken up into cells by transporters such as CorA, MgtE, and transient receptor potential melastatin (TRPM) 6/7 [24]. The incorporated magnesium ions activate adenylate cyclase (AC) [25]. Furthermore, activation of AC increases cAMP and phosphorylates the transcription factor CREB through activation of PKA [19, 20]. Moreover, it has been found that phosphorylated CREB promotes AQP transcription and increases its expression level [17, 18]. Therefore, we investigated the mechanism by which magnesium acetate increases AQP3 expression, focusing on the cAMP/PKA pathway. The results showed that treatment with magnesium acetate enhanced the phosphorylation of CREB (Figure 4). Moreover, it was revealed that the increase in AQP3 expression levels due to the addition of magnesium acetate was suppressed by treatment with H-89, a PKA inhibitor (Figure 5). These results suggested that magnesium acetate may be involved in CREB phosphorylation, which is associated with the activation of the cAMP/PKA signaling pathway in epidermal keratinocytes.

The results of this study showed that the rate of increase in AQP3 levels among the magnesium compounds was the highest with magnesium acetate. As mentioned above,

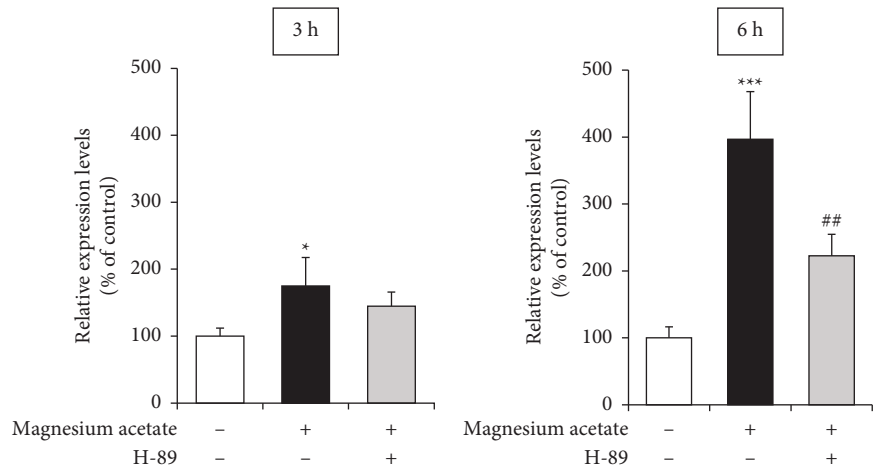


Figure 5: Effects of PKA inhibitors on the magnesium acetate-induced upregulation of AQP3 expression. HaCaT cells were treated with magnesium acetate (25 mM) alone or together with H-89 (30 $\mu$M). After 3 or 6 hours, the mRNA expression level of AQP3 was analyzed by real-time RT-PCR. The mRNA expression levels were normalized to RPL30, and the mean value of the control was expressed as 100% (mean ± S.D., $n = 4$, $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control, $^{\#\#}p < 0.01$ vs. magnesium acetate alone).

magnesium acetate dissociates into acetate ions and magnesium ions. Acetic acid was not found to increase AQP3 expression levels (data not shown). Therefore, it was considered unlikely that acetate ions upregulate the expression of AQP3. In addition, we measured the pH of the mediums containing each magnesium compound, since changes in pH alter the function of AQP3 [26]. However, in each medium, the pH was approximately 7.8, which was comparable to that of the medium containing no magnesium compound (data not shown). Therefore, it was considered that the change in AQP3 expression by magnesium acetate was not caused by pH. This study was not able to identify the mechanism by which magnesium acetate significantly increased AQP3 expression levels relative to the increases in AQP3 expression levels caused by other magnesium compounds, and this is a subject for future investigation.

Magnesium-rich Dead Sea therapy support that magnesium is useful in treating skin conditions [11, 27]. The results of this study showed that one of the mechanisms by which magnesium improves skin function is the upregulated expression of AQP3, a skin function molecule. Thus far, it is known that oral administration of magnesium causes hypermagnesemia in elderly individuals, which induces hypotension, respiratory depression, and cardiac arrest. Studies of treating human skin with magnesium chloride have confirmed that magnesium permeates throughout the skin [28]. Therefore, topical application of magnesium compounds is considered to be more effective and safer than oral administration.

AQP3 is involved in the transport of glycerol in addition to water molecules. It has been previously reported that wound healing is delayed in AQP3-knockout mice due to decreased keratinocyte proliferation, and this decreased proliferation is reversed by glycerol supplementation [29]. In addition, cutaneous AQP3 is decreased in the diabetic model rats, suggesting that this is involved in impaired wound healing [30]. Therefore, it was thought that magnesium compounds may have a certain effect on wound healing.

Decreased AQP3 levels in the skin are not only present in age-associated xeroderma but also in diseases that cause skin dryness [31–35]. For example, cutaneous AQP3 decreases in diabetes [34, 35], vitiligo [32, 33], and psoriasis [31], which causes dry skin. It has also been shown that AQP3 in the skin decreases during treatment with epidermal growth factor receptor tyrosine kinase inhibitor, an anticancer drug that causes skin dryness as a side effect [36]. Therefore, investigations for various substances and natural products that upregulate AQP3 expressions have been carried out [37, 38]. The results of this research suggest that magnesium compounds may become a useful tool for functional cosmetics in the future.

## 5. Conclusions

Magnesium compounds increased the expression level of AQP3 in epidermal keratinocytes and may have a skin-moisturizing effect. The magnesium-induced phosphorylation of CREB may be associated with the activation of the cAMP/PKA pathway. Overall, magnesium compounds may be useful for the prevention and treatment of age-associated xeroderma.

## Data Availability

All data generated or analyzed during this study are included within the article.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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