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Collagen peptide and vitamin C additively attenuate age-related skin atrophy in *Sod1*-deficient mice

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Age-related skin thinning is correlated with a decrease in the content of collagen in the skin. Accumulating evidence suggests that collagen peptide (CP) and vitamin C (VC) transcriptionally upregulate type I collagen *in vivo*. However, the additive effects of CP and VC on age-related skin changes remain unclear. We herein demonstrate that CP and a VC derivative additively corrected age-related skin thinning via reduced oxidative damage in superoxide dismutase 1 (*Sod1*)-deficient mice. Co-treatment with these compounds significantly normalized the altered gene expression of *Colla1*, *Has2*, and *Cil*, a proton-coupled oligopeptide transporter, in *Sod1*^{−/−} skin. The *in vitro* analyses further revealed that collagen oligopeptide, a digestive product of ingested CP, significantly promoted the bioactivity of the VC derivative with respect to the migration and proliferation of *Sod1*^{−/−} fibroblasts. These findings suggest that combined treatment with CP and VC is effective in cases of age-related skin pathology.

Key words: collagen peptide; vitamin C; skin atrophy; superoxide dismutase; proton-coupled oligopeptide transporter

The skin is the largest organ in the body and it plays an essential role in the barrier function in protecting the body from environmental factors such as infection and UV radiation. Since the skin is abundantly composed of collagen fibers, collagen matrix homeostasis plays a central role in maintaining the skin function. In human studies, skin collagen components show age-dependent reductions in both male and female subjects, resulting in age-related skin thinning in older individuals.¹⁾ Therefore, preventing age-related collagen declines helps to maintain skin integrity.

When heated, collagen is denatured and becomes water-soluble gelatin. Collagen peptide (CP) prepared

via the enzymatic hydrolysis of gelatin is widely used as a food ingredient. Accumulating evidence indicates that the dietary supplementation of CP promotes extracellular matrix biosynthesis in the joints, nails, and hair.^{2,3)} Proksch et al. reported that CP intake increases skin elasticity in elderly females.⁴⁾ Oral treatment with CP and/or gelatin also increases the type I collagen expression in the bones and skin.^{5–7)} Notably, Sato and colleagues identified that CP ingestion increases collagen oligopeptides (COPs) composed of two or three amino acids, including Ala-Hyp-Gly, Pro-Hyp, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp, and Phe-Hyp, in human blood,^{8,9)} suggesting that ingested CP is at least, in part, digested and absorbed as COP. Furthermore, Shigemura et al. reported that COPs, such as Pro-Hyp and Hyp-Gly, exhibit bioactivity for skin fibroblasts.^{10,11)} In addition, vitamin C (VC) is a strong antioxidant that regulates the synthesis of collagen, as well as the post-translational modification of collagen by lysyl oxidase as a co-factor.^{12,13)} In fact, VC application upregulates the mRNA levels of collagen types I and III in human skin fibroblasts.^{14,15)} However, it is unclear whether this activity is augmented by simultaneous treatment, thereby modulating the skin function *in vivo*.

Using an antioxidant gene-targeting strategy to induce redox imbalance *in vivo*, we previously demonstrated that *Sod1* deficiency causes skin thinning associated with collagen dysregulation.^{16–18)} Recently, we further established hairless *Sod1*^{−/−} double mutant mice in order to avoid variations in skin morphology during the hair cycle (unpublished results). Therefore, double mutant mice constitute a valuable mouse model mimicking age-related skin thinning. In this context, we herein evaluated the hypothesis that treatment with both CP and VC delays skin aging by attenuating oxidative damage. In the present study, we investigated the additive effects of CP and VC on hairless *Sod1*-deficient skin phenotypes.

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Abbreviations: COP, collagen oligopeptide; CP, collagen peptide; Hyp, hydroxyproline; MDA, malondialdehyde; POT, proton-coupled oligopeptide transporter; ROS, reactive oxygen species; VC, vitamin C.

Materials and methods

Materials. CP (fish-derived, MW 3000–5000, Nippi, Tokyo, Japan) was used. Two COPs, Hyp-Gly and Pro-Hyp, were purchased from Bachem (Bubendorf, Switzerland). Another six COPs, Ala-Hyp, Ser-Hyp, Leu-Hyp, Ala-Hyp-Gly, Phe-Hyp, and Pro-Hyp-Gly, were purchased from AnyGen (Gwangju, Korea). COP mixture was mixed with the above eight types of COP and used at a final concentration of 50 μ M of Hyp-Gly, 5 μ M of Ala-Hyp, 5 μ M of Ser-Hyp, 100 μ M of Pro-Hyp, 5 μ M of Leu-Hyp, 5 μ M of Ala-Hyp-Gly, 5 μ M of Phe-Hyp, and 5 μ M of Pro-Hyp-Gly. Casein peptide (Emulup, molecular weight 4600, lipid: 1.0%, protein: 89.0%, water: 4.0%, ash: 4.5%) was purchased from Morinaga Milk Industry Co, LTD. (Tokyo, Japan). The VC derivative (L-ascorbyl 2-phosphate 6-palmitate trisodium salt) was provided by Showa Denko K.K. (Tokyo, Japan). The VC derivative was prepared by additionally conjugating VC with a phosphate group and a long hydrophobic chain to enhance stability and liposolubility.¹⁹⁾

Animals. *Sod1*^{−/−} mice and Hos:HR-1^{hr/hr} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and Hoshino Laboratory Animals, Inc. (Ibaraki, Japan), respectively. The *Sod1*^{−/−} mice were backcrossed with C57BL/6NcrSlc mice (Japan SLC, Shizuoka, Japan) for five or six generations. Hairless *Sod1*^{−/−} double mutant mice were generated by crossbreeding Hos:HR-1^{hr/hr} with *Sod1*^{−/−} mice. Genotyping of the *Sod1*^{−/−} allele was performed using genomic PCR with genomic DNA isolated from the tail tip, as previously reported.²⁰⁾ The animals were housed under a 12-h light/dark cycle and fed *ad libitum*. The mice were maintained and studied according to protocols approved by the Animal Care Committee of the Tokyo Metropolitan Institute of Gerontology and Chiba University.

Administration of CP and the VC derivative. CP and the VC derivative were co-administered to hairless *Sod1*^{−/−} and *Sod1*^{+/+} mice of both sexes at 17–20 weeks of age for eight weeks. Collagen or casein peptides were dissolved in MilliQ water at 2% (w/w) and orally administered via gavage (0.2 g/kg/day). Following the administration of CP or casein peptides, 0.1% (w/w) solution of the VC derivative (200 μ L/mouse) or MilliQ water (200 μ L/mouse) was applied onto the back skin once a day at 1:00 pm each weekday. Casein peptide and MilliQ water were used as placebo controls for CP and the VC derivative, respectively. We previously demonstrated that a low dose (0.1%) of the VC derivative, as well as 1.0 or 10% solution, significantly rescued skin atrophy in *Sod1*^{−/−} mice against a C57BL/6 background.¹⁶⁾ The preliminary experiments, however, revealed that the administration of 0.1% of the VC derivative showed limited effects on skin thickness in the hairless *Sod1*^{−/−} mice (data not shown). The limited effects of the VC derivative on skin thinning may be due to the differences in histological changes in the skin of the hairless *Sod1*^{−/−} mice compared to that observed in the C57BL/6 *Sod1*^{−/−} mice. In fact, we

found that hairless *Sod1*^{−/−} mice, but not C57BL/6 *Sod1*^{−/−} mice, exhibit marked formation of dermal cysts (unpublished results). In this context, we herein applied 0.1% of the VC derivative in order to clarify the additive effects of treatment with CP and the VC derivative on age-related skin atrophy.

Histology. For the histological observation, skin specimens obtained from the backs of the mice were dissected, fixed in a 20% formalin neutral buffer solution (Wako, Osaka, Japan) overnight, embedded in paraffin and sectioned on a microtome at a thickness of 4 μ m using standard techniques. Hematoxylin and eosin staining was performed as previously described.^{21,22)} The thickness of the skin tissue (epidermis and dermis) was measured using the Leica QWin V3 image software program (Leica, Germany).

Oxidative stress markers. The back skin tissues of male mice were homogenized with radioimmunoprecipitation assay buffer [150 mM NaCl, 10 mM MTris–HCl, pH7.4, 0.1% SDS, 1% triton X-100, 5 mM EDTA, 1% deoxycholic acid] containing protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan). The homogenate was centrifuged at 1600 $\times$ g for 10 min at 4 °C, and the total supernatant was used for the assay. The malondialdehyde (MDA) level was measured using the TBARS Assay Kit (Cayman Chemical Company, MI, USA) according to the manufacturer's instructions. In order to measure the 8-isoprostene content, the back skin tissues were homogenized with 0.1 M phosphate buffer, pH 7.4, containing 1 mM of EDTA (Dojindo Laboratories, Kumamoto, Japan) and 50 μ g/mL (w/w) of dibutylhydroxytoluene (Wako). The homogenate was centrifuged at 8000 $\times$ g for 10 min at 4 °C, and the total supernatant was used for the assay. The 8-isoprostane level was measured using the 8-isoprostane EIA Kit (Cayman Chemical Company) according to the manufacturer's instructions. The supernatant was also assayed for the protein concentration using the DC Protein Assay Kit (BioRad, Hercules, CA, USA), and the MDA and 8-isoprostane levels were normalized to the protein level.

Quantitative PCR. Total RNA was extracted from back skin using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μ g of total RNA using reverse transcriptase (ReverTra Ace qPCR RT Master MIX, TOYOBO, Osaka, Japan). Real-time PCR was performed on a Mini OpticonTM (Bio-Rad) with the SYBR GREEN PCR Master Mix (Bio-Rad) according to the manufacturer's instructions. All data were normalized to the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The *Gapdh* data with large variation ($\pm$ 5-fold change to mean value) were omitted as low RNA quality samples. The following primer sets were used: *Gapdh* forward, 5'-ATGTGTCGTCGTGGATCTGA-3' and reverse, 5'-TGCCTGCTTACCACCTTCT-3'; *Coll1a1* forward, 5'-CATGTTTCAGCTTTGTGGACCT-3'

and reverse, 5'-GCAGCTGACTTCAGGGATGT-3'; *Mmp2* forward, 5'-TAACCTGGATGCCGTCGT-3' and reverse, 5'-TTCAGGTAATAAGCACCCCTTGAA-3'; *Has2* forward, 5'-CGGTCTCTCAAATTCATCTG-3' and reverse, 5'-ACAATGCATCTTGTTCAGCTC-3'; *Ki67* forward, 5'-GCTGTCTCAAGACAATCATCA-3' and reverse, 5'-GGCGTTATCCCAGGAGACT-3'; *Pept1* forward, 5'-TCACAGACCACGACCACAAT-3' and reverse, 5'-CCCCGATGATAGCCAAATAA-3'; *Pept2* forward, 5'-ATCCTGCAGTGCATTGTGAA-3' and reverse, 5'-CTGCTGCTGTAACCAGGACA-3'; *Phl1* forward, 5'-CCGTGTTCTTGGCTCTGATT-3' and reverse, 5'-CCACCAGCTTGTCCTTCAGT-3'; *Ci1* forward, 5'-GTTGCGGTGATCCTGATTCT-3' and reverse, 5'-AGCTGAGGCACTGTCTGGTT-3'.

Cell culture. The skin specimens of *Sod1*^{-/-} neonates obtained at 4–6 days after birth were sterilized with 70% ethanol and dissected into small pieces. The primary dermal fibroblasts were isolated via dissociation in 0.2% collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37 °C for 60 min. The cells were maintained in α -MEM (Life Technologies Corporation) supplemented with 20% FBS (Life Technologies Corporation), 100 units/ml of penicillin (Sigma-Aldrich, MO, USA), and 0.1 mg/mL of streptomycin (Sigma-Aldrich) at 37 °C in a humidified incubator with 5% CO₂ and 1% O₂ to prevent damage by O₂ and to maintain viability of *Sod1*^{-/-} fibroblasts. During the subsequent experiments, the cells were cultured to investigate cellular phenotypes under a general condition (20% O₂).

Outgrowth assay. The back skin was sterilized with 70% ethanol, rinsed with PBS (Takara Bio Inc., Shiga, Japan) and punched out into disks measuring 5 mm in diameter using Dermalpunch (Nipro, Tokyo, Japan). The punched skin disks were placed into a 24-well culture plate (Falcon BD, Franklin Lakes, NJ) and cultured with or without COP or the VC derivative in α -MEM containing 20% FBS, 100 units/ml of penicillin, and 0.1 mg/mL of streptomycin at 37 °C in a humidified incubator with 5% CO₂ and 20% O₂. The number of outgrowth fibroblasts originating from the mouse skin disks was directly counted at 96 h after culture.

In vitro scratch wound assay. Fibroblasts were preincubated with or without COP or the VC derivative in α -MEM containing 20% FBS, 100 units/mL of penicillin and 0.1 mg/mL of streptomycin at 37 °C in a humidified incubator for 24 h with 5% CO₂ and 1% O₂ to prevent damage by O₂. The fibroblast monolayer was artificially injured by scratching across the surface of the plate with a pipette tip. After washing to remove detached cells and debris, the wells were further cultured in medium with or without COP or the VC derivative at 37 °C in a humidified incubator with 5% CO₂ and 20% O₂. Following incubation, the fibroblasts were incubated with a calcein-AM fluorescent probe (1 μ g/mL, Dojindo) for 30 min. The degree of migration of the fibroblasts was determined relative to the average gap at 12 h after

the creation of the scratch wounds using fluorescent microscopy.

Statistics. The statistical analyses were performed using Student's *t*-test for comparisons between two groups and Tukey's test for comparisons between three groups. Differences in the data were considered to be significant for *p* values less than 0.05. All data are expressed as the mean $\pm$ SD.

Results

CP and the VC derivative additively increased the skin thickness of the hairless Sod1^{-/-} mice

In the present study, in order to elucidate the beneficial effects of CP and VC on skin thinning, we performed co-treatment with oral CP and the transdermal VC derivative in the hairless *Sod1*^{-/-} mice for eight weeks. As shown in Fig. 1, the co-treatment significantly increased the epidermal and dermal thickness in the *Sod1*^{-/-} mice compared to that observed in the hairless *Sod1*^{-/-} mice administered the casein peptide only. On the other hand, treatment with the casein peptide plus the VC derivative, the casein peptide alone or CP alone failed to change the skin thickness of the hairless *Sod1*^{-/-} mice (Fig. 1(A)–(C)). In order to exclude the adverse effects of the administration of CP and the VC derivative on the skin tissues, we next investigated histological changes in the epidermis and dermis in wild-type mice (Fig. 1(D) and (E)). The co-administration of these compounds showed no effects on the skin thickness of the hairless wild-type mice (Fig. 1(D) and (E)). Similarly, the other three treatments also showed no adverse effects on skin morphology in the hairless wild-type mice (Fig. 1(D) and (E)). These findings demonstrate that combination treatment with CP and the VC derivative significantly improved skin atrophy in the hairless *Sod1*^{-/-} mice.

CP and the VC derivative significantly decreased oxidative damage in the skin of the hairless Sod1^{-/-} mice

We previously demonstrated that *Sod1* loss induces oxidative damage in several organs, including the skin and bones.^{17,23} In order to evaluate oxidative damage in the skin, we measured the lipid peroxidation levels in the skin tissues (Fig. 2). Regarding the MDA content, co-treatment with the above compounds significantly reduced the MDA levels in the skin tissues of the mutant mice (Fig. 2(A)). In contrast, the other three treatments, including the administration of casein peptide only, CP only and casein peptide plus the VC derivative, did not decrease the MDA levels in the skin tissues (Fig. 2(A)). Regarding the 8-isoprostane levels, co-treatment with CP and the VC derivative also significantly decreased the 8-isoprostane content in the skin extracts (Fig. 2(B)). Interestingly, treatment with CP only and casein peptide plus the VC derivative, but not casein peptide only, significantly downregulated the 8-isoprostane levels in the skin lysates (Fig. 2(B)). These data indicate that the combined treatment mitigated oxidative damage in the skin of the hairless *Sod1*^{-/-} mice.

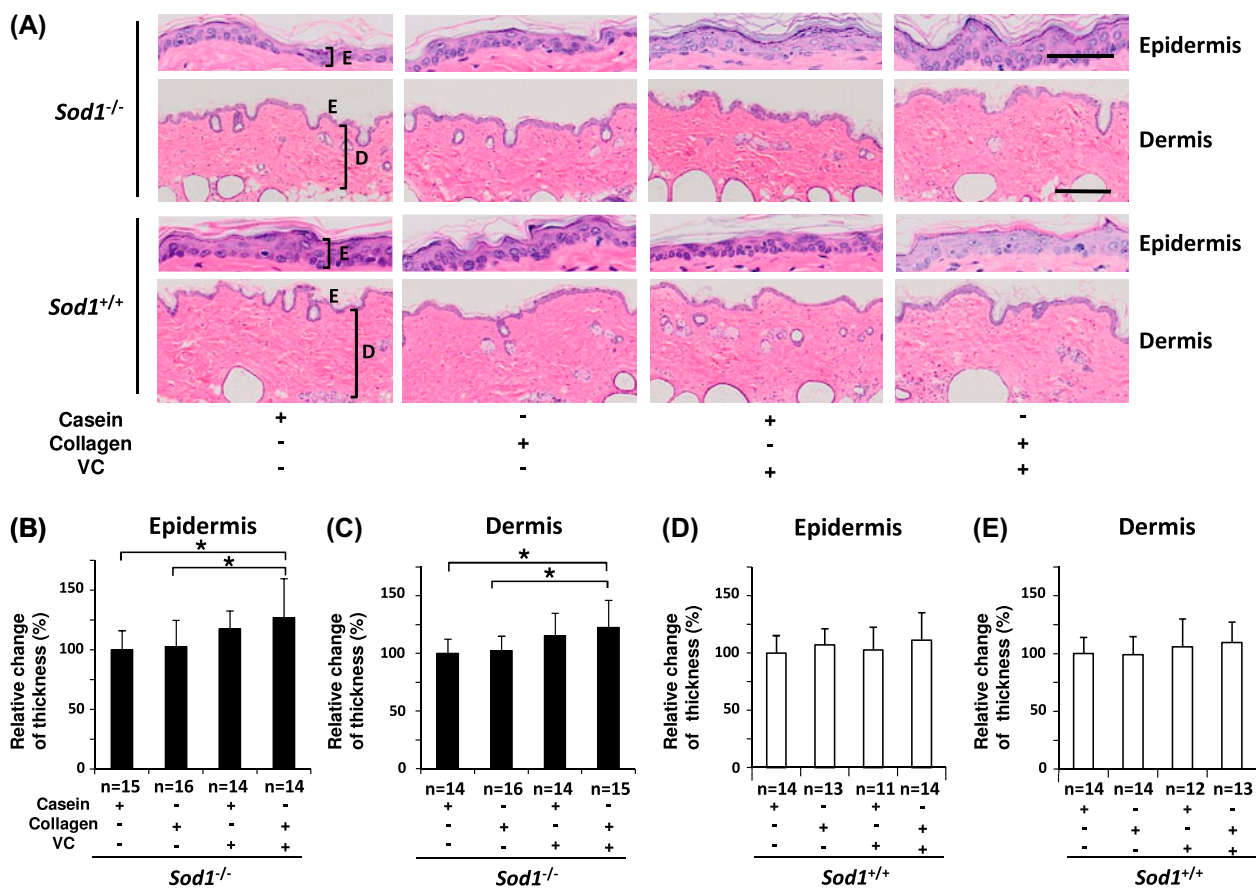


Fig. 1. Co-treatment with CP and the VC derivative additionally thickened the epidermal and dermal layers in the hairless *Sod1^{-/-}* mice.

Notes: (A) Hematoxylin and eosin staining of the back skin of hairless *Sod1^{-/-}* and *Sod1^{+/+}* mice treated with the casein peptide (casein), CP (collagen), casein peptide plus the VC derivative (VC) and CP plus the VC derivative. Casein peptide or CP was orally administered, while the VC derivative was transdermally applied on the skin once a day for eight weeks. E, epidermis; D, dermis. The scale bar represents 50 μ m (top) and 200 μ m (bottom). The thickness of the epidermis (B) and dermis (C) of the back skin of the hairless *Sod1^{-/-}* mice. The thickness of the epidermis (D) and dermis (E) of the back skin of the hairless *Sod1^{+/+}* mice. The data indicate the mean $\pm$ SD; * p < 0.05.

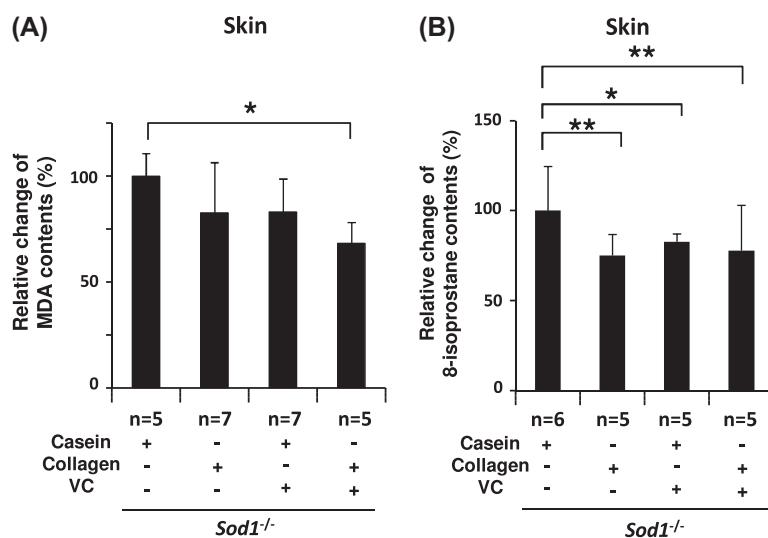


Fig. 2. Treatment with CP and the VC derivative significantly decreased lipid peroxidation in the hairless *Sod1^{-/-}* skin.

Notes: The (A) MDA and (B) 8-isoprostane content in skin lysates obtained from hairless *Sod1^{-/-}* male mice treated with the casein peptide only, CP only, casein peptide plus the VC derivative and CP plus the VC derivative. The data indicate the mean $\pm$ SD; * p < 0.05, ** p < 0.01.

CP and the VC derivative partially normalized the transcriptional profiles of skin-related genes in the hairless *Sod1*^{-/-} skin

In order to investigate the molecular mechanisms underlying the rescue effects of co-treatment with CP plus the VC derivative on skin thinning in hairless *Sod1*^{-/-} mice, we analyzed the expression patterns of extracellular matrix-related genes in the skin. In the hairless *Sod1*^{-/-} mice, the skin mRNA levels of the type I collagen gene (*Col1a1*) were significantly down-regulated, while those of the matrix metalloproteinase 2 gene (*Mmp2*) were significantly upregulated compared to that observed in the hairless *Sod1*^{+/+} mice, indicating reduced collagen biosynthesis (Fig. 3(A) and (B)). Moreover, the mRNA levels of the hyaluronan synthase 2 gene (*Has2*) were also significantly downregulated in

the mutant skin (Fig. 3(C)). In contrast, the mRNA levels of *Ki67*, a marker of the proliferation of skin cells, showed no alterations in their expression between the hairless *Sod1*^{+/+} and *Sod1*^{-/-} mice (Fig. 3(D)). These results suggest that *Sod1* deficiency causes skin thinning due to dysregulation of the extracellular matrix. Among the altered genes, the combined treatment significantly corrected the mRNA levels of *Col1a1* and *Has2*, but not *Mmp2*, (Fig. 3(A)–(C)), suggesting that the co-treatment increased components of the extracellular matrix, such as collagen and hyaluronic acid, thereby normalizing the skin thickness.

Proton-coupled oligopeptide transporter (POT) regulates the transport of oligopeptides into cells.²⁴ Since ingested CP is digested and absorbed partly as an oligopeptide, we next analyzed the expression profiles of

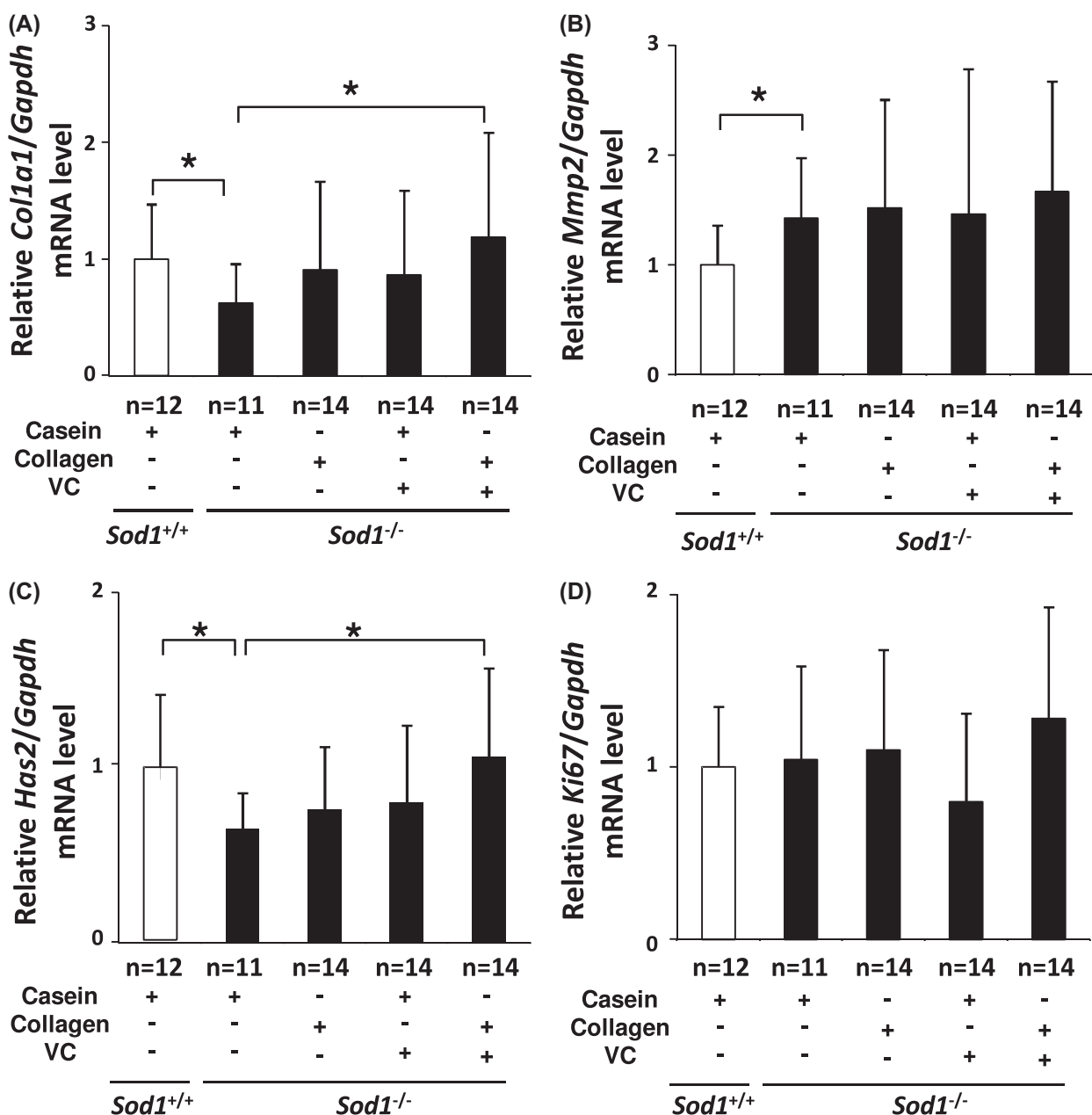


Fig. 3. Treatment with CP and the VC Derivative partially corrected the transcriptional profiles of skin-related genes in the skin tissues of the hairless *Sod1*^{-/-} mice.

Notes: The relative mRNA expression levels of (A) *Col1a1*, (B) *Mmp2*, (C) *Has2* and (D) *Ki67* were determined using qRT-PCR. The data indicate the mean $\pm$ SD; **p* < 0.05.

POT genes, including *Pept1*, *Pept2*, *Pht1*, and *Cil* (PHT2 in humans), in the skin tissues. As shown in Fig. 4, the mRNA levels of *Pept1*, *Pept2*, and *Pht1* were constitutively expressed in the skin in both the hairless *Sod1*^{+/+} and *Sod1*^{-/-} mice. Furthermore, these three genes were not altered by any of the treatments (Fig. 4(A)–(C)). In contrast, the *Cil* gene expression was significantly upregulated in the skin of the hairless *Sod1*^{-/-} mice compared to that observed in the hairless *Sod1*^{+/+} mice (Fig. 4(D)). Interestingly, co-administration selectively normalized the *Cil* expression in the hairless *Sod1*^{-/-} skin (Fig. 4(D)).

*COP mixture and the VC derivative cooperatively attenuated the impairment of cellular phenotypes in the *Sod1*^{-/-} dermal fibroblasts*

We determined plasma levels of the eight types of COP in human blood at 1 hr after ingestion of 25 g CP (unpublished results). Based on these data, the eight

types of COP were mixed to prepare a COP mixture at the concentration described in Materials and Methods. Shigemura et al. reported that Pro-Hyp and Hyp-Gly promote cellular proliferation in dermal fibroblasts.^{10,11} On the other hand, we previously reported that treatment with VC and/or its derivatives significantly promotes the proliferation of *Sod1*^{-/-} fibroblasts.^{17,25} In this context, we investigated whether the COP mixture interacts with the effects of the VC derivative in promoting the proliferation of *Sod1*^{-/-} fibroblasts *in vitro*. The organ culture experiments using skin disks revealed that the *Sod1*^{-/-} fibroblasts demonstrated marked suppression of their outgrowth capacity compared to that observed in the *Sod1*^{+/+} mice, indicating impairment of the migration and proliferation of *Sod1*^{-/-} fibroblasts (Fig. 5(A) and (B)). In order to analyze the combined effects of the COP mixture and the VC derivative *in vitro*, we added the COP mixture and/or the VC derivative into the organ culture of *Sod1*^{-/-} skin disks (Fig. 5(C)). Treatment with the VC deriva-

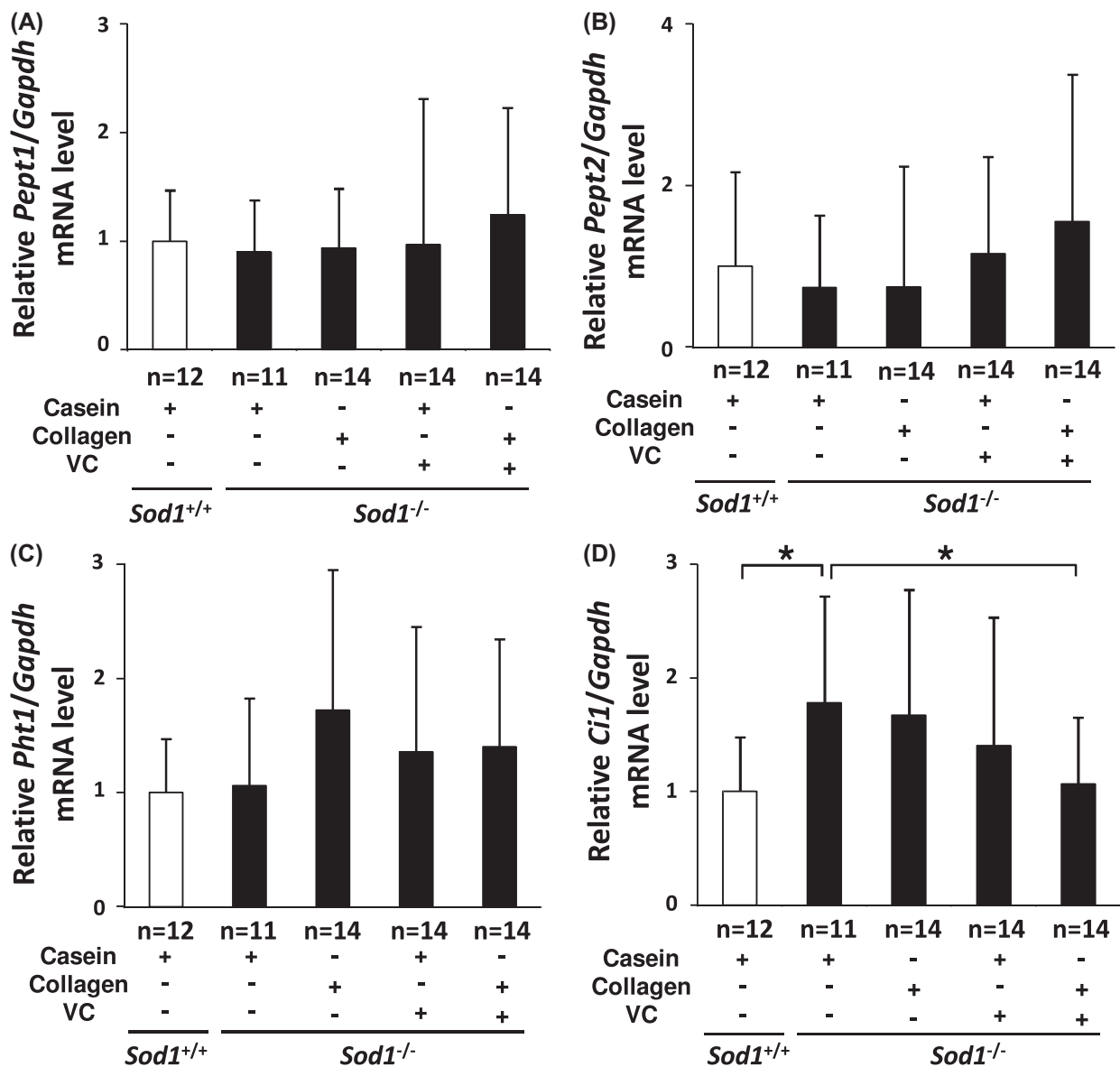


Fig. 4. Treatment with CP and the VC derivative partially normalized the transcriptional profiles of POT family genes in the skin tissues of the hairless *Sod1*^{-/-} mice.

Notes: The relative mRNA expression levels of (A) *Pept1*, (B) *Pept2*, (C) *Pht1*, and (D) *Cil* were determined using qRT-PCR. The data indicate the mean $\pm$ SD; * p < 0.05.

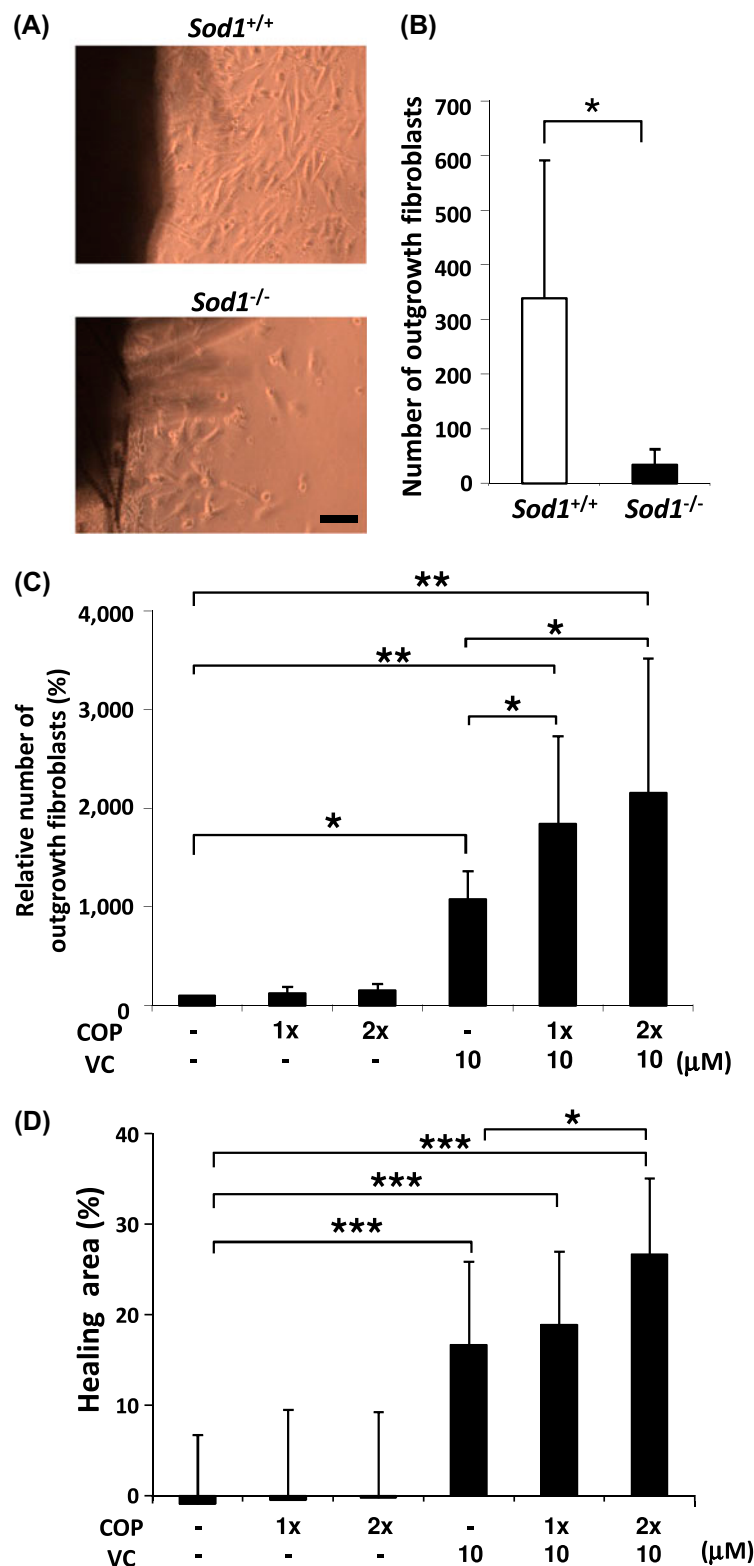


Fig. 5. COP mixture and the VC derivative cooperatively attenuated the impairment of the migration and proliferation of *Sod1*^{-/-} fibroblasts *in vitro*.

Notes: (A and B) Number of outgrowth fibroblasts of *Sod1*^{+/+} and *Sod1*^{-/-} mice in the organ culture. (C) Treatment with the COP mixture plus the VC derivative cooperatively promoted the outgrowth activity of the skin fibroblasts. (D) The scratch wound assay allowed for the direct evaluation of migration *in vitro*. (D) Fibroblasts of *Sod1*^{-/-} mice were scratched with a pipet tip and cultured for 12 h. (D) Following incubation, the healing area was quantified by detecting calcein-labeled fibroblasts in the scratch area. 1x and 2x COP indicate one- or twofold concentrations of the COP mixture, respectively. The data indicate the mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The scale bars represent 100 μ m.

tive only, not the COP mixture only, significantly promoted fibroblast outgrowth from the skin disks. Interestingly, the combined treatment cooperatively enhanced the outgrowth activity (Fig. 5(C)). In the

scratch wound assay, co-treatment with the COP mixture and the VC derivative also significantly facilitated the healing activity of the *Sod1*^{-/-} fibroblasts (Fig. 5(D)). These findings collectively suggest that the

COP mixture promoted the bioactivity of the VC derivative with respect to the migration and proliferation of *Sod1*^{-/-} fibroblasts *in vitro*.

Discussion

In the present study, we demonstrated that co-treatment with CP and the VC derivative prevented skin thinning via upregulation of the *Colla1* and *Has2* expression in *Sod1*^{-/-} skin (Figs. 1 and 3). Furthermore, we confirmed that treatment with either CP or VC only failed to attenuate the skin pathology of the hairless *Sod1*^{-/-} mice (Fig. 1). On the other hand, several reports have shown that treatment with CP ingestion only significantly improves epidermal thickening and impairment of skin elasticity in UVB-irradiated hairless mice.^{26–28} These data indicate that treatment with CP only discriminates rescue effects between hairless *Sod1*^{-/-} and UVB-irradiated hairless wild-type mice. In fact, *Sod1*^{-/-} fibroblasts exhibit marked loss of cell viability and a significant increase in apoptotic cell death associated with a 10-fold increase in intracellular reactive oxygen species (ROS) generation.²⁵ In contrast, UV irradiation results in a 50% reduction of cell viability associated with an only threefold increase in ROS generation.²⁹ These differences in the magnitude of ROS generation and cellular damage may account for the limited effects of treatment with CP only in hairless *Sod1*^{-/-} mice. VC treatment attenuates excess oxidative stress both *in vivo* and *in vitro*; therefore, CP functions as a bioactive peptide in *Sod1*^{-/-} mice.

Alternatively, Mendis et al. reported that CP functions as an antioxidant *in vitro*.³⁰ This phenomenon may partly explain why CP ingestion attenuates UV-induced skin pathology. On the other hand, CP-derived COP has the ability to stimulate skin cells. For example, Pro-Hyp and Hyp-Gly stimulate the proliferation of skin fibroblasts *in vitro*.^{10,11} It is noteworthy that treatment with CP alone induced a significant decrease in the levels of 8-isoprostane, a marker of lipid peroxidation, in the skin of the hairless *Sod1*^{-/-} mice (Fig. 2(B)). This finding suggests that CP ingestion alone improves, at least in part, age-related oxidative damage in the skin *in vivo*. We also cannot exclude that VC enhanced the absorption or bioavailability of digested CP, thus promoting bioactivity of COP in *Sod1*^{-/-} mice.

As shown in Fig. 3, *Sod1* loss induced the transcriptional downregulation of the *Colla1* gene. Proinflammatory cytokines, including TNF- α , inhibit the type I collagen expression in skin fibroblasts.³¹ Garela and colleagues recently reported that the downstream molecule, NF- κ B, suppresses *COL1A1* gene transcription in human dermal fibroblasts³² and accumulates prior to activation in the nucleus in association with the downregulation of the *COL1A1* gene in aged human fibroblasts.³³ Since oxidative stress generally activates NF- κ B, co-treatment with CP and VC may modulate NF- κ B-mediated *Colla1* repression, resulting in improvements in collagen homeostasis in *Sod1*^{-/-} skin.

Peptide uptake systems are conserved in all organisms, including bacteria, mammals, and plants.³⁴ In these systems, the POT family, including PEPT1,

PEPT2, PHT1, and PHT2 (*Cil* in mice), plays an important role as oligopeptide transporters.²⁴ These POT genes can recognize approximately 8400 types of di/tripeptides and play a pivotal role in the transmembrane transport of oligopeptides in mammals.³⁵ PEPT1 expressed in the epithelium of the small intestine plays a functional role in the absorption of nutritional peptides,³⁶ while PEPT2 expressed in the renal tubules is involved in the reabsorption of peptides from primitive urine.³⁷ In addition, PHT1 has been isolated as a neuro-specific peptide/histidine transporter.³⁸ Furthermore, a novel peptide/histidine transporter, PHT2, that is primarily expressed in the lymphatic system has been cloned in rats.³⁹ We herein identified four POT genes constitutively expressed in murine skin tissues (Fig. 4(A)–(D)). Interestingly, only the *Cil* gene was selectively upregulated in the *Sod1*^{-/-} skin (Fig. 4(D)) and normalized by co-treatment with CP and the VC derivative in the *Sod1*^{-/-} mice (Fig. 4(D)). These results suggest that intrinsic oxidative stress induces the *Cil* expression and may alter the transport profile of COP uptake in skin cells. Further analyses are needed to clarify the physiological functions of POT genes, particularly *Cil*, in collagen biosynthesis and the maintenance of skin thickness.

In conclusion, co-treatment with CP and VC effectively protects against intrinsic oxidative stress-induced skin atrophy. The use of combinatory therapy, such as that involving antioxidants and active substances, provides a novel strategy for treating age-related tissue damage.

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