Original Article

A novel synthetic compound shows antioxidant and anti-inflammatory activity and alleviates cognitive deficits in rats for the treatment of Alzheimer's disease

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Background: Alzheimer's disease (AD) is a progressive dementia, and β-amyloid (Aβ) accumulation is widely regarded as the primary pathogenesis of AD. A new synthetic compound, 8-hydroxyquinoline-resveratrol derivative (E)-5-(4-hydroxystyryl)quinolin-8-ol (10c) was evaluated as a possible anti-AD agent.

Method: (I) The total amount of ROS in SH-SY5Y cells was detected by dichlorofluorescein diacetate (DCFH-DA), and the antioxidant activity and neuroprotective effect of 10c in SH-SY5Y cells were evaluated; (II) Griess reagent was used to test the activity of Compound 10c against NO production in LPS-induced BV-2 microglial cells; (III) An automatic digital stereotaxic instrument was used to inject $A\beta_{25-35}$ into the brain to establish an AD animal model to evaluate the protective effect of compound 10c on $A\beta_{25-35}$-induced learning and memory dysfunction in rats.

Results: 10c exhibited far more potent antioxidant activity for both exogenous and endogenous reactive oxygen species (ROS) than trolox, resveratrol, and CQ (ROS production: 10c with 26.23% at 1.5 μM; resveratrol with 82.17% at 2.5 μM; CQ with 78.52% at 10 μM). 10c also shows good neuroprotective effects as an endogenous antioxidant in neuroblastoma cells. Moreover, Compound 10c also demonstrated effective inhibition of nitric oxide (NO) production (IC_{50} =3.10 μM) and IL-1β production in BV-2 microglial cells which were treated with lipopolysaccharide (LPS). In the water maze test, the numbers of rats who crossed the former platform were increased significantly in both the 10c group (5.7±1.6) and positive control group (CQ, 5.1±1.7). Meanwhile, both 10c (43.8±5.5 s) and CQ (44.1±6.6 s) treatment could significantly prolong the time rats spent in the target quadrant compared to the vehicle-treated model group. These results demonstrated that 10c could alleviate the learning and memory dysfunction of rats induced by $A\beta_{25-35}$ to a certain extent.

Conclusions: Altogether, compound 10c is a promising compound for the treatment of AD.

Keywords: Alzheimer's disease (AD); anti-oxidation; anti-inflammation; Morris water maze

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Introduction

Alzheimer's disease (AD) is the most common fatal neurodegenerative disease of older people, and is characterized by loss of memory, cognitive decline, and behavioral abnormalities, eventually leading to death (1). Although the pathogenesis of AD is not fully known, certain hallmarks including β-amyloid (Aβ) deposits, which form extracellular senile plaques, along with cerebral atrophy and intracellular neurofibrillary tangles (NFT) are reported to play prominent roles in the development of AD (2). In the past decades, scientists have developed many approaches to treat AD. However, there is no effective medicine that can definitively cure or delay the process of AD (3).

Aβ accumulation is widely regarded as the primary pathogenesis of AD (4). There is also considerable evidence to support the toxic role of Aβ oligomers in neuronal death (5). Recent studies reported that increased toxic Aβ oligomers induced inflammation in glial cells, as well as increased the expression and production of inflammatory mediators, such as nitric oxide (NO), interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) (6-8). In addition, Aβ-induced oxidative stress refers to cell overoxidation leading to cellular damage, which is carried out by reactive oxygen species (ROS) (9,10). Oxidative damage could lead to mitochondrial dysfunction and cell lysis in AD (11,12). Thus, drugs that scavenge oxygen radicals and reduce the production of inflammatory mediators are speculated to prevent or treat AD to some extent (13,14).

A series of 8-hydroxyquinoline-resveratrol derivatives, which were obtained by combining resveratrol with the metal chelator clioquinol (CQ), were reported to act as antioxidants, biometal chelators, and inhibitors of Aβ aggregation. In particular, (E)-5-(4-hydroxystyryl)quinolin-8-ol (10c) (*Figure 1*), a multi-target-directed small molecules against AD, showed potential anti-oxidant behaviour, good metal chelating ability, inhibition of copper(II)-induced Aβ aggregation with ability to cross blood-brain barrier (15). In this paper, in order to study the pharmacological effects and possible mechanism of 10c in Alzheimer's disease, we further evaluated the antioxidant activity and anti-inflammatory activity of 10c, and also studied the anti-AD effect of 10c in an animal model. A protocol was prepared before the study without registration. We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/apm-21-1983).

Methods

Culture of SH-SY5Y cells and BV-2 cells

The human neuroblastoma cell line SH-SY5Y (Laboratory Animal Service Center at Sun Yat-sen University) was cultured in a 1:1 mixture of F-12 nutrient mixture (Ham12) and Eagle's MEM containing 15 nonessential amino acids, 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Invitrogen, USA), 50 mg/μL penicillin, 50 mg/μL streptomycin, and 1 mM glutamine. Cultures were maintained in a humidified incubator at 37 ℃ with 5% CO_2 and 95% air. For assays, SH-SY5Y cells were plated into 96-well plates (1×10^4 cells/well).

BV-2 microglial cells (BeNa Culture Collection, BNCC) were routinely grown in a humidified incubator at 37 ℃ with 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% heat-inactivated FBS (GIBCO), 50 mg/μL penicillin, 50 mg/μL streptomycin, and 1 mM glutamine. For assays, BV-2 cells were plated into 96-well plates (4×10^4 cells/well).

Determination of ROS

SH-SY5Y cells were plated into 96-well plates at a density of 1×10^4 cells/well, and 24 h later the medium was substituted with the tested compounds and maintained for an additional 24 h at 37 ℃. The cells were washed with phosphate buffer solution (PBS), then loaded with 5 μM of DCFH-DA (a fluorescent probe) in PBS and incubated for 30 min at 37 ℃ in 5% CO_2. After removing DCFH-DA and washing, the cells were treated with 0.1 mM *t*-BuOOH (a compound to induce oxidative stress) in PBS for 30 min. A multifunction microplate reader (Molecular Devices, Flex Station 3) was used to measure the fluorescence intensity of the cells from each well after incubation (λ_{ex} =485 nm, λ_{em} =535 nm).The antioxidant activity, expressed as a percentage of the control cells, was calculated according to the formula (Ft-Fnt)/(Ft'-Fnt) ×100, where Ft = fluorescence intensity of neurons treated with the tested compound, Ft' = fluorescence intensity of neurons treated without the tested compound, and Fnt = fluorescence intensity of neurons untreated with *t*-BuOOH.

Rotenone/oligomycin A-induced death of human neuroblastoma SH-SY5Y cells

The inhibitory effect of 10c on mitochondrial-triggered

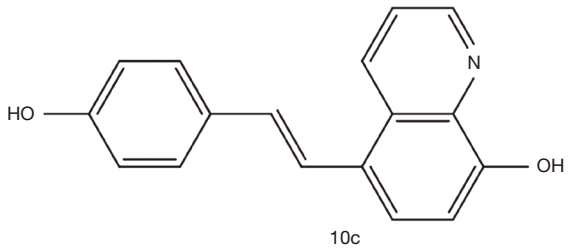

Figure 1 Structure of 10c.

endogenous oxidative stress induced by rotenone/oligomycin A was evaluated with the human neuroblastoma cell line SH-SY5Y, while the mixture of rotenone and oligomycin A acted as the toxic insult (16,17). SH-SY5Y cells were exposed to 30 μM rotenone plus 10 μM oligomycin A for 24 h in the presence or absence of compound 10c and then measured with the MTT reduction method.

Measurement of NO production

The NO concentration was determined according to the Griess reaction (18,19). BV-2 cells were plated into 96-well plates at a density of 4×10^4 cells/well, and 24 h later cells were treated with 1.0 μg/mL lipopolysaccharide (LPS) in the presence or absence of compound 10c for 24 h. Then, 50 μL of culture supernatant was mixed with an equal volume of freshly prepared Griess reagent (1% sulfanilic acid in 5% phosphoric acid, 0.1% N-1-naphthylethylenediamine) at room temperature in the dark. After 10 min, the absorbance at 540 nm was determined by a multifunction microplate reader (molecular devices, flex station 3). Nitrite concentration was calculated with sodium nitrite as a standard. Inhibition (%) = [1– ($A_{LPS+sample}$ – $A_{untreated}$)/(A_{LPS} –$A_{untreated}$)] ×100. These experiments were measured in quintuplicate, and the IC_{50} values were calculated using GraphPad Prism version 5.0. Quercetin was used as a positive control.

Rat IL-1β ELISA method

The quantification of IL-1β was performed using the IL-1β immunoassay kit provided by R&D Systems, which is a 4.5 h solid-phase ELISA designed to measure rat IL-1β levels. A rat IL-1β monoclonal antibody was pre-coated onto a microplate (20). The supernatant of BV-2 cells was treated with 1.0 μg/mL LPS in the presence or absence of compound 10c for 24 h. Then, standards and samples were pipetted into the wells and any rat IL-1β present was bound by the immobilized antibody. After washing to remove any unbound substances, an enzyme-linked polyclonal antibody specific for rat IL-1β was added to the wells. Following washing away any unbound antibody-enzyme reagent, a substrate solution was added to the wells, and the intensity of the color produced, which was measured after the color development was stopped, was proportional to the amount of rat IL-1β bound in the initial step. The experiments were measured in quintuplicate and analyzed using GraphPad Prism version 5.0.

Animal experiments

Chemicals and reagents

$A\beta_{25-35}$ (Cat: RP10008, Sigma) was dissolved in 5 μg/μL sterilized normal saline and cultured in an incubator at 37 ℃ for 7 days to convert it to the neurotoxic form (aggregation form). Compound 10c and CQ were dissolved in 0.5% carboxymethyl cellulose sodium (CMC-Na) salt solution.

Animals

Forty Wistar male rats weighing 250–300 g were purchased from the laboratory animal center of Sun Yat-sen University (Guangzhou, China). The rats were randomly divided into 4 groups: the sham group, the model group, the CQ group (21 mg/kg), and the 10c (35 mg/kg) group, with 10 rats in each group. Rats were provided with sterile food and water according to institutional guidelines, and raised on a 12 h light/dark cycle (light from 07:00 to 19:00) at a temperature of 20–22 ℃ and relative humidity of 60–70%. Rats were fasted overnight and allowed free access to water before each experiment. Animal experiments were performed under a project license (No. 20175000102) granted by the ethics committee of Sun Yat-sen University, in compliance with Chinese national guidelines for the care and use of animals.

Intrahippocampal injection of $A\beta_{25-35}$

Stereotaxic surgery was performed under chloral hydrate (7%, i.p.) anesthesia. $A\beta_{25-35}$ solution (aggregated form, 10 μg/2 μL) was injected by a microsyringe (10 μL gauge) laterally into the hippocampal fissure. The stereotaxic coordinates of the injection were as follows: AP =–4.2 mm, L =3.0 mm from the bregma, H =+4.2 mm from the dura. The injection lasted for 2 min and the needle was left in place for 8 min at the end of injection. At the same time,

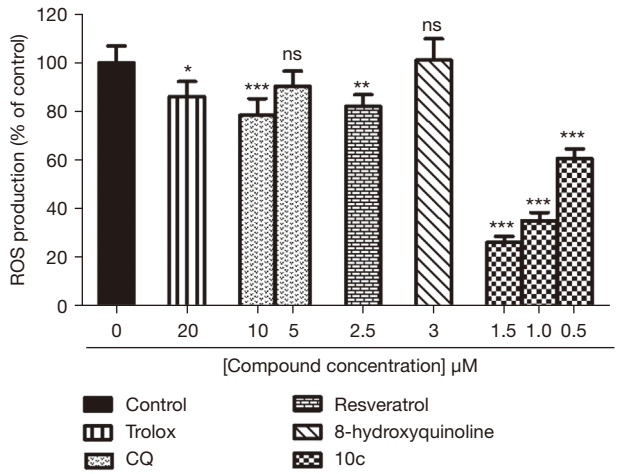

Figure 2 Inhibition of t-BuOOH-induced ROS production by trolox, CQ, resveratrol, 8-hydroxyquinoline, and compound 10c determined by DCFH-DA. Data was presented as mean ± SD minimally from 3 independent experiments and was expressed as percent of the control (untreated with compound). Three independent experiments were performed in sextuplicate. Statistical comparisons with the control were performed with GraphPad Prism 5.0 software by one-way ANOVA, followed by Dunnett's multiple comparison test (levels of significance: *, P<0.05; **, P<0.01; ***, P<0.001, ns = not significant). ROS, reactive oxygen species; CQ, chelator clioquinol.

sham rats were injected with saline following the same experimental procedure. The animals were transferred to individual cages after the surgery. Rats in the sham group and model group were intragastric administrated with 0.5% CMC-Na solution (4 mL/kg), for 28 consecutive days, once per day, CQ group and 10c group were intragastric administrated with CQ (21 mg/kg), and 10c (35 mg/kg) for the 10c group once per day, respectively.

Morris water maze test

The Morris water maze test was performed on day 29 for 6 days. Firstly, rats received visible-platform training for 5 consecutive days (4 trials/day), with the platform placed in a different location every time. Then, rats were allowed to swim to a flag-mounted platform above the water. The rat was permitted to stay on the platform for 15 s, and if it located the platform within 120 s, the seeking time (namely the escape latency) and the path it took were recorded. Otherwise, the rat was directed onto the platform and the searching time was recorded as 120 s. To assess memory retention, a spatial probe trial was performed 24 h after the last visible-platform trial. The platform was removed from the pool and the rats had 120 s to swim to locate the platform. The number of rats crossing the former platform and the time spent in the target quadrant were used to indicate the level of memory retention that occurred after learning.

Statistical analysis

Data were presented as mean ± standard deviation, and results were compared using a one-way ANOVA method with a Fisher LSD post-hoc comparison. A significant difference was defined as P<0.05, and a highly significant difference was defined as P<0.01.

Results

Intracellular antioxidant activity against exogenous ROS

Oxidative stress-induced damage has been proven to be a significant event in AD patients, which is associated with damage to DNA, protein oxidation, and lipid peroxidation. Therefore, the scavenging of ROS by some drugs may be an effective approach for the treatment of AD.

As shown in *Figure 2*, ROS production of SH-SY5Y cells treated with trolox at 20 μM was 86.15% of the control, indicating that trolox had moderate antioxidant activity. In contrast, compound 10c exhibited far more potent antioxidant activity than trolox, resveratrol, and CQ (ROS production-10c: 26.23% of the control at 1.5 μM, 35.13% of the control at 1 μM, and 60.63% of the control at 0.5 μM; resveratrol: 82.17% of the control at 2.5 μM; CQ: 78.52% of the control at 10 μM). The strong antioxidant activity of compound 10c further supports it as a promising compound for the treatment of AD.

Neuroprotective effect of 10c

Rotenone and oligomycin A disrupt ATP synthesis by blocking complexes I and V, respectively, of the mitochondrial electron transport chain (18). Rotenone/oligomycin A is an efficient experimental method to evaluate endogenous mitochondria-triggered oxidative stress. As shown in *Figure 3*, with respect to control, cells treated only with Rotenone/oligomycin (R/O) showed a viability of 35.45%, while 10c could effectively improve cell viability from 1 to 20 μM (cell viability-10c: 41.09% of the control at 1 μM, 51.76% of the control at 2 μM, 71.58%

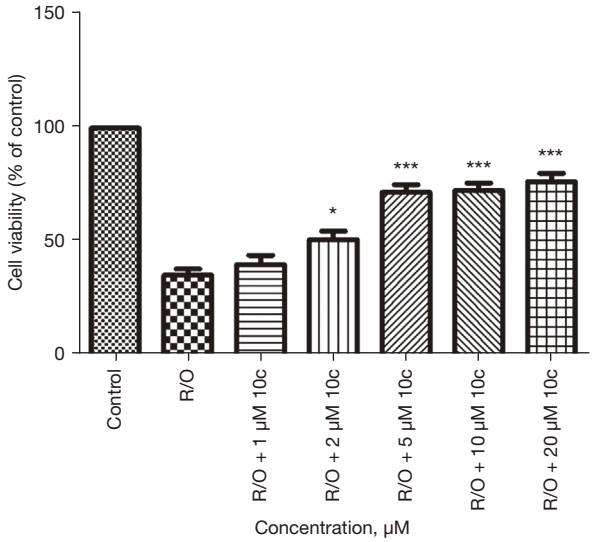

Figure 3 Protection by 10c against rotenone/oligomycin A (R/O)-induced toxic insult in SH-SY5Y cells. Cell viability was determined by the MTT reduction method. Data was presented as mean ± SD minimally from 3 independent experiments and was expressed as percent of control. Three independent experiments were performed in sextuplicate. Statistical comparisons of R/O results were performed with GraphPad Prism 5.0 software by one-way ANOVA, followed by Dunnett's multiple comparison test (levels of significance: *, P<0.05; ***, P<0.001).

of the control at 5 μM, 72.54% of the control at 10 μM, 76.58% of the control at 20 μM). Thus, 10c shows good neuroprotective effects as an endogenous antioxidant in neuroblastoma cells.

Compound 10c suppresses NO level in LPS-induced BV-2 microglial cells

NO, a proinflammatory mediator, is overexpressed in the neuroinflammatory process (21,22). NO and superoxide cause peroxynitrite formation, which leads to oxidative stress causing cell signaling pathway disruption, cellular dysfunction, and brain cell death (23,24). As shown in *Figure 4*, the positive control, quercetin, exhibited moderate activity with an IC_{50} value of 10.00 μM. Compound 10c demonstrated good inhibitory activity against NO production with an IC_{50} value of 3.10 μM, suggesting that it may be an efficient agent for the treatment of neurodegenerative diseases with neuroinflammatory involvement.

Compound 10c reduces IL-1β production in vitro

Neuroinflammation plays an important role in the pathogenesis of AD, and the proinflammatory factor IL-1β plays a central role in immune and inflammatory responses

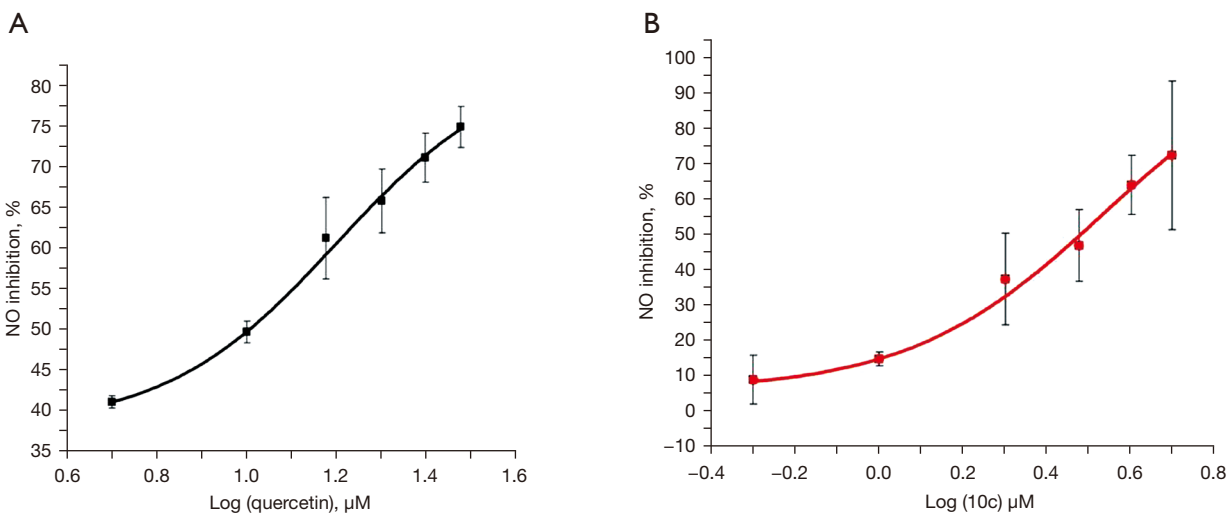

Figure 4 Inhibitory activity against NO production. (A) The inhibitory curves of quercetin (positive control) against LPS-induced NO production in BV-2 cells; (B) the inhibitory curves of 10c against LPS-induced NO production in BV-2 cells. NO, nitric oxide; LPS, lipopolysaccharide.

(25,26). As shown in *Figure 5*, 10c effectively reduced the release of IL-1β from 0 to 20 μM, and it was a potential inhibitor of IL-1β production.

Protective effects of compound 10c on $A\beta_{25-35}$-induced learning and memory dysfunction in rats

In order to evaluate the therapeutic effect of 10c in an AD animal model, we performed the Morris water maze test to assess $A\beta_{25-35}$-induced learning and memory dysfunction in rats treated with compound 10c (27). CQ was used as the reference compound. As shown in *Figure 6A*, the number of model group rats who crossed the former platform was 1.9±1.2 in the spatial probe trial, which was much less than the sham group (7.1±4.4). These results suggest that intrahippocampal injection of $A\beta_{25-35}$ could induce memory and spatial learning deficiency in rats. However, the numbers of rats who crossed the former platform in both the 10c (5.7±1.6) and CQ (5.1±1.7) treatment groups were significantly increased (P<0.05) compared to the model group. Meanwhile, the results of the time spent in the target quadrant were similar to the numbers of rats crossing the former platform. Compared with the sham group (44.8± 11.1 s), the vehicle-treated model group (32.7±4.1 s) showed a significant reduction (P<0.05) in the time rats spent in the target quadrant (*Figure 6B*). Both 10c (43.8±5.5 s) and CQ (44.1±6.6 s) treatment could significantly prolong the time rats spent in the target quadrant compared to the vehicle-treated model group. These results demonstrated that 10c could alleviate the learning and memory dysfunction of rats induced by $A\beta_{25-35}$ to a certain extent. Compared with well-established transgenic mouse model, the animal model we used in this study may just can mimic the early stage of Alzheimer's disease. The long-term evaluation of 10c in AD transgenic mouse model would be carried out in our subsequent work.

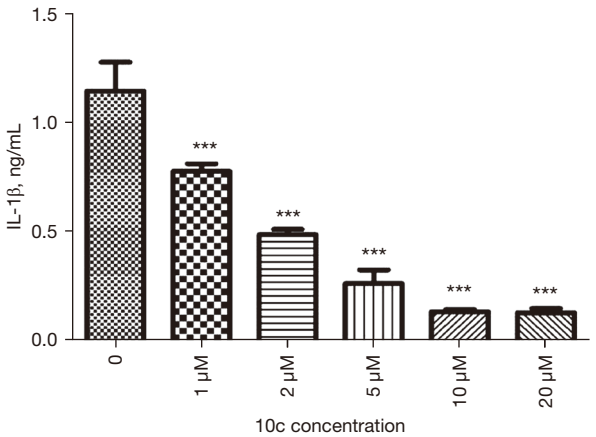

Figure 5 IL-1β secretion. Compound 10c prevented the increase of IL-1β induced by LPS. Cell culture supernatants were harvested to determine IL-1β secretion by ELISA. Values were presented as mean ± SD of 5 independent BV-2 cell replication experiments (levels of significance: ***, P<0.001). LPS, lipopolysaccharide.

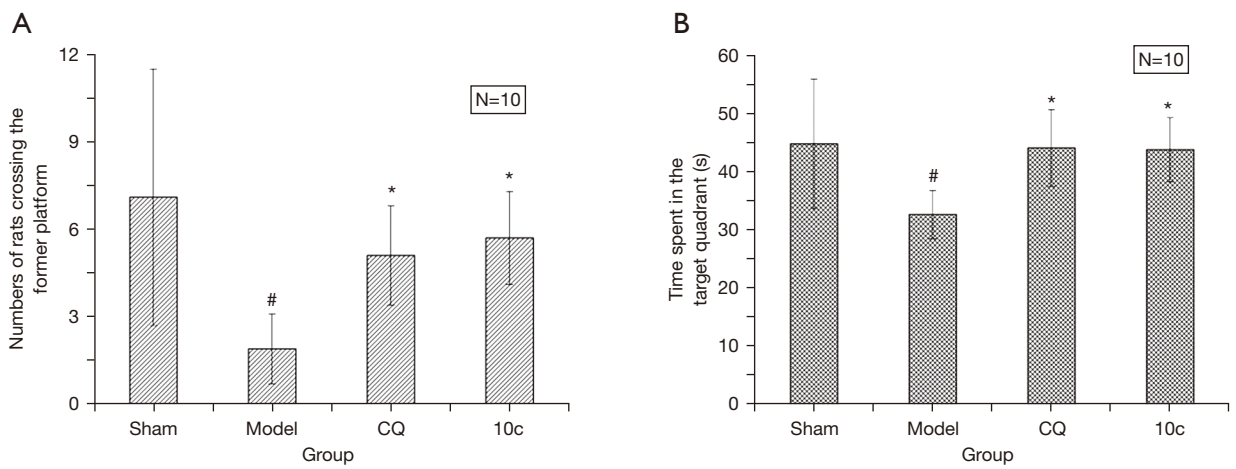

Figure 6 Morris water maze test for rats in the sham group, model group, CQ group, and 10c group. (A) The numbers of rats crossing the former platform in each group in the spatial probe trial; (B) the time each group of rats spent in the target quadrant in the spatial probe trial. #, P<0.05 compared with the sham group; *, P<0.05 compared with the model group (ANOVA for repeated measures). CQ, chelator clioquinol.

Conclusions

Based on previous research, the 8-hydroxyquinoline-resveratrol derivative 10c was studied for its intracellular antioxidative activities, anti-inflammatory activity, and protective effects on $A\beta_{25-35}$-induced learning and memory dysfunction in rats. In summary, 10c showed good activity in inhibiting exogenous and endogenous ROS, and could effectively inhibit the release of proinflammatory mediators such as NO and IL-1β. Importantly, *in vivo* tests indicated that 10c has a good protective effect on the $A\beta_{25-35}$-induced learning and memory dysfunction of rats. Altogether, compound 10c exhibited multi-funtional anti-AD activities, further research will focus on the development of compound 10c as a promising new drug for treatment of Alzheimer's disease.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/apm-21-1983). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 20175000102) granted by the ethics committee of Sun Yat-sen University, in compliance with Chinese national guidelines for the care and use of animals.

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