

# Acceleration of Collagen Breakdown by Extracellular Basic pH in Human Dermal Fibroblasts

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## Key Words

pH · Collagen · Skin fibroblasts · Three-dimensional human skin equivalent system

## Abstract

**Background/Aims:** Wound healing is a complex regeneration process involving the degradation and reassembly of connective tissues and skin layers. Previous studies have shown that pH plays a significant role in both the direct and indirect regulation of cellular processes in the wound, which, in turn, affect the wound healing process. However, the effects of pH on the collagen breakdown component of wound healing have yet to be investigated. Therefore, we investigated the induction of accelerated collagen breakdown by pH imbalance in the skin. **Methods:** Na<sup>+</sup>/H<sup>+</sup> exchanger and metalloproteinase (MMP)-1 were analyzed spectrophotometrically, and the expression of collagen type-I-alpha-1 (COL1A1) and mitogen-activated protein kinase (MAPK) was measured by Western blotting. **Results:** Accelerated collagen breakdown induced by extracellular basic pH via the overproduction of reactive oxygen species (ROS) and MAPK

signaling was examined in skin fibroblasts and in a three-dimensional human skin equivalent system. Basic pH (>7.50) upregulated MMP-1 and downregulated COL1A1 levels via ROS generation and MAPK signaling pathways. Acidic pH (<6.04) slightly upregulated MMP-1 and slightly downregulated COL1A1 levels via ROS generation and the p38 signaling pathway. **Conclusion:** Our results indicate that skin pH is an important effector of collagen formation in wound healing. This finding will aid in the development of new pH-targeted therapeutic strategies.

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## Introduction

Wound healing is a complex physiological process involving skin repair and regeneration [1]. A wound is defined as a break in the continuity of tissue caused by violence or trauma, and is considered healed if the wounded or inflamed tissue is restored to its normal condition [1]. Wound healing is influenced by both intrinsic and extrinsic factors [1–4]. Homeostasis and inflammation, prolifer-

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eration and maturation or remodeling are the distinct, overlapping phases involved in wound healing [3]. Altered or impaired wound healing, as observed in chronic wounds, is marked by an interruption of this process [3]. The presence of bacteria and bacterial products, such as endotoxins, can affect each of the healing processes and impede their orderly progression [4]. The pH within the wound milieu directly and indirectly influences all biochemical reactions involved in the healing process [5]. pH values affect regular cellular events in wound healing. Previous studies have demonstrated that the surface pH of a wound plays an important role in healing the wound as it helps control infection and increase antimicrobial activity, oxygen release, angiogenesis, protease activity and bacterial toxicity [5]. In addition, it has been observed that both acute and chronic wounds with a basic pH exhibit lower healing rates than those with a more neutral pH; wound healing progression decreases when the pH becomes basic [5, 6]. The environment of both acute and chronic wounds progresses from a basic to a neutral state and then to an acidic state when healing commences [6]. In addition, numerous studies have revealed that skin surface pH increases with age. Zlotogorski [7] showed significantly higher skin pH values in subjects >80 years than in younger subjects. Thune et al. [8] demonstrated higher pH in the elderly (age 67–95 years, mean age 81 years) and revealed a positive correlation between age and pH. Moreover, Choi et al. [9] demonstrated elevated pH values throughout all layers of the stratum corneum of aged mice. Thus, pH affects wound progression and skin aging. However, the effects of pH on the collagen breakdown component of wound healing have yet to be investigated in vitro and in a three-dimensional human skin equivalent (3HSE) system. In this study, we examined the acceleration of collagen breakdown induced by an imbalance in extracellular pH in human skin fibroblasts.

## Materials and Methods

### Chemicals

DMEM, penicillin-streptomycin and FBS were purchased from Hyclone Laboratories, Inc. (Logan, Utah, USA). MTT, HCL, NaOH, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and DMSO were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Anti-extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, pERK, pJNK, pp38 and collagen type-I-alpha-1 (COL1A1) were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Anti-rabbit-horseradish peroxidase (HRP) secondary antibody was purchased from Assay Designs (Ann Arbor, Mich., USA).

### Cell Culture and Treatments

The cell culture system was established according to previously published methods [10]. The HS68 cell line, human skin fibroblast cells, was obtained from the American Type Culture Collection (ATCC; Rockville, Md., USA). Primary human dermal fibroblast (PHDF) cells were kindly donated at KIOM in Korea. The human skin keratinocyte (HaCaT) cell line was obtained from the CLS Cell Lines Service GmbH (Eppelheim, Baden-Württemberg, Germany). Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin in a condition of 95% air and 5% CO<sub>2</sub> at 37°C. Adult human dermal fibroblasts (HDFs) were obtained from ScienCell (Carlsbad, Calif., USA) and maintained in fibroblast medium containing fibroblast growth supplement and 10% heat-inactivated FBS and 1% penicillin-streptomycin. Cultures were exposed to culture medium at pH 5.35–8.41 or normal culture conditions.

### 3HSE System

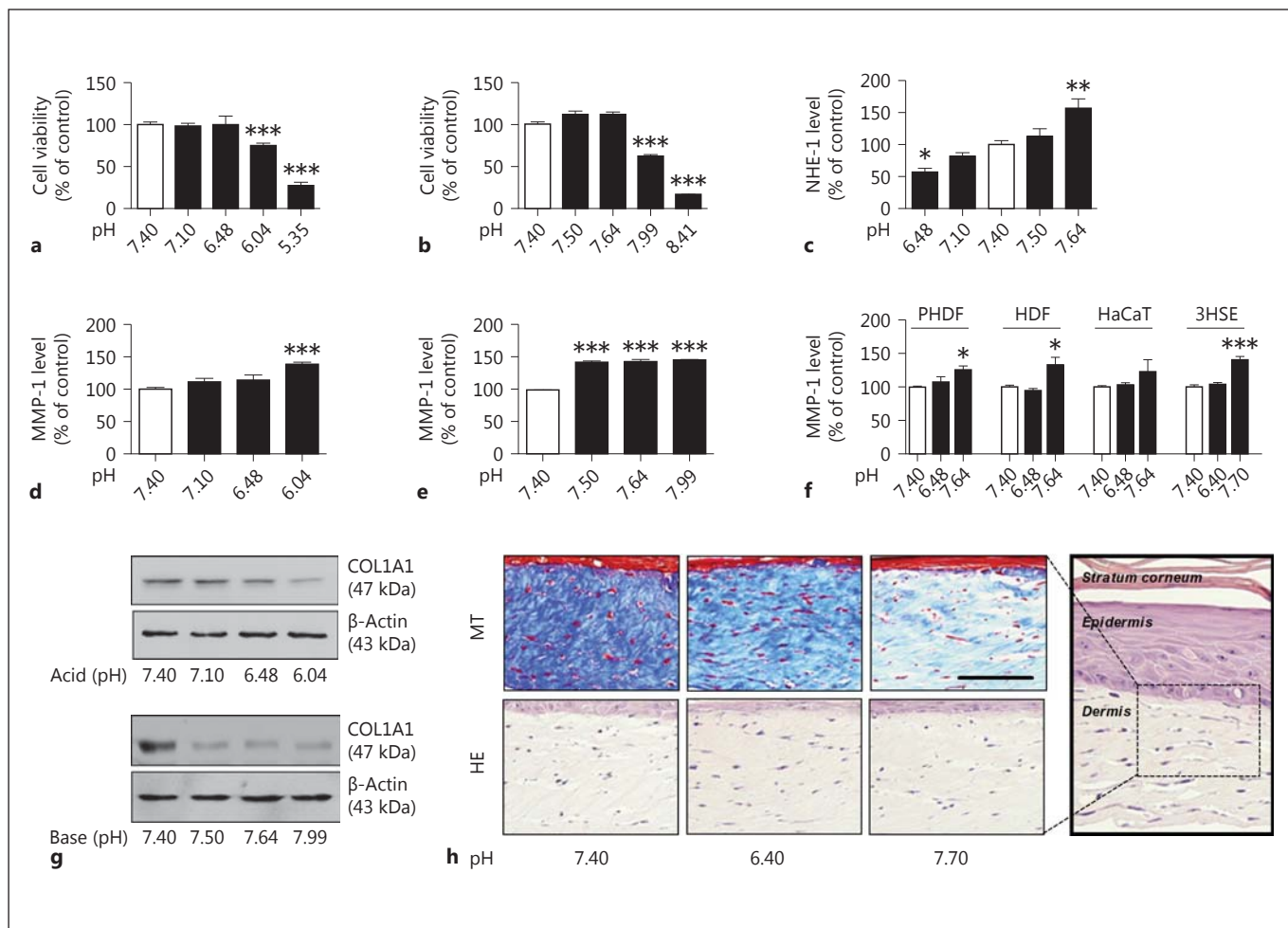
Using Neoderm<sup>®</sup>-ED purchased from TEGO Science (Seoul, South Korea), we generated the 3HSE system. Briefly, HDFs were cultured in collagen matrix for 1 day. Keratinocytes were then seeded on top of the collagen matrix and cocultured for 4 days. Next, the keratinocytes and HDF blocks were lifted and allowed to be exposed to air. The skin equivalent was then treated with pH at 6.40–7.70 for 10 days, and the medium was changed every 2 days for 10 days. The skin equivalent was incubated at 37°C and 5% CO<sub>2</sub>.

### Measuring COL1A1 and MAPK Signaling Pathway by Western Blotting

Western blotting was performed according to previously published methods [11]. The cells and tissues were lysed with protein extraction buffer to obtain whole-protein extracts. The lysates were separated by 12% SDS-PAGE and then transferred to a membrane. The membranes were incubated with 5% skim milk in TBST for 1 h. They were then incubated with COL1A1, ERK, pERK, JNK, pJNK, p38, pp38 and  $\beta$ -actin primary antibody (1:1,000 or 1:5,000 dilutions) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody for 2 h. Immunoreactive bands were detected using an ECL detection kit and an LAS-4000 mini system (Fujifilm Corp., Tokyo, Japan) was used for visualization. The intensities of the bands were normalized to the  $\beta$ -actin, ERK, JNK or p38 and intensity using MultiGauge software (Fujifilm Corporation, Tokyo, Japan).

### Measuring the Generation of Intracellular Reactive Oxygen Species

Reactive oxygen species (ROS) generation was measured as the previously described method [12]. DCFH-DA enters cells passively and is converted into nonfluorescent DCFH, which reacts with ROS to form the fluorescent product DCF. Cells were seeded onto coverslips in 24-well plates and treated with pH 6.48–7.64 for 1 h. The cells were incubated with 25  $\mu$ M DCFH-DA for 30 min. The fluorescence intensity was determined at 485 nm excitation and 535 nm emission using a fluorescence microplate reader (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, Calif., USA). Representative images were taken using a fluorescence microscope (Olympus Microscope System BX51, Olympus, Tokyo, Japan). For the assessment of intraexperimental variability, 3 independent experiments were carried out in triplicate.



**Fig. 1.** Effects of acidic or basic pH on cytotoxicity and NHE-1 levels in skin cells. Cell viability under acidic (**a**) and basic (**b**) conditions was assessed using the MTT assay. **c** The levels of NHE-1 were measured using a kit. The levels of MMP-1 (**d-f**) and COL1A1 (**g**) were measured by kit or Western blotting. Representative histological analysis of section of 3D human skin block treat-

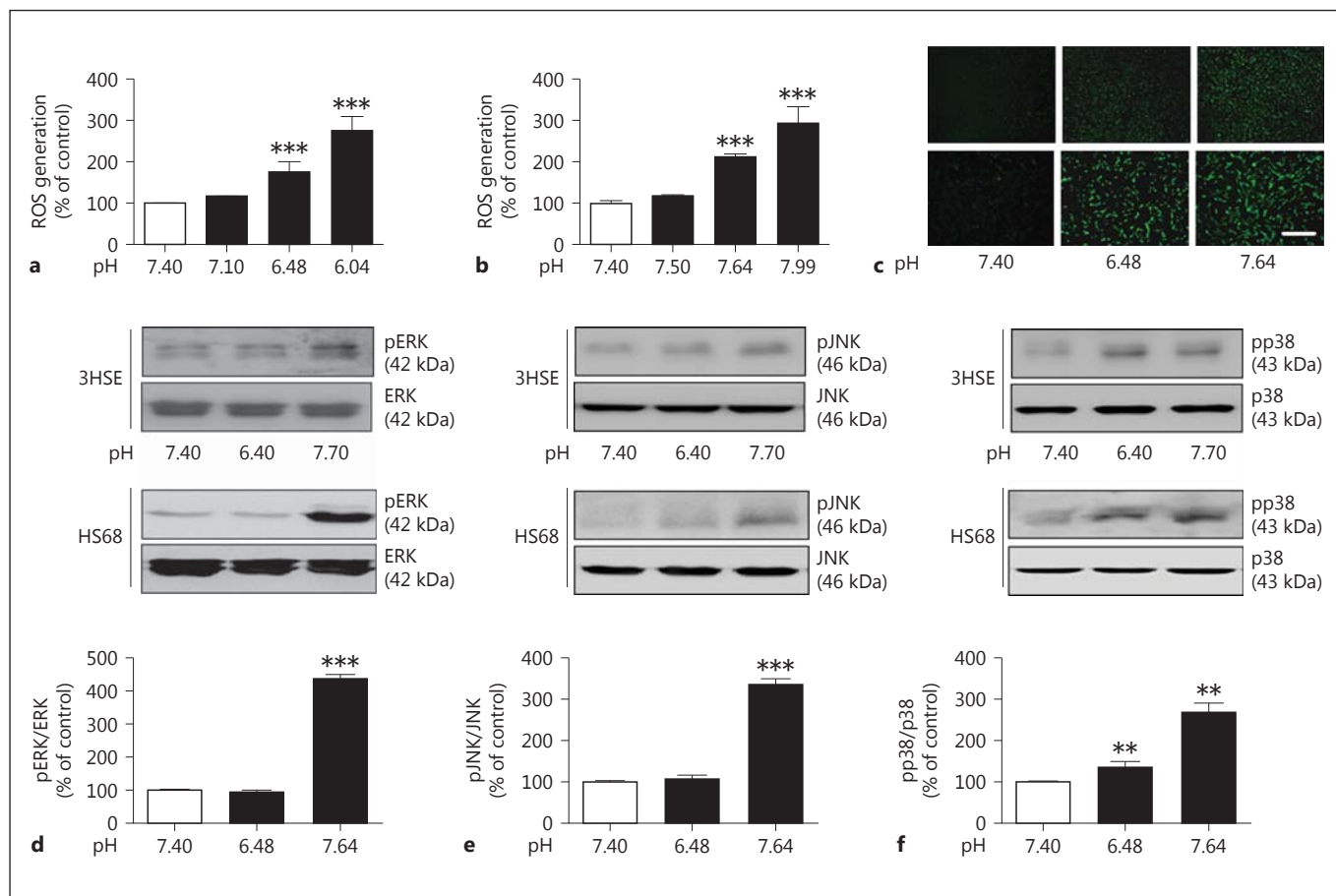
ed with acidic or basic pH. **h** Masson's trichrome (MT) staining was used to identify collagen fibrils and HE was used to identify structures in cells and tissues. Scale bar = 100  $\mu$ m. Values are presented as mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ; one-way ANOVA followed by Tukey's post hoc test, performed with GraphPad Prism software.

#### Measuring Matrix Metalloproteinase 1 and $\text{Na}^+/\text{H}^+$ Exchanger-1 Levels

The tissues were lysed with protein extraction buffer for whole protein. Matrix metalloproteinase (MMP)-1 and  $\text{Na}^+/\text{H}^+$  exchanger 1 (NHE-1) levels were measured using a human MMP-1 ELISA kit (RayBiotech, Norcross, Ga., USA) and NHE-1 kit (Cloud-Clone Corp., Houston, Tex., USA), respectively, according to the instruction manuals.

#### Skin Histological Analysis

Skin sections from the 3HSE were prepared for hematoxylin and eosin and Masson's trichrome staining. Sections (5- $\mu$ m-thick) of 10% neutral formalin solution-fixed paraffin-embedded tissues were cut on saline-coated glass slides, and then deparaffinized 3 times with xylene and dehydrated through a graded alcohol bath. The deparaffinized sections were stained with hematoxylin for 5 min. The slides were then washed and stained with Biebrich scarlet-acid fuchsin. Next, the slides were placed in phosphomolybdic-phosphotungstic acid for 10 min and aniline blue for 5 min to stain the collagen. The slides were then washed and incubated in 1% acetic acid for 15 min. Finally, they were dehydrated and washed. Representative images were taken using a fluorescence microscope (Olympus Microscope System BX51).



**Fig. 2.** Effects of acidic or basic pH on ROS generation in HS68 or 3D human skin equivalent system. **a, b** The level of ROS generation was measured using the DCFH-DA assay. **c** Representative photomicrographs. Scale bar = 50  $\mu$ m. **d–f** The level of the ERK (**d**),

JNK (**e**) and p38 (**f**) MAPKs was measured by Western blotting. Values are presented as mean  $\pm$  SEM. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ; one-way ANOVA followed by Tukey's post hoc test, performed with GraphPad Prism software.

## Results and Discussion

In this study, we examined accelerated collagen breakdown induced by pH imbalance via the overproduction of ROS and MAPK signaling in HS68 skin fibroblast cells. First, in order to evaluate whether pH imbalance impaired collagen expression in the HS68 cells, we measured MMP-1 and COL1A1 levels. Collagen, the protein that provides the skin with its tensile strength, plays a key role in all stages of wound healing [13]. Collagen attracts cells, such as fibroblasts and keratinocytes, to the wound, which encourages debridement, angiogenesis and re-epithelialization [13]. In addition, it provides a natural scaffold or substrate for new tissue growth [14]. Collagen dressings stimulate new tissue growth and encourage the deposition and organization of newly formed collagen fibers

and granulation tissue in the wound bed [14]. These dressings bind chemically to MMPs found in the extracellular fluid of wounds. MMP-1 normally attacks and breaks down collagen; wound dressings containing collagen are thought to provide MMP-1 with an alternative collagen source, leaving the body's natural collagen available for normal wound healing [1, 14]. The MTT assay results demonstrate that the viability of HS68 cells was not affected by 24 h of treatment at pH 6.48–7.64. However, a pH of 6.04–5.35 and 7.99–8.41 caused increased cytotoxicity (fig. 1a, b). The NHE-1 isoform is ubiquitously expressed on the plasma membrane of all cell lines. NHE-1 regulates pH and is involved in the preservation of cell volume under normal physiological conditions. Our results show that NHE-1 expression levels were significantly increased by extracellular basic (7.64) and acid-

ic (6.48) pH (fig. 1c). Similarly, a previous study reported that primary human keratinocyte cultures respond to increased extracellular pH by upregulating NHE-1 protein expression [15]. Thus, all further experiments in this study were performed at pH values of 6.48–7.64. HS68 cells exposed to a basic pH of 7.50–7.99 showed significantly induced MMP-1 levels ( $141.06 \pm 1.63$  to  $144.33 \pm 1.12\%$  of the corresponding level at pH 7.40) and reduced COL1A1 levels (fig. 1d, f). However, cells exposed to an acidic pH of 7.10–6.48 did not exhibit changes in the MMP-1 and COL1A1 levels (fig. 1e, f). Moreover, we confirmed collagen breakdown in another cell type. The result is increased MMP-1 levels in PHDFs, adult HDFs and the HaCaT cell line.

The 3HSE model was developed to replace animal experiments for cosmetic materials and has been shown to have physiologically comparable properties to real skin. It is generated from primary human keratinocytes on a collagen substrate containing HDFs [16, 17]. It is grown at the air-liquid interface where epidermal-dermal interactions occur, and it allows full epidermal stratification. 3HSE is also used to characterize the mode of action of novel agents and their efficacy in skin; it is regarded as a valid alternative for animal testing with numerous applications [16]. Our results show that extracellular acidic (6.40) and basic (7.70) pH-induced collagen degradation (fig. 1g) decreases and MMP-1 expression (fig. 1f) increases in the 3HSE. These results suggest the potential effectiveness of pH if used in a clinical setting. Taken together, these findings indicate that collagen breakdown is accelerated when the pH is increased to basic in skin cells.

ROS have been increasingly implicated in the regulation of signal transduction through the activation of MAPKs [18]. Three distinct, although related, families of MAPKs exist: ERK, JNK and p38 kinase [18]; extensive cross-talk exists between them. The ERK pathway primarily mediates cellular responses to growth factors whereas the JNK and p38 pathways primarily mediate cellular responses to cytokines and physical stress [18]. Several studies have demonstrated that MAPK signaling pathways play an important role in inducing and regulating cell growth and procollagen synthesis in skin cells [11, 19]. In addition, MAPKs are known to play an important role in the signaling pathways that regulate MMP-1 gene expression; specifically, ERK1/2 and p38 regulate MMP gene expression [19]. Therefore, since ROS-MAPK-dependent pathways are involved in collagen regulation, we next measured ROS generation and ERK, JNK and p38 protein levels. HS68 cells exposed to an acidic pH of 6.04–6.48 showed significantly increased ROS genera-

tion ( $177.85 \pm 23.40$  to  $275.36 \pm 33.01\%$  of the corresponding level at pH 7.40) and a slightly elevated p38 protein level at pH 6.48 ( $132.76 \pm 14.66\%$  of the corresponding level at pH 7.40); ERK and JNK protein levels remained unchanged (fig. 2d). HS68 cells exposed to a basic pH of 7.64–7.99 exhibited significantly increased ROS generation ( $214.13 \pm 5.57$  to  $295.30 \pm 39.53\%$  of the corresponding level at pH 7.40) and greatly elevated ERK, JNK and p38 protein levels ( $435.28 \pm 14.03\%$ ,  $333.82 \pm 16.59\%$  and  $267.66 \pm 23.14\%$  of the corresponding levels at pH 7.40, respectively; fig. 2). Moreover, these results were confirmed using a 3HSE model (fig. 2d–f). These results suggest that basic pH conditions increase ROS generation and MAPK signaling, and that weakly acidic pH conditions slightly increase ROS generation and p38 kinase signaling, but not ERK and JNK signaling.

Taken together, the findings of this study, from an in vitro experiment and using a 3HSE system, suggest that collagen maintenance is inhibited in a basic pH environment. Additional studies and similar clinical models will be required to further elucidate the mechanism underlying this phenomenon. Importantly, the inhibition of wound healing through pH imbalance suggests that the development of pH-regulating or maintenance materials may provide effective therapeutic strategies for healthy skin.

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### Statement of Ethics

Ethical approval could be waived, as this is an in vitro study.

### Disclosure Statement

There were no conflicts of interest.

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