Brief Report

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Electrolytic-reduction ion water protects keratinocytes from hydrogen peroxide through radical scavenging activity and induction of AQP3 expression

Hiroyuki Yamamoto[1,2,*], Tokuko Takajo[3], Ami Tsuchibuchi[2], Kaho Makuta[2], Toshiyuki Yamada[2], Mitsuo Ikeda[4], Yoshinao Okajima[4,5], Masahiro Okajima[4,5]

[1] Department of Health and Nutritional Sciences, Faculty of Food and Health Sciences, Aichi Shukutoku University, Aichi, Japan;
[2] Department of Microbiology and Molecular Cell Biology, Nihon Pharmaceutical University, Saitama, Japan;
[3] Division of Physical and Analytical Chemistry, Faculty of Pharmaceutical Sciences, Nihon Pharmaceutical University, Saitama, Japan;
[4] A.I.System Products Corp., Aichi, Japan;
[5] Department of Pharmaceutical Sciences, Meiji Pharmaceutical University, Tokyo, Japan.

SUMMARY Skin exposed to ultraviolet light produces hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) that cause protein denaturation and other disorders. We investigated whether electrolytic-reduction ion water (ERI), which has reducing properties and has been reported to protect skin, exhibits antioxidant activity in skin keratinocytes. The antioxidant activity of ERI was first examined using DPPH assay and Electron Spin Resonance to test for radicals, and using the Amplex Red method to test for H_2O_2. Concentration-dependent scavenging of hydroxyl radical but no H_2O_2 depletion were detected. An investigation of the expression of heme oxygenase-1, which is upregulated by oxidative response in cells, showed an increase through H_2O_2 oxidation, which was inhibited by ERI in a concentration-dependent manner. This suggests that ERI directly removes ROS. Quantitative real-time polymerase chain reaction analysis was performed to determine whether ERI regulates the expression of aquaporin 3 (AQP3), a known H_2O_2 transporter. This analysis revealed that ERI enhances AQP3 expression in a concentration-dependent manner and is involved in the transport of intracellular H_2O_2 to the extracellular space. In addition, ERI inhibited H_2O_2-induced cytotoxicity in a concentration-dependent manner. These results suggest that ERI protects keratinocytes from ROS by directly scavenging them and indirectly by eliminating them through the promotion of the efflux of intracellular H_2O_2.

Keywords antioxidant, electrolytic-reduction ion water, Electron Spin Resonance, heme oxygenase-1, aquaporin 3

1. Introduction

The epidermal layer of the skin plays a fundamental role in the defense against environmental pathogens. In addition, moisture is essential for organisms, and they must have a mechanism to prevent moisture loss from the epidermis. Keratinocytes, which constitute the predominant cell type of the stratum corneum, produce protective factors such as ceramide (*1*) and hyaluronic acid (*2*) to protect against invasion of external substances and moisture loss. Ultraviolet (UV) radiation comprises wavelengths present in sunlight that may cause substantial damage to organisms (*3*) by inducing the generation of H_2O_2 and reactive oxygen species (ROS) (*4*). ROS cause lipid oxidation, protein denaturation, and DNA damage. UV radiation also causes disorders of the skin's defensive system, as well as wrinkle formation and skin hyperpigmentation. Because wrinkling, pigmentation, and sagging are signs of skin aging, UV-induced skin modifications are also referred to as photoaging. Suppressing excessive ROS production in the epidermis to protect the body from UV radiation and other damages is a crucial skin-metabolic function.

Electrolytic-reduction ion water (ERI) contains 0.3% mineral salts, such as sodium, potassium, calcium, magnesium, chlorine, silicon and phosphorus, and has a weak basicity and high reducing properties. Studies have reported beneficial effects of ERI such as improvement in skin burns (*5-7*) and atopic dermatitis (*8*). While

clarifying the mechanism through which ERI improves dermatitis, we reported that ERI enhances the expression of CerS3 (ceramide synthase 3) and ELOVL7 (very long chain fatty acid elongase 7), enzymes involved in ceramide synthesis (*9*). The observed anti-inflammatory effects were believed to be due to the increase in skin ceramide content, which enhances the protective function of the skin. ROS scavenging is also expected to be involved in the skin's protective function, but antioxidant effects of ERI on cells have not yet been reported.

The Nrf2-Keap1 system is a mechanism of biological response of cells to oxidative stress and is responsible for maintaining cellular homeostasis (*10*). In the absence of oxidative stress, Nrf2 is retained in the cytoplasm by the inhibitor Keap1, which inhibits its nuclear translocation, thereby suppressing gene expression. In oxidative stress-exposed cells, the Nrf2-inhibitory mechanism of Keap1 is unlocked and the nucleus-translocated Nrf2 induces the expression of oxidative stress response genes such as glutathione synthetase and heme oxygenase-1 (HO-1) (*11*). Therefore, genes induced by the Nrf2-Keap1 system can be used as indicators of cellular oxidative stress. In the present study, we sought to clarify whether ERI can exert its antioxidant effect on cells by evaluating the expression of HO-1, which is regulated by Nrf2-Keap1, as an indicator of this direct antioxidant effect. In addition, we examined the effect of ERI on the expression of aquaporin 3 (AQP3), a known transporter of H_2O_2, as an indirect mechanism of antioxidant effect.

2. Materials and Methods

2.1. Materials

ERI was obtained from AI System Products (Aichi, Japan). Normal human epidermal keratinocytes (NHEKs) were purchased from PromoCell (Heidelberg, Germany). All other chemicals were of reagent grade. The NHEKs were cultured in Keratinocyte Growth Medium 2 (PromoCell) under humidified air containing 5% CO_2 at 37°C.

2.2. DPPH antioxidant assay

The antioxidant activity of ERI was measured *via* DPPH assay. ERI was mixed with the DPPH (1,1-diphenyl-2-picrylhydrazyl radical; 2,2-diphenyl-1-picrylhydrazyl; Dojindo, Kumamoto, Japan) solution, and the mixture was incubated in the dark at 25°C for 30 min. The antioxidant activity was measured at 520 nm using a microplate reader (VersaMAX, Molecular Devices, San Jose, CA, USA).

2.3. Measurement of hydroxyl radical

Fenton reaction was employed to generate hydroxyl radical ($^{\cdot}OH$) by mixing 1.8 mM DMPO (5,5-Dimethyl-1-pyrroline-N-oxide; Labotec Co. Ltd., Tokyo, Japan), 0.8 mM H_2O_2, various concentrations of ERI and 0.08 mM Fe^{2+}. The mixture solution was transferred to a quartz flat cell and X-band ESR spectra were recorded using a JES-FA 100 ESR spectrometer (JEOL Ltd., Tokyo, Japan). Mn^{2+} was used as the external standard.

2.4. Measurement of hydrogen peroxide

Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine; Chemodex Ltd., St. Gallen, Switzerland) was prepared in DMSO and stored at –20°C. Immediately before use, the thawed stock solution was diluted in 50 mM Tris-HCl buffer, pH7.4. H_2O_2 solution was mixed with various concentrations of ERI, and after respectively 2 and 24 h, the reaction solution containing Amplex Red at 10 µM and 1 U/mL HRP was incubated at room temperature for 10 min. Subsequently, the fluorescence intensity (ex. 563 nm, em. 587 nm) was measured using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan).

2.5. MTT assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Dojindo) assay was used to evaluate the effect of ERI on the cellular activity of keratinocytes and the inhibitory effect of ERI on H_2O_2 injury. To this end, NHEKs were cultured in Keratinocyte Growth Medium 2 (PromoCell) under humidified air with 5% CO_2 at 37 °C. The cells were subsequently seeded into a 96-well plate (10^4 cells/well) and cultured overnight. For the effect of ERI on keratinocytes, we prepared the cells by incubating them for 24 h with various concentrations of ERI. For the inhibitory effect of ERI on H_2O_2 injury, we cultured keratinocytes with 0.1% H_2O_2 and/or various concentrations of ERI. The cells were subsequently treated with 110 µL of Dulbecco's modified Eagle's medium containing 0.5 mg/mL MTT and incubated at 37°C for 4 h. Thereafter, the formazan crystals that formed in metabolically active cells were dissolved with 100 µL of 10% sodium dodecyl sulfate in 10 mM hydrochloric acid. Spectrophotometric absorbance was determined at 560 nm, using a microplate reader, and expressed as a percentage of cellular activity.

2.6. Quantitative real-time polymerase chain reaction

Cell oxidation was assessed by evaluating HO-1 mRNA expression through real-time polymerase chain reaction (RT-PCR). Keratinocytes (10^5 cells/well) were seeded into a 6-well tissue culture plate and incubated overnight. The cells were cultured in media containing 0.03% H_2O_2 and/or various ERI concentrations for 24 h. Total RNA was extracted from the keratinocytes

using Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized from the extracted RNA using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Specific primers were designed to amplify human HO-1 (5′-CTTTCAGAAGGGCCAGGTGA-3′ and 5′-TCCTCCAGGGCCACATAGAT-3′), human AQP3 (5′-CACTCTGGGCATCCTCATCG-3′ and 5′-GCCGGTCCTGGTCAAAGAAG-3′), and human β-actin (5′-AGTCCTGTGGCATCCACGAAAC-3′ and 5′- GCAGTGATCTCCTTCTGCATCC-3′). RT-PCR was performed using the StepOne system (Applied Biosystems, Foster City, CA, USA) or Thermal Cycler Dice Real-Time System III (Takara bio, Tokyo, Japan). Specificity of the PCR products was verified on the basis of the melt curve. Ct values of the samples were normalized to that of β-actin, and the relative expression was calculated using the comparative Ct method.

2.7. Statistical analysis

Data are presented as the mean ± standard deviation (SD). The results were analyzed using the Tukey–Kramer test performed in R (R Development Core Team), and a P-value < 0.05 was considered statistically significant.

3. Results and Discussion

Skin keratinocytes were used in this study to evaluate the antioxidant effect of ERI on intracellular oxidation in the presence of H_2O_2.

First, radical-scavenging activity was evaluated using the DPPH method (Figure 1A) and hydroxyl radical was detected using the ESR spin trapping technique (Figure 1B). These are among the common methods for determining the free radical-scavenging capacity of substances. The results revealed that ERI scavenged DPPH radicals and hydroxyl radical in a concentration-dependent manner, with an IC_{50} of respectively 3.37% (v/v) and 6.81% (v/v). We also evaluated the scavenging capacity of H_2O_2 when mixed with ERI. No decrease in H_2O_2 was present in the ERI even when reacted at concentrations of 50% (v/v) ERI for up to 24 h (Figure 1C).

Next, we evaluated the intracellular antioxidant activity of ERI by way of keratinocytes. To determine the required concentration of ERI, the cytotoxic potential of ERI on keratinocytes was evaluated using the MTT test. ERI exhibited no cytotoxicity up to a 5% (v/v) concentration (Figure 2A), and this value was therefore used as a maximum concentration in subsequent experiments. To assess intracellular antioxidant activity, we used HO-1, the expression of which is upregulated by the Nrf2-Keap1 system, as an indicator of cellular oxidative stress. We used 0.03% H_2O_2 as an ROS donor. As shown in Figure 2B, stimulation with 0.03% (w/v) H_2O_2 increased HO-1 mRNA expression by approximately 2.57-fold. ERI decreased this expression in a concentration-dependent manner.

Because AQP3 expressed in keratinocytes uses water, glycerol, and H_2O_2 as substrates (*12*), AQP3 plays a role in skin moisturization and suppresses intracellular antioxidant activity to efflux intracellular excess H_2O_2. Therefore, we examined whether ERI exerts a cytoprotective effect against indirect oxidative stress through AQP3 expression (Figure 2C). H_2O_2 did not affect AQP3 mRNA expression, but ERI enhanced the expression of AQP3 in a concentration-dependent manner. This result suggests that ERI exhibits a cytoprotective function that is mediated by AQP3. Unlike HO-1 expression, gene expression is not affected by intracellular oxidation induced by H_2O_2 treatment, which suggests that a different mechanism is involved; however, this mechanism remains unknown to date. AQP3 in keratinocytes is upregulated by retinoic acid, and AQP3 expression is believed to be enhanced through

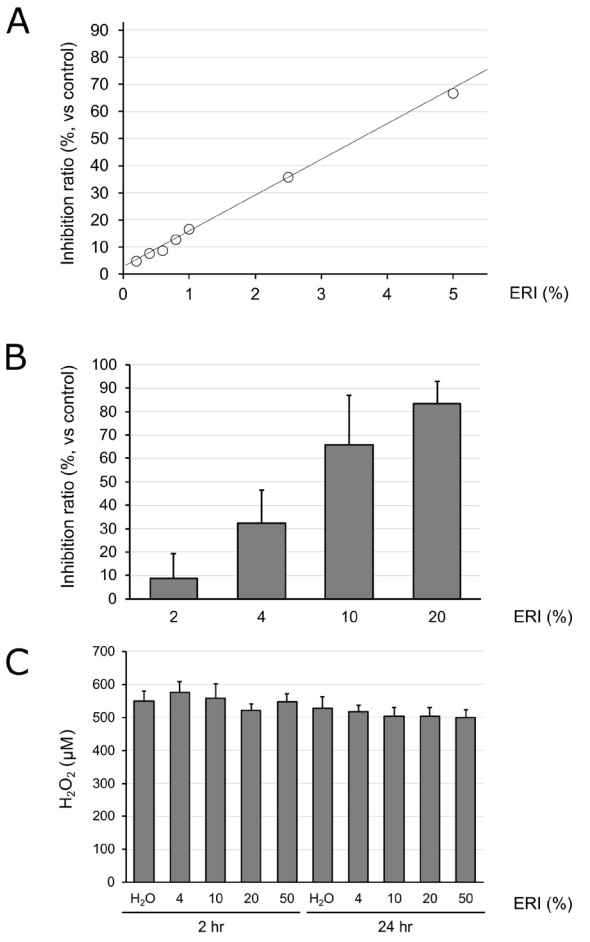

Figure 1. Scavenging activity of ERI on free radicals and hydroxy peroxide. The scavenging properties of ERI for radicals were measured using DPPH radicals (**A**) and hydroxyl radicals (**B**), *vs*. ERI non-additive as control (implied 100%). Data are shown as the mean ± SD (n = 3-4). To assess the decrement activity of hydroxy peroxide of ERI, concentrations of hydroxy peroxide were measured with Amplex Red (**C**). Data are shown as the mean ± SD (n = 4-6).

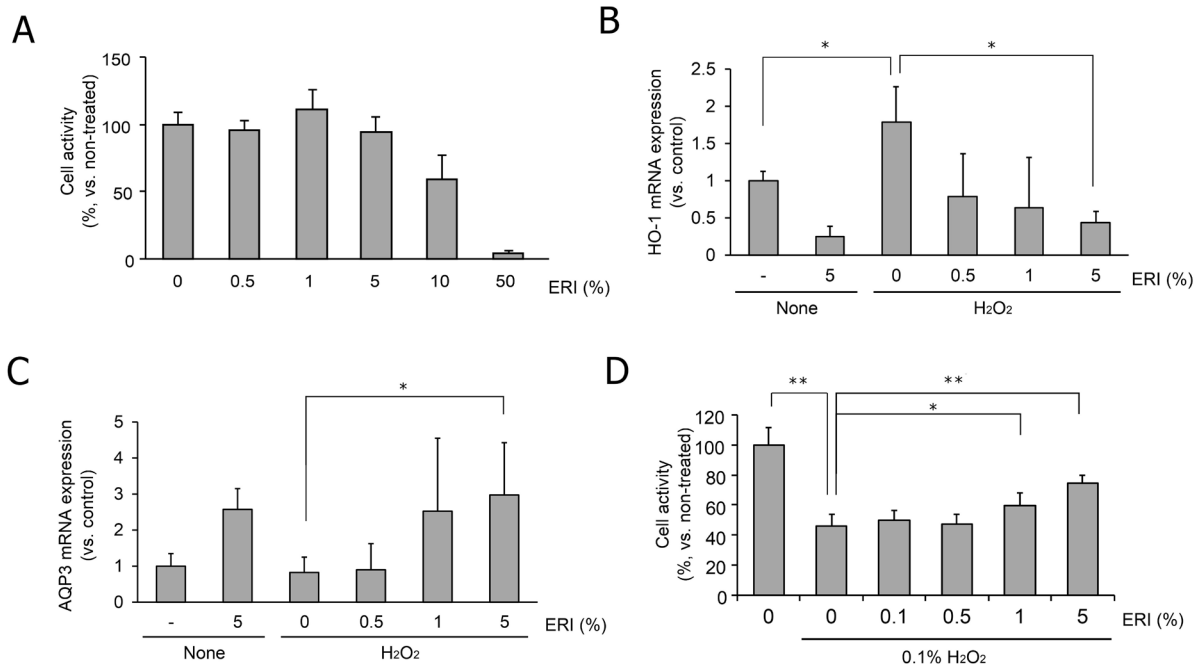

Figure 2 Activity of ERI on normal skin keratinocytes. The effect of ERI on keratinocytes was measured using an MTT assay (**A**). Data are shown as the mean ± SD of eight biological replicates. "100%" indicates an untreated control. HO-1 (**B**) and AQP3 (**C**) mRNA expression levels. mRNA was extracted from NHEKs treated with 0.5%, 1%, or 5% ERI and/or 0.03% H_2O_2 added to the cell culture medium for 24 h. mRNA expression was determined *via* RT-PCR. The expression levels were normalized to that of β-actin. Data are shown as the mean ± SD of three biological replicates. * $P < 0.05$. The inhibitory effect of ERI on 0.1% H_2O_2-induced injury of keratinocytes was measured using an MTT assay (**D**). Data are shown as the mean ± SD of eight biological replicates. "100%" indicates an untreated control. *: $P < 0.05$, **: $P < 0.01$.

the retinoic acid receptor (RAR) (*13*). Therefore, ERI can be assumed to enhance AQP3 expression through RAR. Further verification is required to validate this conclusion.

Finally, the inhibitory effect of ERI on 0.1% H_2O_2-induced keratinocyte injury was evaluated *via* an MTT assay. ERI inhibited cell injury caused by H_2O_2 in a concentration-dependent manner (Figure 2D). This result suggests that ERI may reach the keratinocytes in the epidermal layer and protect the skin when the stratum corneum is damaged by UV exposure, atopic dermatitis, burns, and factors such as H_2O_2 and ROS generated by inflammation. In addition, ERI has been used to deliver drugs through the skin, and dissolving drugs in ERI reportedly enhances their absorption efficiency without damaging the skin (*14*). Although the mechanism of skin-permeability enhancement by ERI has not been fully clarified, ERI holds promise as a vehicle with the ability to scavenge H_2O_2 and ROS.

In summary, our results indicate that ERI exerts its protective effect on keratinocytes by reducing oxidative stress through two pathways: one pathway in which ERI acts directly on intracellular oxidants and electrophiles to reduce oxidative damage, and another pathway in which intracellular H_2O_2 is eliminated through AQP3.

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References

1. Madison KC, Wertz PW, Strauss JS, Downing DT. Lipid composition of cultured murine keratinocytes. J Invest Dermatol. 1986; 87:253-259.
2. Brown KW, Parkinson EK. Glycoproteins and glycosaminoglycans of cultured normal human epidermal keratinocytes. J Cell Sci. 1983; 61:325-338.
3. Claesson S, Juhlin L, Wettermark G. The reciprocity law of Uv-irradiation effects; damage on mouse skin exposed to Uv-light varied over a 107-fold intensity range. Acta Derm Venereol. 1958; 38:123-136.
4. Parat MO, Richard MJ, Leccia MT, Amblard P, Favier A, Beani JC. Does manganese protect cultured human skin fibroblasts against oxidative injury by UVA, dithranol and hydrogen peroxide? Free Radic Res. 1995; 23:339-351.
5. Okajima M, Shimokawa K, Ishii F. The healing effect of electrolytic-reduction ion water on burn wounds. Biosci Trends. 2010; 4:31-36.
6. Shu T, Okajima M, Shimokawa K, Ishii F. The treatment effect of the burn wound healing by electrolyticreduction ion water lotion. Biosci Trends. 2010; 4:1-3.
7. Shu T, Okajima M, Wada Y, Shimokawa K, Ishii F. The treatment effect of the burn wound healing by electrolytic-reduction ion water lotion combination therapy. Part 2: Two degree burn of forearm to the dorsum of the hand. Biosci Trends. 2010; 4:213-217.
8. Shu T, Okajima M, Wada Y, Shimokawa K, Ishii F. The treatment effect of the atopic dermatitis by electrolytic-reduction ion water lotion. Drug Discov Ther. 2010;

4:499-503.
9. Yamamoto H, Ikeda M, Okajima Y, Okajima M. Electrolytic-reduction ion water induces ceramide synthesis in human skin keratinocytes. Drug Discov Ther. 2021; 15:248-253.
10. Jaiswal AK. Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med. 2004; 36:1199-1207.
11. Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem. 1999; 274:26071-26078.
12. Miller EW, Dickinson BC, Chang CJ. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. Proc Natl Acad Sci U S A. 2010; 107:15681-15686.
13. Bellemère G, Von Stetten O, Oddos T. Retinoic acid increases aquaporin 3 expression in normal human skin. J Invest Dermatol. 2008; 128:542-548.
14. Kitamura T, Todo H, Sugibayashi K. Effect of several electrolyzed waters on the skin permeation of lidocaine, benzoic acid, and isosorbide mononitrate. Drug Dev Ind Pharm. 2009; 35:145-153.

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**Address correspondence to:*
Hiroyuki Yamamoto, Department of Health and Nutritional Sciences, Faculty of Food and Health Sciences, Aichi Shukutoku University, 2-9 Katahira, Nagakute City, Aichi, 480-1197, Japan.
E-mail: yamahiro@asu.aasa.ac.jp

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