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Paper

Walnut oil: a promising nutraceutical in reducing oxidative stress and improving cholinergic activity in an *in vitro* Alzheimer's disease model

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Improving the quality of life in elderly patients and finding new treatment options for neurological diseases such as Alzheimer's has become one of the priorities in the scientific world. In recent years, the beneficial effects and therapeutic properties of natural foods on neurological health have become a very remarkable issue. Walnut oil (WO) is a promising nutraceutical, with many phytochemicals and polyunsaturated fatty acids and is thought to be promising in the treatment of many neurological diseases and cognitive deficits, such as Alzheimer's disease (AD). Polyphenolic compounds found in WO enhance intraneuronal signaling and neurogenesis and improve the sequestration of insoluble toxic protein aggregates. The objective of this study was to investigate the potential protective and therapeutic effects of WO in a model of AD induced by retinoic acid (RA) and brain-derived neurotrophic factor (BDNF). In order to achieve this, the experimental groups were formed as follows: Control group, WO group, Alzheimer's disease (AD) group, AD + WO applied group (AD + WO). WO supplementation almost significantly reduced oxidative stress in the AD model, providing 2-fold protection against protein oxidation. Additionally, WO showed a significant reduction in tau protein levels (2-fold), increased acetylcholine (ACh) levels (12%), and decreased acetylcholine esterase (AChE) activity (~50%). Since it has been known for centuries that WO does show any adverse effects on human health and has neuroprotective properties, it may be used in the treatment of AD as an additional nutraceutical to drug treatments.

Key words: Walnut oil; Alzheimer's disease; AChE and ach; neuroprotection.

Introduction

Alzheimer's disease is an escalating global health crisis with profound implications for both individuals and society. As the population ages, the prevalence of AD continues to rise, imposing an immense burden on healthcare systems and caregivers worldwide.[1] AD is a neurological disease that impairs memory, thinking, and day-to-day functioning and is defined by a progressive cognitive decline. It is estimated that over 50 million people worldwide are affected by AD and this number is projected to be tripled by 2050 if effective interventions are not developed. The development of external amyloid-beta (Aβ) plaques and intracellular Tau hyperphosphorylation, both of which compromise the integrity and function of neurons, are two of the disease's defining characteristics.[2,3]

The development of neurodegenerative illnesses has been attributed to oxidative stress, which is defined by an imbalance between an excessive presence of reactive oxygen species (ROS) and a restricted capacity for scavenging within the body.[2] A plausible explanation might be that the brain exhibits a heightened susceptibility to oxidative damage, a condition commonly observed during the first phases of these disorders.[4] In addition, it should be noted that oxidative stress can potentially induce brain damage through increases in lipid peroxidation products and the facilitation of protein and DNA oxidation. These processes have been strongly associated with advancing neurodegenerative disorders.[5] Therefore, protecting the brain from oxidative damage might alleviate the effects of neurodegenerative disorders.

In age-related neurodegenerative disorders like AD, cholinergic degradation and the resulting decline in cognitive function progress rapidly. AD is characterized by well-documented dysfunctions in both the glutamate and cholinergic systems. Excitatory neurotransmitters glutamate (Glu) and acetylcholine (ACh) function via metabotropic receptors (mR) and ionotropic receptors (iR). The neurotransmitter systems of AD patients are found

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to impaired.[6] Cognitive deficits in AD, including memory loss, results from the gradual cholinergic and glutamate signaling deficiencies in the central nervous system. Cholinergic neuron reduction is closely linked to cognitive impairments, and the absence of a functional cholinergic system is a key factor in cognitive decline.[7] AD is characterized by cholinergic neuron degeneration, leading to both structural and functional impairments and accelerating the neurodegenerative process. The substantial cognitive and behavioral decline in AD is attributed to ACh deficiency. An increase in connection within the cerebellar default mode network in AD may potentially result from damage to the cholinergic system.[8]

In the quest for effective interventions against AD, the role of natural compounds, particularly those derived from plants, such as phytochemicals and polyphenols, has emerged as a promising avenue of research.[9] These compounds have garnered significant attention for their potential neuroprotective properties, which include modulating various cellular pathways associated with neurodegeneration, such as inflammation, oxidative stress and protein misfolding.[10,11] Natural products are said to have preventative qualities because of their anti-oxidative or anti-neuroinflammatory actions, which enhance cholinergic transmission and prevent the aggregation of Tau and Aβ peptides. By targeting multiple aspects of the disease, including Aβ plaque formation and Tau hyperphosphorylation, natural compounds offer a recommendable approach to neuroprotection.[12] This multifaceted approach suggests that natural compounds capable of addressing several pathogenic pathways may hold the potential to reduce, delay, or even prevent the occurrence and progression of AD.[13]

WO is a phytochemical-rich substance containing significant amounts of polyunsaturated fatty acids that promise to improve brain health. The polyphenols within walnuts have been proven to mitigate oxidant and inflammatory stress on brain cells, as well as improve interneuronal communication, enhance the generation of new neurons, and increase the elimination of insoluble toxic protein clusters.[14,15] Several recent studies have indicated that WO can greatly reduce cognitive deficits. While scientific evidence regarding the effectiveness of such a treatment for AD is limited, studies have suggested that the unsaturated fatty acids found in WO—specifically linoleic acid (Omega-6), linolenic acid (Omega-3), and oleic acid (Omega-9)—may contribute to improved cognitive function.[16] Nevertheless, the full extent of effects of WO on AD remains unclear and therapeutic benefits have only been investigated in a handful of studies.

Concerning all the available data, this study aimed to investigate the antioxidant and cholinergic effects of WO on the differentiated SH-SY5Y model of AD.

Materials and Methods

Chemicals, reagents, and kits

Brain-derived neurotrophic factor (BDNF) was obtained from Sigma-Aldrich (Mannheim, Germany). 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and cell lysis buffer were also purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium Ham's F12 with L-Glutamine and 25 mM Hepes (DMEM-F12), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Biowest (Riverside, MO). Malondialdehyde (MDA) assay kit, protein carbonyl assay kit, total protein assay kits and all-trans retinoic acid (RA) were purchased from Cayman Chemical (Ann Arbor, MI). The intracellular ROS assay kit was purchased from Sigma-Aldrich (Mannheim, Germany). The human AChE kit, the ACh, and the human Tau protein (MAPτ) ELISA kits were from Elabscience (Houston, TX). Walnut Oil Cold Pressed Chemical Free was purchased from Arifoglu (Istanbul, Turkey). The gas chromatography (GC) capillary column and Rt-2560 100 m, 0.25 mm ID, 0.20 um, was from Restek Corporation (Bellefonte, PA) while the Supelco® 37 Component FAME Mix was purchased from Sigma-Aldrich. The human neuroblastoma SH-SY5Y cell line (ATCC® CRL-2266™) was obtained from the American Tissue Culture Collection (Manassas, VA).

Analysis of walnut oil's fatty acid content

The gas chromatography- flame ionization detection (GC-FID, Agilent Technologies 7890 A GC System) with the G4513A Injector was used to determine the fatty acid content of WO. Initially, 0.1 g of WO was placed into a 20 mL test tube and 5 mL methylation reagent comprising methanol, hexane, and sulphuric acid (75:25:1, v/v/v). The test tube was then heated using a reflux condenser in a boiling water bath, for 30 min while maintaining the water bath temperature at $\geq$90 °C. Afterwards, the mixture was transferred into a separating funnel (50 mL) using 10 mL of distilled water and 10 mL of petroleum ether. The funnel was shaken thoroughly for ensuring the phases to separate. The aqueous phase was then withdrawn, and the ether layer was cleansed twice with 20 mL of distilled water. Subsequently, anhydrous sodium sulphate (Na_2SO_4) was added to the separating funnel, shaken and left to settle for a few minutes. After filtering the mixture, the filtrate was collected in a 15 mL test tube with a conical base. With the assistance of a nitrogen current, the solvent was evaporated over a water bath. The resulting mixture was analyzed for fatty acid content after adding 1 mL of CH_2Cl_2.

Alzheimer's disease model and cytomorphological assessment

The study utilized a modified cell differentiation methodology based on the work of de Medeiros et al.,[17] as outlined by Sanajou et al.,[2] and Demirel et al.[18] Cells were cultured in a standard growth medium for 24 h and were subsequently induced for differentiation by decreasing the FBS ratio to 1% and introducing RA (10 μM) on the first day after plating. Technical term abbreviations, such as FBS, will always be elaborated upon during their first usage for clarity. Cells were treated with RA and BDNF (2.5 ng/mL) starting on day 4. On the seventh day, the process of differentiation was finished, and cells and culture supernatants were collected.

The study consists of four groups:

1) **The control group** was treated with FBS + penicillin/streptomycin.
2) **The WO group** was exposed to WO (15.56 μg/mL) for 24 h.
3) **The AD group** which was treated with RA (10 μM) + BDNF (2.5 ng/mL).
4) **The AD-WO group** was treated with RA (10 μM) + BDNF (2.5 ng/mL) + WO (15.56 μg/mL).

Photographs of the four group cells within the flask were obtained using a camera connected to a phase contrast microscope (Leica, Wetzlar, Germany).

The viability and proliferation of SH-SY5Y cells

To assess the cytotoxic effects of WO, MTT assay was conducted. The cells were seeded in 96-well plates and incubated at 37 °C with 5% CO_2 for 24 h. Following the initial incubation, the cells were

treated with various concentrations of WO (15.56, 31.12, 62.5, 125, 250, 500, and 1,000 μM) for an additional 24 h.

After incubation, cell viability was determined by adding 100 μL of MTT solution (1 mg/mL) to each well. The plates were then incubated at 37 °C, and the formazan crystals formed were dissolved by adding 150 μL of DMSO to each well. The absorbance was measured at 570 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

The optical density (OD) values were normalized and expressed as the percentage difference from the control group. The experiments were repeated three times on different days, with two replicates performed on the same day. The mean of all the experiments was calculated.

Determination of intracellular ROS

To measure intracellular ROS production, a one-step fluorometric assay kit was used. SH-SY5Y cells were seeded in a black/clear bottom 96-well plate with a volume of 100 μL per well. After seeding, the cells were incubated at 37 °C with 5% CO_2 for 24 h.

At the end of the incubation period, the master reaction mix was added to each well and the plate was incubated at 37 °C in 5% CO_2 for 24 h. Fluorescence was measured using an excitation wavelength (λ_{ex}) of 520 nm and an emission wavelength (λ_{em}) of 605 nm.

The intracellular ROS production by the control cells was considered 100%, and the ROS production by the other cells was calculated as a percentage compared to the control.

Cell lysis

After incubation, the cells were washed with pre-cooled PBS and detached using trypsin. The cell suspensions were then collected and centrifuged at 1,000 × *g* for 5 min. The supernatants were removed, and the cells were resuspended in 800 μL of PBS. To completely lyse the cells, a freeze–thaw process was repeated several times. After cell lysis, the samples were centrifuged at 1,500 × g for 10 min at 2–8 °C, and the resulting supernatant was collected for further experiments. The collected supernatant was stored at −80 °C until analysis.

Measurement of MDA

Lipid peroxidation was measured in MDA Colorimetric Assay Kit Elabscience (E-BC-K025-M). Lipid peroxidation can result in the generation ofMDA. A reaction between thiobarbituric acid and MDA takes place under high temperatures and in an acidic environment to measure MDA levels. The formation of this complex can be measured colorimetrically at 530 nm. The results were reported as nmol/mg protein.

Measurement of protein oxidation

Protein oxidation was measured in Protein Carbonyl Colorimetric Assay Kit Elabscience (E-BC-K117-S). Protein oxidation, which peroxides or superoxide anions can induce, can result in the formation of protein carbonyls. A hydrazone product is created when the chemical 2,4-dinitrophenylhydrazine interacts with protein carbonyls. The amount of this product formed was measured at 370 nm. The results were given as nmol/mg protein.

Measurement of AchE, ACh and Tau levels

Tau protein levels, ACh levels, and AchE activities in the study groups were measured in an ELİSA reader using commercial Sandwich-ELISA kits. The micro-ELISA plate in these kits is precoated with an antibody specific to the measured parameter. After incubation, the cells were washed with precooled PBS and trypsinised. The cell suspensions were then collected and centrifuged at 1,000 × g for 5 min. After removal of the supernatants, 800 mL of PBS was used to resuspend the cells. Several freeze–thaw cycles were performed to completely lyse the cells. After lysis, the cells were centrifuged at 1,500 × g for 10 min at 2–8 °C and the supernatant was collected for experiments and stored at −80 °C until analysis.

Table 1. The composition of fatty acids in walnut oil.

Fatty Acids	Percentage (%)
C16:0 (Palmitate)	6.89 ± 0.47
C18:0 (Stearate)	3.15 ± 0.20
C18:1 (C9) (Oleate)	29.14 ± 3.29
C18:2 (C9, C12) (Linoleate)*	55.73 ± 2.78
C18:3 (C6, C9, C12) (Liolenate)	1.41 ± 0.20
C20:2 (C11, C14) (Eicosadienoate)	3.00 ± 0.24
C20:3 (C8, C11, C14) (Eicosatrienoate)	0.64 ± 0.23

Statistical evaluation

Version 17.0 of the Statistical Package for Social Sciences (SPSS) software, created by SPSS Inc. (Chicago, IL), was used to conduct the statistical analysis. To evaluate the differences between the groups, a one-way Kruskal-Wallis analysis of variance was performed. For post-hoc analysis, the Mann–Whitney U test was utilized. The mean ± standard deviation (SD) is used to present the results. P-values were regarded as statistically significant if they were less than 0.05.

Results

Fatty acid content of walnut oil

Table 1 provides a comprehensive breakdown of the fatty acid contents of WO. The percentages (%) of fatty acids present in WO were 6.89 ± 0.47% for C16:0 (palmitate); 3.15 ± 0.20% for C18:0 (stearate); 29.14 ± 3.29% for C18:1 (oleate), 55.73 ± 2.78% for C18:2 (linoleate); 1.41 ± 0.20% for C18:3 (liolenate); 3.00 ± 0.24% for C20:2 (eicosadienoate); and 0.64 ± 0.23% for C20:3 (eicosatrienoate).

Cytomorphology of the cell groups

A varied group of cells was observed in the control group, comprising both epithelial-like and neuronal-like cells with short neurites. The AD group showed a decrease in the number of cells and epithelial-like cells compared with the control and WO treatment groups. The cells in the AD group had a slender, spindle-like shape with longer neurites. Furthermore, vacuoles were scattered within the cytoplasm of cells in the AD group (Fig. 1).

The viability and proliferation of SH-SY5Y cells

The maximum non-cytotoxic dose of WO was 15.56 μg/mL and was used in all in vitro experiments, see Fig. 2.

Intracellular reactive oxygen species levels

Figure 3 shows the intracellular ROS levels for each research group. Compared to the control group, a significant increase in ROS levels was observed for all groups. The AD group exhibited a higher increase in intracellular ROS levels (155%) than the WO group (87%) ($P < 0.05$), as shown in Fig. 2.

MDA levels

The AD group displayed significantly greater lipid peroxidation levels than both the control group (a 2-fold increase) and the WO group (a 3-fold increase) (Fig. 4).

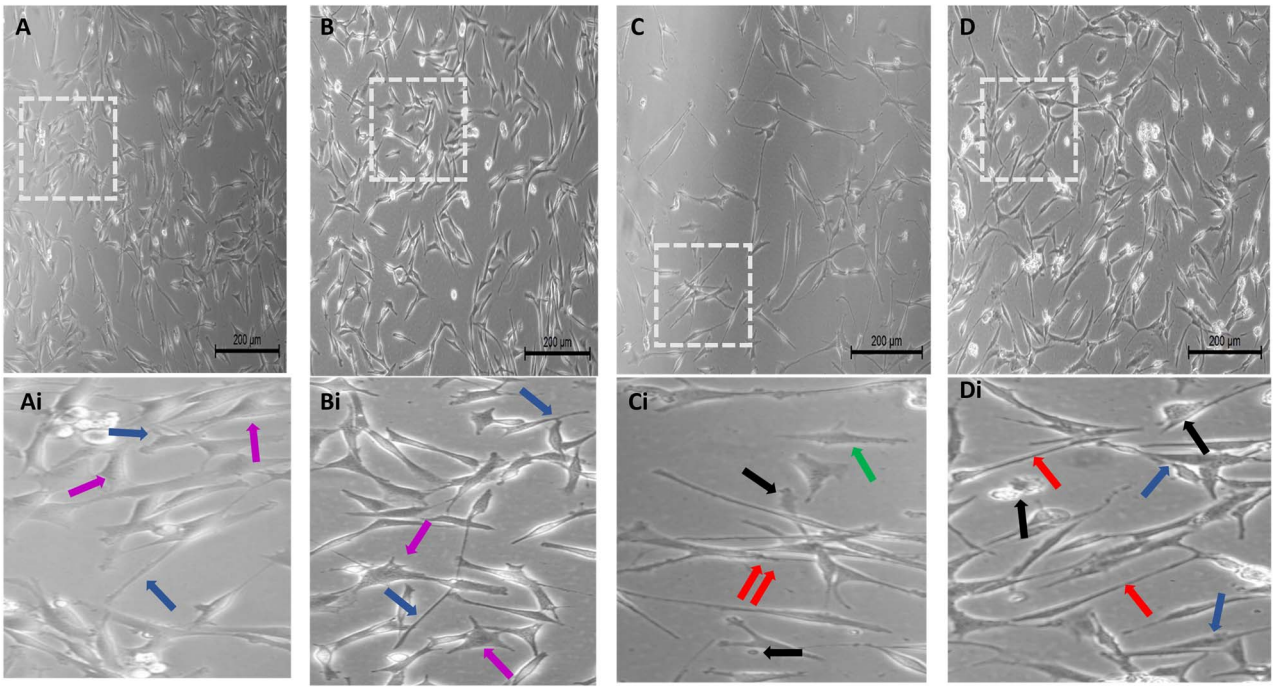

Fig. 1. Cellular morphology in study groups. A) Control group. B) WO group. C) AD group. D) AD + WO group. The epithelial-like cells (arrow) and neuronal-like cells (arrow) with short neurites in the control group and walnut oil group. The pyramidal shaped cells with longer neurites, vacuoles (arrow) in the cytoplasm of the cells and varicosities (arrow) along the thin long neurites in AD group. Many degenerated cells (arrow) are also observed in these groups. In the AD + WO group, fewer degenerative neurons (arrow) and pyramidal shaped cells with longer neurites, vacuoles (red arrow) were observed than the AD group and neuron-like cells were also seen (arrow).

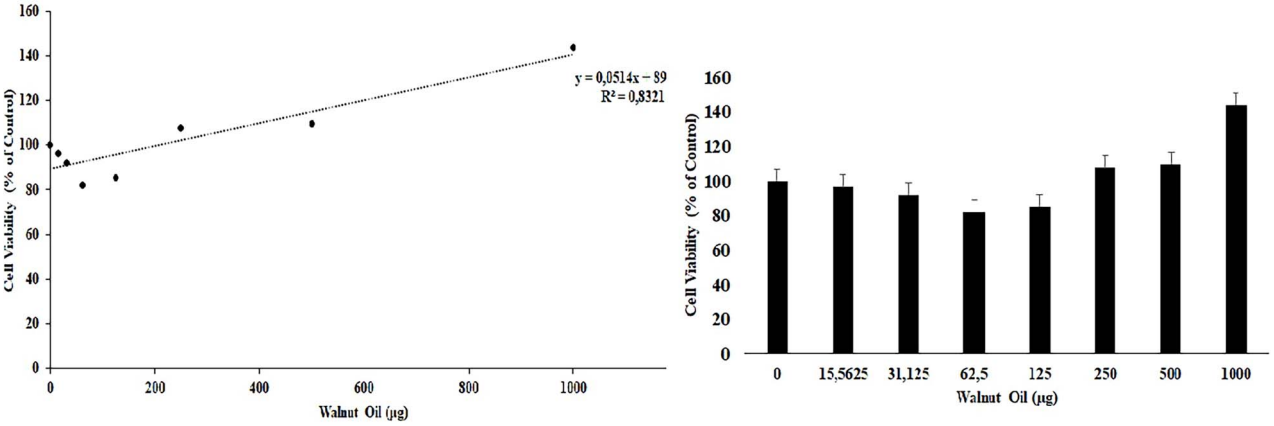

Fig. 2. Cell viability at different concentrations of walnut oil. *$P < 0.001$ represents a significant difference between control and individual treatment. Values are given as mean ± SD. *$P < 0.05$.

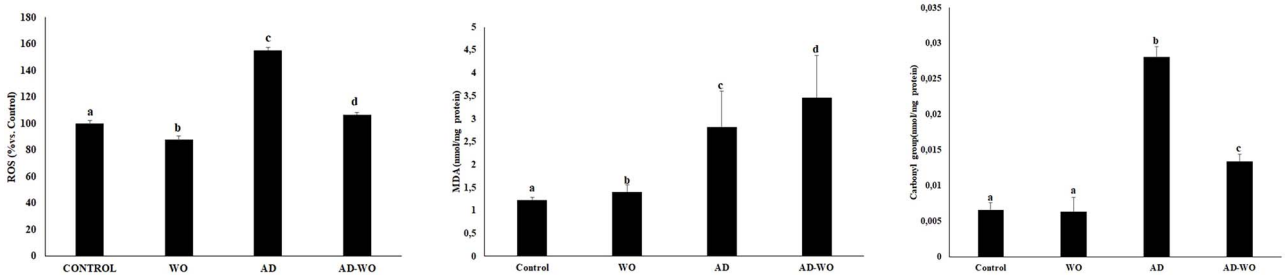

Fig. 3. A) ROS levels; B) MDA levels; C) Protein oxidation levels. a, b, c and d Bars that do not share the same letter (superscripts) are significantly different than each other ($P < 0.05$).

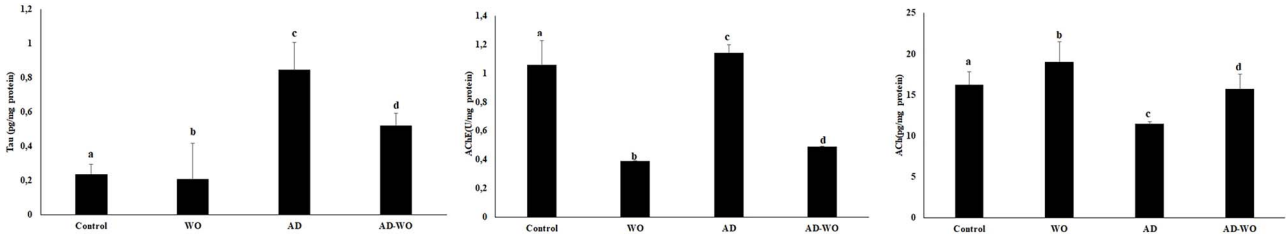

Fig. 4. A) Tau protein levels; B) AChE activity; C) ACh levels. a, b, c, and d Bars that do not share the same letter (superscripts) are significantly different than each other ($P < 0.05$).

Protein carbonyl levels

The AD group had considerably greater amounts of protein carbonyl in comparison to the WO and AD-WO groups. Treatment with WO resulted in a 2-fold decrease in protein carbonyl levels compared to AD ($P < 0.05$) (Fig. 3).

AchE, ACh and Tau levels

A statistically significant increase in Tau protein levels (2-fold) was observed in the AD-WO group and 4-fold was determined in the AD group ($P < 0.05$). In contrast, ACh levels decreased by 32% in the AD group statistically significant ($P < 0.05$). Meanwhile, in WO group, Tau protein levels decreased by 13% ($P > 0.05$). Treatment with WO in the AD model reduced AChE levels by ~50%, while the AChE levels in the AD model increased by 10% vs. control ($P < 0.05$) (Fig. 4).

Discussion

Many of the phytochemicals found in walnuts may be beneficial to human health. Among nut species, walnuts have the highest concentration of phenolic antioxidants, making them one of the most important antioxidant-rich foods.[19]

The results indicate the positive effects of WO treatment on the AD model. The WO treatment caused significant alterations in cell morphology in the AD group. In particular, the AD group had a lower count of epithelial-like cells compared to the control and WO treatment groups. The cells in the AD group displayed slender, spindle-like shapes with elongated neurites. Additionally, their cytoplasm contained a wide distribution of vesicles. The results of this study suggest that WO treatment may positively impact cell morphology in the AD model, the mechanism of which should be investigated with further studies.

AD development is significantly influenced by oxidative stress. The intracellular ROS levels increased in the AD model. Furthermore, compared to the control and WO groups, there was a discernible increase in MDA levels in the AD group. Consequently, these results imply that WO may be able to lessen oxidative stress, which is a major factor in the development of AD. We found that the AD model's intracellular ROS levels were elevated during the investigation. Furthermore, the AD group showed a substantial rise in MDA levels relative to the control and WO groups, suggesting higher levels of lipid peroxidation. According to these findings, WO may be able to lessen oxidative stress since it contains phytochemicals that allow for the lowering of ROS levels through a variety of ways.

Numerous studies have demonstrated the possible advantageous effects of WO for the prevention or protection against AD. These studies mainly focused on the influence of WO's components on AD and disclosed their capacity to combat the disease's pathophysiology by reducing Aβ accumulation and decreasing oxidative stress[20] as well as their ameliorating effects on cognitive functions.[21,22] Our findings are consistent with the results of these comparative studies, suggesting that WO has beneficial effects on AD by addressing key aspects of its pathophysiology. These specifically include the reduction of oxidative stress, regulation of β Aβ accumulation, and improvement in neurotransmitter levels. These combined findings indicate that WO holds great potential as a natural oil for treating AD.

There is strong evidence that both Aβ accumulation and intracellular neurofibrillary tangle (NFT) formation in AD causes an increase in ROS generation, which leads to a vicious cycle where ROS production significantly exceeds the capacity of antioxidant defense system.[23] Direct suppression of ROS, inhibition of ROS-producing enzymes, chelating transition metal ions, hydrogen atom transfer and vitamin E regeneration can impede these reactions.[24] In an in vitro study performed on PC12 cells, walnut extract inhibited membrane damage, attenuated DNA damage, and reduced ROS formation and oxidative stress, which all served to protect against Aβ-induced cell death.[24] Our study have also shown that WO protects the cells from intracellular ROS production which in turn can result in reduced Tau protein levels.

Due to their high instability, ROS can promptly interact with lipids, proteins, and nucleic acids. While lipids are highly susceptible to free radical damage, they play a vital role in initiating and sustaining a chain of events that result in a range of secondary lipid oxidation products such as endoperoxides, hydroperoxides, and oxysterols. These substances break down to produce a variety of reactive carbonyl intermediates, which lead to severe cellular toxicity. The intermediate substances such as α,β-unsaturated aldehydes (4-HNE, acrolein), di-aldehydes (MDA, glyoxal), and keto-aldehydes (4-oxo-trans-2-nonenal, isoketals) can be formed. Herein, lipid peroxidation was significantly increased in the AD group. Our findings support prior research evidencing that the brains of AD patients exhibit higher lipid peroxidation levels compared to those of age-matched controls.[12]

Proteins can be independently modified by ROS and lipid peroxidation by products such as MDA and HNE to produce carbonyl derivatives. Carbonyl groups can develop on proteins through the oxidation of certain amino acid residues (arginine, lysine, proline, and threonine), glutamyl residues, or through the breaking of peptide bonds by means of the α-amidation pathway.[25] Abnormal protein cross-linking may derive from the oxidative conversion of protein side chains to reactive carbonyl or acylating moieties.[26] The production of insoluble fibrils results from protein cross-linking due to oxidative changes. Pathologic fibril formation from protein aggregation is seen in AD. The pathogenesis of AD has been linked to protein oxidation, wherein the hyperphosphorylation of Tau protein results in the assembly of paired helical filaments and the accumulation of proteins. To quantify the extent of protein oxidative damage, measuring protein carbonyl concentration is a reliable biomarker. In the present work, we found increased protein carbonyl content in the AD group and this

may indicate higher protein oxidation within this study group. Our study supports previous research showing that protein oxidation increases with age in rat models[27] and in the brains of AD patients compared to age-matched controls.[21]

Cholinergic pathway has an essential role on the onset of memory impairment.[28] In addition, animal memory impairment is linked to cholinergic function alteration.[29] Ach is produced at the end of synapses from various cortical and subcortical sites and this neurotransmitter is crucial for cognitive processes like memory and learning.[30] At the synaptic ends of cholinergic neurons, choline acetyl transferase (ChAT) normally mediates the transfer of the acetyl group from acetyl CoA to choline, thereby has a crucial role in the synthesis of ACh.[31] In this study, treatment with WO considerably increased AChE protein levels while also restoring the cholinergic system in AD model. Low levels of Ach were observed in the AD group compared to the control group. However, treatment with WO markedly increased Ach levels. Additionally, WO treatment decreased AChE levels in the AD model, indicating that WO may act as a cholinesterase inhibitor and improve cognitive function by regulating neurotransmitter levels[32] Rats that received oral walnut extract had significantly enhanced memory function and increased ACh levels.[33]

The co-occurrence of elevated Aβ levels and Tau hyperphosphorylation can be observed in AD. Our findings reveal a substantial increase in Tau protein levels in the in vitro model compared to control cells. AChE levels was found to be an indicator of neuroinflammation, which also leads to increased oxidative stress and Tau hyperphosphorylation.[34] Our research clearly demonstrates the impact of WO on the inhibition of AChE.

Conclusion

In conclusion, increasing amount of research highlights the exceptional ability of walnuts to improve many characteristic features of AD. According to this study as well as several other studies, walnuts can successfully lower oxidative stress and ROS levels, which are major factors in the onset of AD. WO can markedly lower Tau protein levels and increase cholinergic activity by reducing oxidative stress, which are both critical factors for the maintenance of cognitive function. Given the encouraging findings of these studies, adding walnuts to an AD patient's diet should be considered an additional therapeutic approach. Nevertheless, it is imperative to recognize that even while the results are promising, further clinical research is required to determine the ideal dosages, long-term effects, and drug interactions. A deeper comprehension of the function walnuts in managing AD should be investigated with both in vitro and in vivo models. Walnuts represent a natural and easily obtainable nutritional choice in the fight against AD, providing hope for enhancing the quality of life for people living with AD. The potential for walnuts to be a vital part of AD care can become more intriguing as research in this area progresses.

Conflict of interest statement

All authors declare that they have no conflicts of interest.

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Author contributions

G.D. conceived of the study and performed methodology, investigation, writing-original draft. S.S. Performed writing-original draft, investigation and validation. A.Y.: performed formal analysis and methodology. D.A.Ç. performed validation and investigation. A.B. performed the investigation. A.B.Ö. performed data analysis T.B. performed supervision and visualization. P.E. Performed project administration, conceptualization, visualization, writing-reviewing and editing.

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