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ORIGINAL PAPER

The effect of calcium gluconate with natural extracts on skin toxicity of hydrofluoric acid

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Abstract

Backgrounds: Hydrofluoric acid (HF) is a highly corrosive acid. The conventional treatment for HF burn is topical application of calcium gluconate (CG). Our study aimed to assess the synergistic effects of natural extracts (*Centella asiatica*, *Portulaca oleracea*, and blueberry extracts) in combination with CG in healing HF induced burn wound.
Methods: We investigated the effects of different topical CG formulations with natural extracts using cell proliferation assay, western blot, reverse transcription-polymerase chain reaction, human skin equivalent model (HSEM), and mouse model.
Results: Topical CG formulations with natural extracts showed better recovery from HF burns shown by greater collagen expression in the dermal fibroblast model, HSEM and mouse model.
Conclusion: Our study demonstrated that CG in combination with natural extracts show superior wound healing in treating HF burn compared to CG monotherapy.

Keywords: Calcium gluconate, Human skin equivalent, Hydrofluoric acid, Natural extracts, Wound healing

Introduction

Hydrofluoric acid (HF) is a corrosive inorganic acid ubiquitously used in many commercial industries[1]. Due to its widespread application, injuries related to HF have been relatively common in workplace and even in homes[2]. Being an inorganic acid, not only can HF cause topical chemical burns, but in severe cases cause systemic toxicity[3,4]. HF burn is characterized by rapid tissue necrosis with severe pain[5]. There are two primary mechanisms behind HF burn injury with the first step being coagulation necrosis through dehydration followed by delayed tissue destruction through deep dermal penetration. The latter step is responsible for the hallmark severe pain of HF burns, which is a result of local hyperkalemia secondary to calcium depletion by binding of the fluoride ions to the tissue calcium ions[6]. The morbidity of the burn is directly proportional to the concentration of HF, the duration of exposure, and the appropriate emergency measures taken after contact with the acid[1,7]. The main objective in the treatment for HF burns is to neutralize the fluoride ions into insoluble salts as much as possible before they penetrate deeper into the tissue[8]. Currently, the recommended first-aid therapy is topical application of calcium gluconate (CG)[9]. The medicinal herb *Centella asciatica* contains active compounds, asiaticoside and madecassoside, known to facilitate burn wound healing through antioxidative activity, collagen synthesis, and angiongenesis[10-12]. *Portulaca oleracea*, is another herb known to possess anti-oxidative properties and accelerate the wound healing process[13]. Blueberries contain anthocyanin and polyphenols well known for their antioxidative properties[14]. Up to date, there have been no studies regarding combination therapy in the treatment of HF burns. In our study, we observed the synergistic effects of topical CG combined with the natural extracts *Centella asiatica*, *Portulaca oleracea*, and blueberry extracts in healing burn wounds induced by HF using various methods including cell prolifer-

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Table 1. Sample designation and composition.

Sample \ Composition	Calcium gluconate 2.5%	*Centella asciatica* extract 0.2%	*Portulaca oleracea* extract 0.9%	Blueberry extract 0.3%
CGF-0	–	–	–	–
CGF-C	○	–	–	–
CGF-1	○	○	–	–
CGF-2	○	○	○	–
CGF-3	○	○	○	○

ation assay, western blot, reverse transcription-polymerase chain reaction, human skin equivalent model (HSEM), and mouse model.

Materials & Methods

Chemical reagents

The anti-human pro-COL1A1 (N-17), anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CGF-C is composed of 2.5% calcium gluconate. CGF-3 is composed of *Centella asiatica* extract, *Portulaca oleracea* extract, and blueberry extracts in addition to 2.5% calcium gluconate (see Table 1).

Test solutions

The following concentrations of HF (0.005% and 20%) were produced by diluting 40% HF (Sigma-Aldrich, St Louis, MO) in deionized water.

Cell cultures

The Chamzone Corporation R&D Center (Wonju, Korea) provided HSDF (derived from neonatal foreskin). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone) with 100 U/mL penicillin and 100 mL streptomycin (HyClone). The ambient condition for cell line growth was at 37°C with a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, cells were dissociated with 0.1% trypsin immediately before the transfer and were counted by a hemocytometer.

Quantitative real-time reverse transcription-PCR

Subconfluent cell cultures were treated with HF. After extraction of total RNA (Qiagen), reverse transcription was performed using the Omniscripts RT Kit (Qiagen) according to the manufacturers' protocol. Real-time PCR was performed using the Bio-Rad iQiCycler Detection System (Bio-Rad) with SYBR green Master Mix (Bio-Rad), The following primers were used: Procollagen type I (COL1A1) amplification was performed using the following primers: 5′-GATGTTGAACTTGTTGCTGAGG-3′ (forward) and 5′-TCTTTCCCCATTCATTTGTCTT-3′ (reverse), β-actin: 5′-ACAGGAAGTCCCTTGCCATC-3′ (forward) and 5′-AGGACCAAAAGCCTTCA-3′ (reverse). Reactions were terminated by performing a melting curve analysis using the iCycler iQTM Real-Time Detection System software (Bio-Rad). The delta-delta CT method was applied for the relative quantification of each gene expression using β-actin expression as control. All reactions were carried out as triplicates.

Western blot analysis

Cells were washed with PBS and lysed in lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 100 μg/mL PMSF, and protease inhibitors). Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard. Protein lysates (50 μg) were resolved by 15-18% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking, the PVDF membranes were incubated overnight with indicated primary antibodies on a shaker at 4°C. After extensive washing, membranes were incubated with secondary peroxidase-linked IgG (1:5000, Abcam, Cambridge, MA, USA) for 1 h. After washing 3 times for 10 min with TBST at room temperature, the immunoreactivity was detected by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech).

3D Epidermis

The human reconstituted epidermis 3D EpiDerm™ models (EPI-200) were purchased from MatTek Corporation (Ashland, MA, USA). HSEMs resemble normal human epidermis and consist of multilayered, differentiated tissue, including basal, spinous, granular, and cornified layers. HSEMs were incubated with assay medium at 37°C and 5% CO_2.

Hematoxylin and Eosin staining (H&E staining)

HSEMs and BALB/C mice were used in each experimental group, and each experiment was repeated at least 3 times. After exposure, the 3D EpiDerm™ and mice sample were collected and fixed immediately in 10% formalin for H&E staining microscopy analysis. The 5 μm sections of 3D EpiDerm™ and mouse samples were fixed in 10% formalin in PBS and stained with H&E.

Masson's Trichrome staining (MT staining)

Formalin-fixed tissue was embedded in paraffin and 5 μm sections were stained with Masson's Trichrome reagent to demonstrate collagen. The procedure was as follows: 1) fix in Bouin's or Zenker's liquor for one night, 2) wash in running water until the yellow color disappears and rinse in two changes of distilled water, 3) stain with Mayer's Hematoxylin for 5 min, 4) place in 0.5% hydrochloric acid in 70% alcohol for 5 s, 5) wash in running tap water for 30 s and rinse in two changes of distilled water, 6) stain with acid ponceau for 5 to 10 min, 7) rinse in three changes of distilled water, 8) dissolve in 1% Phosphomolybdic acid aqueous solution, 9) stain with aniline blue or brilliant green for 5 min, 10) dissolve in 1% glacial acetic acid for 5 min, 11) dehydrate in 95% ethyl alcohol for several times, followed by anhydrous alcohol, 12) hyalinize with dimethylbenzene, and 13) seal with neutral balsam. Collagen fibers are stained blue, cytoplasm, muscle fibers and red blood cells red and the nuclei black.

Animal experiments

Female BALB/c mice (8 weeks old) were obtained from Orient Bio (Seong-nam, Korea) and maintained at the KUIACUC. Mice were given cutaneous exposures of 20% hydrogen fluoride for 5 min. After immediate washing, mice were applied with CGF-C or CGF-3 daily up to 7 consecutive days. The day after final treatment, samples were collected from the back of the mice and immediately fixed in 10% formalin solution for H&E staining and MT staining.

Statistics

All experiments were performed in triplicate and independently repeated at least 3 times. The statistical significance of the data was determined by one-way ANOVA using GraphPad PRISM version 4.02 for Windows (GraphPad Software, San Diego, CA). The results are expressed as mean ± SD. The level of significance was set at $P < 0.05$.

Results

Assessment of the induction of expression of COL1A1 (collagen type I alpha I) in skin fibroblast by CGF following HF burn

Using MTT assay, we determined the concentration of CGFs exposed to the fibroblast to be 0.05%, at which the cell viability was maintained at 70-80% (data not shown). After exposure to 0.005% HF, subsequent treatment with different treatment samples were performed. As shown in Figure 1, there was no difference in the COL1A1 mRNA levels between the different concentrations of CGF 6 h after treatment. However, after 12 h of treatment, COL1A1 mRNA level was significantly increased in the CGF-3 group compared to the other treatment groups (Figure 1). Levels of COL1A1 protein expression was subsequently observed using Western blot method. In accordance to the increase in the COL1A1 mRNA levels, significant CO-

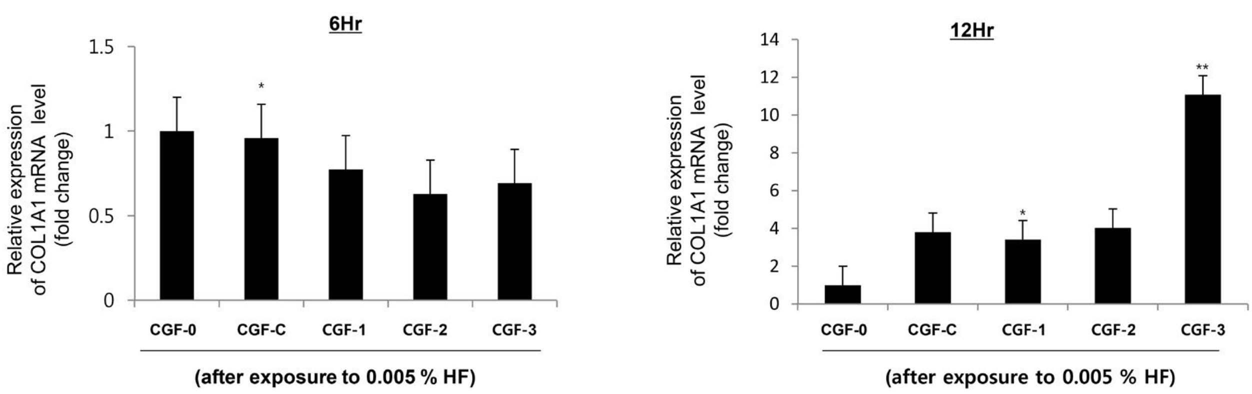

Figure 1. The expression of COL1A1 mRNA levels by CGF treatment in dermal fibroblasts. The mRNA levels of COL1A1 were measured by real-time PCR after treatment of dermal fibroblasts with different CGF for 6 or 12 h after 0.005% HF exposure. Statistical analysis was performed using one-way ANOVA. Data are shown as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

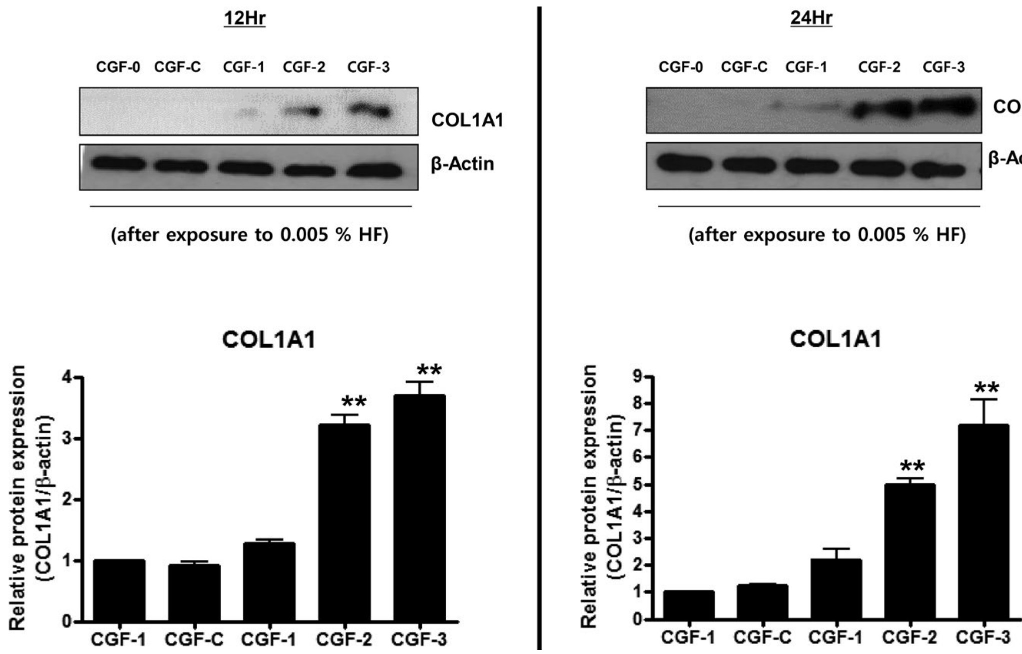

Figure 2. The expression of COL1A1 protein levels by CGF treatment in dermal fibroblasts. The dermal fibroblast was treated with 0.005% HF. The COL1A1 protein expression was detected by western blot 12 or 24 h after sample treatment. Statistical analysis was performed using one-way ANOVA. Data are shown as means ± SD, $^{**}P < 0.01$ compared to control.

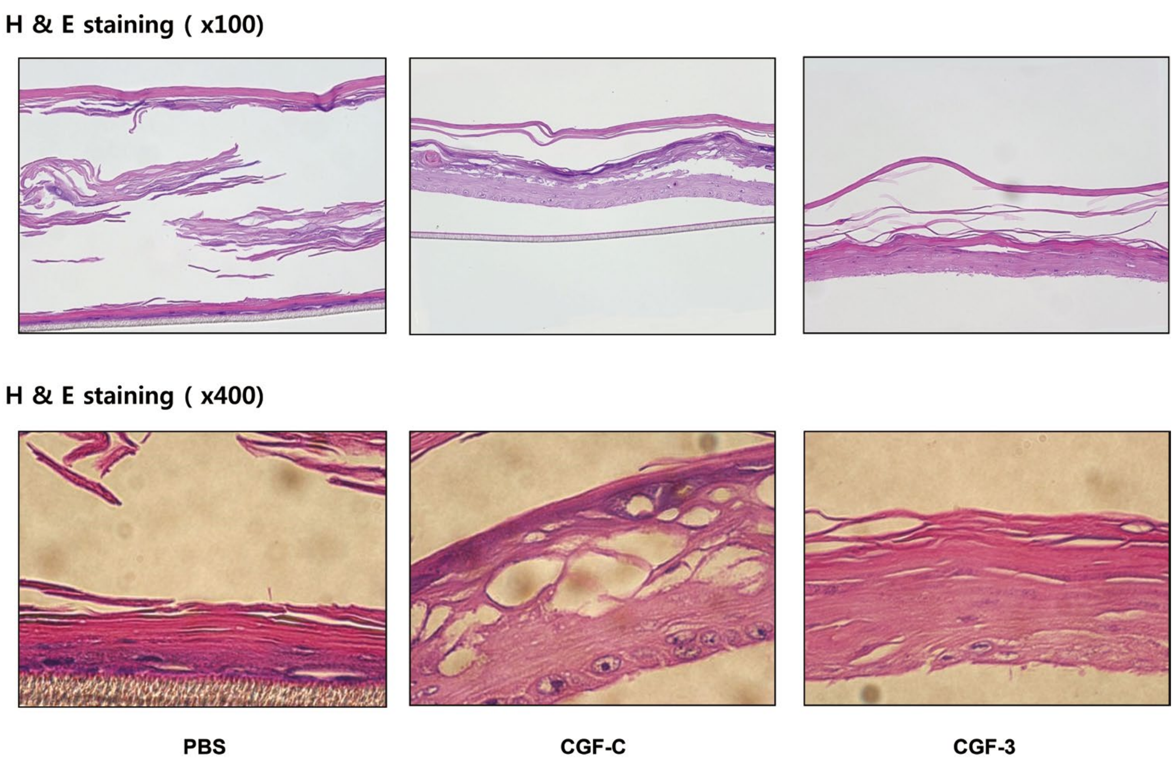

Figure 3. Immunohistochemical analysis (H&E) of HSEM. Histopathology of 20% HF-exposed HSEM treated with PBS, CGF-C, and CGF-3 were observed using H&E stain. Different wound recovery states observed depending on the treatment sample used (H&E × 100, × 400).

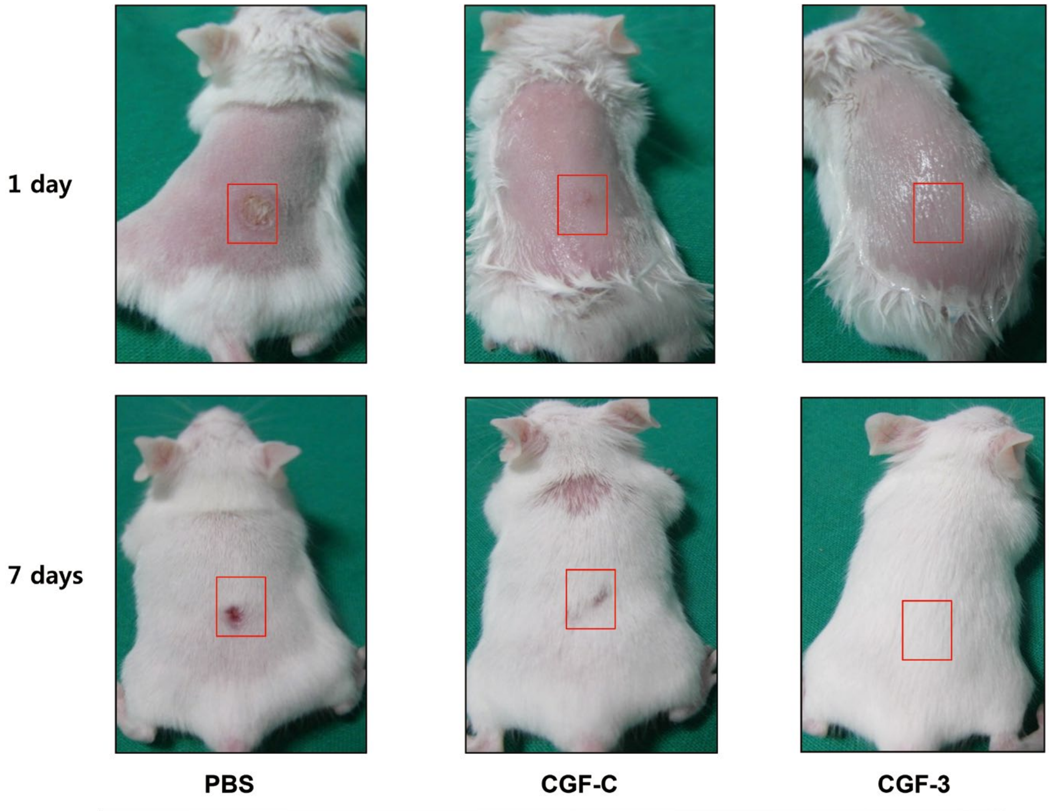

Figure 4. Cutaneous manifestations of mice after exposure to 20% HF and treatment. Burn wound was induced on the dorsal skin of mice using 20% HF. Respective treatment with PBS, CGF-C, and CGF-3 were performed twice-daily 12 h apart for 7 consecutive days and observed visible wound healing.

L1A1 protein expression is observed after 12 to 24 h in the group treated with CGF-3 (Figure 2). CGF-2 group also shows increased COL1A1 protein expression at 12 to 24 h but to a lesser extent than CGF-3 group. This result indicates that the greater number of natural extracts included promotes better wound recovery.

Assessment of immunohistologic effects of CGF following 20% HF exposure using HSEM

As seen in Figure 3, HSEM exposed to HF without treatment showed significant destruction of the epidermis including separation of stratum corneum and severe granular layer defects (Figure 3, PBS). These results indicate that HF exposure induces persistent detachment of the stratum corneum and granular layer leading to severe epidermal destruction. Treatment with CGF-3 attenuated the destruction of epidermis showing increased tissue recovery (Figure 3, CGF-3).

Evaluation of *in vivo* effects in a mouse model

After exposing the dorsum of mouse to 20% HF, burn wound becomes visible. As seen in Figure 4, compared to the untreated (PBS) and calcium gluconate monotherapy (CGF-C) group, CGF-3 group have almost no visible wound one day after treatment. At day 7, the wound in CGF-3 mice is completely covered with normal hair compared to the CGF-C, PBS mice, where remnant wound is depicted by the hairless areas (Figure 4).

Histopathologic evaluation using Hematoxylin & Eosin (H&E) & Masson's trichrome (MT) staining in mouse model

Histopathology shows damage throughout the dermal layers with damaged collagen fibers with stratum corneum hyperplasia in the untreated mice (PBS). CGF-C treated mice shows partial hyperplasia of stratum corneum. Adding natural extracts (CGF-3) restores relative normal stratum corneum, shown by remarkably increased collagen fibers (Figure 5).

Confirmation of target gene expression using RT-PCR in mouse model

Natural extracts induce better HF burn wound recov-

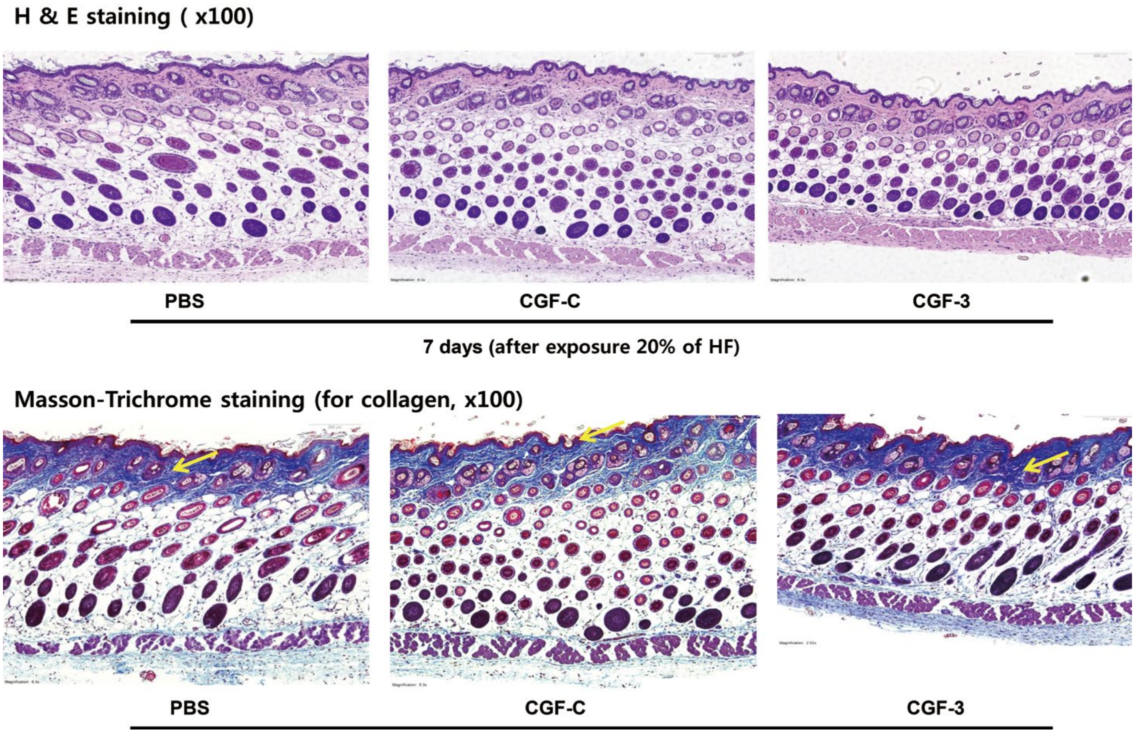

Figure 5. Immunohistochemical analysis (H&E, M-T stain) of skin samples from mice. Biopsy samples harvested from the wound area after 7 days of respective treatment. Changes in stratum corneum observed with H&E staining. Collagen fiber recovery was observed using M-T staining (H&E × 100; M-T × 100).

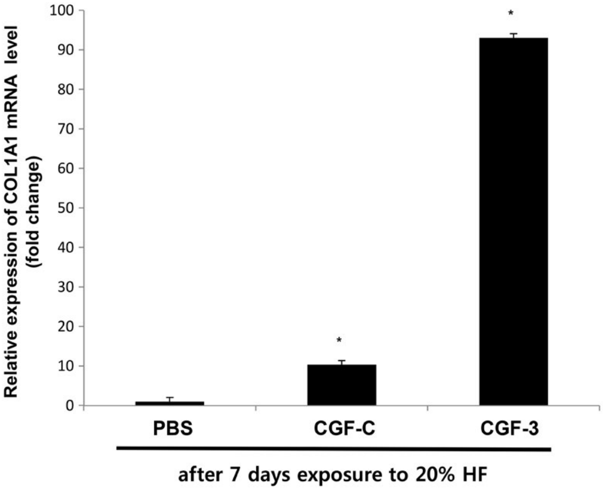

Figure 6. The COL1A1 mRNA expression of mouse skin samples. The mRNA levels of COL1A1 measured by real-time PCR from the skin samples harvested from the wound area after 7 days of respective treatment. Statistical analysis was performed using one-way ANOVA. Data are shown as means ± SD, $*P < 0.05$ compared to control (PBS).

ery as shown by an increase up to 9-fold greater in the mRNA levels of COL1A1 in those treated with CGF-3 compared to CGF-C (Figure 6).

Discussion

Hydrofluoric acid (HF) is a dangerous acid and the chemical burn it causes may have devastating consequences[1]. The symptoms are dependent on the HF concentration. In concentrations greater than 50%, the contact symptoms are usually immediate pain and visible burn, while in concentrations between 20% and 50%, the symptoms may appear after a few hours[15]. In lower concentrations, the symptoms may take up to 24 h to become apparent[16,17]. The mechanism of the symptoms is due to HF dissociating into hydrogen ions and fluoride ions upon absorption by tissues[4]. In higher concentrations, HF acts similar to other strong acids in causing burn and corrosion[8,18]. This superficial burn is a rather minor component of overall HF burn injury, as the more important threats are related to the fluoride ions responsible for the delayed destruction associated with HF burns[19]. Fluoride ions being highly permeable and lipophilic, easily penetrates the tissue and binds to the divalent cations calcium and magnesium depleting the local storage of these ions[5,9,20]. Local calcium depletion increases membrane permeability to potassium resulting in local hyperkalemia and depolarization which is responsible for progressive liquefaction necrosis, bone decalcification and the "pain out of proportion", a hallmark excruciating pain in HF burns[1,8,21]. Therefore, immediate emergency actions are important in minimizing damage, with the first step being thorough washing of the affected area with ample running water[5,9,22]. The goal of HF burn treatment lies on neutralizing the fluoride ions as much as possible, before it infiltrates deeper into the tissue[8]. Typically used topical applicators include calcium gluconate (CG), magnesium pastes, and ammonium compounds[22-27]. Studies have shown that 2.5% CG is the most effective topical treatment[6,22,24,27-29]. CG acts as a calcium depot and trap the fluoride ions preventing it from binding to the tissue calcium ions[5]. Although there are reports that combination treatment shows synergistic effects, there have been none regarding the additive effects of natural extracts in aiding HF induced wound healing[6,27]. To our knowledge, this study is the first to compare the effects of different formulations of calcium gluconate including natural extracts on skin.

Studies have reported that *Centella asiatica*, containing the active compounds asiaticoside and madecassoside, promote healing of burn wounds through inducing collagen synthesis, anti-oxidative activity, angiogenesis and anti-inflammatory effects[10-12,30-33]. In addition, extracts from the herb *Portulaca oleracea* is known for its anti-inflammatory and analgesic effects[13,34,35]. Disi *et al*.[36] reported through an *in vivo* study using mouse that *Portulaca* extracts accelerate the wound healing process by decreasing wound surface area and increasing tensile strength. Anthocyanin and polyphenols in blueberries show antioxidant properties[14,37,38]. We observed the synergistic effects of the three natural extracts combined with CG in treating HF induced burn wound.

Fibroblasts are the main cell type composing the dermis and produces collagen, an important structural protein in skin. They are responsible for the production and remodeling of extracellular matrix (ECM) during wound healing[39]. Type I collagen, a triple helix molecule composed of two $\alpha 1$ chains and one $\alpha 2$ chain, is the most abundant type of collagen found in most connective tissues. The COL1A1 gene encodes the pro-$\alpha 1$ (I) chain, one of the three components of type I collagen[40]. In this regard, the basis of wound recovery in our study was observing the expression of COL1A1 mRNA and protein. In MTT cytotoxic assay, the fibroblasts maintained a viability of 70-80% when exposed to CGF concentrations of 0.05% (data not shown). Using RT-PCR, we observed no difference between the CGF-C (control) and CGF-3 group in expression of COL1A1 mRNA levels 6 h after treatment. As Braken *et al*.[6] observed ongoing coagulative necrosis and collagen loss in the dermis from 15 min to 6 h after HF exposure on mice, we suspected dermal damage to be ongoing at 6 h, even with the treatment. Interestingly at 12 h, there was a 10-fold increase in COL1A1 mRNA expression in the CGF-3 treated group compared to only 4-fold increase in control. This was consistent with the results of western blot, where an increased expression of COL1A1 protein was seen in the CGF-3 group compared to the control group at both 12 h and 24 h. The addition of natural extracts has shown synergistic effects in the treatment of HF burn as the COL1A1 protein and mRNA expression was more evident compared to the control. There was no big difference in the increase in COL1A1 mRNA expression among control, CGF-1, and CGF-2 at 12 h. However, the increase in COL1A1 protein expression was more prominent in the group with more natural extracts added (CGF-3 > CGF-2 > CGF-1), proving the added benefits of these compounds in wound healing.

Wiemer *et al*.[41] have reported the similarities of HSEM to the natural human skin and its use as a suitable alternative for animal experiments. Derived from actual human epidermis, experiments using HSEM is considered a more attractive model for evaluating dermal toxicity and testing skin integrity functions than animal models.[42] To confirm the results from our experiment using fibroblasts, we compared the effects of CGF-3 and control after inducing HF burn in HSEM. Extensive disruption of the epidermis was seen in the PBS group, showing the destructive effects of HF when left untreated. While the CGF-C group showed an improvement and rather intact skin structure, the CGF-3 group resulted in a more stable skin structure. This can be partly attributed to the compounds found in *Centella asciatica*, asiaticoside and madecassoside, which stimulates collagen synthesis as shown in the *in vivo* study by Hou *et al*.[11]. Liu *et al*.[32] also confirmed this notion that madecassoside involve several mechanisms including anti-oxidative activity, collagen synthesis and angiogenesis in accelerating burn wound healing. Burgher *et al*.[43] mentioned that there is still a lack of knowledge on tissue damage regarding the time needed for full-thickness skin penetration of concentrated HF and various authors used different concentrations of HF and exposure times in their *in vivo* study for evaluating efficacy of treatment options[6,27-29,44]. Kono *et al*.[27] reported a profound electrolyte imbalance including hypocalcemia, hyponatremia in the animals exposed to 50% HF. Our animal experiment protocol was exposure of 20% HF for 5 min, as exposure to higher concentrations of HF in real life may require more than topical treatment[1,7,8]. The mice treated with CGF-3 showed complete recovery of wound after 7 d of treatment. Hair growth was more profound compared to the control implying a good recovery of the dermis where hair follicles are embedded. Histopathologic examinations showed a normalized stratum corneum and increased dermal collagen in the CGF-3 group compared to the control treated group. Lastly, COL1A1 gene expression was almost 9-fold greater than the control treated group suggesting a better recovery.

Conclusion

Our study demonstrated that topical calcium gluconate in combination with natural extracts (*Centella asiatica*, *Portulaca oleracea*, and blueberry) show superior wound healing compared to the conventional topical CG monotherapy in the treatment of HF induced burns.

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Conflicts of Interest Jung Jin Shin, Hana Lee, Sang Hoon Jeong, Ji Hyun Kim, Myungjin Lee, and Sang Wook Son have no conflicting interests to declare.

Human and animal rights All animal experiments were approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC-2015-156). All procedures were conducted in accordance with recommendations for the proper animal care and use.

Author contributions Jung Jin Shin participated in writing the article and analysis and interpretation. Hana Lee conducted in data collection and statistical analysis. Sang Hoon Jeong and Ji Hyun Kim critically reviewed the study proposal. Myungjin Lee served as scientific advisor. Sang Wook Son suggested the conception and design.

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