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Research report

Quercetin and rutin prevent scopolamine-induced memory impairment in zebrafish

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ABSTRACT

Demographic aging gives rise to a growing population with age-associated behavioral and cognitive deficits that may be associated at least partially to the increasing prevalence of neurodegenerative disorders, such as Alzheimer's disease (AD). In this disease, it has been observed a decrease in the cholinergic system, which is crucial to memory formation. Scopolamine-induced amnesic effect, through the disruption of the cholinergic neurotransmission, is one of the approaches used to investigate the mechanisms involved in cognitive impairment observed in AD. The aim of our study was to investigate the potential protective role of quercetin and rutin against scopolamine-induced inhibitory avoidance memory deficits in zebrafish. Scopolamine (200 μM dissolved in the tank water for 1 h) given pre-training hindered memory formation while both quercetin and rutin pretreatments (50 mg/kg, single injection, i.p.) prevented the scopolamine-induced amnesia. None of the compounds affected zebrafish general locomotor activity. Together, these results contribute to the increase of the knowledge about plant compounds applicability as medicines to prevent and treat neurodegenerative diseases, like Alzheimer's disease.

1. Introduction

Aging in humans is accompanied by structural and neurophysiological changes in the brain and variable degrees of cognitive decline. A central issue arises when these behavioral and cognitive deficits are accompanied with severe dementia, impacting individuals and their caring personal life quality. Demographic aging, in combination with population increasing life expectancies [31], favors the manifestation of neurodegenerative diseases since the best known risk factor for age-related diseases, including Alzheimer's disease (AD), is aging itself [2].

The Alzheimer's disease is a multifactorial disorder with a complex combination of genetic and non-genetic components [45,54], whereas the non-genetic or sporadic form represents the majority of the cases [54] and involves inflammation, glutamatergic toxicity, mitochondrial and proteassomal dysfunction, the activation of apoptosis pathways and oxidative stress [35,38,46]. Despite these components, AD patients experience disturbances in various neurotransmitter systems. Although deficits in glutamatergic, serotoninergic, and catecholaminergic systems are common in this pathology, functional deficits in the cholinergic system are strongly related to AD symptoms [7,18,21,27]. These changes include a reduction in acetylcholine (ACh) production, leading to a decreased availability of ACh at the neuronal synapse [5,12,28]. This reduction is believed to contribute to memory decline characteristic of AD. Drugs that antagonize cholinergic transmission, such as the muscarinic antagonist scopolamine, have profound amnesic effects in a variety of learning paradigms and the use of this drug is considered an useful approach to investigate the mechanisms involved in cognitive impairment observed in AD [29,32]. Furthermore, AD patients also present an augmented clearance of acetylcholine in the synaptic cleft, as a result of an increase in the enzymatic activity of the protein responsible for the hydrolysis of this neurotransmitter, acetylcholinesterase (AChE) [48]. These findings resulted in the establishment of the AD cholinergic hypothesis, in 1982 by Raymond T. Bartus [3].

This hypothesis was the basis for the development of drugs prescribed until today for AD treatment, called acetylcholinesterase inhibitors (AChEi), which prolong the action of acetylcholine in

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the synapse by preventing its breakdown. This strategy results in improvements of cognition, mood, and behavior [37]. Even though there are modest benefits following the treatment with AChEi to the progression of AD, its use is limited because of the occurrence of severe side effects [9]. The identification of effective treatments will require a better understanding of the physiological mechanisms involved and the development of multi-targeted drugs as a result of innovative approaches [38].

There is a growing interest in the potential of phytochemicals to improve memory, learning, and general cognitive ability [45,50]. There is a large amount of excellent reviews highlighting the effects of phytochemicals as modulators of brain function [55–58]. The main dietary sources of these phytochemicals are fruits, vegetables and plant-derived beverages such as tea and red wine (for a complete review see [54]). This wide class of phytochemicals includes the polyphenols, the most abundant dietary antioxidants [27]. Some studies already reported a possible relationship between polyphenol ingestion and the prevention of AD [25,54]. The polyphenols are a large group of compounds that can be divided into several subgroups, including the flavonoids. Two of the flavonoids most widely and abundantly present in herbs and plants consumed by men are quercetin and rutin [49,54].

Quercetin has been reported to exert numerous pharmacological activities, such as free radical scavenging [24,57,58] and anticarcinogenic effects [8]. In addition, quercetin has the potential to bind to the ATP-binding sites of a large number of proteins [10]. Studies have suggested that quercetin and other polyphenolic substances can inhibit ecto-5'-nucleotidase/CD73 activity [26]. These actions can affect the cell function by altering the phosphorylation state of target molecules and/or by modulating gene expression [66]. In addition, rutin, studied since 1946, is a quercetin glycoside which has been reported to increase the scavenge of free radicals [24,40,41,53], vascular resistance [52], decrease hepatic and blood cholesterol levels, and also shows antiplatelet activity [40,53]. Furthermore, there is a large amount of data reporting the behavioral effects induced by polyphenols [1,21,27,28,56,58,63], including quercetin and rutin [44]. Most of these studies have shown a positive effect of these compounds in ameliorating rodents learning, memory, and cognition through different behavioral tasks [1,21,27,28,44,56,58,63].

There has been a growing interest in the development of novel animal models that could simulate human disease features, contributing to the increase in the knowledge about cellular mechanisms involved in these pathologies. Zebrafish, together with forward genetics and pharmacological interventions, has become a promising model to many human diseases, such as neurodegenerative diseases [33,51]. As a result, zebrafish has already been suited for studies regarding Huntington Disease, Parkinson Disease, Schizophrenia, and AD [reviewed in 4]. Recent study [29] has shown that scopolamine impaired the acquisition and retention of the avoidance response in zebrafish, and physostigmine, one of the available drugs for AD treatment, reversed scopolamine-induced learning deficits when treated prior to the administration of scopolamine, contributing to the suitability of this model to research on AD cognitive aspects. Therefore, considering that: (i) AD is a multifactorial disease which prevalence is expected to increase throughout the years, (ii) available treatments present modest cognitive benefits but severe side effects, a fact that claims for the development of other strategies to treat this neurodegenerative disease, (iii) recent studies suggest the multitargeted action of polyphenols and, finally (iv) polyphenols are natural compounds that do not present collateral effects, we sought to investigate the effects of acute quercetin and rutin treatment in a model of pharmacological cognitive impairment achieved with scopolamine, an antimuscarinic drug, in zebrafish.

2. Materials and methods

2.1. Animals

Wild-type adult (<8 months old) zebrafish of both sexes were obtained from specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept in 50 L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O_2/l) at 25 ± 2 °C, under a 14–10 h light/dark photoperiod in at a density of up to five animals per liter. Animals were acclimated for at least 2 weeks to acclimate before the experiments. They were fed three times a day with TetraMin Tropical Flake fish®. The procedures were previously approved by the Animal Ethics Committee of Pontifical Catholic University of the Rio Grande do Sul (PUCRS) under the number 109/09-CEUA.

2.2. Chemicals

Quercetin ($C_{15}H_{10}O_7$, CAS number 117-39-5), Rutin hydrate ($C_{27}H_{30}O_{16}{\cdot}H_2O$, CAS number 207671-50-9), (−)-scopolamine hydrobromide trihydrate ($C_{17}H_{21}NO_4{\cdot}HBr{\cdot}3H_2O$, CAS number 6533-68-2), benzocaine ($C_9H_{11}NO_2$, CAS number 94-09-7), Tween 20 ($C_{58}H_{114}O_{26}$ CAS number 9005-64-5) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.3. Animal procedures

In order to guarantee the polyphenol doses administered, quercetin and rutin were suspended in Tween 20 (1%) in saline. The doses of the polyphenols were chosen based on a previous study that have demonstrated neuroprotective actions of i.p. administration of quercetin and rutin in rodents [44]. In that study, quercetin and rutin (at 50 mg/kg) reduced the spatial memory impairment and neuronal death induced by repeated cerebral ischemia in rats. Here, we used the same dose adjusted to the fish bodyweight (mean injection volume was 10 μL) in a 20 mL/kg regimen. I.p. injections were conducted using a 3/10-ml U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16″) × 31G Short Needle (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps et al. [42]. Briefly, anesthesia of the animals prior to the injection was obtained by its immersion in a benzocaine solution (1 mM in MeOH 1%) until the animal shows lack of motor coordination and reduced respiration rate. The anesthetized animal was gently put in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). The needle was inserted parallel to the spine into the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted to guarantee that the animal do not spend more than 10 s out of the water. After the injection the animals were placed in a separate tank with highly aerated unchlorinated tap water (25 ± 2 °C) to facilitate the animals recovery from the anesthesia. Quercetin and rutin were injected 2 h before the beginning of experiment. One hour before the beginning of the training session, the animals were transferred another tank to receive the second treatment, consisted of the scopolamine treatment (200 μM dissolved in the water for 1 h) as described previously [29]. The animals that did not receive scopolamine were also transferred to another tank filled with water to ensure the homogeneity of stress presented to the fish. After the training the animals were transferred to another empty tank equal to the previous one. Tween 20 was used as control. Both drugs and vehicle were prepared freshly in the experimental day. All the animals have recovered after 2–3 min following the injection. Animals that did not recover during this period were discarded.

2.4. Behavioral analysis

2.4.1. Inhibitory avoidance

Zebrafish were individually trained and tested for long-term memory in the inhibitory avoidance paradigm as described in detail by [6]. Briefly, an 18 cm L × 9 cm W × 7 cm H glass tank divided in two equally sized compartments, designated hereon as dark and white, by a sliding guillotine-type partition (9 cm × 7 cm) was used. The tank water level was 3 cm and the partition raised 1 cm above the tank floor to allow zebrafish to swim freely from one side of the tank to the other. Two electrodes extending through the wall height and placed on each end side of the dark walls attached to an 8 V stimulator administered a final 3 ± 0.2 V AC shock when manually activated. On training session, animals were placed in the white side of the tank while the partition between compartments was closed. After 1 min of familiarization with the new environment the partition was raised, allowing fish to cross to the dark side of the tank. When animals entered the dark side with their entire body the sliding partition was closed and a pulsed electric shock administered for 5 s. Fish were then removed from the apparatus and placed in the dedicated temporary tank. Animals were tested 24 h after training. The test session repeated the training protocol except that no shock was administered and animals immediately removed from the dark compartment. The latency to completely enter the dark compartment was measured on both sessions and the test latencies used as an index of retention. The saline control and Tween 20 control groups did not differ on any measure; therefore, the saline treated-groups were excluded from the

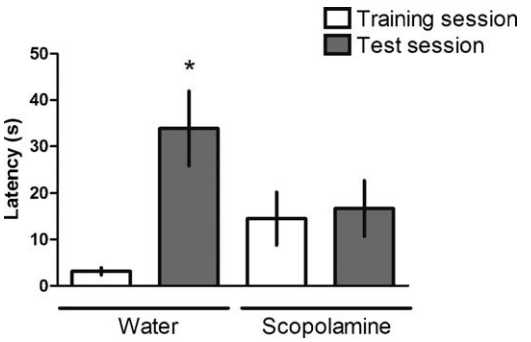

Fig. 1. Scopolamine induces long-term memory deficits in inhibitory avoidance. Effect of scopolamine on latency to cross to dark compartment in training and long-term memory test sessions in the inhibitory avoidance task. Animals were pre-treated with scopolamine 1 h prior to training session. Control animals were manipulated similarly but exposed to water only. Data are presented as mean ± S.E.M. ($N = 7$ per group). *Indicates $p < 0.05$ between training and test sessions compared by Wilcoxon matched pairs test. No differences were found between training performance among all groups by Kruskal–Wallis test.

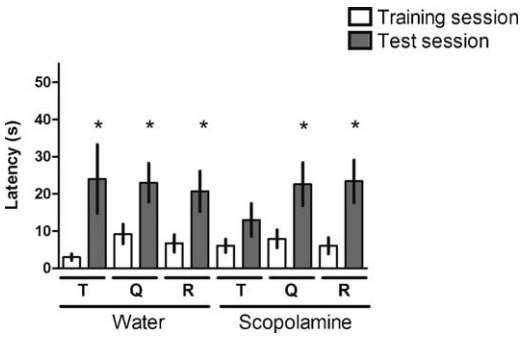

Fig. 2. Scopolamine induced cognitive deficits are prevented by quercetin and rutin. Effects of scopolamine on latencies to enter the dark compartment for animals trained on the inhibitory avoidance task. T, tween-treated animals, Q, quercetin-treated animals, R, rutin-treated animals. Animals received single intraperitoneal injection of Tween 20 (1%), quercetin or rutin (50 mg/kg) 2 h before the training session. The i.p. treatment was combined by water or scopolamine 1 h exposure prior to test. Control animals were manipulated equally as treated animals, except that they received water instead scopolamine. Data are presented as mean ± S.E.M ($N > 7$ per group). *Indicates $p < 0.05$ between training and test sessions for each group compared by Wilcoxon matched pairs test. No differences were found between training performance among all groups by Kruskal–Wallis test.

graphic representation of the results. Tween 20 will be referred to as the control group.

2.4.2. Exploratory assessment

Behavioral testing of drug effects took place during the light phase between 10:00 a.m. and 12:00 a.m. The behavioral screening test was performed 30 min after the injection (saline, Tween 20, quercetin or rutin) or after the beginning of the second drug treatment (water or scopolamine), on the inhibitory avoidance training day. The animals were individually transferred to a 2.7 L tank (24 cm L × 8 cm W × 20 cm H) with laterals and bottom white covered, except of the front to avoid any visual disturbances, and were first habituated to the tank for 30 s, as previously described [17]. There was no drug exposure during behavioral experiments. The locomotor activity of the animals was video recorded using Logitech Quickcam PRO 9000 for 5 min after the habituation period and further analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). The tank was divided into two equal sections with one horizontal line, and the following behavioral patterns were measured: distance traveled, mean speed, number of line crossings (horizontal line), absolute turn angle, and time spent in upper and lower half.

2.5. Statistical analysis

Inhibitory avoidance memory data are presented as mean ± S.E.M. Training and test sessions for each group were compared by Wilcoxon matched pairs test. Latencies of multiple groups were compared using Kruskal–Wallis followed by Mann–Whitney *U* tests. Data of the exploratory assessment were expressed as mean ± S.E.M. of 4 different animals for each group and were analyzed by one-way ANOVA test followed by Tukey test as post-test, considering treatment as factor. *$p < 0.05$ denotes a significant difference from the control group.

3. Results

3.1. Scopolamine induces memory deficits in an inhibitory avoidance paradigm

Adult (>8 months old) male zebrafish long-term memory was tested in the inhibitory avoidance apparatus developed by [6]. To first establish the effectiveness of the scopolamine in the one-trial inhibitory avoidance task, we have treated the animals with scopolamine (200 μM dissolved in tank water for 1 h) for 1 h previous to training session (Fig. 1). While water-exposed animals showed a robust retention of memory on the test session performed 24 h after training when training and test latencies were compared ($p < 0.05$), pre-training scopolamine hindered memory formation ($p = 0.7792$, comparison between training and test session of scopolamine-treated animals). Importantly, no differences were found between training latencies for both groups (Mann–Whitney test). Scopolamine's ability to induce memory deficits when given pre-training at the inhibitory avoidance has been shown in rodents and our data in zebrafish supports its effect to evaluate memory enhancing drugs.

3.2. Scopolamine deficits are prevented by quercetin and rutin treatment

The ability of quercetin and rutin to prevent scopolamine-induced inhibitory avoidance memory deficits was evaluated by combining an initial quercetin and rutin i.p. treatment with the pre-training scopolamine exposure (Fig. 2). Tween 20 was used as the vehicle for quercetin and rutin and it did not differ from water being therefore considered the standard control treatment of the experiment. Animals receiving Tween 20 (control group), quercetin or rutin only effectively learned, showing significant differences from their respective training and test sessions ($p < 0.05$ for each group analyzed separately). Scopolamine pre-training again hindered memory formation, as observed in the lack of long-term memory retention when training and test sessions were compared in animals that received Tween 20 i.p. followed by scopolamine. Acute single pre-treatment with quercetin or rutin 1 h before the beginning of scopolamine treatment prevented the memory impairment caused by scopolamine, as shown by a statistically different latency between training and test sessions for both rutin and quercetin-treated animals ($p < 0.05$ for either group).

3.3. Scopolamine and polyphenols effects on exploratory assessment

Distinct parameters of zebrafish swimming activity were examined in the tank diving behavioral test. No evident effect of the i.p injections and previous anesthesia were observed and probably it did not interfere in the data analysis since all animals were submitted to the same manipulation. As indicated by the distance traveled, mean speed and line crossings, no differences were found in the locomotor activity of animals receiving any of the treatments when compared to the control group (Fig. 3 A–C, respectively). None of the treatments have affected the swimming coordination neither the general swimming pattern of the animals, as shown by the absence of statistically different alterations in the absolute turn angle (Fig. 3D). No differences in the time spent in the upper half or the lower half were found in treated animals in comparison with the control group (Fig. 3E and F, respectively) showing no anxiogenic properties of any treatment.

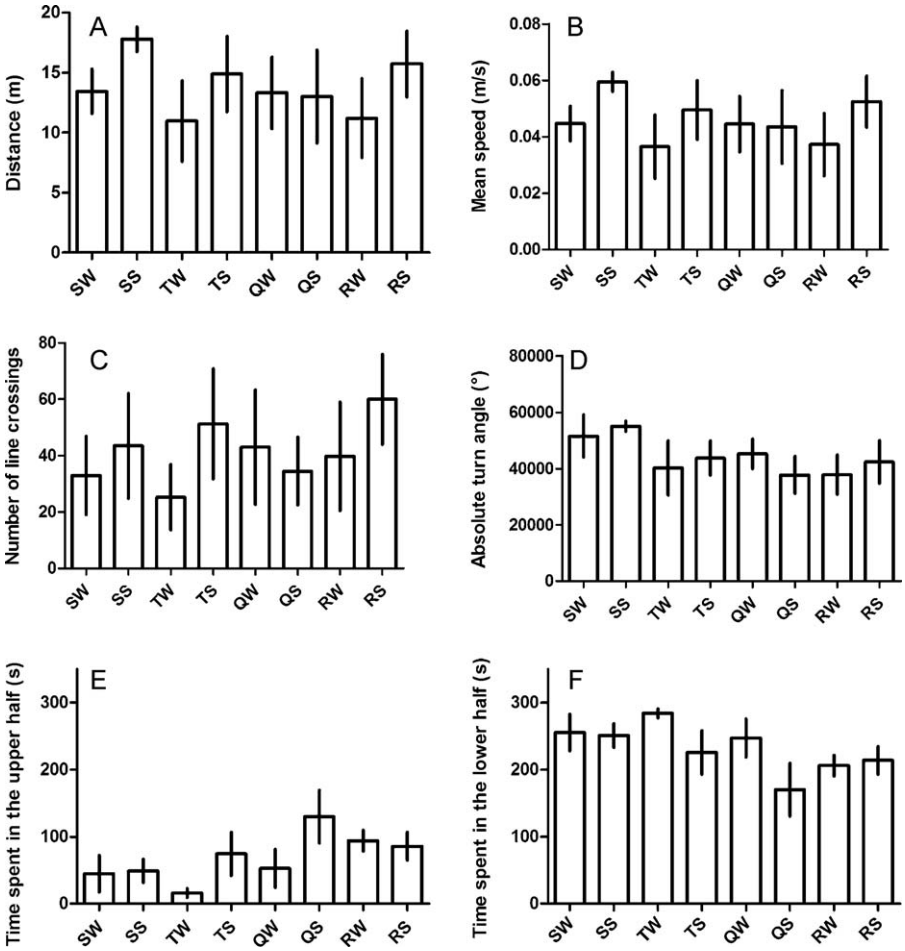

Fig. 3. Neither scopolamine nor quercetin or rutin affects zebrafish locomotor activity. Effect of exposure to saline, Tween 20, quercetin, and rutin, followed by water or scopolamine exposure on the distance traveled (A), mean speed (B), number of line crossings (C), absolute turn angle (D), time spent in the upper zone (E), and in the lower zone (F) determined during 5 min of video recording in the tank diving behavioral test. SW, saline + water, SS, saline + scopolamine, TW, tween + water, TS, tween + scopolamine, QW, quercetin + water, QS, quercetin + scopolamine, RW, rutin + water, RS, rutin + scopolamine. Animals received single intraperitoneal injection of Saline, Tween 20 (1%), quercetin and rutin (50 mg/kg) 2 h before the beginning of the video recording, and were transferred to the second treatment (water or scopolamine 200uM dissolved in the tank water) 1 h before the video recording. Data were expressed as mean ± S.E.M. of 4 different animals for each group and were analyzed by one-way ANOVA test.

4. Discussion

In the present study, we have evaluated the potential preventive role of the flavonoids quercetin and rutin to prevent the scopolamine-induced memory deficits in zebrafish. In accordance with previous studies [29], our results have shown that scopolamine induced robust impairment of memory in the inhibitory avoidance behavioral task. Interestingly, we have found that one single intraperitoneal injection of 50 mg/kg of quercetin or rutin at 1 h before the scopolamine treatment prevented scopolamine induced memory deficits. Scopolamine, the tropane alkaloid originally isolated from classical nightshade, such as *Atropa belladonna* L. [66] is a competitive antagonist of the muscarinic acetylcholine receptor (mAChR) [62]. Besides its use as a treatment for central nervous system dysfunctions such as motion sickness, shaking palsy and oppioid addiction [65], it became widely used as a standard/reference drug for inducing cognitive deficits in a wide range of animal models, especially after the postulation of the cholinergic hypothesis of geriatric memory dysfunction [3].

Despite the scopolamine classical use as an amnesic agent, there is a lot of discrepancy in relation to scopolamine effects in the locomotion. Some studies, in fact, challenge the viability of scopolamine use as a cognitive impairer, questioning if the alterations in behavior are related to peripheral locomotor effects, instead of memory disruption [for a review, see 32]. To address this problem we have performed a general analysis on zebrafish locomotor behavior. As shown in the Fig. 3, there were no changes in none of the parameters analyzed. We have also shown that pre-exposure to scopolamine for 1 h immediately before inhibitory avoidance training did not impact training performance, since control and scopolamine-treated animals training session latencies did not differ. In light of this evidence we believe that the scopolamine-induced memory deficits observed were solely due to the drug effect on the cholinergic system.

The cholinergic system is involved in many physiological processes, including synaptic plasticity and learning and memory [14,43,59,64]. Cholinergic agonists can facilitate memory, whereas cholinergic antagonists can impair memory [35]. Studies of the effects on brain plasticity of cholinergic agents, particularly those engaging muscarinic receptors, have provided robust and clarifying information about learning and memory processes [23]. In addition, the cholinergic hypothesis of geriatric memory dysfunction and evidence of the involvement of this system in the etiology of AD have brought global attention to cholinergic interventions as a treatment for this disease.

In that sense, molecules that could modulate the cholinergic hipoactivity related cognitive effects have potential clinical use [13]. Cholinesterase inhibitors, such as Tacrine, Galantamine, Physostigmine, and Donepezil were designed to ameliorate cholinergic deficits by slowing the rate of acetylcholine degradation after

its synaptic release, but the use of these medicines is not always well accepted by the patients due to their severe side effects [9,47], high cost, and scarcity of robust benefits [7,34].

Quercetin and rutin, natural compounds widely found in the diet, have been studied for a long time and shown to have wide physiological effects [54]. The effects of flavonoid-rich diet on cognitive function have been linked to the ability of flavonoids to interact with the cellular and molecular framework involved in learning and memory, including synaptic potentiation and plasticity [22]. Flavonoids also have known antioxidant abilities, effectively protecting neurons against neurotoxins, suppressing neuroinflammation, and enhancing neuronal function [61,63], stimulating neuronal regeneration and revascularization [22,54]. Flavonoids have also been reported to act as AChEi [1].

Here we have shown that these polyphenols could protect against scopolamine-induced cognitive deficits, using an inhibitory avoidance task. Aversive conditioning tasks, such as the inhibitory avoidance, have been shown useful to analyze cholinergic effects on memory, as muscarinic and nicotinic neurotransmission have been demonstrated to affect every aspect of aversive conditioning. Compelling evidence shows that cholinergic manipulations can affect memory acquisition, consolidation, and retrieval in inhibitory avoidance behavioral task, since cholinergic neurons are presented in areas engaged in learning and memory processes from this task in rodents [60].

Zebrafish is a powerful animal model in many areas of biological research. Its use ranges from toxicology, developmental biology, biomedicine, neurophysiology, drug discovery [51] model for human diseases [4,33], and behavioral analysis [6,15–17,36]. In addition to the known advantages of its use, such as the small size and maintenance cost, the transparency of embryos and larvae, and the speed at which these develop *ex utero* [19,20], zebrafish has been suited for large throughput screening for drug discovery, including those from natural sources [11,15]. Zebrafish combines the ability to perform large scale screenings yet requiring a smaller infrastructure when compared to rodents, bringing new perspectives for the drug discovery process to yet untreatable diseases, such as Alzheimer's disease. Recently, the evaluation of learning deficit induced by scopolamine [29] and PTZ [30] as well as the development of tau-transgenic zebrafish as a model of tauopaties [39] reinforce the idea that zebrafish represents an interesting model for studying the neurodegenerative disease and their related mechanisms.

According to our results, quercetin and rutin administration prevented scopolamine-induced memory deficits in zebrafish, suggesting that these flavonoids might be a preventive strategy against the development of AD. These findings, although restrict to behavioral analysis, raise a new perspective to the prevention and treatment of AD. More experiments are already being conducted to investigate candidate biochemical targets of polyphenols in the scopolamine-induced memory deficits.

Conflict of interests

The authors declare no conflict of interests.

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