Matcha and Its Components Control Angiogenic Potential

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Summary  The brain needs the appropriate capillary networks to maintain normal brain function. Since previous studies showed age-related decrease in the cortical capillaries, it is suggested that protection against capillary aging is critical for maintaining brain function. Epidemiological studies have indicated that brain functions were protected from age-related decline by the long-term consumption of matcha. However, whether matcha has protective effects on capillary aging has not been studied yet. In this study, we utilized Flt1-DsR mice that expressed a red fluorescent protein in vascular endothelial cells to visualize cortical capillaries clearly. We found that cortical capillary density decreased in aging Flt1-DsR mice. Our results of the aortic ring assay and tube formation assay revealed that matcha and its components vitamin K1 and lutein, which are abundant in matcha powder, enhanced the angiogenic potential. Moreover, we evaluated the effect of long-term ingestion of matcha on mouse cortical capillary aging by using imaging experiments. The capillary density of the Flt1-DsR mice, which were fed matcha-containing food, indicated the protective effects of matcha ingestion on capillary aging in a limited cortical layer. These results suggest that biological regulation of matcha and its components affect the angiogenic potential, which is related to the prevention of capillary aging.

Key Words  brain, neocortex, aging, vascular, capillary, matcha, vitamin K1, lutein

The human brain uses 20% of the oxygen that is consumed by the whole body (1). To meet the enormous energy demand, the human brain contains more than 600 km of capillaries. Our recent study revealed that in the developing cortex, a vascular niche contributes to neural differentiation (2, 3), and for appropriate differentiation (4–6), vascularization is strictly controlled spatio-temporarily (2). In the adult brain, amyloid beta was eliminated from the cortex vascularly, and clearance efficiency decreased due to declining vascular function (7–9). These studies indicate that forming and maintaining an appropriate vascular plexus in the cortex is essential to maintain normal brain functions. On the other hand, many studies showed that the vascular plexus collapsed with aging; for example, the vascular density decreased 16% in gray matter and 20% in white matter in human brains, and the vascular number decreased around 20% in the rodent cortex or hippocampus (10–14). It is well-known that the cerebral cortex consists of a six-layer structure. In each layer, neurons have unique properties, such as morphology, gene expression, or neural projection, and these layers form a small functional and structural unit (15, 16).

Previous studies indicated that neurons in layers 3, 5, and 6 were more vulnerable to hypoxia than the other layer neurons (17, 18), and in layers 2, 3, and 5, neurons were degenerated more severely in the Alzheimer’s disease patient’s cortex (19). Therefore, it is important to know how and where vascular degeneration in the brain is proceeded with aging preferentially.

Epidemiological studies have indicated that long-term green tea ingestion prevents cognitive function decline (20). Animal experiments showed that long-term ingestion of green tea catechins have protective effects for brain functions (21). Moreover, many studies have suggested that green tea has protective effects for vascular health (22, 23). Therefore, green tea is expected to have an anti-aging effect on cortical capillary. However, whether matcha prevents capillary aging has not been studied yet. Matcha is a kind of green tea. While other green teas are consumed as the extract of tea leaves, matcha is consumed as a suspension of tea leaves. Furthermore, tea leaves for matcha is richer in amino acids such as theanine than general green tea, because of its special cultivation method which the leaves are shaded from the sun for a few weeks before harvest, and its enriched umami taste enables matcha to drink at least three times higher dose per serving than general green tea. Therefore, matcha contains higher concentrations of active ingredients than other green teas, and intake of matcha is expected to have a stronger effect on the aging brain than ingestion of other green teas. In this
study, to observe capillary clearly, we used Flt1-tandem dsRed bacterial artificial chromosome transgenic mice (Flt1-DsR) that expressed a red fluorescent protein in vascular endothelial cells (2, 24, 25). We analyzed the effects of matcha and its components vitamin K1 and lutein on angiogenesis with an aortic ring assay and tube formation assay. Moreover, we used imaging experiments to assess the protective effects of matcha ingestion on cortical capillary aging.

**MATERIALS AND METHODS**

**Animals.** All mice were bred under specific pathogen-free conditions in a temperature- and humidity-controlled room with a 12-h light/dark cycle. All experiments were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Kobe Gakuin University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Kobe Gakuin University (A19-48, A20-43, and A20-56). For feeding experiments, five to six 7-wk-old maternity mate Flt1-DsR mice were bred together. Mice ingested either a normal diet (MF, Oriental Yeast Co., Ltd.) or a normal diet with the addition of 2% matcha green tea powder (Kyoeisei-
Deeply anesthetized Flt1-DsR mice were perfused with 50 mL phosphate buffered saline (PBS) with 20 mL 4% paraformaldehyde (PFA). Mouse brains were harvested and fixed in 4% PFA for 2 nights at 4 °C. Following 30% sucrose replacement, fixed brains were embedded in an optimal cutting temperature compound (Sakura Tissue-Tek), and sections were prepared on a cryostat. Cryostat sections (50 μm) were washed three times in PBS and treated with a blocking buffer (10% donkey serum and 0.3% Triton X-100) for 1 h at room temperature, followed by incubation with primary antibodies, mouse anti-RFP (1:500; MBL), at 4 °C. Immunolabeled sections were washed three times in 0.1% Triton-X100 (PBS-T) and incubated with secondary antibodies (Invitrogen) for 1 h at room temperature. After washing twice in PBS-T, sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature. Sections were mounted under a glass cover with a mounting medium. All images were acquired with a confocal microscope (FV-1000, Olympus). Fluoview software was used to acquire all images. Images were processed using Adobe Photoshop or Image J. The capillary density was quantified based on a previous report (26). DAPI-stained nuclei images and DsRed-labeled vasculature images were acquired from five sections taken every 400 μm (1.6 mm total width), centered on the Bregma −1.0 mm point. Both sides of the somatosensory cortex were acquired from each section. Imaging stacks of vasculature or nuclei were maximally projected in the z plane. Each layer of the cortex or whole cortex was enclosed into a region of interest (ROI) using maximally projected DAPI images. To measure the capillary density only, relatively large Flt1-positive blood vessels (thicker than 5 μm diameter) was removed from the ROI manually using maximally projected DsRed images. Next, maximally projected DsRed images were given a thresholded using the Image J auto local threshold function mean, which was computed for each pixel according to the mean of the greyscale value within a radius of 30 pixels (approximately 35 μm) according to previous reports (27). The vascular signal (number of pixels) was measured, and the percent of vascular signal was calculated for each ROI. To measure the vascular length, threshold DsRed images were skeletonized. The percent of skeleton signal (number of pixels) was measured for each ROI. The side data from each section was averaged, and the average of 5 sections was calculated. The result was considered to be the capillary density or length of a mouse brain. After calculating the results from all mice, the result was standardized with the control result. 3D images of the confocal images were observed using Fluoview or Image J software.

**Aortic ring assay.** Procedures were performed as previously described (28). Three to four months old Flt1-DsR mice were deeply anesthetized and perfused in 20 mL PBS. Thoracic aortae were harvested and removed carefully from the fat layer and adventitia. Aortae were sliced into approximately 1 mm rings. Rings were embedded in the type 1-A collagen gel (Nitta Gelatin) containing 5 ng/mL vascular endothelial growth factor (VEGF) (Prospec) and incubated in EGM-2 medium (Lonza) added Humedia-EG Kit (Kurabo) and 5 ng/mL VEGF at 37 °C. Matcha powder, vitamin K1, and lutein were added in both collagen gel and the medium. After 2 wk. images were acquired on a phase-contrast microscope. Rings were fixed in 4% PFA for 1 h at 4 °C. Next, rings were washed twice in PBS and treated with a blocking buffer containing 10% donkey serum and 0.1% Triton X-100 for 2 h at 4 °C. Rings were incubated with primary antibodies, mouse anti-RFP (1:2,000; MBL), and rabbit anti-laminin (1:2,000; Sigma) overnight at 4 °C. Immunolabeled rings were washed three times in PBS-T and incubated with secondary antibodies overnight at 4 °C. After washing three times in PBS-T, rings were incubated with DAPI for 30 min at room temperature. Rings were mounted under a glass cover with a mounting medium. Sprouting cells with Flt1-DsRed fluorescence were inspected under a phase microscope over a period of 6–14 d. Images were acquired with a confocal microscope. To measure the angiogenic potential, sprouting blood vessels were traced using ImageJ line- or area-selection tools from phase-contrast or confocal images, and the nuclei of sprouting vessels were counted. The quantified results were standardized to the control values.

| Components | (mg/100 g) |
|------------|------------|
| Catechin   | 35         |
| Catechin-gallocate | 4.90       |
| Epicatechin | 360        |
| Gallocatechin | 120       |
| Gallocatechin-gallocate | 67         |
| Epigallocatechin | 1,900     |
| Epigallocatechin-gallocate | 5,900     |
| Epicatechin-gallocate | 890       |
| Theanine   | 1,800      |
| Vitamin A  | 2.81       |
| α-Carotene | 12.10      |
| β-Carotene | 27.40      |
| Cryptoxanthin | 0.50      |
| Vitamin B1 | 0.44       |
| Vitamin B2 | 1.46       |
| Vitamin B6 | 0.67       |
| Vitamin K1 | 3.24       |
| Total chlorophyll | 996       |
| Chlorophyll-a | 684       |
| Chlorophyll-b | 312       |
| Total carotenoid | 154       |
| Zeaxanthin  | 1.40       |
| Lutein     | 79.60      |

Table 2. Content of vitamins, minerals, and amino acids in matcha powder. Matcha includes abundant vitamin K1 and lutein. ± represents the standard error of the mean (SE).
Matcha Control Angiogenesis

**Tube formation assay.** Procedures were performed as previously described (28). One hundred fifty microliters Matrigel (Corning) was plated into a 48-well plate and incubated for 30 min at 37°C. Human umbilical vein endothelial cells (HUVEC) were plated on the gel and cultured in an EGM-2 medium with an additive factor kit (Lonza) and vitamin K₁ (Nacalai Tesque, Inc.), lutein (San-Ei Gen F.F.I.), or dimethyl sulphoxide (DMSO; as the negative control). After 6–8 h incubation, microscopic images of tubes were acquired on a phase con-
trast microscope. Then, 4–5 images were captured in a well, and all conditions were analyzed in duplicate. The total length of the tubes and branch points were quantified from the images. In this study, HUVEC cells up to passage number 4 were used.

Statistical analysis. Statistical analysis was performed using SPSS. Student’s t-test and one-way ANOVA followed by post hoc Dunnett-t3 test as stated in previous experiments were used to test the significance. Error bars represent the standard error of the mean (SE).

**RESULTS**

Decreased cortical capillary density in the deep layer

First, we observed and compared the structure of the cortical capillary plexus between the upper layer (UL) (layers 2–4) and deep layer (DL) (layers 5–6) from 3 mo old Flt1-DsR mice (Fig. 1A). In the UL, capillaries tend to form vertical and straight plexuses (Fig. 1A’–Supplemental Online Material, Movie S1), while capillaries tend to form a tortuous plexus in the DL (Fig. 1A’–Supplemental Online Material, Movie S2). Next, to assess capillary aging, we compared the capillary density and length of young (3–4 mo) and aged (11–12 mo) brains of Flt1-DsR mice (Fig. 1B). The result of quantification of the capillary density and length showed that the capillary density decreased significantly in the aged cortex, although the capillary length did not change significantly (p<0.05; Fig. 1C, D). Moreover, we found that the capillary density and length significantly decreased in the DL preferentially, while there were no significant differences in the UL (p<0.05; Fig. 1E, F). Then, we observed the capillary morphology. Highly magnified images showed aberrant capillaries in the aged mouse brain (Fig. 1G). Orthogonal views indicated that the capillary lumen collapsed in the twisted segment (Fig. 1G’–1G”). These results indicate that Flt1-positive capillaries aging occurred in the DL of the cortex preferentially.

**Matcha and its components vitamin K1 and lutein increased angiogenic potential**

To evaluate the protective effect of matcha on capillary aging, we cultured thoracic aortae with 20 μg/mL matcha powder and quantified the sprouting cell region. We found that the sprouting endothelial cell region was significantly increased by culturing with matcha (Fig. 2A, B). Therefore, we focused on the components of matcha powder (Table 2), and selected vitamin K1 and lutein as candidate components that were likely increase angiogenic potential, because previous studies demon-

|                      | Control   | Matcha    |
|----------------------|-----------|-----------|
| Body weight (g)      | 53.9±5.6  | 57.6±4.0  |
| Brain (mg/g)         | 9.92±1.12 | 8.95±0.74 |
| Lung (mg/g)          | 6.66±0.63 | 7.97±0.93 |
| Heart (mg/g)         | 4.84±0.33 | 4.77±0.27 |
| Kidney (mg/g)        | 15.61±1.43| 14.17±0.65|
| Liver (mg/g)         | 44.35±1.50| 44.73±1.18|

![Fig. 3. Daily matcha intake increases the capillary density in layer 1.](image-url)

(A) Experimental schedule. (B) Representative cortical capillary images of matcha-fed reporter mouse brains. Scale bar=50 μm. (C) Quantitative data of the capillary density of the whole cortex. n=5 for the control and n=6 for matcha. (D) Quantitative data of the capillary density of each layer. *p<0.05, n=5 for the control and n=6 for matcha. (E) Quantitative data of the capillary length of the whole cortex. n=5 for the control and n=6 for matcha. (F) Quantitative data of the capillary length of each layer. n=5 for the control and n=6 for matcha.
strated their protective effects on vascular health (29, 30). To evaluate their potential to induce angiogenesis, they were added to the medium for HUVEC (Fig. 2C).

The result of the tube formation assay (tube length and branch point) revealed that vitamin K1 enhanced angiogenic potential (Fig. 2C–E). Next, we cultured thoracic aortae with 100 μM vitamin K1, 10 μM lutein, and quantified the sprouting vascular region and number of nuclei of sprouting endothelial cells. Under the vitamin K1-added condition, the number of cell nuclei of sprouting endothelial cells significantly increased (Fig. 2F–H). On the other hand, the addition of lutein did not significantly affect the vascular region and the number of nuclei, although lutein tended to enhance angiogenic potential (p<0.05; Fig. 2I–K).

**Long-term matcha ingestion has partial protective effects on capillary aging**

A protective effect on cognitive function decline by long-term green tea ingestion has been confirmed (20). As we described above, we found that aberrant capillaries appear in the aged brain. Therefore, we tested the effect of long-term matcha ingestion. Young (7 wk-old) F1t1-Dsr mice were fed 2% matcha-containing diet for 30 wk (9 mo old) (Fig. 3A). We noted that long-term ingestion of matcha did not affect the weight of the body or major organs (Table 3). We assessed the cortical capillary density and length of mouse brains after matcha ingestion (Fig. 3B), and found that matcha ingestion induced a significant increase in the capillary density and length of the whole cortex (Figs. 3C, D). On the other hand, we found that the capillary density of layer 1 was significantly higher in matcha ingested mouse brains than that of the control mouse brains (Fig. 3E, F).

**DISCUSSION**

In this study, we analyzed matcha and its components vitamin K1 and lutein to assess the angiogenic potential in vitro, ex vivo, and in vivo experimental systems.

Our in vivo study showed that the capillary structures of the UL and DL were different, and the DL cortex has more tortuous capillaries than that of the UL cortex. A previous study showed that in the Alzheimer’s model mouse brain, venules were more tortuous than healthy mouse brain venules, and it became a risk for deposition of amyloid beta because blood flow decelerated at a tortuous segment (31). Therefore, these reports suggest that DL capillaries have a higher risk of deposition of blood consumptions than do UL capillaries. Whether differences in the vascular plexus or cell properties increase vulnerability for hypoxia and amyloid beta accumulation have not been completely revealed (32). Our results suggest that the capillary structure might be associated with neural vulnerability.

In previous studies, decreased capillary density associated with aging in the cortex was reported for an 18 mo-old mouse (33–35). In this study, we found that the cortical capillary density decreased by 11 mo-old. In addition, the physiological importance of spontaneous capillary obstructions at an early age, which lead to vascular aging, has been demonstrated previously (26, 36).

We focused on matcha because previous reports showed that long-term intake of green tea has protective effects on brain function and vascular health (20–23, 29, 30). First, we showed that matcha induced angiogenesis in an ex vivo experimental system. Next, in vitro and ex vivo experiments of the matcha components showed that vitamin K1 and lutein enhanced the angiogenic potential, although statistical analyses did not show the significant effects of lutein. Previous epidemiological reports indicated that Alzheimer’s disease patients ingested a significantly lower amount of vitamin K1 than that of healthy same-age subjects (37), and cognitive deficits were protected by the intake of vitamin K1 (38). Moreover, a clinical study revealed that vitamin K1 has beneficial effects for the elastic properties of the vessel wall (29), and vitamin K1 inhibits vascular calcification (39, 40). Lutein is a carotenoid, and epidemiologic study showed that lutein increased cognitive function (41, 42). Moreover, lutein is highly concentrated in the brain and indicates protective effects for neurons (43). Since lutein has been indicated to possess anti-oxidant properties, it inhibits atherosclerosis formation, and reduced the risk of age-related macular degeneration (22, 44). Therefore, vitamin K1 and lutein are one of the candidate components that were likely increase angiogenic potential.

In this study, we examined the effect of long-term matcha ingestion on capillary aging using a 2% concentration of matcha green tea powder. The 2018 National Health and Nutrition Examination Survey reported that the three major nutrient intake for adult women are 332 g (men are 365 g). Therefore, 2% feed is equivalent to 6.6–7.3 g of matcha and is converted into the drink for approximately four cups per day, considering that the food intake amount of the mouse is different from that of humans by approximately 1,000 times. For ex vivo and in vitro experiments, 1–100 μM vitamin K1 and 0.1–10 μM lutein was used to assess the angiogenic potential. Approximately 100 nM vitamin K1 is incorporated into the cultured cells by the treatment of 100 μM vitamin K1 (45). In the human body, the level of vitamin K1 is 4 nM in the aged brain (46). In addition, it was revealed that the base plasma level of lutein was 0.2 μM, and after ingestion of 10 mg/d lutein increased the plasma level of lutein to 1 μM in human studies (47). Since lutein is highly accumulated in the brain (48), the brain contains higher level of lutein than the serum. These informations indicate that vitamin K1 and lutein are not the only active components in matcha that increase angiogenic potential; therefore, further studies will be needed to clarify the effect of matcha and its components on the angiogenic potential.

Interestingly in vivo experiments showed that long-term ingestion of matcha protected the capillary density in layer 1 of the cortex. Many neurons, including DL neurons, sprout dendrites to layer 1. Moreover, it has
been reported that the basal dendrites of the DL neurons decreased with aging (42). Therefore, protection of capillary aging by matcha ingestion may contribute to maintaining the functions of the DL neurons by protecting their dendrites in layer 1. Moreover, in the UL and layer 1 of the cortex, spontaneous obstructions associated with capillary pruning occurred preferentially from 3 mo old, and neural death occurs with atrophy (26, 49). These studies indicated that capillaries had a higher risk for aging due to the obstruction in the UL and layer 1 (26). On the other hand, we suggested that capillaries might be vulnerable to aging caused by deposition of blood consumptions in the DL. These informations imply that capillary aging progresses due to different causes in the brain. This is one of the reasons why the ingestion of matcha had protective effects on capillary aging in layer 1 but not the DL. Therefore, it is recommended that the details of the capillary aging of the brain need further elucidation in the future.

A more recent study demonstrated that F1t1 has a key role in the amyloid beta oligomer induced aging process in cultured endothelial cells in vitro experiments (50). Therefore, assessing capillary aging based on the decrease in F1t1-positive capillaries is critical for understanding the pathophysiology of Alzheimer’s disease, thus indicating the protective effects of matcha ingestion on the physiology of brain aging and diseases.

In conclusion, we found that matcha and its components vitamin K₁ and lutein enhanced angiogenic potential in some experimental systems, thus affirming that daily nutrition is important to prevent vascular aging.

Authorship

Research conception and design: TI, YS, TO, YF, MI, and KM; experiments: RI; statistical analysis of the data: RI; writing of the manuscript: RI and KM. All authors discussed the results and commented on the manuscript.

Disclosure of state of COI

All authors declared no competing interests.

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Supporting information

Supplemental online material is available on J-STAGE.

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