Inhibitory effect of vitamin C on intrinsic aging in human dermal fibroblasts and hairless mice

Jae-Hong Jeong¹ · Mi-Bo Kim¹ · Changhee Kim¹ · Jae-Kwan Hwang¹

Received: 22 July 2017 / Revised: 14 September 2017 / Accepted: 22 October 2017 / Published online: 22 November 2017
© The Korean Society of Food Science and Technology and Springer Science+Business Media B.V. 2017

Abstract Vitamin C significantly reduced senescence-associated β -galactosidase (SA- β -gal) activity, with both the suppression of cell-cycle inhibitors (p53, p21, p16, and pRb) and stimulation of cell-cycle activators (E2F1 and E2F2). Vitamin C also effectively attenuated the hyperactivation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase-B (AKT) signaling pathway. The expression of the longevity marker, the mammalian target of rapamycin (mTOR), was down-regulated by vitamin C while the expressions of forkhead box O3a (FoxO3a) and sirtuin1 (SIRT1) were up-regulated by vitamin C. In the middle-aged (MA) mice, oral administration of vitamin C significantly inhibited wrinkle formation, skin atrophy, and loss of elasticity through increasing collagen and elastic fiber. The increase in transepidermal water loss and the decrease in skin hydration were recovered by vitamin C treatment in the MA mice. Overall, vitamin C effectively prevents cellular senescence in vitro and in vivo suggesting it has protective potential against natural aging of the skin.

Keywords Vitamin C · Intrinsic aging · Cellular senescence · Hairless mice

Introduction

Human skin cells are constantly exposed to stress from exogenous and endogenous factors, resulting in extrinsic aging caused mainly by ultraviolet radiation (UVR) and

intrinsic aging caused mainly by cellular senescence [1]. Cellular senescence can be divided into two categories based on the mechanism involved: telomere-initiated or replicative senescence and stress-induced or premature senescence [2]. Accumulation of oxidative stress over time leads to the DNA-damage response which triggers premature senescence through the p53/p21 and p16/pRb pathways [3, 4]. These two major pathways are directly associated with cell cycle arrest, which is the most evident sign of cellular senescence. p53 is a transcription factor that results in cellular senescence by inducing the expression of p21, a cyclin-dependent kinase (CDK) inhibitor. p16 is another CDK inhibitor that suppresses pRb phosphorylation. Cell proliferation is regulated by pRb activity which inhibits an E2F transcription factor, controlling gene expression in the cell cycle [4].

Oxidative stress also leads to stimulation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase-B (AKT) signaling pathway, which controls lifespan and cellular senescence [5]. The forkhead transcription factor FoxO3a plays an essential role in the PI3K/AKT signaling pathway [6]. Down-regulation of FoxO3a in fibroblasts causes many senescent phenotypes such as an increase of senescence-associated β -galactosidase (SA- β -gal) activity and enhancement of p53 and p21 expression [7]. Thus, resistance to oxidative stress increases FoxO3a activity, which attenuates cellular senescence through the p53/p16-pRb and PI3K/AKT signaling pathways.

Senescent cells secrete inflammatory cytokines and other molecules that induce deleterious local tissue changes. The most significant of these effects is known as the senescence-associated secretory phenotype (SASP) [8]. Accumulation of oxidative cellular damage, which occurs with the passage of time, is a primary driving force for aging. The cellular damage causes activation of nuclear

✉ Jae-Kwan Hwang
jkhwang@yonsei.ac.kr

¹ Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul 03772, Korea

factor-kappa B (NF- κ B), which acts as an SASP inducer to regulate the secretion of pro-inflammatory cytokines. Interleukin-6 (IL-6) and IL-8 play a crucial role as pro-inflammatory cytokines in premature senescence initiation and maintenance [9].

Vitamin C, known as ascorbic acid, plays a pivotal biological role by acting as an antioxidant donating two electrons and scavenging many reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [10]. Vitamin C also provides various cutaneous effects such as the promotion of collagen synthesis, inhibition of the collagen-degrading enzyme matrix metalloproteinase-1 (MMP-1), photoprotection against UVR, and anti-inflammatory effects [11]. However, it has not been reported whether vitamin C has a preventive effect on cellular senescence in human dermal fibroblasts (Hs68) and middle-aged (MA) hairless mice. In this study, the inhibitory effect of vitamin C on intrinsic aging was investigated by evaluating its ability to attenuate hyperactivation of the PI3K/AKT signaling pathway, SA- β -gal activity, cell cycle arrest, and inflammatory cytokines *in vitro* and *in vivo*. Moreover, the effect of vitamin C on skin aging phenotypes was evaluated in MA hairless mice.

Materials and methods

Chemical reagents

Vitamin C (purity $\geq 98\%$) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied by Hyclone Laboratories (Logan, UT, USA). Antibodies against p53, p21, p16, pRb, PI3K, phospho-AKT (p-AKT), AKT, phospho-FoxO3a (p-FoxO3a), FoxO3a, phospho-mTOR (p-mTOR), mTOR, SIRT1, NF- κ B, and α -tubulin were supplied by Cell Signaling Technology (Beverly, MA, USA).

Cell culture and H_2O_2 exposure

Hs68 human dermal fibroblasts (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM, supplemented with penicillin (120 units/ml), streptomycin (75 $\mu\text{g}/\text{ml}$), and 10% FBS, under an atmosphere of 5% CO_2 at 37 °C. At 70% confluency, the fibroblasts were treated with 600 μM H_2O_2 (Sigma-Aldrich) for 2 h to induce the senescent state. The culture medium was changed every 12 h during the experimental period, to maintain a consistent effect of vitamin C on Hs68 fibroblasts.

Cell viability assessment

Cell viability was evaluated by MTT colorimetric assay. Hs68 cells (1×10^5 cells/well) were pretreated with various concentrations of vitamin C for 24 h. The cells were exposed to 600 μM H_2O_2 for 2 h in order to induce growth arrest, and then treated with or without vitamin C, for an additional 24 h. After vitamin C treatment, the cell viability was assessed by MTT colorimetric assay according to our previous method [12]. No significant cell viability was observed at less than 500 μM vitamin C, compared to the control (data not shown). The following studies were conducted at less than 500 μM vitamin C.

Animal experiments

Eight-week-old female albino hairless mice (SKH-1; Orient Bio Inc., Seongnam, Korea) were housed in Yonsei Laboratory Animal Research Center (YLARC; Seoul, Korea) at 23 ± 2 °C and $55 \pm 10\%$ relative humidity, with a 12 h day/night cycle for 24 weeks. The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of YLARC (Permit number 2013-0113). Mice were randomly assigned to three MA groups as follows: (1) MA group (MA), (2) MA and 50 mg/kg/day vitamin C administrated group (Vit C 50), and (3) MA and 200 mg/kg/day vitamin C administrated group (Vit C 200). Vitamin C, which was dissolved in saline, was orally and daily administrated to the mice for 24 weeks using oral feeding needles. The volume for oral administration was 200 μl per mouse. The mice in MA group received the saline instead of vitamin C. For the young model, 7-week-old female albino hairless mice (Orient Bio Inc.) were purchased 1 week before sacrifice and housed in the same condition for a week. The mice were euthanized by intraperitoneal injection of a mixture of Zoletil (Virbac, Carros, France) and Rompun (Bayer Korea Ltd., Seoul, Korea). Skin biopsy tissue samples of the hairless mice were collected and kept at -70 °C.

Senescence-associated β -galactosidase assay

The SA- β -gal activity of Hs68 cells and skin tissues was evaluated using a 96-well cellular senescence assay kit (SA- β -gal staining; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, Hs68 cells and the dorsal skin tissues were treated with cold $1 \times$ cell lysis buffer. The whole lysate was centrifuged at 4 °C and $16,500 \times g$ for 10 min and the supernatant was separated. The cell lysate (50 μL) was mixed with an equal amount of 2X assay buffer and incubated in the dark at 37 °C for 1 h. The SA- β -gal activity of the lysate was assessed using a fluorescence plate reader (GloMax-Multi

Microplate Reader; Promega, Madison, WI, USA) at 360 nm (excitation)/465 nm (emission).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of Hs68 cells and homogenized skin tissues were assessed by RT-PCR, according to our previous method [12]. PCR amplification was conducted in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). Primers were designed according to Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and our previous study [13]. The following primer pairs (Bioneer, Daejeon, Korea) were used: human p53 (forward, 5'-ACA CGC TTC CCT GGA TTG G-3'; reverse, 5'-CTG GCA TTC TGG GAG CTT CA-3'), human p21 (forward, 5'-GTC AGT TCC TTG TGG AGC CG-3'; reverse, 5'-GGA AGG TAG AGC TTG GGC AG-3'), human p16 (forward, 5'-GGG TCC CAG TCT GCA GTT AAG-3'; reverse, 5'-CAG TAG CAT CAG CAC GAG GG-3'), human pRb (forward, 5'-TTT ATT GGC GTG CGC TCT TG-3'; reverse, 5'-CAG TTG GTC CTT CTC GGT CC-3'), human E2F1 (forward, 5'-CCG CCA TCC AGG AAA AGG TG-3'; reverse, 5'-GCT ACG AAG GTC CTG ACA CG-3'), human E2F2 (forward, 5'-GAC TAG AGA GCG AGC CGC AA-3'; reverse, 5'-GAG CAG AGA GCA GCG CTT AG-3'), human SIRT1 (forward, 5'-ACC GAG ATA ACC TTC TGT TCG-3'; reverse, 5'-CAC CCC AGC TCC AGT TAG AA-3'), human IL-6 (forward, 5'-ATG AGG AGA CTT GCC TGG TG-3'; reverse, 5'-ACA ACA ATC TGA GGT GCC CA-3'), human IL-8 (forward, 5'-CCA GGA AGA AAC CAC CGG AA-3'; reverse, 5'-CCT CTG CAC CCA GTT TTC CT-3'), human GAPDH (forward, 5'-CTC CTG TTC GAC AGT CAG CC-3'; reverse, 5'-TCG CCC CAC TTG ATT TTG GA-3'). mouse p53 (forward, 5'-CTT GGC TGT AGG TAG CGA CT-3'; reverse, 5'-CAG CAA CAG ATC GTC CAT GC-3'), mouse p21 (forward, 5'-CGG TGT CAG AGT CTA GGG GA-3'; reverse, 5'-AGG CCA TCC TCA AAT GGT GG-3'), mouse p16 (forward, 5'-TGG TGA AGT TCG TGC GAT CC-3'; reverse, 5'-CCA GCG GAA CGC AAA TAT CG-3'), mouse pRb (forward, 5'-TTT TGT AAC GGG AGT CGG GT-3'; reverse, 5'-AAG ATG CAG ATG CCC CAG AG-3'), mouse E2F1 (forward, 5'-CCA CGA GGC CCT TGA CTA TC-3'; reverse, 5'-GGG ACA GAG GGT ATG GAT CG-3'), mouse E2F2 (forward, 5'-ACT AGA GGG GTG AAC GCA GA-3'; reverse, 5'-CGG AAT TCA GGG ACC GTA GG-3'), mouse GAPDH (forward, 5'-ACC ACA GTC CAT GCC ATC AC-3'; reverse, 5'-TCC ACC ACC GGT TGC TGT A-3'). The PCR products were analyzed by 1.5% agarose gel electrophoresis and detected using a G:BOX Image Analysis System, with Gene Snap Program (Syngene, Cambridge, UK).

Western blot analysis

The concentration of lysate protein from Hs68 cells and skin tissues homogenized by NP-40 protein lysis buffer (ELPIS-Biotech) with protease inhibitor cocktail, were quantified by the Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). The proteins were detected by Western blot assay according to our previous method [12]. Primary antibodies against p53, p21, p16, pRb, PI3K, p-AKT, AKT, p-FoxO3a, FoxO3a, p-mTOR, mTOR, SIRT1, NF- κ B, and α -tubulin (1:1000 dilution), were used. The bound primary antibodies were incubated with horseradish peroxidase-linked secondary anti-rabbit or anti-mouse antibodies (1:5000 dilution; Bethyl Laboratories, Montgomery, TX, USA). Blotted antibody signals were detected with the enhanced chemiluminescence (ECL) detection system (Amersham ECL Western Blotting Detection Reagents; GE Healthcare, Uppsala, Sweden) and were visualized by the G:BOX Image Analysis System (Syngene).

Evaluation of skin wrinkle formation

Replicas of hairless mice dorsal skin were prepared using a Silflo kit (CuDerm Corporation, Dallas, TX, USA) and analyzed with Visioline VL650 (CK Electronics GmbH, Cologne, Germany). The skin wrinkle parameters were represented as wrinkle number, depth, length, and the total area of the wrinkles.

Evaluation of skinfold thickness

Skinfold thickness of the dorsal skin in hairless mice was measured using a caliper (Ozaki MFG Co., Ltd., Tokyo, Japan) at 24 weeks. The dorsal skin of hairless mice was manually lifted by pinching at the neck and base of the tail, and skinfold thickness was measured at the mid-back. At least three measurements were taken at close proximity and the mean value was calculated.

Hydroxyproline assay

The hydroxyproline content was determined using a hydroxyproline content assay kit (QuickZyme Biosciences, Leiden, Netherlands) according to the manufacturer's protocol.

Histological analysis

The skin tissues were fixed in 10% formalin for 24 h. Hematoxylin and eosin (H&E) stain for observing skin thickness, Masson's trichrome (M&T) stain for assessing collagen fiber, and Verhoeff-van Gieson's stain for

determining elasticity, were conducted in dorsal skin tissue. The stained skin sections were visualized under an inverted microscope (Nikon Eclipse TE2000U) with twin CCD cameras (Nikon, Tokyo, Japan).

Evaluation of skin hydration and transepidermal water loss (TEWL)

The level of skin hydration was determined using a Corneometer[®] CM 825. The TEWL, a marker of water barrier function of epidermal skin, was observed by Tewameter[®] TM 300, which was mounted on a Multi Probe Adapter[®] MPA5 (CK Electronics GmbH).

Statistical analysis

Data were reported as the mean $\pm$ standard deviation (SD) of triplicate independent experiments. All experimental groups were compared by one-way analysis of variance (ANOVA), followed by Scheffe's test and unpaired *t* test, using IBM SPSS Statistics 21 software (SPSS, Chicago, IL, USA). ^{###}*p* < 0.01, [#]*p* < 0.05, ^{**}*p* < 0.01, and ^{*}*p* < 0.05 were considered to be statistically significant.

Results and discussion

Vitamin C reduces SA- β -gal expression and recovers cell-cycle arrest in vitro and in vivo

Overexpression and accumulation of endogenous lysosomal SA- β -gal, is a typical characteristic of senescence [14]. The activity of SA- β -gal increased in the H₂O₂ control but the activity was dose-dependently decreased by vitamin C treatment in H₂O₂-induced Hs68 cells (Fig. 1A). In addition, the MA control mice showed increased SA- β -gal activity whereas this activity was significantly diminished in vitamin C-treated MA mice (Fig. 1B). The data show that vitamin C effectively attenuates senescence in vitro and in vivo. Increased SA- β -gal activity is the most common hallmark of cell senescence and its activity is directly associated with cell cycle arrest [14]. One of the characteristics of senescence is a change in gene expression, including cell-cycle inhibitors (p53, p21, p16, and pRb) and activators (E2F1 and E2F2) [15]. Vitamin C diminished the mRNA and protein expression of cell-cycle inhibitors, including p53, p21, p16, and pRb in the H₂O₂ control of the Hs68 cells (Fig. 1C, D) and MA mice group (Figs. 1E, 2F). However, the cell cycle activators including E2F1 and E2F2, were increased by vitamin C treatment in

H₂O₂-induced Hs68 cells (Fig. 1C) and MA mice (Fig. 1D). These data indicate that vitamin C prevents cell cycle arrest through regulating the cell cycle inhibitors and activators in H₂O₂-induced Hs68 cells and MA mice. Therefore, vitamin C protects against intrinsic aging through inhibiting cell cycle arrest.

Vitamin C inhibits the PI3K/AKT signaling pathway and increases SIRT1 expression in vitro and in vivo

Oxidative stress-induced cellular senescence stimulates PI3K activity, which triggers AKT hyperactivation [5]. PI3K and AKT downstream markers, such as mTOR and FoxO3a, are known as the regulators of the cell cycle and mediators of lifespan extension [6]. The protein levels of PI3K and p-AKT increased in H₂O₂ control and MA mice group but were effectively decreased by vitamin C treatment without changes in the t-AKT level. p-mTOR protein expression was increased in the H₂O₂ control of Hs68 cells and MA mice group. However, vitamin C treatment significantly reduced p-mTOR expression, without changes in the t-mTOR level. In addition, vitamin C treatment increased FoxO3a protein expression but decreased p-FoxO3a expression compared to the H₂O₂ control of the Hs68 cells and MA mice group (Fig. 2A, B). These data show that vitamin C attenuates cellular senescence by regulating the PI3K/AKT signaling pathway.

Calorie restriction (CR) is also related to the deceleration of aging, depending on SIRT1 expression, which is also associated with cellular senescence regulation. SIRT1 is associated with lifespan and acts as an anti-aging factor in cellular senescence [16]. SIRT1 protein expression was decreased in the H₂O₂ control of Hs68 cells and the MA mice group. In contrast, vitamin C treatment significantly increased SIRT1 protein expression compared to the H₂O₂ control of Hs68 cells and MA mice group (Fig. 2C, D), suggesting vitamin C confers resistance against aging. Research attempts have been made to postpone aging by changing senescence-associated signaling pathways, using pharmacological and natural agents [17]. CR has received particular attention because it can increase the lifespan of model organisms [18]. Accordingly, the search for natural CR mimetics has become a promising area in anti-aging studies. Resveratrol is known as an effective CR mimetic. The senescence-reversing activity of resveratrol is mainly SIRT1-dependent exhibiting beneficial effects against various age-related diseases such as cardiovascular diseases, diabetes, obesity, and cancer [19]. In the same context, vitamin C considerably elevated the protein level of SIRT1 in Hs68 cells and hairless mice. Thus, vitamin C

Fig. 1 The effect of vitamin C on SA-β-gal activity and cell cycle arrest. **(A)** SA-β-gal activity was detected by a fluorescence reader in vitro and **(B)** in vivo. **(C)** The mRNA expression of cell cycle inhibitors (p53, p21, p16, and pRb) and cell cycle activators (E2F1 and E2F2) was investigated by RT-PCR in vitro and **(D)** in vivo. **(E)** The protein level of the cell cycle inhibitors was investigated by

Western blotting in vitro and **(F)** in vivo. Data are represented as the mean ± SD from triplicate independent experiments. ^{##}*p* < 0.01 (normal versus H₂O₂ control); ^{**}*p* < 0.01 (H₂O₂ control versus sample-treated cells). Data are represented as the mean ± SD of five mice per group. ^{##}*p* < 0.01 (young mice versus MA mice); ^{**}*p* < 0.01 (MA mice versus sample-treated mice)

Fig. 2 The effect of vitamin C on the PI3K/AKT signaling pathway and longevity activity. **(A)** The protein expression levels of the PI3K/AKT signaling pathway-associated markers were evaluated by

Western blotting in vitro and **(B)** in vivo. **(C)** The protein expression level of SIRT1 was also investigated by Western blotting in vitro and **(D)** in vivo

inhibits intrinsic aging and increases lifespan via regulating the PI3K/AKT pathway in vitro and in vivo.

Vitamin C reduces inflammatory responses in vitro and in vivo

Senescent cells secrete various pro-inflammatory factors and thus, the condition has been called the SASP, which affects neighboring cells. SASP is a distinct indication of senescence, observed both in vitro and in vivo [8]. When cells were exposed to H₂O₂, IL-6 and IL-8 were overexpressed. However, vitamin C treatment dose-dependently inhibited IL-6 and IL-8 mRNA expression, compared to the H₂O₂ control of Hs68 cells (Fig. 3A). The protein level of NF-κB, an SASP inducer, was also gradually decreased by vitamin C treatment compared to the H₂O₂ control of Hs68 cells and MA mice group (Fig. 3B, C). These data imply that vitamin C serves as an effective anti-aging agent through its anti-inflammatory activities. Therefore, vitamin

C inhibits the maintenance and initiation of cellular senescence by down-regulation of SASP expression.

Vitamin C improves wrinkle formation and skin thickness in vivo

Aged skin is characterized by thin, smooth and soft wrinkles and shows decreased skin thickness [20]. To investigate the inhibitory effect of vitamin C on wrinkle formation, wrinkle parameters were analyzed using photography and skin replica. Various wrinkle parameters, including wrinkle number, length, and depth, and total wrinkle area, were analyzed using photography and skin replica (Fig. 4A). All wrinkle parameters were significantly attenuated by administration of vitamin C compared to the MA mice group (Fig. 4B). Extrinsic aging induced by UVR, exhibits skin hypertrophy, whereas, intrinsic aging induces skin atrophy. The MA mice group showed decreased skin thickness, as represented by the thinner-

Fig. 3 The anti-inflammatory effects of vitamin C. **(A)** The mRNA expression of IL-6 and IL-8 were detected by RT-PCR in vitro. **(B)** The level of NF- κ B protein was evaluated by Western blotting in vitro and **(C)** in vivo

stained skin area while vitamin C administration (50 or 200 mg/kg/day) increased skin thickness (Fig. 4C, D). These data indicate that the skin thickness is significantly increased by vitamin C treatment. Therefore, vitamin C improves morphologic and structural changes, such as wrinkle formation and skin thickness.

Vitamin C inhibits collagen degradation and elasticity reduction in vivo

Collagen is the predominant extracellular matrix component in the skin. Compared to non-aged skin, aged skin shows loss collagen in the extracellular matrix, resulting in dermal atrophy [21]. The MA mice group showed degradation of collagen fibers while vitamin C effectively prevented collagen degradation (Fig. 5A). Hydroxyproline content also supported the inhibitory effect of vitamin C on collagen degradation (Fig. 5B), suggesting vitamin C inhibits collagen degradation and promotes collagen synthesis. Aged skin dermis shows a destruction in the elastic fiber network, resulting in decreased skin elasticity [22]. In order to evaluate the effect of vitamin C on elastic fibers in aged skin, we used a Verhoeff–van Gieson's staining which is a method to evaluate the elastic fibers in skin [12, 13, 23]. As compared to the MA mice group, vitamin C administration (50 or 200 mg/kg/day) increased elastic fibers (Fig. 5C), which imply that vitamin C enhances synthesis of elastic related fibers in the skin. In human skin dermis, intrinsic aging is represented by features, including

the destruction of the elastic fiber network and loss of skin atrophy of the skin dermis due to the loss of collagen [24]. Thus, it is conceivable that the reduction in the biosynthetic capacity of fibroblasts in the aged state can be rejuvenated by vitamin C.

Vitamin C improves skin barrier function in vivo

Skin acts as a barrier, protecting against dehydration. Skin hydration and TEWL represent water-holding properties [21]. These are indicators of skin barrier function. Skin hydration level was increased in the vitamin C administered groups, compared to the MA mice group (Fig. 6A). Although the TEWL level of the MA mice group was increased, vitamin C treatment effectively attenuated the TEWL level in the skin of hairless mice (Fig. 6B). Accordingly, vitamin C has an improvement effect on skin barrier function by increasing skin hydration and decreasing TEWL.

In summary, vitamin C does not only inhibit cellular senescence associated with the PI3K/AKT and the p53/p16-pRb pathways, but also up-regulates the expression of other longevity factors such as mTOR and FoxO3a. Furthermore, vitamin C inhibits morphologic and structural changes, such as wrinkle formation, loss of collagen, and elastic fibers. Overall, vitamin C can serve as an effective CR mimetic and has the potential to be an effective antagonist to natural aging in skin.

Fig. 4 The effect of vitamin C on wrinkle formation and skinfold thickness. (A) Before sacrifice, replica samples of dorsal skin surface were taken. (B) Wrinkle values were obtained from the skin replica analysis. (C) Skin sections were stained with H&E. (D) Skinfold thickness was measured midway between the neck and hips, before

sacrifice, with a caliper. Data are represented as the mean $\pm$ SD of five mice per group. # $p < 0.05$ or ## $p < 0.01$ (young mice versus MA mice); * $p < 0.05$ or ** $p < 0.01$ (MA mice versus sample-treated mice)

Fig. 5 The effect of vitamin C on collagen content. **(A)** Skin sections were stained with Masson's trichrome stain for collagen fibers. **(B)** Hydroxyproline content was assessed at 24 weeks. **(C)** Skin tissue sections were stained with Verhoeff-van Gieson's stain. Data are

represented as the mean $\pm$ SD of five mice per group. [#] $p < 0.05$ (young mice versus MA mice); ^{*} $p < 0.05$ (MA mice versus sample-treated mice)

Fig. 6 The effect of vitamin C on skin barrier function. **(A)** Skin hydration and **(B)** TEWL, as markers of skin barrier function, were measured using Corneometer[®] and Tewameter[®], respectively, 1 day

before the animals were sacrificed. Data are represented as the mean $\pm$ SD of five mice per group. ^{##} $p < 0.01$ (young mice versus MA mice); ^{**} $p < 0.01$ (MA mice versus sample-treated mice)

Acknowledgements This work was supported by a Grant from the Kwang Dong Pharmaceutical Co., Ltd. (2013-8-1178).

Compliance with ethical standards

Conflict of interest The authors declares that they have no conflict of interest.

References

- Naylor EC, Watson RE, Sherratt MJ. Molecular aspects of skin ageing. *Maturitas* 69: 249–256 (2011)
- Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. *Genes Dev.* 24: 2463–2479 (2010)
- Gambino V, De Michele G, Venezia O, Migliaccio P, Dall'Olio V, Bernard L, Minardi SP, Fazio MAD, Bartoli D, Servillo G. Oxidative stress activates a specific p53 transcriptional response that regulates cellular senescence and aging. *Aging Cell* 12: 435–445 (2013)
- Campisi J, d'Adda di Fagnana F. Cellular senescence: when bad things happen to good cells. *Nat. Rev. Mol. Cell Biol.* 8: 729–740 (2007)
- Nogueira V, Park Y, Chen CC, Xu PZ, Chen ML, Tonic I, Unterman T, Hay N. Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. *Cancer Cell* 14: 458–470 (2008)
- Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *EMBO J.* 23: 212–220 (2004)
- Kim HK, Kim YK, Song IH, Baek SH, Lee SR, Kim JH, Kim JR. Down-regulation of a forkhead transcription factor, FOXO3a, accelerates cellular senescence in human dermal fibroblasts. *J. Gerontol. A Biol. Sci. Med. Sci.* 60: 4–9 (2005)
- Campisi J. Cellular senescence: putting the paradoxes in perspective. *Curr. Opin. Genet. Dev.* 21: 107–112 (2011)
- Ren JL, Pan JS, Lu YP, Sun P, Han J. Inflammatory signaling and cellular senescence. *Cell. Signal.* 21: 378–383 (2009)
- Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, Chen S, Corpe C, Dutta A, Dutta SK. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* 22: 18–35 (2003)
- Farris PK. Topical vitamin C: a useful agent for treating photoaging and other dermatologic conditions. *Dermatol. Surg.* 31: 814–818 (2005)
- Kim J, Kim MB, Yun JG, Hwang JK. Protective effects of standardized *Siegesbeckia glabrescens* extract and its active compound kirenol against UVB-induced photoaging through inhibition of MAPK/NF- κ B pathways. *J. Microbiol. Biotechnol.* 27: 242–250 (2016)
- Park JE, Woo SW, Kim MB, Kim C, Hwang JK. Standardized *Kaempferia parviflora* extract inhibits intrinsic aging process in human dermal fibroblasts and hairless mice by inhibiting cellular senescence and mitochondrial dysfunction. *Evid. Based Complement. Altern. Med.* 2017: 6861085 (2017)
- Sikora E, Arendt T, Bennett M, Narita M. Impact of cellular senescence signature on ageing research. *Ageing Res. Rev.* 10: 146–152 (2011)
- Polager S, Ginsberg D. p53 and E2f: partners in life and death. *Nat. Rev. Cancer* 9: 738–748 (2009)
- Blagosklonny MV. Calorie restriction: decelerating mTOR-driven aging from cells to organisms (including humans). *Cell Cycle* 9: 683–688 (2010)
- López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* 153: 1194–1217 (2013)
- Brooks CL, Gu W. How does SIRT1 affect metabolism, senescence and cancer? *Nat. Rev. Cancer* 9: 123–128 (2009)
- Chung JH, Manganiello V, Dyck JR. Resveratrol as a calorie restriction mimetic: therapeutic implications. *Trends Cell Biol.* 22: 546–554 (2012)
- El-Domyati M, Attia S, Saleh F, Brown D, Birk D, Gasparro F, Ahmad H, Uitto J. Intrinsic aging versus photoaging: a comparative histopathological, immunohistochemical, and ultrastructural study of skin. *Exp. Dermatol.* 11: 398–405 (2002)
- Tagami H. Functional characteristics of the stratum corneum in photoaged skin in comparison with those found in intrinsic aging. *Arch. Dermatol. Res.* 300: 1–6 (2008)
- Farage MA, Miller KW, Elsner P, Maibach HI. Characteristics of the aging skin. *Adv. Wound Care* 2: 5–10 (2013)
- Kazlouskaya V, Malhotra S, Lambe J, Idriss MH, Elston D, Andres C. The utility of elastic Verhoeff-Van Gieson staining in dermatopathology. *J. Cutan. Pathol.* 40: 211–225 (2013)
- Uitto J. The role of elastin and collagen in cutaneous aging: intrinsic aging versus photoexposure. *J. Drugs Dermatol.* 7: s12–6 (2008)