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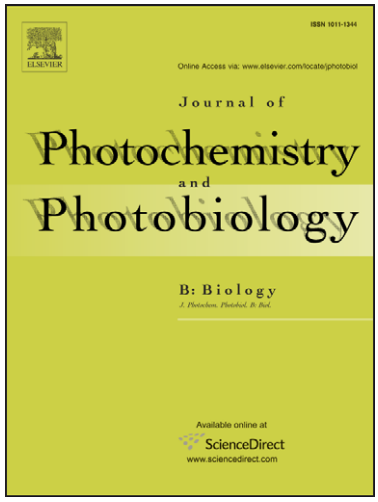

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Hydrogen-rich electrolyzed warm water represses wrinkle formation against UVA ray together with type-I collagen production and oxidative-stress diminishment in fibroblasts and cell-injury prevention in keratinocytes

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ABSTRACT

Hydrogen-rich electrolyzed warm water (HW) was prepared at 41ºC and exhibited dissolved hydrogen (DH) of 1.13 ppm and an oxidation-reduction potential (ORP) of -741 mV in contrast to below 0.01 ppm and +184 mV for regular warm water (RW).

Fibroblasts OUMS-36 and keratinocytes HaCaT were used to examine effects of HW against UVA-ray irradiation. Type-I collagen was synthesized 1.85- to 2.03-fold more abundantly by HW application for 3-5 days than RW in OUMS-36 fibroblasts, and localized preferentially around the nuclei as shown by immunostain. HW application significantly prevented cell death and DNA damages such as nuclear condensation and fragmentation in UVA-induced HaCaT keratinocytes as estimated by WST-1 and Hoechst33342 assays. HW significantly suppressed UVA-induced generation of intracellular superoxide anion radicals in both the cell lines according to NBT assay. Wrinkle repression was clinically assessed using a HW-bathing. Six Japanese subjects were enrolled in a trial of HW-bathing (DH, 0.2-0.4 ppm) every day for 3 months. HW-bathing significantly improved wrinkle in four subjects on the back of neck on 90th day as compared to 0 day. Thus, HW may serve as daily skin care to repress UVA-induced skin damages by ROS-scavenging and promotion of type-I collagen synthesis in dermis.

Keywords:

hydrogen water, UV-A, collagen type-I, fluorescent immunostaining, oxidative stress, hydrogen water bathing, wrinkle repression

1. Introduction

The generation of reactive oxygen species (ROS) is thought to cause oxidative damage in various biomolecules [1,2]. Hydrogen gas (H_2)-dissolved water [3,4] or electrolyzed reduced water [5,6] has attracted much attention because of its antioxidant property. Molecular hydrogen in H_2-dissolved water serves as an antioxidant that reduces hydroxyl radical (·OH) but not the other physiological ROS such as superoxide anion radical (O_2^{-}·) or hydrogen peroxide (H_2O_2), which is thought to be therapeutically beneficial in Parkinson's disease etc [4,7]. Whereas, Shirahata et al. proposed that the ideal scavenger for ROS should be 'active hydrogen (atomic hydrogen)', which was produced near the cathode during electrolysis of water, and the electrolyzed-reduced water exhibited superoxide dismutase (SOD)-like activity [8]. It is reported that the electrolyzed reduced water scavenges H_2O_2 from a human lung adenocarcinona cell line (A549) and inhibits tumor angiogenesis [9]. We also found previously that hydrogen-enriched electrolyzed water achieved tumor-preferential clonal growth inhibition and invasion together with scavenging of intracellular ROS [10].

Exposure of human skin to solar radiation induces skin aging [11], and the loss of collagen is considered the characteristic histological finding in aged skin [12]. The

clinical manifestations of UV/ROS induced disturbances result in photoaged skin with wrinkle formation, laxity, leathery appearance as well as fragility, impaired wound healing and higher vulnerability [13]. Type-I collagen is the major structural component of the dermis, but when confluent fibroblast monolayers were irradiated with UVA, collagenase mRNA was induced, which is probably contributing to the actinic damage to the dermis [14]. Supplementation of the antioxidants such as glutathione, alpha-tocopherol, ascorbate or beta-carotene is successful against photodamage of skin, however the valance among the different antioxidants in the skin is very important, because too much of a single component could even have deleterious effects [15]. Therefore one strategy for preventing photo-aging is using retinoids to inhibit collagenase synthesis and to promote collagen production in combination with other antioxidants to neutralize free radicals [16]. Although hydrogen acts as a scavenger of ROS, it is not ascertained whether HW is beneficial for UV-protective skin care. In the present study, we examined the effects of HW on type-I collagen synthesis in normal human embryo fibroblasts OUMS-36 and prevention of cell death in human skin keratinocytes HaCaT against the exposure to daily dose of UVA, then clinically assessed the effects of HW-bathing on repressive-wrinkle formation.

2. Materials and methods

2.1. Cell culture

Normal human embryo fibroblast cells OUMS-36 were provided by the Cell Bank, Japanese Collection of Research Bioresources (Osaka, Japan). OUMS-36 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui pharmaceutical Co. Ltd., Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biological Industries Ltd., Kibbutz Beit-Haemek, Israel), and 4 mM L-glutamine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37ºC in a 95% humidified air and 5% CO_2. Human skin epidermal keratinocytes HaCaT were kindly provided by Prof. Norbert E. Fusenig, German Cancer Research Center (Heidelberg, Germany) [17]. HaCaT cells were cultured in DMEM supplemented with 10% FBS and 4 mM L-glutamine similarly as mentioned above.

2.2. Preparation of Hydrogen-rich Electrolyzed Warm Water (HW) and HW-containing medium

Hydrogen-rich electrolyzed warm water (HW) was prepared by electrolyzing reversed-osmosis ultra-pure water containing 0.21 mM $NaHCO_3$ at 100 V for 10 min using an electrolyzing device equipped with stainless electrodes (The Chugoku Electric

Manufacturing Co. Inc., Hiroshima, Japan). HW initially contained 1.13 ppm of dissolved hydrogen as measured with a DH meter (DH-35A, DKK-TOA Corp., Tokyo, Japan) and achieved to 41ºC. A batch type electrolyzing device was used, which consists of electrodes wrapped with a semi-permeable film to remove oxygen. Reversed-osmosis ultra-pure water containing 0.21 mM $NaHCO_3$ was warmed to 41ºC with a water bath (Personal-11, Taitec Co., Ltd., Saitama, Japan) and applied as a regular warm water (RW). pH and dissolved oxygen (DO) were measured with a pH/DO meter (D-55, Horiba Inc., Kyoto, Japan), and oxidation-reduction potential (ORP) was measured using an ORP meter (RM-20P, DKK-TOA Corp.). These parameters of HW and RW were measured immediately after preparation and every 30 min up to 180 min of incubation at 37ºC as shown in Fig.1.

Fig. 1 Parameters of hydrogen-rich electrolyzed warm water (HW) and regular warm water (RW) measured immediately after preparation and every 30 min up to 180 min of incubation at 37ºC. Abbreviations represent Control: Reversed-osmosis ultra-pure water, HW: Hydrogen-rich electrolyzed warm water, RW: Regular warm water.

The HW-containing medium was prepared in the procedure that HW (pH 9.0) of 4 mL

was neutralized to pH 7.3-7.4 by adding of 0.5 mL of 10-fold concentrated DMEM (pH 7.2) and 0.5 mL of FBS. The RW-containing medium was prepared in the same manner.

2.3. Application of Hydrogen-rich electrolyzed warm water (HW) and Immunostaining for type I collagen in OUMS-36 fibroblasts

To investigate the effects of HW on type-I collagen production, OUMS-36 fibroblasts of 2.0×10^3 were seeded in a chamber slide (Lab-Tek II, Nalge Nunc^{TM}, Rochester, NY, USA) and incubated for 21 hr at 37ºC in a 95% humidified air and 5% CO_2. Then half volume of the cell culture medium of 1 mL was replaced to HW-or RW-containing medium (pH 7.3-7.4) of 500 L twice daily in the morning and evening for 3-5 days at preliminary to UVA (315-400 nm)-irradiation. UVA was irradiated by 1.2 J/cm^2 using a UV spot curing system Hypercure-200 (Yamashita Denso Corp., Tokyo) emitted from a Hg-Xe lamp UXM-200YA (Yamashita Denso Corp.). OUMS-36 fibroblasts were obtained after 3- and 5-day incubation and the biosynthesized type-I collagen was visualized by fluorescent immunostaining. The adhesive OUMS-36 fibroblasts were fixed with 4% paraformaldehyde for 30 min, then rehydrated with three successive phosphate buffered saline [PBS (-), Nissui pharmaceutical Co. Ltd., Tokyo] washing.

Blocking was conducted with 1% BSA/PBS (-) for 10 min. The primary antibody (a dilution ratio of 1:500, F-56 Anti-hCl [I], purified IgG, Daiichi Fine Chemical Co. Ltd., Toyama, Japan) against type-I collagen was added to the OUMS-36 fibroblasts and incubated for 1 hr at 37ºC, followed by washing with PBS (-) three times. Then OUMS-36 fibroblasts were incubated at 37ºC with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (a dilution ratio of 1: 200; sc-2010, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as the secondary antibody for 45 min. Slides were washed between each step in three shifts of PBS (-) for the total the 15 min. The mounting medium with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Ultracruz Mounting Medium, sc-24941, Santa Cruz Biotechnology Inc.) was applied to each slide. Four sites were randomly selected in slide glasses and observed with a fluorescent microscope (ECLIPSE E600, Nikon Corp., Tokyo) at Ex = 450-490 nm, Em = 520 nm (FITC) and Ex = 365 nm, Em = 400 (DAPI) in connected to a digital camera (C4742-95, Hamamatsu Photonics K.K., Shizuoka, Japan), then amount of type-I collagen was measured using Scion Image software (Scion Corp., Frederick, MD, USA).

2.4. Intracellular ROS-generation examined by NBT assay in OUMS-36 fibroblasts

Nitroblue tetrazolium (NBT) reduction assay was used to determine the intracellular ROS mainly consists of $O_2 \cdot^-$[18]. OUMS-36 fibroblasts of 2.4×10^4 were seeded in a 24-well plate (Becton Dickinson and Company, Sparks, MD, USA) and incubated for 24 hr at 37ºC in a 95% humidified air and 5% CO_2. The cell culture medium was replaced to HW-or RW-containing medium (pH 7.3-7.4) of 500 L and incubated for 5 min, subsequently exposed to UVA-irradiation of 12 J/cm^2 and incubated for 25 min. Then the medium was replaced to 0.2% NBT (Biotium Inc., Hayward, CA, USA) solution of 300 L, which is dissolved in phenol red-free DMEM (Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with 10% FBS. After 1.5 hr of incubation at 37ºC in a 95% humidified air and 5% CO_2, 0.2% NBT solution was aspirated, and the reaction was stopped by addition of 500 L of prechilled PBS (-). OUMS-36 fibroblasts were fixed with 70% methanol (-10ºC) for 1 min. The unreduced NBT dye was completely removed by three-time washing of wells with 500 L of cold PBS (-). Then, 300 L of cold PBS (-) was added into each well, and photomicrographs of NBT-reducing cells were prepared using a Hoffman modulation contrast microscope (ECLIPSE TE-2000-S, Nikon Corp., Tokyo, Japan). The supernatant was aspirated, and formazan formed from NBT was dissolved in 250 L of a 1:1 v/v mixture of 4 M KOH: dimethyl sulphoxide (DMSO) in each well. The plate was set on an orbital

shaker for 30 min, and the nitroblue formazan in the extract was determined at 560 nm with a microplate reader (FLUOstar OPTIMA, BMG Labtech GmbH, Offenburg, Germany).

2.5. Cell viability measured by WST-1 assay in relation to DNA -cleavage and intracellular ROS generation in HaCaT keratinocytes

The cell viability (% of the control) was measured by water-soluble tetrazolium salt (WST-1) method [19]. WST-1 solution was prepared by 5.6 mM WST-1 (Dojindo laboratories, Kumamoto, Japan) in PBS (-) and 2 mM 1-Methoxy-5-methylphenazinium methylsulfate (1-Methoxy PMS, Dojindo laboratories, Kumamoto, Japan) at the volume ratio of 9:1. HaCaT keratinocytes of 8.0×10^3 were seeded in a 24-well plate and incubated for 24 hr at 37ºC in a 95% humidified air and 5% CO_2. The cell culture medium was replaced to HW-or RW-containing medium (pH 7.3-7.4) of 500 L and subsequently exposed to UVA-irradiation of 6 J/cm^2. This procedure of HW-or RW-administration and UVA-irradiation was conducted repeatedly three times every 1.5 hr, namely UVA-irradiation was summed up to 18 J/cm^2. After 18-hr incubation, Hoffman modulation contrast micrographs of HaCaT keratinocytes were prepared using

a microscope (ECLIPSE Ti, Nikon Corp.). Then the medium was replaced with 30 L of the formazan-forming redox indicator dye WST-1 solution and 300 L of phenol-red-free DMEM supplemented with 10% FBS in each well. After incubation for 3 hr, the absorbance of each well was measured at 450 nm with a microplate reader to evaluate the cell viability.

In relation to cell viability, intracellular ROS was also determined. HaCaT keratinocytes of 1.6×10^4 were seeded in a 24-well plate and HW- or RW-administration and UVA-irradiation was conducted as similar to WST-1 assay. After third UVA-irradiation (sum: 18 J/cm^2), intracellular ROS was determined by NBT assay as mentioned above.

Furthermore, morphological changes that are characteristic of apoptosis in the cell were visualized by Hoechst 33342 staining [20]. HaCaT keratinocytes of 8.0×10^3 were seeded in a 24-well plate and HW-or RW-administration and UVA-irradiation was conducted as similar to WST-1 assay. After third UVA-irradiation (sum: 18 J/cm^2) and 18-hr incubation, HaCaT cells were fixed with 4% paraformaldehyde (pH7.4) for 20 min, then washed with PBS(-) three times and exposed to 2 g/mL Hoechst 33342

(Dojindo Laboratories, Kumamoto, Japan) at room temperature, in the dark for 10 min. HaCaT keratinocytes were observed using a fluorescence microscope with Ex=365 nm, Em=400 nm as similarly to mentioned above. At least 85 cells from six different view fields were evaluated, and the percentage of apoptotic cells was calculated.

2.6. Clinical evaluation of repressive-wrinkle formation by daily HW-bathing

Five healthy female volunteers at the age of 14-65 years and one healthy male volunteer at the age of 55 years participated in this study. They did not have any specific diseases based on detailed questionnaires and examination by physicians. HW-bathing was prepared by electrolysis of tap-water using the device for HW-production (The Chugoku Electric manufacturing Co. Inc., Fig. 7). HW-bathing had properties that dissolved-H_2: 0.19-0.41 ppm (RW: < 0.01 ppm), temperature: 41ºC, and oxidation-reduction potential: -150~ -300 mV (RW: +150 mV). The subjects took a bath filled with HW daily for 3-months from 15th December 2009 to 15th March 2010. All subjects took a bath filled with HW daily more than 5 min at home.

Replicas of the back of neck skin were obtained using silicone resin (Skin Cast, Raptor Survey Institute, Ltd., Shizuoka, Japan). The replicas were set on a horizontal stand, and wrinkle shadows were produced by illumination with light of a fixed intensity at an

angle of 20º using a cold lighting system (PICL-NEX, Nippon P· I Co., Ltd., Tokyo). The shadow images were photographed with a CCD camera (Leica Z16 APO, Leica Microsystems GmbH, Wetzlar, Germany). For the image analysis of wrinkles by the skin replica, shadow areas were measured using Scion Image software (Scion Corp.) at the same level of threshold.

2.7. Statistical analysis

Results of the experiments were expressed as means ± SD, for n = 4 or 6, and evaluated by the Student's *t*-test. The *p*-values of which were at least smaller than 0.05 were regarded as being statistically significant.

3. Results

3.1. Effects of HW on type-I collagen production and antioxidant activity against UVA in OUMS-36 fibroblasts

In order to evaluate type-I collagen production by OUMS-36 fibroblasts, we performed fluorescent immunostaining to visualize type-I collagen and the amount of type-I collagen was expressed as an area (m^2) which is corresponding to type-I collagen

per one hundred cells on randomly selected four sites (350 m×350 m). The control (reversed-osmosis ultra-pure water) exhibits measurement values that dissolved H_2 = 0.01 ppm, oxidation-reduction potential = +363 mV and temperature = 29.5ºC. Immediately after preparation using an electrolyzing device equipped with stainless electrodes (The Chugoku Electric Manufacturing Co. Inc.), HW exhibits measurement values that dissolved H_2 = 1.13 ppm, oxidation-reduction potential = -741 mV and temperature = 41.0ºC, whereas RW exhibits each of them was at 0.01 ppm, +184 mV and 41.0ºC (Fig. 1).

Fig 2 shows the distribution and amount of type-I collagen produced by OUMS-36 fibroblasts. In the H_2-lacking regular warm water (RW) supplementations during the cell culture resulted in fewer type-I collagen producing around OUMS-36 fibroblasts compared to the HW-supplementations (Fig. 2). Amount of type-I collagen production (m^2/100 cells) was compared between HW and RW. After 3-day culture on exposures to UVA of 1.2 J/cm^2 × twice/day × 3 days (sum: 7.2 J/cm^2), despite the low cell density, value of amount of type-I collagen (m^2/100 cells) was 2312.95 ± 86.61 with RW and 4816.78 ± 384.41 with HW, that is, HW significantly enhanced type-I collagen biosynthesis more than RW by 2.03-fold (Fig. 2). Furthermore, 5-day culture on

exposures to UVA-irradiation of 1.2 J/cm^2 × twice/day × 5 days (sum: 12 J/cm^2), the value was 3255.50 ± 345.52 with RW and 5998.40 ± 1127.54 with HW, that is, type-I collagen production was significantly increased with HW than RW by 1.85-fold (Fig. 2). Thus, the significantly more amount of type-I collagen was found with HW than RW during the cultivation.

Fig. 2 Distribution and amount of type-I collagen produced by OUMS-36 fibroblasts as detected by fluorescent immunostaining using an antibody against type-I collagen with accompanying DAPI staining for cell nuclei. OUMS-36 fibroblasts were treated with hydrogen-rich electrolyzed warm water (HW) or regular warm water (RW) and subjected to 1.2 J/cm^2 of UVA-irradiation at the rate of twice /day for 3-5 days. Magnification: × 200, Scale bars = 50 m. Amount of type-I collagen was evaluated as an area which is corresponding to type-I collagen per one hundred cells (m^2/100 cells). Values are means ± SD, n = 4, **p* < 0.05 (vs. RW).

After 5-day culture and UVA-irradiation of 1.2 J/cm^2× twice/day×5 days (sum: 12 J/cm^2), type-I collagen was not nearly found in control, but slightly produced with RW and significantly more increased with HW (Fig. 3). Namely, OUMS-36 fibroblasts

began to produce type-I collagen more precociously with HW than RW, and type-I collagen construction was promoted much more with HW than RW over the 5-day (Fig. 2 and 3). Type-I collagen was localized preferentially around the nuclei of OUMS-36 fibroblasts except nuclei inside (Fig. 3).

Fig. 3 Distribution of type-I collagen produced by OUMS-36 fibroblasts as detected by fluorescent immunostaining using an antibody against type-I collagen with accompanying DAPI staining for cell nuclei. OUMS-36 fibroblasts were treated as the same as described in Fig. 2. Relative luminance of test/control indicates the type-I collagen and nucleus on OUMS-cells with white dashed line. Magnification: × 400, Scale bars = 30 m.

The intracellular ROS-generation in UVA-irradiated OUMS-36 fibroblasts was examined by NBT-method. When the pale-yellow NBT reagent reacts with $O_2 \cdot^-$, a dark-blue insoluble formazan is produced [18,21,22]. In UVA-nonirradiated control, OUMS-36 fibroblasts showed slightly blue based on NBT-formazan, which is caused by a physiologically trace level of ROS (Fig. 4). The UVA-irradiation of 12 J/cm^2 increased amount of NBT-formazan ($A_{560\ nm}$) by 6.8% (vs. the UVA-nonirradiated

control) with RW, and cytoplasmic region around the outside of nuclei became appreciably blue (Fig. 4). When the cells were treated with HW for 30 min from preliminary to posterior UVA-irradiation, amount of NBT-formazan increased modestly by 1.9% (Fig. 4). HW could significantly suppress UVA-induced intracellular ROS production in OUMS-36 fibroblasts (Fig. 4).

Fig. 4. UVA-induced intracellular reactive oxygen species (ROS) generation in OUMS-36 fobroblasts, treated with HW or RW as examined by NBT method. OUMS-36 fibroblasts were treated with hydrogen-rich electrolyzed warm water (HW) or regular warm water (RW) and subjected to 12 J/cm^2 of UVA-irradiation. Then NBT was added in each well and incubated for 1.5 hr. Magnification:× 100 (on the upper line), × 200 (on the bottom line), Scale bars = 50 m. Amount of NBT-formazan was evaluated as an absorbance at 560 nm which is related to intracellular ROS in OUMS-36 fibroblasts. Values are means ± SD, n = 4, *$p < 0.05$ (RW vs. HW).

3.2. Defensive effects of HW against UVA-induced injury in HaCaT keratinocytes

The cell viability (% of Control) was measured by WST-1 method, which is based on

intracellular mitochondrial reducing ability and correlated linearly with the number of viable cells [19]. Three times of UVA-irradiation by 6 J/cm^2 (sum: 18 J/cm^2) induced markedly cell death and reduced the cell viability to 29.7%. When HW was administered preliminary to every UVA-irradiation, cell viability was significantly retained to 35.0% (Fig. 5). Cell viability increased slightly to 30.8% with RW, which showed no significant differences as compared to no-additive control. Morphological aspects of HaCaT keratinocytes exposed to UVA-irradiation are in accordance with cell viability (Fig. 5). Under the same conditions as WST-1 assay, we examined intracellular ROS in HaCaT keratinocytes by NBT method. Three-times of UVA-irradiation by 6 J/cm^2 (sum: 18 J/cm^2) increased amount of NBT-formazan ($A_{560\ nm}$) by 10.2% (vs. the UVA-nonirradiated control), and cytoplasmic region around the outside of nuclei became appreciably blue with no additive control (Fig. 5). When the cells were treated with HW preliminary to every UVA-irradiation, amount of NBT-formazan decreased to -3.9%, which is the same level as non UVA-irradiated control, but RW increased intracellular ROS modestly to 1.9% (Fig. 4). Thus HW could significantly suppress UVA-induced intracellular ROS production in HaCaT keratinocytes over the RW (Fig. 5).

Fig. 5 Cell morphological aspects of HaCaT keratinocytes at 21 hr after UVA-irradiation for WST-1 assay and 1.5 hr after UVA-irradiation for NBT assay. HaCaT keratinocytes were treated with hydrogen-rich warm water (HW) or regular warm water (RW) repeatedly and subjected to 6 J/cm^2 of UVA-irradiation at the rate of three-times at 1.5 hr intervals. Magnification: × 200, Scale bars = 50 m. Values are means ± SD, n = 4, *$p < 0.05$ (vs. No additive control).

Furthermore, morphological changes that are characteristic of apoptosis, such as DNA cleavage, karyorrhexis or pycnosis in the cell were visualized by Hoechst 33342 staining [20]. The nuclei in normal cells exhibited the diffused staining of chromatin (Fig. 6). Three-times UVA-irradiation by 6 J/cm^2 (sum: 18 J/cm^2) caused typical morphologic changes of apoptosis such as condensed chromatin and shrunken nucleus (Fig. 6). When the cells were treated with HW preliminary before every UVA-irradiation, the abnormality of nuclei was repressed, but RW did not show so much cytoprotection against UVA. The percentage of apoptotic cells increased by UVA-irradiation, but it was suppressed significantly by HW as compared to RW (Fig. 6). HW exhibited notable defense of DNA as detected by Hoechst 33342 stain.

Fig. 6 Fluorescence micrographs of HaCaT keratinocytes with Hoechst 33342 staining. HaCaT keratinocytes were treated as the same as described in Fig. 5. The condensed chromatin and shrunken nucleus were indicated by white open triangles. Magnification:× 100 (on the upper line), × 1000 (on the bottom line), Scale bars = 100 m (on the upper line), 10 m (on the bottom line). And the percentage of apoptotic cells of each treatment were shown as means±SD, n=6. *$p < 0.05$ (vs. No additive control)

3.3. Clinical evaluation of repressive-wrinkle formation by HW-bathing

Five healthy female volunteers at the age of 14-65 years and one healthy male volunteer at the age of 55 years were enrolled in a clinical study of repressive-wrinkle formation by HW-bathing. The device for HW-bathing (Fig. 7) supplied HW by electrolysis of tap-water and hold dissolved hydrogen at the level of 0.19-0.41 ppm (RW: < 0.01 ppm), temperature: 41ºC, and oxidation-reduction potential at -150~-300 mV (RW: +150 mV) in bath-tub by circulation. About four female (14-46 years, 31.5 ± 11.4 years), HW-bathing exhibited improvement of wrinkles as shown in Fig. 8. The

wrinkle-area ratio ($m^2/mm^2/100$) of them decreased with HW-bathing time dependently from 3041.7 ± 151.6 at 0 day to 2471.3 ± 596.9 at 45^{th} day and 1770.7 ± 547.7 at 90^{th} day, and there was significant difference at 90^{th} as compared to 0 day (Fig. 8). Whereas two subjects (female: 65 years, male: 55 years) did not show improvement of wrinkles.

Fig. 7 The device for HW-bathing and dissolved hydrogen level (ppm) of bathwater in supplying horse and bath-tub. This device can supply HW by electrolysis of tap-water and hold dissolved hydrogen at the level of 0.19-0.41 ppm in bath-tub by circulation.

Fig. 8 Improvement examples by photographic assessment of wrinkle and two-dimensional image analysis of replica in the back of neck area treated with daily HW-bathing before application (0 day) and after application at 45^{th} and 90^{th} day. Wrinkle degree was expressed as relative value of wrinkle-area ratio at 0 day = 100. Scale bars = 3 mm. Values are means ± SD, n = 4, $*p < 0.05$ (vs. 0 day).

4. Discussion

The aim of the present study was to examine the promotive effects of HW on type-I collagen synthesis in OUMS-36 fibroblasts and preventive effects against UVA-induced cell death and intracellular ROS in HaCaT keratinocytes as compared to RW. We also clinically assessed the effect of daily HW-bathing on repressive-wrinkle formation with six subjects for three months. It is reported that UVA-irradiation causes a small decrease in cellular growth rate and in the degree of type I-collagen fibril formation *in vitro*[23]. Based on immunostaining, HW increased type-I collagen production in OUMS-36 fibroblasts. When OUMS-36 fibroblasts were exposed to repetitive UVA-irradiation by 1.2 J/cm^2× twice/day, which is within the range of daily UVA-dose, the amount of biosynthesized type-I collagen with HW increased by 2.03-fold greater than RW on 3rd day, and 1.85-fold greater than RW on 5th day (Fig. 2). Thus HW assisted production of type-I collagen, and H_2-lacking RW did not show a noteworthy increase of type-I collagen. It is reported that intracellular type-I collagen is mostly localized preferentially around the nuclei of juvenile rabbit tenocytes [24], type-III collagen is more diffused in the cytoplasm and type-V collagen is detected in fibrillar and vesicular forms in the cytoplasm [25]. In agreement with this, we observed type-I collagen was distributed around the outside of nuclei of OUMS-fibroblasts with fluorescent microscope (Fig. 2 and 3). The UVA-irradiation of 12 J/cm^2 increased

intracellular ROS by 6.8% with RW at cytoplasmic region around the outside of nuclei in OUMS-36 fibroblasts (Fig. 4). But HW could significantly suppress UVA-induced intracellular ROS production to 1.9% (Fig. 4). These results suggest that dissolved hydrogen in HW could restrain type-I collagen destruction owing to scavenge UVA-induced intracellular ROS, thereafter promote type-I collagen biosynthesis in exposure to the daily dose of UVA.

Then, in HaCaT keratinocytes, three-times of UVA-irradiation by 6 J/cm^2 (sum: 18 J/cm^2) increased intracellular ROS by 10.2% and reduced the cell viability to 29.7%. HW-administration preliminary to every UVA-irradiation decreased intracellular ROS decreased to the same level as non UVA-irradiated control and significantly retained cell viability to 35.0% (Figs. 4 and 5). Meanwhile, RW increased intracellular ROS modestly to 1.9%, and also increased cell viability slightly to 30.8% (Figs. 4 and 5). There was no significant difference between RW-administration and no additive control (Figs. 4 and 5). Furthermore, HW repressed the UVA-induced abnormalities of nuclei in HaCaT keratinocytes, but RW did not show so much cytoprotection against UVA. These results indicate that application of HW could significantly attenuate UVA-induced cell death, as well as inhibit the UVA-induced DNA damages and

intracellular ROS in HaCaT keratinocytes over the RW (Figs. 4 and 5). These results suggest that HW protects HaCaT keratinocytes from UVA-induced cell damage by preventing UVA-derived intracellular ROS generation owing to its high level of dissolved hydrogen: 1.13 ppm and low oxidation-reduction potential: -741 mV.

Wrinkles are modifications of the skin associated with cutaneous aging and preferentially on sun-exposed skin with decrease in collagen fiber [26]. It is reported that L-ascorbic acid stimulates the synthesis of type-I collagen in primary cultures of dermal fibroblasts, and which decreases in a statistically significant linear manner with donor age [27]. If there is an ideal useful material for anti-skin aging, it needs to be biological safety and restore the impaired function in skin without excessive activation. It is noteworthy that the electrolyzed reduced water treatment is effective in palliating chronic hemodialysis-evoked oxidative stress without side effects of oxalate accumulation which is easily induced by L-ascorbic acid [28]. All collagen molecules are made within fibroblasts [29]. It is reported that exposure of cultured human dermal fibroblasts to ultraviolet radiation (UVA, UVB) increases ROS formation and reduces collagen type-I *in vitro*[30]. Destruction of collagen is a hallmark of photoaging [31], and ROS are thought to be a major factor to initiate the up-regulation of

matrix-metalloproteinases (MMPs) in not only fibroblasts but also in keratinocytes to degrade fiber components in dermis, leading to wrinkle formation [32]. Our data supports the hypothesis that UVA-irradiation induces intracellular ROS, but antioxidant response of HW will quench the increased levels of intracellular ROS, inhibit cell death and DNA damage and promotes type-I collagen production. The effects of HW on type-I collagen production and cytoprotection against UVA may be due to the antioxidant activity of dissolved-H_2, as HW was found to be antioxidative but not with H_2-lacking RW *in vitro* by NBT assay. Since intracellular ROS was increased modestly with H_2-lacking RW after UVA-irradiation, it is suggested that 'warm' are not playing a major role in this cytoprotective response (Figs. 4 and 5).

Natural evolution of wrinkles starts from a reducible to a permanent skin invagination with differences among individuals depending on age [33], whereas substantial repair is occurred in hairless mice skin after stopping UV-exposure *in vivo*[34]. In the three-months clinical study on 6-healthy Japanese volunteers (age: 14-65 years) in HW-bathing of 0.19-0.41 ppm of dissolved H_2, the wrinkles in back of the neck were improved significantly on 4-subjects (age: 14-46 years) at 90^{th} day as compared to 0 day. It is reported that hydrogen water is rapidly absorbed and increased breath hydrogen to

the maximal level of approximately 40 ppm for 10-15 min after ingestion [35]. The repressive-wrinkle effect of HW-bathing could be attributed to dissolved-H_2 which was penetrated into the skin thorough dilated follicles by warming with HW at 41ºC and scavenge ROS in the skin. It means that the combination of dissolved-H_2 and warm temperature at 41ºC synergistically improved the mechanical properties of skin due to facilitate a ROS-scavenging reaction of dissolved H_2. *In vitro*, HW was set to contain 1.13 ppm of dissolved-H_2 and applied for 3-5 days test in OUMS-36 fibroblasts and HaCaT keratinocytes. However in clinical study, HW-bathing was containing 0.19-0.41 ppm of dissolved-H_2, but HW-bathing was applied daily for 3-months, which would result in improvement of wrinkles. Thus, HW has potential anti-aging effects to serve for a daily skin care by repressing UVA-induced skin damages by ROS-scavenging and promotion of type-I collagen synthesis in dermis.

5. Conclusions

In this study, we demonstrated that HW promotes the type-I collagen synthesis in OUMS-36 fibroblasts and decreases cell death in HaCaT keratinocytes, each of them is accompanying by scavenging UVA-derived intracellular ROS. In addition, a clinical study of HW-bathing using image analysis showed the repressive-wrinkle formation on

90^{th} day as compared to 0 day. Therefore, continuous application of HW reduces oxidative stress in the skin, and expects to prevent the wrinkle formation. HW-bathing could be applicable for anti-skin aging.

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Fig. 1

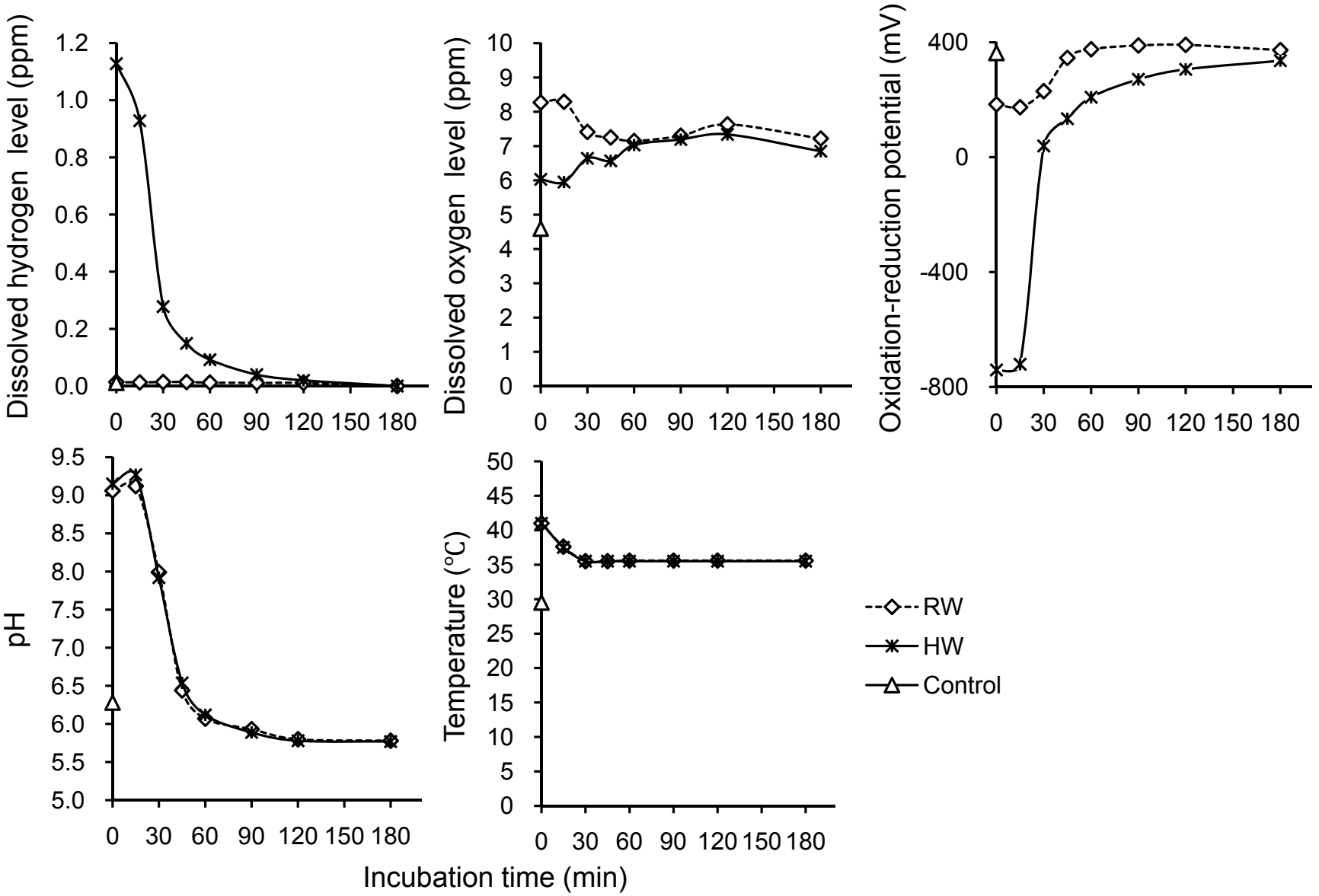

Fig. 2 OUMS-36 Cllgn-I

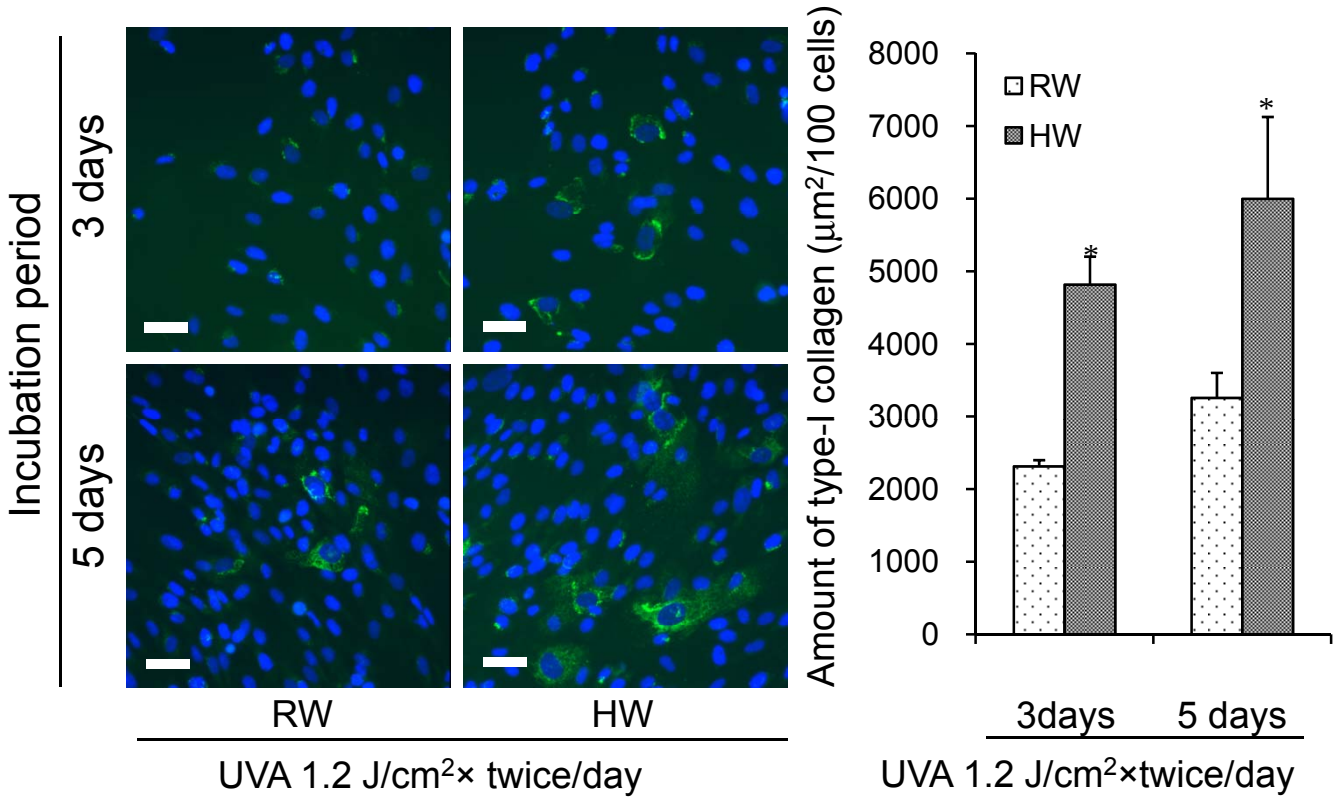

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Fig. 3 OUMS-36 Cllgn-I

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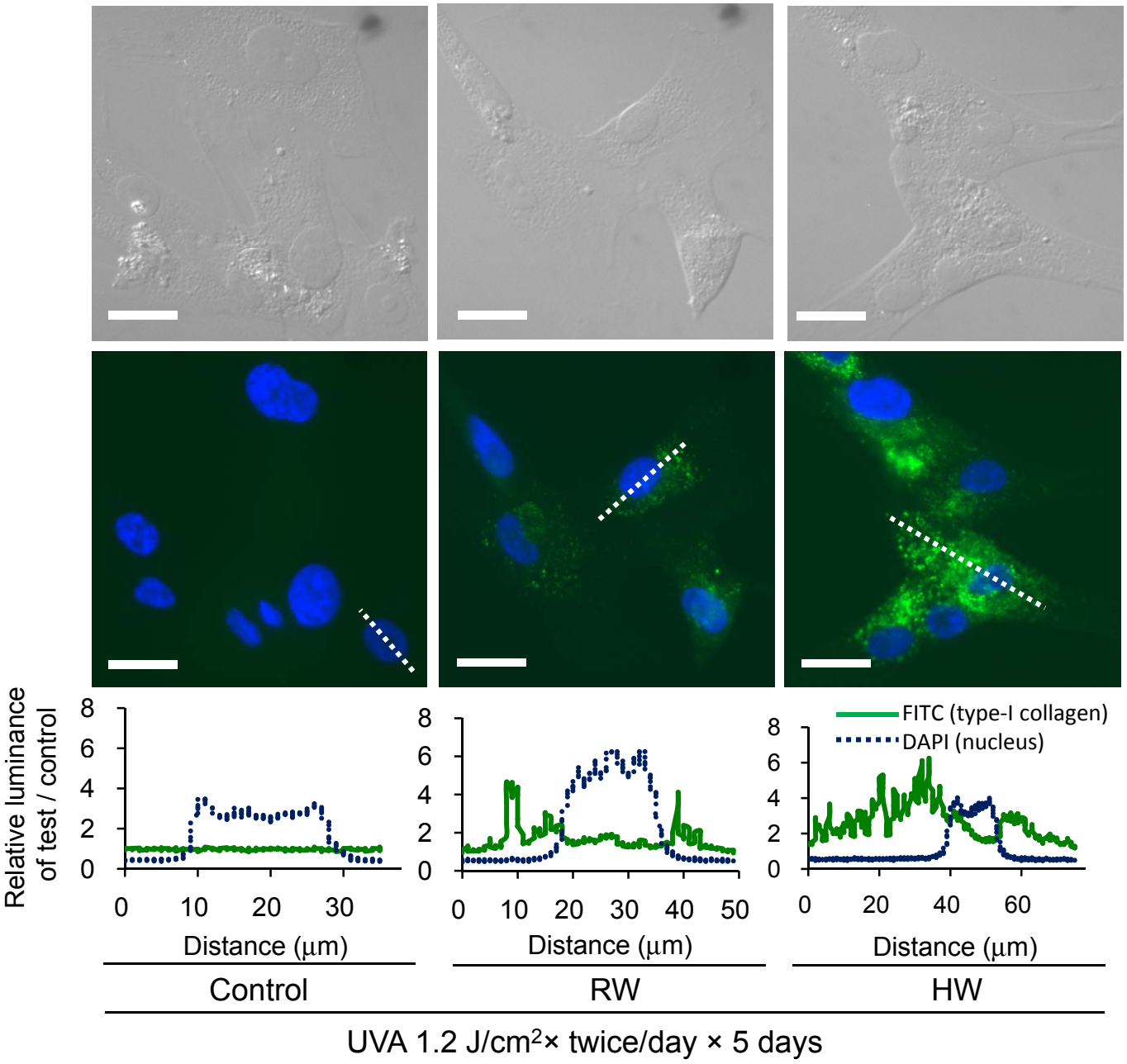

Fig. 4 OUMS-36 NBT

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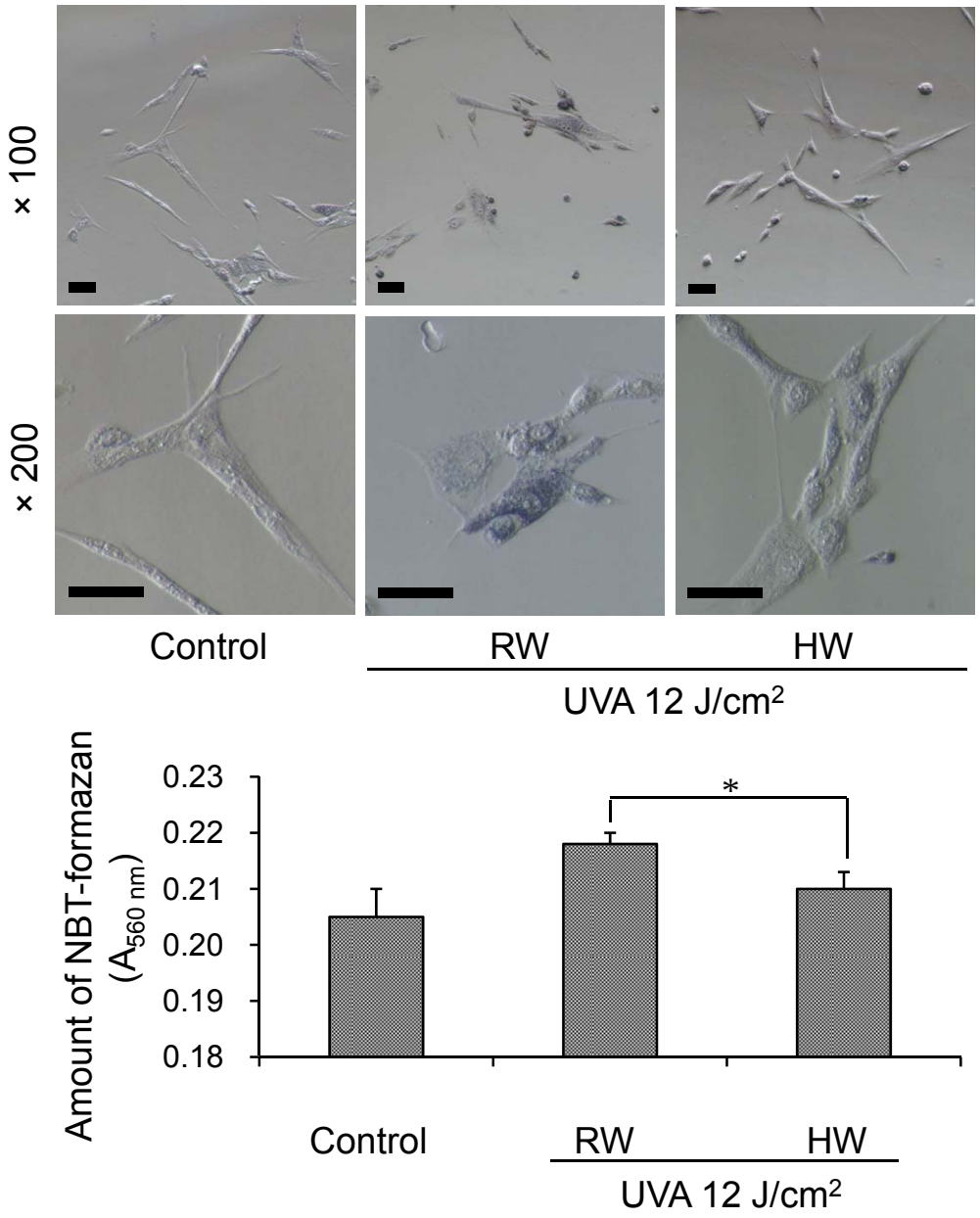

Fig. 5 HaCaT WST-1&NBT

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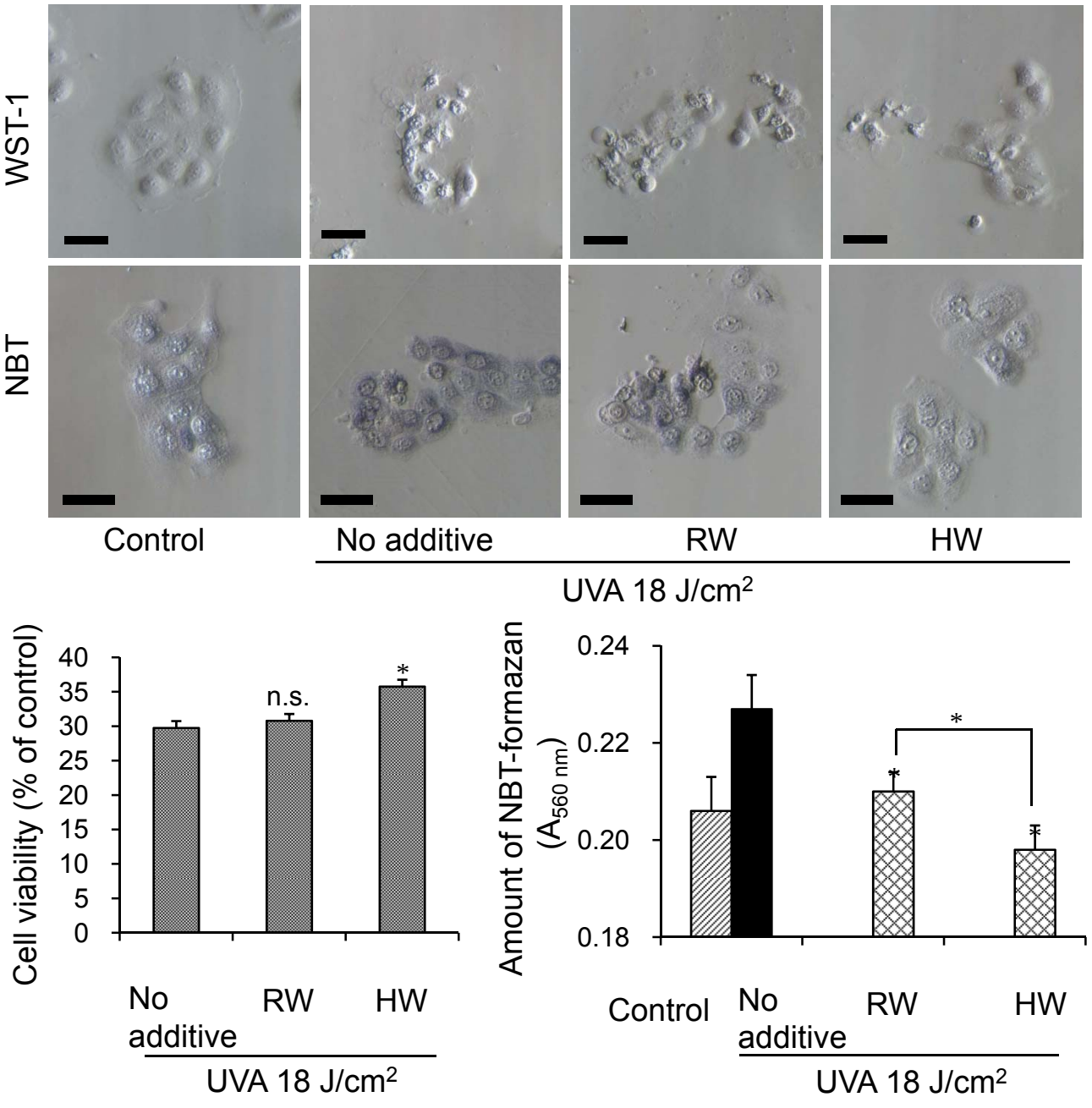

Fig. 6 HaCaT Hoechst33342

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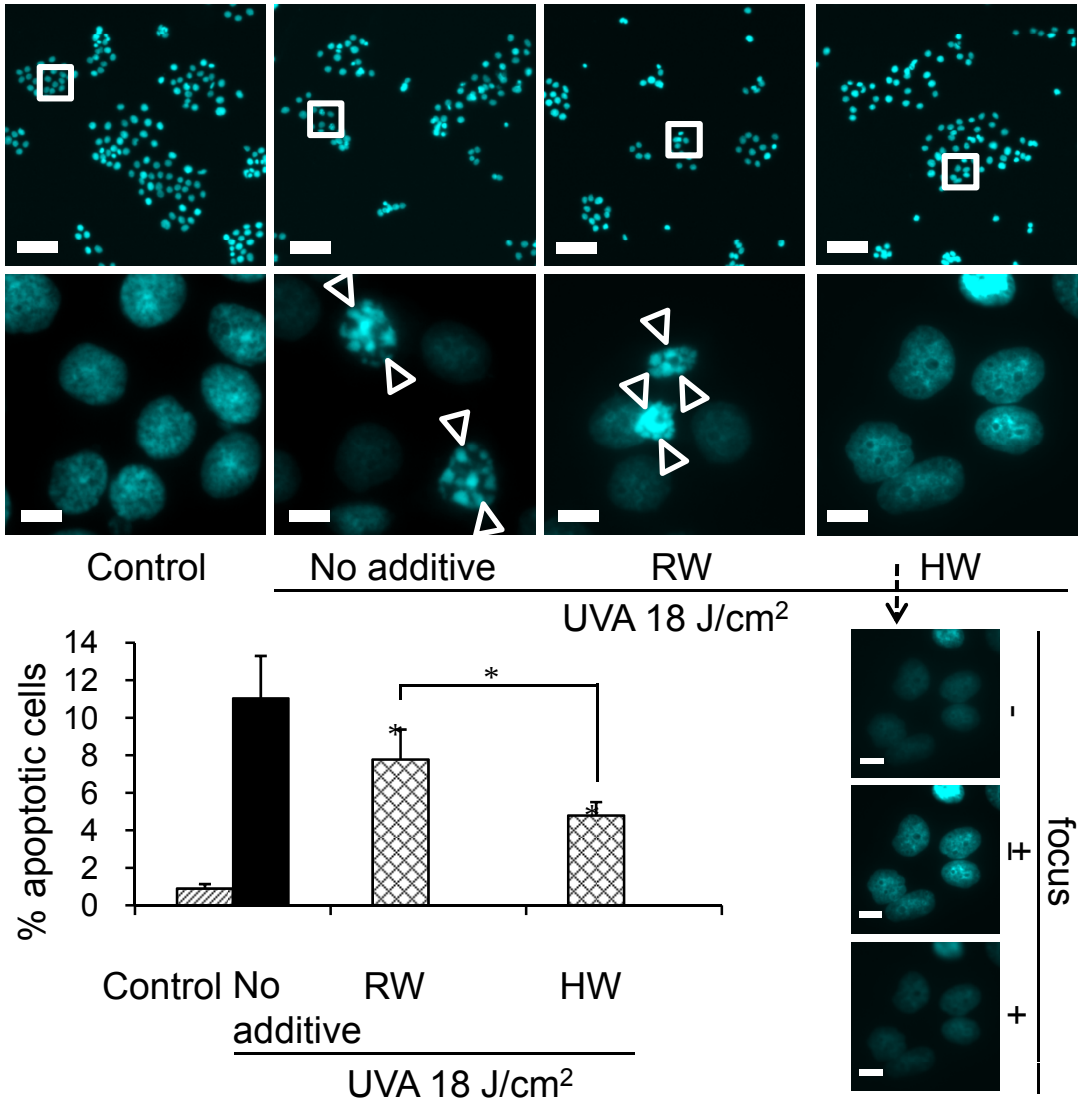

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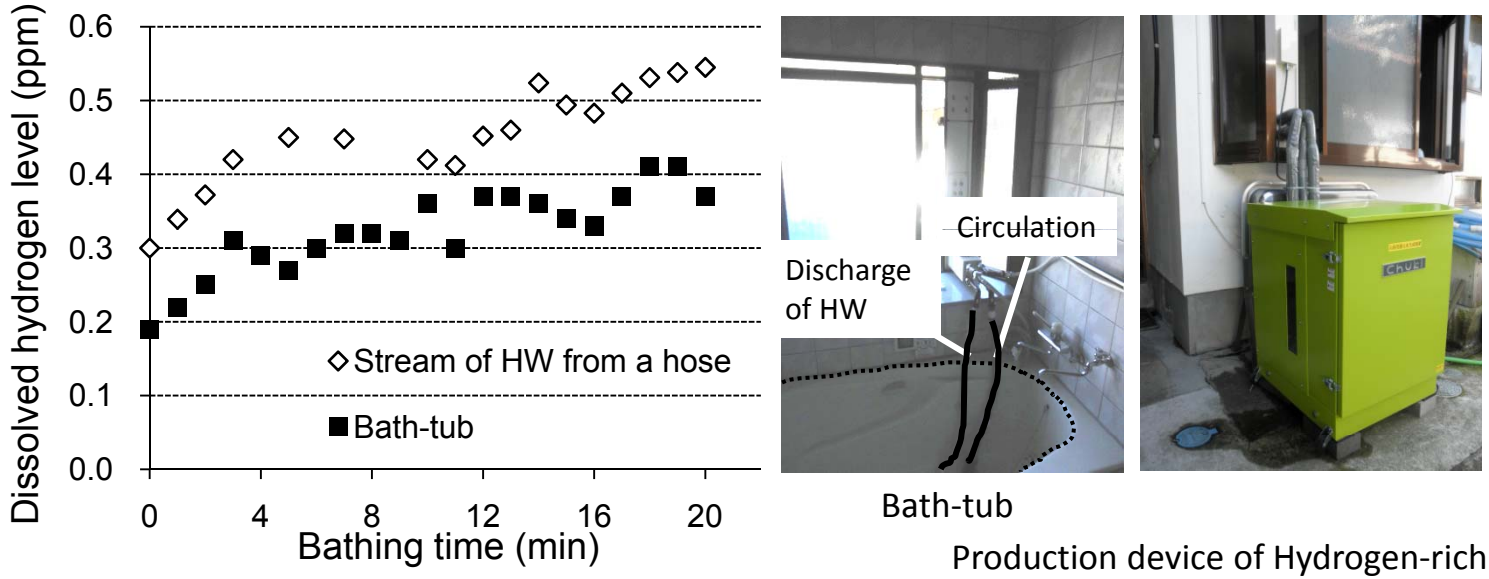

Bath-tub

Production device of Hydrogen-rich electrolyzed warm water for domestic use

Fig. 8

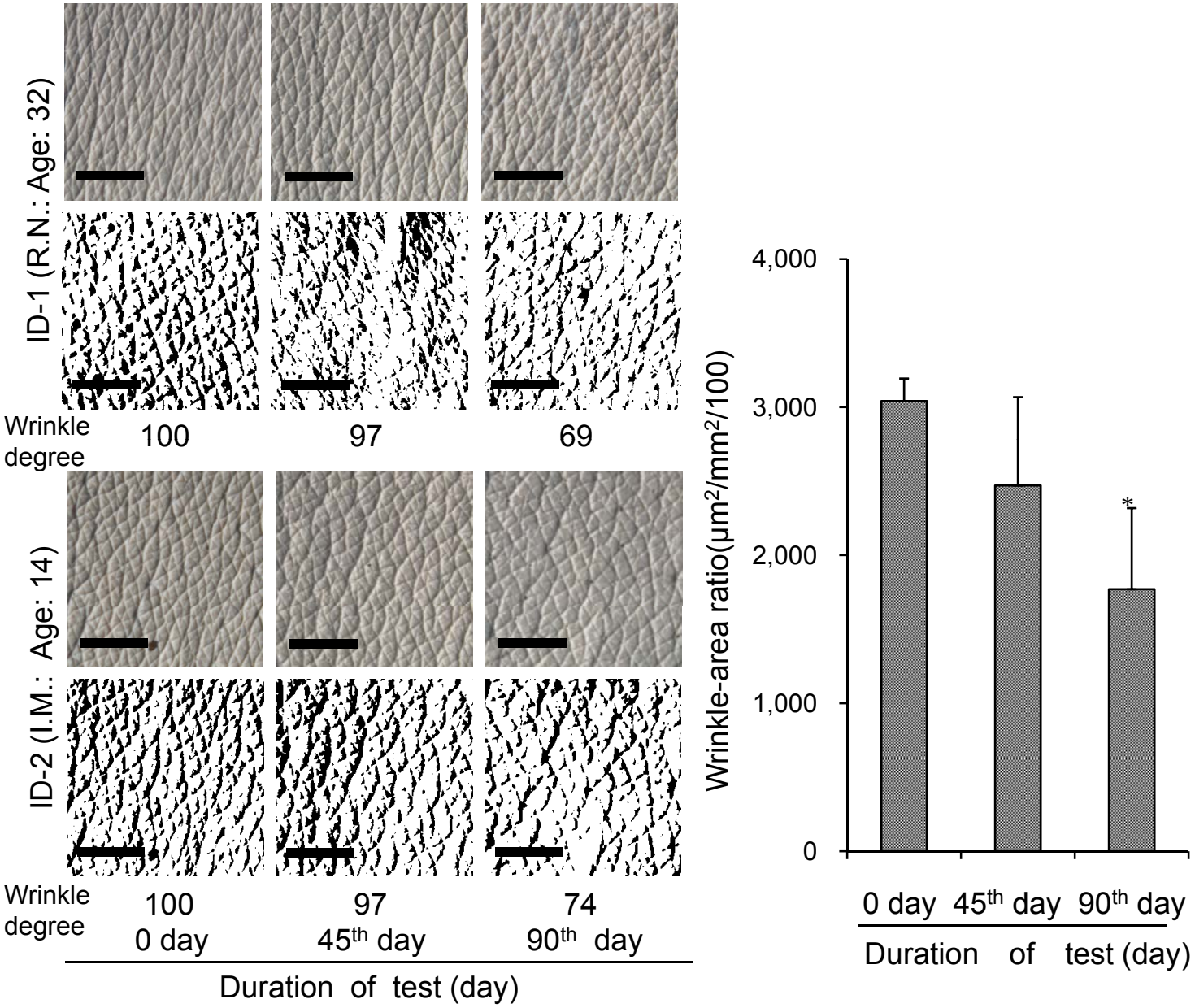
ID-1 (R.N.: Age: 32)
Wrinkle degree
100
97
69
ID-2 (I.M.: Age: 14)
Wrinkle degree
100
0 day
97
45th day
74
90th day
Duration of test (day)
Wrinkle-area ratio(μm²/mm²/100)
4,000
3,000
2,000
1,000
0
*
0 day
45th day
90th day
Duration of test (day)

Fig. 2 OUMS-36 Cllgn-I

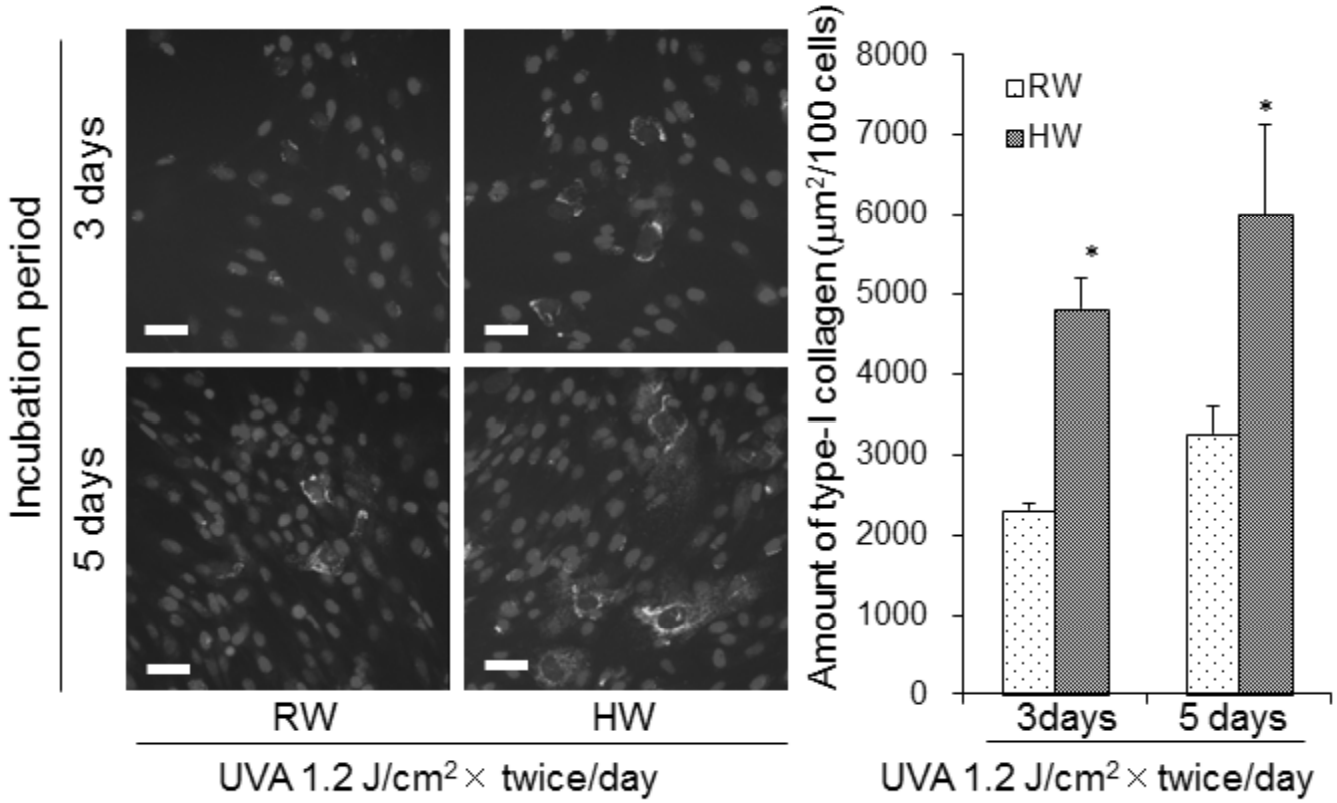

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Fig. 3 OUMS-36 CIlgn-I

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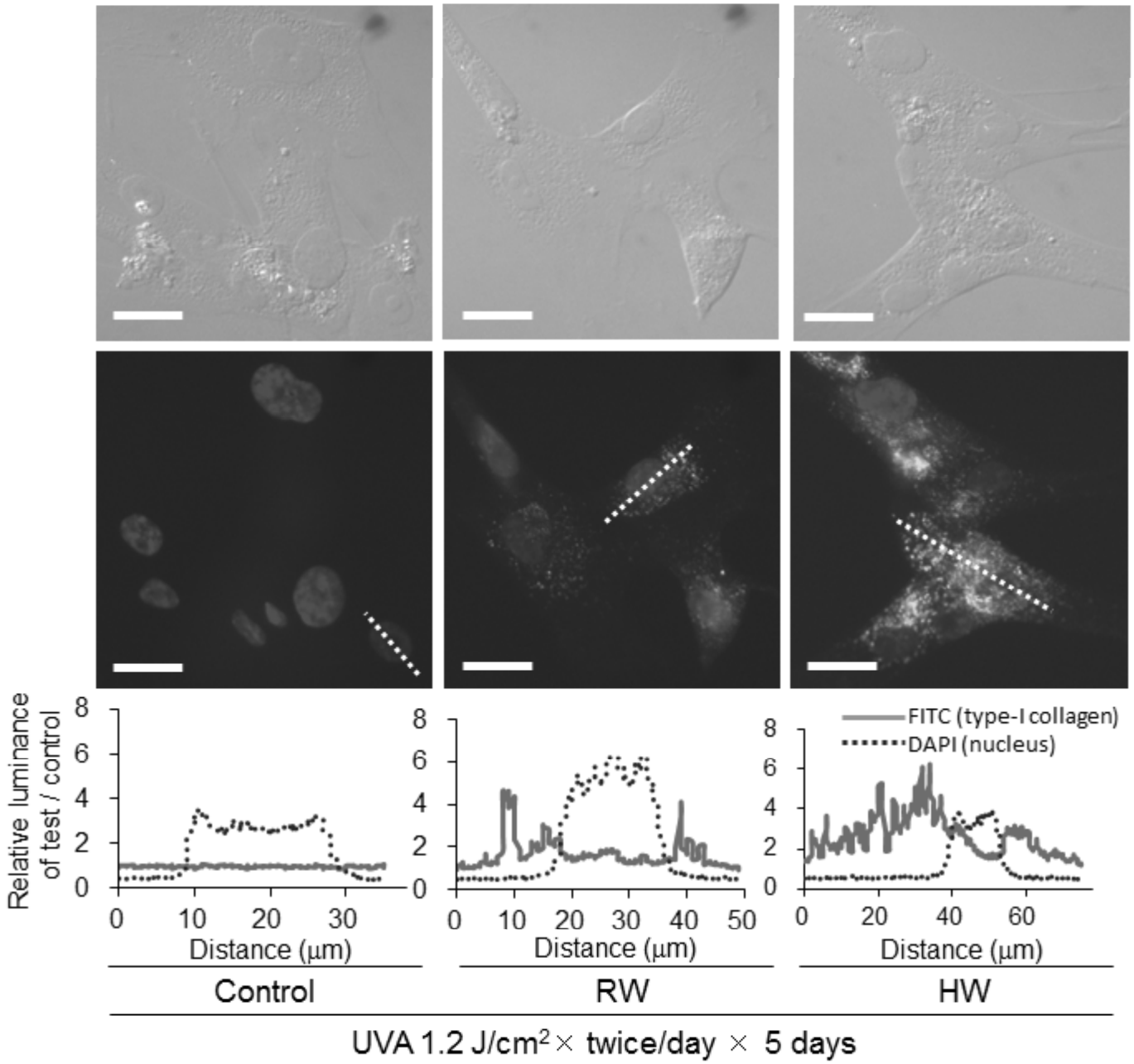

Fig. 4 OUMS-36 NBT

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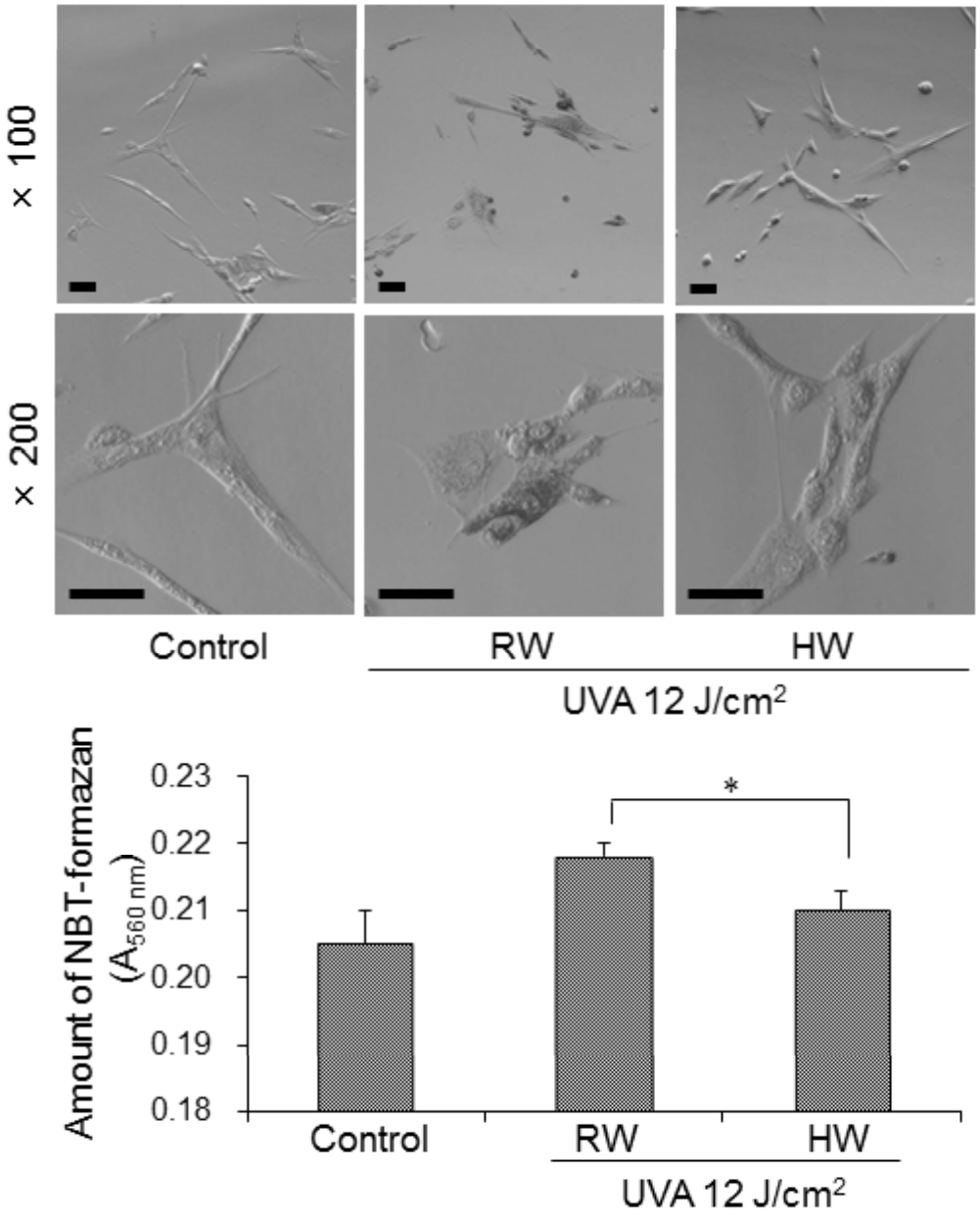

Fig. 5 HaCaT WST-1&NBT

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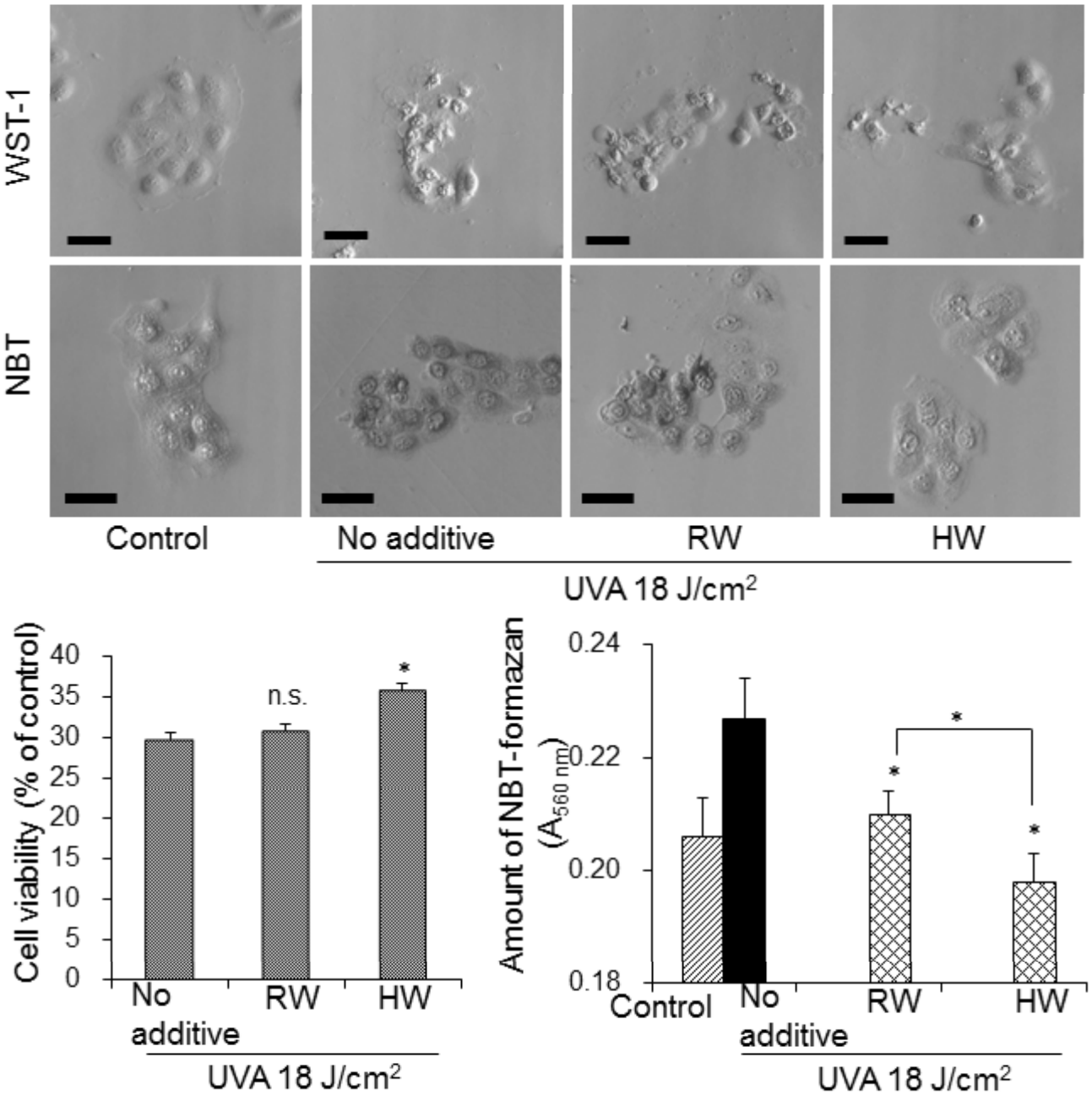

Fig. 6 HaCaT Hoechst33342

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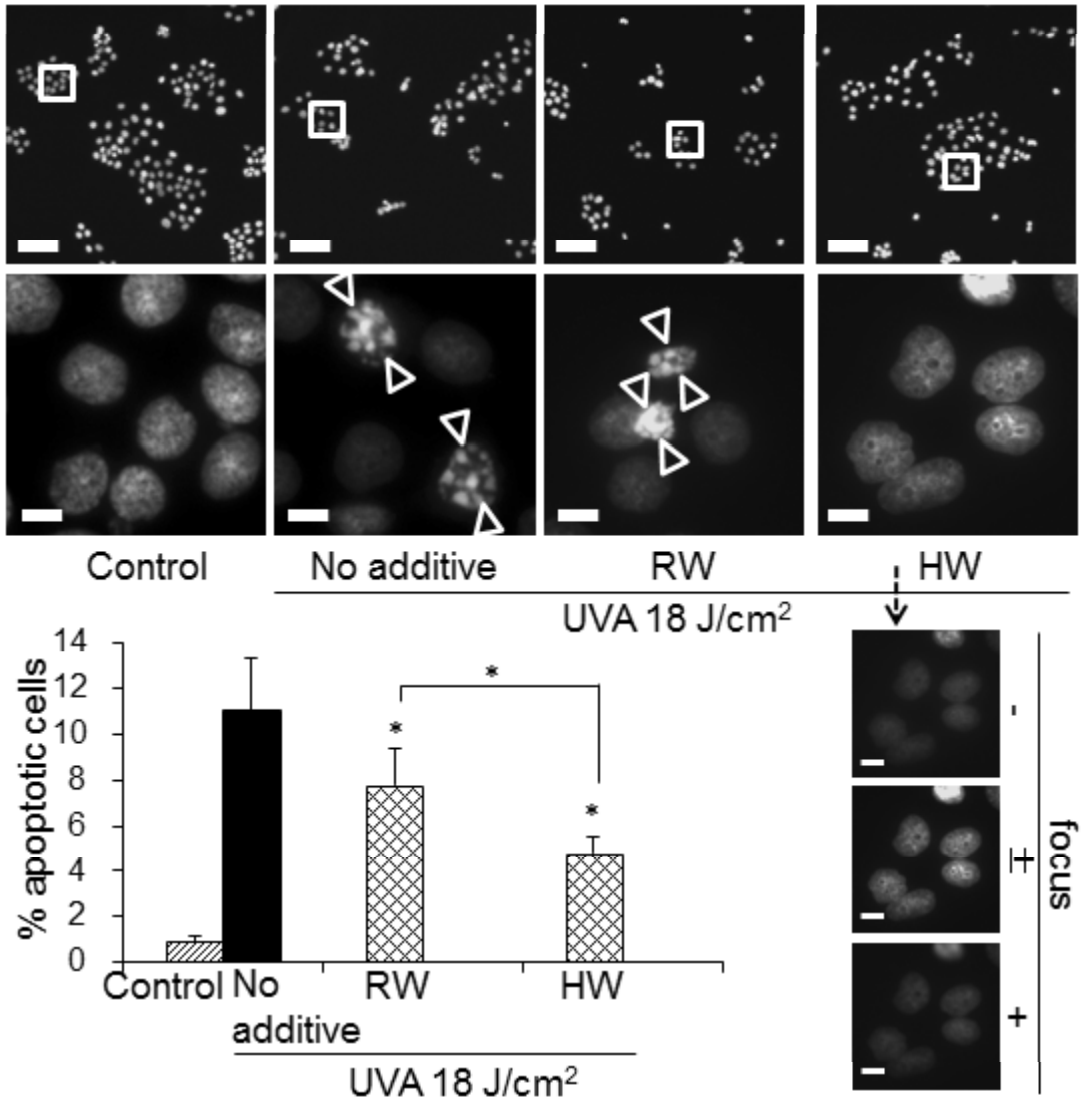

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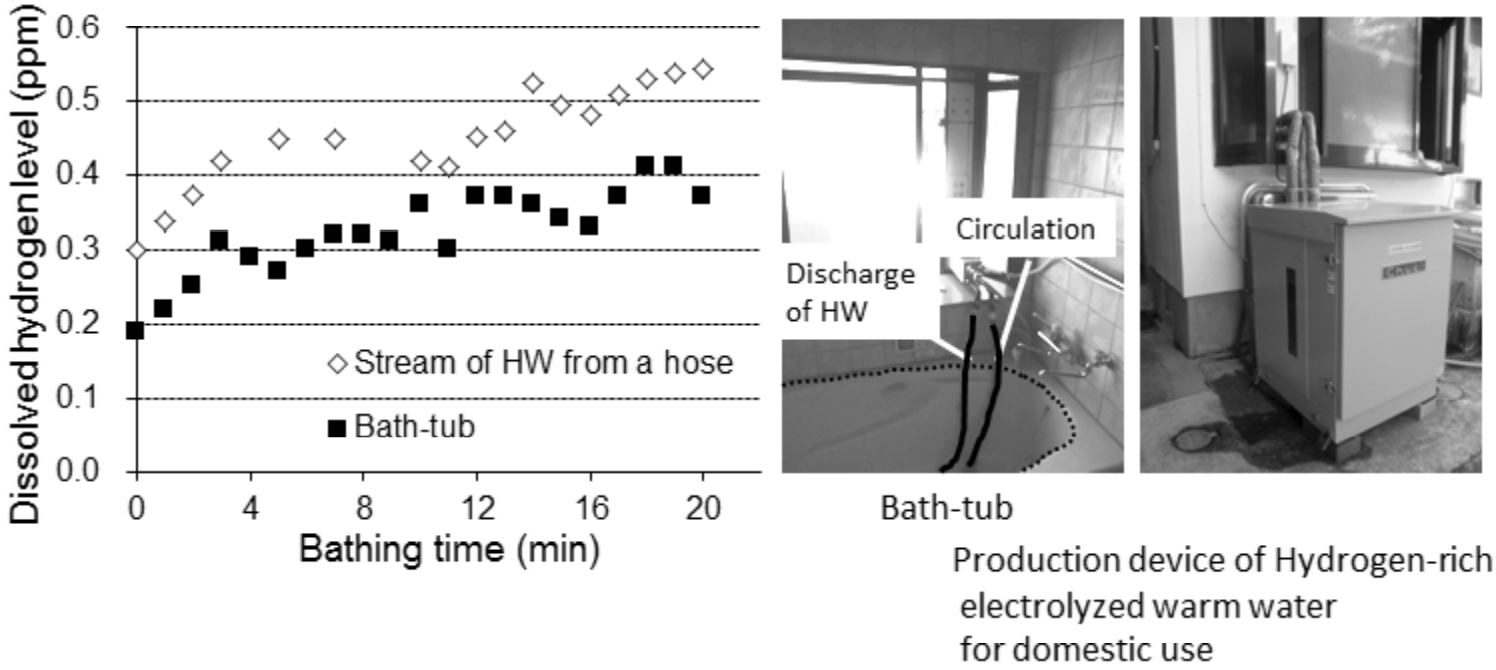

Production device of Hydrogen-rich electrolyzed warm water for domestic use

Highlights

►Hydrogen-rich electrolyzed warm water (HW) promotes the type-I collagen synthesis in fibroblasts and suppresses cell death in keratinocytes, in accompanying with quenching UVA-derived intracellular reactive oxygen species. ►In addition, a clinical study of HW-bathing demonstrated the repressive-wrinkle formation on 90^{th} day as compared to 0 day. ►Therefore, continuous application of HW reduces oxidative stress in the skin, and expects to prevent the wrinkle formation.