Original Article

Anti-aging and functional improvement effects for the skin by functional foods intakes : clinical effects on skin by oral ingestion of preparations containing Astaxanthin and Vitamins C and E

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Abstract

We studied the long term effects of the oral ingestion of liquid preparations containing Astaxanthin (AX), Vitamin C (VC) and Vitamin E (VE) on anti-aging and functional improvements of human facial skin. The subjects were 66 healthy Japanese female volunteers (mean age 37.26 years) who gave informed consent. The subjects were divided into 3 groups after preliminary skin measurements to minimize differences in their average age and the quantity of the stratum corneum layer water. The first group ingested a liquid preparation containing AX (6 mg) + VC (1000 mg) + VE (10 mg) ; the second group ingested a liquid preparation containing VC (1000 mg) + VE (10 mg), while the third group (the control group) ingested only the liquid preparation used for the first two groups. Liquid preparations were all normalized to 100 ml, 22 Kcal and each subject took one portion once every day (January-June, 2007) for 20 weeks. The experiment was carried out using double-blind test methodology. Skin measurements were taken 5 times, at 0, 4, 8, 12 and 20 weeks. The environmental conditions of the skin measurements were as follows ; each subject washed her face, with no cosmetics applied, and then seated at rest for 20 minutes in an environmental test room controlled at a temperature of 22±1° C and 50±5% relative humidity. Measurements carried out included determining the water content of the stratum corneum, skin elasticity, wrinkle area / volume, 3 dimensional measurements of wrinkle replicas, and optical observation of the skin through photography. Ocular inspection (skin photographs and 3 dimensional images of replicas) and wrinkle values (wrinkle areas and wrinkle volume rates) revealed significant improvements in the AX+VC+VE group compared to the control group. On the other hand, in the VC+VE group, there was no significant difference compared with the control group. Thus, the results show that the supplementation of AX has significant anti-aging and functional improvements of the skin.

(Keywords : skin aging, food supplements, antioxidants, astaxanthin, vitamin C)

I. Introduction

Many of the active oxygen species in the skin are said to be generated by ultraviolet radiation and are involved in the pathogenesis of a variety of skin disorders, such as lipid peroxidation, sunburn, phototoxicity and photoaging[1,2]. Anti-oxidants are believed to effectively clear active oxygen species, and their inhibitory effects on photoaging have been investigated[3]. Astaxanthin (hereafter, 'AX')[4,5], one such antioxidant, is a carotenoid, i.e., a red pigment present in a wide variety of marine animals including crustaceans, such as shrimp, crabs and so on, in addition to salmon and their roe and sea bream. Unlike β-carotene, AX does not display provitamin A effects[6], and its anti-oxidant effect is about 40 times greater than that of β-carotene. Inhibitory effects on

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photoaging have been reported *in vitro* and *in vivo* following topical application of AX[7,8]. AX has also been reported to show anti-inflammatory[9], anti-atherogenic[10] and de-stressing[11] effects *in vitro* in addition to reducing eye strain and muscle fatigue[12,13]. However, statistically robust *in vivo* analyses of those effects have not been performed, and the effects of long-term oral administration of AX on anti-aging and skin function are not known. This study examined the long-term effects of ingesting foods containing AX on skin function via double-blind testing of clinical subjects in accordance with ethical guidelines.

II. Methods

This experiment conformed to the 'ethical guidelines for clinical research (Ministry of Health, Labour and Welfare Notification No. 459 of 2004)' and was performed after approval was granted by an Ethics Committee established at the FGC Research Institute (Tokyo, Japan). That Committee considered the validity of the research, specimen safety, consideration of the subjects and the management of personal information. All subjects provided their informed consent to participate in the study. The personal rights and safety of each subject were considered thoroughly. Each subject was allocated into an intake group and was contacted by personnel other than the examiner. A double-blind comparative controlled study was performed where neither the examiners nor the subjects were informed of their intake group. The double-blind state was maintained with encoded names used for specimen numbers and aggregation of measured data for statistical analysis. Even after disclosure of the subject intake group, an external individual performed the examination to maintain the double-blind state for the analysis. The testing method is described below.

A. Test specimens and intake method

The test specimens were prepared in a liquid form (Lotte Co., Tokyo, Japan), and subjects were instructed to consume a 100 ml dose (22 Kcal) each day after dinner or before going to sleep. The active and base ingredients of each test specimen are shown below. Haematococcus algae colour ASUTA Red No. 37101 (an oil phase preparation emulsified in sucrose fatty acid ester ; SaneigenFFI Co., Osaka, Japan) was used as AX.

Test specimen 1 : 6 mg AX, 1000 mg vitamin C (hereafter, 'VC'), and 10 mg vitamin E (hereafter, 'VE') + liquid base material

Test specimen 2 : 1000 mg VC, 10 mg VE + liquid base material

Test specimen 3 : liquid base material only

Liquid base ingredients : Orange juice, glucose-fructose liquid sugar, acidulant, sweeteners (acesulfame K, stevia, sucralose), essence

B. Subjects

The subjects were 66 healthy Japanese women in their late thirties (average age : 37 ± 2 years) who presented with skin dryness, blemishes and wrinkles and had a body mass index (BMI) (weight [kg]/height^2 [m^2]) between 18 and 25. Subjects under certain conditions were excluded from the study such as those who were receiving hormone replacement therapy, who were pregnant or lactating, who had previous experience of cosmetic medicine (s) that may have affected the test site, who had sensitive skin or allergies such as to hay fever, and who were habitual users of drugs or supplements for a chronic disease. In addition, although no 'normal' habits related to diet, exercise, going out, sleeping and so on were specified, the subjects maintained a diary of their daily activities. The subjects were equally divided into 3 groups after preliminary skin assessments to minimize differences in average age and the quantity of stratum corneum water (electrical conductivity, μS). The average age of the subjects who received test specimen 1 (AX+VC+VE group) was 37.24 years, that of the subjects who received test specimen 2 (VC+VE group) was 37.27 years and that of the subjects who received test specimen 3 (the control group) was 37.27 years. One of the subjects from the AX+VC+VE group was unable to complete the skin analysis tests performed after the 12th week because she was transferred ; therefore, only data from 21 subjects were used for that group.

C. Method of assessing skin properties

C-1. Environmental conditions : A room with a fixed temperature, humidity and lighting condition was used. The environmental test chamber was set to 22°C ± 1°C with a relative humidity of 50% ± 5%. These conditions did not change throughout the test period.

C-2. Conditions prior to measurement : Each subject washed her face with cleanser (Unilever Japan K.K, Tokyo, Japan) and facial wash (Kao Co., Tokyo, Japan) and did not use any cosmetics prior to assessment. Measurements were taken after each subject had rested in the fixed-environment test chamber for 20 min to acclimatize to the conditions. Each subject's posture and position was aligned at the start of the experiment and during the measurement of each point.

C-3. Experimental period : This experiment was performed over 20 weeks from January–June 2007. Skin measurements were taken prior to the start of the experiment (week 0) and at the 4th, 8th, 12th and 20th week ; skin metabolism and menstruation were taken into consideration. The same sites of the face were measured each time. Each subject's self-assessment of her skin condition was determined by surveys collected 2 weeks after the intake period.

D. Skin assessment, measurement items and equipment used[14, 15]

The skin measurements were non-invasive and were performed using validated equipment. Details of the equipment used, skin sites assessed and assessment methods used are listed below.

D-1. Stratum corneum water quantity

Stratum corneum water quantity was measured with a high-frequency electrical conductivity method using a Skin Surface Hygrometer SKICON-200EX[16] (I.B.S Co., Hamamatsu Japan). The measurement sites included the outer corner of the eye and the central cheek area. Each site was measured 5 times and the average value (μS) after the minimum and maximum values were excluded was used.

D-2. Skin elasticity

Skin elasticity was measured using a Cutometer SEM575[17] Body No.S/N49012293 Prove No.S/N49012294 (Courage and Khazaka, Cologne, Germany). The measurement site was the upper zygomatic region and displacement was measured during 5 s of aspiration at 300 mbar from a 2 mm-diameter suction port and during the 5 s following aspiration for a total of 10 s. Each measurement was made 5 times. The parameters obtained were calculated as values as reported by Agache et al.[18], namely Ue (instantaneous elastic displacement, mm), Uv (delayed elastic displacement, mm), Ur (instantaneous recovery displacement, mm) and Uf (final elastic displacement, mm) as well as Ur/Uf (biological elasticity, no unit), which is a physical indicator of skin aging.

D-3. Skin surface characteristics 1 (Photographic analysis)

SLR film cameras (Medical Nikoll Camera ; Nikon Co., Tokyo, Japan and Fujicolor Super G Ace 100 ; Fujifilm, Tokyo, Japan) were used for close-up photographs of the skin. The focal length of the cameras was set to a constant in order to ensure that pictures were taken at the same magnification. Each subject's chin was placed on an ophthalmic fixed base and scale photographs of the skin surface of the full face, cheek area on both sides, skin below the eyes and eyelids were taken with an attached colour swatch. The colour of each photograph was calibrated according to the colour swatch when developing the film. The photographs were evaluated by five researchers of skin measurement and cosmetic assessment who visually examined the following seven aspects : (F1) overall impression, (F2) wrinkles at the outer corners of the eyes, (F3) wrinkles in the lower eyelid area, (F4) dark circles under the eyes, (F5) skin redness, (F6) skin condition, and (F7) skin elasticity. Photographs taken at weeks 4, 8, 12 and 20 were compared with those taken before the start of the experiment (week 0) to evaluate the efficacy. The photographs were rated as follows : 1, worse ; 2, slightly worse ; 3, no change ; 4, slight improvement ; 5, clear improvement. The extent of wrinkles was evaluated by 5 assessors based on the wrinkle grade (graded from 0 to 7) according to the Japanese Cosmetic Science Society, Guidelines for Evaluation of Cosmetic Functions average values were then calculated.

D-4 : Skin surface characteristics 2 (Replica image analysis)

A reflective replica analysis system, 3D Skin Roughness Analysis Measurement ASA-03R (Asahi Biomed Co., Kanagawa, Japan), was used. A shaded image corresponding to the shape of the wrinkles was obtained by irradiating the collected Replica Kit ASB-01 replicas with 30 parallel lights (Asahi Biomed Co., Kanagawa, Japan) and photographing the replicas with a CCD camera. Images were processed using 2-dimensional imaging analysis[19]. The parameters obtained were : wrinkle groove width (W', μm), wrinkle depth (D', μm), rectangular width (X, μm) and number of lines (Y). The wrinkle area ratio is represented by the formula : $\sum W'/XY/100$ ($\mu m^2/100$, $\mu m \times 10^{-2}$ or $10^{-2}\mu m^2$). The average wrinkle depth is the sum of the depth of the wrinkles found in the rectangular area divided by the number of wrinkles. The wrinkle volume indicates the volume of the wrinkles within the rectangular area and was calculated as width (1/2) × cumulative depth. Wrinkles were approximated as isosceles triangles for the calculation of the formula : $\sum W'/D'/XY/100$ ($\mu m^3/100$, $\mu m \times 10^{-2}$ or $10^{-2}\mu m^3$). In the processed images, wrinkles were ternarized using multistage filters and a determination process ; the identified width is displayed in green and shadows (depth) are in brown.

E. Statistical processing

Except for keratinocytes and visual observations of the skin surface, variations in all measurements, including the subjects' questionnaire results, were compared before the start of the experiment (week 0) and after 4, 8, 12 and 20 weeks for the control group, the AX+VC+VE and the VC+VE groups. The average values and standard deviations (SD) were calculated. One-way ANOVA with Tukey's post-hoc multiple comparison test was used for comparisons between 3 or more groups and calculations were made with JMP6 software for Windows. A significant difference is normally indicated with a hazard ratio of $<1\%$ or $<5\%$ when determining the efficacy of a food item for health-related purposes (Department of Food Safety in the Pharmaceutical and Food Safety Bureau in Japan, notification No. 0201002, 1 February 2005). However, significances with a hazard ratio of $<10\%$ in randomized controlled trials can be used as material related to the efficacy of the food for health-related use. In this study, hazard ratios of $<1\%$, $<5\%$ and $<10\%$ were used, and efficacy was confirmed primarily from the perspective of being a study on effectiveness of food intake on health.

III. Results

The water content of the stratum corneum of the cheek measured by high-frequency conductivity, the Ue parameter indicating skin elasticity of the cheek, the Ur/Uf physical indicator, wrinkle grades of the outer corner of the eye assessed from photographs of the skin taken under fixed conditions, wrinkle area ratios ($\mu m^2/100$) determined using a reflective replica analysis system and the volume of wrinkles ($\mu m^3/100$) observed at the outer corner of the eye are displayed below. Subjects were equally divided into 3 groups after preliminary skin measurements (prior to intake) to minimize differences in the average age and the quantity of stratum corneum water (electric conductivity μS). Because differences between the groups in terms of other skin measurements were not examined prior to the start of the experiment, the only other item for which a difference between groups was identified was the wrinkle grade. In brief, the initial wrinkle grade values of the AX+VC+VE group were significantly lower (<5% hazard ratio) than those of the control and the VC+VE (<1% hazard ratio) groups. No other parameter of skin measurement was significantly different between the 3 groups. All test specimens were consumed for 20 weeks, and no systemic or skin-related side effects were observed.

A. Water content of the stratum corneum

Changes over time relative to the initial water content values of the stratum corneum of the cheek in all 3 groups are shown in Figure 1. An increase of water content of the stratum corneum over the 20 weeks of the study was observed in all groups, but no clear difference could be seen between groups.

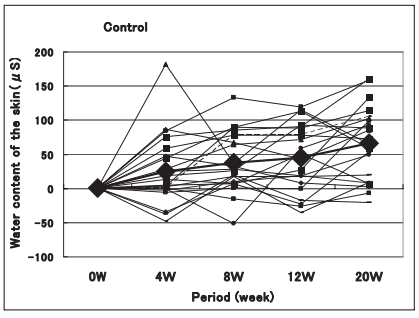

Fig.1-a：The control group

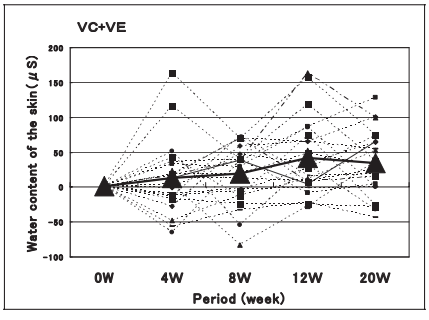

Fig.1-b：Control +VC+VE drink intake group

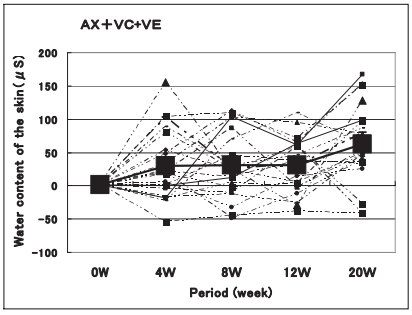

Fig.1-c：Control +AX +VC+VE drink intake group

Figure 1：The water content of the stratum corneum of the skin , cheeks (μS)

The average water content values of the stratum corneum of the cheek and the differences between the initial values at week 0 and those at the 4th, 8th, 12th and 20th week are shown in Figure 2. The water content of the stratum corneum increased over time in all groups；the level of the increase in the VC + VE group was significantly lower than that in the control group (<5% hazard ratio). The water content of the stratum corneum of the AX+VC+VE group showed an increase similar to the control group, and the level of increase was significantly higher than that in the VC+VE group (<10% hazard ratio). There was no significant difference in the rate of increase for other comparisons between the groups. The average water content values of the stratum corneum of the outer corner of the eye increased in all groups, but no significant difference was observed between any of the groups.

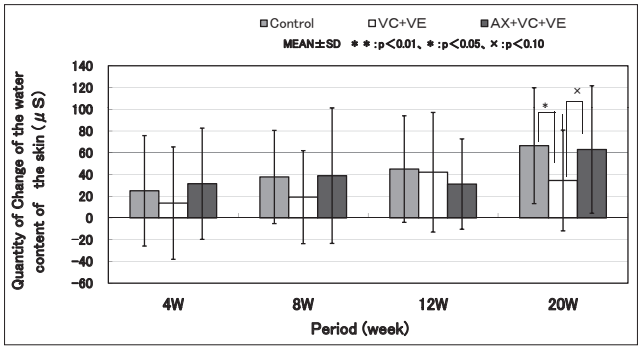

Figure 2：Results of the water content of the stratum corneum of the skin , cheeks (μS)
The difference with the measurement value before the examination

B. Skin elasticity

The average difference between the initial values of the Ue parameter and the Ur/Uf physical indicator was obtained to investigate the effect of intake.

A higher Ue parameter indicates higher skin flexibility. The average change in Ue values before the start of the study (week 0) and at the 4th week in the VC+VE group had significantly decreased compared to the control group

(<5% hazard ratio). After 8 weeks, the values had increased compared to the initial values in all 3 groups, and no significant difference was observed between any of the groups. After 12 and 20 weeks, the values decreased compared to the initial values in all 3 groups, and no significant difference was observed between any of the groups (Fig. 3).

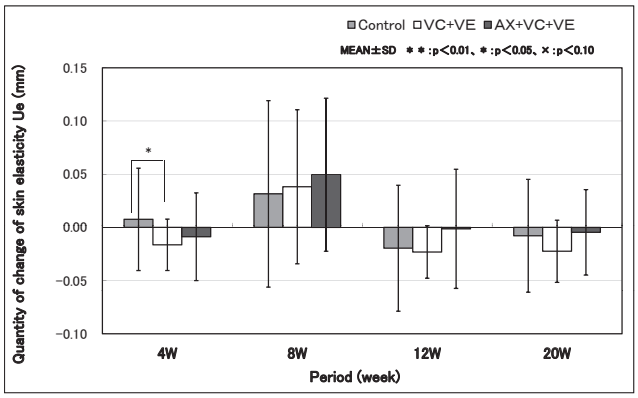

Figure 3: Results of the skin elasticity of displacement at a moment; Ue, cheeks (mm)

Furthermore, a higher Ur/Uf physical indicator indicates a higher resiliency of skin elasticity. The average change in Ur/Uf values before the start of the experiment and at the 4th week in the VC+VE group decreased significantly compared with the control group (<10% hazard ratio). After 8 weeks, the values decreased from the initial values in all 3 groups, and no significant difference was observed between any of the groups. After 4, 12 and 20 weeks, the values increased compared to the initial values in all 3 groups, and no significant difference was observed between any of the groups (Fig. 4).

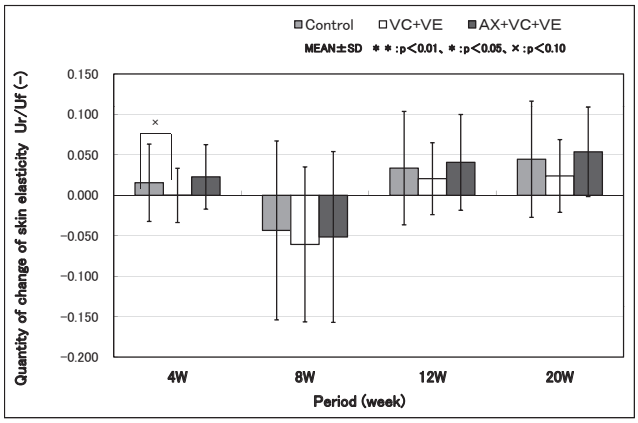

Figure 4: Results of the skin elasticity of cheeks; Ur/Uf (no unit; -)

C. Wrinkle morphology assessed using photographs of the skin taken under fixed conditions

The wrinkle grade of the outer corner of each subject's eyes was assessed using photographs of the skin taken under fixed conditions (Japanese Cosmetic Science Society Cosmetic Function Assessment Method guidelines) and ranged from 1 to 3 with an average value of 1.68. As seen in Figure 5, the wrinkle grade of the outer corner of the left eye in the initial measurements of the AX+VC+VE group was significantly lower than that of the VC+VE (<5% hazard ratio) and the control (<1% hazard ratio) groups.

In terms of the average change in visual assessment of wrinkles at the outer corner of the eye (F2) relative to the initial values, a significant improvement was seen after 8 weeks in the AX+VC+VE group compared with the control group (0.0911; <10% hazard ratio) (Fig. 6). Moreover, although no difference in the visual assessment of small wrinkles in the lower eyelid area (F3) was observed between the groups after 12 weeks, the AX+VC+VE group had improved significantly compared with the VC+VE group (<10% hazard ratio) after 20 weeks (Fig. 7).

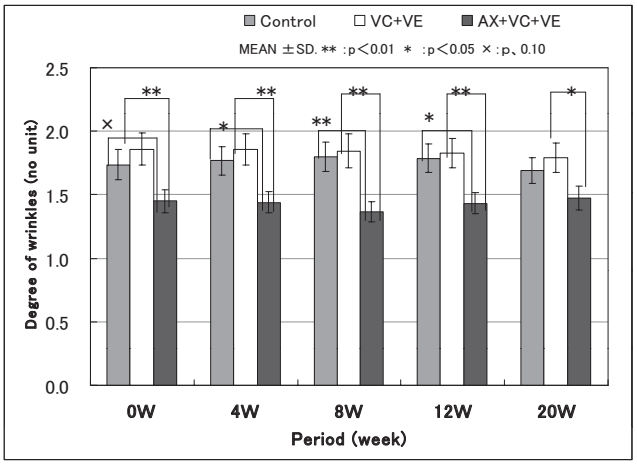

Figure 5: Results of the degree of visually observed wrinkles at the outer corner of the eyes (-)

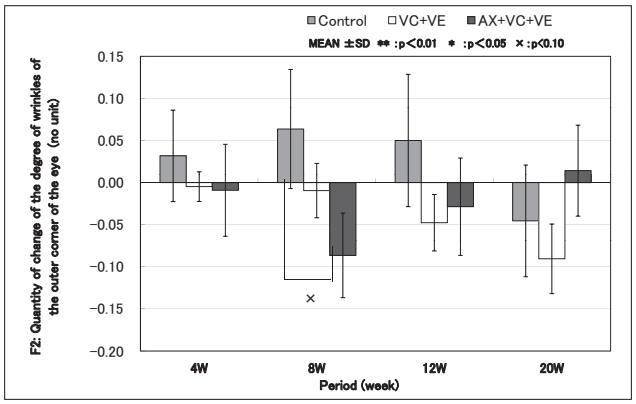

Figure 6: Results of the degree of visually observed wrinkles at the outer corner of the eyes (-)
The difference with the measurement value before the examination

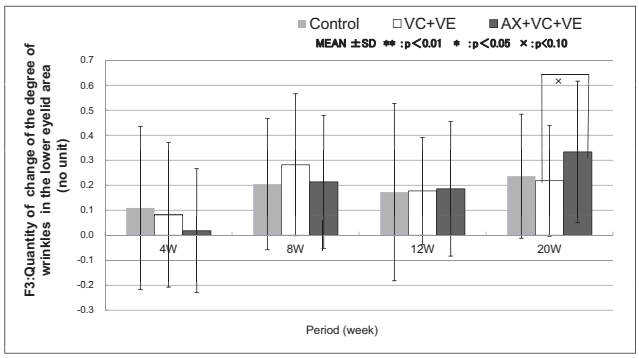

Figure 7：Results of the degree of visually observed wrinkles in the lower eyelid area (-)
The difference with the measurement value before the examination

D. Wrinkle morphology assessed using images taken using a reflective replica analysis system

Figure 8 displays a typical time-varying image of subjects from each group captured using a reflective replica analysis system. The wrinkle area ratios of the outer corners of the eye (μm^2/100) obtained from replica images over time are expressed as values relative to the initial values and are displayed in Figure 9. In all groups, there was a slight increase in the wrinkle area ratio at the outer corners of the eye (μm^2/100) over time. However, as seen in Figure 10, the average increase in the wrinkle area ratio at the outer corners of the eye over time was significantly suppressed in the AX+VC+VE group compared with the control group after 8 weeks (<10% hazard ratio), 12 weeks and 20 weeks (<5% hazard ratio). In addition, as seen in Figure 11, there was an increase in wrinkle volume (μm^3/100) over time in all groups. However, the average increase in wrinkle volume over time was significantly suppressed in the AX+VC+VE group compared with the control group after 8 weeks (<10% hazard ratio), 12 weeks (<1% hazard ratio) and 20 weeks (<10% hazard ratio).

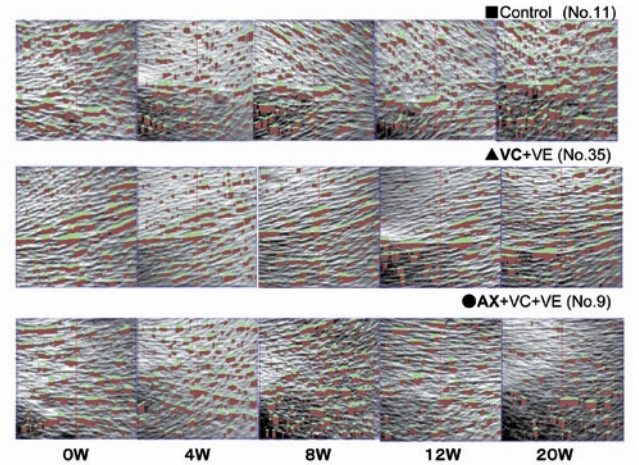

Figure 8：Representative replica images of the wrinkles at the outer corner of the eye

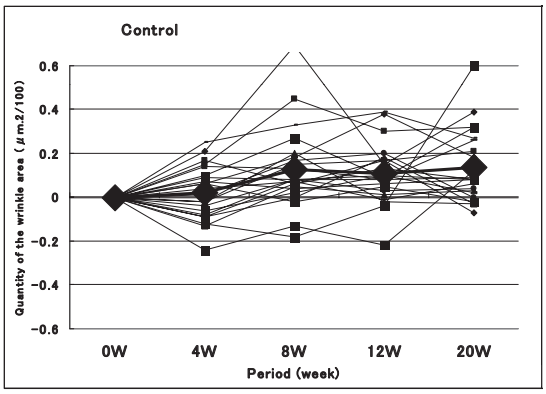

Fig. 9-a：The control group

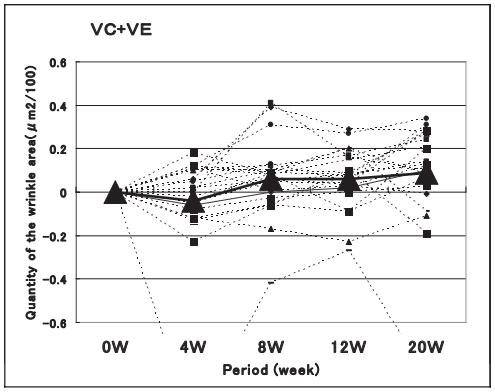

Fig. 9-b：Control +VC+VE drink intake group

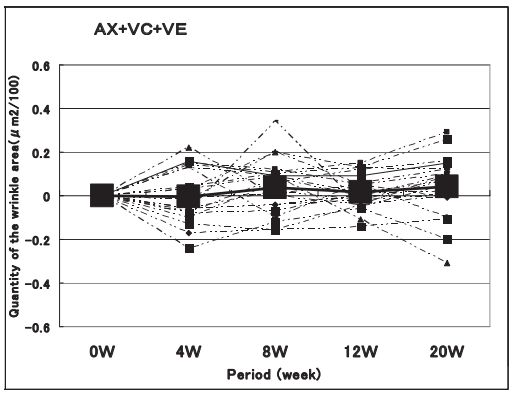

Fig. 9-c：Control +AX +VC+VE drink intake group

Figure 9：The replica image analysis of the wrinkle area at the outer corner of the eye (μm^2/100)

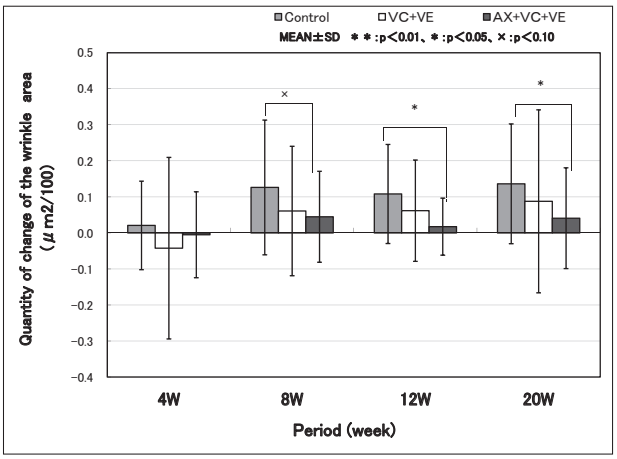

Figure 10：Results of replica image analysis of the wrinkle area at the outer corner of the eye (μm^2/100)
The difference with the measurement value before the examination

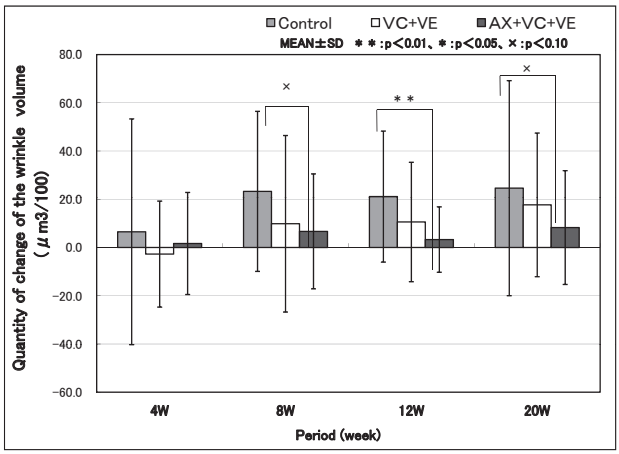

Figure11：Results of replica image analysis of the wrinkle volume at the outer corner of the eye (μm^3/100)

IV. Discussion

This study investigated the effects of a liquid solution containing AX (6 mg/day), VC (1000 mg/day) and VE (10 mg/day) consumed over an extended period on aging and function of the skin. Statistically significant differences in wrinkle measurements (wrinkle area ratio, wrinkle volume) were observed between the AX+VC+VE and the control groups; there was no significant difference between the VC+VE and the control groups. An increase in wrinkle measurements over time was observed in all groups; however, this increase was very slight in the AX+VC+VE group, where significant suppression was observed. Essentially, the experimental period of January–June overlapped with an increase in ultraviolet light irradiation, resulting in a trend of increased facial wrinkling. However, this increase was significantly repressed in the group that consumed the test specimen containing AX; it is thought that this was an effect of the long-term intake of the test solution.

Because the subjects in this study were middle-aged (average age: 37.2 years), wrinkling at the outer corners of their eyes was minor. However, it is believed that the prevention or suppression of aging of the skin in response to ultraviolet irradiation observed in the AX+VC+VE group is an intrinsic effect of the oral ingestion of AX. On the other hand, although improvement has previously been reported with topical moisturizers[20], no significant difference in the water content of the stratum corneum was observed in this experiment. This suggests that the wrinkle suppressing effect arising from the oral consumption of AX is due to a mechanism other than increased stratum corneum moisture. In addition, the water content of the stratum corneum was significantly reduced in the VC+VE group compared with the other groups at 20 weeks after the start of the experiment. Moreover, the skin elasticity measurements of the VC+VE group were significantly lower in comparison to the control group at 4 weeks. The reasons for this are unclear. After the 4th week, no significant difference in skin elasticity measurements was observed between any of the groups. Further investigations using the same measurement methods and sites are required.

In humans, the skin around the corner of the eyes in particular, is most exposed to sunlight and is seriously affected by ultraviolet radiation because of its position on the face and its angle relative to the sun. Active oxygen radicals generated by exposure to ultraviolet radiation not only reduce the amount of collagen (a dermal fibrillary element), which plays an important role in maintaining the elasticity of the skin, but also promotes the cross-linking of collagen resulting in reduced dermal flexibility and elasticity. UV radiation has also been reported to promote the activation of enzymes that destroy elastic fibres and collagen making the skin more prone to wrinkling[21]. Furthermore, it is known that elastolytic (elastase SFE) and collagenolytic (collagenase type-1 MMP-1) enzyme activities increase in fibroblasts exposed to ultraviolet radiation as a result of active oxygen species, such as hydroxyl radicals and singlet oxygen[22-24]. However, significant suppression of increased MMP-1 activity is seen in fibroblasts of AX-coated skin[7]. The authors have also confirmed a significant suppression of the increase in collagenase type-1 MMP-1 activity when AX is added to fibroblasts exposed to ultraviolet radiation[25].

Photolesions on the skin resulting from aging and ultraviolet radiation are reportedly involved in the over-production of certain types of active oxygen species. Accordingly, as seen in this study, the long-term intake of AX, which removes active oxygen, is likely to counter the effects of active oxygen species in the body and facial skin exposed to ultraviolet radiation and to reduce the extent of wrinkling.

The sum of these results suggests that foods with high anti-oxidant constituents, such as AX, can have anti-aging effects on the skin and can bring about an improvement in skin function.

V. Declaration of Interest

The authors have no conflict of interest to declare.

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特定の機能性食品摂取による皮膚の抗老化と機能改善作用の検討 抗酸化機能を有するアスタキサンチンおよびビタミンC，E 含有飲料長期摂取（20週間）がおよぼす皮膚作用について

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要　約

我々は，Astaxanthin（以下 AX），Vitamin C（以下 VC），Vitamin E（以下 VE）を含む飲料を，長期間摂取した場合の皮膚の抗老化と機能改善作用について検討した。同意を得た一般健常女性66名（平均年齢37.26歳）を，初回計測値の角層水分量，平均年齢に差が生じないように３群に分け，AX６mg，VC1000mg，VE10mg 含飲料摂取群，VC1000mg，VE10mg 含飲料摂取群，飲料基剤のみ摂取するコントロール群とした。飲料はすべて100ml，22Kcal に調整し，20週間（2007.１～６月）毎日１回摂取した。試験は二重盲検法で実施。皮膚計測は，開始前，４，８，12，20週目の計５回，洗顔後化粧品無塗布で恒温恒湿室内20分間座位安静後に行なった。計測項目は，角層水分量，皮膚弾力，皮膚光学写真観察，シワレプリカ３次元計測によるシワ面積・シワ体積である。その結果，目尻の皮膚拡大写真の目視評価およびレプリカ画像解析によるシワ計測値（面積率，体積率）において AX+VC+VE 群は，コントロール群に比べ有意な改善がみられた。VC+VE 群はコントロール群との有意差が認められなかった。これらの試験結果から，AX を含む機能性食品を長期に摂取することによる皮膚の抗老化および機能改善作用が示唆された。
（キーワード：皮膚老化，機能性食品，抗酸化剤，アスタキサンチン，ビタミンC）

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