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Efficacy of *Piper Longum* Extract in a Streptozotocin-Induced Mouse Model of Sporadic Alzheimer's Disease Combined with Chronic Restraint Through Lipocalin-2-Associated Neuroinflammatory Mechanism

Hyun-Yee Ha¹, Seung-Hyeon Han¹ and Seung-Hun Cho^{1*}

Abstract

Background Alzheimer's disease (AD) is a neurodegenerative disease that is often accompanied by neuropsychiatric problems, including depression. Lipocalin-2(LCN2), a proinflammatory mediator, exacerbates the symptoms of depression and memory impairment through neuroinflammation. This study investigated the potential of *Piper Longum* extract(PLE), a traditional Indian medicinal plant, in the treatment of AD and comorbid depression.

Methods In this study, we investigated memory impairment and depression-like behaviors through behavioral experiments such as the Y-maze test, Morris water maze test, open field test (OFT), forced swim test (FST), and tail suspension test (TST). We used Western blot, RT-PCR analysis, and immunofluorescence to identify changes in AD-related markers, such as amyloid beta (A β) protein, tau phosphorylation, tumor necrosis factor-alpha (TNF- α), and LCN2-related astrocytes, in the brain of Streptozotocin/Chronic Restrain Stress (STZ/Res) mice treated with PLE.

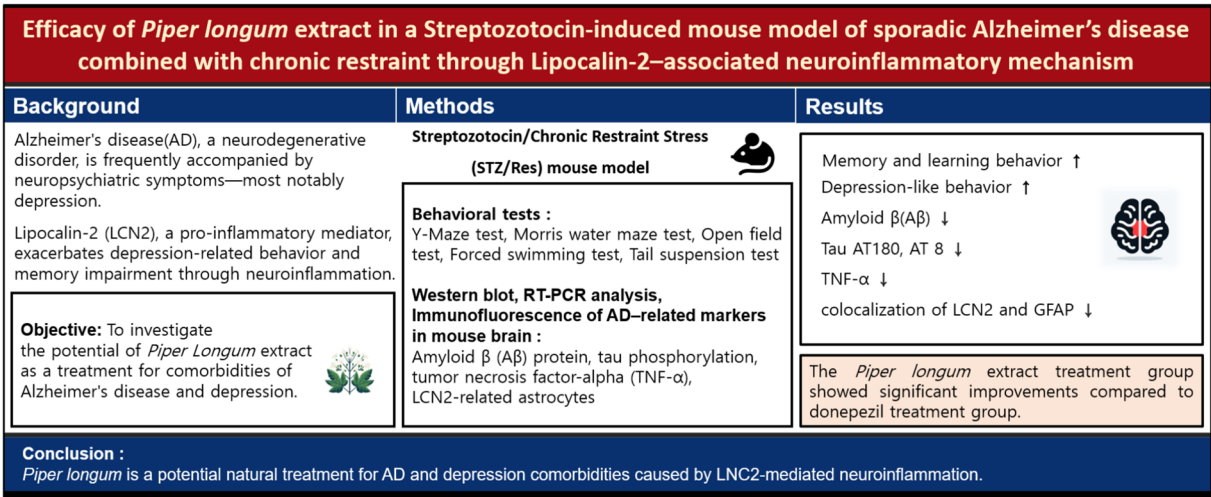
Results The PLE group showed significant improvements in the Morris water maze, Y-maze, FST, and TST. A β protein, hyperphosphorylation of tau, TNF- α level decreased when treated PLE compared to Donepezil(DPZ). LCN2 merged with GFAP and colocalization was significantly decreased in the PLE-treated group.

Conclusion Our findings suggest that *Piper longum* is a potential natural treatment for AD and depressive comorbidities caused by LCN2-mediated neuroinflammation.

Keywords *Piper longum*, Alzheimer's disease(AD), depression, Comorbidities, Amyloid β (A β), Tau phosphorylation, Tumor Necrosis Factor-alpha(TNF- α), Lipocalin2(LCN2), Streptozotocin(STZ), Neuroinflammation

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Graphical Abstract



1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that impairs cognitive function [1]. AD is neuropathologically defined by extracellular amyloid β (A β) plaque deposition and intracellular accumulation of hyperphosphorylated tau as neurofibrillary tangles [2, 3]. AD patients are accompanied by various neuropsychiatric problems, including anxiety, depression, and delusions [4]. Neuropsychiatric symptoms (NPS)—including depression, anxiety, apathy, agitation, and delusions—affect up to 90% of AD patients, with depressive and anxiety symptoms reported in 50–65% or more depending on assessment methods. Patients with AD have a particularly high prevalence of depression, and chronic restraint stress can accelerate cognitive dysfunction [5].

Although cognitive decline remains the hallmark of AD, comorbid depressive behavior reflects a complex interplay of mechanisms: monoaminergic dysregulation such as serotonergic and noradrenergic dysfunction, hippocampal atrophy, neuroinflammation, HPA axis hyperactivity, reduced BDNF, and dysfunction in prefrontal–limbic circuits [5, 6]. In addition, psychosocial stressors such as declining autonomy, social withdrawal, and caregiver/environmental pressures further exacerbate depressive symptoms. These biological and psychosocial factors form a bidirectional feedback loop, wherein mood disturbances accelerate cognitive and memory decline—underscoring depressive behavior as not merely comorbid, but integral to the AD phenotype [7–10].

The proinflammatory mediator lipocalin-2(LCN2) is a key proteins that causes neuroinflammatory and

degenerative changes in the brain [11]. LCN2 is associated with neuroinflammation by activating astrocytes, microglia, and proinflammatory cytokines in AD [12]. Studies have shown that LCN2 has been found to be involved in the morbidity of both AD and depression. Depression and memory impairment are exacerbated by LCN2-mediated neuroinflammation. Depression increases LCN2 production in the prefrontal cortex and induces neuroinflammatory [13], thereby promoting the rapid progression of memory disorders [14]. However, there have been no reports of appropriate therapeutic substances based on these mechanisms.

Regarding the complex etiology of AD and depression comorbidities, it is important to develop treatments that simultaneously address the pathological changes in AD and depression-like behavior [15]. However, combinations of anti-AD drugs and antidepressants are often limited by the risk of adverse drug-drug interactions and side effects [16]. Considering the unsatisfactory efficacy of treatments for AD and depression, multi-target directed ligands (MTDLs) that simultaneously regulate multiple factors are being studied as novel treatment approaches [17]. However, the disadvantages of MTDL are its large molecular weights, ranging from 500 to 700, which can weaken drug degradability and reduce drug efficacy [18]. Therefore, safe and effective therapeutic drugs are urgently required to prevent or slow the disease progression.

Piper longum commonly known as long pepper, belongs to the Piperaceae family [19]. *Piper longum* is an important plant in traditional Indian medicine [20]. Several studies have investigated the pharmacological effects

of *Piper longum* on AD and depression [21]. According to a recent study, *Piper longum* contains rich amide alkaloids, including Piperine (PPR), piperlonguminine (PPL), and $\Delta\alpha,\beta$ -dihydropiperlonguminine (DPPL) [22]. These ingredients have antioxidant, anti-inflammatory, antitumor and antidepressant effects [23, 24]. However, there have been no studies on the effectiveness of *piper longum* as a therapeutic agent for memory deficiency symptoms and depression-like behavioral comorbidities [25], or on the effectiveness of the neuroinflammatory mechanisms associated with LCN2.

The streptozotocin (STZ) mouse model is a typical gene mutation model used in experiments that cause sporadic AD, the most common type of AD [26]. Res mouse model has been used to induce depressive behavior in mice [27]. In the current study, STZ/Res mouse model that induced cognitive decline and depressive behavior simultaneously were used. We observed improvements of *Piper longum* extract on changes in amyloid beta, tau phosphorylation, inflammatory indicators Tumor Necrosis Factor- α (TNF- α), LCN2-related astrocytes, and depressive-like behavior. As a result, *piper longum* extract was effective in improving AD triggers and also in depressive behavior. Therefore, we propose the use of *Piper longum* as a natural treatment for AD and depression comorbidities caused by LNC2-mediated neuroinflammation.

2 Materials and Methods

2.1 Reagents

Piper longum (made in Indonesia) were purchased from local herbal medicine shop in South Korea. Streptozotocin Sigma (St. Louis, MO, USA), and donepezil (DPZ) was purchased from Cayman (Ann Arbor, MI, USA). LCN2 (R&D Systems, CAS Number: AF1857) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). β -actin (Santa Cruz, CAS Number: sc-69879) and GFAP (Santa Cruz, CAS Number: sc-33673) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Ionized calcium-binding adapter molecule 1 (IBA1; Invitrogen, CAS Number: PA5-27,436) was purchased from Invitrogen (Carlsbad, CA, USA). Tau Monoclonal Antibody (T46) (Invitrogen, CAS Number: 13-6400) was obtained from Invitrogen (Carlsbad, CA, USA). Phospho-tau (Thr231) (AT180) (Invitrogen, CAS Number: MN1040) and phospho-tau (Ser202, Thr205) (AT8) (Invitrogen, CAS Number: MN1020) monoclonal antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

2.2 Animals

Eight-week-old male C57Bl/6 mice (Young Bio, Inc., Korea) weighing 20–22 g were used for this experiment. The mice were housed in acrylic cages (26×42×18 cm)

of continuous automated monitoring system with controlled temperature ($22\pm2^{\circ}\text{C}$) and relative humidity ($60\pm10\%$), free access to water and food, twelve-hour light/dark cycle. All behavioral tests were conducted between 9:00 and 17:00. All experimental procedures were approved by KyungHee University Medical Center Institutional Animal Care (approval number; KHMC-IACUC 2019–030).

2.3 Drug Administration

Mice were randomly distributed into four groups: (i) Sham control (Sham), distilled water per os (po) + sham surgery ($n=10$); (ii) STZ (3 mg/kg) + Res + Vehicle (Veh), distilled water po ($n=10$); (iii) STZ + Res + DPZ (1 mg/ml) po ($n=10$), (iv) STZ + Res + PLE po ($n=10$). PLE and, DPZ were completely dissolved in distilled water and orally administered to the mice. Oral administration was continued until the probe test in the Morris water maze was complete. All the experiments followed the schedule shown in Fig. 1.

After one week of daptation, STZ (3 mg/kg; Sigma) was infused bilaterally at a rate of 0.5 $\mu\text{l}/\text{min}$ for three minutes using an injection needle and a micro-drive pump (Pump 11 Elite Nanomite; Harvard Apparatus, Holliston, MA, USA). It was administered twice over three days. Mice in the veh, DPZ, and PLE groups were injected with STZ into the Lateral ventricle (AP – 0.5 mm; ML $\pm$ 1.1 mm; DV – 2.8 mm) through the previously implanted. The sham mice did not undergo injection implantation.

2.4 Behavioral Tests

2.4.1 Y-Maze Test

The spontaneous alteration behavior of mice in the Y-maze was used to measure short-term memory. Each mouse ($n=9$ –10/group) was placed in a specific arm of the apparatus and allowed to move freely in the maze during an 8-min session. Entry into all three arms (each arm was designated A, B, and C) on consecutive choices was defined as an actual alteration (i.e., ABC, CAB, or BCA, but not BAB). Between the tests, the maze arms were cleaned to remove residual odors.

2.4.2 Morris Water Maze Test

A circular water tank (90 cm in diameter, 32.5 cm in height) was filled with water, which was rendered opaque by white ink and was kept at $23\pm1^{\circ}\text{C}$, was used. An escape platform (10 cm in diameter) was hidden 1 cm below the water surface and placed at the midpoint of each quadrant. Each mouse ($n=9$ –10/group) was trained to find the platform with starting positions in different quadrants in each trial. The latency to located the escape platform was recorded for each trial. The training lasted for five consecutive days. After 24 h after the last training

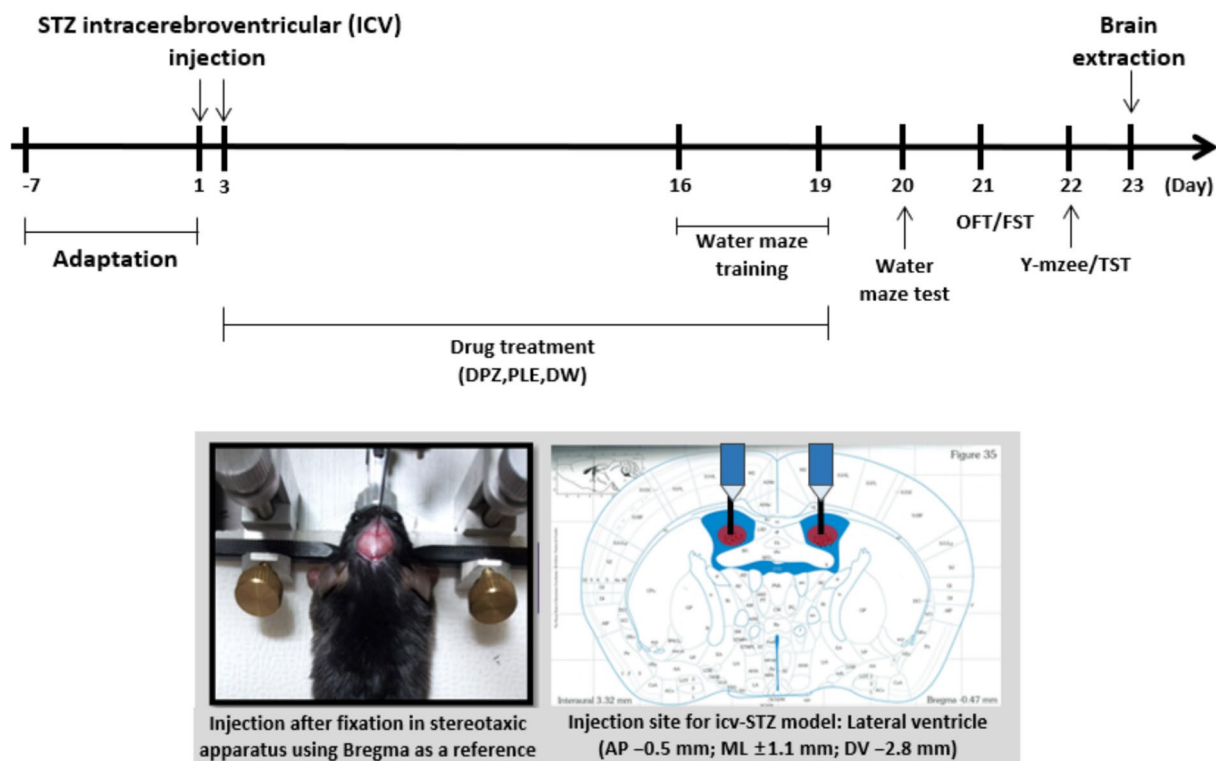


Fig. 1 Intracerebroventricular Injection of STZ

session, a probe test was performed to evaluate the consolidated memory. The escape platform was removed and each subject was allowed to swim freely in the tank for 90 s. The time spent by each mouse in the quadrant in which the platform was previously located was measured. All data were recorded using video-tracking software (SMART 3.0, Panlab Harvard Apparatus, MA, USA).

2.4.3 Open Field Test(OFT)

The OFT was conducted in a quiet, dark room using identical apparatus (50×50×50 cm acrylic boxes with overhead video recording). Mice (n=10/group) were gently placed in the central grid and allowed to explore freely. In the OFT, the total distance traveled was analyzed for 10 min using video-tracking software.

2.5 Tissue Collection

After the behavioral tests were completed, mice were anesthetized and sacrificed. Some mice underwent rapid removal of the hippocampus and prefrontal cortex (PFC) from the brains. The hippocampi and PFC were dissected and stored at -80°C for western blotting analysis. The remaining mice in each group were anesthetized using diethyl ether and were perfused with phosphate-buffered saline, followed by 4%

paraformaldehyde solution. Collected brains were subsequently submerged in 4% paraformaldehyde for 24 h at 4°C and transferred to a 20% sucrose solution. The brains were stored at -80°C for immunohistochemical analysis.

2.6 Western Blot

Hippocampal and PFC tissues were homogenized in 1×RIPA buffer (Pierce Biotechnology, IL, US). The homogenate was centrifuged at $12,000\times g$ for 15 min at 4°C , and the supernatant was collected. Proteins were quantified using the BCA protein assay kit (Pierce Biotechnology). The protein extract (40 μg per lane) was loaded onto 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel followed by transferring onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with the primary antibody (1:1000) against overnight at 4°C . The membranes were then incubated with goat antimouse IgG or goat antirabbit secondary antibodies (1:5000) for one hour at room temperature. Immunoreactive bands were detected using an ECL kit (CAS number: 34577; Sigma-Aldrich). The blots were imaged using Davinch-Chemi (Celltagen, Seoul, Korea) and quantified using the ImageJ v.1.44 software (NIH).

2.7 RT-PCR

RNA was extracted from the brain tissue soaked in TRIzol, and the concentration of RNA was determined using a NanoDrop machine. RT-PCR was performed to synthesize cDNA using the measured RNA concentrations. The RT-PCR sequences are listed in Table 1.

2.8 Immunofluorescence (IF)

The tissue slides were air-dried for 1 h and then immersed in 1×Tris–EDTA buffer (pH 9.0). Heating was carried out at 70–80 °C for 30 min for antigen retrieval. The slides were then rinsed with 1×TBST and a circle was drawn around the tissue attached to the slide using a liquid blocking pen. The tissues were blocked using Dailuent (ScyTek Laboratories, CAS number: ADT999, USA) for 15 min. After dissolving the primary antibody “A” in Dailuent, process the tissue and incubate at 4 °C for 24 h. The primary antibody “A” was diluted in diluent and applied to the tissue, followed by incubation at 4 °C for 24 h. Wash with 1X TBST after primary antibody treatment. After washing with 1×TBST, the secondary antibody was diluted in TBST and applied, followed by incubation for 20 min in the dark. Shake off the secondary antibody, treat with another primary antibody “B” and incubate at 4 °C for 24 h. After discarding the secondary antibody, a second primary antibody “B” was applied and incubated at 4 °C for another 24 h. Similarly, wash after secondary antibody treatment, treat with Dailuent, cover slides, and check colocalization of “A” and “B” through fluorescence microscopy. Finally, the sections were washed, treated with Dailuent, mounted with coverslips, and the

colocalization of antibodies “A” and “B” was assessed using fluorescence microscopy.”

2.9 Statistical Analysis

Data were assessed using one-way analysis of variance followed by Tukey’s post-hoc test comparisons for behavioral and biochemical analyses. All results were expressed as means±standard error of mean, *p* values<0.05 were considered statistically significant, and all calculations were made using SPSS version 22.0 (Chicago, IL, USA).

3 Result

3.1 Body Weight Change

Body weight was maintained on day 11 at a level similar to the initial weight in all three STZ/Res groups compared to the continuous increase in body weight in the sham group (Fig. 2). Data are expressed as mean±SD. *N*=10 per group.

3.2 Effect of PLE on Memory and Learning Behavioral Test in STZ/Res Mouse Model

3.2.1 Morris Water Maze Test

Time spent in the quadrant were significantly lower in the STZ/Res+veh group (*p*<0.05) than in the Sham group. The DPZ-treated (*p*<0.05) and PLE-treated groups (*p*<0.05) increased to a higher level than the sham group, and the two values were similar (Fig. 3). Data are expressed as mean±SD. *N*=10 per group.

The Morris water maze test was performed to evaluate reference memory. The movement distance in the quadrant was significantly decreased in the STZ/Res+veh group (*p*<0.001) compared to the sham group. In comparison, the movement distance increased significantly in the DPZ-treated group (*p*<0.0001) compared to the sham group. In the PLE-treated group (*p*<0.0001), the distance traveled was further increased compared to that

Table 1 RT-PCR sequence

No	Name	Sequence (5'→3')	GenBank No
1	TNF-α(F)	ATGGCCTCCCTCTCAGTTC	NM_013693
2	TNF-α(R)	TTGGTGGTTTGCTACGACGTG	
3	IL-6(F)	AGTTGCCTTCTGGGACTGA	NM_031168
4	IL-6(R)	TCCACGATTCCCAGAGAAC	
5	GAPDH(F)	TGGGCTACACTGAGCACCAG	NM_008084
6	GAPDH(R)	GGGTGTCGCTGTTGAAGTCA	
7	Lipocalin-2(F)	ATGTCACCTCCATCCTGGTC	AA087193
8	Lipocalin-2(R)	CACACTCACCACCCATTCTCAG	
9	C1qa(F)	CAGTTTGATCGGACCAACGGA	NM_007572.2
10	C1qa(R)	CAAGCGTCATTGGGTTCTGC	
11	C3(F)	ACCTTACCTCGGCAAGTTTCT	NM_009778.3
12	C3(R)	TTGTAGAGCTGCTGGTCAGG	
13	C5aR(F)	GAAGCGGCAACCTGGGGATGT	NM_007577.4
14	C5aR(R)	CGTCTGGCTCGAAGGCTGTCAC	
15	C8g(F)	CCCAGGTCAATTCAGTGCT	NM_027062.2
16	C8g(R)	GGTACAGGATGGCAAAGCTC	

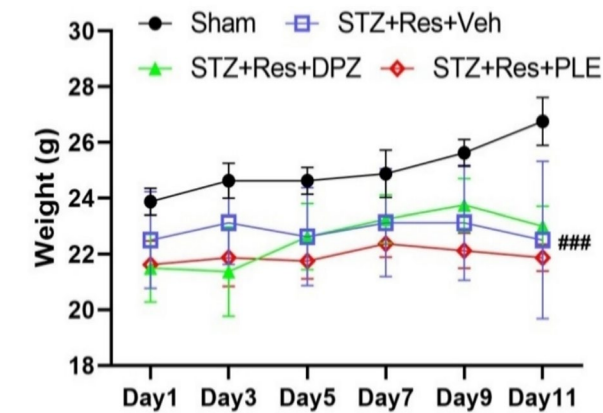


Fig. 2 Body weight changes in 11 days

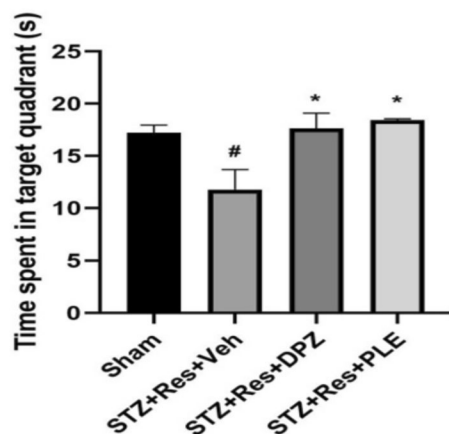


Fig. 3 Time spent in target quadrant of the Morris water maze test

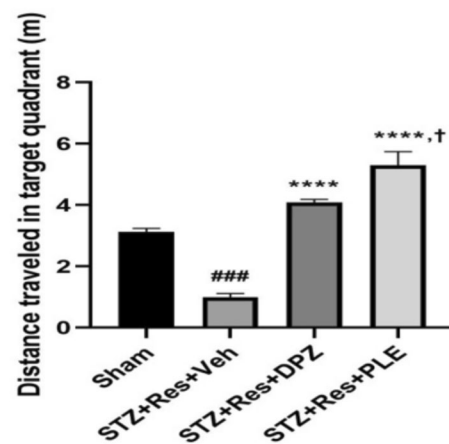


Fig. 4 Distance traveled in target quadrant of the Morris water maze test

in the DPZ-treated group (Fig. 4). Data are expressed as mean $\pm$ SD. $N=10$ per group.

3.3 Y-Maze Test

The Y-maze test results confirmed that the movement time in the quadrant was significantly reduced in the STZ/Res+veh group compared to that in the sham group. In comparison, the STZ/Res+DPZ group increased to a higher level than that in the sham group. In addition, the PLE-treated group showed a significant increase compared to the STZ/Res+DPZ group ($p<0.05$) (Fig. 5). Data are expressed as mean $\pm$ SD. $N=10$ per group.

3.4 Effect of PLE on Depression-like Behavior Test in STZ/Res Mouse Model

3.4.1 Open Field Test

The OFT was performed to evaluate whether the treatments affected the motor function of each group. There

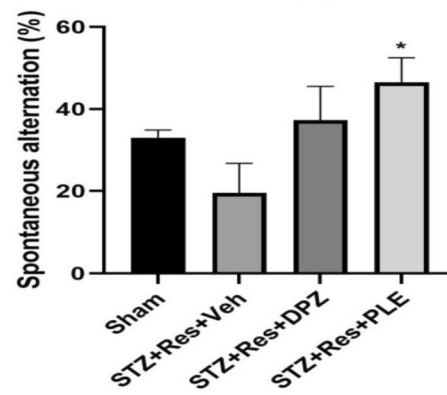


Fig. 5 Spontaneous alternation in Y-maze test

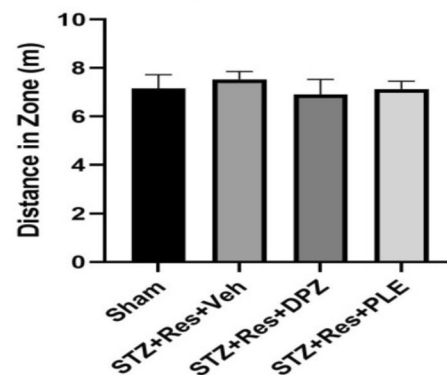


Fig. 6 Distance in zone of the OFT

was no significant difference in the total distance traveled between the groups in the OFT (Fig. 6). Data are expressed as mean $\pm$ SD. $N=10$ per group.

3.4.2 Forced Swim Test

FST were performed to explore the effect of recovery on depression-like behavior in STZ/Res mice. In the FST, immobility was found to be increased in the STZ/Res+veh group ($p<0.05$) compared to the sham group and significantly decreased in the STZ/Res+DPZ group ($p<0.0001$). There was a greater reduction in the immobility time in the STZ/Res+PLE group ($p<0.0001$) than in the STZ/Res+DPZ group (Fig. 7). Data are expressed as mean $\pm$ SD. $N=10$ per group.

3.4.3 Tail Suspension Test

In the TST, the immobility time of the vehicle group was significantly increased compared to that of the sham group ($p<0.01$), and the immobility time of the DPZ-treated group was significantly decreased compared to that of the sham group ($p<0.0001$). Immobility was significantly reduced in the PLE-treated group compared

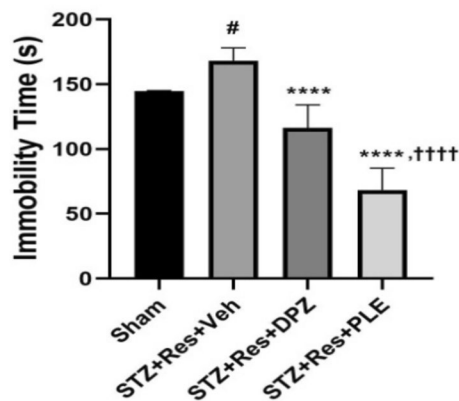


Fig. 7 Immobility time of the FST

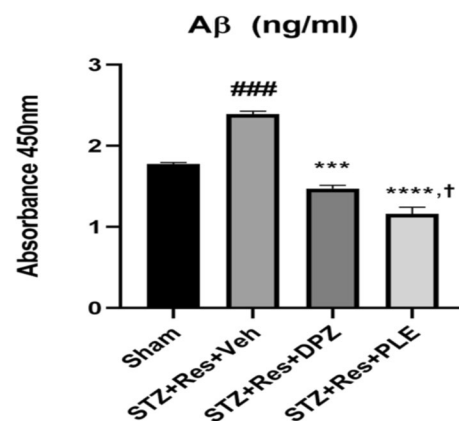


Fig. 9 The Amyloid β ($A\beta$) protein level measured by ELISA at 450 nm absorbance

to the DPZ-treated group ($p < 0.0001$) (Fig. 8). Data are expressed as mean $\pm$ SD. $N = 10$ per group.

3.5 Efficacy of PLE and DPZ on AD-Related Markers

3.5.1 Efficacy of PLE and DPZ on Amyloid β Protein

The $A\beta$ protein level was significantly increased in STZ/Res+veh group ($p < 0.001$) compared to the sham group. In contrast, the protein expression level of $A\beta$ was significantly decreased in STZ/Res+DPZ ($p < 0.001$) and PLE treatment group ($p < 0.0001$) was lower than the STZ/Res+DPZ group (Fig. 9). Data are expressed as mean $\pm$ SD. $N = 10$ per group.

3.6 Efficacy of PLE and DPZ on Tau (AT180/AT8) Protein.

To determine the effect of PLE on the hyperphosphorylation of tau protein, western blots for AT180 and AT8 were performed (Fig. 10). Data are expressed as mean $\pm$ SD. $N = 10$ per group.

In the STZ/Res+veh group ($p < 0.05$), AT180 was significantly higher than that in the sham group. AT180

relative intensity was significantly reduced in the STZ/Res+DPZ and STZ/Res+PLE groups ($p < 0.01$). The PLE-treated group showed lower AT180 levels than the DPZ-treated group.

Similarly, in the case of AT8, a significantly higher relative intensity was observed in the STZ/Res+veh group ($p < 0.01$) than in the sham group. AT8 levels decreased in the STZ/Res+DPZ ($p < 0.05$) and STZ/Res+PLE ($p < 0.01$) groups, with the PLE-treated group showing lower levels than the DPZ-treated group ($p < 0.01$).

3.6.1 Efficacy of PLE and DPZ on TNF- α

The level of TNF- α in the prefrontal cortex was significantly increased in the STZ/Res+veh group compared to the sham group ($p < 0.05$). TNF- α decreased in the DPZ-treated group ($p < 0.01$) and PLE-treated group decreased to a lower level ($p < 0.001$) compared to the DPZ-treated group. It can be inferred that PLE played a role in the recovery of increased TNF- α in the prefrontal cortex (Fig. 11). Data are expressed as mean $\pm$ SD. $N = 10$ per group.

3.7 Efficacy of PLE and DPZ on Colocalization of LCN2 with GFAP and Iba-1 in PFC

The expression levels of LCN2 and GFAP, an astrocyte marker, were increased in the STZ/Res+veh group compared to the sham group. The colocalization of LCN2 with GFAP was significantly increased in the vehicle group, reduced in the DPZ group, and markedly decreased in the PLE-treated group. LCN2 colocalization with Iba-1, a microglial marker, showed a slight increase in the vehicle group and was reduced in both DPZ and PLE groups. While Iba-1 is specific to microglia, its colocalization with LCN2 appeared less distinct than with GFAP. Immunofluorescence was performed using standard double-labeling protocols with appropriate primary

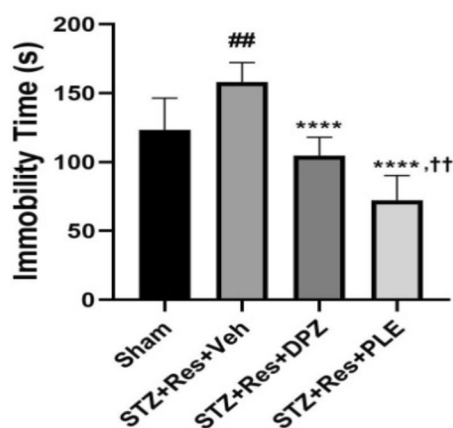


Fig. 8 Immobility time of the TST

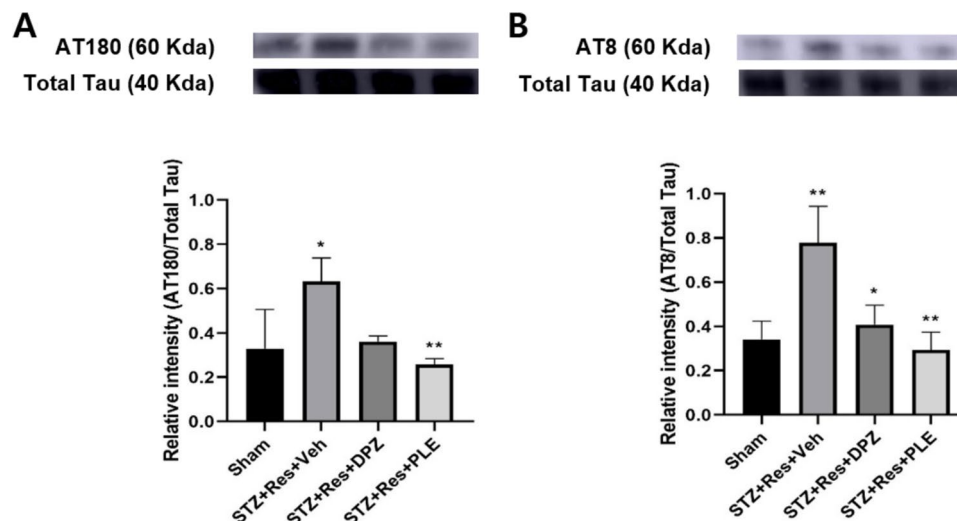


Fig. 10 Relative intensity of **A** AT180, and **B** AT8 compared to total tau

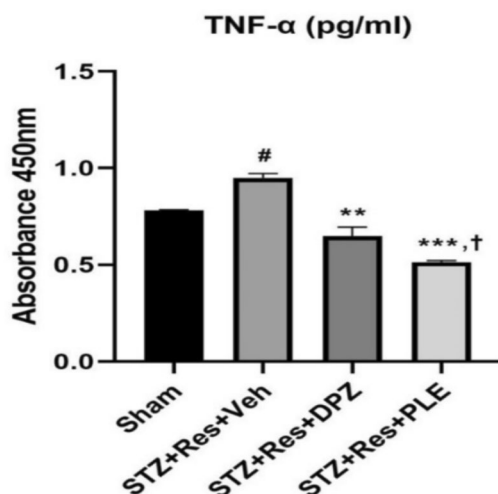


Fig. 11 The level of TNF-α at absorbance 450nm

and secondary antibodies, followed by confocal imaging (Fig. 12). Representative images from each group are shown; immunofluorescence was performed on brain tissue from $n = 10$ mice per group.

4 Discussion

The prevalence of depression is high in people with Alzheimer's disease, and depression can lead to serious cognitive impairment [28]. However, the effectiveness of this treatment for coexisting memory deficits and depressive behaviors is unclear. Therefore, it is particularly important to identify the biological mechanisms underlying the coexistence of AD and depression and to identify novel and effective treatments. In this study, we experimentally

demonstrated that PLE, an herb with anti-inflammatory and antidepressant effects, improves learning and memory deficits, as well as depression-like behavior in an STZ/RES-induced mouse model.

Piper longum, a fruit of the pepper family, has long been used in traditional medicine [29]. In addition to being used in cooking, it has antioxidant, anticancer, anti-inflammatory, antianxiety, antidepressant, and neuroprotective effects [21]. As with the known anti-inflammatory and antidepressant effects of piper longum and its components, similar clinical effects were obtained in both cognitive impairment and restraint stress in the STZ/RES mouse model. In the current study, experiments were conducted on the effects of PLE on regulatory factors such as Aβ, tau protein, TNF-α, and LCN-2, which cause cognitive impairment in AD [30]. In addition, the clinical effects of depressive behavior were tested using the OFT, FST, and TST, which confirm chronic restraint stress [27].

According to the previous studies, piperine, one of the main active molecules in *Piper longum*, improves cognitive function and restores neurotransmission in SZT-induced AD [31]. Studies have shown that piperine attenuates the increase in IL-6 and TNF-α levels and that it regulates anti-inflammatory cytokines in the hippocampus [32]. Additionally, piperine and black pepper fruit essential oils have been reported to have strong antidepressant effects in depressive behavioral studies such as the FST, TST, and OFT [33, 34]. In patients at high risk for major depressive disorder, anxiety and depression scores were significantly reduced in patients treated with piperine-containing curcuminoids [35]. This is consistent with the current research results showing that piper

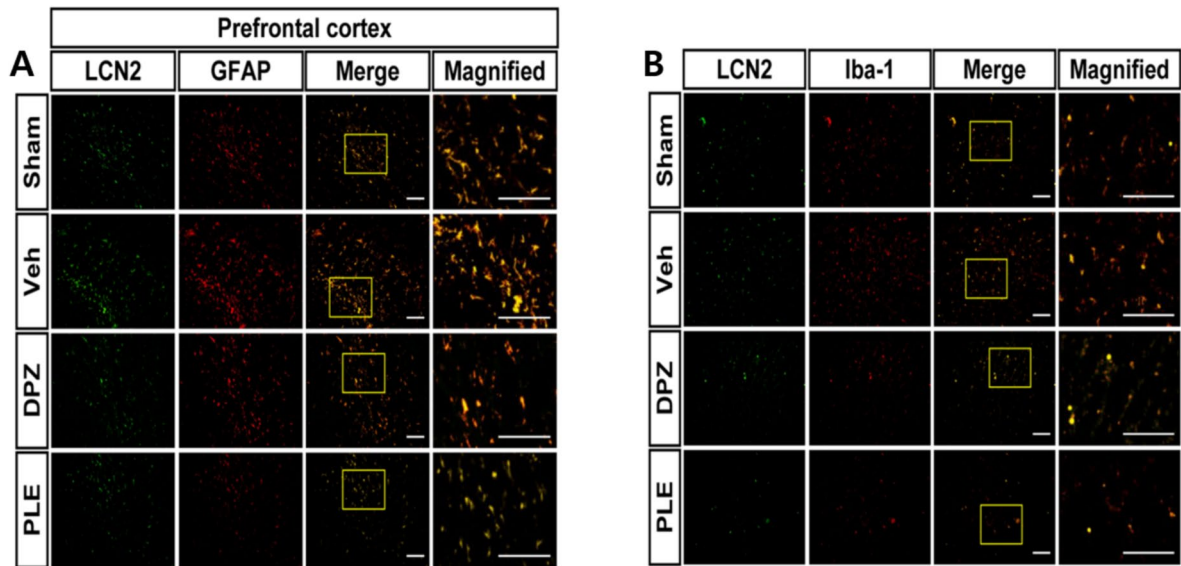


Fig. 12 Representative immunofluorescence images showing the colocalization of LCN2 with GFAP **A** and Iba-1 **B** in the prefrontal cortex

longum had a positive effect on improving not only cognitive impairment but also antidepressant behavior.

In AD, the soluble portion of A β is involved in hyperphosphorylation of tau [36]. A β induces hyperphosphorylation of tau, increasing the glutamate release and enhancing long term depression induction [37]. Studies have shown that black pepper consumption reduces cholinesterase levels and amyloid plaque formation, provides neuroprotection, and improves memory function [38–40]. In accordance with the previous studies on the association between these two key pathological proteins, we confirmed the regulatory effect of PLE on A β levels and hyperphosphorylation of tau [41].

Ethanollic PLE inhibit neuroinflammation through various mechanisms [42]. This may affect cognitive changes and reduce the incidence of neurological disorders, in which inflammation is a key factor [43]. TNF- α , an inflammatory cytokine marker, is associated with AD because it can impair neurological function [44]. It is known to be a multifactorial cytokine that acts by inducing apoptosis through TNF- α receptor 1 or activating inflammatory survival genes of TNF- α receptor 2 [45]. According to studies, ethanollic Piper nigrum and piper longum extract had anti-inflammatory effects and inhibited the expression of cytokines Interleukin-4(IL-4), IL-6, IL-1 β , and TNF- α [46, 47]. Piperine also reduced the levels of proinflammatory cytokine TNF- α expression [48].

Previous studies have reported increased cytokine production and oxidative stress following ICV-STZ injection [49]. Piperine attenuates the oxidative stress induced by ICV-STZ injection and promotes the production of anti-inflammatory cytokines [50]. Recently, piperine

treatment has been shown to improve STZ-induced cognitive impairment and restore hippocampal neurotransmission [51]. Piperine attenuated the expression of inflammatory cytokines IL-1 β , IL-6, and TNF- α , and significantly increased anti-inflammatory cytokines IL-4 and IL-10 in the STZ-injected AD mouse model [52]. These actions of piperine strongly suggest that it may contribute to improvements in cognitive function.

LCN2 is an inflammatory protein that functions in Central Nervous System (CNS) cell death, survival, and migration by upregulating the secretion of mediators, such as chemokines, in the brain [53]. LCN2 is secreted by glia under various conditions and studies have shown that TNF- α signaling in astrocytes is associated with LCN2 expression [54]. Studies have shown that TNF- α stimulates LCN2 and protects human prostate cancer cell line PC-3 from apoptosis [55]. LCN2, released by astrocytes regulates cellular functions, affecting neuroinflammation, neurodegeneration, cognitive impairment, neuronal health, and synapses [56]. LCN2 is a key marker of neuroinflammation associated with sporadic AD and chronic restraint stress [13, 57]. Studies have shown that the absence of LCN2 causes behaviors such as anxiety and depression, and impairs cognitive function [58]. This suggests that LCN2 may act as a central regulator of emotional and cognitive behavior.

Several studies have shown that piper longum and its active ingredients, piperine and piperlongumin, are effective in improving depression and suppressing AD neuroinflammation in geriatric neurological diseases [50]. However, little is known about the effects of *piper longum* on LCN2-mediated neuroinflammation [57]. In

this study, immunofluorescence colocalization increased when LCN2 merged with GFAP and Iba-1, decreased in the PLE-treated group. These results indicate that PLE can be an effective therapeutic agent for improving cognitive function and depressive behavior caused by LCN2-mediated neuroinflammation. However, since the current study only included representative fluorescence, future work would be better served by including quantitative analysis, such as integrated density or area fraction, would provide more objective support for our conclusions.

Patients with AD are usually prescribed acetylcholinesterase inhibitors N-methyl-D-aspartate antagonists [59]. DPZ is a representative acetylcholinesterase inhibitor widely used to reduce symptoms [60]. Although these drugs have side effects such as nausea, vomiting, diarrhea, and insomnia, they are currently used as treatments for Alzheimer's disease [61]. A previous study suggested that a traditional formulation containing *Piper longum* L. could be developed for the treatment for AD patients with AD. The study showed that intake of combination treatment improved memory impairment in the brain of Tg-APPswe/PS1dE9 mice and suppressed A β levels and plaque deposition as much as the DPZ treatment group [62]. In an efficacy comparison, the combination drug was more effective in protecting against A β -induced cell death than of the *piper longum* alone group and the DPZ group.

However, DPZ treatment focuses on AD, and it is difficult to discuss the effects of DPZ on comorbid diseases such as AD and depression. In the current study, we set up an experiment compared the effects of PLE and DPZ treatment in an STZ/RES-induced mouse model. The PLE group showed better results than the DPZ group. Compared to DPZ, PLE showed remarkable improvement in AD incidence factors and depressive behavior experiments. Therefore, the potential use of PLE as a natural pharmacological agent for AD prevention and treatment can be confirmed.

In the current study, we focused primarily on the time spent and distance traveled in the target quadrant as primary indicators of spatial memory. Unfortunately, data on escape latency and swimming paths were not recorded in a format suitable for presentation. We acknowledge that inclusion of such parameters would have provided a more comprehensive assessment of spatial learning.

As mentioned above, *Piper longum* consists of various bioactive components [63]; however, the detailed alkaloid components could not be distinguished and used in this experiment. In future studies, it will be necessary to analyze which alkaloids are more effective for AD and depression comorbidities. Additional studies are needed to compare the efficacy of specific bioactive ingredients

and PLE. As a potential treatment for the coexistence of AD and depressive behavior, the optimal effect of the PLE dose should also be considered in future studies.

5 Conclusion

The objective of this study was to explore the efficacy of PLE on cognitive function and depression. We found that PLE improved not only learning and memory, but also depression-like behavior in STZ/RES mice, most likely through LCN2-mediated neuroinflammation. Better results were obtained compared with the effectiveness of DPZ, which is a powerful treatment for AD. Therefore, PLE can be used as a therapeutic agent for comorbid memory deficits and depression.

Abbreviations

A β	β -Amyloid
AD	Alzheimer's disease
CNS	Central Nervous System
DPZ	Donepezil
DPPL	Dihydropiperlonguminine
FST	Forced Swim Test
GFAP	Glial Fibrillary Acidic Protein
Iba-1	Ionized Calcium-Binding Adaptor Molecule 1
ICV	Intracerebroventricular Injection
IF	Immunofluorescence
LCN2	Lipocalin2
MTDLs	Multi-target directed ligands
OFT	Open Field Test
PC-3	Prostate cancer cell line
PFC	Prefrontal Cortex
PLE	<i>Piper Longum</i> Extract
PPL	Piperlonguminine (PPL)
PPR	Piperine
Res	Chronic Restraint Stress
STZ	Streptozotocin
STZ/Res	Streptozotocin/Chronic Restraint Stress
TNF- α	Tumor Necrosis Factor-alpha
TST	Tail Suspension Test
Veh	Vehicle

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Author Contributions

HY Ha interpreted the data and contributed significantly to the writing of the manuscript text. SH Han performed the experiments and wrote Material and Methods text and Fig. 1 and Table 1. SH Cho designed the experimental model and provided important ideas. All authors read and approved the final manuscript.

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Availability of Data and Materials

No datasets were generated or analyzed during the current study.

Declarations

Conflict of Interest

The authors declare no competing interests.

Ethical Approval

This research was approved by the Institutional animal care and use committee, Department of Neuropsychiatry in Korean Medicine, Kyung Hee University Medical Center, Seoul, Korea.

Consent to Participate

This research was approved by the Institutional animal care and use committee, Department of Neuropsychiatry in Korean Medicine, Kyung Hee University Medical Center, Seoul, Korea.

Consent for Publication

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