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ORIGINAL ARTICLE

The Effect of Curcumin on Aβ, Akt, and GSK3β on the Brain and Retina of APP/PS1 Mice and in the Blood of Alzheimer's Patients with Early-stage Disease

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ABSTRACT

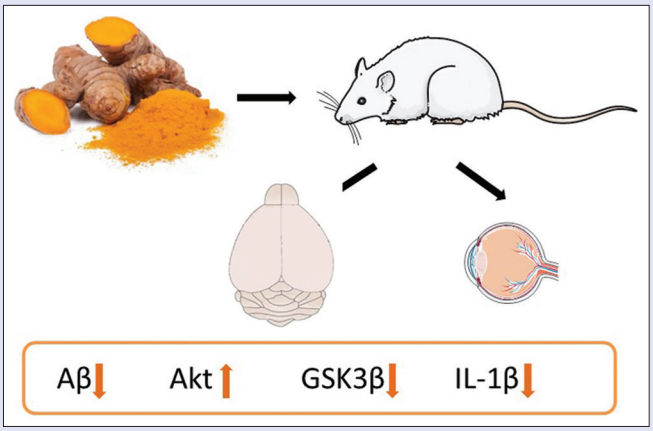
Background: Curcumin possesses multifunctional pharmacological properties, including antioxidant, anti-inflammatory, and antidiabetic properties. We investigated whether curcumin can improve pathological changes associated with Alzheimer's disease (AD), including amyloid β (Aβ), protein kinase B (PKB, also termed Akt), and glycogen synthase kinase 3β (GSK3β) levels and expression. **Materials and Methods:** Alzheimer's transgenic APP_{SWE}/$PS1\Delta_{E9}$ mice and wild-type mice were treated with curcumin by intragastric administration for 2 weeks at 2 and 5 months of age, respectively. Aβ plaques and contents in the brain and retina were measured by immunohistochemistry and enzyme-linked immune sorbent assay, respectively, while the expression of Akt and GSK3β was tested by RNA isolation and quantitative real-time polymerase chain reaction. Blood of patients with AD and age-matched healthy controls was used to determine the contents of Aβ, Akt, and GSK3β. **Results:** Curcumin treatment decreased Aβ accumulation in the early stages of AD at 5 months ($P < 0.001$). It also improved AD-associated pathological changes, including upregulation of Akt ($P < 0.01$) and downregulation of GSK3β ($P < 0.01$). In addition to AD-associated changes, the proinflammatory cytokine interleukin (IL)-1β was significantly decreased with curcumin treatment ($P < 0.05$). **Conclusion:** In the early stage of AD, curcumin can suppress Aβ accumulation, upregulate the expression of Akt, downregulate the expression of GSK3β, and inhibit the proinflammatory cytokine IL-1β. But in the late stage, curcumin has an insignificant inhibitory effect on GSK3β. In patients with AD, a low expression of Akt and a high expression of GSK3β were observed. Curcumin may have a similar effect on patients with AD by regulating these protein expressions and can be used to improve the pathological features of AD in the early stages of the disease.

Key words: Aβ, Akt, brain, curcumin, early stage of AD, GSK3β, retina

SUMMARY

- Curcumin appears to suppress Aβ accumulation during the early stage of AD, upregulate the expression of Akt, downregulate the expression of GSK3β, and inhibit the proinflammatory cytokine IL-1β.

Abbreviations used: AD: Alzheimer's disease; Aβ: amyloid β; PKB, also termed Akt: protein kinase B; GSK3β: glycogen synthase kinase 3β; APP/PS1: APPSWE/PS1ΔE9; RT-qPCR: real-time quantitative polymerase chain reaction; WT: wild type; HCs: healthy controls; PBS: phosphate-buffered saline; DAPI: 4,6-diamidino-2-phenylindole; ELISA: enzyme-linked immune sorbent assay; MMSE: mini-mental state examination.



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INTRODUCTION

In Alzheimer's disease (AD), the accumulation of amyloid β (Aβ) is one of the key processes in disease progression and a key mediator of synaptic dysfunction and cognitive impairment.[1] Aβ regulates protein kinase B (PKB, also termed Akt) and glycogen synthase kinase 3β (GSK3β) signaling, which interact mutually in the AD brain.[2,3] Additionally, Aβ production may also be regulated by GSK3.[4] Previous studies have suggested that the Akt/GSK3β pathway plays a crucial role in the maintenance of neuronal survival and neuronal networks in AD.[5,6] Pharmacological activation of Akt rescued memory impairment in Aβ-injected AD model mice.[7] Disturbance of the AKT/GSK-3b signaling pathway is a key mechanism that underlies the pathophysiology of both AD and diabetes mellitus (DM).[8,9]

Curcumin, the main active component of the traditional Chinese medicine turmeric, has anti-inflammatory, antioxidant, and antitumor effects.[10] Previous curcumin human tolerance tests and clinical research on the effect of curcumin treatment on AD have revealed a very small probability of digestive tract intolerance.[11] Animal experiments showed that after 1 week of curcumin treatment, the number of Aβ plaques in the brains of APP_{SWE}/$PS1\Delta_{E9}$ (APP/PS1) transgenic mice was substantially

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reduced.[12,13] These studies used transgenic mice; the genotype of these animals expressed a chimeric mouse/human amyloid precursor protein and human presenilin.[14] Curcumin may effectively clear Aβ due to its ability to break down products within the body.

In recent years, the neuroprotective effect of curcumin has drawn researchers' interest. Studies showed that curcumin could improve cognition and inhibit inflammation by regulating related cell pathways, including brain-derived neurotrophic factor (BDNF) and Akt/GSK3β signaling pathways; it thus improves cognitive decline in rats caused by intracerebroventricular injection of $A\beta_{42}$.[15] Studies on curcumin treatment of spinal injuries reveal that curcumin can regulate the Toll-like receptor 4 (TLR4)/nuclear factor kappa-B (NF-κB) signaling pathway by downregulating the levels of TLR4 and NF-κB, thereby protecting individuals against spinal injury.[16] In Aβ-stimulated microglia, curcumin can block NF-κB, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPK) signaling pathways, which produce anti-inflammatory effects and protect hippocampal HT-22 cells from the neurotoxic effects because of microglial activation.[17] Curcumin can also regulate the phosphatase and tensin homolog (PTEN)/Akt/GSK3β pathway and inhibit tau hyperphosphorylation caused by Aβ in SH-SY5Y cell lines.[18] Curcumin has also been shown to inhibit inflammatory gene expression and proinflammatory pathways by inhibiting NF-κB activity.[19]

To date, several studies have reported that curcumin improves the cognitive function by inhibiting Aβ accumulation in APP/PS1 mice.[20] However, it remains undefined whether curcumin can prevent neuronal injury via the AKT/GSK-3b signaling pathway. In the present study, we investigated the effect of curcumin on Aβ *in vivo* and the protein expression of AKT and GSK-3b by immunohistochemistry (IHC) and real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

MATERIALS AND METHODS

Animals

APP/PS1 transgenic mice and age-matched wild-type (WT) mice were provided by the Model Animal Research Center of Nanjing University (Nanjing, China). To exclude the effect of sex on the results, only male mice were used. Estrogens play a crucial physiological function in AD.[21,22] Female mice were excluded due to the effect of estrogen on AD. Animals were housed in cages in a room maintained at 22°C ± 2°C and 60% ± 5% relative humidity under a 12-h light–dark cycle (lights on at 6:00 am). Water and food were made available *ad libitum*. Animal experiments were conducted outside the housing area in a separate room.

Patients

Nineteen AD patients and 20 age-matched healthy controls (HCs) were recruited for the study. AD patients with no retinal diseases aged 60–88 years were diagnosed by two research psychiatrists according to the standards of the National Institution of Neurologic and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDSADRDA) and Diagnostic and Statistical Manual of Mental Disorder, (DSM) 5.0 version.

Drug preparation and administration

Curcumin (pure curcumin ≥80%; Hushi, Shanghai, China) was dissolved in phosphate-buffered saline (PBS; 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.1 M KCl, and 0.1 M NaCl, pH 7.4). Animals were divided at the age of 2 and 5 months into four experimental groups (n = 15 mice/group) as follows: (1) APP/PS1 mice treated with curcumin (0.1 mg/g) dissolved in PBS; (2) APP/PS1 mice treated with a similar volume of only PBS; (3) WT mice treated with curcumin (0.1 mg/g) dissolved in PBS; and (4) WT mice treated with a similar volume of only PBS. All treatments were administered intragastrically for 2 weeks. Dosage range was 0.4–0.6 ml depending on the mouse body weight. The dosage interval was 24 h.

Aβ immunohistochemistry

Briefly, after anesthetization, the mice were perfused with saline until the limbs and the liver turned white, and then perfused with 4% paraformaldehyde until the tail stiffened. The brain tissue was dissected and incubated with 4% paraformaldehyde for 1 day. After washing with PBS, the tissue was placed in a centrifuge tube containing 30% sucrose solution until the brain tissue sunk to the bottom. A cryostat was used to cut the brain tissue into 25-μm-thick sections. The sections were incubated in 1% bovine serum albumin (BSA) for 1 h and then incubated with Aβ antibody (1:500, Cell Signaling Technology) at 4°C overnight. The sections were washed three times with PBS, rinsed, and then incubated with the secondary antibody at 37°C for 1 h. After staining with 4,6-diamidino-2-phenylindole (DAPI) for 1 min, the sections were washed again three times with PBS and imaged using a confocal fluorescence microscope.

Determination of parameters in animals

Enzyme-linked immunosorbent assay

The Aβ and interleukin (IL)-1β levels in the mouse brain, retina, and human blood were measured by enzyme-linked immunosorbent assay (ELISA) (MyBioSource, San Diego, CA, USA). Absorbance at 450 nm (at a reference wavelength of 690 nm) was measured using an absorbance reader (Sunrise™; Tecan, Geneva, Switzerland). The absorbance value was transformed into a concentration value by reading the absorbance of pure samples on a standard curve. RNA isolation and RT-qPCR were used to determine Akt and GSK3β expression. Total cellular RNA was isolated using RNAiso Plus reagent (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions.

Determination of parameters in human beings

Serum Aβ, Akt, GSK3β, and IL-1β levels

Serum Aβ, Akt, GSK3β, and IL-1β levels were estimated using an ELISA kit (MyBioSource). All procedures were performed according to the manufacturer's instructions. Mini-mental state examination (MMSE), which involves scores ranging from 0 to 30, was adopted to evaluate the cognitive levels of all subjects. Lower MMSE scores indicated poorer cognitive performance.

Statistical analyses

Data are presented as the mean ± standard error (SE). Prism v7.0 (GraphPad, San Diego, CA, USA) was used for statistical analyses. Differences among multiple mean ± SE values were assessed by one- and two-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test. Differences between two mean ± SE values were assessed using unpaired t-tests. Statistical significance was set at $P < 0.05$.

RESULTS

Curcumin reduced Aβ protein levels in mice with AD

Aβ plaques were stained by IHC [Figure 1], and the levels of total Aβ in the cortex analyzed using a specific sandwich ELISA are shown in Figure 2. Curcumin treatment reduced the amount of positive staining for Aβ in the cortex of APP/PS1 mice at 5 months, which represents the early stage of AD [Figure 1].

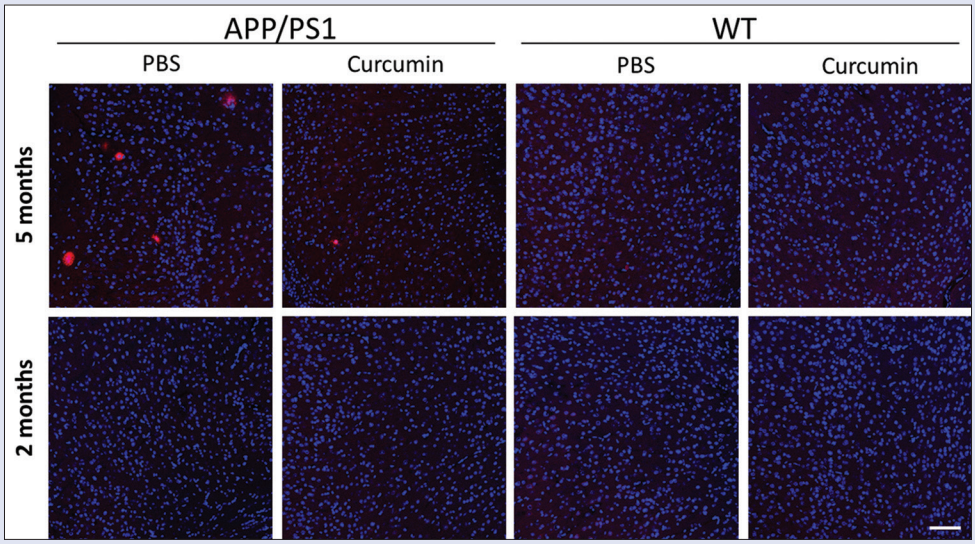

Figure 1: Immunohistochemistry in the cortex of APP/PS1 and WT mice at 2 and 5 months of age (red: Aβ; blue: DAPI). WT exhibits no positive staining for Aβ in either age group; APP/PS1 at 5 months shows positive Aβ staining. Scale bar = 100 μm. Aβ = amyloid β, APP/PS1 = APPswe/PS1ΔE9, DAPI = 4,6-diamidino-2-phenylindole, PBS = phosphate-buffered saline, WT = wild type

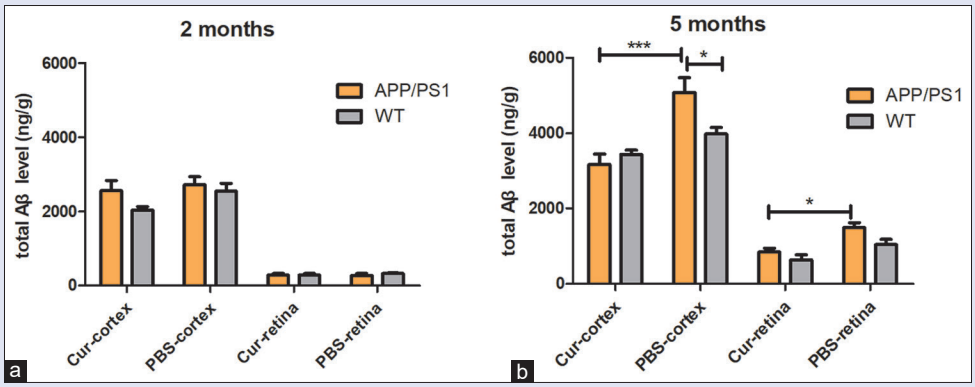

Figure 2: Aβ levels in the cortex and retina of APP/PS1 and WT mice at 2 (a) and 5 (b) months of age. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Aβ = amyloid β, APP/PS1 = APPswe/PS1ΔE9, PBS = phosphate-buffered saline, WT = wild type

There was no obvious effect of curcumin on Aβ levels in the 2-month-old mice [Figure 2]. However, in the 5-month-old mice, curcumin treatment decreased the Aβ levels in both the cortex and retina of the APP/PS1 group ($P < 0.05$).

Curcumin modulates the Akt and GSK3β expressions in AD mice

Compared to WT, the gene expression of Akt was significantly decreased ($P < 0.01$), whereas the gene expression of GSK3β was significantly increased ($P < 0.05$) in the brain and retina of APP/PS1 mice [Figure 3]. After administration of curcumin, the APP/PS1 mice showed a significant decline in the expression of GSK3β at 2 months ($P < 0.01$) and an increase of Akt gene expression at both 2 and 5 months ($P < 0.01$).

Effect of curcumin on the inflammatory mediators in AD mice

Levels of IL-1β measured from the soluble fraction of the brain and retina of mice at 2 and 5 months are shown in Figure 4. Compared to the WT group, APP/PS1 mice showed significantly increased levels of IL-1β ($P < 0.001$). Moreover, compared to the APP/PS1 group, the curcumin treatment group exhibited a significant decrease in IL-1β levels ($P < 0.001$).

The difference in Aβ, Akt, GSK3β, and proinflammatory cytokines between AD patients and healthy subjects

The demographics and clinical characteristics of the study subjects are detailed in Table 1. The mean age of AD was 72.56 ± 10.27 years, 55.56% of subjects were male, body mass index (BMI) was 21.89 ± 2.78, number of years of education was 10.33 ± 3.08 years, MMSE score was 10.89 ± 7.52, and mean disease course was 7.22 ± 2.82 years. There were significant differences in the mean MMSE scores between the AD and HC groups ($P < 0.01$). There were no differences in age, BMI, or education level between the two groups ($P > 0.05$).

Figure 5 shows the levels of Aβ, Akt, GSK3β, and IL-1β in the serum of AD patients and HCs. There was a significant difference in the Akt

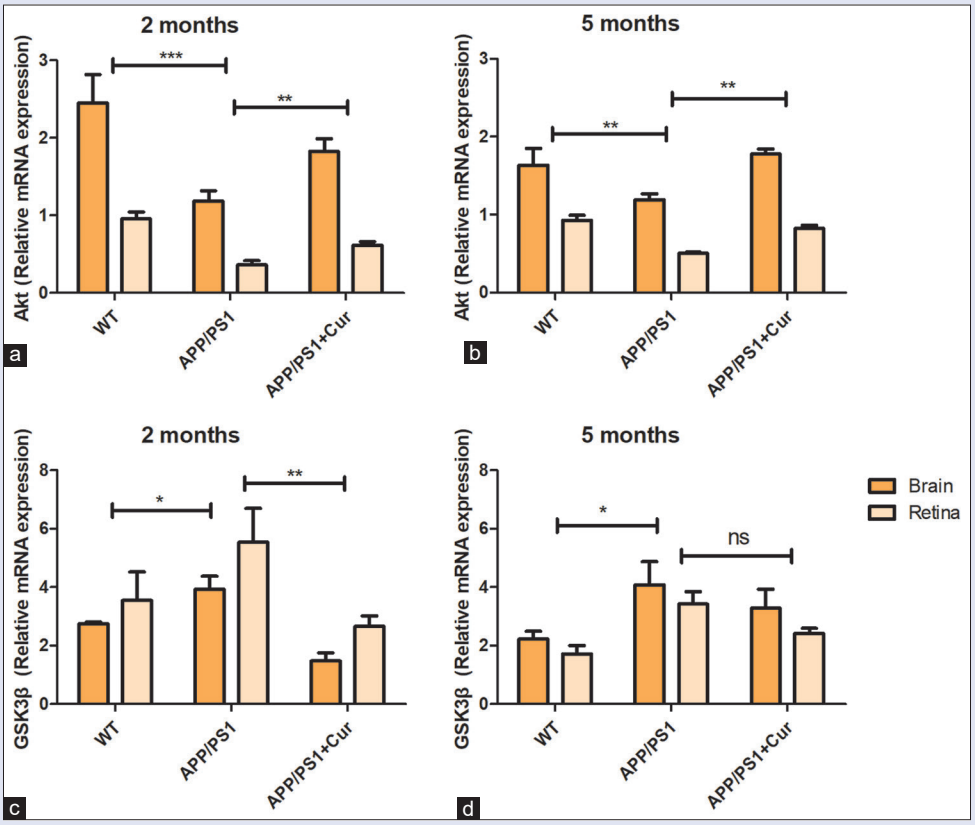

Figure 3: Effect of curcumin administration for 2 weeks on the gene expression of Akt at 2 months (a) and 5 months (b) and GSK3β at 2 months (c) and 5 months (d) in APP/PS1 and WT mice. Values represent the mean and standard deviation, using two-way ANOVA followed by Bonferroni's *post hoc* test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Akt = protein kinase B, ANOVA = analysis of variance, APP/PS1 = APPswe/PS1ΔE9, GSK3β = glycogen synthase kinase 3β, WT = wild type

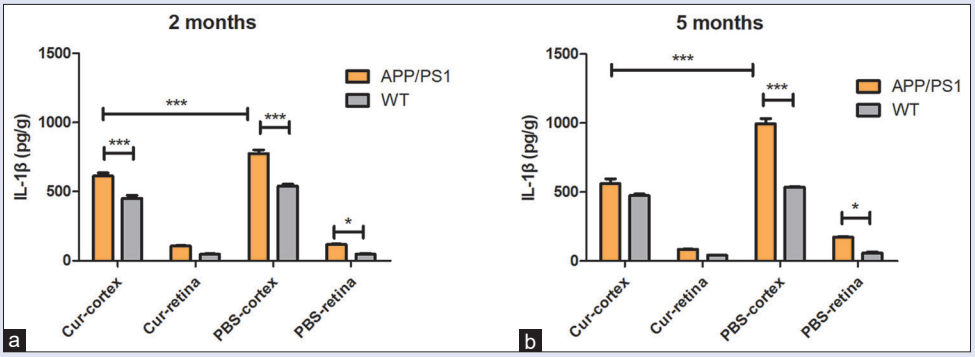

Figure 4: Curcumin shows a significant decrease in IL-1β levels in APP/PS1 mice at both 2 months (a) and 5 months (b). A two-way ANOVA (genotype × treatment) reveals a significant effect of curcumin treatment. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ANOVA = analysis of variance, APP/PS1 = APPswe/PS1ΔE9, IL-1β = interleukin-1β, PBS = phosphate-buffered saline, WT = wild type

levels between groups ($P < 0.05$); however, no difference was found in Aβ, GSK3β, and IL-1β expression.

DISCUSSION

Neurodegenerative diseases can affect both brain and retina. Although the neuroprotective effect of curcumin has already been studied, it remained unknown whether curcumin could directly influence Aβ accumulation, proinflammatory cytokines, and related proteases. The current study investigated the effect of curcumin on Aβ, Akt, and GSK3β in the brain and retina of APP/PS1 mice.

The aging process can cause a large amount of Aβ accumulation that may disrupt the immune response and produce local inflammation.[8] Additionally, Aβ clearance can be decreased due to insulin resistance.[23] In this study, we found that Aβ plaques were observed in APP/PS1 transgenic mice largely at 5 months of age compared to 2 months of age. We noticed that the amount of Aβ in the cortex of WT mice also increased with mouse age. Aβ content increased, but Aβ plaques had not yet formed. Similarly, the proinflammatory cytokine IL-1β also significantly

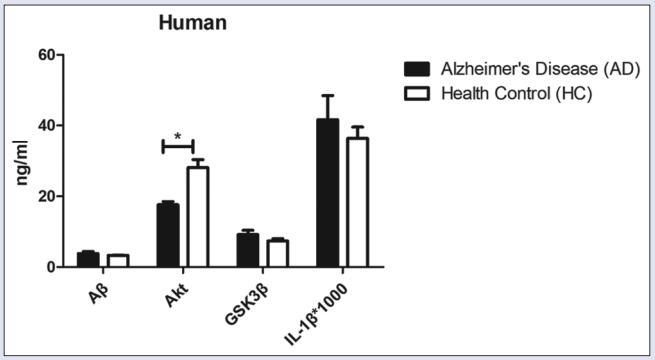

Figure 5: Levels of Aβ, Akt, GSK3β, and IL-1β in the serum of AD patients and HC. Aβ = amyloid β, AD = Alzheimer's disease, Akt = protein kinase B, GSK3β = glycogen synthase kinase 3β, HC = healthy controls, IL-1β = interleukin-1β

Table 1: Demographic and clinical characteristics of all subjects

Variables	AD patients (*n*=9)	Healthy controls (*n*=10)	*P*
Age (years)	72.56±10.27	73.91±7.82	0.368
Sex (male)	5	6	NA
BMI	21.89±2.78	21.64±2067	0.815
Study duration (years)	10.33±3.08	10.46±2.58	0.505
MMSE	10.89±7.52	27.82±1.78	0.001
Comorbidity			
Hypertension	2	1	NA
Hyperlipemia	1	0	NA
Diabetes mellitus	1	0	NA
Disease duration (years)	3.22±1.64	0	0.000
Treatment			
Memantine	5	NA	NA
Donepezil	4	NA	NA

AD=Alzheimer's disease, BMI=body mass index, MMSE=mini-mental state examination, NA=not applicable

increased with mouse age (775.8 ± 45.9 in 2 months vs. 994.1 ± 65.9 in 5 months, $P = 0.009$). Previous studies have suggested that sustained inflammation results in chronic Aβ deposition, which eventually leads to AD.[24] In patients with insulin resistance, there may be an inflammatory microenvironment.[25]

GSK3β plays a key role in insulin resistance. One of the features of insulin resistance is the impaired insulin/P13K/Akt pathway, which leads to an increase in GSK3β.[26] In the current study, in APP/PS1 mice, the gene expression of GSK3β was significantly increased ($P < 0.05$) in the brain and retina, whereas the gene expression of Akt was significantly decreased ($P < 0.01$). Besides its role in AD, inhibition of GSK3β activity can also prevent pathological changes in patients with DM.[27] Growing evidence suggest a close relationship between DM and AD.[28] GSK3β was highly expressed in the patients with AD in this study, which corroborated previous studies.[29,30] The therapeutic potential of GSK3β inhibition has been investigated in several preclinical AD studies, which show that levels of tau phosphorylation and Aβ deposition can be suppressed by GSK3β inhibitors.[31,32] Compared with normal individuals, Akt expression was significantly decreased in AD patients in our study. Akt activator influences AD-like memory impairment and long-term potentiation (LTP) impairment.[7]

Curcumin has multifunctional pharmacological effects, including antioxidant, anti-inflammatory, and antidiabetic properties.[33-36] The current study found an inhibitory effect of curcumin on brain and retina Aβ levels. These results were consistent with previous studies.[37] Moreover, we investigated the neuroprotective mechanism and the improvement of brain diseases that may result from this inhibitory effect. After administration of curcumin, Akt level increased and GSK3β decreased significantly in 2-month-old APP/PS1 mice. But in 5-month-old mice, the inhibitory effect of curcumin on GSK3β was not significant. Curcumin can also be used as an imaging agent for Aβ plaques.[13] Therefore, in addition to investigating the neuroprotective effects of curcumin, further research on the diagnosis and treatment of eye- and brain-related diseases with curcumin is required in the future.[38,39]

The limitation of this study is that changes only in Aβ, Akt, and GSK3β were investigated. The interaction between Akt and GSK3β will be investigated in the future. It is important to investigate a full range of pathological pathways in the AD brain to enable development of more therapeutic targets for AD treatment.

CONCLUSION

In conclusion, this study demonstrated that curcumin appears to suppress Aβ accumulation during the early stage of AD, upregulate the expression of Akt, downregulate the expression of GSK3β, and inhibit the proinflammatory cytokine IL-1β. But in late stage, the inhibitory effect of curcumin on GSK3β was not significant. In patients with AD, there were also a low expression of Akt and high expression of GSK3β. Curcumin may have a similar effect on AD patients by regulating the expression of these proteins. These results suggest that curcumin treatment may be useful to improve the pathological features of AD in the early stages of the disease.

Acknowledgements

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Statement of ethics

All animal experiments were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Medical School of Ningbo University (Ningbo, China).

This study was approved by the local ethics committee of Ningbo Kangning Hospital (NBKNYY-2018-LC-21) and registered as a clinical trial in the China Clinical Trial Registry (CHICTR: ChiCTR2000035243). All procedures of the study were in accordance with the Helsinki Declaration (2014) ethical standards and regulations for human research.

Author contributions

XM, XL, and MY performed animal experiments, data collection, and wrote the manuscript. XM, CQ, and JH performed data analysis. CZ and ZC proofread the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

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