Chronic cerebral hypoperfusion in a mouse model of Alzheimer’s disease: An additional contributing factor of cognitive impairment

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The purpose of the present study was to evaluate whether chronic cerebral hypoperfusion would affect cognitive status in an Alzheimer mouse model. Behavioral tests and histological evaluations were performed using female Tg2576 mice eight weeks after right common carotid artery occlusion (rCCAO), which is known to induce a type of vascular dementia without neuronal necrosis in nontransgenic mice. Positron emission tomography with 18F-fluorodeoxyglucose (FDG-PET) was utilized to evaluate metabolic status in the rCCAO-operated brain of nontransgenic mice. Escape latency from the Morris water maze test was not significantly different between rCCAO- and sham-operated mice. However, the learning curve was impaired in rCCAO-operated transgenic mice while it was preserved in sham-operated transgenic or rCCAO-operated nontransgenic mice. Histological examination revealed no evidence of cell death in the rCCAO-operated brains, and the extent of amyloid deposition was not different in rCCAO- and sham-operated mice. The brain of rCCAO-operated mice showed metabolic deficits in the ipsilateral parietal cortex through FDG-PET. In conclusion, further cognitive decline which is more comparable to typical Alzheimer’s disease was induced by chronic cerebral hypoperfusion in an Alzheimer mouse model. This aggravation might be associated with hypometabolism via chronic cerebral hypoperfusion.

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Alzheimer’s disease (AD) has been reported to be associated with vascular disease in the brain. Among various vascular risk factors, chronic cerebral hypoperfusion (CCH) is related to the pathogenesis of AD as well as a type of vascular dementia (VD) [1,2,6,8,12]. In this respect, reduced cerebral perfusion in AD could cause mixed dementia-like cognitive abnormalities. Nevertheless, cognitive changes induced by CCH have not been extensively studied in AD transgenic mouse models.

The Tg2576 mouse is an AD animal model which overexpresses human APP695 with the “Swedish” mutation [5]. The mice present amyloid plaques with corresponding memory deficits, but neuronal death is not generally induced [5,11]. Memory impairments are usually revealed in a serial Morris water maze test which reflects deficits in the hippocampus [5,11]. One important point is that the learning curve is spared in Tg2576 mice even though their escape performance is worse in comparison to the control mice [9]. On the other hand, a recent study reported that right common carotid artery occlusion (rCCAO) in mice could induce a deficit on the non-spatial working memory one month post-operation and that these deficits were related to cortical-subcortical circuit damage [12]. Although their regional cerebral blood flow remained low until later, there was no corresponding neuronal death. The authors suggested that these changes resulted from CCH which had been induced by rCCAO. In the current study a mixed dementia model with both AD and VD was used. Given that extensive necrosis may mask important pathological features, a mild ischemic model without inducing a gross infarction was preferable for evaluating both pathological and cognitive changes. The present study was performed to examine whether CCH could cause further cognitive deficits in an AD mouse model. We also evaluated regional glucose metabolism using 18F-fluorodeoxyglucose positron emission tomography for animals (18F-FDG microPET) in order to document a causative factor which induced cognitive deficits in rCCAO-operated mice.

All experiments were performed in accordance with the Guidelines for Animal Experiments of Ajou University. Female Tg2576 (Tg+) mice (C57BL/6 background) and their littermates (Tg−) weighing 23–29 g (age, 14–15 months) were used (Taconic, NY). To evaluate basic findings of rCCAO-operated brain in nontransgenic mice, female C57BL/6 mice weighing 18–26 g (age, 10–11 months)
were rapidly frozen in powdered dry ice and stored at −80°C for anesthesia maintenance in 30% O2/70% N2O through a face-mask. To establish the rCCAO model, the right CCA was carefully isolated from the adjacent vagus nerve after midline neck incision and completely ligated with 6-0 silk sutures. As a sham operation, the mice were subjected to the same surgery without the ligation. Body temperature was maintained at 37.5 ± 0.5°C with heating pads until the animals recovered from surgery. Tissues for histological examination were obtained from brain sections of animals sacrificed eight weeks after surgery. Under deep anesthesia with chloral hydrate, blood was washed out and brain tissues were fixed by transcardiac perfusion with phosphate-buffered saline (PBS) and 4% paraformaldehyde (Sigma–Aldrich, MO), respectively. For the study of blood–brain barrier permeability, 3% Evans blue (Sigma–Aldrich, 3 ml/kg) was intravenously injected 20 min prior to perfusion fixation. Whole brains were then removed and kept in 4% paraformaldehyde at 4°C for 24 h. Twenty-four hours later, brains were immersed in 30% sucrose for 3–4 days at 4°C. Subsequently, brains were rapidly frozen in powdered dry ice and stored at −70°C. The brains were then sectioned with a cryostat to a thickness of 30 µm.

An object recognition test was performed six weeks after surgery to examine the non-spatial working memory. The test was carried out using a modification of a method described in previous studies [3,12]. Briefly, the apparatus used in the test was constructed using an opaque acrylic box (30 cm × 45 cm × 30 cm). The objects to be discriminated were made of acryl and were of different shapes and colors (green pyramid and black cubes). The day before the test, the mice were allowed to explore the box free of any objects for 5 min. On the day of the test, a session of two trials was conducted with an inter-trial interval of 60 min. In the first trial, two identical objects (black cubes) were presented on two opposite sides of the box, and the mice explored for 10 min. Object recognition was defined if the center of the mouse body was at a distance 5 cm from the object. In the second trial, one of the objects presented in the first trial was replaced with a new object (green pyramid) and the mice were placed in the box for 3 min. The time spent for exploring familiar (F) and new (N) objects was automatically recorded by EthoVision 3.0, a video tracking system (Noldus Information Technology, The Netherlands). An discrimination index was calculated as (N − F/N + F) for intergroup comparisons. Successively, a Morris water maze test was performed seven to eight weeks after surgery to examine spatial memory. An 85-cm circular swimming pool was used to measure learning acquisition and memory retention after surgical procedures. For the purposes of analysis, the pool floor was divided into four zones: Z (I), Z (II), Z (III), and Z (IV). An indiscernible 9-cm platform was positioned 1.5 cm below the water surface in Z (II). Testing involved three trials per day over 5 days. In the course of daily testing, animals were successively admitted into the opposite quadrant, Z (IV), and allowed to swim for a maximum of 60 s. On locating the platform (or being guided to the platform after 60 s), the animal was permitted to remain on the platform for 30 s prior to the next trial on the first day. Latency of finding the platform and remaining there for 10 s or more was estimated for each of the three trials, and the average of the latencies, which had been recorded for each animal by EthoVision 3.0, were calculated. Following the 5 days of acquisition testing, memory retention was determined in a single 60-s probe trial. For this trial, the submerged platform was removed from the water maze and the animal was released into the quadrant opposite that into which the submerged platform had been placed.

Acid fuchsin (Sigma–Aldrich) or 0.5% cresyl violet (Sigma–Aldrich) staining was used to detect neuronal death. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Millipore, MA) was performed to detect apoptotic neuronal death. Proteinase K (20 µg/mL) was applied to the specimens for 15 min at room temperature and then 3% hydrogen peroxide in PBS was added for 5 min at room temperature. Successively, equilibration buffer, working strength TdT enzyme, Stop/Wash buffer, and anti-digoxigenin conjugate were applied. After being washed in PBS, color was developed with peroxidase substrate and counterstaining. Luxol fast blue (Sigma–Aldrich) staining was used to observe features of oligodendrocytes or myelin in the white matter. Tissues were soaked into 100% ethanol for 5 min and then 95% ethanol for 10 min. Luxol fast blue was then applied at 37°C overnight. Tissues were washed with distilled water and then dipped into 0.05% lithium carbonate solution for 20 s. Color differentiation was done with 70% ethanol and then the specimens were dehydrated and mounted. With respect to immunohistochemistry, brain sections were washed in PBS and then incubated in 0.25% Triton X-100 mixed with 0.3% hydrogen peroxide in at room temperature for 10 min. Subsequently they were reacted with 10% normal goat serum for 1 hour and exposed to the primary antibodies (1:200) overnight at 4°C. A monoclonal antibody to pan-axonal neurofilament marker (Invitrogen Corporation, CA) was used as the primary antibody. The sections were then incubated in a biotinylated secondary anti-rabbit antibody (1:200, Vector Laboratories, CA) for 2 h at room temperature. For 3,3′-diaminobenzidine tetrahydrochloride (DAB kit, Vector Laboratories) staining, Avidin–biotin peroxidase complex solution (Vectastain ABC kit, Vector Laboratories) was applied. The DAB reaction was stopped by rinsing with PBS. Mounted sections were observed under a bright field microscope. To examine the amyloid plaques, thioflavine S staining was used. Briefly, brain tissues were washed with 0.1 M phosphate buffer for 5 min and then stained in freshly prepared and filtered 1% thioflavine S (Sigma–Aldrich) solution in 0.1 M phosphate buffer for 5 min. These sections were rinsed with 0.1 M phosphate buffer and distilled water and mounted with the mounting medium (Vector Laboratories). Sections were visualized and images were captured with a microscope and digital camera (Olympus, Japan) attached to a computer and saved as Tagged Image Format files. Computer-aided quantification of amyloid plaques was performed using the MetaMorph 7.0 software (Universal Imaging Corp, PA). Plaque counts and percentage occupied by the thioflavine S positive plaques were quantified in the ipsilateral brain sections. The region of interest was drawn manually under 100-fold magnification and the threshold was kept constant throughout the analysis.

Regional glucose metabolism in female C57BL/6 mice, weighing 23–29 g (age, 14–15 months), were evaluated using 18F-FDG microPET imaging eight weeks after sham or rCCAO surgery. Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) and intravenously injected with 55.5 MBq of 18F-FDG through the tail vein. After an uptake period of 60 min, animals were placed in a spread prone position and scanned with the eXplore Vista (GE Healthcare, WI), consisting of a 11.8-cm diameter ring with 36 position-sensitive γ-ray scintillation detectors (dual layer [GSO, LYSO crystal] phoswich detectors), providing a 6.7 cm transaxial and a 4.8 cm axial field of view, with image resolution of <1.0 mm. A 45-min static acquisition was performed in three-dimensional mode, and images were reconstructed by a maximum–a-posteriori probability algorithm. Corrections for dead time, random, scatter, decay, and normalization were performed.

Data are presented as the mean ± standard error of the mean (SEM). A repeated measures analysis of variance (ANOVA) was used to compare the serial mean latency among groups in the Morris water maze test. Bonferroni’s test was used for post hoc comparisons after repeated measures ANOVA. To estimate whether learning curve of Tg+ groups was preserved or impaired, additional repeated measures ANOVA for single group analysis was performed. One-way ANOVA was performed to compare memory
repetition in the Morris water maze test and the discrimination index in the object recognition test. Scheffe’s test was used for post hoc comparisons after one way ANOVA. A t-test was used to compare the amount of amyloid plaques between Tg+ sham- and rCCAO-operated mice. It was also used as a post hoc test in cases where the Scheffe’s test did not show any significant difference between groups. Differences of p < 0.05 were considered to be statistically significant. Statistical analyses were performed using a commercially available software package, SPSS version 12.0 for Windows (SPSS Inc., IL) and graphs were drawn using SigmaPlot version 9.0 (Systat Software Inc., IL).

With regard to basic characteristics in rCCAO-operated mice, mice with rCCAO did not exhibit necrosis seven days post-operation by 0.5% cresyl violet staining (Fig. 1A). Similarly, Evans blue staining for examination of the blood brain barrier revealed that it was intact in mice with rCCAO (Fig. 1B). As for perfusion status, about 40% drop in CBF was revealed in rCCAO-operated mice when it was measured in the ipsilateral hemisphere by laser Doppler flowmetry (data not shown). On paw grip endurance testing for which mice were placed on the wire-lid of a conventional housing cage trying (data not shown). On paw grip endurance testing for which latency to escape to a hidden platform in the Morris water maze was assessed, a significant difference in the latency among the groups was revealed (repeated measures ANOVA, F = 8.700, p < 0.001) (Fig. 2A). This latency to escape was delayed in the Tg+ mice with or without rCCAO as compared to the Tg- sham mice (post hoc, Bonferroni’s test; Tg+ sham vs Tg− sham, p = 0.008; and Tg+ rCCAO vs Tg− sham, p = 0.001). The performance was not statistically different between the Tg+ rCCAO- and sham-operated mice (post hoc, Bonferroni’s test, p = 1.000); however, the Tg+ rCCAO group showed an impairment of the learning curve (repeated measures ANOVA for a session analysis of the Tg+ rCCAO group, F = 0.884, p = 0.420) whereas the learning curve of Tg+ sham or Tg− rCCAO group was preserved (repeated measures ANOVA for a session analysis; Tg+ sham group, F = 6.647, p = 0.003; Tg− rCCAO group, F = 5.114, p = 0.025). A probe test revealed a statistical difference among the groups in terms of the time spent in the target quadrant where the platform had been removed (mean time spent in the target quadrant (s) ± SEM; Tg− sham, 15.39 ± 1.25; Tg+ rCCAO, 14.00 ± 1.80; Tg+ sham, 8.94 ± 1.92; Tg+ rCCAO, 7.66 ± 2.33; one-way ANOVA, F = 3.500, p = 0.025) (Fig. 2B). Memory retention of Tg+ mice exhibited greater impairment as compared to the Tg− mice (post hoc, t-test; Tg+ sham vs Tg− sham, p = 0.017; Tg+ rCCAO vs Tg− sham, p = 0.021). However, there was no difference between Tg+ rCCAO- and Tg+ sham-operated mice (post hoc, Scheffe’s test; Tg+ rCCAO vs Tg+ sham, p = 0.972). Non-spatial working memory was evaluated using the object recognition test. The discrimination index was significantly different among the groups (mean of the discrimination index ± SEM; Tg− sham, 0.80 ± 0.10 for Tg− rCCAO, 0.42 ± 0.20 for Tg+ rCCAO, and Tg− sham, and −0.01 ± 0.19 for Tg+ rCCAO; one-way ANOVA, F= 3.269, p = 0.035) (Fig. 2C). Tg− or Tg+ rCCAO-operated mice had lower indices compared with Tg− sham mice (post hoc, t-test, Tg− rCCAO vs Tg− sham, p = 0.005; Tg+ rCCAO vs Tg− sham, p = 0.007). There was no significant difference between the Tg+ rCCAO- and sham-operated mice (post hoc, t-test; p = 0.146).

Histological studies were done eight weeks after surgery. No cell death was observed in the brain sections of the Tg+ and Tg− mice upon acid fuchsin examination (Fig. 3A) and TUNEL staining...
Fig. 2. Loss of learning curve on the Morris water maze test in Tg+ rCCAO-operated mice. (A) A Morris water maze test revealed that the latency to escape to the platform was prolonged in the Tg+ mice as compared to the Tg− mice (repeated measures ANOVA, F=8.700, p<0.001). Among Tg+ mice, the learning curve of rCCAO-operated mice was impaired (* F=0.884, p=0.420) whereas that of sham-operated mice was preserved (** F=6.647, p=0.003). (B) A probe test revealed that the time spent in the target quadrant decreased in the Tg+ mice compared to the Tg− mice (ANOVA, F=3.500, p=0.025) but it was not different between Tg+ rCCAO- and sham-operated mice. (C) The object recognition test revealed that the discrimination index for both Tg+ and Tg− rCCAO-operated mice significantly decreased when compared to the Tg− sham-operated mice (one way ANOVA, F=3.269, p=0.035; post hoc, t-test, Tg− rCCAO vs Tg− sham, p=0.005; Tg+ rCCAO vs Tg− sham, p=0.007).

Fig. 3. No evident neuronal death in the coronal brain sections were observed eight weeks after surgery. (A) Acid fuchsin, right parietal cortex (bar = 50 μm). (B) TUNEL, right parietal cortex (bar = 50 μm). (C) Monoclonal antibody to the pan-axonal neurofilament, right corpus callosum (bar = 50 μm). (D) Luxol fast blue, right corpus callosum (bar = 100 μm).
significant neuronal death, exhibited hypometabolism in the ipsilateral brain sections (Fig. 3C) or Luxol fast blue (Fig. 3D). Amyloid plaques were detected using thioflavin S fluorescence staining and the plaque burden on the right hemisphere sections was subsequently quantified. There was no statistical difference in the plaque burden between the Tg+ rCCAO and Tg+ sham mice (mean area ±SEM; sham, 39971.35 ± 10380.34 μm² vs rCCAO, 39023.56 ± 11797.44 μm²; t-test, p = 0.952) (Fig. 4).

Our overall results suggested that cognitive impairment of Tg+ rCCAO-operated mice is more than simple addition of those of Tg+ mice and those of rCCAO-operated mice. The Tg+ rCCAO-operated mice exhibited distinct characteristics of both AD and VD. Moreover, learning impairment became more evident after rCCAO surgery in Tg+ mice. The Tg2576 mouse is a good model of AD in that amyloid plaques are induced and cognitive deficits accompany the pathological changes. Although mice exhibit impaired performance on the escape latency of Morris water maze test, learning ability is preserved [9]. Most graphs related to the Morris water maze test for AD models revealed that learning curves of Tg+ mice were intact even in aged mice with decreased memory [5,11]. In the current study, the results were similar in that Tg+ mice without rCCAO revealed lower performance in the escape latency but showed preserved learning ability. However, this learning ability was lost in Tg+ mice upon rCCAO-operation, suggesting that the aggravation in learning impairment might result from a synergistic effect of CCH on AD cognition.

The presence of cognitive impairment without neuronal death in rCCAO-operated mice has been questioned. Our preliminary microPET study suggested that cerebral hypometabolism is associated with rCCAO. Mice with rCCAO-operation, which had dissociation between cognitive impairment and the absence of significant neuronal death, exhibited hypometabolism in the ipsilateral cortex on 18F-FDG microPET images. These features might reflect that the ipsilateral brain with rCCAO is in the status between the benign oligemia and ischemic penumbra. Without neuronal death, CCH can affect dendritic arborization and synaptic contact [4]. A decline in the levels of the microtubule-associated protein 2, which plays a role in dendritic branching, remodeling or plasticity, and a reduction in the levels of the synaptophysin protein have been reported to be associated with CCH [7,10]. In addition, early hypometabolic changes prior to neurodegenerative damage occurs without senile plaque formation or cerebral ischemic injury in aged rats with bilateral CCAO and this phenomenon has been reported to be associated with astrocytosis, oxidative stress, protein abnormalities and endothelial cell damage [2].

PET studies for Tg+ mice, unfortunately, were not available with old Tg2576 mice since it requires more than a year of breeding time. The negative result of axonal and demyelinating injury is not conclusive because relatively thick sections (30 μm) were used. A previous study used 2 μm of paraffin sections for revealing no white matter damage [12]. Regardless of some limitations, the finding that CCH itself caused cognitive aggravation without increasing amyloid plaques and cellular death is a significant concept in the understanding of the pathophysiology and clinical manifestations of vascular influences on AD.

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