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ARTÍCULO ORIGINAL

Quercetin ameliorates inflammation in CA1 hippocampal region in aged triple transgenic Alzheimer´s disease mice model.

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Introduction: Alzheimer's disease is the most common form of dementia. It is characterized by histopathological hallmarks such as senile plaques and neurofibrillary tangles, as well as a concomitant activation of microglial cells and astrocytes that release pro-inflammatory mediators such as IL-1β, iNOS, and COX-2, leading to neuronal dysfunction and death.
Objective: To evaluate the effect of quercetin on the inflammatory response in the CA1 area of the hippocampus in a 3xTg-AD male and female mice model.
Materials and methods: Animals were injected intraperitoneally with quercetin every 48 hours during three months, and we conducted histological and biochemical studies.
Results: We found that in quercetin-treated 3xTg-AD mice, reactive microglia and fluorescence intensity of Aβ aggregates significantly decreased. GFAP, iNOS, and COX-2 immunoreactivity also decreased and we observed a clear tendency in the reduction of IL-1β in hippocampal lysates.
Conclusion: Our work suggests an anti-inflammatory effect of quercetin in the CA1 hippocampal region of aged triple transgenic Alzheimer's disease mice.

Key words: Alzheimer disease; quercetin; microglia; astrocytes.

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La quercetina disminuye la inflamación en la región CA1 del hipocampo en un modelo de ratón triple transgénico para la enfermedad de Alzheimer.

Introducción. La enfermedad de Alzheimer es la forma más común de demencia; se caracteriza por la presencia de marcadores histopatológicos, como las placas seniles y los ovillos neurofibrilares, así como por una activación concomitante de células microgliales y astrocitos que liberan mediadores proinflamatorios, como IL-1β, iNOS y COX-2, lo cual conduce a la disfunción y la muerte neuronal.
Objetivo. Evaluar el efecto de la quercetina sobre la reacción inflamatoria en el área CA1 del hipocampo en un modelo de ratones 3xTg-AD.
Materiales y métodos. Los animales se inyectaron intraperitonealmente con quercetina cada 48 horas durante tres meses, y se hicieron estudios histológicos y bioquímicos.
Resultados. Se encontró que en los animales 3xTg-AD tratados con quercetina, la microglía reactiva y la intensidad de fluorescencia de los agregados Aβ disminuyeron significativamente, y que hubo una menor reacción de GFAP, iNOS y COX-2, así como una clara tendencia a la reducción de la IL-1 β en lisados de hipocampo.
Conclusión. Los resultados del estudio sugieren un efecto antiinflamatorio de la quercetina en la región CA1 del hipocampo en un modelo en ratón triple trasgénico para la enfermedad de Alzheimer.

Palabras clave: enfermedad de Alzheimer; quercetina; microglía; astrocitos.

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Alzheimer´s disease is the most common form of dementia, which is characterized by a progressive loss of memory and other cognitive functions. Its main histopathological hallmarks are extracellular beta-amyloid (βA) accumulation in senile plaques and intracellular hyperphosphorylated tau protein

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Felipe Vargas-Restrepo: experiments and drafting of the manuscript
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All authors analyzed the data and participated in the review and approval of the final version of the manuscript.

forming neurofibrillary tangles (1). These aggregates induce an inflammatory response by the microglia and astrocytes that allow a gradual activation and subsequent production of pro-inflammatory mediators leading to neurodegeneration (2).

Microglial cells and astrocytes are involved in the inflammatory response in the central nervous system (CNS). Microglia remain in a resting state exhibiting a branched morphology; once they are activated by the presence of βA the size of the soma in the cell increases and the number of processes decreases acquiring an amoeboid form with absence or presence of shorter branches (3). Their ability to engulf βA peptides reduces while the production of pro-inflammatory mediators increases (3,4). Astrocytes also have a ramified morphology when they are in resting state, but after the onset of the Alzheimer's disease, they acquire a hypertrophic morphology with a reduction of branches and processes, and an increased cell soma (5). As is the case for microglia, astrocytes increase the production of pro-inflammatory mediators when they are activated (6).

Among the most important inflammatory mediators produced by microglial cells and astrocytes in Alzheimer's disease are the interleukin 1β (IL-1β), the inducible nitric oxide synthase (iNOS) and the cyclooxygenase 2 (COX-2). IL-1β is produced by those cells surrounding βA plaques (7), and it can modulate tau hyperphosphorylation through activated microglia and p38-MAPK activation (7,8). The other inflammatory mediator involved in neuroinflammation in the disease is iNOS. This enzyme is expressed in response to immunological challenges or by damaged tissue producing nitric oxide (NO) (9), which causes DNA damage and induces the production of peroxynitrites that destroy the mitochondria and reduce ATP formation (10). High levels of βA increase iNOS expression and NO production in microglia and astrocytes (9,11). COX-2 is an inducible enzyme in pathological conditions and it catalyzes the synthesis of prostaglandins, many of which are neurotoxic, such as prostaglandin E_2 (PGE_2), which is the main produced inflammatory prostaglandin (12). βA peptides induce the activation of PGE_2 in astrocytes and microglia (6,12), which can produce an increase in astrocyte proliferation *in vivo* and diminish the ability of microglia to phagocytose βA peptides (13,14).

Quercetin is a molecule with neuroprotective properties which may increase the neuronal resistance against oxidative stress by βA (15,16). In our previous studies, we have demonstrated that quercetin reduces histopathological hallmarks improving cognitive and emotional skills in a 3xTg-AD mice model (17). Given that the hippocampus CA1 region is a vulnerable area for excitotoxicity and neuronal death in Alzheimer's disease (18), in this study we evaluated the effect of quercetin on the pro-inflammatory response.

Materials and methods

Animals

Male and female homozygous 3xTg-AD for APP (Swe), tau (P301L), PS1 (M146V), and knocking-PS1 mice (Non-3xTg) (M146V) (19) from our in-house colony were maintained at the specific pathogen-free vivarium of the *Sede de Investigación Universitaria* at the *Universidad de Antioquia* in Medellín. We assigned animals of 18 to 21 months of age randomly to the vehicles (DMSO) or quercetin groups regardless of their transgenic or non-transgenic condition (non-Tg). 3xTg-AD mice had a homogenous β-amyloidosis and tauopathy penetrance.

The animals were handled according to Colombian standards and guidelines (Law 84/1989 and Resolution 8430/1993); the protocol was approved by the Ethics Committee of the *Universidad de Antioquia* for animal experimentation. Special care was taken to minimize animal suffering and to reduce the number of animals used.

Administration of drugs

The 3xTg-AD and non-Tg mice received 25 mg/kg intraperitoneal injections of quercetin or 0.1% DMSO every 48 hours for three consecutive months, as previously described (17).

Histology and immunohistochemistry

Animals were intracardially perfused using 4% paraformaldehyde, and 50 µm coronal sections were used for Nissl (toluidine blue) staining and immunohistochemistry evaluation as previously described (17). We assessed the CA1 region at bregma -1.82 and -2.06 mm. Anti-GFAP (1:1000, Sigma # G3893), anti-iNOS (1:250, C-11, Santa Cruz Biotechnology, Sc # 7271) (permeabilizing tissues 10 mM Tris pH 6, overnight at 4°C) were the mouse primary antibodies used together with the Iba1 anti-rabbit primary antibody (1:500, Wako # 019-19741) as microglia and COX-2 markers (1:500, # AB15191, Abcam).

To determine immunoreactivity (IR) densitometry we used a 10X objective and we analyzed it with the Fiji ImageJ 1.45 software (NIH, USA) based on

staining intensity. The number of animals per group was three in non-Tg quercetin animals and four in the other groups.

Immunofluorescence

We rinsed 50 µm coronal sections at bregma -1.82 and -2.06 mm in 0.1 M PBS following a previously published protocol (20). Sections were incubated with β-amyloid anti-mouse primary antibody (1:500, β amyloid 1-16 (6E10) # SIG-39320, Covance), and Iba1 anti-rabbit primary antibody (1:500, # 019-19741, Wako). We analyzed the sections by using a motorized spinning disk confocal microscope (Olympus IX81-DSU). The omission of the primary antibodies resulted in no staining. Camera exposure time and gain were adjusted so that no pixel saturation was present in any channel, and identical camera settings were used for all images in each experiment.

Immunofluorescence (IF) was determined using a 10X objective and analyzed by Fiji ImageJ 1.45 software (NIH, USA) based on staining intensity. We processed all experimental groups at the same time for minimizing variability. The number of animals per group was three for non-Tg quercetin animals and four for the other groups.

ELISA IL-1b

We measured IL-1β using the Quantikine ELISA Mouse IL-1β kit™ (Cat. # MLB00C, RyD Systems, Minneapolis, USA) following the manufacturer's protocol with a peptide concentration of 50 µg/ml. The number of animals per group was two for 3xTg-DMSO animals and four for the other groups.

Western blotting

The procedure was performed as described previously (17). Briefly, anti-NOS2, anti-COX2, and anti-Tubulin (1:10000, monoclonal anti-βIII tubulin, # G712A, Promega, AB_430874) were used as loading control, and CW IRDye 680 goat anti-mouse or rabbit 800 (LI-COR, diluted 1: 10000) were used as secondary antibodies. Fluorescence intensity was analyzed using the Odyssey Infrared Imaging System™ application software, version 3.0 (LI-COR, ODY-1735). We used four animals in each group.

Statistical analysis

We randomly processed the data collected. We used at least three mice in each group for histological evaluation and four in each group for biochemical analyses. We evaluated data with a normal distribution using analysis of variance (ANOVA) to compare the four experimental groups, and then Tukey's test as post-hoc multiple comparison when appropriate. When the conditions of normality of the data distribution and variances were not normal we used the nonparametric Kruskal-Wallis test. The statistical analysis was performed using GraphPad Prism software (version 6.0), and results were considered to be significant at $p \leq 0.05$. The values were expressed as the mean ± SEM.

Results

Quercetin reduces β-amyloid aggregation and microglial immunoreactivity in aged 3xTgAD mice.

We confirmed an increased fluorescence intensity (FI) of the microglial population and βA plaques in 3xTg-AD mice compared to non-Tg animals (figure 1A). Interestingly, quercetin treatment reduced significantly the Iba-1 (34%) and βA (48%) FI in the CA1 region of aged 3xTg-AD mice compared to untreated 3xTg-AD mice (figure 1B,C). Microglia showed increased cell body size similar to amoeboid shape in untreated 3xTgAD mice, which was blocked by the quercetin treatment in aged animals (figure 1A).

Quercetin ameliorates astrogliosis in aged 3xTgAD mice.

We qualitatively assessed pyramidal layer of the CA1 area in relation to the cell cytoarchitecture and the presence of microglial cells using a Nissl-Iba1 IR counterstaining. We detected an increased cellular condensation and irregular morphology in aged 3xTgAD mice surrounded by Iba1+ cells (figure 2A). These observations were supported by hypertrophied astrocytes, which showed a significant increase in the GFAP immunoreactivity (33%), compared with untreated and treated control groups (figure 2B). However, with the quercetin treatment mice recovered a similar morphological shape and reactivity in the CA1 area to those of control groups (figure 2 A, B).

Inflammatory mediators are down-regulated by quercetin in the CA1 area of 3xTgAD mice hippocampus.

The hippocampus CA1 area of 3xTgAD mice showed a significant increase of iNOS immunoreactivity with a diffuse distribution in the parenchyma (figure 3A, B). COX-2 reactivity also showed a vessel-like elongated pattern, which significantly increased in the Alzheimer's disease model (figure 3A, C).

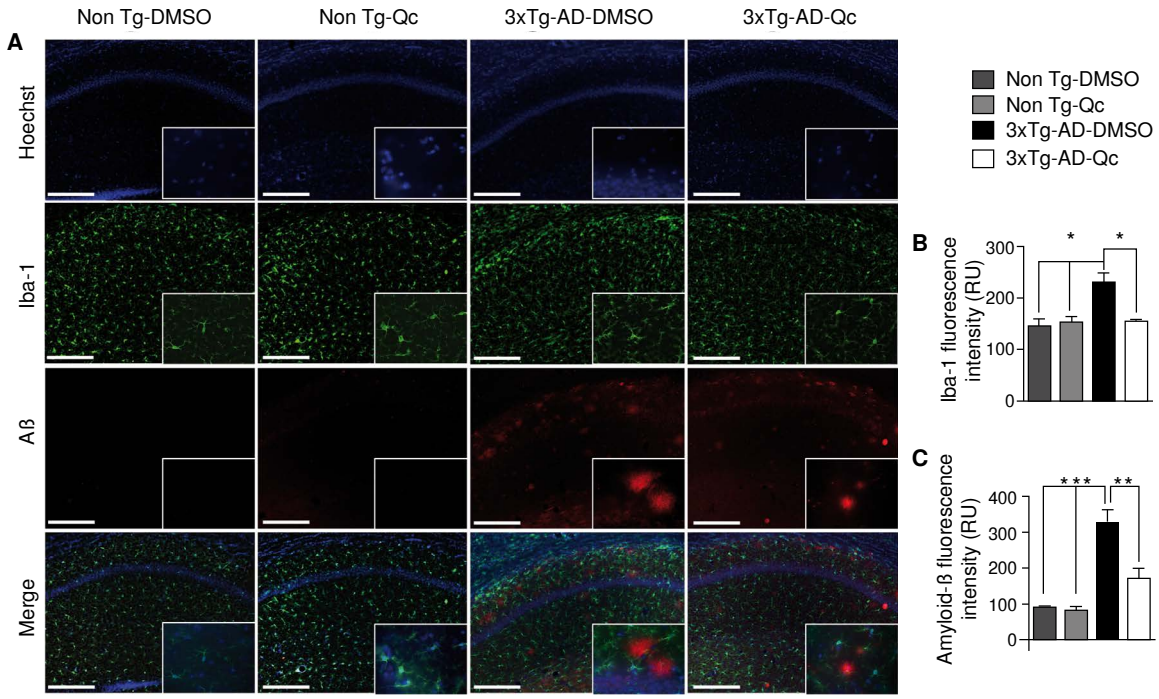

Figure 1. Quercetin reduced the Iba-1 and βA fluorescence intensities in CA1 area of aged 3xTg-AD mice. **(A)** Immunofluorescence microglial (green) and Aβ plaques (red) from CA1 region. 10X and 60X; scale bar: 50µm and 5µm, respectively. **(B)** Fluorescence intensity quantification of Iba-1 positive cells. 10X. **(C)** Fluorescence intensity quantification in aggregates Aβ 10X. Data presented as mean ± SEM. n: 3-4, * (p<0.05) ** (p=0.001), *** (p<0.001)

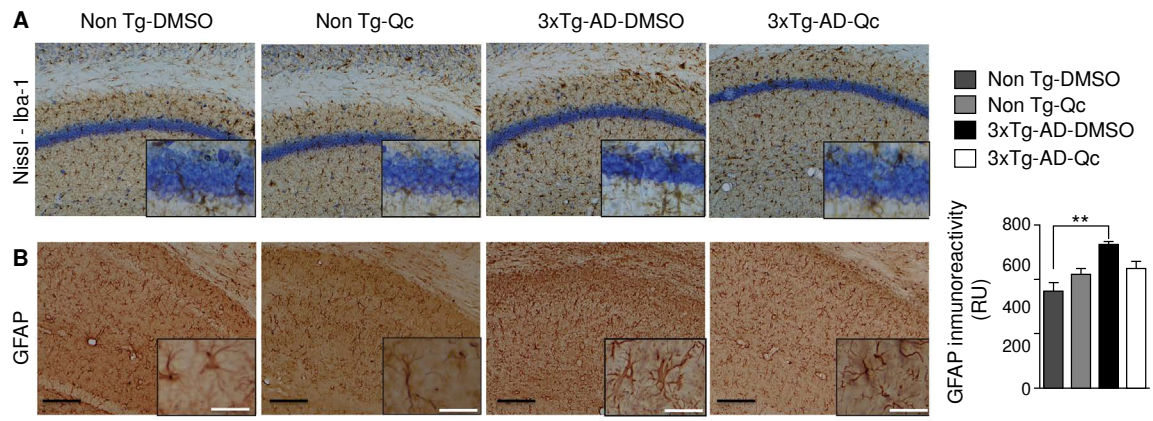

Figure 2. Quercetin recovered altered cell cytoarchitecture and ameliorated astrogliosis in the CA1 area from aged 3xTgAD mice. **A)** Representative images of Nissl-Iba-1 counterstaining. **B)** GFAP immunohistochemistry for astrocytes, and quantification of GFAP immunoreactivity shown as densitometric relative units (RU) in CA1. 10X and 40X; scale bar: 50 µm and 15 µm, respectively. Data expressed as the mean ± SEM. n: 3-4. **p<0.01

Quercetin-treated mice presented a significant reduction in immunostaining in the CA1 area (figure 3A,B,C), supported by normal IL-1β levels in comparison to the untreated 3xTg-AD group and similar to the control groups (figure 3 D). However, hippocampal total lysates did not show significant changes in the iNOS and COX-2 protein levels (figure 3 E).

Discussion

Findings suggested a reduction of the pro-inflammatory response in the CA1 hippocampal region of aged 3xTg-AD mice with the use of quercetin, confirming our recent results where quercetin reversed β-amyloidosis and tauopathy associated to cognitive and emotional behavioral improvement (17).

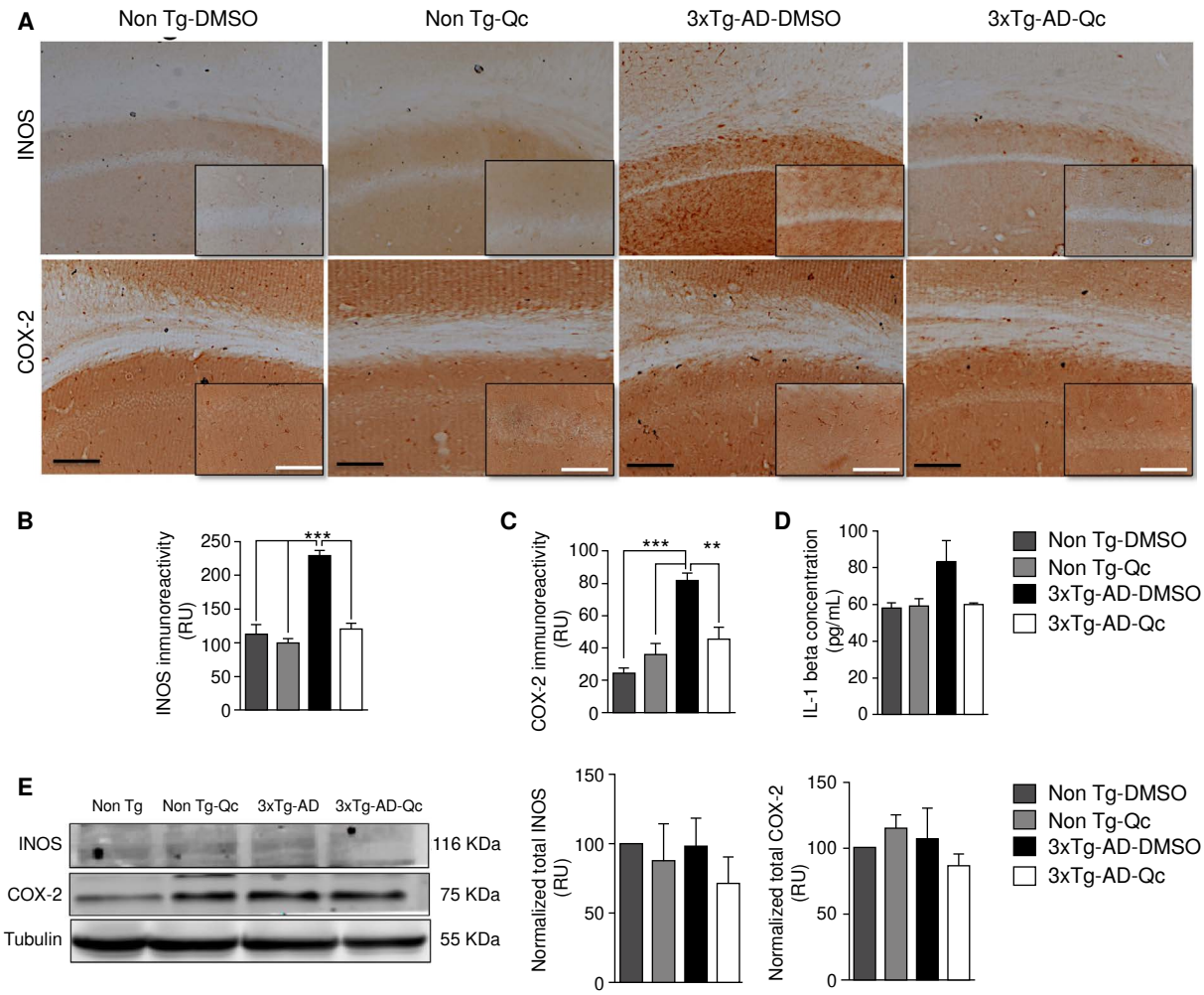

Figure 3. Proinflammatory indicators were reduced by quercetin treatment in the CA1 area of 3xTgAD mice hippocampus. **A)** iNOS and COX-2 immunohistochemistry in CA1 region.10X and 40X; scale bar: 50 µm and 15 µm, respectively. **B)** iNOS, and **C)** COX-2 immunoreactivity quantification at 10X. **D)** IL-1β hippocampal lysate quantification in non-Tg and Tg mice with DMSO and QC treatment. **E)** Representative bands of iNOS and COX-2 in hippocampal lysates and densitometric quantification of iNOS and COX-2. Tubulin was used as control load. Immunohistochemistry (n: 3-5) and ELISA (n: 2-4). Data presented as mean ± SEM. * (p<0.05) ** (p=0.001), *** (p<0.001). For Western blotting, data are presented as mean ± SEM. n=4, (p<0.005)

In a pathological context, βA aggregates can activate microglia cells and astrocytes generating local inflammation and amplifying neuronal death signaling (21). In our study, the CA1 hippocampal area of aged 3xTgAD mice presented a pro-inflammatory environment marked by β-amyloid plaques surrounded by microgliosis associated to hypertrophied astrocytes and condensed pyramidal layer. These changes were accompanied by the up-regulation of IL-1β, COX-2, and iNOS, which could be specific for the CA1 area, as they were not detected in the total hippocampal lysates.

Human Alzheimer's disease and models are characterized by a high microglial hyperreactivity (1,3), which increases the release of proinflammatory cytokines and decreases βA clearance (4,22). For this reason, IL-1β stimulates the production of COX-2 by microglial cells in brains affected by Alzheimer's disease (23), which favors the expression of iNOS through PGE_2 production (24), although IL-1β also directly activates before iNOS (7). In a positive feedback, PGE_2 also induces microgliosis and this promotes astrocyte proliferation (13). Recently, it was found that microglial-specific deletion of PGE_2 restores microglial chemotaxis and βA clearance suppresses toxicity of the exacerbated pro-inflammatory response and microglial activation (25,26). On the other hand, astrocytes reactivated by their interaction with βA release IL-1β, iNOS, and COX-2 amplifying the immune response (5,6,12). This

exacerbated reactivity causes astrocyte atrophy, which may also result in a reduced proteolytic clearance of βA and contribute to the extracellular βA accumulation and the decrease of neuronal support (27-29).

Our findings suggest that the quercetin treatment induced an anti-inflammatory response confirming previous studies where the compound decreases the production of inflammatory mediators such as iNOS, NO, COX-2, PGE_2, and IL-1β and reduces the activation of microglia and astrocytes (30-33). Furthermore, quercetin contributes to the reduction of oxidative stress, since it increases the production of antioxidant enzymes in astrocytes, microglia and neurons (34,35). Thus, quercetin might help to inhibit the feedback among proinflammatory mediators and glial cells avoiding the spreading of neuronal damage.

Interestingly, our results showed that the quercetin treatment also reverses the immune response in an advanced stage of the disease in the model under study. This suggests that the induction of βA phagocytosis (4,36) and the decrease in the release of neurotoxic cytokines (2) are mediating the protective action of quercetin, because in our previous observations quercetin did not regulate typical tauopathy mediator enzymes, such as CDK5 and GSK3-beta (17). However, quercetin might reduce tauopathy by the regulation of IL-1 β/p38 MAPK activation (8) and, thus, improve cognitive performance (17). Other protective effects have been described for quercetin in restoring the expression of genes perturbed by βA accumulation including DNA replication, cell cycle proteins, hypoxia response, *de novo* pyrimidine deoxyribonucleotide biosynthesis, p53 pathway and βA metabolism regulation and in decreasing βA40 and βA42 species by the stabilization of astrocytes-derived apolipoprotein E (37,38).

Our work suggests an anti-inflammatory effect of quercetin in hippocampal CA1 region in a model for Alzheimer's disease of triple transgenic aged mice by reducing β-amyloid plaques aggregation and microglial and astroglial reactivity as reflected in the decrease of IL-1β/ COX-2/ iNOS pro-inflammatory signaling, which could be closely related to previous findings on the reversal of tauopathy, as well as emotional and cognitive impairment.

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Conflicts of interest

The authors declare they have no competing interests.

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