Journal of Functional Foods 91 (2022) 105021

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Dietary consumption of desert olive tree pearls reduces brain Aβ content and improves learning and memory ability in aged mice

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ARTICLE INFO

Keywords:
Desert Olive Tree Pearls
Aging
Spatial learning and memory
Amyloid-β
Neuroinflammation

ABSTRACT

Recently, the number of patients with neurodegenerative diseases, such as Alzheimer's disease (AD), has increased with aging of the population. Desert Olive Tree Pearls (DOTP), one of the olive oil products rich in functional compounds such as polyphenols is considered to have beneficial effect on neuronal activities. In this study, our study showed that pre-treatment of SH-SY5Y cells with DOTP ameliorated Aβ-induced cytotoxicity. Moreover, the Morris water maze test indicated that DOTP improves learning and memory in aged mice. Also, DOTP induced a decrease in $A\beta_{42}$ and inflammatory cytokine levels and increase in neurotransmitter levels in limbic system and serum of aged mice. These results suggest that DOTP may have anti-oxidative effects and decreased oxidative stress associated with overproduction of pro-inflammatory cytokines in Aβ accumulations by aging, thus protecting neurons. Generally, the results of the present study suggest that DOTP is a promising dietary ingredient for improvement of cognitive function.

1. Introduction

Currently, approximately 50 million individuals worldwide have dementia-related problems. Due to aging populations, this number is expected to reach 66 million by 2030 and 115 million by 2050 (Peprah and McCormack, 2019). Much research has been conducted on the pathology of Alzheimer's disease (AD), but treatment for the most common forms of dementia is still unclear. Current treatments target symptoms and do not affect the disease progression. AD is characterized by accumulation of amyloid β (Aβ) plaques in the brain parenchyma and cerebral blood vessels (Selkoe, 2001). Accumulation of Aβ is associated with pathological changes associated with AD (Hardy, 2009). Aβ is a product of amyloid precursor protein (APP) that is processed to produce $A\beta_{40}$ and $A\beta_{42}$. Currently, acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine) and glutamate receptor antagonists (memantine) are available as treatments for symptoms of AD (Casey et al., 2010). However, these treatments result in several improvements in symptoms but are limited, and none of them have therapeutic or disease-modifying effects (Casey et al., 2010). Therefore, much research is needed to develop therapeutic approaches that have AD-modifying effects to prevent the pathology of AD and enhance cognitive function.

Diet therapy is one of the oldest and traditional methods of prevention and cure of all known diseases that makes up the majority of some Greek, Arab, and Islamic health systems based on herbs and diets (Saad, 2015). Particularly, several epidemiological studies have reported various health benefits of the Mediterranean diet. Interestingly, the prevalence of AD is lower in the Mediterranean region than in other countries (Solfrizzi et al., 2003; Panza et al., 2004; Scarmeas et al., 2009). Several systematic reviews and meta-analyses have also shown that the Mediterranean diet reduces the incidence of AD (Singh et al., 2014; Aridi et al., 2017; Hill et al., 2019). One of the most representative components of the Mediterranean diet is olive oil products such as olive oil and Desert Olive Tree Pearls (DOTP), which have many bioactive compounds, especially hydroxytyrosol, with many health benefits. Recently, several reports of Mediterranean diet have suggested that its consumption ameliorates and decrease the risk for many chronic diseases, such as cardiovascular disease (Estruch et al., 2013), diabetes (Filippatos et al., 2016), and age-related memory decline (Feart et al., 2013). Moreover, hydroxytyrosol, main component of DOTP, have the potential to exert the neuroprotective effect due to its potent anti-

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https://doi.org/10.1016/j.jff.2022.105021
Received 17 December 2021; Received in revised form 26 February 2022; Accepted 27 February 2022

oxidant and anti-inflammatory activities (Omar et al., 2017). In our previous study, we reported for the first time the neuroprotective effect against Aβ-induced neuronal cell death in olive oil (Villareal et al., 2016). However, although DOTP, which also contains many bioactive compounds such as hydroxytyrosol related to antioxidants and anti-inflammatory compounds, is expected to have the potential to exert physiological activities equal to or higher than that of olive oils, there are no reports about its physiological activities.

Thus, in the present study we studied the effects of DOTP on cell viability and neuroprotection against $A\beta_{42}$-induce neuronal cell death in SH-SY5Y human neuro-blastoma cells. Moreover, to investigate the possibility of using DOTP as a medical food in AD, in the present study, we hypothesized that oral administration of DOTP enhances spatial learning and memory ability by reducing $A\beta_{42}$ content and attenuating $A\beta_{42}$-associated biomarker changes. To the best of our knowledge, this is the first study to investigate the effect of using DOTP as a possible medical food with current AD medication.

2. Materials and methods

2.1. Desert Olive Tree Pearls (DOTP) samples

The Desert Olive Tree Pearls (DOTP) used in this study was obtained from ATLAS OLIVE OILS company in Morocco. DOTP, one of the olive oil products, is made from leaves, baby leaves, and fruits of olive trees by cold agitation and high-pressure mechanical manufacturing methods and contains a lot of polyphenols, such as hydroxytyrosol, tyrosol, oleocanal, and oleacein (analyzed using HPLC by Sidi Mohamed Ben Abdellah University, Morocco) (Table 1). Particularly, DOTP contains high amount of hydroxytyrosol (16.12 mg/g) compared with other polyphenols (Table 1). In the present study, for in vitro experiments, DOTP was dissolved in serum-free Eagle's minimum essential medium (OPTI-MEM; Gibco, Japan) with homogenization and sonication and for in vivo experiment, DOTP was dissolved in milliQ water with homogenization and sonication.

2.2. SH-SY5Y cell culture

The human neuroblastoma SH-SY5Y cell line was purchased from the American Type Culture Collection (ATCC). SH-SY5Y cells were cultured in a 1:1 (v/v) mixture of Dulbecco's modified Eagle Medium and Ham's F-12 medium (Gibco, Japan) supplemented with 15% heat-inactivated fetal bovine serum (Bio West, U.S.A) and 1% penicillin (5000 μg/ml)-streptomycin (5000 IU/ml) (PS) (Lonza, Japan) at 37 °C in a humidified atmosphere of 5% CO_2 in air. SH-SY5Y cells were cultured in 100-mm petri dishes or 96-well plates. Serum-free Eagle's minimum essential medium (OPTI-MEM; Gibco, Japan) was used to culture the cells for the cell viability assay.

2.3. MTT assay

Cell viability and mitochondrial activity were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to check for effects of DOTP (10, 25, 50, 75, and 100 μg/mL) and $A\beta_{42}$ (3 μM) on cytotoxicity. SH-SY5Y cells were seeded at 2×10^5 cells/mL in 96-well plates (BD BioCoat, U.S.A.) and incubated for 24 hr. After 24 hr incubation, SH-SY5Y cells were treated DOTP for 72 hr. To evaluate the neuroprotective effects of DOTP against Aβ-induced cytotoxicity, SH-SY5Y cells were pre-treated with DOTP for 24 hr before 3 μM $A\beta_{42}$ monomer treatment. After sample treatment, a solution of 5 mg/ml MTT dissolved in PBS was added (10 μl/well) and incubated for another 24 hr. The resulting MTT formazan was dissolved in 100 μl of 10% SDS (w/v) and the absorbance was measured using a microtiter plate reader (Dainippon Sumitomo Pharma Co., Ltd., Japan).

Table 1
Polyphenols contents in Desert Olive Tree Pearls (DOTP).

	Content (mg/g)
Hydroxtyrosol	16,12
Tyrosol	3.2
Oleocantal	4.46
Oleacein	1.68
Total polyphenols	55.44

2.4. RNA isolation from SH-SY5Y cells

SH-SY5Y cells were seeded at 3.7×10^5 cells/mL in a 10 cm^2 dish (BD BioCoat) and incubated at 37 °C for 24 h. The SH-SY5Y cells were then pre-treated with DOTP (100 μg/mL) at 37 °C for 24 hr. After DOTP treatment, $A\beta_{42}$ solution (final concentration: 3 μM) was added and incubated for a further 24 hr at 37 °C. Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions as per a previous study (Villareal et al., 2016).

2.5. Measurement of MAPK-related genes using real-time reverse transcription polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed to evaluate the effect of DOTP on MAPK-related genes expression in SH-SY5Y cells. In this study, 100 μg/mL DOTP showed the highest effect on neuroprotective effect against $A\beta_{42}$ toxicity. Thus, we used 100 μg/mL of DOTP for the effect of DOTP on MAPK-related genes expression. The TaqMan probe (ThermoFisher Scientific, USA) was used for the quantification of gene expression. Using a superscript III reverse transcriptase kit (ThermoFisher Scientific) a complementary DNA (cDNA) solution was synthesized following the manufacturer's instructions. For quantification of transcript amounts, TaqMan real-time RT-PCR amplification reactions were performed using the Applied Biosystems 7500 Fast Real-Time System (ThermoFisher Scientific). All primer sets and the TaqMan Universal PCR Master Mix were obtained from ThermoFisher Scientific. Specific primers for actin beta (Hs1060665_m1), Poly (ADP-ribose) polymerase 1 (PARP1) (Hs00242302_m1), Mitogen-Activated Protein Kinase Kinase 4 (MAP2K4) (Hs00387426_m1), Mitogen-activated protein kinase 14 (MAPK14) (Hs01051152_m1), Mitogen-Activated Protein Kinase 8 (MAPK8) (Hs01548508_m1), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) (Hs00907957_m1), AKT Serine/Threonine Kinase 1 (AKT1) (Hs00178289_m1), and caspase-3 (CASP3) (Hs00234387_m1) were used.

2.6. Animals and sample treatment

Male C57BL/6J mice, aged 10 weeks and 18 months, were obtained from Japan SLC, Inc. (Shizuoka, Japan) and housed under conditions of controlled temperature and humidity and unrestricted access to food and water with a 12-hr light/dark cycle. The protocol for this animal experiment was approved by the Animal Care and Use Committee of the University of Tsukuba (19–363). Because estrogens are well known for their robust enhancement on cognition about learning and memory in female rodents (Korol & Pisani, 2015), to avoid the effect of estrogens, we used male mice in the present study. After 1 week of acclimatization to the laboratory conditions, the mice were divided into four groups: 10-week-old water-administered group (n = 8) (Young control), 10-week-old DOTP-administered group (n = 8) (Young test), 18-month-old water-administered group (n = 8) (Aged control), and 18-month-old DOTP-administered group (n = 8) (Aged test). In this study, based on the previous study about animal experiment of olive oil study (Hernández-Rodas et al., 2017) and morris water maze (MWM) experiment of aging study (Sasaki et al., 2019; Sasaki et al., 2021), the concentration and the period of oral administration of DOTP were decided. The DOTP-administered group was administered 50 mg/kg DOTP mixed with MilliQ water for 30 days by oral gavage using a tube and syringe.

Water-administered groups were administered an equivalent volume of MilliQ water.

2.7. Morris water maze (MWM) test

To evaluate the effects on spatial learning and memory of DOTP administration, the MWM test was performed as previously described (Sasaki et al., 2019; Sasaki et al., 2021). The apparatus consisted of a circular water tank (120 cm in diameter and 45 cm in height) that contained water (23 ± 2 °C) to a depth of 30 cm and divided into four quadrants: north, east, west, and south. The escape platform (10 cm in diameter) was submerged 1 cm below the water surface and placed at the midpoint of any quadrant so that it was invisible at the water level. All mice received training for 5 consecutive days using a single hidden platform in one quadrant, with the start point rotating around the other three quadrants. The latency to escape from the water maze (find the hidden escape platform) was measured for each trial. Then, the probe test was conducted on day six after the MWM test training session, the platform was removed, and each mouse was allowed to swim freely for 60 s. The respective time spent by each mouse in the target quadrant and the number of crossings over the platform location (where the platform was located during the training) were calculated. The time spent by each mouse in the target quadrant and the number of times crossing the virtual platform were considered to represent the degree of memory consolidation.

2.8. Protein extraction from the limbic systems of mouse brain

Furthermore, to evaluate the molecular markers of spatial learning and memory improvement effects of DOTP, animals were sacrificed, and the brain was carefully removed after the behavioral test. The limbic system of the mouse brain was carefully dissected, frozen in liquid nitrogen, and stored at −80 °C until use. Moreover, 100 mg of the limbic system was homogenized in 1 mL of RIPA buffer with protease inhibitor (Santa Cruz Biotechnology, Japan) using a glass-Teflon homogenizer and centrifuged (10000*g*, 30 min, 4 °C). After centrifugation, the supernatant was collected for protein samples. Protein estimation was conducted using 2-D quant kit (GE Healthcare Inc., Japan) according to procedure of the kit, and the data were expressed as pg/μg protein.

2.9. $A\beta_{42}$ analysis in the limbic systems of mouse brain

$A\beta_{42}$ levels in the limbic system of the mouse brain were measured using commercial ELISA kits (Wako, Japan) in accordance with the manufacturer's instructions. Briefly, protein samples or standards were used to determine $A\beta_{42}$ levels. After treatment with the $A\beta_{42}$ antibody, a second incubation was performed with streptavidin-horseradish peroxidase conjugate solution. After addition of substrate and stop solution, $A\beta_{42}$ levels were determined by measuring the absorbance at 450 nm. $A\beta_{42}$ levels were expressed as nmol/μg protein.

2.10. TNF-α and IL-6 analysis in the serum and limbic systems of mouse

Commercial ELISA kits (R&D Systems, USA) were used to measure TNF-α and IL-6 levels in the serum and limbic systems of the mouse brain. Briefly, serum, protein samples, or standards were used according to the manufacturer's instructions for TNF-α or IL-6 ELISA kit. TNF-α and IL-6 levels were computed as pg/mL (serum) or pg/μg protein (protein).

2.11. Dopamine, noradrenaline, serotonin, gamma-aminobutyric acid (GABA), and glutamate analysis in the limbic systems of mouse brain

Dopamine, noradrenaline, serotonin, GABA, and glutamate levels in the limbic systems of the mouse brain were measured using commercial ELISA kits (ImmuSmol, Inc., France). Briefly, protein samples or standards were used for catecholamine extraction, acylation, and determination according to the manufacturer's instructions. After optimal color development, the reaction was stopped, and the absorbance at 450 nm was recorded. Dopamine, noradrenaline, serotonin, GABA, and glutamate levels were computed by correcting for the protein concentration, and the data were expressed as ng/μg protein.

2.12. Pro-BDNF and BDNF analysis in serum and the limbic systems of mouse brain

Pro-BDNF and BDNF levels in the serum and limbic systems of the mouse brain were measured using commercial ELISA kits (R&D Systems, Inc., Minneapolis, USA) in accordance with the manufacturer's instructions. Briefly, serum, protein samples, or standards were used to determine the Pro-BDNF and BNDF levels in the brain. After treatment with the antibody, a second incubation was performed with streptavidin-horseradish peroxidase conjugate solution for 60 min. After addition of substrate and stop solution, Pro-BDNF and BNDF levels were determined by absorbance at 450 nm. The pro-BDNF and BNDF levels were normalized to the determined protein concentration. The data for pro-BDNF and BNDF were expressed as pg/mL (serum sample) and pg/μg protein (protein samples).

2.13. Statistical analysis

Statistical analysis of the results obtained from the MWM was conducted using two-way ANOVA followed by the Tukey's post-hoc test. In another experiment, comparisons between treatment groups of animal models were performed using one-way ANOVA followed by Tukey's post hoc test for normally distributed data. was also conducted. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Desert Olive Tree Pearls (DOTP) induced cell viability and inhibited Aβ42-induced cell death on human neuroblastoma SH-SY5Y cells

SH-SY5Y cells were treated with DOTP (10, 25, 50, 75, and 100 μg/mL) for 72 hr and cell viability measured with the MTT assay. Our MTT results showed that. only DOTP (25, 50, 75, and 100 μg/mL) treated cells induce a significant increase in cell proliferation (120.8 ± 4.3%, 127.8 ± 7.8%, 139.7 ± 7.1%, and 156.3 ± 8.8%, respectively) ($P < 0.01$) compared with non-treated cells (Fig. 1A). Moreover, to evaluate the neuroprotective effect of DOTP SH-SY5Y cells were pre-treated with DOTP (10, 25, 50, 75, and 100 μg/mL) for 24 hr. And then, $A\beta_{42}$ was added (final concentration: 3 μM) and co-treated with DOTP for 48 hr; and cell viability was measured with the MTT assay. In our present study, when we assessed the optimal concentration of $A\beta_{42}$, 3 μM-treatment showed a reduction in cell viability of approximately 50% (Data was not shown). Therefore, we decided that 3 μM $A\beta_{42}$ treatment is optimal concentration for evaluating neuroprotective effect. As shown in Fig. 1B, the $A\beta_{42}$-treated group showed a significantly reduction in cell viability compared to the non-treated group (54.6 ± 1.7%). In contrast, pre-treatment with DOTP (25, 50, 75, and 100 μg/mL) ameliorated $A\beta_{42}$-induced cytotoxicity (114.2 ± 5.9%, 121.9 ± 7.4%, 147.3 ± 7.9%, and 175.8 ± 11.2% compared to 100% in $A\beta_{42}$-treated cells, respectively, $P < 0.01$) (Fig. 1B).

3.2. Effect of Desert Olive Tree Pearls (DOTP) on gene expression related MAPK signaling in $A\beta_{42}$-treated SH-SY5Y cells

To evaluate the effects of DOTP on MAPK-related genes expression was measured by real-time RT-PCR in $A\beta_{42}$-treated SH-SY5Y cells. SH-SY5Y cells were treated with 100 μg/mL of DOTP for 24 hr. After DOTP treatment, SH-SY5Y cells were stimulated with $A\beta_{42}$ for further 24 hr. As shown in Fig. 2, the $A\beta_{42}$-treated cells showed significantly

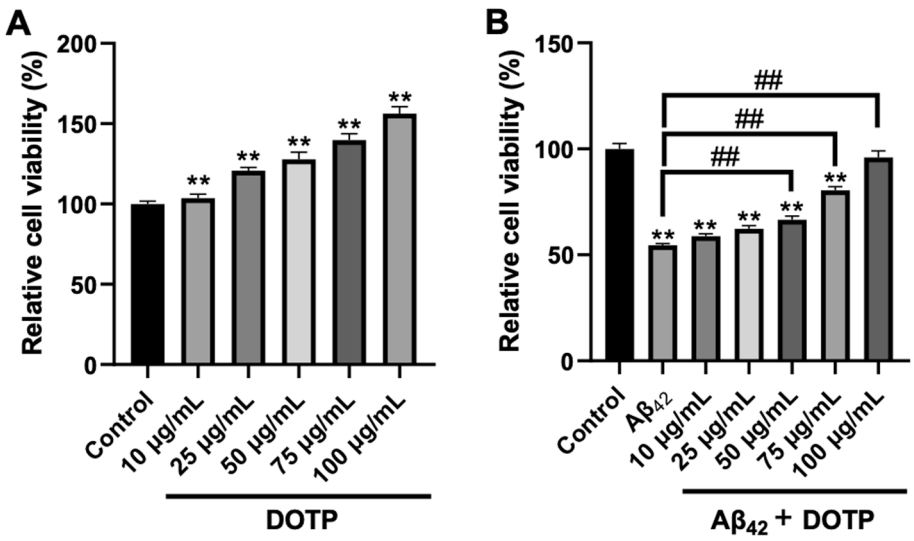

Fig. 1. Effect of Desert Olive Tree Pearls (DOTP) on (A) the cell viability and (B) Amyloid-β (Aβ)-induced changes in human neuroblastoma SH-SY5Y cells viability. For the evaluation of cell viability, SH-SY5Y cells were treated with DOTP for 72 hr. And for the evaluation of neuropro-tection, SH-SY5Y cells were pre-treated with DOTP for 24 hr, and then, the cells were treated with 3 μM Aβ for 48 hr. Each bar represents the mean ± SEM (n = 5 independent experiments). ** $P < 0.01$ vs control cells, ## $P < 0.01$ vs $A\beta_{42}$-treated cells.

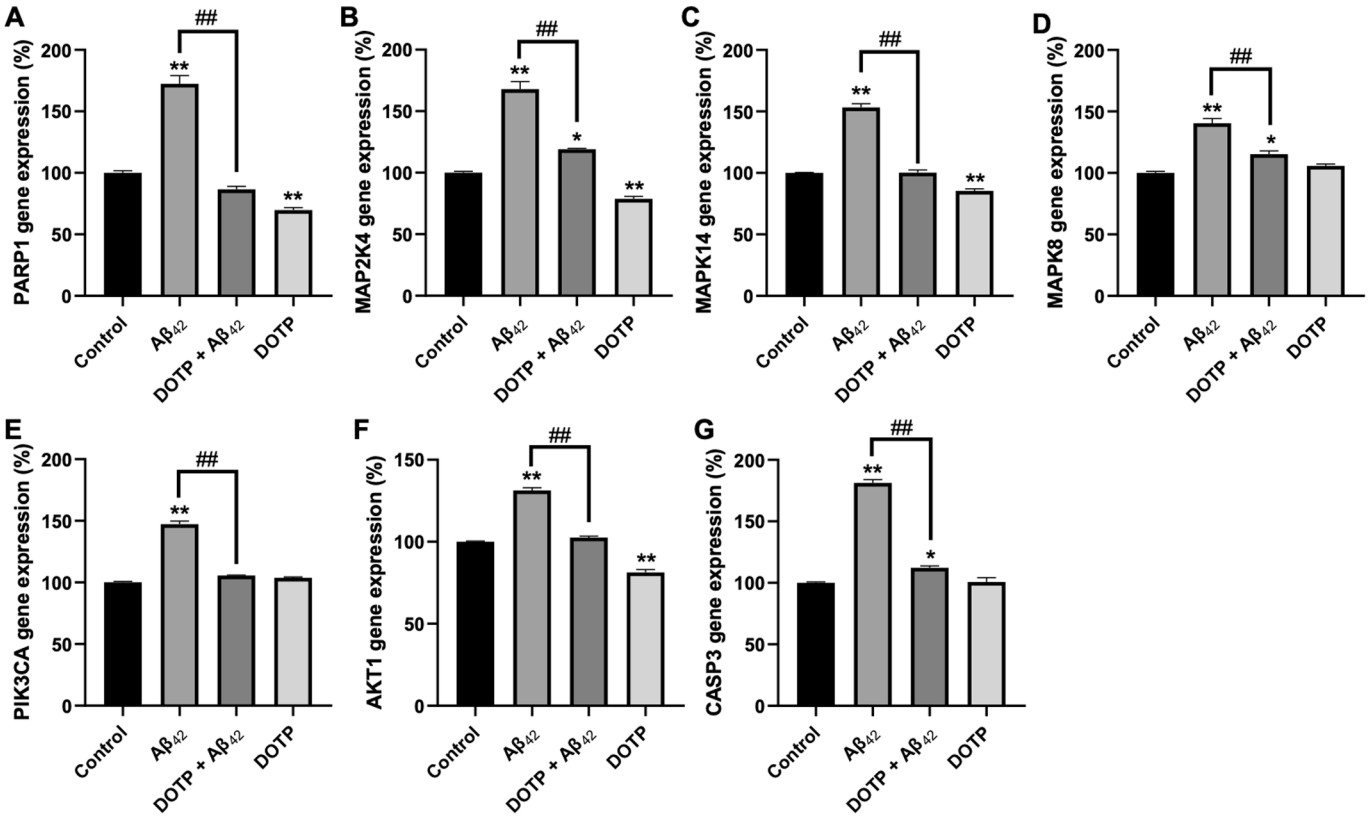

Fig. 2. Effect of Desert Olive Tree Pearls (DOTP) on gene expression of (A) PARP1, (B) MAP2K4, (C) MAPK14, (D) MAPK8, (E) PIK3CA, (F) AKT1, and (G) CASP3 in human neuroblastoma SH-SY5Y cells. SH-SY5Y cells were pre-treated with 100 μg/mL DOTP for 24 h. Following that, cells were treated with $A\beta_{42}$ (final concentration: 3 μM) for further 24 h. After treatment, the gene expression of PARP1, MAP2K4, MAPK14, MAPK8, PIK3CA, AKT1, and CASP3 were evaluated using real-time RT-PCR. Values are expressed as the mean ± SD of triplicate experiments and are expressed relative to the percentage of control cells. ** $P < 0.01$ vs control cells, ## $P < 0.01$ vs $A\beta_{42}$-treated cells.

increase ($P < 0.01$) PARP1 gene expression by 172.4 ± 11.7% (Fig. 2A), MAP2K4 (Fig. 2B) gene expression by 167.9 ± 10.6%, MAPK14 (Fig. 2C) gene expression by 153.3 ± 5.2%, MAPK8 (Fig. 2D) gene expression by 140.5 ± 6.5%, PIK3CA (Fig. 2E) gene expression by 147.3 ± 4.2%, AKT1 (Fig. 2F) gene expression by 131.3 ± 2.8%, and CASP3 (Fig. 2G) gene expression by 181.2 ± 4.8% compared to that in non-treated cells. However, the 100 μg/mL DOTP-treated cells showed a significantly lower gene expression of PARP1 by 50.2% (Fig. 1A), MAP2K4 (Fig. 2B) by 71.0%, MAPK14 (Fig. 2C) by approximately 65.4%, MAPK8 (Fig. 2D) by 82.0%, PIK3CA (Fig. 2E) by 71.7%, AKT1 (Fig. 2E) by 78.0%, and CASP3 (Fig. 2F) by approximately 62.0% compared to that in $A\beta_{42}$-treated SH-SY5Y cells.

3.3. Administration of Desert Olive Tree Pearls (DOTP) improved spatial learning and memory in aged mice

To evaluate the potential effect of DOTP on spatial learning and memory impairment, 50 mg/kg of DOTP was orally administered to 10-week-old mice (young mice) and 18-month-old mice (aged mice) for 30 days. Spatial learning and memory were evaluated using the Morris water maze (MWM) test, a widely used tool to assess spatial learning and memory in rodents. As shown in Fig. 3A, the escape latency time of the Aged control group did not show any decrease from the 1st to 5th day training (Day 1, 50.11 ± 6.31 s; Day 2, 50.87 ± 7.27 s; Day 3, 46.83 ± 6.83 s; Day 4, 49.08 ± 4.23 s; Day 5, 46.13 ± 5.75 s). However, on day 5, both the Young control group (31.75 ± 3.57 s) and Aged test group (33.0 ± 5.27 s) showed a statistically significant decrease in escape latency compared with the Aged control group (46.13 ± 5.75 s) (Fig. 3A). In contrast, in the Young test group (Young mice fed with DOTP group) also showed a decrease (31.33 ± 4.89 s) in escape latency; however, this decrease was not different from that in the Young control group (31.75 ± 3.57 s).

Moreover, to verify whether the effect of spatial learning and memory improvement by administration of DOTP is sustainable and whether learning and memory ability of mice was dependent on spatial information, a probe test was conducted. In the probe test, the observation of the behavior of the mouse is performed under the same condition except removing the platform. As a result of the probe test, the Aged test group showed a significantly increased swimming time in the quadrant (28.75 ± 4.25 s) compared with the Aged control group (20.50 ± 3.51 s). Furthermore, the Aged test group showed an increase in the number of crossings of the platform, but the difference was not significant. Moreover, Young control group and Young test group showed a significant increase both in the number of crossing times across the virtual platform (Young control group, 1.75 ± 0.50; Young test group, 1.67 ± 0.26) and the swimming time in the quadrant (Young control group, 29.25 ± 3.92; Young test group, 30.50 ± 3.96) where the platform was installed compared with Aged control group (number of crossing of the platform, 0.75 ± 0.29; time spent in quadrant, 20.50 ± 3.51 s) (Fig. 3B and 3C). Conversely, there was no difference between the Young control and Young test groups in terms of both the number of crossings of the platform and time spent in quadrant.

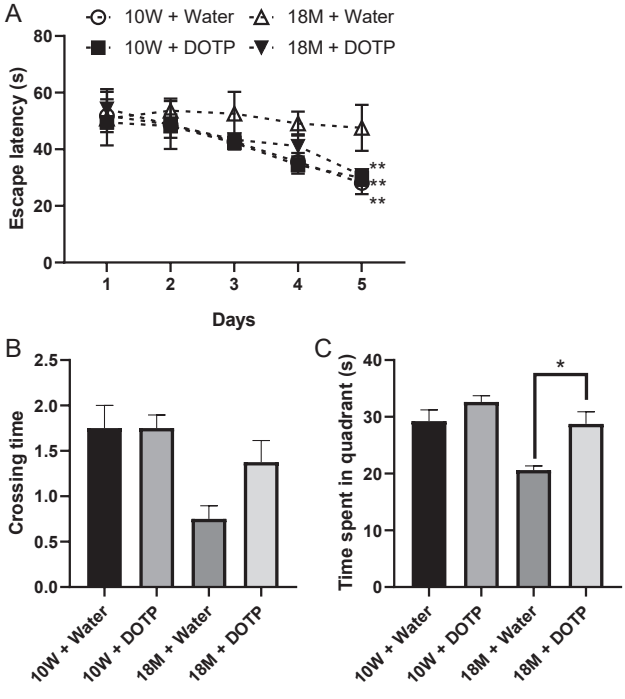

Fig. 3. Effect of Desert Olive Tree Pearls (DOTP) administration on spatial learning and memory as evaluated by escape latency of young (10 weeks) mice, aged (18 months) mice, and mice 50 mg/kg DOTP - and water-treated group by Morris water maze test (A). Effect of DOTP on numbers of crossings of platform (B). Effect of DOTP on the time spent in the target quadrant by DOTP -treated or water-treated mice (C). *P < 0.05, **P < 0.01 Compared with Aged control group by one-way ANOVA.

3.4. Administration of Desert Olive Tree Pearls (DOTP) reduced $A\beta_{42}$ content in limbic systems of mouse brain

Then, we assessed whether oral administration of DOTP reduced the levels of $A\beta_{42}$ in the limbic system of the mouse brain. The ELISA results showed that the $A\beta_{42}$ levels in the limbic systems of mouse brains were significantly ($P < 0.05$) increased in the Aged control group (301.41 ± 36.06 pmol/μg protein) compared to that in the Young control group (123.69 ± 12.44 pmol/μg protein) (Fig. 4). However, administration of DOTP reversed this increase of $A\beta_{42}$ levels (173.31 ± 9.54 pmol/μg protein) in aged mice (Aged test group). Furthermore, in young mice (10-weeks-old mice), $A\beta_{42}$ levels (Young control group, 123.69 ± 12.44 pmol/μg protein; Young test group, 119.13 ± 17.05 pmol/μg protein) compared with the Aged control group, but between the water-treated group and DOTP-treated group, there was no significant difference (Fig. 4).

3.5. Desert Olive Tree Pearls (DOTP) suppressed the levels of neuroinflammatory factors in serum and limbic systems of mouse brain

To investigate if altered neuroinflammation by the increase in $A\beta_{42}$ may have contributed to spatial learning and memory improvement of DOTP, quantification of inflammatory cytokines in serum and limbic systems of mice brain was performed. Quantification using the commercial ELISA kits showed that TNF-α and IL-6 levels, which are major inflammatory cytokines, were higher in the serum of the Aged control group (TNF-α, 42.30 ± 7.86 pg/mL; IL-6, 455.92 ± 48.10 pg/mL) than in the Young control groups (TNF-α, 20.52 ± 1.57 pg/mL; IL-6, 197.83 ± 30.38 pg/mL) (Fig. 5A and 5B). However, in the Aged test group, a decrease in the serum TNF-α levels (19.67 ± 5.44 pg/mL) was observed (Fig. 3A). Moreover, administration of DOTP significantly decreased serum IL-6 levels (57.56% compared to 100% in the SAMP8 water-administered group) (Fig. 5C).

Next, we evaluated whether administration of DOTP could reduce the TNF-α and IL-6 levels in the limbic systems of aged mice brains. As

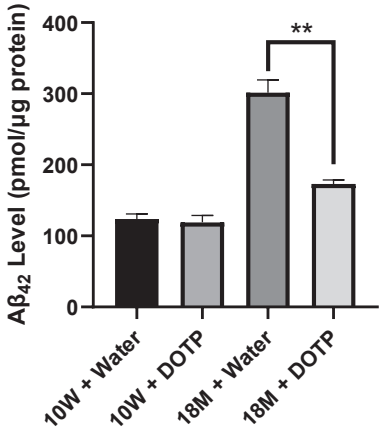

Fig. 4. Effect of Desert Olive Tree Pearls (DOTP) on $A\beta_{42}$ level in the limbic systems of young (10 weeks) and aged (18 months) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. The $A\beta_{42}$ level was determined by ELISA kit. Each value is presented as mean ± SD. **P < 0.01 compared to Aged control group by one-way ANOVA.

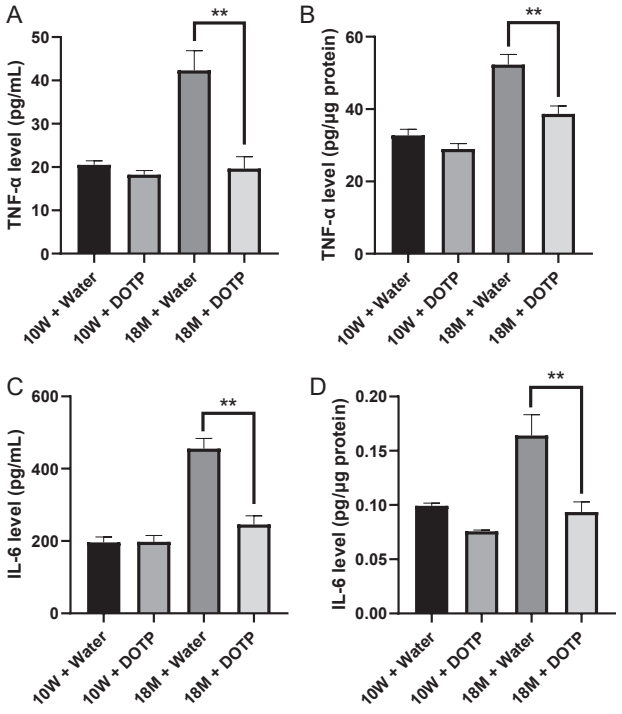

Fig. 5. Effect of Desert Olive Tree Pearls (DOTP) on levels of (A, B) tumor necrosis factor-α (TNF-α) and (C, D) interleukin-6 (IL-6) in serum and the limbic systems of young (10 weeks old) and aged (18 months old) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. The TNF-α and IL-6 levels were determined by ELISA kit. Each value is presented as mean ± SD. **P < 0.01 compared to Aged control group by one-way ANOVA. ## P 0.01 compared to Young control group by one-way ANOVA.

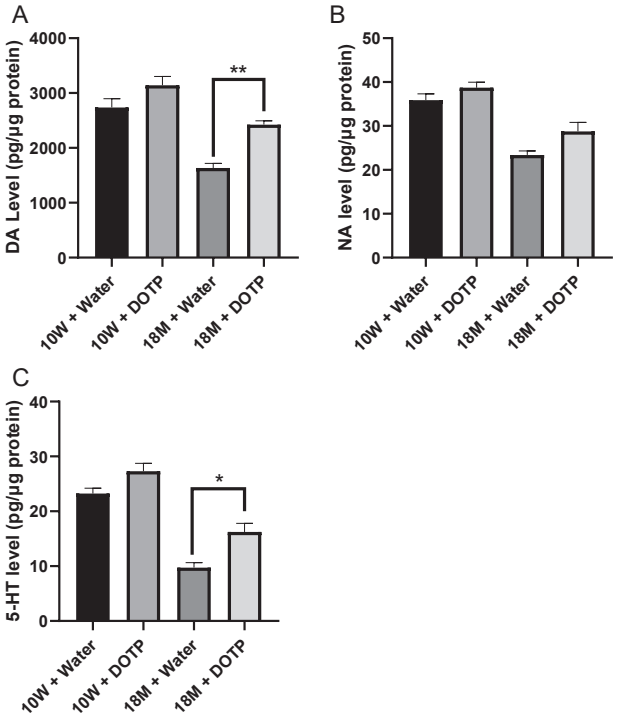

Fig. 6. Effect of Desert Olive Tree Pearls (DOTP) on (A) dopamine (DA), (B) noradrenaline (NA), and (C) serotonin (5-HT) levels in the limbic systems of young (10 weeks) and aged (18 months) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. These neurotransmitters levels were determined by ELISA kit. Each value is presented as mean ± SD. **P < 0.01 compared with Aged control group by one-way ANOVA.

shown in Fig. 5B and 5D, the TNF-α and IL-6 levels in the limbic systems from the Aged control group (TNF-α, 52.32 ± 6.31 pg/μg protein; IL-6, 0.164 ± 0.033 pg/μg protein) were significantly higher than those in the Young control group (TNF-α, 32.74 ± 3.34 pg/μg protein; IL-6, 0.099 ± 0.005 pg/μg protein). In contrast, the TNF-α and IL-6 levels in the Aged test group (TNF-α, 38.69 ± 3.83 pg/μg protein; IL-6, 0.094 ± 0.016 pg/μg protein) were significantly reduced compared with the Aged control group ($p < 0.01$).

3.6. Desert Olive Tree Pearls (DOTP) attenuated the decline in neurotransmitter level in the limbic systems of mouse brain by aging

To further probe the potential mechanisms contributing to the spatial learning and memory improvement of DOTP, we quantified neurotransmitters (dopamine, noradrenaline, serotonin) in the limbic systems of mice brain. Using commercial ELISA kits, we found that the levels of dopamine (1635.41 ± 165.15 ng/μg protein), noradrenaline (23.40 ± 2.03 ng/μg protein), and serotonin (9.75 ± 1.57 ng/μg protein) in the limbic systems were significantly lower in the aged mice (Aged control group) compared with those in the young mice (Young control group) (2739.23 ± 275.37 ng/μg protein, 35.88 ± 2.78 ng/μg protein, and 23.25 ± 1.69 ng/μg protein, respectively) (Fig. 6). However, in the limbic systems of the Aged test group, dopamine (2426.14 ± 120.80 ng/μg protein) and serotonin (16.26 ± 2.67 ng/μg protein) levels were significantly higher compared with those in the Aged control group (Fig. 6). The noradrenaline levels (28.75 ± 3.57 ng/μg protein) in the Aged test group were also higher compared with those in the Aged control group, but this was not significant. In contrast, we did not find any significant change in these neurotransmitters between the Young control and Young test groups.

3.7. Desert Olive Tree Pearls (DOTP) enhanced the GABA production in the limbic systems of mouse brain

In parallel with the increased dopamine and serotonin levels, we also evaluated the effect on glutamate and GABA levels in the limbic systems of the mouse brain. In the Aged control group, the GABA level (Fig. 7A) was significantly decreased (8.13 ± 0.47 ng/μg protein), and the glutamate level (Fig. 7B) was significantly increased (72.32 ± 5.78 ng/μg protein) compared to those in the Young control group (11.27 ± 0.91 ng/μg protein; 50.01 ± 3.24 ng/μg protein). DOTP treatment induced an increase in GABA levels (11.45 ± 0.89 ng/μg protein) in the limbic

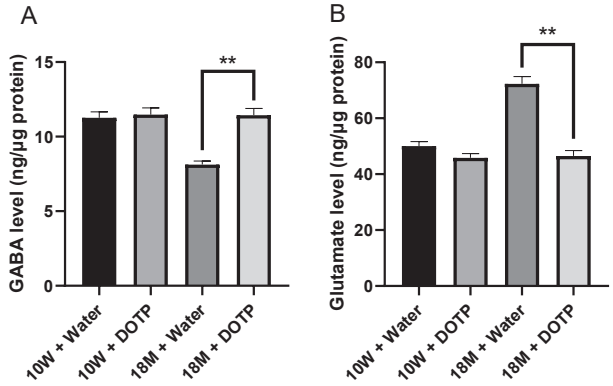

Fig. 7. Effect of Desert Olive Tree Pearls (DOTP) on (A) GABA and (B) glutamate levels in the limbic systems of young (10 weeks) and aged (18 months) mice brain. Mice were orally administered with 50 mg/kg DOTP for 30 days. These neurotransmitter levels were determined by ELISA kit. Each value is presented as mean ± SD. ** P < 0.01 compared with Aged control group by one-way ANOVA.

systems of 18-month-old mice compared with the Aged control group. Furthermore, administration of DOTP induced the decrease in glutamate level (46.51 ± 4.79 ng/μg protein) in limbic systems of 18-month-old mice compared with Aged control group.

3.8. Desert Olive Tree Pearls (DOTP) enhanced the brain-derived neurotrophic factor (BDNF) production in serum and limbic systems of mouse brain

Commercial ELISA assays were run to measure the proBDNF and BDNF levels in the serum of young (10 weeks) and aged (18 months) mice with or without DOTP. As shown in Fig. 8A and 8B, there was a significant increase in the serum proBDNF level and a significant reduction in the serum BDNF level in the Aged control group (proBDNF, 33.59 ± 3.07 pg/mL; BDNF, 40.14 ± 6.49 pg/mL) compared with the Young control group (proBDNF, 17.07 ± 3.56 pg/mL; BDNF, 82.40 ± 8.97 pg/mL). Further analyses of the DOTP-treated group revealed that the proBDNF levels in serum in the Aged test group (25.52 ± 2.12 pg/mL) were significantly lower than those in the Aged control group. Moreover, the serum BDNF levels in the Aged test group (66.58 ± 8.19 pg/mL) were significantly higher than that in the Aged control group.

We further analyzed the protein levels of proBDNF and BDNF in the limbic systems of young and aged mice with or without DOTP (Fig. 8C and 8D). The proBDNF levels in the limbic systems of the Aged control group (0.318 ± 0.013 pg/μg protein) was significantly higher than that in the Young control group (0.231 ± 0.023 pg/μg protein). The BDNF levels in the limbic systems of the Aged control group (0.127 ± 0.007 pg/μg protein) were significantly lower than those in the Young control group (0.171 ± 0.012 pg/μg protein). However, the Aged test group showed significantly decreased proBDNF levels (0.225 ± 0.029 pg/μg protein) and increased BDNF levels (0.159 ± 0.009 pg/μg protein) in the limbic systems of the brain compared to the Aged control group.

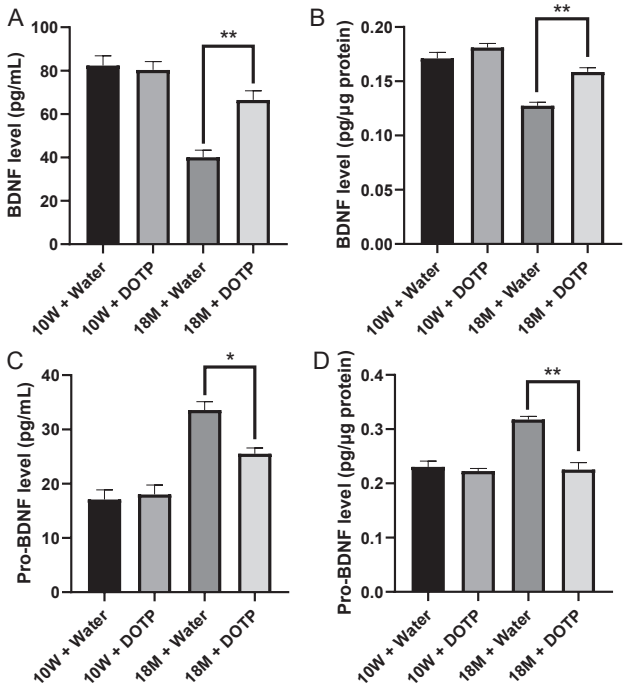

Fig. 8. Effect of Desert Olive Tree Pearls (DOTP) on levels of (A, B) brain-derived neurotrophic factor (BDNF) and (C, D) BDNF precursor (proBDNF) in the serum and limbic systems of young (10 weeks) and aged (18 months) mice. Mice were orally administered with 50 mg/kg DOTP for 30 days. The BDNF and proBDNF levels were determined by ELISA kit. Each value is presented as mean ± SD. **P < 0.01 compared to Aged control group by one-way ANOVA.

4. Discussion

Dementia triggers behavioral disorders and memory impairment and is generally caused by neurodegeneration and age-related neuronal cell death. Among the dementias, AD is the major neurodegenerative disease associated with aging. Aging is the most important risk factor for the onset of neurodegenerative diseases, and most neuro-degenerative disorders develop in the elderly population (Kritsilis et al., 2018). Thus, there is an urgent need to develop neuroprotective agents against Aβ-induced toxicity from natural products to prevent damage or death of neural cells, both neurons and glia. Several previous studies have reported that natural products, especially from plant sources, show biological activities, such as neuroprotective effects (Daulatzai, 2013; Pak et al., 2016). Natural resource-derived products have multiple bioactive properties and could be useful for the prevention of neurodegeneration and improvement of cognitive function (Sowndhararajan and Songmun, 2017). Moreover, olive oil products have been established as a dietary ingredient that promotes health. Evidence reports the beneficial properties of olive oil products in the prevention of cardiovascular disorders and improvement of cognitive function (Scarmeas et al., 2011). In the present study, we focused on Desert Olive Tree Pearls (DOTP), which is one of the olive oil products, that are rich in natural bioactive substances, such as hydroxytyrosol (233 mg/kg). It has been reported that hydroxytyrosol treatment showed neuroprotective effect due to the reduction of aggregates-membrane interaction on SH-SY5Y cells by stabilizing $A\beta_{42}$ oligomers and fibrils (Leri et al., 2019). We evaluated the effect of DOTP on learning and memory ability by MWM and $A\beta_{42}$ levels in aged mice. We also focused on major pro-inflammatory cytokines and neurotransmitters, which are affected by $A\beta_{42}$ toxicity. Our MWM test results showed that 30 days administration of DOTP improves spatial learning memory in aged mice. Moreover, the probe test results showed that spatial learning and memory ability were improved by DOTP administration. Therefore, our present result of MWM revealed that DOTP has the potential to prevent or improve the loss of learning and memory ability by aging.

As aging progresses, the production of Aβ, which leads to a large number of amyloid plaques, was increased. This overproduction can reduce the number and plasticity of synapses, and the resulting synapses can also be abnormal in shape and composition. It is well known that the most important Aβ peptides in AD are the proteolytic products of APP metabolism, $A\beta_{40}$ and $A\beta_{42}$. Particularly, $A\beta_{42}$ is more hydrophobic and viscous than $A\beta_{40}$, and because it has high amyloid-forming properties, it easily forms oligomers (Jeong, 2017; Takahashi et al., 2017). In the present study, treatment of DOTP showed the neuroprotection against $A\beta_{42}$-induce neurotoxicity in human neuroblastoma SH-SY5Y cells. Moreover, DOTP also induced the regulation of up-regulation of genes-related MAPK signaling, which activate $A\beta_{42}$-treatment. Therefore, we focused on the effect of DOTP on $A\beta_{42}$ levels in limbic systems of the mouse brain. Ahlemeyer and colleagues reported that Aβ was present at high levels in the 15-month-old mice and they also suggest the aged mouse as a model to study Aβ plaque formation (Ahlemeyer et al., 2018). Actually, our results showed that $A\beta_{42}$ level was significantly increased in the limbic systems of 18-month-old mice (aged mice) brain com-pared with 10-week-old mice (young mice). Therefore, we considered that because $A\beta_{42}$ is a common feature found in both our in vitro neuroprotection model and in vivo aged mice model, the results from in vitro and in vivo are correlated. In the present study, oral administration of DOTP induced a decrease in $A\beta_{42}$ levels in the limbic systems of mice brain compared with aged mice treated with water. Many natural food-derived compounds have been reported may be useful for AD therapeutics because its effect on Aβ. For example, food derived polyphenols such as rosmarinic acid, ferulic acid and curcumin showed the significantly inhibition of Aβ aggregation (Ono et al., 2004) and (-)-epicatechin, abundant in various fruits, showed the reduction of Aβ in a transgenic mouse model of AD (Cox et al., 2015). Thus, our results suggest that the decrease in the amount of $A\beta_{42}$ in the brain due to

administration of DOTP contributed to the improvement of spatial learning memory in aged mice. However, further mechanistic analysis of the reduction in $A\beta_{42}$ levels in the brain following DOTP administration is needed for future investigation.

Aged mice develop cognitive impairment due to the damage of oxidative stress and neuroinflammation due to an increase in inflammatory cytokines (Perkins et al., 2021), which are relatively similar to the physiological symptoms found in AD. Neuroinflammation and overactivation of microglia are closely interrelated, and the overactivation of microglia is widely accepted as a major hallmark of ROS generation in the central nervous system (Block et al., 2007; Sorce and Krause, 2009). Intercellular ROS generation leads to the release of inflammatory mediators via MAPKs and NF-κB signaling molecules in the microglia (Pan et al., 2008). Microglia-derived increase in free radical generation damages neuronal cells and is implicated in neurodegeneration (Parvathenani et al., 2003). It has also been reported that excessive production of inflammatory cytokines, which are downstream molecules of MAPK and NF-κB signaling, induces mitochondrial dysfunction and damage to dopaminergic neurons (Hunter et al., 2007). In the present study, we confirmed that aged mice treated with water had significantly increased TNF-α and IL-6 levels in the serum and limbic systems of mouse brain. However, DOTP administration decreases the levels of these pro-inflammatory cytokines in aged mice. Actually, DOTP treatment induced the decrease of the increase MAPK-related genes expressions on $A\beta_{42}$-treated SH-SY5Y cells in this study. And we also found that DOTP administration regulated the decreases in dopamine, serotonin, and GABA levels in aged mice. Moreover, oral administration of DOTP induced a decrease in glutamate levels in the limbic systems of mouse brain. In the brain, dopamine is synthesized by specific dopamine neurons and plays several important roles in the brain through four distinct pathways: mesolimbic, mesocortical, nigrostatial, and tuberoinfundibular pathways. These pathways are related to the regulation of mood and aid in cognitive and motor function. Impairment of this dopaminergic system potentially causes depression (Dailly et al., 2004), memory loss (Sawaguchi et al., 1988), and impaired motor control in patients with AD. Serotonin is involved in a wide range of physiological, emotional, and cognitive functions (Lucki, 1998). Serotonin dysfunction has been implicated in several psychiatric and neurological diseases across the life span (Lucki, 1998), including age-related diseases, such as AD (Blin et al., 1993). Glutamate is a key molecule in cellular metabolism, the most abundant excitatory neurotransmitter in the brain, and the principal neurotransmitter of cortical efferents. GABA is the main inhibitory neurotransmitter of the central nervous system, which allows them to communicate with both the immune (Lee et al., 2011) and nervous systems (Heja et al., 2012). Generally, defective functioning of brain glutamate and GABA is also considered responsible for AD pathology (Zhang et al., 2016). Thus, our results of the neuro-transmitter quantification indicated that oral administration of DOTP may have the potential to improve the impairment of neurotransmitters in aged mice. Taken together, our results suggest that DOTP may have anti-inflammatory effect associated with the regulating the overproduction of inflammatory cytokines during aging, thus protecting neurons through suppression of neuroinflammation.

BDNF, a member of the neurotrophin family of proteins, is an important component of brain function, including neuronal survival, learning and memory, and synaptic plasticity (Arancio & Chao, 2007). BDNF is involved in the pathophysiology of neuropsychiatric disorders, such as depression (Castrén & Kojima, 2017), anxiety (Wang et al., 2015), schizophrenia (Zhang et al., 2012), and various neurodegenerative disorders, such as Parkinson's disease (Wang et al., 2016) and AD (Hock et al., 2000). The BDNF gene product is composed of three different forms, including mature BDNF, BDNF precursor (proBDNF), and BDNF prodomain, and performs various biological functions (Hempstead, 2015). Mature BDNF is well known for its neurotrophic and neuroprotective functions, but proBDNF and pro-domains may have opposite functions to BDNF (Hempstead, 2014). Thus, the results of many studies suggest that an imbalance or insufficient proBDNF transformation into mature BDNF plays a key role in the pathogenesis of neurodegenerative disorders by impairing neuronal plasticity (Wang et al., 2021). In fact, we found that the BDNF levels decreased and proBDNF levels increased in both the serum and limbic systems of aged mice. Moreover, treatment with DOTP ameliorated this change in BDNF and proBDNF levels. Therefore, our findings suggest that administration of DOTP improves the imbalance between BDNF and proBDNF due to aging.

5. Conclusion

All these findings suggest that the Desert Olive Tree Pearls (DOTP), one of the olive oil products, probably has an effect on anti-neuroinflammation and neuroprotection associated with Aβ reduction and accumulation by aging and increases learning and memory and brain neurotransmitters. To best of our knowledge our present study may be the first report on the anti-neuroinflammatory effect of olive oil products (DOTP) associated with a decline of brain Aβ in aged mice. It could be used as a new therapeutic agent for the treatment and prevention of neurodegenerative diseases related to the aging process. And it has also been reported that the phenolic fraction of DOTP contains antioxidants and anti-inflammatory compounds, such as hydroxytyrosol, which are associated with the reduction of Aβ pathology (Leri et al., 2019; Nardiello et al., 2018). Thus, it is crucial to continue the study and discovery of the effects of DOTP and its active compounds on other aspects of neural health and pathology.

6. Ethics statement

Animal experiment was approved by the Animal Care and Use Committee of the University of Tsukuba (19–363).

CRediT authorship contribution statement

Kazunori Sasaki: Investigation, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Hiroko Isoda:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank Mr. Othmane Aqallal, director of the Company Atlas Olive Oils from Morocco for the preparation of the Desert Olive Tree Pearls product (DOTP) (OLIVIE PowerUp: https://www.olivie.ma/en/olivie-products/olivie-powerup/).

Funding information

This study was partially supported by the project of the Japan Science and Technology Agency (JST) Science and Technology Research Partnership for Sustainable Development (SATREPS).

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