Protective effects of *Perilla frutescens* seed oil on cognitive function, oxidative stress and acetylcholinesterase activity in a D-galactose-induced accelerated aging model in rats

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Abstract. Aging-related cognitive decline is closely associated with oxidative stress, cholinergic dysfunction and hippocampal vulnerability. Perilla seed oil (PSO), a functional food rich in α-linolenic acid and antioxidant phytochemicals, may have cognitive benefits; however, its efficacy and underlying mechanisms in experimental models of accelerated aging remain insufficiently understood. The present study aimed to investigate the effects of PSO supplementation on cognitive performance and neurobiological alterations in a D-galactose (D-Gal)-induced accelerated aging model in rats. Wistar rats were injected subcutaneously with D-Gal (300 mg/kg) daily for 8 weeks and simultaneously treated orally with PSO (100 or 500 mg/kg), fish oil (500 mg/kg) or vehicle. Cognitive function was evaluated using the Morris water maze and novel object recognition tests. Oxidative stress markers, including malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD), as well as acetylcholinesterase (AChE) activity, were assessed in the hippocampus. Neuronal cell density in the CA1 and CA3 regions was examined using Nissl staining. PSO supplementation significantly improved spatial memory performance and recognition memory, increased hippocampal SOD activity and reduced AChE activity compared with the D-Gal group. Although MDA and GSH levels did not differ significantly, both exhibited a tendency toward normalization. In addition, neuronal density in the CA3 region was significantly reduced in the D-Gal group compared with the control group, whereas no significant differences were observed in the CA1 region. These findings suggest that PSO attenuates D-Gal-induced cognitive impairment, which may be partially associated with enhanced antioxidant enzyme activity and modulation of cholinergic function, rather than with restoration of neuronal density. PSO may therefore represent a potential nutritional intervention for supporting cognitive function during aging-related neurobiological changes.

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Introduction

The global population is aging at an unprecedented rate, with the number of individuals aged ≥60 years projected to >2 billion by 2050 (1). Aging is accompanied by progressive deterioration of physiological homeostasis and functional decline across multiple organ systems, with the brain being particularly vulnerable. Age-related brain dysfunction is characterized by metabolic dysregulation, chronic neuroinflammation and impaired cellular stress-response mechanisms, all of which contribute to neurodegenerative processes and cognitive decline (2-4). Among the various mechanisms implicated in aging, oxidative stress has been widely recognized as a central pathological contributor (5-7). Oxidative stress occurs when the generation of reactive oxygen species (ROS) exceeds the capacity of endogenous antioxidant defenses, resulting in oxidative damage to DNA, proteins and lipids (7,8). These molecular insults are associated with age-related neurodegeneration and impairments in learning and memory (9,10).

The D-galactose (D-Gal)-induced model is widely employed as an accelerated aging-like paradigm to investigate the mechanisms underlying age-associated cognitive dysfunction in rodents. Chronic administration of D-Gal leads to excessive ROS production, oxidative damage, mitochondrial dysfunction and neuroinflammation, thereby recapitulating key pathological features of natural aging (11-15). In addition to oxidative injury, D-Gal has been shown to disrupt

cholinergic neurotransmission, thereby impairing synaptic plasticity and cognitive performance (14,15). Given the important role of the cholinergic system in learning and memory and its vulnerability to oxidative stress, interventions that attenuate oxidative damage while preserving cholinergic function are of considerable interest in aging and neurodegeneration research.

Perilla seed oil (PSO), commonly known as Nga-mon in Thailand, is derived from *Perilla frutescens* (L.) Britt., a plant belonging to the mint family (*Lamiaceae*). PSO has been traditionally consumed throughout Asia as both a medicinal herb and a functional food. PSO is particularly rich in α-linolenic acid (ALA), an essential omega-3 fatty acid (16). In addition, it contains phenolic and flavonoid bioactive compounds (17). These constituents have been reported to possess antioxidant properties, with ALA in particular exhibiting anti-inflammatory activity and being associated with improvements in memory and cognitive performance (17-21). Notably, previous clinical studies have demonstrated that PSO supplementation attenuates age-related cognitive decline in healthy older individuals, improving memory, attention and processing speed while reducing subjective forgetfulness. These cognitive benefits were accompanied by enhanced systemic antioxidant capacity and reduced oxidative stress biomarkers, supporting a potential neuroprotective role for PSO (19,22).

Despite these clinical observations, the efficacy of PSO in experimental models of accelerated aging remains insufficiently investigated, particularly in the D-Gal-induced rat model. Moreover, the mechanisms by which PSO may influence cognitive decline, including modulation of oxidative stress, preservation of cholinergic neurotransmission and changes in hippocampal neuronal integrity, remain incompletely elucidated. Therefore, the present study aimed to investigate whether PSO supplementation could attenuate D-Gal-induced cognitive impairment in rats and to examine the associated mechanisms, with a particular focus on oxidative stress, cholinergic function and hippocampal neuronal integrity.

Materials and methods

Preparation of PSO. Seeds of *P. frutescens* were collected from cultivated areas in Mae Hong Son Province, Thailand. The seeds were cleaned, air-dried, mechanically crushed and subjected to cold-press extraction. The extracted oil was centrifuged at 4,000 x g for 10 min at 4˚C, filtered, and stored in airtight amber glass bottles at 4˚C until use. The chemical composition of the PSO used in the present study was not reanalyzed because the oil was obtained from the same production lot previously characterized by Kangwan *et al* (20), and the present study was designed to evaluate the biological effects of PSO rather than to perform additional compositional characterization. Using gas chromatography-mass spectrometry analysis, that PSO lot was shown to contain ALA (59.20±0.11%) as the major fatty acid, together with linoleic, oleic, palmitic and stearic acids. Accordingly, the administered doses in the present study should be interpreted as PSO doses rather than as individually quantified active constituents.

Animals. Male Wistar albino rats (6-8 weeks old; n=35; initial body weight, 150-180 g) were obtained from Nomura Siam. The animals were acclimatized for 1 week under controlled environmental conditions (temperature, 25±2˚C; relative humidity, 60±10%; and a 12-h light/dark cycle). Throughout the experimental period, the rats had ad libitum access to a standard laboratory diet (feed food no. 082; Charoen Pokphand Foods PCL) and reverse osmosis water. Body weight was recorded weekly throughout the experimental period. All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Phayao (approval no. 1-004-66).

Experimental groups and treatments. After a 1-week acclimatization period, the rats were randomly assigned to five experimental groups (n=7 per group) and treated for 8 weeks. The control sham group received daily subcutaneous injections of normal saline at the nape of the neck and oral administration of corn oil (CO). The D-Gal group received 5% D-Gal (300 mg/kg/day) subcutaneously in combination with oral CO. For the positive control group, rats received 5% D-Gal (300 mg/kg/day) subcutaneously together with fish oil (FO; 500 mg/kg/day, orally). The PSO treatment groups received 5% D-Gal (300 mg/kg/day) subcutaneously in combination with PSO at doses of 100 or 500 mg/kg/day, orally. A total of two doses of PSO were included to assess dose dependence and define an effective range in the D-Gal-induced accelerated aging model, while FO was used as a positive control omega-3 intervention to validate model responsiveness and benchmark the effects of PSO. Cognitive function was evaluated using the Morris water maze (MWM) on days 50-54, followed by a probe trial on day 55 and the novel object recognition (NOR) test on day 56, prior to sacrifice. The experimental timeline is illustrated in Fig. 1.

Cognitive function test

MWM. The MWM was used to assess spatial learning and memory, following a protocol adapted from Jeefoo *et al* (15). The apparatus consisted of a circular pool (150 cm in diameter and 45 cm in height) painted dark blue and divided into four quadrants. The pool was filled with a starch solution to a depth of 25 cm and maintained at 25˚C. A circular escape platform (12.5 cm in diameter) was submerged 2 cm below the water surface and positioned in a fixed target quadrant. Each rat underwent three training trials per day, with a maximum duration of 90 sec per trial, for 5 consecutive days (days 50-54). The animals were released from different starting positions facing the pool wall, while the platform location remained constant throughout the training period. Escape latency, defined as the time required to locate the hidden platform, was recorded as an index of spatial learning. On day 55, a probe trial was conducted by removing the platform. Each rat was released from the quadrant opposite the target quadrant and allowed to swim freely for 90 sec. Spatial memory retention was assessed by measuring the time spent in the target quadrant using an overhead video tracking system connected to a computer.

NOR. Recognition memory was assessed using the NOR test in a gray plastic arena (54x90x50 cm). On day 55, the rats were allowed to explore the empty arena freely for 5 min to habituate, then returned to their home cages for 5 min. During the training phase, each rat was placed in the arena containing

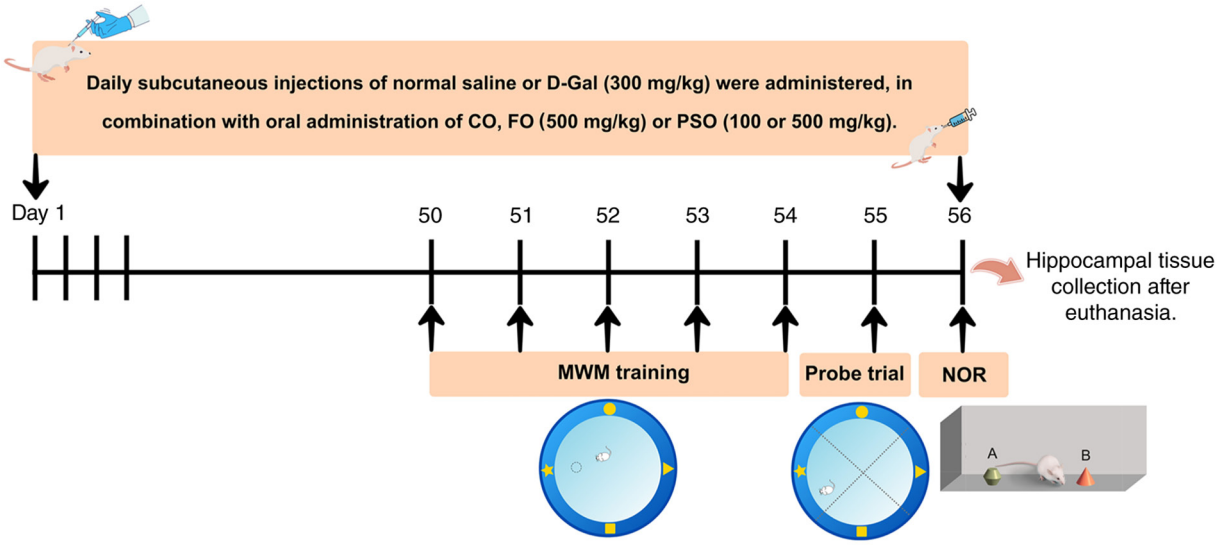

Figure 1. Experimental design of the animal study. Male Wistar rats were randomly assigned to five groups: Control sham, D-Gal, D-Gal plus FO and D-Gal + PSO at doses of 100 or 500 mg/kg. Treatments were administered for 8 weeks. Cognitive function was evaluated using the MWM (days 50-54), followed by a probe trial (day 55) and the NOR test (day 56) prior to sacrifice. CO, corn oil; D-Gal, D-galactose; FO, fish oil; MWM, Morris water maze; NOR, novel object recognition; PSO, Perilla seed oil.

two identical objects (A1 and A2) and allowed to explore for 5 min. Object exploration was defined as directing the nose toward an object at a distance of ≤2 cm. A total of 24 h after the training session, one of the familiar objects was replaced with a novel object (B), and the test phase was conducted for 5 min. Recognition memory was quantified using the discrimination ratio (DR), calculated as DR=(TB-TA)/(TB + TA), where TA and TB are the times spent exploring the familiar and novel objects, respectively (23). A higher DR value indicates improved recognition memory performance.

Hippocampal tissue preparation. At the end of the experimental period, the rats were deeply anesthetized intraperitoneally with thiopental sodium (100 mg/kg; Jagsonpal Pharmaceuticals Ltd., India) prior to transcardial perfusion with normal saline. Adequate depth of anesthesia was confirmed by absence of tail reflex before perfusion. After perfusion, mortality was confirmed by cessation of respiration and heartbeat. The brains were then rapidly removed, and the cerebral hemispheres were separated along the midline. The left hippocampi were immediately microdissected on an ice-cold glass plate, homogenized in 10% (w/v) PBS (1 M, pH 7.4) using a handheld homogenizer (D-160; DLAB Scientific Co., Ltd) and centrifuged at 8,000 x g for 15 min at 4˚C. The resulting supernatants were collected and stored at -80˚C for subsequent lipid peroxidation and enzymatic activity assays.

In parallel, the right cerebral hemispheres were fixed overnight in 4% buffered formalin at 4˚C, cryoprotected in 30% sucrose for 48 h at 4˚C and sectioned coronally at 30-μm thickness using a cryostat microtome (CM1950; Leica Microsystems). The sections were stored in antifreeze solution at 4˚C until further processing. For histological analysis, sections were stained with 0.2% cresyl violet (MilliporeSigma) for 10 min at 25˚C, dehydrated through graded ethanol solutions (70, 95 and 100%), cleared in xylene and covered with a coverslip using DPX mounting medium (Distyrene-Plasticizer-Xylene). All images were captured using the ECLIPSE Ni-U | Upright Microscopes (Nikon Corporation).

Biochemical assays

Lipid peroxidation. Lipid peroxidation was assessed by measuring malondialdehyde (MDA), a major end product of lipid peroxidation, as an index of oxidative stress. MDA levels were determined using the thiobarbituric acid-reactive substances assay, as previously described (15). Briefly, hippocampal tissue supernatants were mixed with SDS, acetic acid and thiobarbituric acid, followed by incubation in a boiling water bath for 60 min. After cooling, the absorbance of the resulting chromogen was measured at 540 nm using a microplate reader. MDA concentrations were expressed as μmol per mg protein.

Reduced glutathione (GSH). GSH levels in hippocampal tissue homogenates were measured using a commercially available assay kit (GSH Assay Kit; cat. no. DIGT-250; BioAssay Systems), according to the manufacturer's instructions. GSH concentrations were expressed as nmol per mg protein.

Superoxide dismutase (SOD) activity. SOD activity in hippocampal tissue homogenates was determined using a commercially available assay kit (SOD Assay Kit-WST; cat. no. S311-500; Dojindo Laboratories, Inc.), according to the manufacturer's instructions. Enzyme activity was expressed as the percentage inhibition of water-soluble tetrazolium salt-1formazan formation.

Acetylcholinesterase (AChE) activity. AChE activity was determined according to the method described by Nakdook *et al* (24), with minor modifications. The reaction mixture (225 μl) consisted of hippocampal tissue supernatant, 0.1 M phosphate buffer (pH 7.4), 1 mM acetylthiocholine iodide (ATCI) and 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The change at 405 nm was monitored every 10 sec for 3 min using a microplate reader. Enzyme activity was expressed as nmol of ATCI hydrolyzed per min per mg protein.

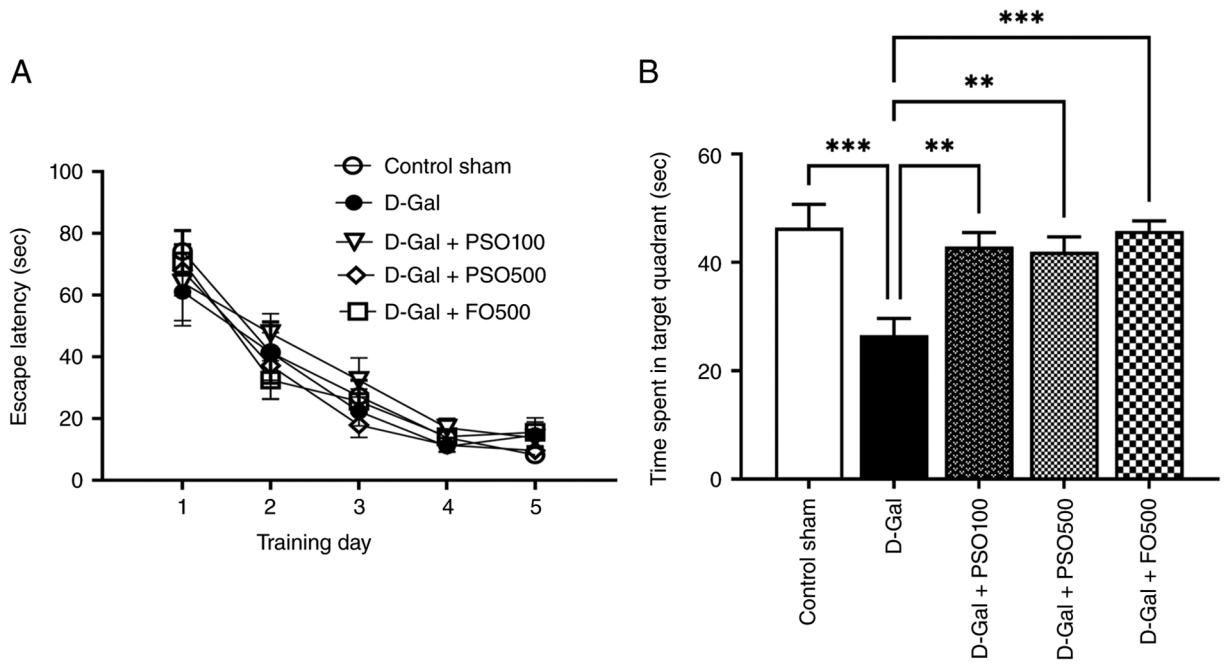

Figure 2. Effects of PSO on spatial memory in rats. (A) Escape latency during the Morris water maze training trials. (B) Time spent in the target quadrant during the probe trial. Data are presented as the mean ± SEM. **P<0.01 and ***P<0.001 compared with the D-Gal group. D-Gal, D-galactose; FO500, fish oil 500 mg/kg; PSO100, Perilla seed oil 100 mg/kg; PSO500, Perilla seed oil 500 mg/kg.

Cell count analysis. Coronal hippocampal sections (30-μm thick) were stained with cresyl violet (Nissl staining) for 10 min at 25˚C. Images of the pyramidal cell layers in the cornu ammonis 1 (CA1) and cornu ammonis 3 (CA3) subregions were captured under bright-field microscopy at x40 magnification using a Nikon microscope and analyzed using NIS-Elements imaging software version 5 (Nikon, Corporation) (25). Neuronal cell bodies were manually counted within a fixed-size region of interest (ROI; 100x100 μm) positioned over the pyramidal cell layer. Only clearly identifiable neuronal cell bodies with a visible nucleus and nucleolus within the pyramidal layer were included in the counts. For each animal, one anatomically matched coronal section at the mid-hippocampal level was selected for analysis, based on the Paxinos and Watson rat brain atlas (26). A total of two non-overlapping fields were analyzed for each hippocampal subregion (CA1 and CA3) and the mean value for each region was calculated per animal for subsequent statistical analysis. Cell counting was performed by an investigator blinded to the experimental groups. Neuronal density was expressed as cells/mm^2, calculated from the mean number of neurons counted within fixed-size ROIs (100x100 μm).

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software; Dotmatics). Data are presented as mean ± SEM. Escape latency during the acquisition phase of the MWM was analyzed using a two-way repeated measures ANOVA (Group x Day). Probe trial data, DR, biochemical parameters and histological data were analyzed using one-way ANOVA followed by Dunnett's post hoc test. Paired Student's t-tests were used only for within-group comparisons of object exploration times during the NOR test, specifically between A1 and A2 during the training phase and between the familiar object (A) and the novel object (B) during the test phase, within the same animals in each group. These tests were not used for between-group comparisons or as post hoc tests following ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

No significant differences in body weight were observed among experimental groups throughout the study period (data not shown).

Effect of PSO on cognitive function

MWM test. During the acquisition phase of the MWM test, escape latency progressively decreased across the training days in all experimental groups (Fig. 2A). Two-way repeated measures ANOVA revealed a significant effect of day [F(2.548, 76.45)=88.15; P<0.001], but no significant effect of group [F(4, 30)=0.3644; P>0.05] or group x day interaction [F(10.19, 76.45)=0.6452, P>0.05] (Table SI). Thus, all groups showed improvement during the acquisition phase, with no significant differences among groups. The day-by-day escape latency data for the acquisition phase are provided in Table SI. By contrast, significant between-group differences were observed in the probe trial (Fig. 2B). In the probe trial, rats in the D-Gal group spent significantly less time in the target quadrant (26.60±3.09 sec) compared with the control sham group (44.44±4.29 sec; P<0.001), indicating impaired spatial memory (Fig. 2B). Treatment with PSO at doses of 100 or 500 mg/kg significantly increased the time spent in the target quadrant (42.92±2.59 sec and 41.95±2.77 sec, respectively) compared with the D-Gal group (P<0.01 for both). Similarly, administration of FO at a dose of 500 mg/kg significantly increased target quadrant time (45.83±1.86 sec) compared with the D-Gal group (P<0.001; Fig. 2B).

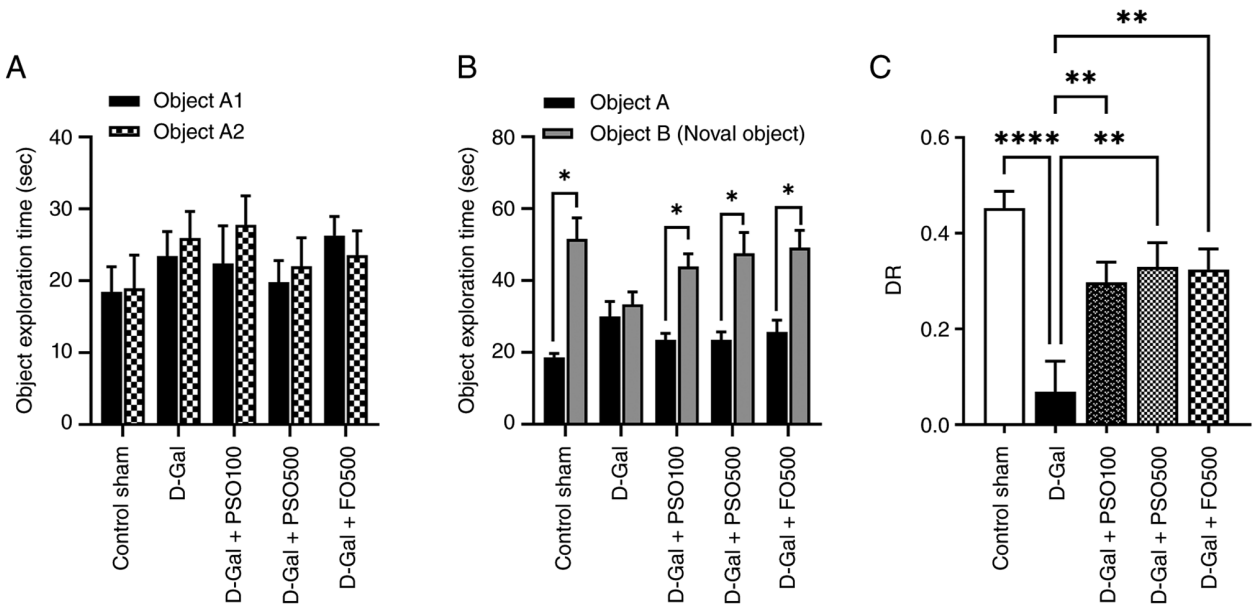

Figure 3. Effect of PSO on recognition memory in rats. (A) Exploration time of the two identical objects (A1 and A2) during the training phase of the novel object recognition test. (B) Exploration time of the familiar object (A) and the novel object (B) during the test phase. (C) DR calculated as (TB-TA)/(TB + TA). Data are presented as the mean ± SEM. In panel B, *P<0.05 indicates a significant within-group difference between the familiar and novel objects (paired Student's t-test). In panel C, *P<0.05, **P<0.01 and ****P<0.0001 compared with the D-Gal group (one-way ANOVA followed by Dunnett's post hoc test). D-Gal, D-galactose; DR, discrimination ratio; FO500, fish oil 500 mg/kg; PSO100, Perilla seed oil 100 mg/kg; PSO500, Perilla seed oil 500 mg/kg.

NOR test. Recognition memory was evaluated using the NOR test (Fig. 3). During the training phase, no significant within-group differences were observed between exploration of the two identical objects (A1 and A2) in any group (Fig. 3A). During the test phase, control sham rats and D-Gal-treated rats receiving PSO (100 or 500 mg/kg) or FO (500 mg/kg) showed significantly greater exploration of the novel object than the familiar object within the same group (Fig. 3B). By contrast, the D-Gal group showed no significant preference for the novel object. Comparisons of DR among groups are shown in Fig. 3C. The DR of the D-Gal group (0.07±0.06) was significantly lower compared with that of the control sham group (0.45±0.04; P<0.0001), indicating impaired recognition memory. Treatment with PSO at doses of 100 or 500 mg/kg significantly increased the DR values (0.30±0.04 and 0.33±0.05, respectively) compared with the D-Gal group (P<0.05 and P<0.01, respectively). Similarly, FO treatment (500 mg/kg) significantly increased the DR value (0.32±0.04) compared with the D-Gal group (P<0.01).

Effect of PSO on oxidative stress markers in the hippocampus. As shown in Fig. 4, hippocampal MDA levels were significantly higher in the D-Gal group (0.47±0.09 μmol/mg protein) compared with the control sham group (0.14±0.01 μmol/mg protein; P<0.05; Fig. 4A). In addition, the antioxidant parameters GSH and SOD activity were significantly decreased in the D-Gal group (4.49±0.67 nmol/mg protein and 88.71±1.92%, respectively) compared with the control sham group (6.99±0.65 nmol/mg protein and 95.61±0.38%; P<0.05 and P<0.001, respectively; Fig. 4B and C). Treatment with PSO at 500 mg/kg significantly increased SOD activity (93.76±0.94%) compared with the D-Gal group (P<0.05). Similarly, FO (500 mg/kg) treatment significantly increased SOD activity (94.40±1.05%) relative to the D-Gal group (P<0.01; Fig. 4C). Although PSO or FO treatment did not result in statistically significant changes in MDA levels or GSH activity compared with the D-Gal group (P>0.05), both parameters exhibited an improving trend and did not differ significantly from control sham values (Fig. 4A and B).

Effect of PSO on hippocampal AChE activity. Hippocampal AChE activity was significantly higher in the D-Gal group (0.90±0.11 nmol of ATCI hydrolyzed/min/mg protein) compared with the control sham group (0.49±0.04 nmol of ATCI hydrolyzed/min/mg protein; P<0.001; Fig. 4D). Treatment with PSO at doses of 100 or 500 mg/kg significantly reduced AChE activity (0.54±0.06 and 0.54±0.06 nmol of ATCI hydrolyzed/min/mg protein, respectively) compared with the D-Gal group (P<0.01). Similarly, FO (500 mg/kg) treatment significantly decreased AChE activity (0.50±0.03 nmol of ATCI hydrolyzed/min/mg protein) relative to the D-Gal group (P<0.001; Fig. 4D).

Effect of PSO on hippocampal neuronal density. Representative Nissl-stained images of the pyramidal cell layers in the CA1 and CA3 regions are shown in Fig. 5A. Neuronal density was quantified and expressed as cells/mm^2. In the CA1 region (Fig. 5B), neuronal density did not differ significantly among the experimental groups (P>0.05). The control group showed a neuronal density of 2,229±207.80 cells/mm^2, whereas the D-Gal group exhibited 1,657±148.20 cells/mm^2. Treatment with PSO at 100 and 500 mg/kg resulted in neuronal densities of 1,693±221.00 and 1,721±290.70 cells/mm^2, respectively, while the FO-treated group showed 1,929±277.3 cells/mm^2.

By contrast, in the CA3 region (Fig. 5C), neuronal density was significantly reduced in the D-Gal group compared with the control group (1,786±70.47 vs. 1,136±105.10 cells/mm^2; P<0.001). Treatment with PSO (100 mg/kg), PSO (500 mg/kg)

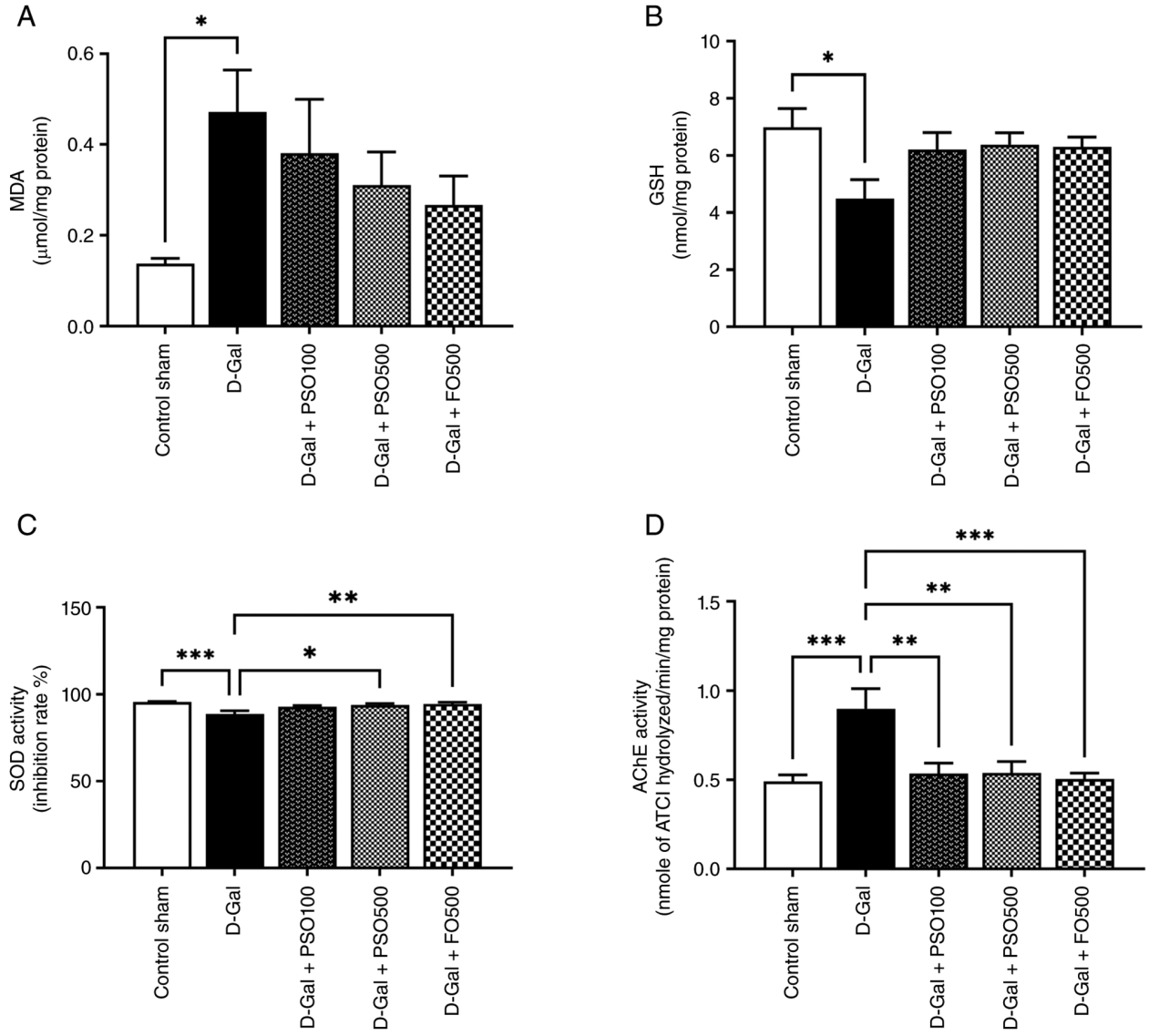

Figure 4. Effects of PSO on oxidative stress markers and AChE activity in the hippocampus. (A) MDA levels. (B) GSH levels. (C) SOD activity. (D) AChE activity in the hippocampus. Data are presented as the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 compared with the D-Gal group. AChE, acetylcholinesterase; D-Gal, D-galactose; FO500, fish oil 500 mg/kg; PSO100, Perilla seed oil 100 mg/kg; PSO500, Perilla seed oil 500 mg/kg; GSH, reduced glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

and FO (500 mg/kg) resulted in neuronal densities of 1,364±81.31, 1,271±116.90 and 1,386±116.40 cells/mm^2, respectively. However, none of these treatment groups differed significantly from the D-Gal group (P>0.05).

Discussion

The present study demonstrated that PSO supplementation attenuated cognitive impairment induced by chronic D-Gal administration in rats. PSO treatment improved spatial learning and memory performance in the MWM probe trial and enhanced recognition memory in the NOR test. Notably, no significant group differences were observed during the acquisition phase of the MWM, whereas PSO- and FO-treated rats showed improved performance in the probe trial. This pattern may suggest that PSO exerted a greater influence on memory retention or consolidation than on the initial acquisition of spatial learning. At the biochemical level, PSO (500 mg/kg) significantly increased SOD activity, and both doses reduced hippocampal AChE activity. Although MDA and GSH levels did not differ significantly from those in the D-Gal group, both parameters showed a tendency toward normalization, suggesting a partial improvement in hippocampal redox balance. Histological analysis of the hippocampus revealed region-specific changes in neuronal density: no significant differences were observed in the CA1 region, whereas neuronal density in the CA3 region was significantly reduced in the D-Gal group compared with the control group. Consistent with this observation, PSO treatment did not markedly alter neuronal cell counts in these regions. Collectively, the results indicate that PSO mitigates D-Gal-induced cognitive dysfunction, potentially by enhancing antioxidant enzyme activity and modulating cholinergic signaling, but does not significantly restore hippocampal neuronal density. Importantly, these findings suggest that cognitive and neurochemical dysfunctions may co-occur with region-specific structural vulnerability

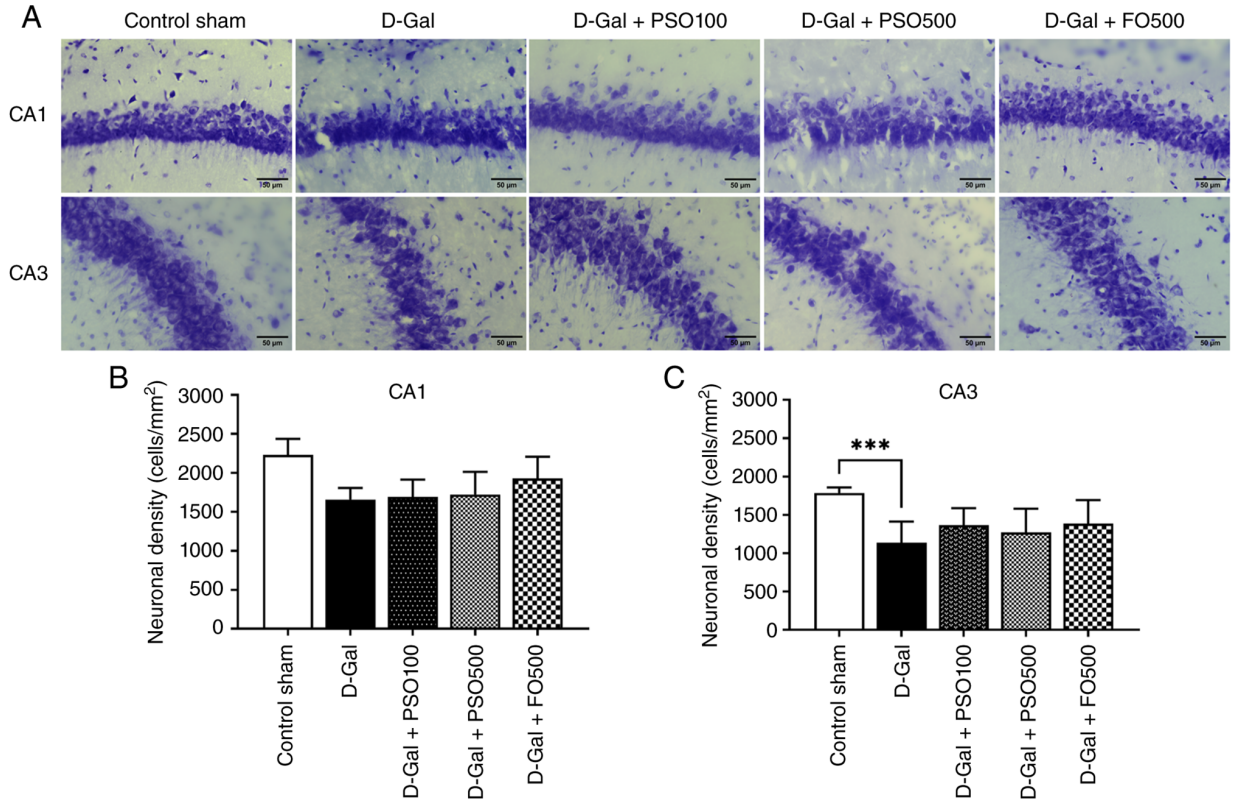

Figure 5. Effects of PSO on hippocampal neuronal cell density in CA1 and CA3 regions. (A) Representative Nissl-stained images of the CA1 and CA3 pyramidal cell layers. (B) Quantification of neuronal cell density in the CA1 region (cells/mm²). (C) Quantification of neuronal cell density in the CA3 region (cells/mm²). Data are presented as the mean ± SEM. ***P<0.001 compared with the D-Gal group. Scale bar, 50 μm. D-Gal, D-galactose; FO500, fish oil 500 mg/kg; PSO100, Perilla seed oil 100 mg/kg; PSO500, Perilla seed oil 500 mg/kg.

during early-stage brain aging, underscoring the relevance of functional, biochemical and histological markers as complementary indicators of brain aging.

Although systemic senescence-associated biomarkers were not directly measured in the present study, the D-Gal-induced accelerated aging model has been extensively characterized in previous investigations. Chronic D-Gal administration has been reported to induce oxidative stress, mitochondrial dysfunction, neuroinflammation and increased expression of senescence-related markers (27). A previous systematic review and meta-analysis further supported this model by demonstrating consistent effects on cognitive impairment and oxidative imbalance across multiple experimental conditions (27). In the present study, the observed cognitive deficits, increased MDA levels, reduced antioxidant capacity and elevated AChE activity collectively support the presence of aging-associated alterations under the experimental conditions used.

The detrimental effects of D-Gal administration on cognitive performance and oxidative balance observed in the present study are consistent with previous reports demonstrating that chronic D-Gal exposure induces oxidative stress and memory impairment (14,15). Protective effects on cognitive function associated with antioxidant-related mechanisms have also been reported for various nutraceutical interventions, including resveratrol (28), curcumin (29) and omega-3 fatty acids (30,31). In addition, ALA, the predominant fatty acid component of PSO, has been associated with enhanced cognitive performance and resistance to oxidative stress-related neuronal dysfunction (32,33). In particular, Lee *et al* (32) demonstrated that ALA derived from PSO attenuates amyloid-β-induced memory impairment. Although the individual contributions of ALA and minor bioactive constituents such as tocopherols and polyphenols were not directly assessed in the present study, their combined presence may contribute to the overall biological effects of PSO.

Chronic D-Gal exposure has been shown to disrupt endogenous antioxidant defenses, promote oxidative damage and neuroinflammation and thereby contribute to age-related cognitive decline (14,15,34). In the present study, PSO supplementation at the higher dose significantly increased hippocampal SOD activity, suggesting enhancement of enzymatic antioxidant defenses, particularly SOD activity. By contrast, alterations in MDA and GSH levels were less pronounced, with PSO- and FO-treated groups exhibiting only a tendency toward normalization compared with the D-Gal group. These findings indicate that the observed biochemical effects of PSO may be more closely associated with enhancement of enzymatic antioxidant activity, particularly SOD at the higher dose, rather than changes in non-enzymatic antioxidant components such as GSH under the present experimental conditions. Similar findings in the FO-treated group are consistent with previous reports demonstrating that omega-3 fatty acids can enhance antioxidant enzyme activity in D-Gal-induced aging models (35-37). Because SOD reflects enzymatic antioxidant defense, whereas MDA represents a downstream product of lipid peroxidation, the significant

increase in SOD activity without a corresponding reduction in MDA may suggest that PSO enhanced antioxidant capacity before producing a measurable reduction in accumulated oxidative damage. The lack of statistical significance in MDA and GSH levels may be partially attributable to the relatively small sample size (n=7 per group), which may limit the statistical power to detect modest biochemical changes. In addition, the 8-week experimental duration may have been insufficient to induce more pronounced lipid peroxidation or depletion of non-enzymatic antioxidant reserves.

In addition, the antioxidant-related effects observed in the present study are in agreement with previous evidence indicating that ALA, the major fatty acid constituent of PSO, suppresses lipid peroxidation and enhances antioxidant enzyme activities in experimental models of amyloid-β-induced neurotoxicity (32,33). Although inflammatory markers were not directly evaluated in the present study, prior investigations have suggested that PSO and ALA can modulate inflammatory signaling pathways, including the NF-κB pathway, and reduce pro-inflammatory cytokine expression in cellular and animal models (38,39). Therefore, it is plausible that the beneficial effects of PSO observed in the present study may also involve interactions between oxidative stress regulation and inflammatory processes. Moreover, minor bioactive constituents of PSO, such as polyphenols and tocopherols, possess antioxidant properties (16-19) and may collectively contribute to the overall biological effects of PSO. From a translational perspective, modulation of oxidative and inflammatory balance at this functional stage may be particularly relevant for delaying or mitigating subsequent neurodegenerative processes.

In addition to oxidative and inflammatory mechanisms, disruption of cholinergic neurotransmission is a well-recognized contributor to age-related cognitive decline. Increased AChE activity accelerates acetylcholine degradation and has been associated with memory deficits in D-Gal-induced aging models (14,15,40). In the present study, supplementation with PSO and FO significantly reduced hippocampal AChE activity compared with the D-Gal group, suggesting a preservation of cholinergic function. The inclusion of two PSO doses allowed assessment of dose dependency, while the FO group served as a benchmark omega-3 intervention to contextualize the magnitude of PSO effects. This reduction was accompanied by improvements in spatial learning and recognition memory, indicating that modulation of cholinergic activity may contribute to the observed cognitive benefits. Similar cholinergic-related cognitive benefits have been reported for other omega-3-rich interventions. For example, krill-derived phosphatidylserine improved learning and memory performance while modulating hippocampal choline acetyltransferase and AChE activity (41), and omega-3 fatty acids were shown to prevent ketamine-induced elevations in AChE activity (42). Together, these findings suggest that omega-3 fatty acids, including ALA-rich PSO, may support cognitive function through combined antioxidant-related and cholinergic-modulatory effects. Supporting this concept, a previous *in vitro* study have demonstrated that docosahexaenoic acid (DHA)-containing phospholipids, such as 1,2-di-DHA-phosphatidylcholine, directly inhibit AChE activity and exhibit antioxidant properties, underscoring the close interplay between redox regulation and cholinergic signaling (43).

Histological analysis revealed region-specific alterations in hippocampal neuronal density. While no statistically significant differences were observed in the CA1 region among the experimental groups, neuronal density in the CA3 region was significantly reduced in the D-Gal group compared with the control group. These findings indicate that chronic D-Gal exposure under the present experimental conditions induced selective structural vulnerability within the CA3 subregion of the hippocampus. This region-specific pattern may be biologically plausible, as recent evidence indicates that hippocampal vulnerability can vary across experimental models and that CA3 damage may, under some conditions, be more pronounced than CA1 depending on factors such as species, age, experimental protocol and observation period (44). In addition, CA3 neurons may exhibit increased susceptibility to metabolic and oxidative stress due to differences in intrinsic cellular properties, including higher metabolic demand and lower calcium-buffering capacity (45). Although PSO and FO treatment did not significantly restore neuronal density in the CA3 region, improvements in cognitive performance and biochemical parameters were observed in the treated groups. This discrepancy suggests that the beneficial effects of PSO on cognitive function may be more closely associated with modulation of oxidative stress and cholinergic signaling than with robust structural recovery of neuronal populations in the hippocampus.

Several limitations of the present study should be acknowledged. First, although the D-Gal paradigm is widely used as an accelerated aging-like model, systemic senescence-associated biomarkers were not assessed in the present study. Future studies should include direct assessment of established aging/senescence-associated markers, such as p16, p21, SA-β-galactosidase activity and inflammatory cytokines, to strengthen validation of the accelerated aging-like phenotype. Second, although oxidative stress and cholinergic activity were assessed, inflammatory markers and synaptic plasticity-related markers, such as synaptophysin and PSD-95, were not directly examined, limiting mechanistic insight into the molecular pathways underlying the observed effects of PSO. Third, although fatty acid composition data for the PSO production lot used in the present study were available from prior characterization, total polyphenol content and tocopherol levels were not determined for this lot. This limits the interpretation of the possible contributions of other bioactive constituents in PSO in addition to its fatty acid composition. Fourth, histological evaluation was restricted to neuronal cell density in the CA1 and CA3 regions, and additional analyses of dendritic architecture or synaptic markers may provide a more comprehensive understanding of hippocampal integrity. In addition, neuronal quantification was performed using a standardized sampling approach based on anatomically matched sections and fixed regions of interest rather than unbiased stereological methods. Although this approach is commonly used for comparative analyses, a more comprehensive stereological assessment across multiple serial sections and larger sample sizes would provide greater sensitivity for detecting subtle neuronal alterations in future studies. Finally, the present study focused on a single time point of D-Gal exposure; longer treatment periods may be required to determine whether prolonged oxidative stress ultimately leads to structural neuronal loss. Future studies incorporating extended aging paradigms and additional

molecular endpoints will be valuable for further elucidating the neurobiological effects of PSO in brain aging. In addition, food intake was not quantitatively monitored, as it was not included in the original experimental design, therefore no food intake records were available. This should be considered a limitation when interpreting potential confounding effects related to nutritional intake. Moreover, only male rats were included in the present study. Considering that sex hormones influence oxidative stress (46), cholinergic signaling (47) and cognitive aging (48), future studies incorporating female models, particularly those mimicking perimenopausal or postmenopausal conditions, would provide valuable insight into potential sex-specific effects of PSO.

In conclusion, the present study demonstrates that PSO supplementation attenuates D-Gal-induced cognitive impairment in rats and is associated with partial attenuation of oxidative imbalance, reflected by increased hippocampal SOD activity, together with modulation of cholinergic function. Histological analysis revealed region-specific alterations in hippocampal neuronal density: Significant neuronal loss in the CA3 region following D-Gal administration, with no significant differences in the CA1 region. Although PSO treatment did not significantly restore neuronal density, improvements in cognitive performance and biochemical parameters suggest that its beneficial effects may be associated, at least in part, with modulation of antioxidant enzyme activity and cholinergic signaling. These findings highlight the potential of PSO as a nutritional intervention to support cognitive function amid aging-related neurobiological changes. Given the known influence of sex hormones on cognitive aging (48), the potential relevance of PSO for the cognitive health of women warrants further investigation, particularly in female models reflecting perimenopausal or postmenopausal conditions. Further studies are warranted to elucidate the long-term effects and molecular mechanisms underlying the beneficial effects of PSO in aging-related cognitive decline.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WPJ conceived and designed the study, developed the methodology and provided project supervision. WPJ, RS and SP performed the animal experiments and behavioral assessments. WPJ, AK, NK and JK conducted the biochemical assays. ST performed histological staining and guided image interpretation. SP and JK conducted neuronal cell counting and WPJ acquired and compiled histological images and data. WPJ, AK, ST and NK analyzed and interpreted the data. WPJ was responsible for statistical analysis, project administration, funding acquisition and overall supervision. WPJ drafted the original manuscript. WPJ, ST, AK and NK reviewed and edited the manuscript. WPJ and ST confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethical approval and consent to participate

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Phayao, Thailand (approval no. 1-004-66). All methods were carried out in accordance with the relevant guidelines and regulations for the care and use of laboratory animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, ChatGPT (OpenAI) was used solely to improve the readability and language of the manuscript. The authors subsequently reviewed and edited the content as necessary and take full responsibility for the final content of the manuscript. No artificial intelligence tools were used in data analysis, interpretation, or generation of figures.

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