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RESEARCH ARTICLE

Age and Dietary Vitamin C Intake Affect Brain Physiology in Genetically Modified Mice Expressing Human Lipoprotein(A) and Unable to Synthesize Vitamin C

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ARTICLE HISTORY

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Abstract: ***Aims*:** Lipoprotein (a) deposition in coronary vascular plaques and cerebral vessels is a recognized risk factor for cardiovascular disease, and research supports its role as a "repair factor" in vascular walls weakened by vitamin C deficiency.

***Background*:** Humans depend on dietary vitamin C as an important antioxidant and as a cofactor in collagen synthesis, yet are prone to vitamin C deficiency. The brain is the one with the highest vitamin C content, owing to its high oxygen consumption and oxidative stress. It has been shown that brain aging is accompanied by accumulated oxidative damage, which can lead to memory decline and neurological diseases.

***Objective*:** Our transgenic mouse, Gulo (-/-); Lp(a)+, presents a unique model for the study of key aspects of human metabolism with respect to a lack of internal vitamin C synthesis and the production of human lipoprotein(a).

***Methods*:** This mouse model was used in our study to investigate the effects of prolonged intake of low and high levels of vitamin C, at different ages, on oxidative damage, cholesterol levels and lipoprotein(a) deposition in the brain.

***Result*:** The results show that a long-term high vitamin C intake is important in maintaining brain cholesterol homeostasis and preventing oxidative damage in Gulo(-/-);Lp(a)+ mice as they age. Moreover, we observed that the formation of brain lipoprotein(a) deposits was negatively correlated with brain level of vitamin C, thereby confirming its role as a stability factor for an impaired extracellular matrix.

***Conclusion*:** Our study emphasizes the critical role of vitamin C in protecting brain health as we age.

***Other*:** Our findings show that optimal vitamin C intake from early life to old age is important for brain health as it prevents oxidative stress damage and maintains cholesterol homeostasis in the brain. More importantly, the negative correlation between brain ascorbic levels and the formation of Lp(a) deposit on the choroid plexus further emphasizes the critical role of vitamin C in protecting brain health throughout the normal aging process.

Keywords: Vitamin C, human lipoprotein(a), brain physiology, transgenic mice, Gulo (-/-);Lp(a)+, antioxidant.

1. INTRODUCTION

Age-associated accumulation of oxidative damage is a contributing factor to memory decline and increased risk of neurological diseases [1, 2]. Vitamin C deficiency, which is implicated in reduced antioxidant defense in the brain, is related to increased risk of cognitive problems and cerebral diseases as well as accelerated amyloid accumulation in the course of normal aging [3-6]. Higher vitamin C intake in elderly individuals is associated with reduced cognitive decline and a lower risk of Alzheimer's disease [7-9].

Vitamin C is not only an important antioxidant but it also regulates collagen production and serves as a cofactor in the hydroxylation of proline and lysine to form a triple helix during collagen biosynthesis [10]. This makes vitamin C essential in maintaining the stability of connective tissue and the integrity of the vascular walls.

Surprisingly, vitamin C deficiency has been frequently reported in Western populations [11-13]. An estimated 25%

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of men and 16% of women in the low-income population in the UK had plasma vitamin C levels lower than 11umol/L, which is characteristic of scurvy [14]. A study in Canadian men and women aged 20-29 years showed that about 1 in 7 had vitamin C deficiency [15]. Aging is also associated with a risk of vitamin C deficiency owing to eating habits, accompanying diseases, and physiological changes [16].

Human dependency on dietary vitamin C intake stems from an inability to synthesize vitamin C endogenously, owing to the loss of the gene encoding for gluconolactone oxidase (Gulo), a key enzyme in the ascorbic acid biosynthesis pathway. This genetic change, which occurred in our primate ancestors about 60 million years ago, coincided with the emergence of Lp(a).

Lp(a) is a low-density cholesterol (LDL)-like lipoprotein, which contains a large protein, apolipoprotein(a) (apo(a)), linked by a disulfide bridge to the LDL protein component, apolipoprotein B-100 (apo(B)). The presence of Lp(a) is characteristic of human metabolism, and has been detected only in a very few animal species within a subset of primates [17]. Rath and Pauling suggested that owing to the presence of a large adhesive apo(a) protein, the Lp(a) can function as a repair molecule in the vascular walls, compensating for extracellular matrix (ECM) weakness, most often triggered by vitamin C deficiency [18, 19]. As this biological repair process overshoots, the large amount of lipids carried by this lipoprotein can turn this initial vascular repair process into atherosclerotic plaques [19]. We have shown earlier that loss of vascular endothelial integrity (increased endothelial gaps), observed during vitamin C deficiency, results in increased Lp(a) binding and the formation of atherosclerotic deposits in the vascular wall [20, 21].

Most studies of Lp(a) relate to coronary atherosclerosis. However, a study of random autopsies indicated that deposition of apo(a) was positively correlated with the degree of atherosclerosis detected in cerebral blood vessels [22]. Lp(a) has also been considered a risk factor for cerebrovascular disease and Alzheimer's disease [23, 24]. While apo(a) transcripts have been found in the brain and other organs [25], circulating apo(a) is synthesized mainly by the liver and secreted into the bloodstream [26]. There, it binds to circulating LDL to generate complete Lp(a) particles [27]. High Lp(a) concentration was found in patients with vascular dementia with cerebral infarction resulting from large artery occlusion [28]. Some studies suggested that abnormally high levels of serum Lp(a) are associated with cerebrovascular disease [29] and vascular dementia [30].

The brain contains approximately 25% of the total body cholesterol [31], which plays an essential role in brain development and function, including plasma membrane formation and synapse development. Numerous studies have shown that many pathological changes in the brain are associated with changes in brain cholesterol metabolism [32, 33]. It has been established that oxidative stress negatively impacts central nervous system (CNS) functions and is involved in neurodegenerative disorders, such as Alzheimer's disease [34]. Dixit *et al*. found that chronic vitamin C deficiency not only accelerated oxidative stress in mouse brains but also increased amyloid plaque production and cognitive impairment [35]. It has been suggested that high doses of vitamin C could be protective against Alzheimer's disease-like pathologies [36]. Thus, the optimal intake of vitamin C is necessary to protect the brain from age-related oxidative stress as well as cerebrovascular disease.

To our knowledge, the information on the effects of chronic vitamin C deficiency in relation to the presence of Lp(a) and cholesterol metabolism in the brain during normal aging is scarce, mainly due to a lack of suitable animal models that can mimic human metabolism in respect of the Lp(a), coupled with a lack of vitamin C synthesis.

We developed a unique humanized mouse model unable to synthesize vitamin C (Gulo (-/-)) and at the same time carrying two mutations resulting in the synthesis of human apo(a) (h-apo(a)) and human apo(B)-100 (h-apo(B)-100) to form human Lp(a). This Gulo (-/-); Lp(a)+ mouse model has shown dietary vitamin C-related changes in collagen synthesis, Lp(a) production and atherogenicity, and cancer metastasis mediated through connective tissue stability [37].

In this study, we used the Gulo (-/-); Lp(a)+ mouse model to investigate the effects of the extended intake of low and high dietary vitamin C on age-related changes with respect to Lp(a) deposition in the brain, total brain cholesterol levels, and oxidative stress.

2. MATERIALS AND METHODS

2.1. Breeding and Genotyping of Transgenic Mice

Human Gulo (-/-); Lp(a)+ mice were generated as described by Cha *et al.* [20]. Briefly, homozygous Gulo (-/-) mice were generated by breeding heterozygous Gulo (+/-) mice BALB/cBy-Gulosfx/J (Jackson Laboratory, Sacramento, CA). Then homozygous Gulo (-/-) mice were bred with human apo(a) [h-apo(a)] transgenic mice (Mutant Mouse Regional Resource Center, Columbia, MO) and human apoB-100 (h-apoB-100) transgenic mice (Taconic Farms Inc., Hudson, NY) separately to produce Gulo(-/-); h-apo(a)+ mice and Gulo(-/-); h-apoB-100+ mice. These two transgenic mice were then bred to generate the Gulo (-/-); Lp(a)+ strain.

Genotyping was performed by TaqMan FAM probe Real Time-PCR at Transnetyx (Cordova, TN) using mouse tail clips. Genotyping ensured homozygosity of the Gulo locus knockout and the presence of h-apo (a) and h-apo(B)-100 genes. All animal experiments were conducted with humane and customary care, and followed a protocol approved by the internal Institutional Animal Care and Use Committee. All mice were housed in a barrier facility with a 12-hour light/12-hour dark cycle.

2.2. Experimental Design

Experiments were undertaken on both male and female Gulo (-/-); Lp(a)+ mice. Three groups of animals were used, aged 8-9 months (32-36 weeks), 1 year (52 weeks) and 2 years (104-116 weeks), at the time of harvesting. Twelve animals of each gender were randomly assigned to each age group. In each age group, 6 animals were assigned to a high vitamin C (H-VC)-supplemented diet, a modified LabDiet® Laboratory Rodent Diet 5001 with 1000 PPM vitamin C with distilled water, and 6 animals were assigned to a vitamin C-deficient (L-VC) diet (LabDiet® Laboratory Rodent

Diet 5001) with 30mg/L vitamin C added in distilled water. The H-VC diet provided the mice with approximately 4mg ascorbic acid daily, while the L-VC diet, with 30mg/L vitamin C added in distilled water, provided the mice with approximately 0.12mg ascorbic acid daily. Diet and water were provided *ad libitum*. The only source of vitamin C intake for the experimental Gulo (-/-);Lp(a)+ mice was the diet and water. We did not observe significantly different diet consumptions during the 20 weeks of the experiment. After 20 weeks, the mice were harvested for blood and tissues.

2.3. Sample Preparation

Serum was collected from blood drawn *via* cardiac puncture at the end of the experiment. Mouse midbrains were cut to preserve the hippocampus and were preserved in 10% neutral buffered formalin. Forebrain, hindbrain, and liver were collected, fast frozen in liquid nitrogen, and stored at -80°C until use.

2.4. Ascorbic Acid Measurement

Frozen mouse liver and brain were weighed and homogenized in Millipore water. The homogenates were then centrifuged at 2000rpm at 4 °C for 20 mins. The serum samples and tissue supernatants were used for ascorbic acid determination by a BioVision Ferric Reducing Ascorbate (FRASC) Assay Kit (Milpitas, CA), and expressed as nmole/mL for serum samples and nmole/mg tissue weight for brain samples.

2.5. Serum Apolipoproteins Measurement

Serum h-apo(a) levels were determined by using the IBL International GmbH Lp(a) Enzyme Immunoassay (Hamburg, Germany). Serum h-apoB-100 was determined by using the Assaypro AssayMax Human Apolipoprotein B Enzyme Kit (St. Charles, MO).

2.6. Brain Cholesterol Measurement

Brain total cholesterol levels were determined by Abcam HDL and LDL/VLDL Cholesterol Assay Elisa Kit (Cambridge, MA).

2.7. Brain 8-OHdG

Brain 8-OHdG levels were determined by BioVision QuickDetect™ 8-OHdG (Mouse) Elisa Kit (Milpitas, CA).

2.8. Immunohistology

Mouse midbrains were fixed in 10% neutral buffered formalin. The midbrains were then embedded in paraffin, sectioned and stained for hematoxylin and eosin (HE), h-apo(a), and h-apo(B)-100 at Histotox Labs, Inc. (Boulder, CO).

Immunohistochemical analysis was performed using ImageScope. The regions of interest (ROI) were the hippocampus region (1000 X 2800 microns) and the choroid plexus region (45X45 microns). Deposition of Lp(a) was indicated by the collocation of h-apo(a) and h-apo(B)-100. The extent of positive stains of h-apo(a) and h-apo(B)-100 was measured in the ROI for 3 mice per group. The data were expressed as the ratio of the number of positive pixels (positive stains) over the total number of pixels (positive+negative). The deposition of Lp(a) was calculated as the percentage of positive h-apo(a) stain over positive h-apo(B) stain at the same ROI.

2.9. Statistical Analysis

All data are presented as means ± standard deviation. Significant differences between means were determined by student's t-tests at a significance level of 0.05 with Microsoft Excel. The student's t-tests were computed between groups differing by a single characteristic. Specifically, t-tests were computed for each biomarker between H-VC and L-VC groups of the same age and sex, between age groups of the same vitamin C diet and gender, and between genders of the same vitamin C diet and age. Semi-partial correlation analysis between Lp(a) deposition rate in choroid plexus and brain vitamin C levels was performed by R package ppcor (Kim S., 2015).

3. RESULTS

3.1. Serum Ascorbic Acid Levels

Age-related changes in ascorbic acid levels in the serum and brain tissue of Gulo (-/-); Lp(a)+ mice kept on diets supplemented with a high dose of vitamin C (H-VC) and a minimal dose of vitamin-C (L-VC) for 20 weeks are shown in Fig. (**1**).

Fig. (**1A**) shows that serum ascorbic acid levels were significantly lower in both male and female L-VC mice compared with mice consuming an H-VC diet ($p<0.05$). As such, serum ascorbic acid levels in L-VC males at the ages of 8-9 month, 1 year, and 2 years were 3.3, 1.8, and 2.7nmol/ml, respectively, and in L-VC females at the equivalent ages were 1.1, 1.2, and 1.5nmol/ml, respectively. Serum ascorbic acid levels in H-VC mice of corresponding sex and age were for males: 72.9, 81.5, and 63.9nmol/ml, respectively; while for females: 80.6, 103.6, and 121.9nmol/ml, respectively. Although serum ascorbic acid levels appear to increase with age in H-VC female mice, the differences did not reach statistical significance ($p>0.05$). However, female mice at the ages of 1 year and 2 years had significantly higher serum ascorbic acid levels than male mice of corresponding ages ($p<0.05$).

As shown in Fig. (**1B**), the brain ascorbic acid levels were several times lower in both male and female L-VC mice at the age of 8-9 months, 1 year and 2 years (male: 0.1, 1.2, and 0.5nmol/mg, respectively; female: 0.03, 0.6, and 0.4nmol/mg, respectively; $p<0.05$) compared with H-VC mice in corresponding sex and age groups (male: 2.4, 2.4, and 2.4nmol/mg, respectively; female: 2.6, 2.7, and 2.4nmol/mg, respectively). Whereas we had seen age-related changes in serum ascorbic acid levels, the H-VC mice maintained similar brain ascorbic acid levels across different age groups in both males and females. Interestingly, 1- and 2-year-old L-VC mice had significantly higher ascorbate contents in brain tissue than 8-9-month-old L-VC mice of both genders ($p<0.05$).

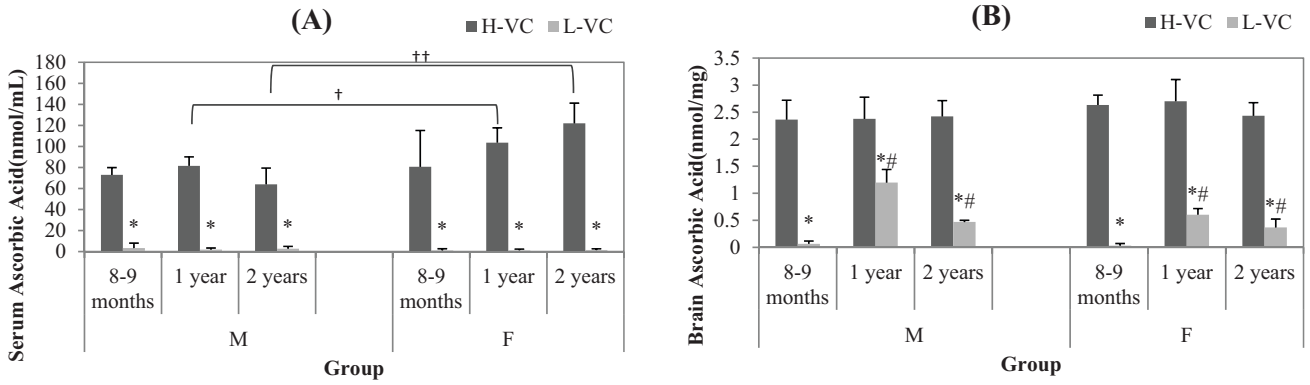


Fig. (1). Ascorbic acid levels in serum (A) and brain (B) in each age group and gender. Data are expressed as Mean ± SD. * represents statistically significant difference between H-VC groups and L-VC groups of the same age and gender at the significance level of 0.05; # represents statistically significant difference between age groups of the same gender and diet at the significance level of 0.05; † represents statistically significant difference between genders of the same age and diet at the significance level of 0.05; ††indicates $p<0.01$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

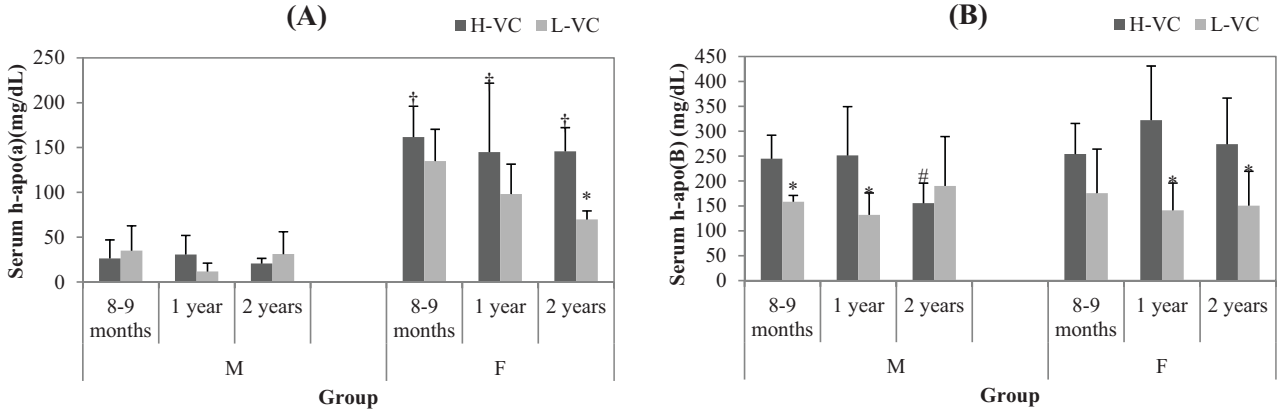


Fig. (2). Serum levels of h-apo(a) (A) and h-apo(B) (B) in each age group and gender. Data are expressed as Mean ± SD. * represents statistically significant difference between H-VC groups and L-VC groups of the same age and gender at the significance level of 0.05; # represents statistically significant difference among age groups of the same gender and diet at the significance level of 0.05; † represents statistically significant difference between genders of the same age and diet at the significance level of 0.05. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Serum Lipoproteins

3.2.1. Serum Human Apo(a) Levels

As seen in Fig. (**2A**), serum h-apo(a) levels were significantly higher in female than in male mice of comparable age and dietary vitamin C intake. In L-VC female mice, serum h-apo(a) levels were 134.9, 98.1, and 69.7mg/dL at the age of 8-9 months, 1 year and 2 years, respectively. Serum h-apo(a) levels in H-VC female mice at corresponding ages were higher, at 161.6, 144.9, and 145.7mg/dL, respectively. However, the statistically significant difference in serum h-apo(a) between the L-VC and H-VC mice was observed only in the 2-year-old female mice ($p<0.05$). The L-VC female mice had approximately 2-fold lower serum h-apo(a) levels compared with H-VC female mice at the age of 2 years ($p<0.05$). Serum h-apo(a) levels in male mice did not markedly differ between L-VC groups at the age of 8-9 months, 1 year and 2 years (34.9, 11.7, and 31.1mg/dL, respectively; $p>0.05$) and H-VC groups at corresponding ages (26.3, 30.6, and 20.5mg/dL, respectively).

Interestingly, in both H-VC and L-VC groups, we found that serum h-apo(a) levels were approximately 5-fold higher in female mice than in male mice in all age groups ($p<0.05$). Furthermore, serum h-apo(a) levels did not differ among ages in H-VC and L-VC groups in either gender ($p>0.05$).

3.2.2. Serum H-apo(B) Levels

Fig. (**2B**) shows the values for serum h-apo(B) in Gulo (-/-); Lp(a)+ mice fed an H-VC or L-VC diet for 20 weeks.

Serum h-apo(B) levels in 2-year-old H-VC male mice were significantly lower compared with 8-9-month- and 1-year-old animals (155.5, 244.7, and 251.3mg/dL, respectively; $p<0.05$). However, these differences did not reach statis-

tical significance in H-VC female mice at corresponding ages (273.9, 254.2, and 322.2mg/dL, respectively; $p>0.05$). L-VC female mice had lower serum h-apo(B) levels at the age of 8-9 months, and significantly lower levels at the age of 1 year and 2 years, compared with H-VC animals (175.5, 141.1, and 150.4mg/dL, respectively; $p<0.05$). L-VC male mice at the age of 8-9 months and 1 year had less h-apo(B) than H-VC animals of corresponding ages (158.3 and 131.9mg/dL, respectively; $p<0.05$). However, 2-year-old L-VC male mice had slightly higher serum h-apo(B) (190.1mg/dL) than H-VC animals (155.5 mg/dL).

3.2.3. Brain Total Cholesterol

Fig. (**3A**) shows age-related changes in brain total cholesterol levels in L-VC and H-VC mice.

The brain total cholesterol levels in younger male and female H-VC mice were significantly higher than in 2-year-old animals. As such, males aged 8-9 months and 1 year had cholesterol levels of 3.4 and 3.9 ug/mg, respectively ($p>0.05$), and females 2.6 and 4.2 ug/mg, respectively ($p<0.01$). Brain total cholesterol level was significantly lower in 2-year-old mice of both genders (males: 1.9 ug/mg; and females: 2.5 ug/mg; $p<0.01$).

Brain cholesterol levels in female mice were affected more by age than by vitamin C dietary intake. Brain cholesterol levels in H-VC mice did not significantly differ from those in L-VC mice at the age of 8-9 months and 1 year in either gender. As such, brain cholesterol levels in L-VC males were 2.9 and 4.1ug/mg, respectively; and in L-VC females were 2.9 and 4.7ug/mg, respectively ($p>0.05$). However, the brain cholesterol level was significantly lower in L-VC mice than in H-VC mice at the age of 2 years (males: 1.3 and 1.9ug/mg; females: 1.6 and 2.5ug/mg, respectively; $p<0.05$).

3.2.4. Brain Oxidation

To further evaluate the age-related metabolic changes in the brain of Gulo (-/-); Lp(a)+ mice with high and low dietary vitamin C intake, we examined an important biomarker for cellular oxidative stress - 8-hydroxy-2'-deoxyguanosine (8-OHdG).

Fig. (**3B**) shows that in H-VC groups, brain 8-OHdG levels gradually increased with age in both male and female mice. In H-VC male mice aged 8-9 months, 1 year and 2 years, brain 8-OHdG levels were 5.2, 5.5, and 6.2ng/mg, respectively; and in females were 5.3, 5.9, and 6.2ng/mg, respectively. Brain 8-OHdG levels were significantly higher in male mice aged 2 than in males aged 8-9-months ($p<0.05$), but not in female mice ($p=0.052$).

Compared with H-VC groups, brain 8-OHdG levels significantly increased in 2-year-old L-VC mice of both genders (male: 6.7ng/mg and female: 6.8ng/mg, respectively; $p<0.05$). However, the differences in brain 8-OHdG levels were not statistically significant between mice on H-VC and L-VC diet at the age of 8-9 months and 1 year (male: 4.9 and 6.5ng/mg, respectively; and female: 5.4 and 5.9ng/mg, respectively; $p>0.05$).

3.2.5. Brain H-apo(a) and H-apo(B) Deposition

Mouse brains isolated from both H-VC and L-VC mice displayed areas of microhemorrhages. The microhemorrhages were mostly seen in cortical vessels, as shown in Fig. (**4A**). The collocations of h-apo(a) and h-apo(B)-100 immunostainings indicative of the Lp(a) deposition were found in brain capillaries, hemorrhaged blood vessels, and the choroid plexus, as shown in Figs. (**4B-E** and **5A-B**).

The observed most intense h-apo(a) and h-apo(B)-100 immunoreactivity was related to hemorrhage in cortical vessels and brain capillaries, and to a lesser extent to non-leaky blood vessels. In addition, we observed mild mouse MMP9 stains co-located with h-apo(a) and h-apo(B)-100 deposition at the hemorrhaged blood vessels (picture not shown), indicating a possible injury or blood-brain dysfunction at these areas.

The degree of Lp(a) deposition in the hippocampus was expressed as the percentage of h-apo(a) and h-apo(B)-100 collocation (Fig. **4F**). In H-VC mice, the Lp(a) deposits increased with age both in males and females. As such, the Lp(a) deposit rate increased in H-VC male mice from 37% at 8-9 months, to 53% in 1-year-old mice and 55% in 2-year-old mice. In H-VC female mice of corresponding ages, the Lp(a) tissue deposition increased from 53% to 68%, and to 70%, respectively.

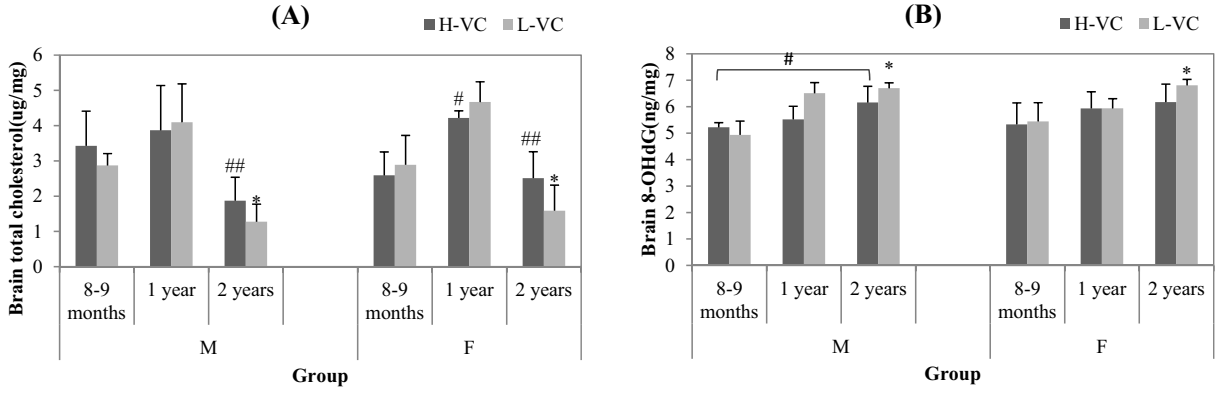


Fig. (3). Brain total cholesterol (A) and brain 8-OHdG (B) levels in each group of both genders. Data are expressed as Mean ± SD. * represents statistically significant difference between H-VC groups and L-VC groups of the same age and gender at the significance level of 0.05; # represents statistically significant difference among age groups of the same gender and diet at the significance level of 0.05; ## indicates $p<0.01$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

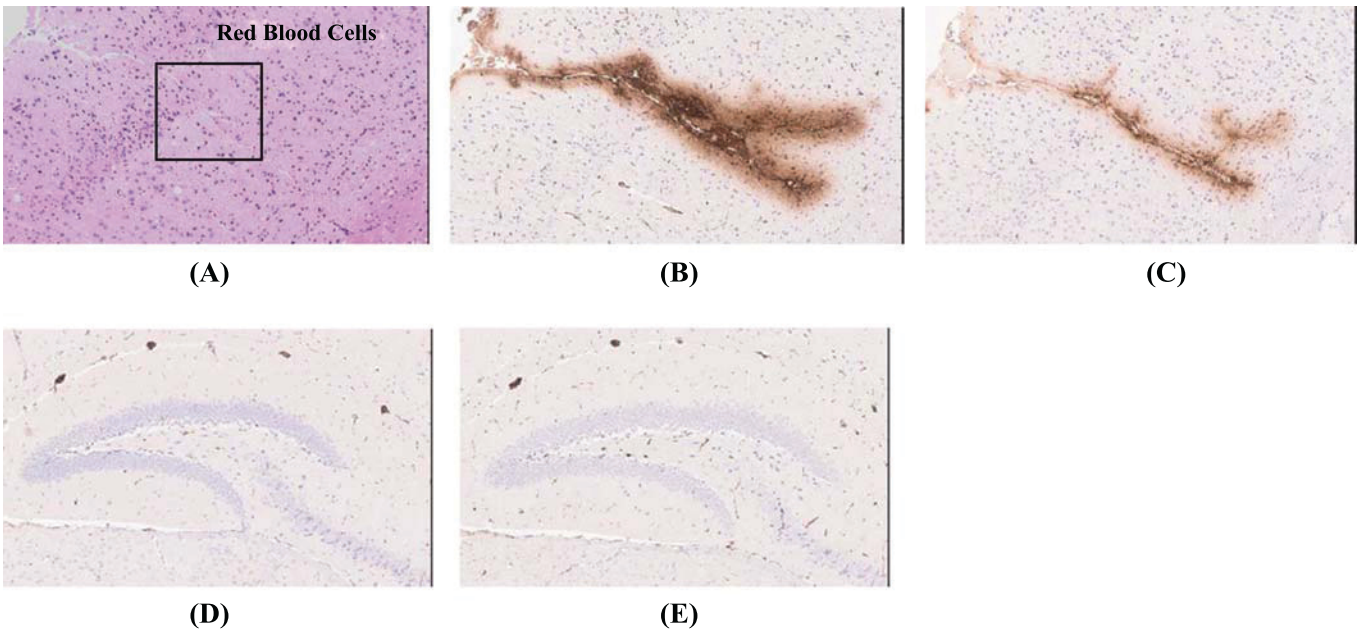


Fig. (4). Examples of microhemorrhage HE staining (**A**), h-apo(a) deposit (**B**), and h-apo(**B**)-100 deposit (**C**) in brain cortical vessels. Examples of h-apo(a) deposits (**D**) and h-apo(B)-100 deposits (**E**) in the hippocampus region. Examples of HE stain. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

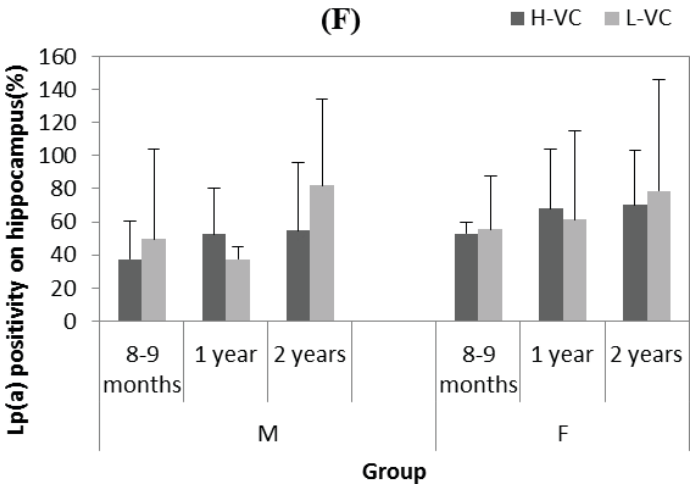


Fig. (4). (**F**) **Quantification of Lp(a) deposit on the hippocampus (A)** The Lp(a) positivity was calculated as the percentage of h-apo(a) stain over h-apo(B)-100 stain at the same areas in the hippocampus. Data are expressed as Mean ± SD. * represents statistically significant difference between H-V C groups and L-VC- groups at the *p* level of 0.05; # represents statistically significant differences between age groups at the *p* level of 0.05. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The differences in Lp(a) deposition in the hippocampus between H-VC and L-VC mice of the same age and diet did not reach statistical significance. We may have been underpowered to detect a difference. However, we could observe an evident trend towards higher Lp(a) deposition in L-VC mice, especially at the ages of 8-9 months and 2 years. As such, Lp(a) deposits in L-VC male mice at the ages of 8-9 months and 2 years were 50% and 82%, respectively, compared with 37% and 55% in male H-VC mice of corresponding age(p>0.05). In L-VC females, the Lp(a) deposit rates in mice aged 8-9 months and 2 years were 56% and 79%, respectively, compared with 53% and 70% in H-VC females of corresponding ages, higher but not statistically different (p>0.05).

The degree of Lp(a) deposition on the choroid plexus was expressed as the percentage of h-apo(a) and h-apo(B)-100 immunostain collocation (Fig. **5B**). The results showed increased deposition of Lp(a) in both male and female mice in

L-VC groups compared with H-VC animals at corresponding ages. In H-VC female mice, the Lp(a) deposits significantly increased from 63% to 90% and 93% in the 8-9 months, 1-year old and 2-year-old animals, respectively ($p<0.05$). However, the Lp(a) deposits in the choroid plexus in H-VC male mice of the corresponding ages were fairly consistent (54%, 53% and 58%, respectively; $p>0.05$).

Lp(a) deposits in the choroid plexus in L-VC mice were significantly higher at the age of 8-9 months, than in H-VC mice of corresponding age, in both genders (males: 118% and 54%, respectively; females: 133% and 63%, respectively; $p<0.05$). L-VC mice aged 2-years also had more Lp(a) deposits compared with H-VC mice of the same age (males: 134% and 58%, respectively; females: 129% and 93%, respectively; $p>0.05$).

The Lp(a) deposition was observed in the stromal space of the choroid plexus in both H-VC and L-VC mice. The representative micrographs of h-apo(a) and h-apo(b)-100 collocation in choroid plexus in each group are shown in Fig. (**5A-B**). The degree of Lp(a) deposition on the choroid plexus was expressed as the percentage of h-apo(a) and h-apo(b)-100 immunostain collocation (Fig. **5C**). In H-VC female mice, the Lp(a) deposits increased from 63% to 82% in the 8-9 months and 2-year-old animals, respectively ($p>0.05$). The Lp(a) deposits in the choroid plexus in H-VC male mice of the corresponding ages were fairly consistent (54%, 53% and 58%, respectively; $p>0.05$).

Lp(a) deposits in the choroid plexus in L-VC mice were significantly higher at the age of 8-9 months, than in H-VC mice of corresponding age, in both genders (males: 118% and 54%, respectively; females: 133% and 63%, respectively; $p<0.05$). Female L-VC mice aged 1 year also showed more Lp(a) deposits compared with H-VC mice of the same age (132% and 65%, respectively; $p>0.05$).

Semi-partial regression analysis of values using individual animals, including both genders, all ages and vitamin C diets, revealed that brain ascorbic acid levels were significantly negatively correlated with Lp(a) deposition in choroid plexus ($r^2=0.15$, $p<0.05$, Fig. **5D**).

(A)

Male mice: Lp(a) deposition in choroid plexus.

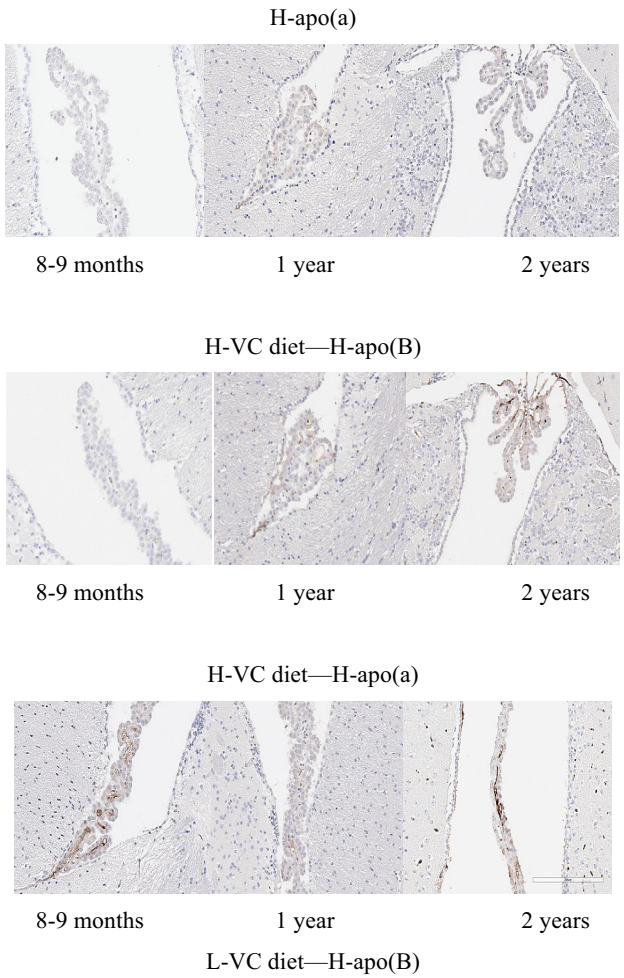
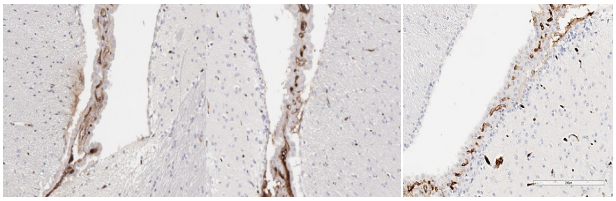


Fig. (5). Contd…

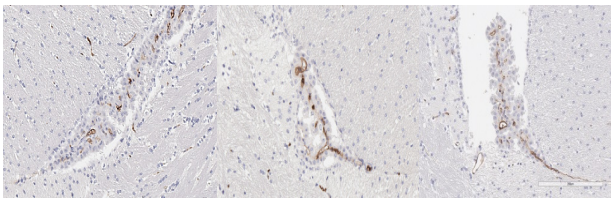


8-9 months 1 year 2 years

(B)

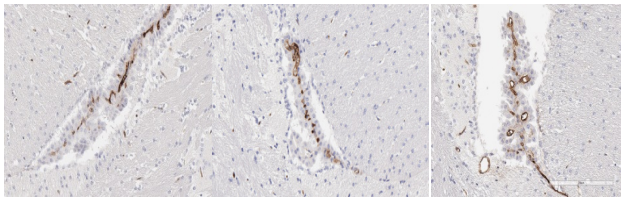
Female mice: Lp(a) deposition in choroid plexus.

H-apo(a)



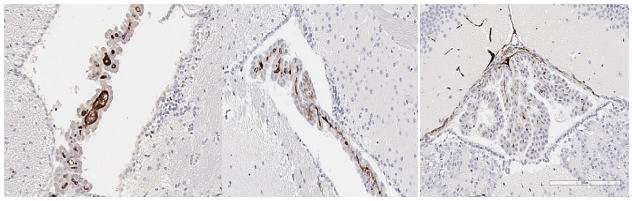
8-9 months 1 year 2 years

H-VC diet—H-apo(B)



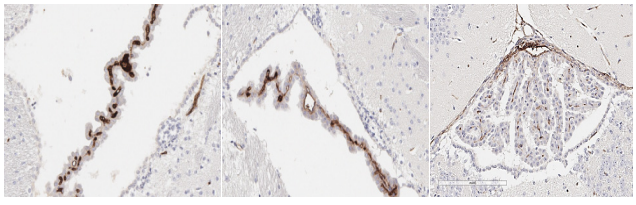
8-9 months 1 year 2 years

H-VC diet—H-apo(a)



8-9 months 1 year 2 years

L-VC diet—H-apo(B)



8-9 months 1 year 2 years

Fig. (5). Contd…

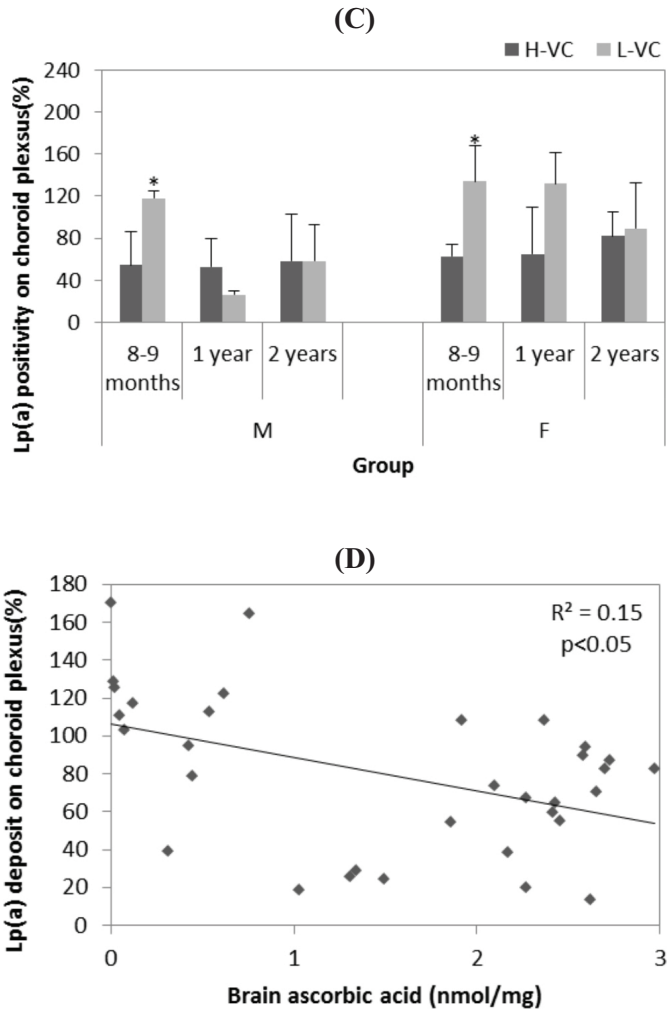


Fig. (5). Representative graphs of h-apo(a) and h-apo(B)-100 collocation in choroid plexus in each group in male (**A**) and female mice (**B**). Scale bars, 200 μm. Quantification of Lp(a) deposition in choroid plexus (**C**). Correlation between brain ascorbic acid levels (nmol/mg) and Lp(a) deposition in choroid plexus (**D**). Data are expressed as Mean ± SD. * represents statistically significant difference between H-VC groups and L-VC groups of the same age and gender at the significance level of 0.05. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

Vitamin C is an essential nutrient and an important anti-oxidant. The dosage of ascorbic acid from the H-VC diet was equivalent to approximately 13mg/kg for humans based on metabolic rate differences, which is sufficient to saturate the total body pool of vitamin C in humans [38]. The 20 weeks of L-VC diet intake of about 0.12 mg/day vitamin C resulted in a dramatic decrease in serum ascorbic acid concentrations in both male and female mice, ranging from 0 to 12.5nmol/ml, which corresponds to the scurvy plasma vitamin C levels in human - 10uM and below [39]. It is anticipated that the 20 weeks of low dietary intake of vitamin C applied in our study will incur profound metabolic changes as it accounts for about 16% of an average lifespan of a wild type mouse (30 months) or 30% of maximum lifespan (16 months) of Gulo (-/-) mice kept on 0.4 mg/day of vitamin C [40]. Most studies in Gulo (-/-) mice have been conducted with shorter (about 4 weeks) vitamin C deprivation. Furthermore, our results showed that serum ascorbic acid levels in 1- and 2-year-old female H-VC mice were almost twice as high as in H-VC males of corresponding ages. This result corresponds with findings by Kuo *et al*. They found that ascorbic acid levels were higher in female adult Slc23a2+/- and Slc23a2+/+ mice than in male mice. These researchers suggested that female mice had a lower vitamin C urinary secretion rate than male mice, which could lead to higher vitamin C concentrations in plasma [41].

The intracellular intake of vitamin C in the brain occurs through sodium-dependent vitamin C transporter (SVCT2), which allows a significant amount of vitamin C to be taken up into the brain [42]. We observed that 20-week supplementation with 4 mg/day of vitamin C (H-VC) increased brain levels of ascorbic acid in both male and female mice, regardless of age, while its low intake (0.12 mg/day) led to a significant depletion of brain ascorbic acid in both males and females of all ages, especially in younger (8-9-month-old) mice.

Interestingly, older Gulo (-/-); Lp(a)+ mice, especially at 1 year of age, kept on a low-ascorbate diet (L-VC), had significantly higher brain ascorbic acid levels than young 8-9-months-old mice. This corresponds to the findings that, in a state of vitamin C deprivation, the SCVT2 expression in the brain of aged animals is upregulated compared with young animals, which contributes to the increased retention of vitamin C in aged brains [43]. It is also possible that adult and aged mouse brains have greater buffering capacity, with regard to vitamin C retention, than the brains of young mice.

Brain cholesterol is synthesized *de novo* and its rate declines with age. Our results show that the brain cholesterol levels in Gulo (-/-); Lp(a)+ mice were significantly lower in 2-year-old male and female mice compared with younger animals in both H-VC and L-VC groups. Vitamin C deficiency did not affect brain cholesterol levels in younger mice, which suggests that cholesterol metabolism in the brain of older mice is more prone to the consequences of scurvy. Although the role of brain cholesterol levels in neurological diseases is still unclear, it has been widely reported that Alzheimer's disease is accompanied by lower brain cholesterol levels [44, 45]. It has further been reported that brains with decreased levels of brain cholesterol are more susceptible to pathological diseases [46].

The brain is an organ with a high rate of oxygen consumption, thus it is prone to oxidative damage [47, 48], and numerous studies have reported an age-related increase of 8-OHdG in the brain [47, 49, 50]. Our results showed that 8-OHdG levels were significantly higher in the brains of 2-year-old Gulo (-/-); Lp(a)+ mice compared with younger mice in both males and females, suggesting increased oxidative damage with age.

We found an increase of brain 8-OHdG levels in 2-year-old male mice and female mice kept for 20 weeks on a low vitamin C diet (L-VC), compared with mice consuming high levels of vitamin C (H-VC). Vitamin C consumption levels were not seen to affect 8-OHdG formation in the brain in younger mice in this study. Age-related changes were less pronounced than the changes observed at different vitamin C intake levels in both male and female mice. It is well established that vitamin C, as an antioxidant, plays an important role in protecting the brain from oxidative stress. When the brain is depleted of antioxidants, it becomes more susceptible to reactive oxygen species-related damage [51]. The accumulation of 8-OHdG in the brain is a contributing factor to neurodegenerative diseases such as Alzheimer's disease [52]. Our results suggest that great attention should be paid to avoiding vitamin C deficiency in mid to old-age to reduce the risk of impaired brain cholesterol levels and to protect against age-related oxidative stress in the brain.

Our results show that female mice had significantly higher serum h-apo(a) levels compared with male mice regardless of age and vitamin C intake. This gender difference was most likely owing to the suppressing effect of testosterone on h-apo (a) expression in male mice [53]. Such effect was supported by findings that the serum levels of h-apo(a) dramatically increase with castration in male h-apo(a) transgenic mice, but no consistent effect of castration was seen on serum levels of h-apo(a) in female h-apo(a) transgenic mice [54]. Human studies have indicated that estrogen can lower Lp(a) levels [55], however, it was not observed in Gulo(-/-) ;Lp(a)+ mice.

The majority of Lp(a) studies relate to its role as a risk factor for coronary heart disease. The cerebral arteries, as proximal arteries, are exposed to relatively high mechanical stress and are at a high risk of atherosclerosis-related diseases, especially in patients with low ascorbate levels [56]. However, the role of Lp(a) in brain aging and its related deposition in cerebral capillaries has not been previously elaborated. In this study, we found that Lp(a) was deposited in hemorrhaged cerebral capillaries and blood vessels, much as we observed in our previous studies showing Lp(a) deposition in the aorta and coronary blood vessels [20, 21]. This may reflect that both tissue repair and an anti-fibrinolytic mechanism as h-apo(a), made of repeated copies of Kringle IV plasminogen, can compete with plasminogen for its binding sites on endothelial cells, thus inhibiting fibrinolysis.

In addition, we found predominant Lp(a) deposition in the stromal side of the choroid plexus, which was significantly negatively correlated with the brain ascorbic acid levels. This finding corroborates our previous study results showing that Lp(a) accumulated in the vascular wall of dietary vitamin C-deficient Gulo(-/-); Lp(a) mice, but was absent in animals with high vitamin C intake [20], as well as is in accordance with Lp(a)'s role as a repair factor for a structurally weakened vascular wall [18]. The choroid plexus consists of many fenestrated capillaries lying on the basal membrane, containing type IV collagen. It has been shown that vitamin C deficiency lowers the expression of type IV collagen, thus affecting blood vessels integrity [57]. Therefore, the basal membrane of the choroid plexus in vitamin C-deficient mice could be damaged owing to chronic vitamin C deficiency. The choroid plexus functions as a barrier between blood and cerebrospinal fluid (CSF). It has been suggested that the cholesterol accumulation on the choroid plexus is one of the signs of choroid plexus damage [58].

CONCLUSION

Our findings show that optimal vitamin C intake from early life to old age is important in brain health to prevent oxidative stress damage and to maintain cholesterol homeostasis in the brain. More importantly, the negative correlation between brain ascorbic levels and the formation of Lp(a) deposit on the choroid plexus further emphasizes the critical role of vitamin C in protecting brain health throughout the normal aging process.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the internal Institutional Animal Care and Use Committee.

HUMAN AND ANIMAL RIGHTS

The animal studies were ethically conducted, and the procedures were followed according to the research standards set by the National Academy of Sciences, The National Academies Press, Washington D.C.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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