Qifu-Yin attenuates AGEs-induced Alzheimer-like pathophysiological changes through the RAGE/NF-κB pathway

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[ABSTRACT] Qifu-Yin (QFY), a widely used formula of traditional Chinese medicine (TCM) derived from “Jingyue Quanshu”, is one of the most commonly used TCM prescriptions for the clinical treatment of Alzheimer disease. The role of advanced glycation end products (AGEs) and its receptor RAGE have attracted increasing attention as the pivotal role of Aβ has been questioned. The present study was designed to test the neuroprotective effects of QFY, and the possible mechanism in AGE-induced Alzheimer model rats. After injection of AGE in the CA3 area of the hippocampus, QFY (8.6, 4.3, and 2.15 g⋅kg⁻¹), and a positive control drug donepezil (2 mg⋅kg⁻¹), and a positive control group donepezil (2 mg⋅kg⁻¹) were administrated through gastric intubation to rats once daily for thirty consecutive days. Another positive control group was the AGE + anti-RAGE group, which was simultaneously inject ed with anti-RAGE antibody before AGE treatment. The control group, sham-operated group, as well as the AGE + anti-RAGE group received saline at the same dosage. The Morris water maze test and the step-down passive avoidance test were conducted to evaluate the cognitive function of the rats. The expression of RAGE and NF-κB were assayed by immunohistochemical staining. The levels of Aβ, TNF-α, and IL-1β in the hippocampus were measured by enzyme-linked immunosorbent assay (ELISA). The results showed that QFY could significantly attenuate the memory impairment induced by AGE, decrease the expressions of RAGE and NF-κB, and reduce the levels of Aβ, TNF-α, and IL-1β in the hippocampus in a dose-dependent manner. Also, the blockage of RAGE could significantly reduce the impairments caused by AGES. In conclusion, QFY could attenuate AGES-induced, Alzheimer-like pathophysiological changes. These neuroprotective effects might be related to the RAGE/NF-κB pathway and its anti-inflammatory activity.

[KEY WORDS] Alzheimer’s disease; Qifu-Yin; Advanced glycation end products (AGEs); RAGE


Introduction

Alzheimer’s disease (AD) is a neurodegenerative disor-
brains, as many studies have indicated that non-demented people may have equivalent densities of neuritic plaques as equal-aged demented individuals [10]. The most important factor may be that many trials against Aβ have not produced positive outcomes in recent years [5]. For example, a six-year immunization trial against Aβ 42 showed that patients had neither a shorter time to severe dementia nor improved survival, even though amyloid plaque was virtually removed completely [6]. In addition, the first passive Aβ immunotherapy, as well as a following study of bapineuzumab, an anti-Aβ monoclonal antibody, had no significant result or clinical benefit [17]. In another Phase II study of an anti-Aβ antibody, solanezumab, no improvement of cognition was observed [10]. These outcomes contributed to the judgment that the field might need to reconsider its direction, that is, amyloid-beta, while certainly involved in the disease, is not an initiating event, but rather is secondary to other pathogenic events [11].

In 1997, it was proposed that the formation of advanced glycation end products (AGEs) might be the chemical process responsible for both of the protein deposition and inflammation in the AD brain [12]. Accumulation of AGEs in cells and tissues is a normal feature of aging, but is elevated in AD. It has been demonstrated that AGEs are co-localized in senile plaques, as well as neurofibrillary tangles (NFTs), from patients with AD [13-14]. Also, several proteins associated with neurodegenerative diseases, such as Aβ, tau, prions, and transthyretin, were found to be glycated in AD patients [15]. Moreover, nucleation-dependent polymerization of Aβ could be significantly accelerated by crosslinking through AGEs in vitro, while formation of the AGE-crosslinked amyloid peptide aggregates could be attenuated by the AGE-inhibitors [16]. These clinical studies and experimental data suggest that AGEs might play an important role in the etiology or progression of AD [17].

When AGEs bind to the receptor for advanced glycation end products (RAGE), they activate redox-sensitive transcription factors such as NF-κB, and subsequently induce the expression of proinflammatory cytokines, such as IL-1β and TNF-α [18-19]. RAGE is a multiligand-binding member of the immunoglobulin superfamily. It can bind with a broad repertoire of ligands, such as Aβ, high mobility group box-1 (HMGB1/amphoterin), and S100/calgranulins, as well as AGEs [15]. This ligand-receptor interaction may activate receptor-mediated signal transduction pathways, such as RAGE/ NF-κB pathway [20]. Moreover, overexpression of RAGE can exaggerate neuroinflammation, as evidenced by increased pro-inflammatory mediator production, Aβ accumulation, impaired learning/memory, and neurotoxicity in an Aβ-rich environment [21], and blockade of RAGE can attenuate the AGE-induced activation [16-19, 22]. Therefore, intervening RAGE/NF-κB signaling pathway and the downstream inflammatory response might provide a potential to attenuate AGEs-induced brain damage.

Qifu-Yin (QFY), comprising Ginseng Radix et Rhizoma, Rehmanniae Radix Praeparata, Angelicae Sinensis Radix, Atrac-

tylodis Macrocephalae Rhizoma, Ziziphi Spinosae Semen, Polygonae Radix and Glycyrrhiza Radix et Rhizoma, is a widely used formula of traditional Chinese medicine (TCM) derived from “Jingyue Quanshu”, a medical classic written by Jingyue Zhang in the Ming Dynasty. QFY is one of the most commonly-used TCM prescriptions in the clinical treatment of AD. However, the effect of Qifu-Yin on AD animal models, and the possible mechanisms are far from being clearly understood.

Previous studies showed that ginseng had therapeutic effects on neurodegenerative diseases, such as AD [23-24], and many components in ginseng had anti-neuroinflammatory effects [25-26]. Many studies in China indicated that Radix Rehmanniae could effectively treat AD in vitro and in vivo [27-28]. In addition, Radix Rehmanniae suppressed the expression of pro-inflammation genes, including TNF-α and RAGE, and the activation of NF-κB induced by AGEs [29]. Angelica sinensis has shown a number of pharmacological effects in treating AD [30], and A. sinensis could decrease β-amyloid-induced neurotoxicity and tau phosphorylation in cultured cortical neurons [13]. Moreover, glycyrrhizic acid, one of the main compositions in Radix Glycyrrhizae, was shown to protect endothelial cells against AGEs-induced endothelial dysfunction through inhibiting RAGE/NF-κB pathway activation [32]. Also, other components in the formula are often used clinically in treating neurological diseases in China. The above mentioned findings provide some pharmacological basis for the dementia prevention function of Qifu-Yin.

The aim of this investigation was to evaluate the effect of Qifu-Yin on AGEs-induced AD rat model, and examine the possible underlying mechanism. The Morris water maze test and step-down type passive avoidance test were conducted to explore the learning performances and spatial memory ability of the experimental animals. Immunohistochemistry for RAGE and NF-κB were measured in the cortex and hippocampus of the rats, and levels of TNF-α, IL-1β, and Aβ in the hippocampus were measured using ELISA kits. The studies showed that QFY attenuated AGEs-induced Alzheimer-like behaviors in rats with the suppression of RAGE and NF-κB expressions and its anti-inflammatory effects in hippocampus.

Methods and Materials

Animals

Adult male Sprague-Dawley rats (280–300 g, Experimental Animal Center in Jiangsu Province, Nanjing, China) were housed for one week to adapt to the environment. They were maintained in a temperature controlled room (25 ± 2 °C) and kept on a 12-h light/12-h dark cycle with food and water available ad libitum. All of the experiments and animal care were performed strictly according to the Provision and General Recommendations of Chinese Experimental Animals Administration Legislation and were approved by the Science and Technology Department of Jiangsu Province.

Preparation of AGEs

The preparation of AGEs proteins, i.e. AGEs-BSA, was
performed as described previously \cite{22}. Incubate 300 mg·mL$^{-1}$ bovine serum albumin (BSA) with or without 0.5 mol·L$^{-1}$ D-glucose in 0.1 mol·L$^{-1}$ phosphate buffer, pH 7.2, at 37 °C for three months under sterile conditions. Unincorporated glucose was then removed by dialysis through a semipermeable membrane against PBS for 48 hours. The prepared AGE were sterilized by filtration and kept at 20 °C. The AGE-specific fluorescence was measured at excitation/emission 370/440 nm using a fluorescence spectrophotometer (PerkinElmer, Waltham, MA, USA).

\textbf{Drugs and treatment}

Ginseng Radix et Rhizoma (Northeast, China), Rehmanniae Radix Praeparata (Henan, China), Angelicae Sinensis Radix (Gansu, China), Atractylodis Macrocephalae Rhizoma (Zhejiang, China), Ziziphi Spinosae Semen (Hebei, China), Polya galae Radix (Shaanxi, China) and Glycyrrhiza Radix et Rhizoma (Inner Mongolia, China), were purchased from Integrated Chinese and Western Medicine Hospital of Jiangsu University, Nanjing, China.

Qifu-Yin is composed of the above seven raw materials in the dry weight ratio of 6 : 9 : 9 : 5 : 6 : 5 : 3. To obtain the aqueous extract of Qifu-Yin, the ingredients were added to ten times of distilled water, heat-extracted for 2 hours the first time and 1 hour the second time, pressure-filtered, concentrated with a rotary evaporator, and stored at 4 °C for use. Anti-RAGE antibodies were purchased from Santa Cruz Biotechnology Inc (Texas, USA). Rat NF-$\kappa$B p65 IgG were from Bioworld Technology Inc (Nanjing, China). ELISA kit for measurement of TNF-$\alpha$, IL-1$\beta$ and A$\beta$ were obtained from R&D Systems Inc (Min, USA).

Rats were anesthetized by intraperitoneal injection of chloral hydrate (320 mg·kg$^{-1}$), and placed into a stereotaxic device. Two holes were made for injection at coordinates –3.4 mm posterior to bregma, ± 2.5 mm lateral to midline, and –3.0 mm dorsal to ventral dura \cite{33}. A sample (3.6 mL) of BSA (500 µg), or AGE (500 µg), or AGE (500 µg) plus anti-RAGE antibody (50 µg) (AGE + anti-RAGE) was injected bilaterally into the CA3 region of the rats. Then the rats were left in a temperature-controlled chamber until they recovered from anesthesia. Penicillin-G 200 000 IU·mL$^{-1}$ was administered after surgery for three days, to prevent infection \cite{34}.

One day after the operation, the AGE-treated rats were randomly divided into five groups (n = 10) as follows: group 1 served as the model group and received saline water; groups 2, 3, and 4 received Qifu-Yin (dissolved in saline water) at doses of 8.6, 4.3, and 2.15 g·kg$^{-1}$, respectively, according to the clinical dosage; group 5 received donepezil hydrochloride of 2 mg·kg$^{-1}$ \cite{34} (dissolved in saline water). Meanwhile, BSA-injected rats served as a sham-operated group, no operated rats served as the control group, and AGE + anti-RAGE injected rats received saline water. All of the drugs were administered orally for 30 consecutive days.

\textbf{Morris water maze test}

Twenty-five days after drug administration, spatial memory ability was detected by the Morris water maze test \cite{34}. The test was carried out in a black circular pool (180 cm in diameter and 60 cm in height) with a featureless inner surface. A round escape platform was placed 1 cm underneath the water surface in the center of one quadrant. The rats were given two trial sessions each day for four consecutive days, with an intertrial interval of five hours, and the escape latencies were recorded. Once the rat located the platform, it was permitted to remain on it for 10 s. If the rat did not locate the platform within 120 s, it was placed on the platform for 15 s and the escape latency was recorded as 120 s. On day 5, the platform was removed and each rat was allowed to swim freely for 120 s as the probe test. The time that rat spent in the target quadrant (where the platform was once hidden) and times of crossing the platform were measured.

\textbf{Step-down type passive avoidance test}

One day after Morris water maze test, the step-down type passive avoidance test was carried out according to the previously described method \cite{35}. At the beginning of training, rats were placed in the box to adapt for 3 min. Then electric currents were delivered and the rats would jump onto the platform to avoid the electric shock, and the electric current was maintained for 5 min. After a 24 h interval, the rats were again placed on the platform, and the latency to step down on the grid with two paws for the first time and the number of errors subjected to shocks within 3 min were measured as learning performances.

\textbf{Preparation of tissue samples}

Rats were decapitated 60 min after the behavioral tests. Brains were removed carefully and hippocampi were immediately dissected on a cold plate, weighed, and quickly homogenized with 0.9% ice-cold saline water. The homogenate was centrifuged at 3 000 r·min$^{-1}$ for 10 min at 4 °C and the supernatants were collected and stored at 4 °C for TNF-$\alpha$, IL-1$\beta$, and A$\beta$ measurement, according to the manufacturer’s instructions.

\textbf{Brain slice collection and immunohistochemistry of RAGE and NF-$\kappa$B}

Two rats selected randomly from each group were deeply anesthetized with chloral hydrate (320 mg·kg$^{-1}$, i.p.) and sacrificed by perfusion transcardially with 0.1 mol·L$^{-1}$ phosphate buffer saline (PBS, pH 7.4), followed by 4% paraformaldehyde in PBS. After 24 h fixation, the brains were dehydrated by serial gradient concentrations of alcohol and then embedded in paraffin blocks. Immunohistochemistry was performed on the paraffin-embedded tissue sections (4 µm each piece) as previously described \cite{36}. After quenching endogenous peroxidase and blocking with normal goat serum, the sections were incubated with rabbit anti-rat antibodies specific against RAGE and NF-$\kappa$B p65 overnight at 4 °C, respectively. After washing with PBS, the sections were incubated for 2 h at 37 °C with the secondary antibodies, and then stained by 3, 3′-diaminobenzidine in chromogen solution. The staining of
RAGE and NF-κB was measured using computerization by Image Pro Plus software (IPP 6.0, Media Cybernetics). Brown staining on the cell membrane or in the cytoplasm represented positive staining, and staining density indicated the expression level of RAGE and NF-κB.

**Statistical analysis**

All values were expressed as the $\bar{x} \pm s$ and analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test using SPSS version 13.0 software; a $P$-value of less than 0.05 was considered significant and $P < 0.01$ was considered to be statistically very significant.

**Results**

**Morris water maze test**

The Morris water maze test was used to assess the spatial learning and memory ability of the rats. As shown in Fig. 1A, the mean latency to find the platform declined progressively during the four training days. No marked difference exists between the control and sham-operated groups. The AGE-treated rats remarkably spent longer time on finding the platform than did the control ones (day 1 to day 4, $P < 0.01$). These results revealed that the AGE-treated rats had obvious cognitive impairment. Moreover, the increase of escape latency was shortened respectively by Qifu-Yin (8.6, 4.3, and 2.15 g·kg$^{-1}$) from the second to fourth days ($P < 0.01$ or $P < 0.05$ vs the model), by donepezil treatment from the third to fourth days ($P < 0.01$ or $P < 0.05$ vs the model), and by the simultaneous application of anti-RAGE antibody from the second to fourth day ($P < 0.01$ or $P < 0.05$ vs the model). Fig. 1B illustrated the swim paths of rats in the second trial on the
second and fourth days. Rats tended to explore all the four quadrants of the pool in the second day. On the fourth day, the control rats swam in the direction of the platform while AGE-treated rats took longer swimming paths. In the probe test, as shown in Fig. 1C and Fig. 1D, the control and sham-operated rats spent more time in the target quadrant (33.2 ± 9.7 s, and 31.8 ± 11.3 s, respectively) and more crossing times (6.1 ± 2.0 s and 5.3 ± 1.6 s, respectively) than the AGE-treated rats did (18.9 ± 5.3 s, P < 0.01, and 1.3 ± 1.0, P < 0.01). Compared with the AGE-treated rats, Qifu-Yin (8.6, 4.3 g kg⁻¹) and the anti-RAGE antibody treated rats, had more crossing times (P < 0.01, P < 0.05, and P < 0.01, respectively). However, as for time in the target quadrant, no significant improvement was observed, except for the Qifu-Yin group at a dose of 8.6 g kg⁻¹. These data suggest that QFY could improve the learning and memory ability of the AGE-induced model rats.

**Step-down type passive avoidance test**

Step-down type passive avoidance test is a behavioral task to evaluate memory ability. Compared with the control rats, the AGE-treated rats exhibited poor performances, whose number of errors increased (P < 0.01) and the latency shortened (P < 0.01) significantly (Fig. 2A; Fig. 2B). Compared with the model rats, Qifu-Yin treatment at the doses of 8.6 and 4.3 g kg⁻¹ significantly prolonged the latency (P < 0.05 and P < 0.05, respectively) and decreased the number of errors (P < 0.01 and P < 0.01, respectively). Meanwhile, donepezil and the blockage of RAGE displayed a similar effect (P < 0.05 and P < 0.01) compared with the model rats. However, a low dose of Qifu-Yin (2.15 g kg⁻¹) failed to alter the latency and the number of errors (P > 0.05 vs the model). These results suggested that QFY could improve the cognitive function of AGE-induced model rats.

![Fig. 2](image)

**Effect of Qifu-Yin on levels of TNF-α, IL-1β, and Aβ in the hippocampus**

Neuroinflammatory activity has been associated with heavy Aβ plaque burden and accumulation of neurofibrillary tangles. Despite the deposition of Aβ, another early pathological feature of AD is the increase in levels of various cytokines, such as TNF-α and IL-1β, classically regarded as pro-inflammatory mediators. Table 1 summarizes the anti-inflammatory and neuroprotective effects of Qifu-Yin in AGES-treated rats. Injection of AGES could significantly increase the levels of Aβ, TNF-α and IL-1β (P < 0.01, P < 0.01 vs the control). QFY at the doses of 8.6 and 4.3 g kg⁻¹, and the pretreatment with anti-RAGE antibody could significantly decrease the protein expression of Aβ, TNF-α, and IL-1β (P < 0.01 or P < 0.05 vs the model). However, despite donepezil and QFY at the dose of 2.15 g kg⁻¹ having a trend to decrease the levels of Aβ and the pro-inflammatory cytokines, no significant difference was observed (P > 0.05 vs the model). These findings suggested that QFY could inhibit AGE-induced neuroinflammation, and had a protective effect on AGE-induced brain damage in a dose-dependent manner.

**Table 1** Effect of QFY on TNF-α and IL-1β levels in the hippocampus of AGE-treated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g kg⁻¹ d⁻¹)</th>
<th>TNF-α (pg mL⁻¹)</th>
<th>IL-1β (pg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>10.63 ± 0.59</td>
<td>1.69 ± 0.11</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>-</td>
<td>11.73 ± 0.35</td>
<td>1.74 ± 0.08</td>
</tr>
<tr>
<td>AGE</td>
<td>-</td>
<td>20.47 ± 2.11**</td>
<td>3.66 ± 0.26**</td>
</tr>
<tr>
<td>AGE + anti-RAGE</td>
<td>-</td>
<td>15.94 ± 0.65*</td>
<td>2.29 ± 0.14*</td>
</tr>
<tr>
<td>AGE + Donepezil</td>
<td>0.002</td>
<td>20.28 ± 1.07</td>
<td>3.45 ± 0.17</td>
</tr>
<tr>
<td>AGE + QFY</td>
<td>2.15</td>
<td>18.54 ± 0.55</td>
<td>2.83 ± 0.09*</td>
</tr>
<tr>
<td>AGE + QFY</td>
<td>4.3</td>
<td>15.77 ± 0.37**</td>
<td>2.28 ± 0.12**</td>
</tr>
<tr>
<td>AGE + QFY</td>
<td>8.6</td>
<td>14.02 ± 0.86****</td>
<td>1.98 ± 0.17****</td>
</tr>
</tbody>
</table>

**Effect of Qifu-Yin on the expressions of RAGE and NF-κB in the cortex and hippocampus**

Studies suggested that RAGE was markedly expressed in neurons, microglia and astrocytes in AD-pathological regions, and that the levels of RAGE proteins positively correlated with the severity of the AD pathology. NF-κB, a family of DNA-binding proteins, is activated under physiological and pathological conditions and involved in regulating many aspects of cellular activity, in stress, injury, and especially in pathways of the immune response. In this experiment, the
expressions of RAGE and NF-κB in the cortex and hippocampus of the rats were analyzed by immunohistochemistry. As shown in Fig. 3 and Fig. 4, brown staining represented the positive expression. The mean densities of RAGE and NF-κB were significantly higher in the cortex and hippocampus, respectively (Fig. 3C; Fig. 4C). Compared with the model group, Qifu-Yin considerably down-regulated the mean densities of RAGE and NF-κB in a dose-dependent manner ($P < 0.01$ or $P < 0.05$). However, donepezil treatment showed no significant difference compared with the model group. These data indicated that QFY might attenuate AGE-induced cognitive impairment through modulating the RAGE/NF-κB pathway.

**Discussion**

Advanced glycation end products (AGEs), the non-enzymatic glycation products of reducing sugar and amino groups of proteins, lipids, etc., have been implicated as important contributors in the pathogenesis of Alzheimer’s disease [40]. Since several trials against anti-Aβ antibody have failed [6-10], the key role of Aβ in AD is questioned, while AGEs and their role in AD receive more attention. AGEs begin to accumulate in neuronal perikarya of the hippocampus and para-hippocampus, as well as in reactive astroglia in human brains after the third decade of age [41]. In AD, this effect is accelerated: AGEs accumulate extracellularly on β-amyloid plaques and intracellularly in neurons and astrocytes [42].
The binding of AGEs to its receptor, RAGE, activates oxidative stress, causing a host of damage, and finally activate redox-sensitive transcription factors such as nuclear factor kappa B (NF-κB), resulting in the up-regulation of cytokines such as IL-1β and TNF-α. In the present study, the potential protective effect of Qifu-Yin on AGE-induced cognitive dysfunction was evaluated, including behavior test and anti-inflammation activity, while the underlying mechanism through attenuating RAGE/NF-κB activation was also explored.

In this study, injection of AGE in the CA3 area of the hippocampus could activate the RAGE/NF-κB pathway and impair the performance of rats in the Morris water maze test and step-down passive avoidance test, which was in agreement with the previous findings. Rats with long-term treatment of QFY showed better cognitive parameters as compared to the AGE-induced model rats in a dose-dependent manner. This result indicates that QFY has the potential to ameliorate cognitive deficits induced by AGEs.

RAGE, one of the immunoglobulin superfamily of cell-
surface molecules, was originally found as an AGEs-binding receptor and also recognized as a pro-inflammatory molecule mediating signal transduction cascade events [45]. In addition, the survey evidence shows that interaction between AGEs and its receptor (RAGE) could activate NF-κB-mediated inflammatory pathway and result in neurodegeneration [39]. This study demonstrated that QFY down-regulated RAGE and NF-κB expressions, attenuated the increasing levels of Aβ, TNF-α, and IL-1β induced by AGEs in a dose-dependent manner.

To further confirm the role of RAGE, an anti-RAGE antibody was used to block the receptor of AGEs. It was found that simultaneous application of anti-RAGE antibody almost abolished the AGE-induced Alzheimer-like pathophysiological changes. These results confirm that AGEs may induce brain damage through up-regulating RAGE, and thus activation of NF-κB.

In the present study, donepezil improved rat behavior against AGEs insult, but no significant effects on regulation of IL-1β and TNF-α production were observed. This result was contradictory to the published study, which showed that donepezil inhibited inflammatory gene expression in a tauopathy mouse model [45]. Although different animal models and molecular targets might be the potential causes for the different results, the anti-inflammatory activity of donepezil in Alzheimer disease remains to be clarified.

It was demonstrated that AGEs could activate RAGE/NF-κB pathway, induce cognitive damage in rats, and contribute to Aβ accumulation and neuroinflammation. However, whether this increase of Aβ was caused directly by the neurotoxicity of AGEs, or indirectly by the increasing levels of pro-inflammatory cytokines like TNF-α or IL-1β, remains unknown. More research is needed since the main effective components in QFY have not been identified. In conclusion, QFY might attenuate AGEs-induced AD-like pathophysiological changes via RAGE/NF-κB pathway, at least partially.

References


