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High-dose of vitamin C supplementation reduces amyloid plaque burden and ameliorates pathological changes in the brain of 5XFAD mice

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Blood–brain barrier (BBB) breakdown and mitochondrial dysfunction have been implicated in the pathogenesis of Alzheimer's disease (AD), a neurodegenerative disease characterized by cognitive deficits and neuronal loss. Besides vitamin C being as one of the important antioxidants, recently, it has also been reported as a modulator of BBB integrity and mitochondria morphology. Plasma levels of vitamin C are decreased in AD patients, which can affect disease progression. However, investigation using animal models on the role of vitamin C in the AD pathogenesis has been hampered because rodents produce with no dependence on external supply. Therefore, to identify the pathogenic importance of vitamin C in an AD mouse model, we cross-bred 5 familial Alzheimer's disease mutation (5XFAD) mice (AD mouse model) with ι-gulono-γ-lactone oxidase (Gulo) knockout (KO) mice, which are unable to synthesize their own vitamin C, and produced Gulo KO mice with 5XFAD mice background (KO-Tg). These mice were maintained on either low (0.66 g/l) or high (3.3 g/l) supplementation of vitamin C. We found that the higher supplementation of vitamin C had reduced amyloid plaque burden in the cortex and hippocampus in KO-Tg mice, resulting in amelioration of BBB disruption and mitochondrial alteration. These results suggest that intake of a larger amount of vitamin C could be protective against AD-like pathologies.

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Alzheimer's disease (AD) is the most common neurodegenerative disorder, characterized by amyloid plaque deposits and elevated oxidative stress.[1–3] As increased oxidative stress is believed to be an early event in AD pathology,[4,5] intake of antioxidants, including vitamin C, from the diet or as supplement can retard the development of AD, possibly by preventing or neutralizing the damaging effects of free radicals.[6] Previous study has shown that acute systemic injection of vitamin C in APP/PSEN1 mice showed the cognitive-enhancing behaviors without changing plaque deposition.[7] Also, it is reported that oral intake of vitamin C for 6 months in AD mouse model showed an attenuation of Aβ oligomerization and behavioral decline, but not reduction of brain plaque deposition.[8]

However, it remains unclear whether vitamin C directly influences brain pathology in AD patients and/or in animal models, as mouse is able to synthesize vitamin C endogenously. To examine the precise effect of vitamin C on AD pathology *in vivo*, we successfully generated a transgenic mice (knockout (KO)-Tg mice) by crossing 5 familial Alzheimer's disease mutation (5XFAD) mice, AD model mice, with mice lacking ι-gulono-γ-lactone oxidase (Gulo), the enzyme required for the biosynthesis of ascorbic acid.[9] These mice have heavy depositions of amyloid plaques in the brain and are unable to synthesize vitamin C *in vivo*.

Vitamin C accumulates in the central nervous system and its level in the brain is much higher than that in plasma or in other organs.[10] Plasma levels of vitamin C are lower in AD patients than in healthy individuals.[11] Furthermore, deficiencies in vitamin C level are related cognitive impairment.[7,8] Recently, it has been reported that treatment with vitamin C prevents compression-induced blood–brain barrier (BBB) disruption and both low and high vitamin C levels have an impact on the number and size of mitochondria.[12,13] As BBB disruption and mitochondrial dysfunction are well-known risk factors for AD pathogenesis,[2,14,15] we hypothesized that vitamin C may be associated with AD-like pathologies.

In KO-Tg mice, the mice were maintained on a low level of vitamin C supplementation (0.66 g/l, 2-fold above the minimum anti-scurvy level) or a high level (3.3 g/l, 10-fold level above the minimum requirement) to determine the direct effect of vitamin C on AD pathology.

We have found that sufficient vitamin C supplementation significantly reduces amyloid deposition in both the cortex and

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Abbreviations: BBB, blood–brain barrier; AD, Alzheimer's disease; Aβ, amyloid β-peptide; 5XFAD, 5 familial Alzheimer's disease mutations; Gulo, ι-gulono-γ-lactone oxidase; TJ, tight junction; GFAP, glial fibrillary acidic protein; KO, knockout; APP, β-amyloid precursor protein
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the hippocampus, ameliorates BBB disruption by preventing tight junction (TJ) structural changes and morphological changes in the mitochondria. Humans cannot synthesize vitamin C themselves *in vivo*,[16,17] therefore, the KO-Tg mouse model is better able to imitate the condition of AD patients. Our data suggest that sufficient dietary vitamin C may be an important factor in preventing the progression of AD pathology.

Results

Amyloid plaque burden is decreased in the cortex and hippocampus of the high-dose supplemented group of 5XFAD mice. The 5XFAD mice used in the present study display amyloid deposition at a very early age.[9,18] Histopathologically, AD is characterized by the presence of extracellular senile plaques consisting of β-amyloid (Aβ) peptide in both the frontal cortex and hippocampus.[19] To evaluate whether vitamin C supplementation can change amyloid plaque burden in the frontal cortex and hippocampus, we quantified deposited Aβ plaques following immunohistochemistry using the Aβ-specific antibody 4G8 in each group. All KO-WT mouse groups showed no Aβ plaque formation and all KO-Tg mice had a number of amyloid plaques in the frontal cortex and other regions (Figure 1a). Areas from the motor (b and c in white dotted line) and somatosensory (a and d in white dotted line) cortices in each hemisphere were magnified. Compared with the low-dose supplemented KO-Tg mice, KO-Tg mice with the high dosage of vitamin C showed a reduced number of plaques in the frontal cortex (Figure 1b). There was no amyloid plaque accumulation in any of the KO-WT mouse group. Decreased amyloid plaques were observed in the hippocampus of the high-dose vitamin C-treated KO-Tg mice (Figure 1c). Magnified images of the hippocampus showed significantly less amyloid plaque deposition in the high-dose vitamin C-treated KO-Tg mice (Figure 1d). The relative area covered with amyloid plaques was then quantified in each cortex and hippocampus (Figure 1e), $n=4$, respectively. There was a significant decrease of 4G8-positive plaques in the cortex (1.6-fold, $P<0.001$) and hippocampus (1.4-fold, $P<0.05$) from the high-dose supplemented group compared with those of the low-dose supplemented group. These findings suggest that the higher dose of vitamin C intake could reduce amyloid plaque deposition in the brain.

Cerebral capillaries are less impaired in the brains from the high-dose supplemented group of 5XFAD mice. Previously, we had observed disrupted microvessels near Aβ plaque-deposited areas in the brains of 5XFAD mice by using an anti-GLUT-1 antibody.[20] Because the expression of GLUT-1 is limited to the endothelium of the BBB, decreased GLUT-1 levels in AD implicate the increased permeability of the BBB,[21] which is the reason that GLUT-1 is used as an endothelial marker. Moreover, reactive oxygen species (ROS) significantly contribute to BBB dysfunction.[22] To assess whether vitamin C affects the change of cerebral capillaries in the KO-Tg mice, we performed immunostaining with both anti-GLUT-1 and anti-Aβ (4G8) antibodies. Both KO-WT mice groups showed long tube-like form and no 4G8 immunoreactivity and there was no difference in the density of GLUT-1-stained vessels in between the low- and high-doses supplemented KO-WT groups (Figure 2A). In contrast, compared with the KO-WT mice, the KO-Tg mice receiving the lower vitamin C dose had disrupted vessels in the cortex near the areas containing amyloid plaques deposits (Figure 2B, yellow dotted circle). Capillaries stained with GLUT-1 in the cortex of KO-Tg mice that received the high-dose vitamin C regimen showed a prevention of disrupted GLUT-1 staining around the areas of decreased plaque burden (Figure 2B, white dotted circle). To clearly observe the modification of cerebral capillaries by senile plaques in the crossed KO-Tg mice model, we used a super-resolution SIM (Nikon, Tokyo, Japan). Although the reconstructed 3D-SIM image showed that cerebral capillaries of the KO-WT mouse group remained intact (Figure 2C), those of the KO-Tg mouse group showed damaged and collapsed structure (Figure 2D). There were less disruptions and gaps in the capillary vessels stained with GLUT-1 in the high-dose vitamin C-supplemented-KO-Tg mice as compared with the low-dose supplemented KO-Tg mice. These results suggest that sufficient vitamin C supplementation ameliorates in impaired cerebral capillaries in the brains of 5XFAD mice.

Alteration of cerebral TJs was decreased in the high-dose supplemented group of 5XFAD mice. The BBB is a specialized brain endothelial system of fully differentiated neurovascular structures, which has an important role in regulating and restricting the transport of various molecules across the brain endothelium.[23,24] TJs between endothelial cells in brain capillaries are the most prominent feature of the BBB and are responsible for its integrity.[20] The structure of TJs in the brain endothelium has been revealed by electron microscopy (EM) in previous studies.[25] The changes of cerebral TJs in the cerebral capillaries of all KO-WT and KO-Tg mice were examined by EM to identify vascular endothelial integrity and the extent of BBB damage. Representative EM images of the TJs of all KO mice groups receiving the low-dose supplementation of vitamin C showed that TJs of KO-Tg mice appeared to be significantly shorter than TJs seen in KO-WT mice (Figure 3a, arrows). Ten TJs from all KO mice used in this experiment were measured. Interestingly, the average length of TJs from the high-dose vitamin C-treated KO-Tg mice (1225.6 ± 553.4 nm) was significantly two times longer than the low-dose supplemented KO-Tg mice (Figure 3b; $n=10$, 624.63 ± 182.94 nm; $P<0.05$).

Gliosis were decreased in the brains from the high-dose supplemented group of 5XFAD mice. Astrocytes, which make up about 50% of the cells in the cortex, regulate BBB function by interacting with endothelial cells and neurons.[23] Neuroinflammation in the area of amyloid plaques was observed in the brains of 5XFAD and AβPP/PS1 mice as demonstrated by astrocytic reactivity.[9,26] To identify the effect of vitamin C supplementation on neuroinflammation, we examined whether activation of astrocytes is altered by using a reactive astrocyte marker protein, GFAP. A representative picture for each group of the low- and high-doses supplemented KO-Tg mice is shown (Figure 4a).

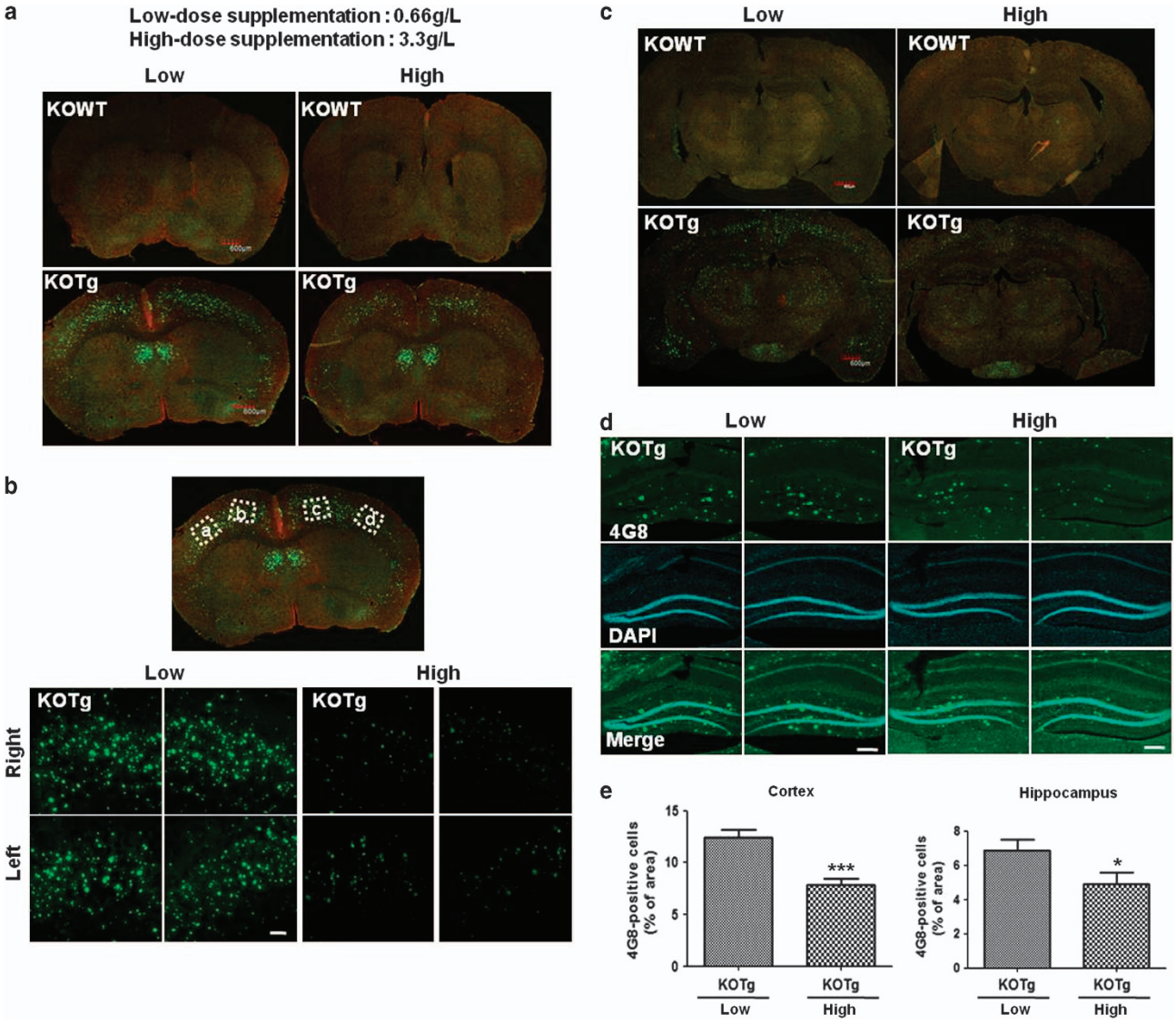
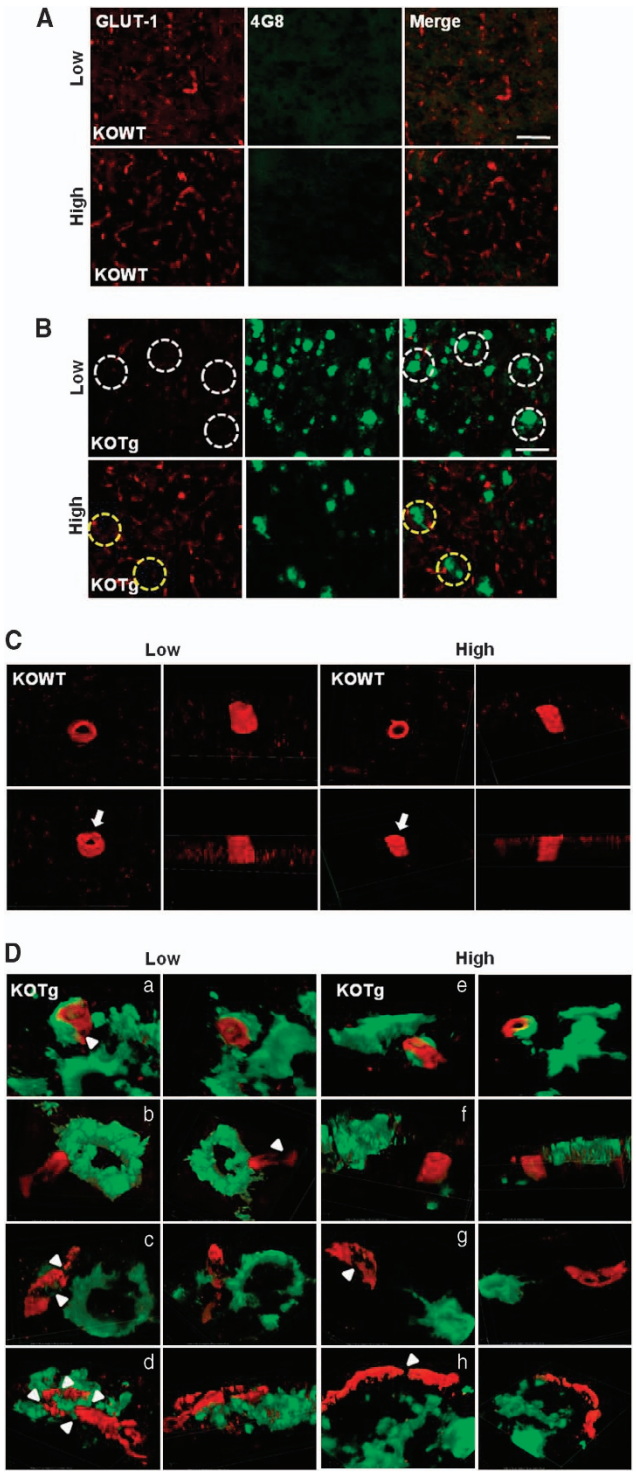


Figure 1 Amyloid plaque burden is decreased in the cortex and hippocampus of the high-supplementation group of 5XFAD mice. (**a**) Coronal serial sections of brains from 6-month-old mice ($n=4$ for each of the groups) were stained with anti-Aβ antibody (green; 4G8) and imaged by confocal microscopy. There were no signals in the frontal cortex of KO-WT mice in either of the two treatment. 4G8-positive areas were decreased in the frontal cortex of KO-Tg on the high-dose vitamin C treatment. Scale bar = 600 μm. The section shown is at the interaural level, 4.78 mm; Bregma, −0.98 mm (Fig. 23 in Franklin and Paxinos[57]). (**b**) Frontal cortex of KO-Tg mice of both treatment groups was magnified. (**a** and **d**) Somatosensory region; (**b** and **c**) motor region. Scale bar = 100 μm. (**c**) Coronal serial sections of brains from 6-month-old mice ($n=4$ for each of the groups) were stained with anti-Aβ antibody (green; 4G8) and imaged by confocal microscopy. There were no signals in the hippocampus of KO-WT mice in either treatment groups. Plaques were particularly decreased in the hippocampus of KO-Tg on the high-dose vitamin C treatment. Scale bar = 600 μm. The section shown is at the interaural level, 1.86 mm; Bregma, −1.94 mm (Fig. 47 in Franklin and Paxinos[57]). (**d**) Hippocampus of KO-Tg mice of both treatment groups was magnified. Nuclei were stained with 4′-6-diamidino-2-phenylindole. Scale bar = 100 μm. (**e**) Compared with the low-dose supplemented KO-Tg mice, the high-dose supplementation KO-Tg mice exhibited significantly less plaque burden in the cortex and hippocampus. Both *P* values (***$P<0.001$ and *$P<0.05$) mean the high-dose *versus* the low-dose supplemented KO-Tg group

Boxed regions of the cortex of these images were magnified, and a significantly reduced GFAP immunoreactivity was observed in the cortex of the high-dose vitamin C-treated KO-tg mice compared with that of the low-dose supplemented KO-Tg mice (Figures 4b and c; $n=4$, 1.5-fold; $P<0.05$).

Abnormal mitochondrial morphology was prevented in the brains from the high-dose supplemented group of 5XFAD mice. Mitochondrial dysfunction *in vitro* and AD mouse models have been reported previously.[27–30] Although a recent study has demonstrated that vitamin C could inhibit mitochondrial membrane potential depolarization by neurotoxicity of Aβ_{42} peptides *in vitro,*[31] the precise effect of vitamin C supplementation on mitochondrial morphology change in the AD mouse model is unknown. We examined the morphology of mitochondria in the brains of all 6-month-old KO mice by EM and observed more distinctive structural changes in some of the mitochondria of the KO-Tg mice receiving low-dose vitamin C-supplemented water. Compared with all KO-WT mouse groups, the

low-dose supplemented KO-Tg mice showed abnormal mitochondrial morphology. In addition, we found that compared with KO-Tg mice receiving low-dose vitamin C, KO-Tg mice receiving high-dose vitamin C had avoided severe alterations of mitochondrial morphology such as loss of cristae like a vacant hole and a smaller diameter (Figure 5).



Discussion

As vitamin C acts as a vital antioxidant molecule in the brain and neurodegenerative diseases typically involve high levels of oxidative stress, a number of reports have suggested that vitamin C has potential therapeutic roles against AD, ischemic stroke, Parkinson's disease and Huntington's disease.[32–35] Although vitamin C treatment showed better amelioration in learning and behavioral deficits in APP/PSEN1 transgenic mice,[7,8,36] there is disagreement about its effects on plaque burden and no evidence about the direct effects on AD-like pathology. Unlike humans, mice can synthesize vitamin C endogenously. In this study, we successfully made the first-line 5XFAD mice lacking Gulo and therefore unable to produce vitamin C endogenously, mimicking the situation in humans and primates. This model is optimal for confirming the direct effect of vitamin C on AD pathology *in vivo*. Moreover, as AD is characterized not only by the presence of amyloid plaque burden but also by BBB disruption and mitochondrial dysfunction, the current results regarding the effect of vitamin C on amyloid plaque burden, BBB alteration and impaired mitochondria suggest that vitamin C has an important role in the pathogenesis of AD.

Although a previous study demonstrated that plaque burden was not altered by the dietary intake or systemic injection of vitamin C,[7,8] the exact effect of vitamin C on amyloid plaque burden is unclear because the mice used in that study could endogenously produce vitamin C. However, in our study, we clearly confirmed that chronic administration with higher dose supplementation of vitamin C significantly decreased the plaque burden in the cortex (by 57.9%) and hippocampus (40.29%) of 6-month-old KO-Tg mice (Figure 1). It has been reported that dehydroascorbic acid modulates Aβ precursor protein (APP) processing by decreasing C-terminal fragment products of both α-amyloid protein and APP and full-length APP, and that adequate dietary dehydroascorbic acid could be protective against Aβ accumulation.[37] Figure 1 shows that vitamin C is directly related to amyloid plaque deposition, suggesting that sufficient dietary intake of vitamin C has a crucial role in protecting against Aβ-induced oxidative stress.

Figure 2 Cerebral capillaries are less impaired in the brains from the high-supplementation group of 5XFAD mice. (**A**) Coronal serial sections of brains from 6-month-old mice ($n = 4$ for each from standard and decreased groups) were co-immunostained with anti-GLUT1 (red) and anti-Aβ (green; 4G8) antibodies and imaged by confocal microscopy. Capillaries stained with anti-GLUT1 antibody (red) showed long tubular-like form in KO-WT mice of both treatment groups. (**B**) KO-Tg mice displayed amyloid plaque deposition (green) and cut capillary forms. Capillaries adjacent to the amyloid plaques displayed disconnected tubular-like form in KO-Tg mice (shown in the yellow dotted circle). Decreased impairment was observed in the high-dose supplemented KO-Tg mice than the low-dose supplemented KO-Tg mice (shown in the white dotted circle). Scale bar = 50 μm. (**C**) 3D-SIM images of the brains from the KO-WT mice in both treated groups. (**D**) 3D-SIM images of the brains from both the low-dose supplemented KO-Tg (a–d) and the high-dose supplemented KO-Tg mice (e–h). Brain slices were recorded on 3D-SIM images along *z* axis with a thickness of 0.15 μm, reconstructed and 3D volume images were created with the alpha blending function. Axial directions are represented on each image. Capillaries stained with anti-GLUT1 antibody (red) and amyloid plaque stained with anti-Aβ (green; 4G8) antibody. Arrow, sectioned *z* axis image; arrow head, damaged micro-vessel. Scale bars = 2 μm. 3D depth, 2.8–4.5 μm in the high-dose supplemented KO-Tg and 3.9–7.6 μm in the low-dose supplemented KO-Tg mice

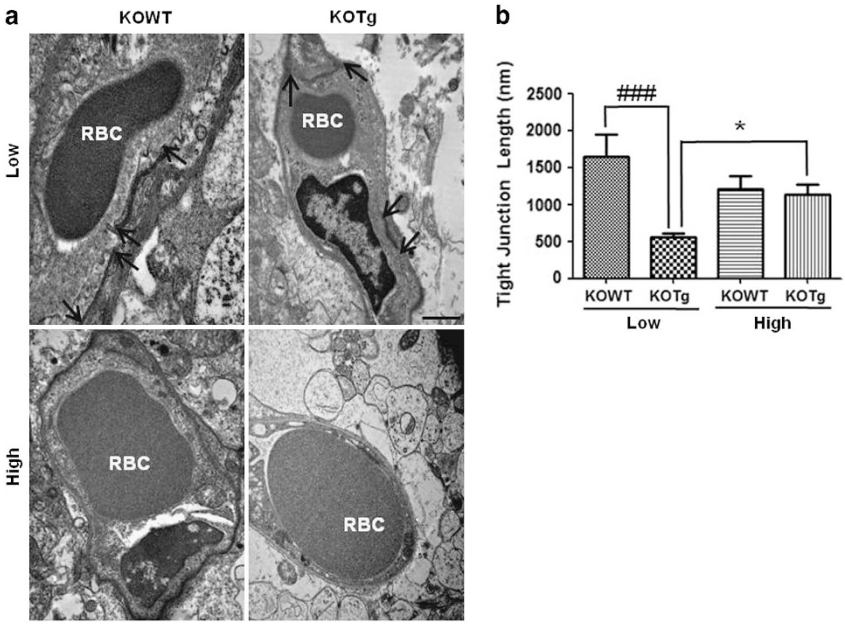


Figure 3 Alteration of cerebral tight junctions (TJs) decreased in the high-supplementation group of 5XFAD mice. (**a**) EM pictures revealed that TJs were altered in the brains of KO-Tg mice as compared with KO-WT mice. TJs of KO-Tg mice were shorter than that of KO-WT mice in the low-dose supplemented groups, and TJs of the high-dose supplemented KO-Tg mice were longer than that of standard vitamin C-treated-KO-Tg mice. $n = 4$ for each cortex from all four groups. Arrow, TJs; RBC, red blood cells. Scale bar $= 2\,\mu$m. (**b**) Length of eight TJs from all four groups was examined and the high-dose supplemented KO-Tg mice had longer (1,225.6 nm) TJs as compared with standard vitamin C-treated-KO-Tg mice (624.2 nm). Graph shows an average of eight capillaries. $^{*}P < 0.05$ means the high-dose *versus* the low-dose supplemented KO-Tg and $^{\#\#\#}P < 0.001$ means the low-dose supplemented KO-Tg *versus* the low-dose supplemented KO-WT mice

Another interesting finding of this study is the influence of vitamin C on cerebral capillaries, which suggests that vitamin C may be more than a micronutrient in the CNS.[38] BBB breakdown is now considered as the basis of initiation and progression of AD, because it reflects abnormal functioning of the BBB that modulates efflux and influx of various molecules including Aβ. Previously, by using a cerebral endothelial marker in brains of 5XFAD mice, we could observe disrupted cerebral capillaries in the location where amyloid plaques were accumulated.[20] Several studies have shown that Aβ induces BBB changes mediated by oxidative stress.[39,40] In this study, we observed that the high dose of vitamin C in KO-Tg mice was associated with an increase of positive GLUT-1 staining and reduced morphological perturbations of capillaries. Using confocal microscopy and 3D-SIM images, we found that cerebral capillaries of high-dose vitamin C-treated KO-Tg mice had scratches over the vessel, but were less damaged than that of low-dose vitamin C-treated KO-Tg mice (Figure 2). In addition, the EM study showed significantly reduced alterations in TJ morphology in the high-dose vitamin C-treated-KO-Tg mice brains (Figure 3). The length of TJs of the capillaries is directly related to endothelial structure and function. We suggest that the longer TJ in the high-dose supplemented vitamin C-treated KO-Tg mice indicates an increase in the number of TJ proteins and the transport of various molecules. Oxidative stress has a key role in the pathogenesis of the AD, contributing to the degeneration of the basal forebrain cholinergic system and general cell death.[41,42] AD patients have been found to have lower plasma and CSF ascorbate levels despite adequate nutritional intake.[43,44] Levels of BACE1 are increased in vulnerable regions of AD brains, but oxidative stress induced JNK-mediated increased expression of BACE1 and promoted production of Aβ levels.[45] ROS generated by oxidative stress alter brain endothelial TJ dynamics via RhoA, PI3 kinase and PKB signaling.[22] TJs are responsible for the BBB integrity and impaired TJ increases paracellular permeability.[46] Therefore, it may be presumed that sufficient vitamin C supplementation may ameliorate BBB alteration by reducing amyloid plaque deposition and ROS generation via above intracellular signaling cascades. Oxidative stress is one of the earliest changes in AD and mitochondria are the major source of ROS.[47] Increased oxidative stress levels and mitochondrial dysfunction have long been implicated in the onset of the familial and sporadic forms of AD.[48,49] It has been reported that vitamin C enters mitochondria via the facilitative glucose transporter (Glut1). In addition, vitamin C that accumulates in mitochondria (mtAA) could protect against oxidative stress by quenching mitochondrial ROS.[50] Little is known about the effects of vitamin C on mitochondrial morphology in AD mice brains. Interestingly, EM evidence indicates that the high-dose vitamin C supplementation in KO-Tg mice leads to the prevention of abnormal mitochondrial morphology (Figure 5). This finding suggests that amyloid plaque disrupts the transport of vitamin C into mitochondria via altered Glut1 and induce extracellular oxidative damage that cannot be blocked by insufficient levels of vitamin C. The structure of mitochondria features a double-membrane construction involving an outer and an inner membrane. Particularly, the inner membrane is the main barrier to metabolites and protein transporter.[51] In addition, the mitochondrial inner

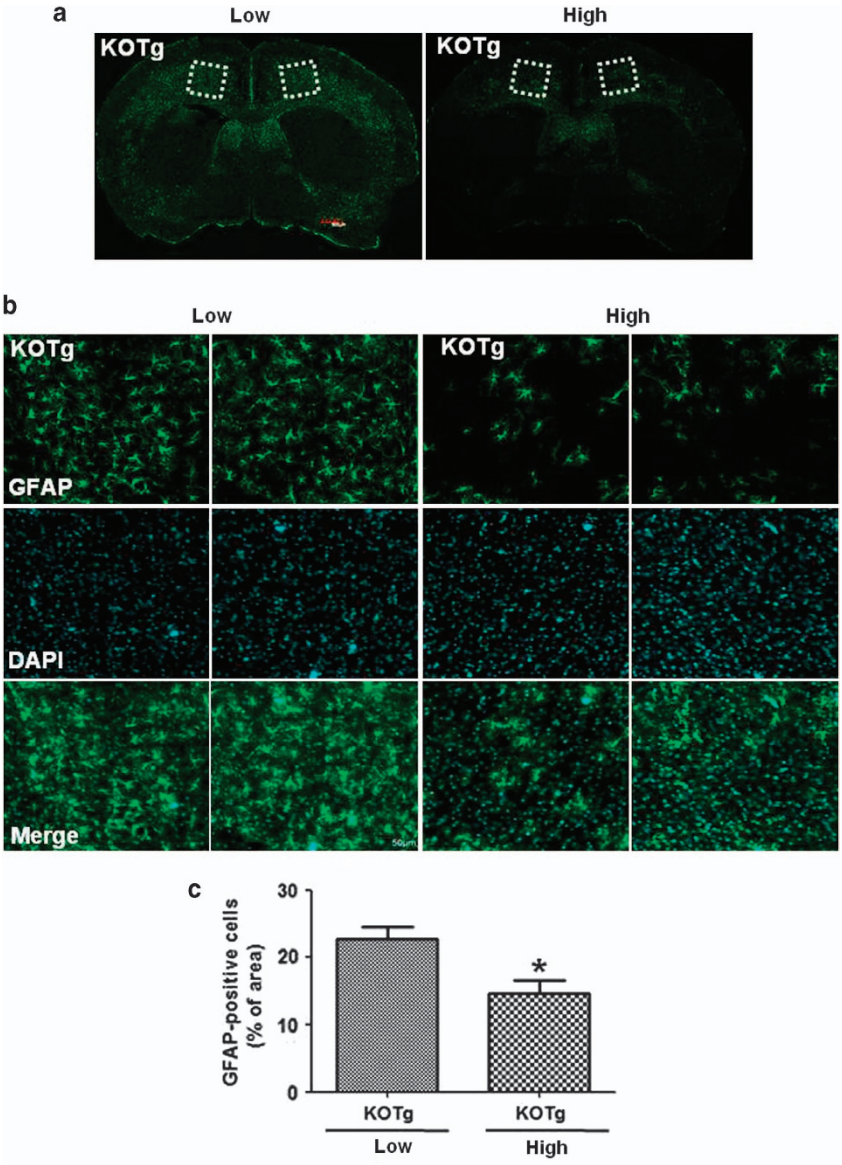


Figure 4 Gliosis decreased in the brains from the high-supplementation group of 5XFAD mice. (**a**) Coronal sections stained with anti-GFAP antibody (green) in the cortex of each KO-Tg group. Scale bar = 600 μm. (**b**) Boxed areas of **a** were magnified. Compared with standard vitamin C-treated KO-Tg mice, the high-dose supplemented KO-Tg had 54.5% less GFAP-positive cells. Nuclei were stained with 4′-6-diamidino-2-phenylindole. Scale bar = 50 μm. (**c**) Quantification of GFAP immunoreactivity. GFAP-positive cells were significantly decreased in the high-dose supplemented KO-Tg mice than standard vitamin C-treated KO-Tg mice (*$P<0.05$)

membrane generates an ATP energy source using a respiratory chain with complexes I–IV.[52] We also confirmed functional changes following mitochondrial morphological changes in *in vitro* AD conditions.[30] These results demonstrate that mitochondrial morphology is responsible to maintain and regulate its function. As mitochondria are also dynamic organelles undergoing fission and fusion,[53] the low dose of vitamin C supplementation to KO-Tg mice may contribute to impaired mitochondrial function caused by an imbalance of mitochondrial fission/fusion. Although we did not perform any experiments involving synaptic plasticity in these mice, brains from the low-dose vitamin C-treated-KO-Tg mice might have the synaptic alteration. This is because synaptic terminals have abundant mitochondria and faulty mitochondria lead to synaptic dysfunction in the axons or dendrites of neurons.[54,55]

Collectively, our data suggest that sufficient vitamin C supplementation results in reduction of amyloid plaque deposition, BBB disruptions and mitochondrial dysfunction in the brains of 5XFAD mice, an animal model for AD. Although the precise mechanism of vitamin C intake-induced changes of AD pathologies shown in KO-Tg remains to be elucidated, these results suggest that vitamin C supplementation could be an important pharmacological avenue for the treatment of AD and the optimal vitamin C dose is necessary for the prevention of AD pathogenesis. How sufficient vitamin C supplementation could ameliorate amyloid plaque generation, BBB disruption and mitochondrial dysfunction will need to be investigated.

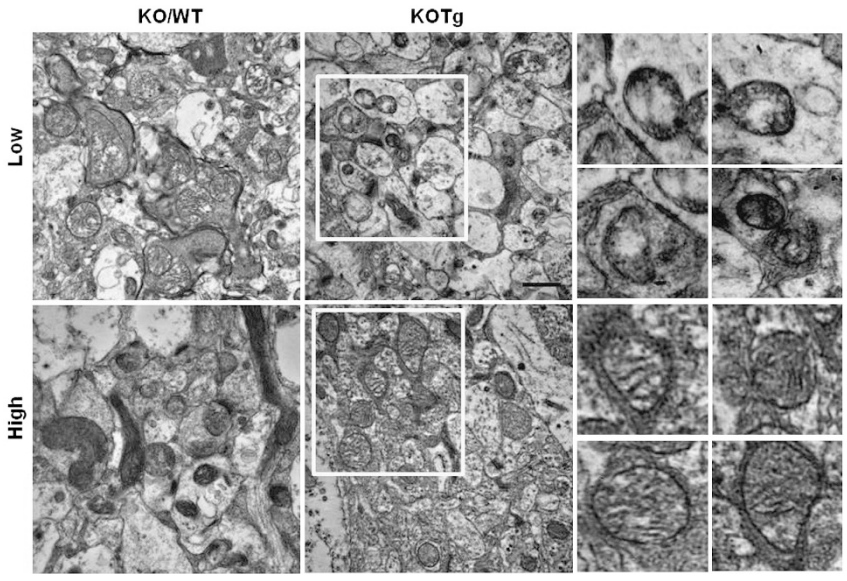


Figure 5 Abnormal mitochondrial morphology was prevented in the brains from the high supplementation group of 5XFAD mice. Representative EM from the brains of all four groups shows mitochondrial morphology. Boxed areas of KO-Tg image were magnified for each figure. KO-WT mice showed no alterations of mitochondrial structure. The high-dose supplemented KO-Tg mice had avoided impaired mitochondria than standard vitamin C-treated KO-Tg mice. Scale bar = 1 μm

Materials and Methods

Animals. Female transgenic mice with five familial AD mutations (5XFAD, mixed b6/SJL background) were purchased from Jackson Lab (Bar harbor, ME, USA). These mice co-express and co-inherit FAD mutant forms of human APP (the Swedish mutation: K670N, M671L; the Florida mutation: I716V, and London mutation: V717I) and PS1 (M146L; L286V) transgenes under transcriptional control of the neuron-specific mouse Thy1 promoter.[9] Homozygote mutant C57BL/6 mice lacking a functional gene for Gulo (L-gulono-γ-lactone oxidase; Gulo KO mice) were provided by Dr. Lee, Wang Jae (Seoul National University, College of Medicine, Seoul, Korea). Heterozygous transgenic 5XFAD mice (B6/SJL hybrid background) were crossbred to Gulo KO mice. The resultant F1 heterozygous *Gulo*$^{+/-}$ 5XFAD was further intercrossed, yielding animals with four different genotypes (wild type, 5XFAD, Gulo KO · wild type and Gulo KO · 5XFAD) in the F2 progeny. For genotyping by PCR, three primers Gulo2 (5′-CGCGCCTTAATTAAG GATCC-3′), Gulo3 (5′-GTCGTGACAGAATGTCTTGC-3′) and Gulo4 (5′-GCATCC CAGTGACTAAGGAT-3′) were used. A 230-bp and/or 330-bp fragment derived from the targeted and endogenous locus was used to distinguish among wild-type, homozygote and heterozygote mice. Kim *et al.*[56] demonstrated that various doses of vitamin C supplementation in *Gulo*$^{-/-}$ mice showed remarkable differences in plasma level of vitamin C. For example, 0.33 g/l vitamin C-supplemented *Gulo*$^{-/-}$ mice: 24.88 μM (similar plasma level to scurvy) and 3.3 g/l vitamin C-supplemented *Gulo*$^{-/-}$ mice: 88.41 μM (similar plasma level to wild-type mice). Most organ concentration of vitamin C in *Gulo*$^{-/-}$ mice supplemented with 0.33 g/l vitamin C was similar to the levels of *Gulo*$^{-/-}$ mice with vitamin C withdrawal for 1–2 weeks. Therefore, we supplemented the drinking water for the Gulo KO mice until 8 weeks of age with vitamin C at 0.66 g/l, twofold above the minimum of 0.33 g/l, to ensure normal development. After 8 weeks of age, the supplementation was elevated to a higher dose of 3.3 g/l to the high-supplementation group. This timing of supplementing different doses was chosen as 5xFAD mice normally start to generate amyloid plaque from 2 months of age. All experiments were performed with 6-month-old male mice. Animal treatment and maintenance were performed according to approved protocols and the principles of laboratory animal care as outlined by NIH publication No. 85-23 (1985 revision) and the Animal Care and Use Guidelines of Seoul National University, Seoul, Korea. Every effort was made to minimize animal distress and to reduce the number of mice used.

Immunohistochemistry. For immunohistochemistry, *Gulo*$^{-/-}$ wild-type (KO-WT; 0.66 g/l), *Gulo*$^{-/-}$ 5XFAD (KO-Tg; 0.66 g/l), *Gulo*$^{-/-}$ wild-type (KO-WT; 3.3 g/l), *Gulo*$^{-/-}$ 5XFAD (KO-Tg; 3.3 g/l; $n = 4$ for each group) mice were killed at 6 months of age. Mice were anesthetized with a mixture of Zoletil 50 (Virbac, Carros, France) and Rompun (Bayer Korea, Seoul, Korea) solution (3 : 1 ratio, 1 ml/kg, i.p.) and perfused transcardially with a freshly prepared solution of 4% paraformaldehyde in PBS. After the mice were decapitated, their brains were dissected from the skull. Serial 30-μm-thick coronal tissue sections were cut using a freezing microtome (Leica, Nussloch, Germany). Free-floating sections were incubated with the following the primary antibodies: biotin-labeled 4G8 (1 : 1000; Covance, Princeton, NJ, USA), rabbit anti-GLUT-1 (1 : 1000; Millipore, Schwalbach, Germany) and anti-glial fibrillary acidic protein (GFAP, 1 : 2000; Zymed Laboratories Invitrogen, Carlsbad, CA, USA) overnight at 4 °C. After washes in PBS, the sections were incubated with the following secondary antibodies: Alexa Fluor 488-conjugated streptavidin (1 : 1000; Invitrogen, Carlsbad, CA, USA), goat anti-rabbit Alexa 594 (1 : 1000; Invitrogen) and goat anti-rat Alexa 488 (1 : 1000; Invitrogen) for 2 h. All sections were counterstained with 4′-6-diamidino-2-phenylindole before mounting and analyzed on a confocal laser scanning microscope (FluoView FV 10i; Olympus, Center Valley, PA, USA)

Quantification of immunoreactivity. Four sections (100-μm apart) from each mouse were used for analysis. Immunofluorescence images of the cerebral frontal cortex and hippocampus were taken using a fluorescence microscope (FluoView FV 10i; Olympus). To analyze amyloid plaque burden and GFAP, the number of immunofluorescence-positive pixels in the cerebral frontal cortex and hippocampus areas from the acquired images was analyzed using the Image J processing software (National Institutes of Health, Bethesda, MD, USA).

3D-SIM image acquisition. To identify the modification of cerebral capillaries by senile plaque, we used the super-resolution Structured Illumination Microscope (Nikon N-SIM, Nikon). 3D-SIM images of each fixed brain slices were taken by moving the stage in the *z*-direction with a step size of 0.15 μm. The sequential *z*-sections were reconstructed to create a 3D-SIM image (*z* axis; brain slices thickness about 5.0 ± 0.4 μm) and produce the 3D-deconvolution with alpha blending function using NIS-E software (Nikon). Images were taken with an Eclipse Ti-E inverted research microscope, using CFI Apo TIRF × 100 oil objective lens (NA = 1.49, Nikon) and 512 × 512-pixel resolution iXon DU-897 EMCCD camera (Andor Technology, Belfast, UK). Multicolor fluorescence analysis was performed using a diode laser (488, 561 nm; exposure time, 40 ms; EM gain, 150; conversion gain, × 1), and images were processed with the NIS-E software (Nikon, Tokyo, Japan) and exported to the Adobe Photoshop program.

Transmission EM. Several cerebral cortex pieces from each mouse group were randomly excised, diced (1 mm^3) and fixed overnight at 4 °C in a mixture of cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 2% paraformaldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.2) and

embedded with epoxy resin. The epoxy resin-mixed samples were loaded into capsules and polymerized at 38 °C for 12 h and 60 °C for 48 h. Thin sections were sliced on an ultramicrotome (RMC MT-XL) and collected on a copper grid. Appropriate areas for thin sectioning were cut at 65 nm and stained with saturated 4% uranyl acetate and 4% lead citrate. The ultrastructure of TJs and mitochondria of the brain was then examined by a transmission EM (JEM-1400, JEOL, Tokyo, Japan). TJs were measured, and the averages were determined ($n = 10$ for each group).

Statistics. All data are expressed as mean ± S.E.M. Statistical analysis was performed using GraphPad Prizm4 (GraphPad, San Diego, CA, USA). The data were analyzed by one-way ANOVA with Tukey's multiple-comparison test or unpaired *t*-test, as appropriate ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Conflict of Interest

The authors declare no conflict of interest.

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