Quercetin promotes learning and memory performance concomitantly with neural stem/progenitor cell proliferation and neurogenesis in the adult rat dentate gyrus

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ABSTRACT

The decline in neurogenesis is a very critical problem in Alzheimer disease. Different biological activities have been reported for medicinal application of quercetin. Herein, we investigated the neurogenesis potential of quercetin in a rat model of Alzheimer's disease induced by amyloid-beta injection. Rats were randomly divided into Control, Alzheimer + Saline and Alzheimer + Quercetin groups. Following the administration of Amyloid-beta, rats in the Alzheimer + Quercetin group received 40 mg/kg/day quercetin orally for one month. Our data demonstrated amyloid-β injection could impair learning and memory processing in rats indicated by passive avoidance test evaluation. We noted that one-month quercetin treatment alleviated the detrimental effects of amyloid-β on spatial learning and memory parameters using Morris water maze analysis. Quercetin was found to increase the number of proliferating neural stem/progenitor cells. Notably, quercetin increased the number of DCX-expressing cells, indicating the active dynamic growth of neural progenitor cells in the dentate gyrus of the hippocampus. We further observed that the quercetin improved the number of BrdU/NeuN positive cells contributed to enhanced adult neurogenesis. Based on our results, quercetin had the potential to promote the expression of *BDNF*, *NGF*, *CREB*, and *EGR*-1 genes involved in regulating neurogenesis. These data suggest that quercetin can play a valuable role in alleviating Alzheimer's disease symptoms by enhancing adult neurogenesis mechanism.

1. Introduction

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder contributes to learning and memory deficits. Impairment of short-term memory is a primary clinical symptom, but long-term memory loss and cognitive impairment occur with progression of neuropathy (Morris, 1996; Price et al., 1993). The main histopathological findings are the formation of amyloid-beta (Aβ) plaques and neurofibrillary tangles, which are the result of the abnormal production of Aβ and hyperphosphorylation of the microtubule-associated protein tau in the brain. These changes result in the degeneration of neurons and removal of synaptic connectivity (Fraser et al., 1997; Goedert, 1998). Previous experiments have shown that adult

hippocampal neurogenesis and neuronal communications can be severely affected by the extracellular accumulation of Aβ (Zhao et al., 2008). Hippocampus, as the most popular region for neurogenesis, has a pivotal role in learning and memory function. However, this region is more susceptible to histological changes in the early stage of AD. Progression of AD and the emergence of dementia lead to reduced adult neurogenesis and degeneration of synapses (Thuret et al., 2009). Besides the existence of histopathological features, some alterations in the level of neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) have been reported. These factors are required for neural stem/progenitor cells (NSC/NPCs) proliferation, migration, and differentiation (Chao, 2003; Hock et al., 2000). Generally, neurotrophic factors can be divided into three groups:

neurotrophins, glial cell-derived neurotrophic factor (GDNF) family ligands, and neuropoietic cytokines. The neurotrophin group includes BDNF, NGF, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Bothwell, 2014; Chao, 2003; Lu et al., 2013). Reduction of peptide translation and neurotrophin mRNA expression can be seen in some brain regions of Alzheimer's disease patients, especially hippocampus (Lee et al., 2005; Phillips et al., 1991). Therefore, the application and regulation of neurotrophic factors could be considered as a potential therapeutic value in AD and other neurodegenerative diseases (Kazim and Iqbal, 2016). Considering the existence of NSC/NPCs in the subventricular zone (SVZ) and subgranular zone (SGZ), many attempts have been made to develop breakthrough treatment approaches against neurodegenerative diseases (Bjorklund and Lindvall, 2000; Brinton and Wang, 2006; Gage, 2000; Kulbatski et al., 2005). For instance, a dynamics simulation of NSC/NPCs is touted as one of the major challenges in the context of CNS regeneration, notably AD. In order to induce recruitment of endogenous NSC/NPCs, application of natural pharmacological compounds seems to be a novel therapeutic strategy (Ke et al., 2006; Obermair et al., 2008; Okano et al., 2007; Picard-Riera et al., 2004). There is strong evidence that natural pharmacological agents such as flavonoids improve learning and memory through modulation of the cellular and molecular mechanisms correlated with the processes of memory consolidation (Spencer, 2008; Youdim and Joseph, 2001). Quercetin (3,3′,4′,5,7-Pentahydroxyflavone dihydrate), is a flavonoid compound seen commonly in fruits, vegetables, leaves and grains (Silva et al., 2008). This herbal compound has important antioxidant and free radical scavenger activities (Saponara et al., 2002). Besides these benefits, the involvement of quercetin in the activation and stimulation of neurogenesis has been considered in recent decades (Tchantchou et al., 2009). The potency of quercetin to easily pass through the blood-brain barrier contributed to the reduction of neurodegenerative changes and age-related neurocognitive impairment (Manach et al., 2004; Rogerio et al., 2007; Youdim et al., 2004). Furthermore, it has been indicated that the quercetin protected neurons against oxidative stress and Aβ accumulation in *in vitro* and *in vivo* conditions (Cho et al., 2006; Zhu et al., 2007). Regarding the lack of enough knowledge about quercetin effect on the learning, memory function, cellular and molecular signaling pathways, we aimed to examine the impact of quercetin on learning and memory impairment in a rat model of the AD by stimulating adult neurogenesis using molecular, cellular and neurobehavioral analyses.

2. Materials and methods

2.1. Ethical issue and animals housing conditions

Adult male Wistar rats (350–400 g) were maintained on a 12 h light–dark cycle in an air-conditioned constant room temperature (23 ± 1 °C), with free access to food and water. They were habituated to the housing conditions for 14 days prior to the beginning of the experimental design process. All experiments were conducted according to international principal guidelines and approved by a local ethics committee of Tabriz University of Medical Sciences. All animals were randomly divided into 3 groups (15 rats per group): Control, Alzheimer + Saline (AD + NS), Alzheimer + Quercetin (AD + Q). Experimental design is presented in Fig. 1.
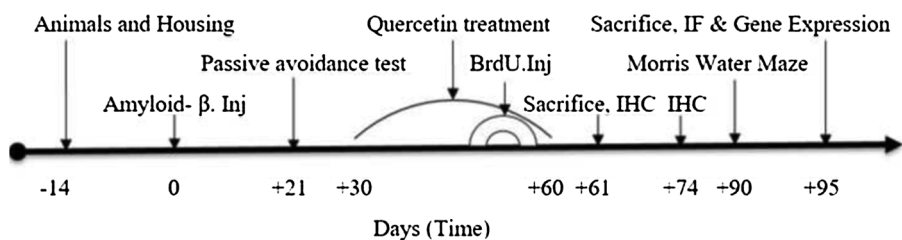
2.2. Stereotaxic surgery and induction of rat model of AD

A rat model of AD was established by direct Aβ (1–42; Sigma) injection. Briefly, the rats in groups (AD + NS and AD + Q) were anesthetized intraperitoneally with chloral hydrate solution and placed into a stereotaxic apparatus (Stoelting, USA) (Amtul et al., 2015; Kim et al., 2014). The stereotaxic apparatus was coordinated to conduct microinjection (AP: -0.8, ML: ± 1.4 and DV: − 4 mm) below dura and standardized using by a stereotaxic atlas of Paxinos and Watson. Rats were injected with 3 μg/μl of Aβ 1–42 into bilateral intracerebroventricular (I.C.V) zones (Esfandiary et al., 2014; Liu et al., 2015; Paxinos and Watson, 2007; Xie et al., 2017). After surgery procedure, animals were placed in heated chambers in a darkened room and allowed to recover with free access to food and water. Twenty-one days after the operation, the passive avoidance test was performed to confirm the induction of AD and memory impairment. All of the behavioral tests were performed by who are blind to the experiments.

2.3. Passive avoidance test

The shuttle-box apparatus was used to evaluate the learning ability and memory function. The passive avoidance test was conducted in adaptation, training and probe trials (for three consecutive days on 19th, 20th and 21st days after Aβ injection)(Liu et al., 2015; Xie et al., 2017). For habituation to new living condition, each rat was placed in the apparatus. On the second day, rats were put in the illuminated chamber for training trial. Once rats entered the dark chamber an electric shock (40 V, 0.5 A. 2 s) was delivered to their feet through the floor grid followed by rats returning to the cage until the retention trial (probe trial) on the third day (21st days after Aβ injection). In the probe trial, each rat was allowed to enter the illuminated chamber and the transition time interval between illuminated and dark chambers recorded as step-through latency. If the animal did not enter the dark chamber within 5 min, the test was terminated and the step-through latency recorded during early 300 s (Abdel-Aal et al., 2011; Esfandiary et al., 2015; Mazzola et al., 2003; Venault et al., 1986; Xie et al., 2017).

2.4. Quercetin treatment protocol

We used oral administration route as for quercetin administration. This approach has been extensively applied in traditional medicine (Halliwell et al., 2000; Singh et al., 2003). Quercetin was dissolved in 0.9% normal saline solution and administered orally at a dosage of 40 mg/kg/day for one month via a gastric tube in the (AD + Q) group (Bhutada et al., 2010). The rats in the (AD + NS) group have received only normal saline solution simultaneously.

2.5. BrdU labeling

10 mg/ml BrdU (5-Bromo-2-Deoxyuridine; Sigma) was dissolved in 0.9% NaCl solution and then sterilized by using 0.22-μm pore size microfilters. All animals received an IP injection of 50 mg/kg body weight per day during the last 7 days of quercetin treatment.

Fig. 1. The experimental schedule used over the course of the study.

Table 1

The list of primers used in the current experiment.

Gene	Primer sequence		T_a (°C)
	Forward	Reverse	
BDNF	5'- GCCTCCTCTGCTCTTTCTG -3'	5'-TTATCTGCCGCTGTGACC-3'	60
NGF	5'-TCCACCCACCCAGTCTTCCA-3'	5'-TCACCTCCTTGCCCTTGATGTC-3'	60
CREB	5'-AGTGACTGAGGAGCTTGTACCA-3'	5'-TGTGGCTGGGCTTGAAC-3'	60
EGR-1	5'-GACCACCTTACCACCCACATCC-3'	5'-GCCTCTTGCGTTCATCACTCCT-3'	60
β-actin	5'-TGTCCACCTTCCAGCAGATGT-3'	5'-TGTCCACCTTCCAGCAGATGT-3'	60

2.6. Morris water maze assessment

Spatial learning and memory capacity were assessed by using Morris water maze behavioral test with some modifications (Morris et al., 1982). In brief, a tank (180 cm in diameter and 60 cm in high) was filled with water (22 ± 1 °C) and a submerged platform 2 cm below the water surface. Visual cues were placed in all directions in the room around the tank. The water maze test was performed 4 weeks following the last BrdU injection. At the beginning of the experiment, rats were habituated to the pool by allowing them to perform 60-sec swimming without the platform. The acquisition training phase was performed for 5 successive days and each rat participated in 4 trials per day. In this phase, two parameters were evaluated; the time latency to reach the platform and the distance traveled by rats. During each trial, rats were allowed to find the hidden platform for 60 s. After mounting the platform, the animals were allowed to stay for 30 s and then placed in a holding cage for 10 min until the start of the next trial. If rats failed to find the platform within 60 ss, they were gently guided to the platform and allowed to stay on it for 30 s. The test phase (probe trial) was performed 24 h after the training sessions one day prior to sacrifice. In the test session, the platform was removed and each rat swam for the 60 ss and the average time spent in the target quadrant was calculated where the platform located on the training sessions.

2.7. Monitoring BrdU and DCX expressing cells by immunohistochemistry assay

In the first and fourteenth days after the last BrdU injection, five rats from each group were anesthetized and transcardially perfused with cold saline followed by 4% paraformaldehyde solution to evaluate the proliferation rate of cells (BrdU positive cells) and neural progenitor cells (DCX positive cells) respectively. After the perfusion-fixation procedure, the hippocampus was immediately removed and post-fixed overnight at 4 °C with the same fixative solution. The next days, tissues were dehydrated in a series of alcohols, cleared by incubations in xylene and finally embedded in paraffin. The blocks were serially sectioned by a rotary microtome (Leica, Austria) and mounted on poly-lysine-coated slides, dried overnight at 4 °C. Following paraffin removal and rehydration, slides were subjected to microscopic evaluation. The twelve serial sections throughout the dentate gyrus were selected for monitoring BrdU- and DCX-positive cells (Kempermann et al. 1997). The sections were washed in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.4, and 0.9% NaCl). Antigen retrieval was done by incubating sections in pre-heated 10 mM sodium citrate buffer for 15 min at 100 °C. For DNA denaturation, the sections were incubated in 2 N HCl bath for 60 min at 37 °C and in 0.1 M boric acid (pH = 8.5) solution for 10 min and then washed in TBS. Blocking endogenous peroxidase step was performed 0.6% H2O2 in TBS for 30 min. After several washes in TBS, sections were incubated in TBS containing 3% donkey serum and 0.3% Triton-X for 1 h. We used primary monoclonal antibodies against BrdU (Rat monoclonal to BrdU, BIO-RAD, cat# OBT0030, Dilution: 1:200,) and DCX (Rabbit polyclonal to Doublecortin, Abcam, cat# ab18723 Dilution: 1:100) for 12 h at 4 °C. Sections were incubated with biotinylated donkey anti-rat IgG secondary antibody (Jackson Immuno

Research Laboratories, Inc, cat# 712-065- 153, Dilution: 1:1000) and biotinylated donkey anti-rabbit IgG secondary antibody (Abcam, cat# ab207999, Dilution: 1:1000) for 1 h. ABC reagent (1 μl/ml, Vectastain Elite, Vector Laboratories) was added to slides and kept for 1 h. Then, the sections were incubated in Diaminobenzidine as a chromogen (0.25 mg/ml in TBS with 0.01% H2O2 and 0.04% nickel chloride, Sigma) for 7 min. After several washes in TBS, sections were mounted, air-dried and coverslipped.

2.8. Detection of BrdU/NeuN double positive cells by immunofluorescence imaging

At the end of the fourth week of the experiment, the rest of the rats were selected for neurogenesis examination. Sections were exposed to a mixture of primary antibodies including mouse anti-NeuN (Millipore, clone A60, cat#: MAB377, Dilution: 1:100, Table 1) and rat anti-BrdU (BIO-RAD, cat# OBT0030, Dilution: 1:200) antibodies. The next day, the sections were incubated with a mixture of secondary antibodies; Donkey anti-Rat IgG Secondary Antibody Alexa Fluor 488 (Invitrogen, cat# A-21208, Dilution: 1:1000,) and Donkey anti-Mouse IgG Secondary Antibody, Alexa Fluor 568 (Invitrogen, cat#A10037, Dilution: 1:1000,) in a humid and dark chamber at RT for one hour, Finally, the slides were visualized with a fluorescence microscope and digitally photographed (Carl Zeiss, Germany).

2.9. Stereological study for quantification of BrdU, DCX and BrdU/NeuN-expressing cells

For detection of BrdU- and DCX-labeled cells, every sixth serial section was selected throughout the rostrocaudal extent of the granule cell layer of the dentate gyrus of the hippocampus per rat (Gundersen et al., 1988; Williams and Rakic, 1988). To determine the reference volume, the granular cell layer volume was measured using a new CAST stereology software system. For this purpose, the area of a traced granule cell layer in every section was measured and the sum of areas multiplied by the distance between selected samples to define reference volume. To quantify BrdU and DCX positive cells/per rat, the number of BrdU- and DCX-labeled cells in the granular cell layer was multiplied by the reference volume. To determine the neuronal differentiation of new generating cells, a series of every twelfth section was evaluated using fluorescence microscopy. Generally, 100 BrdU-labeled cells per animal were analyzed for the co-expression of BrdU and NeuN for neuronal phenotype and the ratio of BrdU-labeling cells co-expressing with NeuN were determined (Kempermann et al., 1997).

2.10. Real-time PCR analysis

For gene analysis, five rats/per group were used. After decapitation, the brain was dissected and immediately hippocampus removed and stored at −80 °C until use. The frozen tissues were used for the gene expression assessment. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). The RNA samples from three individual animals/per group were used for the synthesis of cDNA using Revert AidTM First Strand cDNA Synthesis kit (Fermentas, K1621, K1622) according to the

manufacturer's instructions. Relative gene expression analysis was performed using Maxima™SYBR Green/ROX qPCR Master Mix (2X) kit (Fermentas, K0221). In each PCR reaction, 10 µl Power SYBRH Green PCR Master Mix 2X was mixed with 2 µl cDNA and10 pM /µl of each (Forward and reverse) specific primers in a total volume of 20 µl. Relative quantitative real-time PCR was performed using (ABI PRISM 7500 Sequence Detection System; Applied Biosystems). Gene expression was calculated using the delta-delta cycle threshold ($2^{-\Delta\Delta CT}$) method. All the reactions were performed in triplicate. The endogenous control β-actin was used to normalize quantification of the mRNA target, and nonspecific amplifications were verified by a dissociation curve. Comparative threshold cycle (CT) method was used to determine to mean fold changes in gene expression between the control and target genes. Primer sequences for *BDNF, NGF, CREB, EGR*-1, and *β-actin* were outlined in Table 1.

2.11. Statistical analysis

Data were expressed as mean ± SEM and were analyzed using the SPSS version 16 software. The escape latency and swim distance in the water maze were analyzed by two-way repeated measures ANOVA followed by Tukey's HSD test for between-subject difference among groups and (within subjects) for effects across block interval 1–5 ("BLOCK" effect). The probe trial data for percentage of time spent in target quadrant were analyzed by multivariate ANOVA followed by Tukey's HSD test as a post hoc analysis. For other analyses, we used One-way ANOVA followed by Tukey's HSD multiple comparison tests unless mentioned. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of Aβ injection on learning and memory

Data showed that amyloid administration didn't differ step-through latency in the training (learning) trial among all groups on 20th days after Aβ injection ($p = 0.7$) (Fig. 2). In contrast, a significant difference was observed in mean step-through latency between the control and AD rats in the retention (probe) trial ($p < 0.001$) in which Aβ injected rats developed a significant impairment in retention and probe trial on 21st days after Aβ injection (Fig. 2). Our results confirmed that Aβ successfully induced learning and memory impairments.

3.2. Chronic quercetin treatment improved cognitive performance in AD rats

In this panel, all rats unless AD + NS group showed a reduction in escape latency during 5 blocks recorded and in the distance swim to locate the platform over the course of the acquisition training (BLOCK effect, *p < 0.001*; Fig. 3A and B). Probe trial performance was measured by comparing the time spent in target quadrant with an average
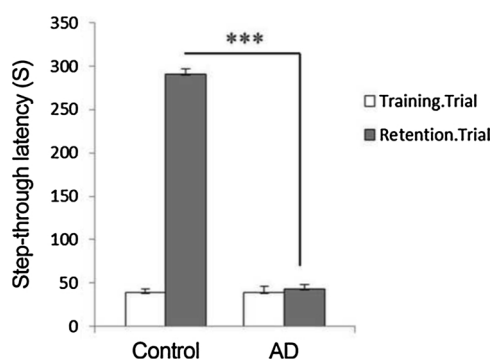

Fig. 2. The effects of amyloid–β on step-through latency (seconds) in the passive avoidance experiment. *t*-student test ***$p < 0.001$.

of time spent in all three non-target quadrants. Data analysis showed a significant difference in probe trial between the rats from different groups ($p < 0.001$; Fig. 3C). Statistical analysis showed that the mean percentage of time spent in target quadrant increased in the control and quercetin-treated rats compared to AD + NS group ($p < 0.001$; Fig. 3C). Taken together, these results showed the potency of quercetin in improving spatial learning and memory.

3.3. Quercetin promoted the proliferation of BrdU-positive cells in the dentate gyrus

To compare the proliferation rate of NSC/NPCs in the subgranular zone of the dentate gyrus, BrdU incorporation was analyzed 24 h after the last BrdU injection (Fig. 4A). Compared to rats from AD + NS, the number of BrdU-positive cells significantly increased in AD + Q group (1885.50 ± 7.96 versus 842.10 ± 9.03, $p < 0.001$; Fig. 4B). Also, we found a significant difference in the number of BrdU-expressing cells between the control and AD + NS ($p < 0.001$). Therefore, these results imply that quercetin could promote the proliferation rate of progenitor cells in the hippocampus of AD rats.

3.4. Quercetin induced DCX protein level and increased the number of neural progenitors cells

Next, we decided to examine the DCX expression in the dentate gyrus of the hippocampus using immunofluorescence staining approach (Fig. 5A). DCX is conceived as a marker of developing neural progenitor cells or migrating neuroblasts in the dentate gyrus (Meyer et al. 2002). Our results showed a significant difference in the number of DCX positive cells in rats received quercetin after 14 days post-BrdU injection. Statistical analysis revealed a significant difference in the number of DCX-expressing cells in AD + Q as compared with AD + NS (1272.80 ± 1.34 versus $842.2 \pm .85$) ($p < 0.001$, Fig. 5B). Commensurate with these data, quercetin effectively increased neural progenitor cells or neuroblasts in the adult dentate gyrus.

3.5. Quercetin treatment stimulated neurogenesis with increased the number of BrdU-NeuN positive cells

To evaluate the effect of quercetin on maturation and differentiation, we examined the differentiation potential of BrdU-positive cells into neurons 4 weeks after the last BrdU injection by cells co-labeling for BrdU and the neuron-specific marker; NeuN (Fig. 6A). The percent of double positive cells per dentate gyrus from different groups were determined; Control: $82.80 \pm 0.24\%$; AD + NS: $77.60 \pm 0.56\%$ and AD + Q: $81.30 \pm 0.39\%$ (Fig. 6B). These results showed that quercetin had potential to increase the percent of the BrdU/NeuN expressing cells in AD + Q in comparison with the group AD + NS ($p < 0.001$), indicating the positive effect of quercetin on neuronal differentiation of NSCs ($p < 0.001$). Collectively, these data showed that quercetin possibly could reduce AD-associated pathologies by the acceleration of neurogenesis in adult rats.

3.6. Quercetin increased the expression of BDNF, NGF, CREB and EGR-1 genes

We decided to investigate whether quercetin could modulate the expression of the *BDNF, NGF, CREB,* and *EGR*-1 in the rat brain. To test this hypothesis, we measured the expression of these genes using quantitative real-time PCR analysis. According to data, the transcript level of *BDNF, NGF, CREB,* and *EGR*-1 increased significantly in AD rats received quercetin compared to rats from AD + NS group ($p < 0.001$; Fig. 7). We also observed a significant difference between the control and quercetin-treated group in terms of all genes expression (*p < 0.001*; Fig. 7). These data indicated that quercetin potently regulates the expression of the *BDNF, NGF, CREB* and *EGR*-1 genes in the
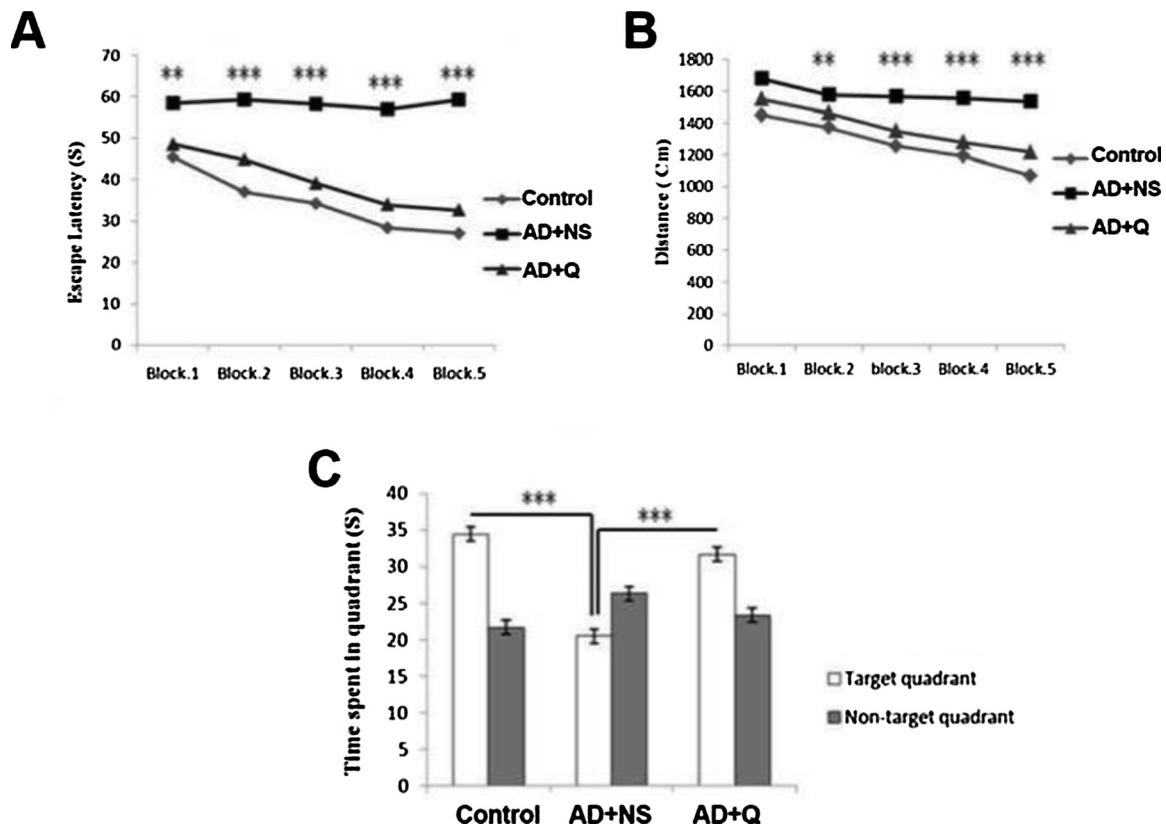
Fig. 3. The behavioral assessment of Morris water maze test(A–C). The effect of chronic treatment with quercetin on the performance of spatial memory acquisition phases (A–B) and probe trial (C). Escape latency (A) Distance latency (B) at different days to reach the platform. Probe trial performance was measured by comparing the time spent in target quadrant with an average of time spent in all three non-target quadrants. (A and B): two-way repeated measures ANOVA; C: Multivariate ANOVA and Tukey post hoc. (**$p < 0.01$; ***$p < 0.001$).

rat model of AD.

4. Discussion

Neurogenesis is defined as the birth of new nerve cells, consisting of proliferation, migration, and differentiation into mature cell type. Adult NSC/NPCs commonly undergo cell proliferation in the SGZ of dentate gyrus; migrate into the granule cell layer with changes in biochemical and morphological levels (Hastings and Gould, 1999; Markakis and Gage, 1999; Stanfield and Trice, 1988). Several studies have shown that there is a close relation between adult hippocampal neurogenesis and learning and memory function (Gould et al., 1999). Hippocampus is a central structure for the formation of certain types of memory such as episodic and spatial memory (Squire, 1992). The decline in neurogenesis rate has been contributed to cognitive impairments and thereby suggested to play a key role in AD (Tatebayashi et al., 2003; Verret et al., 2007). Neurogenesis can be regulated by a vast array of factors, neighboring niche, growth factors as well as different compounds, notably medicinal herbs–derived compounds (Gage, 2002; Gould et al., 1999; van Praag et al., 2000). Our hypothesis in the current project, is whether quercetin could promote learning and memory by stimulating adult neurogenesis? Therefore for response to this question, we herein investigated the beneficial effect of quercetin on dementia and cognitive impairment in the rat model of AD.

Due to lipophilicity and engaging specific efflux transporter such as P-glycoprotein, quercetin is easily absorbed after oral administration and passes from the blood-brain barrier (Lin and Yamazaki, 2003; Youdim et al., 2003). Our experiment showed that the impairment of learning and memory was induced 21 days after Aβ injection. In the next series of the experiment, we found that one-month quercetin treatment reversed the cognitive and behavioral performance deficits.

Consistent with our data, many authorities reported that quercetin could promote learning and memory performance through the regulation of acetylcholine esterase bioactivity, regulation of neurotrophic factors level, control of oxidative stress status, induction of anti-apoptotic genes and the inhibition of Aβ fibril formation (Bhutada et al., 2010; Bournival et al., 2009; Kumar et al., 2008; Liu et al., 2013; Tongjaroenbuangam et al., 2011). In the current experiments, oral administration of quercetin restored hippocampal-dependent learning and memory deficits after one month in AD rats. Along with these observations, we observed an increased proliferation, migration and differentiation of rat NSCs (Clelland et al., 2009; Garthe et al., 2009; Kee et al., 2007). The number of proliferating NSCs was significantly increased 24 h after the last BrdU injection in AD rats received quercetin compared to rats from AD + NS group. In the adult rodent brain, DCX is significantly expressed in the hippocampal dentate gyrus and the SVZ of the lateral ventricle (Brown et al., 2003; Couillard-Despres et al., 2005). During hippocampal neurogenesis, DCX specifies the period between committed NSCs and early maturation stage and is typically expressed two weeks after generation of developing neurons (Brown et al., 2003; McDonald and Wojtowicz, 2005; Plümpe et al., 2006). Additionally, DCX is an interesting marker to study neuronal migration (Couillard-Despres et al., 2005). Quercetin was found to increase the number of DCX-positive cells in the adult dentate gyrus coincided with enhanced neuroblast migration. Quercetin had potential to direct the fate of NSC/NPCs into the neural lineage. An increased number of neurons co-expressing BrdU and NeuN cells could be correlated with an improved neurogenesis rate. In agreement with this finding, previous studies discovered that memory function and performance was proportionally associated with hippocampal neurogenesis in rats (Drapeau et al., 2003). Although quercetin could increase the expression level of some genes involving in neurogenesis, the molecular mechanisms of the
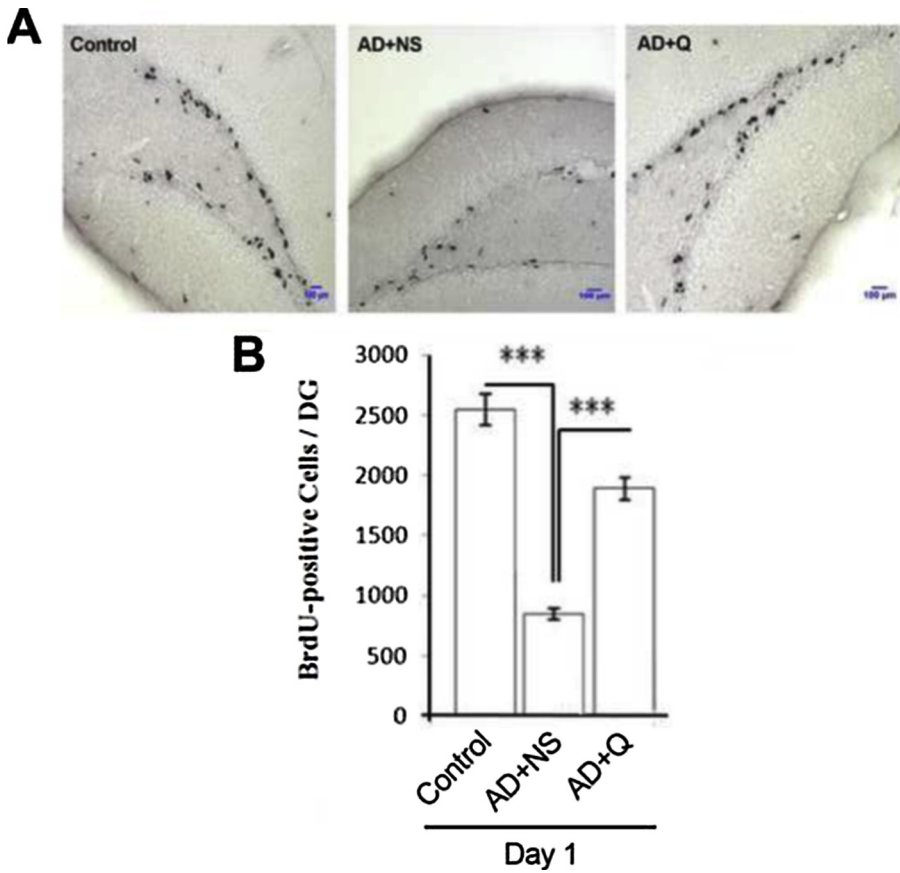
Fig. 4. The effects of the quercetin treatment on NSC/NPCs proliferation (A–B). Immunohistochemistry analysis of BrdU-positive cells in the dentate gyrus on 1 day after the last BrdU injection(A). Histogram of BrdU-positive cells in the DG on day 1 after the last BrdU injection(B). Data analysis showed significant differences in the number of BrdU-positive cells in quercetin-treated rats as compared with AD + NS. One-way ANOVA and Tukey post-hoc test (***$p < 0.001$).

quercetin on neurogenesis has not been well understood. Real-time PCR analysis showed that quercetin was able to improve learning and memory function through the regulation of neurotrophic factor signaling pathways. Neurotrophic factors such as BDNF and NGF are well known to be implicated in adult neurogenesis phenomenon, synaptic plasticity, and memory (Lim et al., 2003; Poo, 2001). Herein, increased transcript levels of BDNF and NGF have confirmed in AD rats received quercetin. In the context of the quercetin signaling pathway, it seems that quercetin interacts primarily with ERK and PKB/Akt pathways and subsequently increases the transcription of CREB. These changes lead to
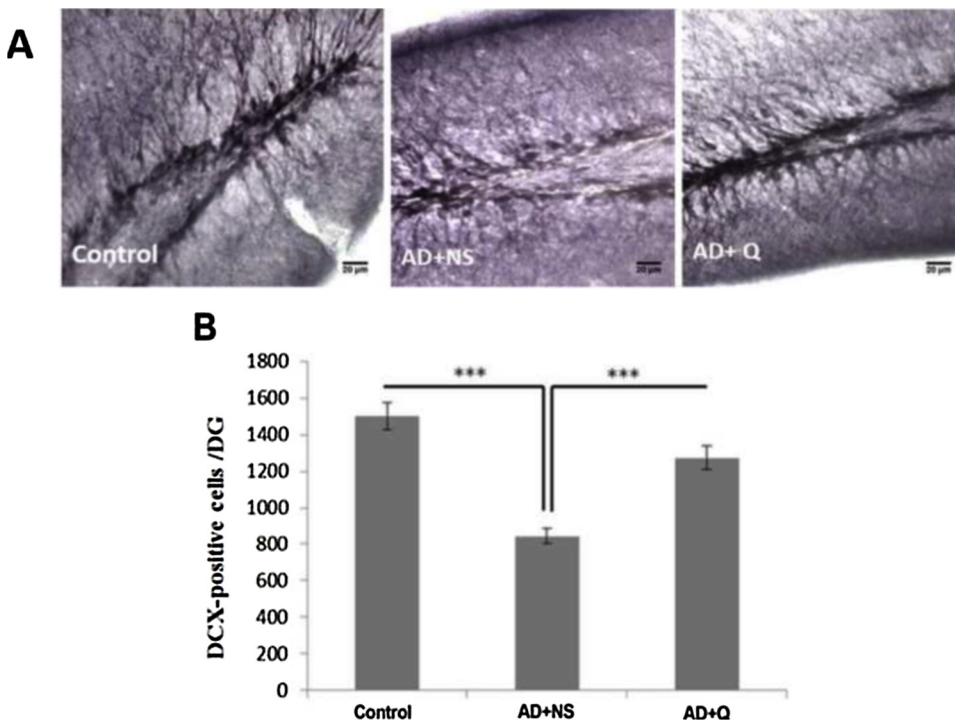

Fig. 5. The effects of the quercetin treatment on neural progenitor cells (A–B). Immunohistochemistry staining for DCX (A). Quantification of cells expressing DCX-positive cells in the DG 14 days after last BrdU injection (B). Our result indicated that quercetin significantly increased the number of expressing DCX-positive cells as compared to cells from control or AD + NS. One-way ANOVA and Tukey post-hoc test (***$p < 0.001$).
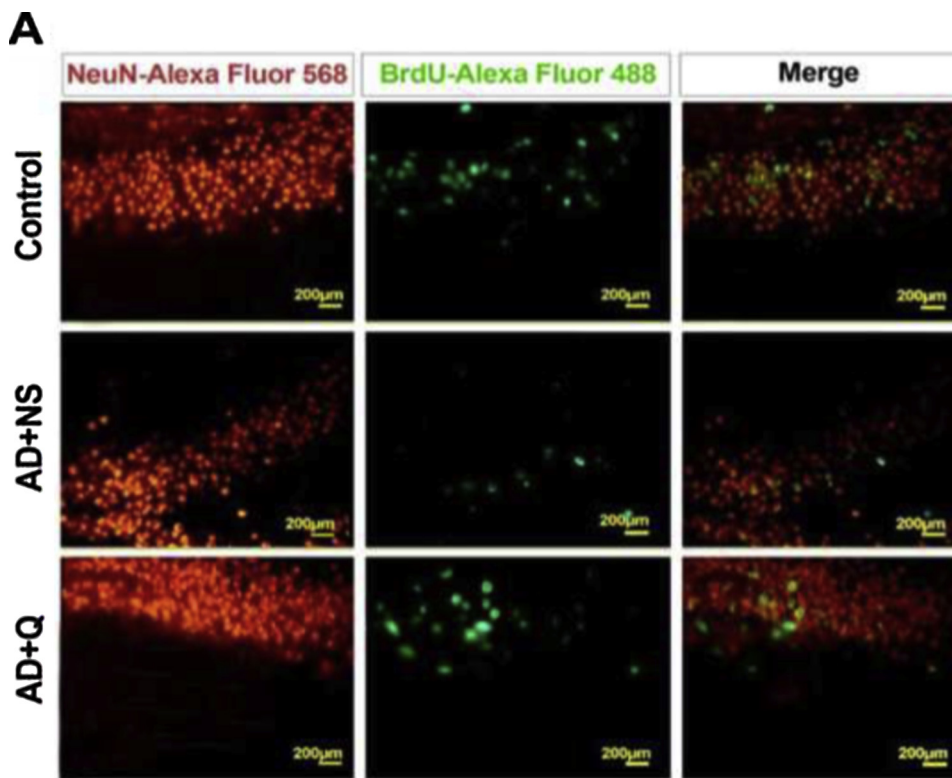
Fig. 6. The representative images of adult neurogenesis analysis (A–C). Double immunostaining showed NeuN/BrdU positive cells in the dentate gyrus 4 weeks after the last injection (A). Quantification of cells expressing NeuN relative to the total number of BrdU-positive cells per DG, 4 weeks after the last BrdU injection (B). Our result indicated that quercetin significantly increased the number of expressing NeuN cells relative to the total number of BrdU-positive cells as compared with AD + Q and control. Quantification of BrdU-positive cells expressing NeuN in the percentage of BrdU-positive cells per DG(C). The result showed that the number of cells co-expressing BrdU-NeuN was significantly increased as normalized to BrdU-positive cells. One-way ANOVA and Tukey post-hoc test (***$p < 0.001$).
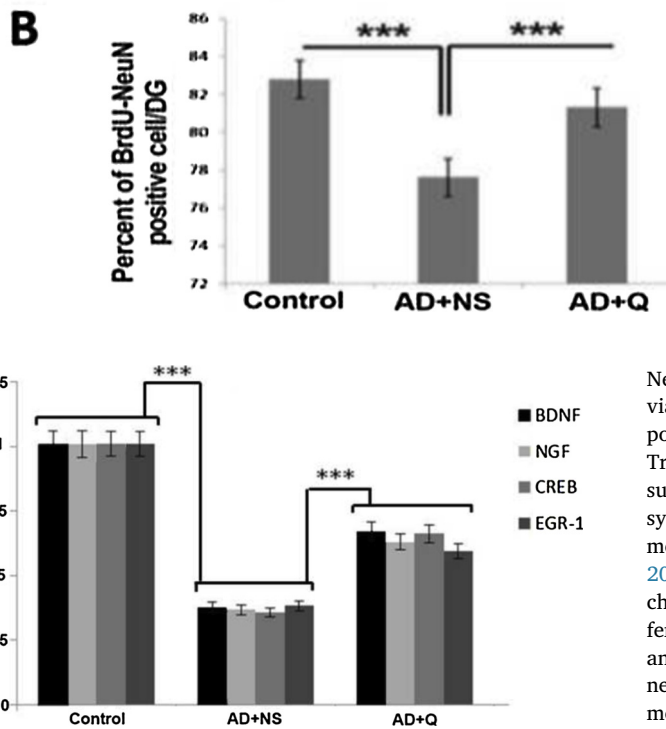

Fig. 7. Real-time PCR analysis of BDN,NGF, CREB and ERG-1 after quercetin treatment. (BDN: $p_{Control}$ *versus* $_{AD+NS} < 0.001$; p_{AD+Q} versus $_{AD+NS} < 0.001$, NGF: $p_{Control}$ *versus* $_{AD+NS} < 0.001$; p_{AD+Q} versus $_{AD+NS} < 0.001$, CREB: $p_{Control}$ *versus* $_{AD+NS} < 0.001$; p_{AD+Q} versus $_{AD+NS} < 0.001$, ERG-1: $p_{Control}$ *versus* $_{AD+NS} < 0.001$; p_{AD+Q} versus $_{AD+NS} < 0.001$). One-way ANOVA and Tukey post-hoc test.

the activation and the production of neurotrophins such as BDNF and NGF which are involved in memory acquisition and consolidation (Spencer, 2007; Spencer et al., 2003). The neurotrophin signaling mediates neuronal survival, proliferation, and differentiation.

Neurotrophins exert their influence on downstream signaling cascade via two receptor types: p75 neurotrophin receptor (p75NTR) and tropomyosin receptor kinase (Trk). BDNF binds to TrkB and NGF binds to TrkA (Chao, 2003). BDNF plays a central role in the process of neuronal survival, differentiation, hippocampal neurogenesis, axon growth and synaptic plasticity which is essential for the learning process and memory establishment (Lee et al., 2002; Lu et al., 2014; Vilar and Mira, 2016; Voineskos et al., 2011). NGF, as a critical neurotrophic factor in cholinergic neurons development, induces neuronal survival and differentiation (Nilbratt et al., 2010). This factor is produced in the cortex and hippocampus and transported to the basal forebrain cholinergic neurons in the nucleus basalis of Meynert required for cognition and memory function (Cattaneo and Calissano, 2012; Triaca et al., 2016). In human with the AD, the NGF level is decreased and contributed to neural tissue degeneration (Francis et al., 1999; Scott et al., 1995).

Our results showed the increase of CREB expression after treatment with quercetin. It has been shown that the expression of neurotrophins and memory molecular transduction are regulated by cAMP response element-binding protein (CREB).

CREB, as a cellular transcription factor, is responsible for the induction of numerous factors involved in neuronal activities, synaptic functions, learning and memory function (Bonni et al., 1995; Lonze and Ginty, 2002; Silva et al., 1998). Inside neurons, CREB binds to certain DNA sequences to increase/decrease the transcription of the

downstream genes (Bonni et al., 1995; Lonze and Ginty, 2002). For instance, CREB downregulation is involved in the pathology of the AD and promoting the expression of CREB considered as a possible therapeutic target for AD (Pugazhenthi et al., 2011). Similar to CREB, the expression of early growth response protein 1 (EGR-1) gene was significantly increased in AD rats given quercetin. CREB initiates intracellular signaling through the activation of EGR-1, which is expressed after synaptic activation and enhances synaptic plasticity. EGR-1 is also known as zinc finger protein (Zif268) and possesses a distinct pattern of expression in the brain and its induction has been shown to be associated with the neuronal activity (Bozon et al., 2002; Knapska and Kaczmarek, 2004). The improvement of learning and memory performance could be mediated by phosphorylation of CREB that is required for the development of dendrites and the upregulation of EGR-1 (Bozon et al., 2002; Tully et al., 2003). The activity of EGR-1 seems to activate downstream target genes which are involved in consolidation or stabilization of long-lasting memory and synaptic plasticity (Davis et al., 2003). In the present study, the promotion of the neurogenesis and synaptic plasticity were associated with functional recovery. These findings highlighted the ability of quercetin to induce neurogenesis as a compensatory mechanism for neuronal death during AD. There are some limitations related to this study. We suggest future investigations to examine the potent effect of quercetin on neurogenesis under physiological condition.

5. Conclusion

In summary, quercetin could enhance the learning and memory performance by the upregulation of neurogenesis in the hippocampus. Considering the changes in molecular, cellular levels and behavioral features, quercetin has the potential to be introduced as a new strategy for the prevention and treatment of AD subjects. However, in order to thoroughly clarify the beneficial effects of quercetin on AD treatment, future studies are required to be done.

Conflict of interest

The authors have no conflicts of interest to disclose.

Acknowledgments

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