Acerola (*Malpighia emarginata* DC.) Juice Intake Suppresses UVB-Induced Skin Pigmentation in SMP30/GNL Knockout Hairless Mice

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Abstract

Background/Aims

Acerola (*Malpighia emarginata* DC.) is a fruit that is known to contain high amounts of ascorbic acid (AA) and various phytochemicals. We have previously reported that AA deficiency leads to ultraviolet B (UVB)-induced skin pigmentation in senescence marker protein 30 (SMP30)/gluconolactonase (GNL) knockout (KO) hairless mice. The present study was undertaken to investigate the effects of acerola juice (AJ) intake on the skin of UVB-irradiated SMP30/GNL KO mice.

Research design/Principal findings

Five-week old hairless mice were given drinking water containing physiologically sufficient AA (1.5 g/L) [AA (+)], no AA [AA (-)] or 1.67% acerola juice [AJ]. All mice were exposed to UVB irradiation for 6 weeks. UVB irradiation was performed three times per week. The dorsal skin color and stratum corneum water content were measured every weekly, and finally, the AA contents of the skin was determined. The skin AA and stratum corneum water content was similar between the AA (+) and AJ groups. The L* value of the AA (+) group was significantly decreased by UVB irradiation, whereas AJ intake suppressed the decrease in the L* value throughout the experiment. Moreover, in the AJ group, there was a significant decrease in the expression level of dopachrome tautomerase, an enzyme that is involved in melanin biosynthesis.

Conclusion

These results indicate that AJ intake is effective in suppressing UVB-induced skin pigmentation by inhibiting melanogenesis-related genes.

Abbreviations: AA, ascorbic acid; AJ, acerola juice; Ccnd1, cyclin D1; Dct, dopachrome tautomerase; DHA, dehydroascorbic acid; DHICA, dihydroxyindole-2-carboxylic acid; ECD, electrochemical detection; Edn1, endothelin 1; GNL, gluconolactonase; KO, knockout; MPA, metaphosphoric acid; SMP30, senescence marker protein 30; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; Tyr, tyrosinase; Tyrp1, tyrosinase related protein-1; UVB, ultraviolet B.

Introduction

Senescence marker protein-30 (SMP30) was originally identified as a 34-kDa protein whose expression in the liver decreases with aging in an androgen-independent manner [1]. Previously, we found that SMP30 acts as a gluconolactonase (GNL) (EC 3.1.1.17) and is responsible for the conversion of L-gulonic acid into L-gulono-γ-lactone during the penultimate step of ascorbic acid (AA) synthesis [2]. Additionally, we demonstrated that SMP30/GNL knockout (KO) mice are unable to synthesize AA *in vivo* and develop scurvy when fed an AA-deficient diet [2]. Because humans cannot synthesize AA due to numerous mutations in the gene for L-gulono-γ-lactone oxidase, which is the terminal enzyme of the AA synthetic pathway, SMP30/GNL KO mice have been utilized to investigate the role of AA *in vivo* [3]. Thus far, we have reported that a complete lack of AA leads to an increase in superoxide generation in the brain [4, 5], an increase in protein oxidation in the liver [6, 7], the development of pulmonary emphysema [8–10], and decreases in the noradrenaline and adrenaline contents of the adrenal glands [11]. In this manner, we have revealed the physiological functions of AA by utilizing SMP30/GNL KO mice as an animal model. Furthermore, to clarify the pivotal role of AA in the skin, we developed and established SMP30/GNL KO hairless mice by mating SMP30/GNL KO mice with hairless mice [12]. The characteristic features of the SMP30/GNL KO hairless mice are as follows: an inability to synthesize AA, hairlessness, and the ability to undergo normal melanogenesis. We have reported that AA-deficient SMP30/GNL KO hairless mice with chronic UVB irradiation exhibit suppressed epidermal hyperplasia and develop excessive skin pigmentation [12]. Because SMP30/GNL KO hairless mice derive AA primarily through dietary intake, SMP30/GNL KO hairless mice can be valuable for evaluating not only AA but also the functional foods that promote AA in the skin.

Acerola (*Malpighia emarginata* DC.) is a fruit that is found throughout Central America and within the northern part of South America. Because acerola has high concentrations of AA in the edible part, it is considered to be one of the best sources of AA [13]. Additionally, acerola contains numerous functional phytochemicals, such as carotenoids and polyphenols [14–16]. Previously, we tested for various polyphenols in acerola and found that cyanidin-3-α-O-rhamnoside, pelargonidin-3-α-O-rhamnoside, quercetin-3-α-O-rhamnoside, kaempferol glycosides, astilbin, and proanthocyanidin are present [14, 15, 17]. In addition to polyphenols, we have reported on the contents of various nutritional components such as glucose, fructose, malic acid and several amino acids in the edible part of acerola [17]. The contents of glucose, fructose, malic acid, total polyphenols and total amino acids in acerola was 15 to 16, 14 to 16, 4 to 5, 1.2 to 1.5, and 1.8 to 2.1 g/kg edible portion, respectively [17]. It has been demonstrated that acerola intake exerts protective effects against DNA damage generated by $FeSO_4$ [18], inhibits lung tumorigenesis induced by nicotine-derived nitrosamine ketone [19], regulates blood glucose levels and exerts anti-inflammatory effects in obese mice [20, 21]. Similarly, we have demonstrated a beneficial effect of acerola in humans; i.e., the intake of acerola juice (AJ) affects the absorption of AA in plasma and minimizes its excretion via urine in healthy Japanese subjects, which suggests improvement of the bioavailability of AA in humans [22]. In the skin, we have previously reported that the intake of acerola polyphenol extracts prevents UVB-induced skin pigmentation in brownish guinea pigs, which possess moderate numbers of melanocytes and melanosomes in the epidermis [23]. Because acerola contains not only a large amount of AA but also beneficial ingredients as noted above, it is important to elucidate the effect of whole juice intake in UVB-irradiated skin. Therefore, in this study, we focused on the effects of AJ intake on UVB-induced skin damage in SMP30/GNL KO hairless mice.

Materials and Methods

Preparation of acerola juice

AJ was prepared from concentrated mature clear juice, which was purchased from Nichirei do Brazil (Recife, Brazil). The concentrated clear juice was diluted with a hydrochloric acid solution and then filtered (No. 5C, Toyo Advantec Co., Tokyo, Japan). The prepared AJ was immediately frozen and stored at—80˚C until use.

Animals

The animal experiments were performed in accordance with the animal care and use protocol approved by the Institutional Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology (TMIG) and in accordance with the Guidelines for the Care and Use of Laboratory Animals of TMIG.

SMP30/GNL KO hairless mice were established as previously described [12]. Briefly, SMP30/GNL KO hairless mice were generated by mating SMP30/GNL KO mice (C57BL/6 background) with hairless mice (Hos:HR-1, Hoshino Laboratory Animals, Ibaraki, Japan). A minimum of three generations of this strain were interbred for the present study. After weaning at 5 weeks old, male SMP30/GNL KO hairless mice were divided into the following three groups: AA deficient [AA (-)], AA supplemented [AA (+)], and AJ supplemented [AJ]. The AA (+) group had free access to water containing 1.5 g/L AA and 10 µM EDTA, whereas the AA (-) group was given water without AA. The AJ group was given 1.67% acerola juice diluted with the same drinking water that was given to the AA (-) group. The diluted AJ contained 1.5 g/L AA. The concentration of AA in the drinking water was sufficient to maintain normal AA levels in many tissues [24, 25]. Throughout the experiment, the drinking water was changed every 3 or 4 days. All mice were fed an AA-deficient diet (CL-2, CLEA Japan, Tokyo, Japan), and a 12-h light/dark cycle was maintained in a controlled environment.

UVB irradiation of SMP30/GNL KO hairless mice

UVB irradiation was performed as previously described [12, 26]. SMP30/GNL KO hairless mice were irradiated with 94 mJ/cm^2 of UVB three times per week for 6 weeks under three UVB lamps (GL20SE, Sankyo, Kanagawa, Japan). The irradiance of the UVB lamps was measured using a UV radiometer (UV-340, Custom, Tokyo, Japan). The UVB lamps emit a continuous light spectrum between 280–380 nm with a peak emission at 306 nm. At the end of the six-week experimental period, the mice were euthanized by cervical dislocation, and their skin was subsequently collected and stored at -80˚C until use.

Measurements of dorsal skin color and stratum corneum water content

During the experiment, the dorsal skin color and stratum corneum water content were measured every week for 6 weeks. The degree of dorsal skin color was quantified using a portable reflectance colorimeter in the CIE-L*, a*, b* system (Minolta CR-200 colorimeter, Konica Minolta, Tokyo, Japan) by measuring the reflected object color. The results are expressed as an L* value, which is a measurement of skin lightness on a continuous black to white scale, where 100 is completely white and 0 is completely black. The stratum corneum water content, as assessed indirectly by high-frequency superficial skin conductance, was measured using a Ski-con-200 (IBS Co., Hamamatsu, Japan).

Measurement of AA and DHA contents in the skin

The skin levels of AA and dehydroascorbic acid (DHA), which is an oxidized form of AA, were measured using HPLC and electrochemical detection (ECD), as previously reported [12, 27]. Briefly, the skin was homogenized with 14 volumes of ice-cold 5.4% metaphosphoric acid (MPA) containing 1 mM EDTA (MPA/EDTA) solution and centrifuged at 21,000 x g for 10 min at 4˚C. The total AA (AA + DHA) and AA contents in the centrifugal supernatants were determined individually as follows, and the DHA content was calculated by subtracting the AA content from the total AA content. To determine of the total AA, the centrifugal supernatants were reduced with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 2 h on ice. After the reduction, the reaction mixture was diluted with 5% MPA/EDTA and analyzed for total AA by HPLC coupled with ECD. For the determination of AA, the non-reduced centrifugal supernatants were diluted with 5% MPA/EDTA and analyzed by HPLC coupled with ECD. Separation was achieved on an Atlantis dC18 5-μm column (4.6 × 150 mm) combined with an Atlantis dC18 5-μm guard column (4.6 × 20 mm) from Nihon Waters (Tokyo, Japan). The mobile phase consisted of 50 mM phosphate buffer (pH 2.8), 540 μM EDTA and 2% methanol. The flow rate was 1.3 mL/min. The temperatures for the column and Waters 2465 electrochemical detector were set at 30˚C, and the temperature for the autosampler thermostat was set at 4˚C. The sample was injected into the HPLC coupled with ECD for the AA analysis. Electrical signals were recorded using an electrochemical detector with a glassy carbon electrode at +0.6 V. All electrical signal data from the electrochemical detector were collected using Waters Empower2 software (Nihon Waters, Tokyo, Japan).

Histology

Dorsal skin tissues were fixed in a 10% formalin neutral buffer solution (Wako Pure Chemicals, Osaka, Japan), embedded in paraffin, and sectioned on a microtome at a thickness of 3 μm. For the hematoxylin and eosin (HE) staining, the sections were deparaffinized with xylene, rehydrated with a graded series of ethanol, and then subjected to HE staining. The epidermal thicknesses were determined using the Multi Gauge, version 3.0 (Fuji Photo Film, Tokyo, Japan).

Quantitative real-time RT-PCR analysis

Total RNA was extracted from the skin tissues using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. The total RNA (1 μg) was reverse transcribed at 37˚C using PrimeScript™ RT Enzyme (Takara, Shiga, Japan). Real-time RT-PCR was performed using a real-time PCR detection system (Takara Bio, Madison, WI, USA), and expression of the following genes was detected in the AA (-), AA (+), and AJ groups: tyrosinase (*Tyr*), tyrosinase related protein-1 (*Tyrp1*), dopachrome tautomerase (*Dct*), *Tnf-α*, endothelin 1 (*Edn1*), and cyclin D1 (*Ccnd1*). The final mixture for RT-PCR consisted of 1× SYBR Premix Ex Taq Mix (Takara), 0.4 μM of primers (forward and reverse), and cDNA. The primer sequences are provided in Table 1. The PCR amplification consisted of 40 cycles (95˚C for 5 s and 60˚C for 30 s) after an initial denaturation step (95˚C for 10 s). The mRNA expression levels were evaluated relative to the level of ribosomal protein, large, P1 (*Rplp1*), and the mRNA levels of the AA (+) group were designated as 1.0.

Statistical analysis

The probability of significant differences between the experimental groups was determined by one-way ANOVA followed by Tukey's HSD post hoc comparisons. Dunnett's test was also used to assess the effects of UVB on the L^* value. KaleidaGraph software was used (Synergy

Table 1. Primer sets used for qPCR analysis.

Target gene		Sequence
Tyrosinase (Tyr)	Forward	5'-CAAGTACAGGGATCGGCCAAC-3'
	Reverse	5'-GGTGCATTGGCTTCTGGGTAA-3'
Tyrosinase-related protein 1 (Tyrp1)	Forward	5'-TGATGCGGTCTTTGACGAATG-3'
	Reverse	5'-GTTGGTAACTGGAGGCCAGAATG-3'
Dopachrome tautomerase (Dct)	Forward	5'-AACCGCAGAGCAACTTGGCTAC-3'
	Reverse	5'-CTCCCAGGATTCCAATGACCAC-3'
Tnf-α	Forward	5'-TTGTTGCCTCCTCTTTTGCT-3'
	Reverse	5'-TGGTCACCAAATCAGCGTTA-3'
Endothelin 1 (Edn1)	Forward	5'-GTGTTCCCTAGCCTGTCTGC-3'
	Reverse	5'-TGGAATCTCCTGGCTCTCTG-3'
Cyclin D (Ccnd1)	Forward	5'-ATTTGCACACCTCTGGCTCT-3'
	Reverse	5'-TCACCTCTTCCCTCACATCC-3'
Ribosomal protein, large, P1 (Rplp1)	Forward	5'-TCCGAGCTCGCTTGCATCTA-3'
	Reverse	5'-CAGATGAGGCTCCCAATGTTGA-3'

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Software, Reading, PA, USA) to perform the one-way ANOVAs. Differences were considered significant at P values of <0.05.

Results

AA concentration in acerola juice

To adjust the AA concentration in the drinking water to 1.5 g/L, we first determined the total AA concentration in the AJ. As illustrated in Table 2, the total AA, AA, and DHA concentrations were 89.5 ± 1.5, 85.9 ± 2.2, and 3.6 ± 0.7 g/L, respectively. Because DHA can be reduced to AA by several enzymes, including glutaredoxin, protein disulfide isomerase, omega class glutathione transferase, 3α-hydroxysteroid dehydrogenase, and thioredoxin reductase [28], we used the total AA concentration in the AJ for the preparation of the drinking water for the AJ group. That is, the AJ group was given 1.67% AJ, which contained 1.5 g/L total AA.

Body weight in skin

To investigate the influence of AJ intake on the growth of the SMP30/GNL KO hairless mice, we measured the body weight changes of the three groups during the experiment. The body weights of the mice did not vary significantly among the three groups from the start date to the fifth week of the experimental period, but the body weight of the AA (-) group was significantly lower than that of the AJ group at 6 weeks (Table 3).

AA content in skin

At the end of the experiment (6 weeks), the AA and DHA content in the skin of the AA (-) group was below the level of detection (Fig 1). In contrast, the AA and DHA content was not significantly different between the AA (+) and AJ groups. The amounts of water consumption

Table 2. Total AA, AA, and DHA concentrations in acerola juice.

	Total AA (g/L)	AA (g/L)	DHA (g/L)
Acerola juice	89.5 ± 1.5	85.9 ± 2.2	3.6 ± 0.7

The data are presented as the mean ± SEM of three acerola juice samples.

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Table 3. Body weight change.

	AA (-) (g)	AA (+) (g)	AJ (g)
Start date	16.7 ± 0.8	16.7 ± 0.8	16.5 ± 1.2
1 w	18.2 ± 1.3	16.3 ± 0.7	18.0 ± 0.8
2 w	20.1 ± 0.9	18.0 ± 0.9	18.8 ± 0.7
3 w	21.3 ± 0.8	19.4 ± 0.9	19.9 ± 0.7
4 w	21.7 ± 0.8	20.7 ± 0.9	21.0 ± 0.5
5 w	19.2 ± 0.9	20.3 ± 1.1	20.2 ± 0.6
6 w	18.6 ± 1.0	21.3 ± 0.9	22.0 ± 0.6*

The values are expressed as the means ± the SEMs of five animals. ANOVA analysis $F_{2, 12} = 4.6$, $P < 0.05$ for 6 weeks. Asterisk indicate significant comparisons (*$P < 0.05$) between the AA (-) and AJ groups by Tukey's HSD post hoc comparisons.

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in the AA (-), AA (+), and AJ groups were 4.9 ± 0.4, 4.1 ± 0.3, and 4.7 ± 0.3 mL/day/mouse, respectively. The consumption of total AA in the AA (+) and AJ groups were 6.2 ± 0.5, and 7.1 ± 0.5 mg/day/mouse, respectively. There was no significant difference in the amount of water ingested during the experiment. Moreover, there was no significant difference in the consumption of total AA between the AA (+) and AJ groups.

Histological skin analysis

To investigate the effects of AJ intake on the histologically evaluated changes in the skin, we performed HE staining and measured the thicknesses of the epidermal skins of all the mice.
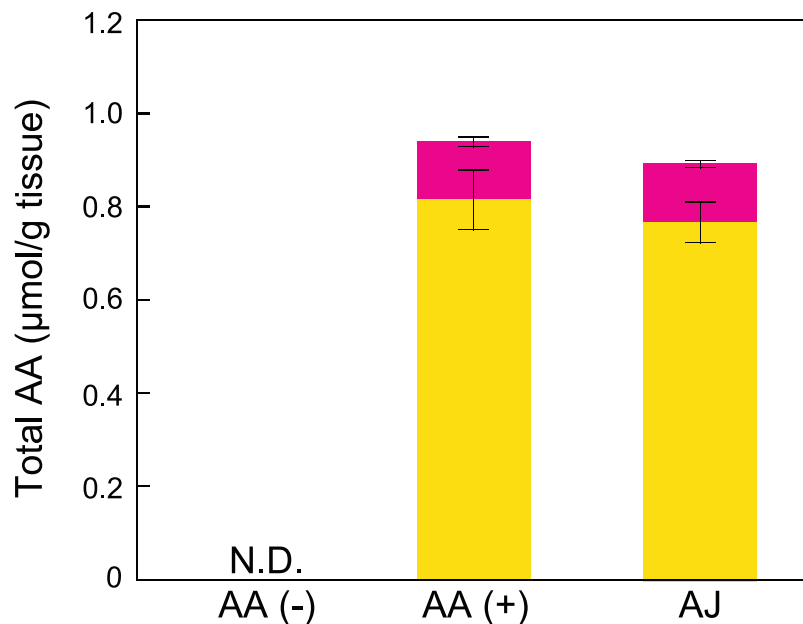

Fig 1. AA and DHA contents. The AA content (yellow bar) was determined by HPLC coupled with ECD, as described in the Materials and Methods section. The total AA (total bar height) content was determined by the reduction of DHA with TCEP. The DHA content (red bar) was calculated by subtracting the AA content from the total AA content determined in a different chromatographic run. N.D. indicates not detected. The values are expressed as the means ± the SEMs of five animals. ANOVA analysis: $F_{2, 12} = 104.3$, $P < 0.001$ for the AA content and $F_{2, 12} = 90.9$, $P < 0.001$ for the DHA content. The error bars represent the SEMs for the DHA and AA. AA, ascorbic acid; AJ, acerola juice.

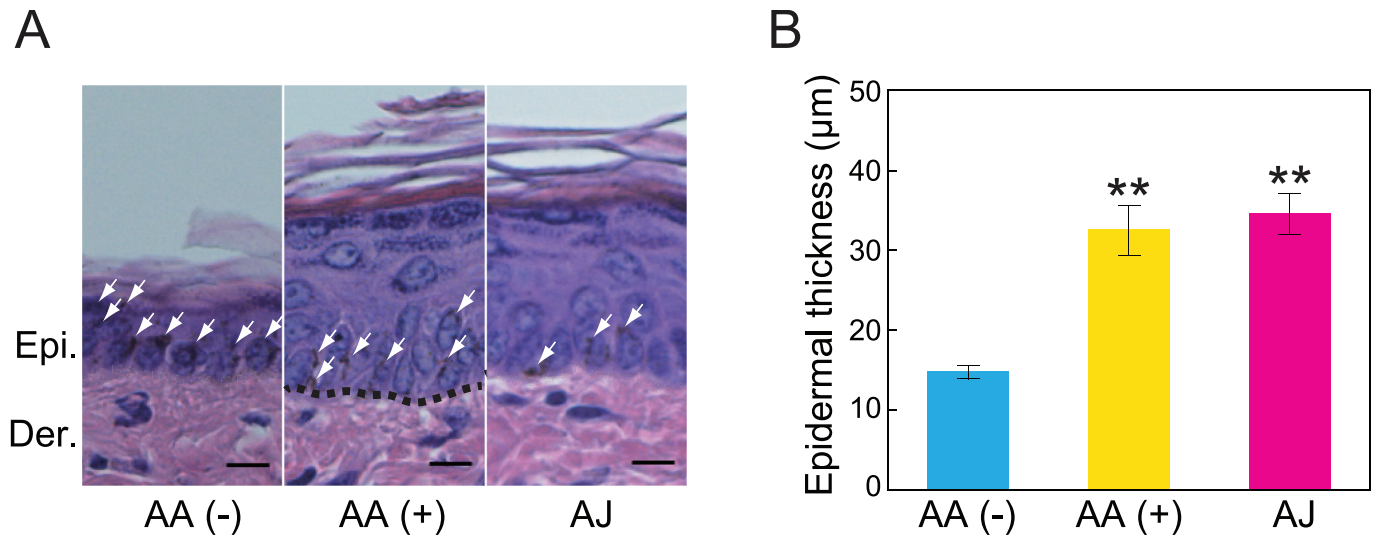doi:10.1371/journal.pone.0170438.g001

Fig 2. The effect of acerola juice intake on epidermal morphology and thickness. (A) High magnifications of the epidermis from the skins of the AA (-), AA (+) and AJ groups are shown. The white arrows indicate the skin pigment. (B) The epidermal thicknesses in the HE-stained sections were measured as the distance between the top of the basement membrane and the bottom of the stratum corneum in five fields randomly selected from each animal. The values are expressed as the means ± the SEMs of five animals. ANOVA analysis: $F_{2,\ 12} = 20.6$, $P < 0.001$. The asterisk indicates a significant difference (**$P < 0.01$) compared with the AA (-) group by Tukey's HSD post hoc comparisons. AA, ascorbic acid; AJ, acerola juice; Der., dermis; Epi., epidermis. Bar = 10 μm.

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Representative histological HE skin staining is provided in Fig 2A. In normal skin, chronic UVB irradiation induces epidermal hyperplasia, which is important for protecting the basal keratinocytes from subsequent DNA damage from UVB [29, 30]. As expected, epidermal hyperplasia was observed in the AA (+) and AJ groups (Fig 2A). The epidermal thicknesses were not significantly different between the AA (+) and AJ groups (Fig 2B). However, the AA (-) group did not manifest epidermal hyperplasia. Massive skin pigmentation was observed in the epidermis in the AA (-) group, but only slight pigmentation was observed in the AA (+) and AJ groups (Fig 2A). The epidermal thickness in the AA (-) group was significantly decreased compared with those of the other two groups (Fig 2B).

Stratum corneum water content

Because chronic UVB irradiation has been known to cause skin barrier dysfunction as well as decreases in the water content of the stratum corneum [31], we measured the water content of the stratum cornea of the three groups during the experiment. As illustrated in Fig 3, the stratum corneum water content decreased gradually in all groups after the initiation of UVB irradiation. The stratum corneum water content was not significantly different between the AA (+) and AJ groups during the experiment. However, the water content of the AJ group was significantly higher than that of the AA (-) group at 5 and 6 weeks. Moreover, the water content of the AA (+) group was significantly higher than that of the AA (-) group only at 6 weeks.

UVB-induced skin pigmentation

We next assessed the effects of AJ intake on the UVB-induced skin pigmentation of the SMP30/GNL KO hairless mice every week using a colorimeter. The L* values decreased gradually in the AA (-) and AA (+) group after the initiation of UVB irradiation (Fig 4A). Compared with the start day in the same group, significant differences were observed after 2 weeks of irradiation in the AA (-) group, whereas such differences were only observed at 3 and 5 weeks in
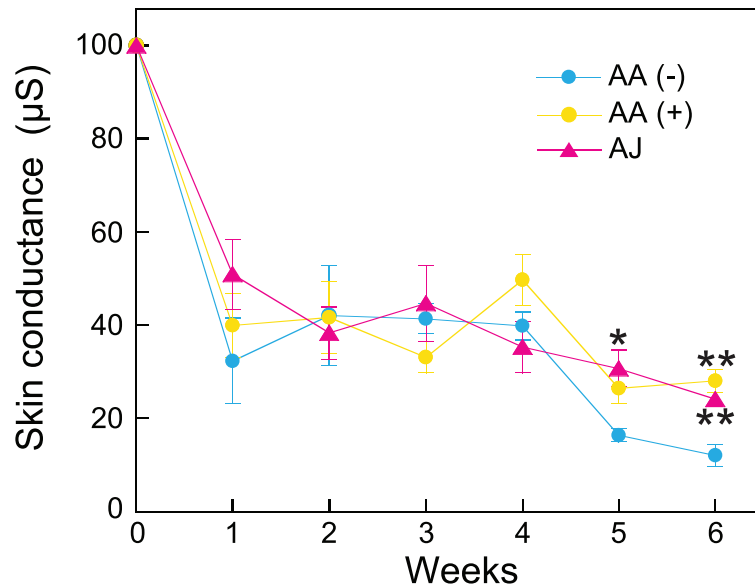
Fig 3. The effect of acerola juice intake on the stratum corneum water content. The stratum corneum water content was measured every week for 6 weeks as described in the Materials and Methods. The values are expressed as the means ± the SEMs of five animals. ANOVA analysis: $F_{2, 12} = 4.6$, $P < 0.05$ at 5 weeks and $F_{2, 12} = 25.2$, $P < 0.001$ at 6 weeks. The asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) compared with the AA (-) group by Tukey's HSD post hoc comparisons. AA, ascorbic acid; AJ, acerola juice.
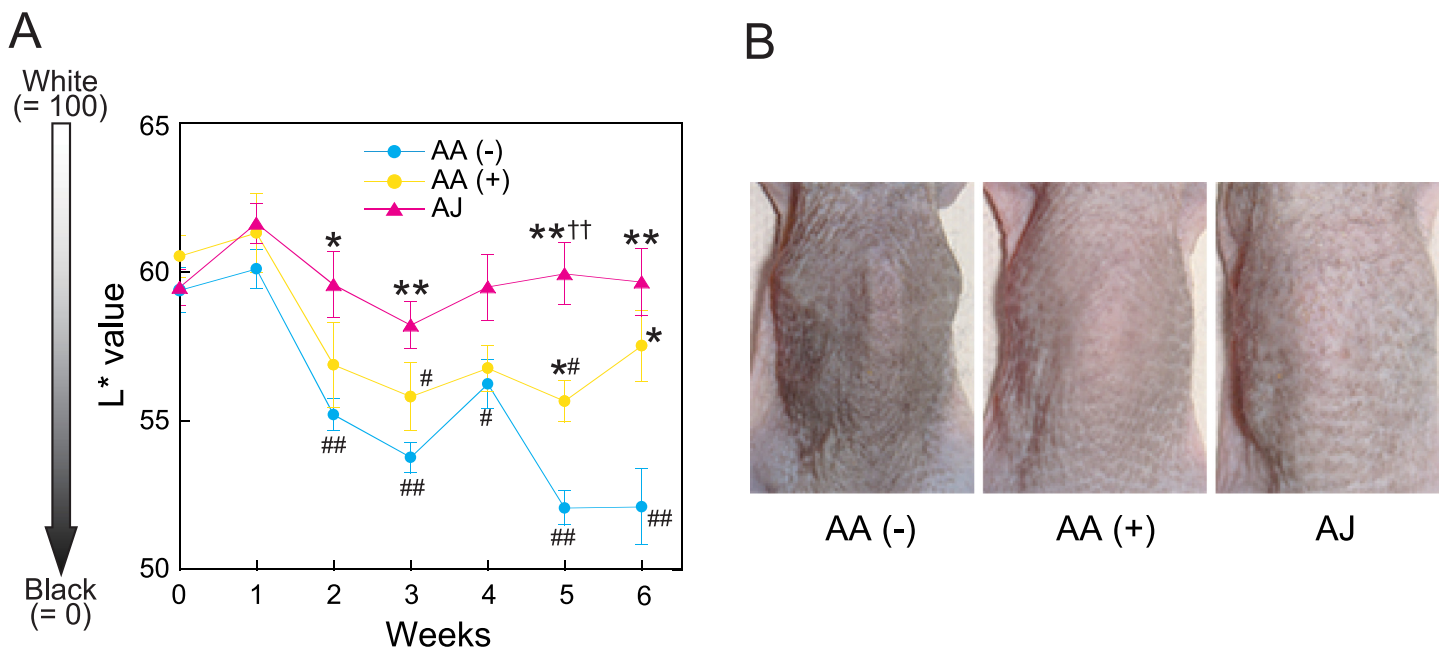
Fig 4. The effects of acerola juice intake on L* value and back skin colors. (A) The time course of the L* value (a measure of skin lightness). (B) Representative photographs of UVB-irradiated areas at 6 weeks. The values are expressed as the means ± the SEMs of five animals. ANOVA analysis: $F_{6, 28} = 17.4$, $P < 0.0001$ for the AA (-) group, $F_{6, 28} = 4.4$, $P < 0.01$ for the AA (+) group, and $F_{6, 28} = 1.1$, $P = 0.39$ for the AJ group. Sharps indicate significant differences (#$P < 0.05$, ##$P < 0.01$) compared with the start date by Dunnett's test for post hoc comparisons. ANOVA analysis: $F_{2, 12} = 4.1$, $P < 0.05$ at 2 weeks, $F_{2, 12} = 6.7$, $P < 0.01$ at 3 weeks, $F_{2, 12} = 3.8$, $P = 0.053$ at 4 weeks, $F_{2, 12} = 24.2$, $P < 0.0001$ at 5 weeks, and $F_{2, 12} = 10.5$, $P < 0.01$ at 6 weeks. The asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) compared with the AA (-) group by Tukey's HSD post hoc comparisons. The double daggers indicate significant differences (††$P < 0.01$) compared with the AA (+) group by Tukey's HSD post hoc comparisons. AA, ascorbic acid; AJ, acerola juice.

the AA (+) group. Surprisingly, the L* value of the AJ group did not change significantly during the experiment compared with the value on the start day. The L* value of the AJ group was significantly higher than that of the AA (-) group after 2 weeks of irradiation, except at week 4. In contrast, the L* value of AA (+) group was significantly higher than that of the AA (-) group at weeks 5 and 6. Comparison between the AJ and AA (+) groups revealed that the L* value of the AJ group was higher throughout the experiment and significantly higher than that of the AA (+) group at 5 weeks. Fig 4B provides representative photographs of the dorsal skin colors at the end of the experiments. There was a visible reduction in the pigmentation of the skin that had been treated with AJ.

Gene expression

To determine the effects of AJ intake on gene expression, we next examined the mRNA expression levels of melanogenesis-related genes (*Tyr*, *Tyrp1*, and *Dct*), inflammatory cytokines (*Tnf-α* and *Edn1*) and a cell cycle-related gene (*Ccnd1*) in the skins of all groups. The *Tyr* mRNA expression levels of the AA (-) and AJ groups were 29% and 32% lower, respectively, than that of the AA (+) group, although these differences were not statistically significant (Fig 5A). Similarly, the *Tyrp1* mRNA expression levels of the AA (-) and AJ group were 27% and 17% lower, respectively, than that of the AA (+) group, although these differences were not statistically significant (Fig 5A). The *Dct* mRNA expression levels of the AA (-) and AJ groups were 29% and 37% lower, respectively. Of note, there was a significant difference in the *Dct* mRNA expression levels between the AA (+) and AJ groups (Fig 5A). As illustrated in Fig 5B, the *Tnf-a* mRNA expression level of the AA (-) group was significantly higher than those of the AA (+) and AJ groups. In contrast, there was no significant difference in the level of *Tnf-α* mRNA between the AA (+) and AJ groups. The *Edn1* mRNA expression level of the AA (-) group was 26% higher than that of the AA (+) group (Fig 5B). Although there was no significant
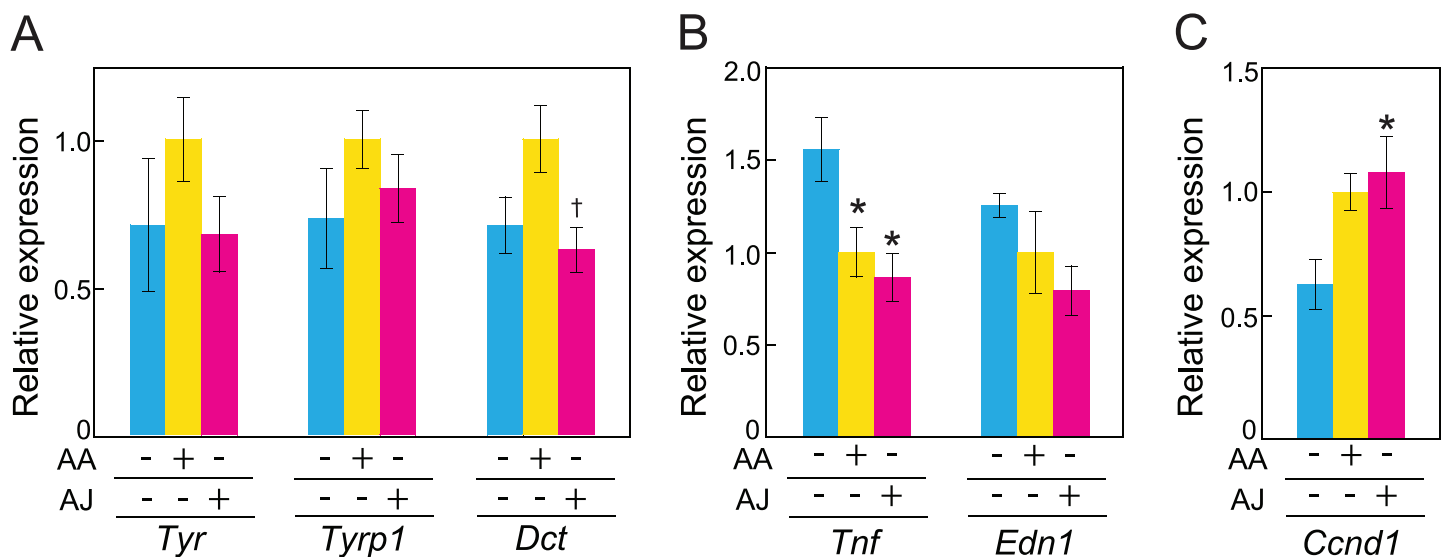

Fig 5. The effect of acerola juice intake on gene expression in the skin. (A) Melanogenesis-related enzyme genes *Tyr*, *Tyrp1* and *Dct*. (B) Cytokine genes *Tnf-a* and *Edn1*. (C) Cell cycle progression gene *Ccnd1*. Quantifications of the mRNA are illustrated relative to the *Rplp1* mRNA. The mRNA levels of the AA (+) group were designated as 1.0. The values are expressed as the means ± the SEMs of five animals. ANOVA analysis: $F_{2, 12} = 1.1$, $P = 0.369$ for the *Tyr* gene, $F_{2, 12} = 1.1$, $P = 0.372$ for the *Tyrp1* gene, $F_{2, 12} = 4.1$, $P < 0.05$ for the *Dct* gene, $F_{2, 12} = 6.3$, $P < 0.05$ for the *Tnf-a* gene, $F_{2, 12} = 2.3$, $P = 0.147$ for the *Edn1* gene, and $F_{2, 12} = 4.7$, $P < 0.05$ for the *Ccnd1* gene. The asterisks indicate significant differences (*$P < 0.05$) compared with the AA (-) group by ANOVA followed by Tukey HSD post hoc comparisons. The daggers indicate significant differences ($^†P < 0.05$) compared with the AA (+) group by ANOVA followed by Tukey's HSD post hoc comparisons. AA, ascorbic acid; AJ, acerola juice.

difference between the AA (+) and AJ groups, the *Edn1* mRNA expression level of the AJ group was 21% lower than that of the AA (+) group. Compared with the AA (+) group, the *Ccnd1* mRNA expression level of the AA (-) group was relatively lower by 37%, although this difference did not reach significance (p = 0.08; Fig 5C). There was no significant difference between the AA (+) and AJ groups in the *Ccnd1* mRNA levels. However, the *Ccnd1* mRNA expression level of the AJ group was significantly higher than that that of the AA (-) group.

Discussion

Acerola is considered a functional fruit due to its strong antioxidant properties and high phenolic contents and is thus consumed worldwide to promote health [14]. Previously, acerola has been demonstrated to exhibit beneficial effects, such as anti-mutagenic activity, an anti-hyperglycemic effect, and an *in vivo* anti-inflammatory effect [18–21]. Because little is known about the protective effects of AJ intake in UVB-damaged skin, we examined the effects of AJ intake on the skin of SMP30/GNL KO hairless mice. In the present study, we demonstrated that AJ intake suppresses excessive UVB-induced skin pigmentation compared with the same amount of AA intake.

In mammalian species, two main types of melanin are generated, that is, a red-to-yellow pheomelanin and a brown-to-black eumelanin [30]. It has been appreciated that melanin is the major pigment of the skin and determines the skin color. Skin pigmentation is the major photo-protective response of the skin against chronic UV radiation [32]. To elucidate the mechanism of the suppressive effect of AJ intake on skin pigmentation, we investigated the effect of AJ intake on the expression of melanogenesis-related genes. Tyrosinase is an essential enzyme in melanogenesis and catalyzes L-tyrosine to L-DOPA and L-DOPA to dopaquinone. Dopachrome tautomerase catalyzes dopachrome to dihydroxyindole-2-carboxylic acid (DHICA). Tyrosinase-related protein-1 is a DHICA oxidase that catalyzes DHICA to eumelanin [32]. We found that AJ intake significantly decreased the expression level of *Dct* and tended to decrease the levels of *Tyr* and *Tyrp1*. We previously investigated variations in the polyphenol composition of acerola and found that cyanidin-3-α-O-rhamnoside, pelargonidin-3-α-O-rhamnoside, quercetin-3-α-O-rhamnoside, kaempferol glycosides, astilbin and proanthocyanidin are present [14, 15, 17]. Additionally, we revealed that polyphenol extracts from acerola and individual polyphenols (cyanidin-3-α-O-rhamnoside, pelargonidin-3-α-O-rhamnoside and Astilbin) inhibit tyrosinase activity [23]. Previous studies have indicated that polyphenol extracts inhibit melanogenesis through the down-regulations of *Tyr*, *Tyrp1*, and *Dct* [33–35]. Therefore, the suppressive effect of AJ intake on skin pigmentation may have been due to the inhibitory effects of polyphenols on melanogenesis.

Moreover, UVB exposure facilitates the release of pro-inflammatory mediators, including TNF-α and Edn1, from keratinocytes [36–38]. TNF-α stimulates the infiltration of immune cells into the skin, and elastases and collagenases are thereby secreted from the infiltrated immune cells, leading to damage in the skin [39]. Considering that there was no difference in *Tnf-a* mRNA expression between the AJ and AA (+) groups, it appears that AJ can suppress the inflammation induced by UVB. Edn1 is considered one of the most potent agonists for stimulating melanocytes to accelerate melanogenesis [40, 41]. Because the *Edn1* mRNA level in the AJ group was lower than that in the AA (+) group, AJ intake may suppress melanogenesis-related cytokines.

Because keratinocytes differentiate and migrate to the upper layers of the epidermis, melanin pigment is removed from the epidermis along with keratinocytes. We found that the *Ccnd1* mRNA expression level of the AJ group was significantly higher than that of the AA (-) group. Cyclin D1 is a critical gene for the progression from the G_0/G_1 to the S phase of the cell

cycle and thus contributes to keratinocyte differentiation [42]. Robles *et al.* reported that the overexpression of cyclin D1 increases the rate of epidermal proliferation in cyclin D1 transgenic mice [43]. Our results revealed that AJ intake may facilitate the removal of melanized keratinocytes from the epidermis.

Another possibility is the involvement of effects of reactive oxygen species (ROS) on melanogenesis in the skin. UV interacts with oxygen to promote the formation of ROS, such as superoxide radicals, hydrogen peroxide, and highly reactive hydroxyl radicals in the skin [30]. It has been reported that the UV radiation-induced proliferation and melanogenesis of melanocytes are reduced by the topical application of an antioxidant, such as AA or vitamin E, to the skin of hairless mice, which suggests the involvement of ROS in melanogenesis in melanocytes [44]. Mitra *et al.* also reported that oxidative stress appears to have a role in pheomelanin-mediated melanogenesis [45]. Our previous study demonstrated that polyphenols isolated from the acerola fruit exert superoxide radical scavenging activity using an electron spin resonance spectrometer [14]. Other studies have also indicated that acerola has antioxidant activity using a 1, 1-diphenyl-2-picrylhydrazyl radical scavenging assay [16, 46, 47]. Some studies have reported that the antioxidant activity of acerola juice depends on its polyphenols and AA contents [21, 48]. Because there was no difference in AA content between the AA (+) and AJ groups, as illustrated in Fig 1, various polyphenols from acerola juice may contribute to the reduction of UVB-induced skin pigmentation.

In addition, it has been reported that cortisol and some neuroendocrine mediators such as melatonin and β-endorphin are involved in melanogenesis and UVB-induced skin damage. Cortisol can be produced in the skin by UVB irradiation [49]. It is suggested that production of cortisol maintains skin homeostasis against UVB-induced skin damage [49]. Melatonin is mainly produced in the pineal gland and retina, however, it is also produced in the skin [50–53]. Janjetovic *et al.* reported that melatonin has strong radical-scavenging activities and has a protective effect against UBV-induced oxidative stress [53]. Moreover β-endorphin can be detected in the components of the skin such as the epidermis, dermis and adnexa [50, 54]. It has been reported that β-endorphin stimulates keratinocyte migration in vitro, induces epidermal and follicular melanogenesis. Additionally, melanocortin receptor 1 is recognized for its role in the regulation of melanin pigmentation [55]. Although the relationship between acerola and these molecules are unclear, acerola may coordinate with these molecules to protect against UVB-induced damage.

We previously reported that AA deficiency suppresses UVB-induced epidermal hyperplasia and increases UVB-induced skin pigmentation [12]. In this study, we confirmed the same results, as illustrated in Figs 2 and 4. Moreover, we found for the first time that AA deficiency decreased the stratum corneum water content and significantly increased the expression level of *Tnf-a*. These results suggest that AA deficiency facilitates the release of pro-inflammatory mediators induced by UVB radiation and leads to epidermal barrier dysfunction. Moreover, our study also indicates that SMP30/GNL KO hairless mice represent an effective animal model for investigating the effects of functional foods on the skin.

In conclusion, our findings in this study strongly indicate that the intake of AJ compared with AA intake in the same amount significantly prevented skin damage induced by long-term UVB irradiation. Our findings strongly suggest that acerola is not only one of the best sources of AA but is also a functional food for the skin. Further study is needed to elucidate the protective effects on human skin in a human trial or using a reconstructed human skin model.

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Author Contributions

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References

1. Fujita T, Uchida K, Maruyama N. Purification of senescence marker protein-30 (SMP30) and its androgen-independent decrease with age in the rat liver. Biochim Biophys Acta. 1992; 1116(2):122–8. PMID: 1581340

2. Kondo Y, Inai Y, Sato Y, Handa S, Kubo S, Shimokado K, et al. Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy. Proc Natl Acad Sci U S A. 2006; 103(15):5723–8. doi: 10.1073/pnas.0511225103 PMID: 16585534

3. Nishikimi M, Yagi K. Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. Am J Clin Nutr. 1991; 54(6 Suppl):1203S–8S. PMID: 1962571

4. Sato Y, Kajiyama S, Amano A, Kondo Y, Sasaki T, Handa S, et al. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. Biochem Biophys Res Commun. 2008; 375(3):346–50. doi: 10.1016/j.bbrc.2008.08.020 PMID: 18706888

5. Kondo Y, Sasaki T, Sato Y, Amano A, Aizawa S, Iwama M, et al. Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice. Biochem Biophys Res Commun. 2008; 377(1):291–6. doi: 10.1016/j.bbrc.2008.09.132 PMID: 18848523

6. Amano A, Sato Y, Kishimoto Y, Takahashi K, Handa S, Aigaki T, et al. Effects of ascorbic acid deficiency on protein and lipid oxidation in livers from SMP30/GNL knockout mice. J Nutr Sci Vitaminol (Tokyo). 2013; 59(6):489–95.

7. Sato Y, Amano A, Kishimoto Y, Takahashi K, Handa S, Maruyama N, et al. Ascorbic acid prevents protein oxidation in livers of senescence marker protein-30/gluconolactonase knockout mice. Geriatr Gerontol Int. 2014; 14(4):989–95. Epub 2013/10/15. doi: 10.1111/ggi.12162 PMID: 24118935

8. Sato T, Seyama K, Sato Y, Mori H, Souma S, Akiyoshi T, et al. Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. Am J Respir Crit Care Med. 2006; 174(5):530–7. doi: 10.1164/rccm.200511-1816OC PMID: 16728709

9. Koike K, Kondo Y, Sekiya M, Sato Y, Tobino K, Iwakami SI, et al. Complete lack of vitamin C intake generates pulmonary emphysema in senescence marker protein-30 knockout mice. Am J Physiol Lung Cell Mol Physiol. 2010; 298(6):L784–92. doi: 10.1152/ajplung.00256.2009 PMID: 20172953

10. Koike K, Ishigami A, Sato Y, Hirai T, Yuan Y, Kobayashi E, et al. Vitamin C prevents cigarette smoke-induced pulmonary emphysema in mice and provides pulmonary restoration. Am J Respir Cell Mol Biol. 2014; 50(2):347–57. doi: 10.1165/rcmb.2013-0121OC PMID: 24032444

11. Amano A, Tsunoda M, Aigaki T, Maruyama N, Ishigami A. Effect of ascorbic acid deficiency on catecholamine synthesis in adrenal glands of SMP30/GNL knockout mice. Eur J Nutr. 2014; 53(1):177–85. doi: 10.1007/s00394-013-0515-9 PMID: 23508458

12. Sato Y, Arai KY, Nishiyama T, Nomura Y, Kishimoto Y, Aizawa S, et al. Ascorbic acid deficiency leads to epidermal atrophy and UVB-induced skin pigmentation in SMP30/GNL knockout hairless mice. J Invest Dermatol. 2012; 132(8):2112–5. doi: 10.1038/jid.2012.105 PMID: 22495180

13. Vendramini AL, Trugo LC. Chemical composition of acerola fruit (Malpighia punicifolia L.) at three stages of maturity. Food Chemistry. 2000; 71(2):195–8.

14. Hanamura T, Hagiwara T, Kawagishi H. Structural and functional characterization of polyphenols isolated from acerola (Malpighia emarginata DC.) fruit. Biosci Biotechnol Biochem. 2005; 69(2):280–6. doi: 10.1271/bbb.69.280 PMID: 15725651

15. Hanamura T, Aoki H. Toxicological evaluation of polyphenol extract from Acerola (Malpighia emarginata DC.) fruit. J Food Sci. 2008; 73(4):T55–61. doi: 10.1111/j.1750-3841.2008.00708.x PMID: 18460146

16. Nóbrega EM, Oliveira EL, Genovese MI, Correia RTP. The Impact of Hot Air Drying on the Physical-Chemical Characteristics, Bioactive Compounds and Antioxidant Activity of Acerola (Malphigia emarginata) Residue. Journal of Food Processing and Preservation. 2015; 39(2):131–41.

17. Hanamura T, Uchida E, Aoki H. Changes of the composition in acerola (Malpighia emarginata DC.) fruit in relation to cultivar, growing region and maturity. Journal of the Science of Food and Agriculture. 2008; 88(10):1813–20.

18. Horta RN, Kahl VF, Sarmento Mda S, Nunes MF, Porto CR, Andrade VM, et al. Protective effects of acerola juice on genotoxicity induced by iron in vivo. Genet Mol Biol. 2016; 39(1):122–8. doi: 10.1590/1678-4685-GMB-2015-0157 PMID: 27007905

19. Nagamine I, Akiyama T, Kainuma M, Kumagai H, Satoh H, Yamada K, et al. Effect of acerola cherry extract on cell proliferation and activation of ras signal pathway at the promotion stage of lung tumorigenesis in mice. J Nutr Sci Vitaminol (Tokyo). 2002; 48(1):69–72.

20. Hanamura T, Mayama C, Aoki H, Hirayama Y, Shimizu M. Antihyperglycemic effect of polyphenols from Acerola (Malpighia emarginata DC.) fruit. Biosci Biotechnol Biochem. 2006; 70(8):1813–20. PMID: 16926491

21. Dias FM, Leffa DD, Daumann F, Marques Sde O, Luciano TF, Possato JC, et al. Acerola (Malpighia emarginata DC.) juice intake protects against alterations to proteins involved in inflammatory and lipolysis pathways in the adipose tissue of obese mice fed a cafeteria diet. Lipids Health Dis. 2014; 13:24. doi: 10.1186/1476-511X-13-24 PMID: 24495336

22. Uchida E, Kondo Y, Amano A, Aizawa S, Hanamura T, Aoki H, et al. Absorption and excretion of ascorbic acid alone and in acerola (Malpighia emarginata) juice: comparison in healthy Japanese subjects. Biol Pharm Bull. 2011; 34(11):1744–7. PMID: 22040889

23. Hanamura T, Uchida E, Aoki H. Skin-lightening effect of a polyphenol extract from Acerola (Malpighia emarginata DC.) fruit on UV-induced pigmentation. Biosci Biotechnol Biochem. 2008; 72(12):3211–8. doi: 10.1271/bbb.80421 PMID: 19060403

24. Iwama M, Shimokado K, Maruyama N, Ishigami A. Time course of vitamin C distribution and absorption after oral administration in SMP30/GNL knockout mice. Nutrition. 2011; 27(4):471–8. doi: 10.1016/j.nut.2010.04.010 PMID: 20708381

25. Kondo Y, Sakuma R, Ichisawa M, Ishihara K, Kubo M, Handa S, et al. Potato chip intake increases ascorbic acid levels and decreases reactive oxygen species in SMP30/GNL knockout mouse tissues. J Agric Food Chem. 2014; 62(38):9286–95. doi: 10.1021/jf502587j PMID: 25180784

26. Naganumaa M, Yagi E, Fukuda M. Delayed induction of pigmented spots on UVB-irradiated hairless mice. J Dermatol Sci. 2001; 25(1):29–35. PMID: 11154861

27. Sato Y, Uchiki T, Iwama M, Kishimoto Y, Takahashi R, Ishigami A. Determination of dehydroascorbic acid in mouse tissues and plasma by using tris(2-carboxyethyl)phosphine hydrochloride as reductant in metaphosphoric acid/ethylenediaminetetraacetic acid solution. Biol Pharm Bull. 2010; 33(3):364–9. PMID: 20190394

28. Linster CL, Van Schaftingen E. Vitamin C. Biosynthesis, recycling and degradation in mammals. FEBS J. 2007; 274(1):1–22. doi: 10.1111/j.1742-4658.2006.05607.x PMID: 17222174

29. Seike M, Ikeda M, Morimoto A, Matsumoto M, Kodama H. Increased synthesis of calcitonin gene-related peptide stimulates keratinocyte proliferation in murine UVB-irradiated skin. J Dermatol Sci. 2002; 28(2):135–43. PMID: 11858952

30. D'Orazio J, Jarrett S, Amaro-Ortiz A, Scott T. UV radiation and the skin. Int J Mol Sci. 2013; 14(6):12222–48. doi: 10.3390/ijms140612222 PMID: 23749111

31. Yamada Y, Obayashi M, Ishikawa T, Kiso Y, Ono Y, Yamashita K. Dietary tocotrienol reduces UVB-induced skin damage and sesamin enhances tocotrienol effects in hairless mice. J Nutr Sci Vitaminol (Tokyo). 2008; 54(2):117–23.

32. Chen H, Weng QY, Fisher DE. UV signaling pathways within the skin. J Invest Dermatol. 2014; 134(8):2080–5. Epub 2014/04/25. doi: 10.1038/jid.2014.161 PMID: 24759085

33. Lee MH, Lin YP, Hsu FL, Zhan GR, Yen KY. Bioactive constituents of Spatholobus suberectus in regulating tyrosinase-related proteins and mRNA in HEMn cells. Phytochemistry. 2006; 67(12):1262–70. doi: 10.1016/j.phytochem.2006.05.008 PMID: 16782143

34. Zi SX, Ma HJ, Li Y, Liu W, Yang QQ, Zhao G, et al. Oligomeric proanthocyanidins from grape seeds effectively inhibit ultraviolet-induced melanogenesis of human melanocytes in vitro. Int J Mol Med. 2009; 23(2):197–204. PMID: 19148543

35. Kim JH, Baek SH, Kim DH, Choi TY, Yoon TJ, Hwang JS, et al. Downregulation of melanin synthesis by haginin A and its application to in vivo lightening model. J Invest Dermatol. 2008; 128(5):1227–35. doi: 10.1038/sj.jid.5701177 PMID: 18037902

36. Bashir MM, Sharma MR, Werth VP. UVB and proinflammatory cytokines synergistically activate TNF-alpha production in keratinocytes through enhanced gene transcription. J Invest Dermatol. 2009; 129 (4):994–1001. doi: 10.1038/jid.2008.332 PMID: 19005488

37. Strickland I, Rhodes LE, Flanagan BF, Friedmann PS. TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. J Invest Dermatol. 1997; 108(5):763–8. PMID: 9129230

38. Imokawa G, Miyagishi M, Yada Y. Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. J Invest Dermatol. 1995; 105(1):32–7. PMID: 7615973

39. Rijken F, Bruijnzeel PL. The pathogenesis of photoaging: the role of neutrophils and neutrophil-derived enzymes. J Investig Dermatol Symp Proc. 2009; 14(1):67–72. doi: 10.1038/jidsymp.2009.15 PMID: 19675558

40. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev. 2004; 84(4):1155–228. Epub 2004/09/24. doi: 10.1152/physrev.00044. 2003 PMID: 15383650

41. Murase D, Hachiya A, Kikuchi-Onoe M, Fullenkamp R, Ohuchi A, Kitahara T, et al. Cooperation of endothelin-1 signaling with melanosomes plays a role in developing and/or maintaining human skin hyperpigmentation. Biol Open. 2015; 4(10):1213–21. doi: 10.1242/bio.011973 PMID: 26340945

42. Fernandez-Hernandez R, Rafel M, Fuste NP, Aguayo RS, Casanova JM, Egea J, et al. Cyclin D1 localizes in the cytoplasm of keratinocytes during skin differentiation and regulates cell-matrix adhesion. Cell Cycle. 2013; 12(15):2510–7. doi: 10.4161/cc.25590 PMID: 23839032

43. Robles AI, Larcher F, Whalin RB, Murillas R, Richie E, Gimenez-Conti IB, et al. Expression of cyclin D1 in epithelial tissues of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia. Proc Natl Acad Sci U S A. 1996; 93(15):7634–8. PMID: 8755527

44. Quevedo WC Jr., Holstein TJ, Dyckman J, McDonald CJ, Isaacson EL. Inhibition of UVR-induced tanning and immunosuppression by topical applications of vitamins C and E to the skin of hairless (hr/hr) mice. Pigment Cell Res. 2000; 13(2):89–98. PMID: 10841030

45. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012; 491(7424):449–53. doi: 10.1038/nature11624 PMID: 23123854

46. Sagar S. Blessy K C, Kuna Aparna. Antioxidant Properties of Acerola (Malpighia Emarginata Dc.) and Acerola squash. International Journal of Science and Research 2014; 3(7):2176–9.

47. Lima VLAGD, Melo EA, Pinheiro IO, Guerra NB. Antioxidant capacity of anthocyanins from acerola genotypes. Food Science and Technology (Campinas). 2011; 31:86–92.

48. Leffa DD, da Silva J, Daumann F, Dajori AL, Longaretti LM, Damiani AP, et al. Corrective effects of acerola (Malpighia emarginata DC.) juice intake on biochemical and genotoxical parameters in mice fed on a high-fat diet. Mutat Res. 2014; 770:144–52. doi: 10.1016/j.mrfmmm.2013.11.005 PMID: 24296107

49. Skobowiat C, Sayre RM, Dowdy JC, Slominski AT. Ultraviolet radiation regulates cortisol activity in a waveband-dependent manner in human skin ex vivo. Br J Dermatol. 2013; 168(3):595–601. doi: 10. 1111/bjd.12096 PMID: 23363016

50. Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketee JD. Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. Adv Anat Embryol Cell Biol. 2012; 212:v, vii, 1–115. PMID: 22894052

51. Slominski A, Fischer TW, Zmijewski MA, Wortsman J, Semak I, Zbytek B, et al. On the role of melatonin in skin physiology and pathology. Endocrine. 2005; 27(2):137–48. doi: 10.1385/ENDO:27:2:137 PMID: 16217127

52. Slominski A, Wortsman J, Tobin DJ. The cutaneous serotoninergic/melatoninergic system: securing a place under the sun. FASEB J. 2005; 19(2):176–94. doi: 10.1096/fj.04-2079rev PMID: 15677341

53. Janjetovic Z, Nahmias ZP, Hanna S, Jarrett SG, Kim TK, Reiter RJ, et al. Melatonin and its metabolites ameliorate ultraviolet B-induced damage in human epidermal keratinocytes. J Pineal Res. 2014; 57 (1):90–102. doi: 10.1111/jpi.12146 PMID: 24867336

54. Tobin DJ, Kauser S. Beta-endorphin: the forgotten hair follicle melanotropin. J Investig Dermatol Symp Proc. 2005; 10(3):212–6. doi: 10.1111/j.1087-0024.2005.10108.x PMID: 16382667

55. Zmijewski MA, Slominski AT. Is Mc1r an important regulator of non-pigmentary responses to UV radiation? Exp Dermatol. 2013; 22(12):790–1. doi: 10.1111/exd.12129 PMID: 24279915