[Original Article]

The Effect of Vitamin C Consumption on the Oxidative Stress Regulation System in SMP30/GNL Knockout Mice

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[ABSTRACT]

We studied how consumption of vitamin C (VC) affects the oxidative stress regulation system. SMP30/GNL knockout mice (males, $n = 33$), which cannot synthesize VC, were randomly divided into three groups: the VC 100 Group consuming 1.5 g/L VC, the VC 2.5 Group consuming 0.0375 g/L VC, and the VC 0 Group consuming 0 g/L VC. To examine the oxidative stress regulation system, the reactive oxygen metabolites (d-ROM test) and biological anti-oxidant potential (BAP test) values were measured, and the BAP/d-ROM ratio was calculated. We obtained measurements at the beginning of the study (5 weeks old: baseline) and after VC consumption for 9 weeks (14 weeks old: 9 wk). For the plasma VC concentration, plasma reduced (ascorbic acid (AA)) and plasma oxidized (dehydroascorbic acid (DHA)) were measured at 9 wk, and the total VC (AA + DHA: total) concentration was calculated. Compared to the other groups at 9 wk, the VC 100 Group showed a significantly lower value for the d-ROM test, a significantly higher value for the BAP/d-ROM ratio, no significant difference in the BAP test, and a significantly lower senescence grading score. The VC 100 Group showed a significantly higher total VC concentration compared with the other groups. Differences in consumption of VC caused a change in the d-ROM test, the BAP/d-ROM ratio, and the plasma VC concentration.

[Key words]

SMP30/GNL knockout mice, vitamin C, oxidative stress regulation system

INTRODUCTION

Oxidative stress leads to oxidative modification of biogenic substances by reactive oxygen species, which impairs mitochondria genes¹⁾. As a result, organ dysfunction and various illnesses such as lifestyle-related diseases occur²⁾, and an association with senescence has been suggested³⁾. Thus, oxidative stress is an unfavorable condition in the body in which the balance between oxidative reactions (oxidative stress) and anti-oxidant reactions (anti-oxidant potential) breaks down. Thus, understanding how the body can improve its defense systems against oxidative stress through anti-oxidant enzyme systems and anti-oxidant-containing foods is important. Although senescence may have an association with an increase in and accumulation of reactive oxygen species in cells or tissues, no reports have been published that scientifically demonstrate that reactive oxygen species themselves are directly involved in senescence processes^{3,4)}.

Anti-oxidant foods contain various substances that protect the body from oxidative stress, ranging from essential nutrients such as vitamin C (VC) and E, and β -carotene to catechin, ubiquinol (reduced coenzyme Q10, H_2CoQ10 : QH), and others. Generally, VC and QH are found in foods that provide sensitivity to oxidative stress and act as radical scavenger anti-oxidants⁵⁾. In particular, the presence of VC and QH controls the generation of lipid peroxides without greatly reducing vitamin E⁵⁾. The brain does not contain VC and shows increased generation of superoxide radicals and reactive oxygen species, indicating an association between VC and reactive oxygen species⁶⁾. The changing states of VC and QH in the body, together with lipid peroxides, are important for assessing oxidative stress. In this study, we used SMP30/GNL knockout

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mice, which lack the ability to generate VC, causing them to consume QH for a long time. Senescence marker protein 30 (SMA30) is a 34-kDa protein whose tissue levels in the liver, kidney, and lung decrease with aging^{4,6}. We used these mice to study oxidative stress (hydroperoxide concentration) and the changing state of QH in the body^{7,8}. VC is a water-soluble, powerful anti-oxidant found in food that efficiently removes reactive oxygen species in cells and the blood⁶. Thus, these mice without the ability to generate VC are thought to be a model for examining the oxidative stress regulation system.

ANIMALS AND METHODS

1 Animals

We used 33 SMP30/GNL knockout mice (4 weeks old; males), which were randomly divided into three groups: the VC 100 Group, consuming 100% of the necessary VC per day (1.5 g/L VC) in their drinking water ($n=11$); the VC 2.5 Group, consuming 2.5% of the necessary VC per day (0.0375 g/L VC) in their drinking water ($n=11$); and the VC 0 Group, consuming 0% of the necessary VC per day in their drinking water ($n=11$). SMP30/GNL knockout mice obtained it than Tokyo Metropolitan Institute of Gerontology (TMIG). The amount of consumed VC was based on our previous study⁴. All mice were kept at room temperature ($20\pm 1^\circ\text{C}$), at a relative humidity of about 50%, and with a light-dark cycle of 12 hours. Solid feed that is free of VC (CL-2, CLEA Japan, Inc.) was provided ad libitum, and no restriction was placed on movements. The mice used in this study are senescence-accelerated mice and are extremely similar to humans that cannot synthesize VC⁹. A report has shown that raising mice on solid feed that is free of VC causes typical scurvy with weight loss and leads to death by the 136th day in all mice¹⁰. A report has shown that raising mice on solid feed that is free of VC causes typical scurvy with weight loss and leads to death by the 136th day in all mice¹⁰. Senescence is accelerated about four times due to the VC deficiency¹⁰.

2 Methods

We measured oxidative stress at the beginning of the study (5 weeks old: baseline) and after VC consumption for 9 weeks (14 weeks old: 9 wk). Seven mice (5 from the VC 2.5 Group, 2 from the VC 0 Group) died of unknown causes during the course of the study.

2.1 Oxidative stress regulation system

Using analytical equipment for reactive oxygen and free

radicals (model FRAS4/FREE, H & D, Italy), we measured oxidative stress (reactive oxygen metabolites test: d-ROM test) and anti-oxidant potential (biological anti-oxidant potential: BAP test), and then the latent anti-oxidant potential (BAP/d-ROM ratio) was calculated. For the anti-ROMs test, the state of rapid anti-oxidant defenses (anti-ROMs test 1) and state of slow anti-oxidant defenses (anti-ROMs test 2) were measured. To determine the plasma VC concentration at 9 wk, we measured the plasma reduced (ascorbic acid (AA)) and plasma oxidized (dehydroascorbic acid (DHA)) VC with HPLC (High-performance liquid chromatography), and then the total VC (AA + DHA: total) concentration was calculated. For the d-ROM test, we collected about 100 μL blood from a tail vein and centrifuged it immediately. Blood plasma was obtained and stored at -80°C until used.

2.2 d-ROM test

For the d-ROM test, the levels of free radicals in the body, especially hydroperoxide concentrations, were measured (units: U.CARR, 1 U.CARR=0.08 bmg/dl hydrogen peroxide) according to the optical measurement method (color reaction). The measured value indicates the degree of oxidative stress (oxidative reaction)^{11,12}.

2.3 BAP test

For the BAP test, the levels were measured (units: μM) by the reducing action of anti-oxidant materials in blood plasma. The measured values indicate the degree of anti-oxidant potential (anti-oxidant reaction). That is, the amount of blood plasma mixed with reagents containing ferric ions that was reduced to ferrous ions was measured at the decoloring level of the color reaction liquid according to the optical measurement method. The amount of reduced iron ions in blood plasma represents the anti-oxidant potential^{11,12}. The BAP/d-ROM ratio, which was calculated on the basis of the values obtained in the BAP test and the d-ROM test, indicates the degree of latent anti-oxidant potential. In other words, the latent anti-oxidant potential indicates the balance between oxidative stress and anti-oxidant potential.

2.4 Anti-ROMs test

For the anti-ROMs test, the anti-oxidant potential was measured according to how much anti-oxidant materials in blood plasma reduce Fe^{3+} to Fe^{2+} , which turns red-purple after reacting with dipyridamole¹³. The values obtained in the anti-ROMs test are the amount of reduced iron ions in blood plasma, and indicate the degree of anti-oxidant potential of rapid reactivity and slow reactivity, depending on the different reaction rates of the anti-oxidant materials. The anti-ROMs test

1 shows values of VC and Trolox (vitamin E-like material), which are quick to respond, and the anti-ROMs test 2 shows those of L-cysteine and uric acid, which are slow to respond (units: $\mu\text{Eq/l}$).

2.5 Plasma VC concentration

It seems like the amount of VC would go down during oxidative stress because VC is consumed to deal with the stress⁵). Thus, the plasma VC concentration is a marker related to oxidative stress⁵).

2.6 Senescence grading scores and weight

Senescence grading scores, which were designed to evaluate senescence more objectively, were graded according to changes in hair luster, lesions around the eyes, bend of the spine, and others¹⁴). Senescence grading scores are generally used as an acceptance criterion for the aging process. Higher scores indicate that the aging process is accelerated. In the present study, we determined the grade of senescence, taking our previous study into account¹⁴). Weight was measured using a scale for animals (KN type, Natsume Corporation).

2.7 Statistical analyses

Numerical values are presented in this study as the mean $\pm$ standard deviation, and a critical rate less than 5% was regarded as significant. SPSS (Version 19.0 for Windows) was used for statistical analyses. After testing normality and homogeneity, we performed multiple comparisons (Steel-Dwass method) based on the result of the Kruskal-Wallis test. The Mann-Whitney U test was used to compare two groups. This

study was conducted with the approval of the Animal Research Committee of Prefectural University (approval number: 50).

RESULTS

1 The oxidative stress regulation system

In the d-ROM test, all three groups showed a significant increase at 9 wk compared with baseline. Also at 9 wk, the VC 100 Group showed a significantly lower level than the other groups (Table 1). In the BAP test at 9 wk compared with baseline, the VC 100 and VC 0 Groups showed a significant decrease, but the VC 2.5 Group showed no change. However, the VC 2.5 Group showed wide fluctuations at baseline. We found no significant difference at 9 wk among the three groups. For the BAP/d-ROM ratio, all groups showed a significant decrease at 9 wk compared with baseline. Also at 9 wk, the VC 100 Group showed a significantly higher values than the other groups. No change was seen between baseline and 9 wk in the anti-ROMs test 1 and the anti-ROMs test 2 in all group. For the plasma VC concentration (total concentration), the VC 100 Group showed a significantly higher level at 9 wk compared with the other groups.

2 Senescence grading scores and weight

For the senescence grading scores, all groups showed a significant increase at 9 wk compared with baseline (Table 1). At 9 wk, the VC 100 Group showed a lower score than the

Table 1 Change in the oxidative stress regulation system and Plasma vitamin C values

	Baseline				9 wk			
	VC 100 Group (n=11)	VC 2.5 Group (n=6)	VC 0 Group (n=9)	<i>p</i> Value	VC 100 Group (n=11)	VC 2.5 Group (n=6)	VC 0 Group (n=9)	<i>p</i> Value
d-ROMs test, U.CARR	91.4 $\pm$ 5.7	89.3 $\pm$ 5.3	96.3 $\pm$ 4.3	0.053	109.7 $\pm$ 6.6*	165.3 $\pm$ 23.7*†	161.3 $\pm$ 49.5*†	<0.001
BAP test, $\mu\text{M/l}$	2624.3 $\pm$ 126.3	2547.0 $\pm$ 278.4	2613.1 $\pm$ 183.8	0.621	2139.8 $\pm$ 191.7*	2279.0 $\pm$ 166.7	2310.3 $\pm$ 168.9*	0.130
BAP/d-ROM ratio	28.81 $\pm$ 2.31	28.77 $\pm$ 4.89	27.20 $\pm$ 2.49	0.417	19.59 $\pm$ 2.23*	14.14 $\pm$ 2.61*†	15.31 $\pm$ 3.49*†	<0.004
anti-ROMs 1 test, $\mu\text{Eq/l}$	90.4 $\pm$ 31.0	113.1 $\pm$ 57.3	125.8 $\pm$ 44.4	0.304	74.4 $\pm$ 28.0	43.8 $\pm$ 24.9	71.6 $\pm$ 16.6	0.131
anti-ROMs 2 test, $\mu\text{Eq/l}$	345.1 $\pm$ 39.5	331.6 $\pm$ 92.8	381.1 $\pm$ 92.8	0.599	282.7 $\pm$ 83.1	257.0 $\pm$ 68.8	320.6 $\pm$ 54.9	0.205
Total vitamin C, μM	—	—	—		64.8 $\pm$ 19.5	9.1 $\pm$ 9.0†	4.3 $\pm$ 1.1†	<0.001
Senescence grading scores, point	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	1.000	4.1 $\pm$ 0.5*	9.4 $\pm$ 0.8*†	11.0 $\pm$ 1.0*†	<0.001
Body-weight, g	20.6 $\pm$ 1.4	19.7 $\pm$ 1.3	19.6 $\pm$ 1.0	0.249	28.5 $\pm$ 2.1*	16.5 $\pm$ 2.5*†	17.0 $\pm$ 2.1*†	<0.001

VC 100 Group: vitamin C 100%, VC 2.5 Group: vitamin C 2.5%, VC 0 Group: vitamin C 0%, d-ROMs test: plasma oxidative stresses, BAP test: plasma anti-oxidant potential, d-ROM/BAP ratio: ratio of d-ROM to BAP, anti-ROMs 1: state of rapid antioxidant defenses, anti-ROMs 2: state of slow antioxidant defenses, Total vitamin C: Asctbic acid values+Dehydroascrbic acid values, Senescence grading scores: age-related changes in senescence grading scores.

Values are mean $\pm$ SD. *p* Value by Kruskal Wallis test for the comparison between 3 groups and Steel-Dwass test for the VC 100 Group vs VC 2.5 Group or VC 0 Group. Mann Whitney test for the intergroup comparison.

* $p < 0.05$ vs baseline.

† $p < 0.05$, VC 100 Group vs VC 2.5 Group or VC 0 Group.

other groups. For the body weight, only the VC 100 Group showed a significant increase at 9 wk compared with baseline, and the other groups showed a significant decrease.

DISCUSSION

VC efficiently scavenges reactive oxygen species in cells and the blood, and the association between VC and reactive oxygen species has become clear⁶⁾. The aim of this study was to show an association between VC and oxidative stress, based on concentration differences of consumed VC. At 9 wk in this study compared with baseline, all three groups showed a significant increase in the d-ROM test and a significant decrease in the BAP/d-ROM ratio. On the other hand, among the three groups at 9 wk, the VC 100 Group showed a significantly lower level in the d-ROM test and a significantly higher BAP/d-ROM ratio. This showed that the d-ROM test value and the BAP/d-ROM ratio changed with aging regardless of concentration differences caused by VC consumption. However, we observed that the VC 100 Group controlled aging changes in the d-ROM test and in the BAP/d-ROM ratio. In other words, the VC 100 Group was able to control not only the increase in oxidative stress that occurs with aging but also the decrease in latent anti-oxidant potential. On the other hand, the VC 2.5 Group and VC 0 Group showed the increase in oxidative stress that occurs with aging and the decrease in latent anti-oxidant potential that is due to VC deficiency or similar conditions. In general, as the products of energy in the body decrease with aging, the function of the anti-oxidant enzyme systems becomes weak, and oxidative stress increases¹⁵⁾. In the presence of continuous exposure to oxidative stress, the scavenging activity of VC¹⁰⁾ and the defense mechanism provided by the coenzyme Q10 redox cycle¹⁶⁾ are working. From our results, we observed that in the VC 100 Group, the increase in oxidative stress was controlled by the scavenging activity of VC, whereas in the VC 2.5 and VC 0 Groups, oxidative stress increased due to a deficiency in the scavenging activity.

In the BAP test, no significant difference was found at 9 wk among the three groups. This showed that the BAP test is unaffected by concentration differences in consumed VC. One report has shown that in general, the d-ROM test reflects endogenous characteristics of lifestyle-related diseases (heredity, severity of disease, sleep disorder), whereas the BAP test reflects exogenous characteristics such as meal content (quantity, quality)²⁾. However, considering that the BAP test evaluates the reduction of iron ions by blood plasma, we observed

the possibility that the BAP test may reflect improvement in the systematically anti-oxidized condition (for example, the influence of anti-oxidant agents such as QH), together with the influence of VC consumption. Hereafter, comprehensively evaluating the coenzyme Q10 redox cycle and anti-oxidant activity by determining plasma QH values will be necessary. Also, at 9 wk compared with baseline, the VC 100 and VC 0 Groups showed a significant decrease in the BAP test, but the VC 2.5 Group showed no change. Generally, the function of anti-oxidant enzyme systems weakens with aging¹⁵⁾. The VC 2.5 Group may have shown no change because of the wide fluctuations at baseline. No change was seen among the groups in the anti-ROMs tests in this study, showing that the anti-ROMs tests are unaffected by concentration differences in consumed VC. In particular, the anti-ROMs test 1 shows values of VC and Trolox, which have quick reactivity. Thus, the anti-ROMs test 1, as with the BAP test, may reflect an improvement in the systematically anti-oxidized condition (for example, the influence of anti-oxidant agents such as vitamin E). Hereafter, comprehensively evaluating the anti-oxidant activity by determining plasma vitamin E values will be necessary.

The VC 100 Group showed a significantly higher plasma VC concentration (total concentration) than the other groups, showing that the plasma VC concentration is affected by concentration differences in consumed VC. Because the VC 100 Group showed a significantly lower level in the d-ROM test than the other groups, we observed an association between the d-ROM test and the plasma VC concentration. In other words, the VC 100 Group may have controlled the increase in oxidative stress that occurs with aging as a result of the scavenging activity of VC¹⁰⁾.

The VC 100 Group showed a significantly lower senescence grading score, as was seen in the d-ROM test, showing that the VC 100 Group controlled the change that occurs with aging. A close association is present between senescence and reactive oxygen species^{3,6,10)}. Thus, the VC 100 Group may have controlled the increase in oxidative stress with VC, resulting in senescence inhibition. However, the results of this study only showed changes that occurred over 9 weeks, and the idea that reactive oxygen species themselves are directly involved in the senescence process remains unproven.

CONCLUSION

We used 33 SMP30/GNL knockout mice (4 weeks old; males). Differences in consumption of VC caused a change in

the d-ROM test, the BAP/d-ROM ratio, and the plasma VC concentration.

GRANT

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要 旨

SMP30/GNL ノックアウトマウスを用いたビタミン C 摂取が 酸化ストレス防御系におよぼす影響

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摂取するビタミンC (VC) が酸化ストレス防御系などへおよぼす影響について検討した。対象は VC ノックアウトマウス (SMP30/GNL, 雄) 33 匹を無作為に, VC 100 Group: 1.5 g/L VC 摂取, VC 2.5 Group: 0.0375 g/L VC 摂取, VC 0 Group: 0 g/L VC 摂取の 3 群に区分した。酸化ストレス防御系は d-ROM test と BAP test を測定し, BAP/d-ROM 比を算出した。測定は研究開始時 (週齢 5 週: Baseline) と VC 摂取 9 週間 (週齢 14 週: 9 wk) の時点とした。また, 血漿 VC 濃度は 9 wk の時点で還元型 (アスコルビン酸: AA) と酸化型 (デヒドロアスコルビン酸: DHA) を測定し, 総 VC (AA+DHA: Total) 濃度を算出した。9 wk において 3 群間を比較すると, VC 100 Group は d-ROM test が有意な低値, BAP/d-ROM 比が有意な高値, BAP test が有意差なし, 老化度評点が有意な低値を認めた。Total 濃度において VC 100 Group は, 他の群と比較して有意な高値を認めた。摂取する VC の違いは, d-ROM test や BAP/d-ROM 比, および血漿 VC 濃度に変化をおよぼすことが明らかとなった。

キーワード: SMP30/GNL ノックアウトマウス, ビタミン C, 酸化ストレス防御系