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Investigation of neuroprotective and antiapoptotic effects of rosmarinic acid in an *in vitro* Alzheimer's disease model

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ABSTRACT

Objective(s): Rosmarinic acid (RA) exhibits anti-oxidant, anti-aging, and anti-inflammatory properties. This study aimed to investigate the effects of RA on amyloid beta (Aß), phosphorylated tau (p-tau), α-synuclein (α-syn), Abelson tyrosine kinase (Abl), and apoptotic markers in an *in vitro* Alzheimer's disease (AD) model.

Materials and Methods: SH-SY5Y cells were differentiated into cholinergic neurons by all-trans retinoic acid (ATRA). CCK-8 assay was performed to determine the concentrations of Aβ, RA, and Aβ+RA on differentiated cells (D-cholinergic neurons). Then, control (D), D+Aβ, pretreatment (D+Aβ+RA), and D+RA groups were formed. Gene expression levels of apoptotic biomarkers were evaluated using qRT-PCR. Aβ, α-syn, Abl, p-tau, caspase-7 (CASP7), BAX, and cytochrome c (CYCS) protein levels were determined by ELISA.

Results: CCK-8 assay showed that RA (0.5–5 μM) was non-toxic to differentiated cells, and the IC_{40} of $A\beta_{1-42}$ was 20 μM. Pretreatment with 1.5 μM RA protected cells from Aβ-induced toxicity. Gene expression analysis revealed that apoptotic markers (BAX, BLC2, CYCS, CASP3, CASP7, FAS, FADD) were significantly increased in the D+Aβ group compared to the control, whereas their levels were markedly reduced in the D+Aβ+RA group relative to the D+Aβ group. ELISA results corroborated qRT-PCR findings for CASP7, BAX, and CYCS. Additionally, RA decreased Aβ, α-syn, and p-tau protein levels, while Abl levels increased only in the D+RA group.

Conclusion: RA exhibits neuroprotective and antiapoptotic effects by modulating apoptotic markers and reducing pathological proteins in an *in vitro* AD model.

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Introduction

The global population is progressively aging, and the average life expectancy continues to increase, leading to a rising prevalence of neurodegenerative diseases in the geriatric population (1). Among these, Alzheimer's disease (AD) stands out as the most common neurodegenerative disease, affecting millions of individuals worldwide. Beyond the neurobiological damage, AD also brings about substantial social and economic burdens, making it a priority in current scientific research (1). AD is characterized by extensive intracerebral Aβ protein aggregation, elevated α-synuclein (α-syn) levels, and neurofibrillary tangles resulting from tau hyperphosphorylation (2, 3). Additionally, cholinergic neuronal damage has been widely documented, and this disruption is associated with the cognitive impairments observed in AD patients (4). Consequently, acetylcholinesterase (AChE) inhibitors are frequently employed in the symptomatic treatment of AD to enhance cholinergic activity (4, 5).

Increased neurotoxicity and apoptosis are also frequently observed among the pathological characteristics of the disease (1).

Tau is a microtubule-associated protein that plays a crucial role in stabilizing axonal microtubule bundles, which serve as essential structural components of the axonal cytoskeleton (6). Tau is highly expressed in many areas of the central nervous system, particularly in distal axons. Tau has multiple phosphorylation sites, and changes in its phosphorylation levels regulate microtubule stability, localization, and functional roles in neurons (7). Hyperphosphorylation causes the dissociation of tau protein from microtubules. This dissociation plays a role in the development of neurodegenerative diseases by destabilizing axonal morphology and transport function (8).

α-syn plays a role in the physiopathology of Parkinson›s disease (PD), dementia with Lewy bodies, and multiple system atrophy (9). High levels of α-syn have been detected in the cerebrospinal fluid (CSF) of individuals with mild cognitive impairment. Recent studies have demonstrated that asymptomatic accumulation of amyloid plaques is associated with elevated CSF α-syn levels in both individuals at risk for sporadic AD and carriers of autosomal-dominant AD mutations (9).

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Apoptosis, which also plays a critical role in maintaining homeostasis, is regulated by a balance between pro-apoptotic and anti-apoptotic mechanisms that act in concert to ensure cellular integrity (10, 11). Dysregulation or aberrant activation of apoptotic pathways can lead to pathological changes, as observed in AD, where extensive neuronal loss is largely associated with apoptosis (11).

The effect of neuronal loss by apoptosis is greater than that of neurofibrillary tangles from Aβ aggregation and tau hyperphosphorylation in AD (11). The apoptosis process in AD is characterized by a loss of function of anti-apoptotic molecules (BCL-XL) and increased activity of pro-apoptotic molecules (BAX/Caspase-3) (12). Simultaneous increased expression of proapoptotic proteins, including caspase-3 (CASP3), is associated with neuronal loss. Caspase 8 and 9 are responsible for mitochondrial dysfunction in AD. BCL2 has an antiapoptotic role and suppresses the activation of CASP3 and other proapoptotic factors (11).

In the current treatment landscape for AD, poly-therapeutic approaches incorporating natural anti-oxidants have demonstrated superiority over monotherapies (13). In this context, rosmarinic acid (RA), a naturally occurring polyphenolic compound found in various plant species, including members of the *Boraginaceae* and *Lamiaceae* families, has garnered significant attention (14). RA has a wide range of notable biological activities, including anticancer, anti-oxidant, cardioprotective, hepatoprotective, antiviral, antibacterial, antidiabetic, nephroprotective, anti-aging, antiallergic, anti-inflammatory, and antidepressant effects (14–16). Recently, the neuroprotective and anti-apoptotic potential of RA has also been the target of research.

Abl, a non-receptor tyrosine kinase, is activated by oxidative stress and phosphorylates α-syn, thereby promoting its aggregation and neuronal degeneration. Based on this information, Abl has recently been identified as a target in AD (17). Increased Abl expression was observed in postmortem brain tissues of experimental AD and PD models (18–20). A study assessing the effects of RA in a PD model using the SH-SY5Y cell line demonstrated that RA exerts neuroprotective effects primarily by suppressing Abl. Furthermore, the results demonstrated that RA significantly preserved cell viability, indicating its protective and antiapoptotic potential against neurotoxicity (21).

Research on the effects of RA in AD is limited. Moreover, the effects of RA on Aβ in AD remain unknown. Thus, this study aimed to investigate the effects of RA on Aβ aggregation, p-tau levels, α-syn and Abl expression, as well as apoptosis in an *in vitro* AD model.

Materials and Methods

Ethics approval

Approval was obtained from the X Non-Pharmaceutical and Non-Medical Device Research Ethics Committee (Date 21.07.2023 and # 2023/4462).

Cell culture

The human neuroblastoma cell line SH-SY5Y (CRL-2266™) was purchased from ATCC (American Type Culture Collection). SH-SY5Y cells were incubated in DMEM F-12 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C, 95% humidity, and 5% CO_2. RA solution (Sigma-Aldrich; Cat# 20283-92-5) was prepared in DMSO according to the manufacturer's instructions.

Differentiation of the SH-SY5Y cell line

For differentiation of SH-SY5Y cells, cells were fed with DMEM F-12 medium containing 1% FBS supplemented with 10 μM ATRA (Targetmol; Cat# T1051) for 4 days (22). On the 5th day, the cell culture medium was replaced with fresh medium, and the cells were cultured for 6 days, and then the formation of D-cholinergic neurons was observed.

CCK-8 assay

Differentiated SH-SY5Y cells were seeded into 96-well plates. After overnight culture, RA was applied at concentrations of 0.5, 1.5, 5-25, 50, 75, and 100 μM for 24 hr. To evaluate the cytotoxic effect of $A\beta_{1-42}$ (Adooq Bioscience; Cat# A14075), differentiated SH-SY5Y cells were seeded in 96-well plates and treated with Aβ at concentrations of 5, 10, 20, 40, 80, and 100 μM for 24 hr. The concentration of Aβ corresponding to approximately 60% viability was determined using the CCK-8 assay (Abbkine; Cat# BMU106-EN).

Differentiated SH-SY5Y cells were seeded at a density of 10^3 per well in a 96-well plate and cultured overnight to investigate the neuroprotective potential of RA. Cells were then treated with RA at various concentrations (0.5, 1, and 5 μM) for 24 hr before Aβ treatment (20 μM). After 24 hr, the medium was aspirated, and 20 μM $A\beta_{1-42}$ was added to the cells. The plate was read on an ELISA reader at 450 nm after 2-hr of incubation. CompuSyn Version 1.0 software was used for concentration calculations.

Experimental groups

A total of four experimental groups were established, including differentiated SH-SY5Y cells (D) as the control, differentiated cells treated with 20 μM $A\beta_{1-42}$ (D+AB), differentiated cells treated with 1.5 μM RA for 24 hr followed by $A\beta_{1-42}$ for 24 hr (D+RA+AB), and differentiated cells treated with 1.5 μM RA for 24 hr (D+RA).

RNA Isolation, cDNA synthesis, and qRT-PCR analysis

Total RNA isolation was performed using TRIzol reagent (QIAzol; Cat# 79306). cDNA synthesis was performed using a first-strand cDNA synthesis kit (Bio-Rad; Cat#1708891) according to the manufacturer's instructions. qRT-PCR analysis was used to determine changes in apoptosis-related gene expression (BAX, BCL2, CASP 3, 7, 8, 9, CYCS, FADD, FAS, and P53). Primer sequences for the target and reference genes used in qRT-PCR (Table 1) were obtained from the literature (23). The qRT-PCR profile and protocol were described elsewhere. (23). ACTB was used for internal control and normalization of qRT-PCR data.

ELISA

Protein level expressions of p-tau (BT Lab; Cat# E5874Hu), Aβ (Finetest; Cat# EH4494), α-syn (BT Lab; Cat# E1313Hu), Abl (BT Lab; Cat# E6357Hu), caspase 7 (CASP7), (BT Lab; Cat# E2257Hu), BAX (BT Lab; Cat# E4977Hu) and CYCS (BT Lab; Cat#E7110Hu) were determined using ELISA kits according to the manufacturer's instructions. Briefly, after adding 10 μl of biotinylated antibody and 50 μl of streptavidin-HRP to 40 μl of the sample, the plate was incubated at 37 °C for 1 hr. After 5 repeated washes, 50 μl of Substrate A and 50 μl of Substrate B solutions were added. After incubation at 37 °C for 10 min, 50 μl of stop solution was added, and absorbance was measured in the ELISA

Table 1. Primer sequences of studied apoptosis and reference genes in qRT-PCR analysis

Gene	Forward primer sequence	Reverse primer sequence	PCR product size (bp)
BAX	GGAGCTGCAGAGGATGATTG	GGCCTTGAGCACCAGTTT	151
BCL2	GTGGATGACTGAGTACCTGAAC	GAGACAGCCAGGAGAAATCAA	125
CASP3	GAGCCATGGTGAAGAAGGAATA	TCAATGCCACAGTCCAGTTC	162
CASP7	CGAAACGGAACAGACAAAGATG	TTAAGAGGATGCAGGCGAAG	169
CASP8	GCCCAAACTTCACAGCATTAG	GTGGTCCATGAGTTGGTAGATT	160
CASP9	CGACCTGACTGCCAAGAAA	CATCCATCTGTGCCGTAGAC	153
CYCS	GGAGAGGATACACTGATGGAGTA	GTCTGCCCTTTCTTCCTTCTT	102
FADD	TGACCGAGCTCAAGTTCCTATG	CCAGGTCGTTCTGCTCCAG	108
FAS	GTGATGAAGGACATGGCTTAGA	GTGTGCATTCCTTGATGATTCC	156
P53	GAGATGTTCCGAGAGCTGAATG	TTTATGGCGGGAGGTAGACT	129
ACTB	AGCACGGCATCGTCACCAACT	TGGCTGGGGTGTTGAAGGTCT	179

CASP7: Caspase-7

plate reader at 450 nm.

Statistical analysis

All experimental procedures were repeated three times. qRT-PCR data were analyzed according to the $2^{-\Delta Ct}$ method. Group comparisons were conducted using SPSS version 26.0. Results are given as the mean value ± standard deviation (Mean ± SD). Differences between the groups were compared using a *post hoc* Tukey test. $P<0.05$ was considered statistically significant in all experiments.

Results

CCK-8 cell viability assay

According to the results of the CCK-8 assay, RA at concentrations of 0.5, 1.5, and 5 µM did not show cytotox[illegible] effects on differentiated cells (Figure 1). The IC_{40} value of Aβ was determined to be 20 µM (Figure 2). Pretreatment of the cells with 1.5 µM of RA followed by exposure to 20 µM Aβ demonstrated the neuroprotective effect of RA (Figure 3).

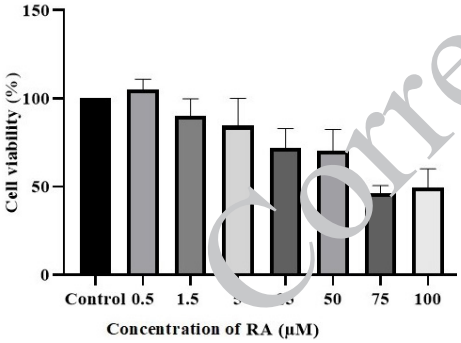

Figure 1. Effect of RA on cell viability in differentiated SH-SY5Y cell line (24 hr)
RA: Rosmarinic acid

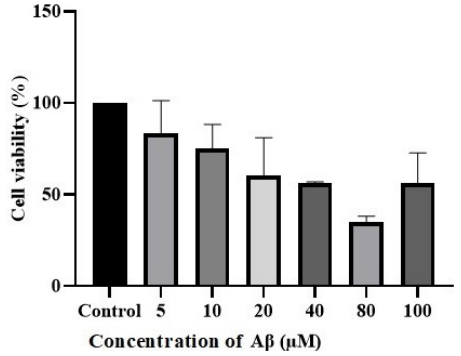

Figure 2. Effect of Aβ on cell viability in differentiated SH-SY5Y cell line (24 hr)
Aβ: Amyloid beta

qRT-PCR analysis

A significant increase in BAX, BCL2, CASP3, CASP7, CASP8, CYCS, FADD, and FAS gene expressions was observed in the D+AB group compared to the D group (control) ($P\leq0.001$, $P\leq0.05$, $P\leq0.001$, $P\leq0.001$, $P\leq0.05$, $P\leq0.001$, $P\leq0.001$, and $P\leq0.001$, respectively) (Figure 4). On the other hand, BAX, BCL2, CASP3, CASP7, CYCS, FADD, and FAS gene expression levels were upregulated in the D+AB+RA group compared to the D+AB group ($P\leq0.001$, $P\leq0.01$, $P\leq0.001$, $P\leq0.001$, $P\leq0.05$, $P\leq0.001$, and $P\leq0.001$, respectively) (Figure 4).

ELISA

p-tau protein level increased from 1268.93±49.65 U/ml in the D group to 1514.93 ±62.93 U/ml in the D+AB group and decreased to 1325.6±62.93 U/ml in the D+AB+RA group ($P\leq0.01$, $P\leq0.05$, respectively) (Figure 5). α-syn protein level increased from 313.47±15.46 pg/ml in D group to 416.8±8.44 pg/ml in D+AB group and decreased to 350.33±12.11 pg/ml in D+AB+RA group ($P\leq0.01$, $P\leq0.01$, respectively) (Figure 5). Aβ protein level increased from 167.333±9.46 pg/ml in the D group to 3166.833±55.75 pg/

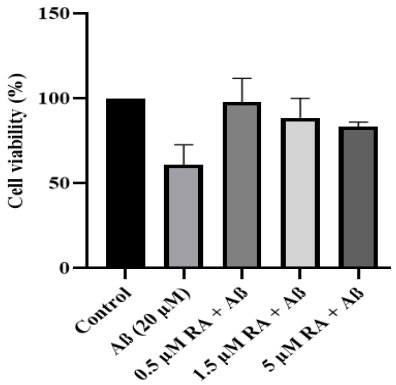

Figure 3. CCK-8 Cell viability assay results
The neuroprotective effect of RA. Pretreatment of cells with 1.5 µM RA, followed by exposure to 20 µM Aβ, demonstrated RA's neuroprotective effect.
RA: Rosmarinic acid; Aβ: Amyloid beta

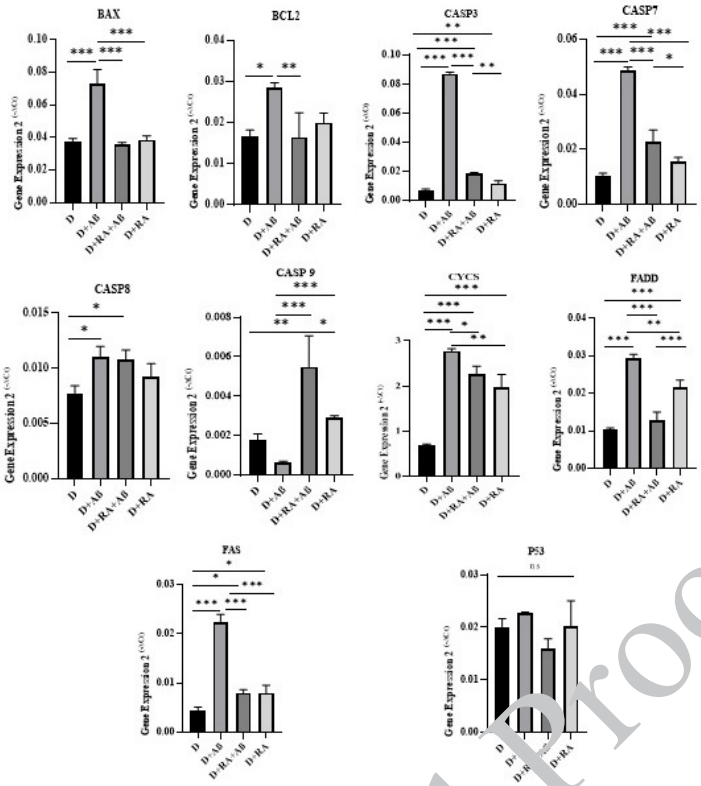

Figure 4. Effect of RA on expression levels of apoptosis-related genes in SH-SY5Y cell lines
*P≤0.05; **P≤0.01; ***P≤0.001. Apoptotic markers BAX, BLC2, CYCS, CASP3, CASP7, FAS, and FADD were significantly increased in the D+Aβ group compared to the control (D) group (P≤0.001, P≤0.05, P≤0.001, P≤0.001, P≤0.05, P≤0.001, P≤0.001, and P≤0.001, respectively). BAX, BLC2, CYCS, CASP3, CASP7, FAS, and FADD levels were markedly reduced in the D+Aβ+RA group relative to the D+Aβ group (P≤0.001, P≤0.01, P≤0.001, P≤0.001, P≤0.05, P≤0.001, P≤0.001, respectively).
RA: Rosmarinic acid; Aβ: Amyloid beta

ml in the D+AB group and decreased to 2079.33±176.72 pg/ml in the D+AB+RA group (P≤0.001, P≤0.001, respectively) (Figure 5). The Abl protein level increased from 42.23±59.14 ng/L in the D group to 442.09±38.23 ng/L in the D+AB group and to 933.04±123.13 ng/l in the D+AB+RA group (P≤0.001, P≤0.001, respectively) (Figure 5).

CASP7 protein level increased from 2000.5±210.77 ng/l in the D group to 3898.83±279.74 ng/l in the D+AB group and decreased to 1620.5±296.52 ng/l in the D+AB+RA group (P≤0.001, P≤0.05, respectively) (Figure 6). BAX protein level increased from 27.73±2.69 ng/l in the D group to 48.75±2.10 ng/l in the D+AB group and decreased to 27.53± 1.52 ng/l in the D+AB+RA group (P≤0.001, P≤0.01, respectively) (Figure 6). CYCS protein level increased from 106.43±15.63 ng/ml in the D group to 178.83± 17.46 ng/ml in the D+AB group and decreased to 69.87±7.97 ng/ml in the D+AB+RA group (P≤0.001, P≤0.001, respectively) (Figure 6).

Discussion

The pathophysiology of AD involves the accumulation of extracellular Aβ plaques and disruption of neuronal microtubules due to tau protein hyperphosphorylation (24). Common pathological characteristics of AD include oxidative stress, lipid peroxidation, inflammation, impaired cellular function, and increased levels of apoptosis (25). Especially pronounced degenerative transformations are formed in cholinergic neurons. This damage is often associated with cognitive decline, which is observed in AD clinics. Therefore, acetylcholinesterase inhibitors are also used to treat AD (24). Thus, we differentiated

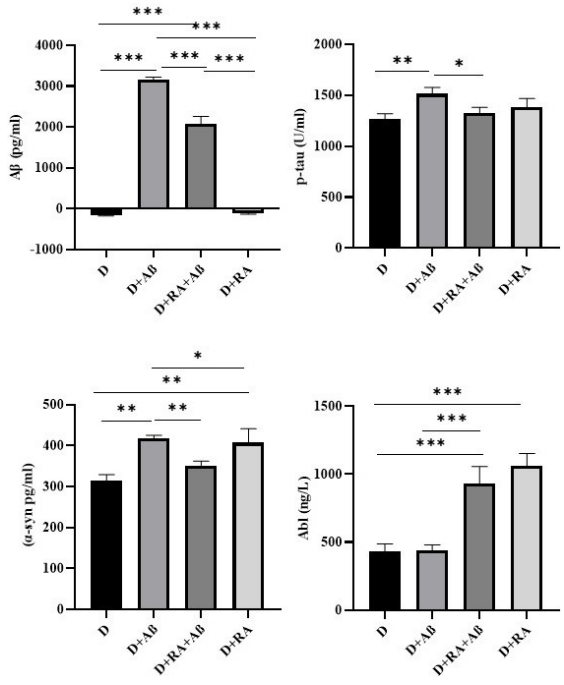

Figure 5. ELISA results demonstrating the effect of RA on Aβ, p-tau, Abl, and α- Synuclein levels in SH-SY5Y cell groups
*P≤0.05; **P≤0.01; ***P≤0.001. In the D+AB group, Aβ, p-tau, and α-syn levels were increased compared to the control group (P≤0.001, P≤0.01, P≤0.01, respectively). In the D+AB+RA group, Aβ, p-tau, and α-syn levels were decreased compared to the control group (P≤0.001, P≤0.05, P≤0.01, respectively). Abl increased in the D+RA+AB group compared to the D and D+AB groups (P≤0.001, P≤0.001, respectively).
RA: Rosmarinic acid; Aβ: Amyloid beta

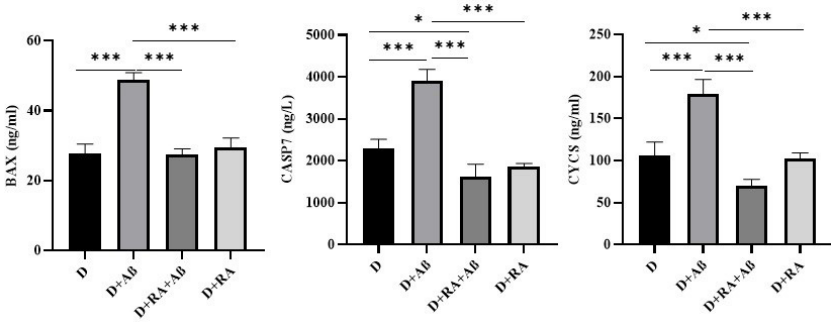

Figure 6. ELISA results demonstrating the effect of RA on CASP7, BAX, and CYCS levels in SH-SY5Y cell lines
*P<0.05; **P<0.01; ***P<0.001. Apoptotic markers BAX, CASP7, and CYCS levels were significantly increased in the D+Aβ group compared to the control (D) group (P≤0.001, P≤0.001, and P≤0.001, respectively). BAX, CASP7, and CYCS levels were markedly reduced in the D+Aβ+RA group relative to the D+Aβ group (P≤0.01, P≤0.05, P≤0.001, respectively).
RA: Rosmarinic acid; Aβ: Amyloid beta

human neuroblastoma cells into cholinergic neurons by administration of ATRA in our study (22). A number of studies have shown that RA reduces or inhibits AChE levels in AD (24–27). Different studies have shown that RA-containing extracts modulate the cholinergic system (28,29). RA administration decreased acetylcholinesterase activity in the rat hippocampus and frontal cortex and improved memory impairment (30).

RA is a bioactive molecule present in various plants of the Lamiaceae family, including rosemary, mint, sage, and lemon balm (31). Previous studies have described multiple impacts of RA, including anti-amyloidogenic, antimicrobial, immunomodulatory, anti-inflammatory, anticancer, anti-oxidant, analgesic, and antidiabetic effects (31). In our study, Aβ levels were higher in the AD model group than in the control group. Compound levels were significantly decreased after RA pretreatment. Similar RA results have been obtained in a few *in vivo* studies. In Tg2576 mice, oral administration of RA reduced amyloid plaques (32). Lee *et al.* also reported that RA administration significantly improved Aβ25-35-impaired cognitive function (33).

AD is distinguished by the deposition of amyloid plaques and the formation of neurofibrillary tangles in the brain, consisting of intraneuronal inclusions of the tau protein associated with microtubules. AD is one of the most common tauopathies, in which tau hyperphosphorylation plays a pivotal role in its pathophysiology (34). Several studies conducted on the SH-SY5Y cell line demonstrated that various phytochemicals exert neuroprotective effects, particularly by enhancing anti-oxidant capacity and reducing Aβ- and tau-associated neurotoxicity (35). In one study, SH-SY5Y cells differentiated in a three-dimensional ECM system with retinoic acid and neurotrophic factors developed a more mature neuronal phenotype, characterized by extensive neurite outgrowth and synapse formation (36). In addition, increased expression of neuron-specific markers and mature tau isoforms was observed, reaching levels comparable to those found in the adult human brain (36). Agholme *et al.* demonstrated that SH-SY5Y cells differentiated with retinoic acid and BDNF exhibited pronounced neuronal morphological changes accompanied by increased expression and enzymatic activity of cholinergic markers, including AChE and ChAT (37). In addition, combined low-dose exposure to Aβ oligomers and okadaic acid reduced neurite density without significantly affecting cell viability, thereby establishing an *in vitro* model that mimics early-stage cholinergic synaptopathy associated with AD (37). In a study investigating the effects of various polyphenolic compounds on an in vitro tau 4R model, a 50 μM concentration of RA significantly inhibited tau aggregation (34). In an experimental AD mouse model, Yamamoto *et al.* showed that RA significantly reduced cognitive loss and tau hyperphosphorylation, which was achieved through modulation of the c-Jun N-terminal kinase pathway (35). In an *in vitro* AD model using the PC12 cell line, Juvenile *et al.* reported that non-phosphorylated tau levels did not change significantly, whereas p-tau levels increased significantly following $A\beta_{1\text{-}42}$ administration (39). In the same study, it was shown that 10^{-5} and 10^{-4} M concentrations of RA reduced the hyperphosphorylating effect of tau protein 10 minutes before Aβ administration (39). In our study, p-tau was increased in the AD model group compared to the control.

In AD patients, increased α-syn was first detected in brain tissue from a single patient by Ueda *et al.* (40). Subsequent autopsy studies corroborated this finding (41). Different clinical and preclinical studies have demonstrated that α-syn levels in CSF and brain tissue are significantly higher in AD (42, 43). In one study, an AD model was established in SH-SY5Y cells by $A\beta_{1\text{–}42}$ treatment, and it was shown that increased Aβ and α-synuclein expression altered the secretion levels of inflammatory proteins (44).

Another AD model on Neuro2a cells showed that RA significantly reduced α-syn, Aβ, and tau protein levels (45). In our study, α-syn levels were higher in the AD model group than in the control group.

In AD, Abl contributes to the formation of neurofibrillary tangles by regulating the cytoskeletal signaling pathway. Immunocytochemical analyses further indicate that Abl is associated with the development of both neuritic plaques and neurofibrillary tangles in the brains of AD patients (46, 47). An AD mouse model showed that Abl inhibition ameliorated Aβ-induced synaptic changes and cognitive impairment (48, 49).

It is known that Abl hyperactivity is associated with α-synuclein-induced neuropathology (50). In mice carrying the hA53T α-syn mutation, which models human α-synucleinopathy, deletion of the Abl gene was found to reduce α-syn aggregation, associated neuropathological aspects, and neurobehavioral deficiencies (50). In the same study, overexpression of Abl in hA53Tα-syn mice facilitated α-syn aggregation, neuropathology, and neurobehavioral

deficits (50). These studies demonstrated that Abl and α-syn play a critical role in neurodegeneration. Furthermore, Abl activation was shown to lead to an age-dependent increase in α-syn (50). As a result of these effects, Abl inhibitors are emerging as a therapeutic target in AD treatment (51). Abl inhibitors are also currently used to treat leukemia in clinical practice. However, most Abl inhibitor drugs used in the treatment of leukemia are not suitable for the treatment of AD as they lack the ability to cross the blood-brain barrier (52, 53). Although Nilotinib and Dasatinib can cross the blood-brain barrier, they lack Abl selectivity and are known to interact with other kinases. (54, 55). In our study, Abl was significantly higher in the pretreatment group compared to the AD model group. The effects of RA on Abl levels, neuroprotection, and α-syn levels differed from those reported in other studies focused on AD.

A study investigating the effects of RA and ursocholic acid in an experimental AD mouse model, RA increased the animals' preference for social interaction compared to the model group and the donepezil-treated group (58). In an in vitro AD model study using PC12 cells, apoptosis was significantly induced 6 hours after administration of 1 μg/ml Aβ1-42, and this could be prevented by administration of 10-6 and 10-5 M RA (39). In our study, RA pretreatment showed a neuroprotective effect by suppressing the apoptosis pathway in an *in vitro* AD model.

Findings support the neuroprotective and anti-apoptotic effects of RA in an *in vitro* AD model. However, further studies are warranted to explore the underlying mechanisms. The observed increase in Abl protein levels in the pretreatment group suggests a possible compensatory or protective response that should be clarified through functional studies with Abl modulators.

Conclusion

In this study, RA exhibited neuroprotective and antiapoptotic effects in an *in vitro* model of AD. The findings indicate that RA may serve as a potential therapeutic strategy for neurodegenerative diseases. Further *in vivo* and *in vitro* studies are necessary to better understand RA's overall influence on neurodegenerative conditions. In particular, *in vivo* studies using experimental AD animal models to investigate the distinct effects of RA would be valuable for confirming the translational relevance of the current findings. Additionally, exploring the comparative or synergistic effects of RA in combination with other known neuroprotective agents could offer further insights into its therapeutic utility.

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Authors' Contributions

A Ş was responsible for writing the original draft, methodology, formal analysis, and conceptualization. C EG contributed to the conceptualization, review, and editing of the writing, methodology, and formal analysis. EN D focused on methodology, formal analysis, and review and editing. E K handled validation, methodology, review, and editing. S K managed validation, methodology, conceptualization, and review and editing.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Declaration

No artificial intelligence tools or technologies were used in the preparation of this article.

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