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Rosa damascena and Ginkgo biloba aqueous extracts inhibited Tau phosphorylation and neurodegeneration in vitro and in vivo

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Abstract

Background: One of the environmental factors leading to Alzheimer's disease (AD) is traumatic brain injury (TBI). Meanwhile, tau protein hyperphosphorylation is known as one of the mechanisms of AD development. In the present study, the effect of Rosa damascena and Ginkgo biloba aqueous extracts on tau hyperphosphorylation was studied on SH-SY5Y cell lines and mouse TBI models.

Methods: Tau protein hyperphosphorylation was induced in SH-SY5Y cells using 10 μM retinoic acid (RA). Then, cells were treated with 500 and 1000 μg/ml aqueous extracts of Rosa damascena and Ginkgo biloba. Cell viability was studied by MTT test and tau protein hyperphosphorylation was studied by western blot and immunostaining techniques. Also, after the induction of TBI by pneumatic cylinder, mice were treated with 500 and 1000 μg/ml aqueous extracts of Rosa damascena and Ginkgo biloba, and the animals were tested for beam balance and walk tests to measure balance and muscle stiffness. Finally, tau protein hyperphosphorylation in the brain was investigated using an immunostaining technique.

Results: Both aqueous extracts of Rosa damascena and Ginkgo biloba were able to improve SH-SY5Y viability. Also, a decrease in phosphorylated tau protein was observed in cells treated with aqueous extracts of Rosa damascena and Ginkgo biloba. Performance improvements in beam balance and walk tests in TBI mice treated with 1000 μg/ml Rosa damascena and Ginkgo biloba aqueous extracts were seen. Also, tau protein phosphorylation was significantly decreased in the brain of TBI rats treated with those aqueous extracts.

Conclusion: aqueous extracts of Rosa damascena and Ginkgo biloba have neuroprotective effects and are beneficial in reducing TBI-induced tau protein hyperphosphorylation, and they can prevent tau pathology.

Keywords: Extract, Hyperphosphorylation, Mouse, Tau. Alphabetical order.

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disorder that eventually leads to cognitive impairment, personality changes, and severe disability (1). This disease imposes heavy costs on the health system and it is estimated that by 2050, one person will be infected with this disease every 33 seconds (2). In AD, areas related to mental functions in the brain such as the neocortex and hippocampus are affected (3). Currently, cholinesterase inhibitors are the first line of treatment, however, these drugs lead to only reductions in AD symptoms and this disease has not been completely cured yet (4).

In AD, factors such as oxidative stress (5), toxicity related to increased glutamate (6), decreased amount of acetylcholine (7),

and brain inflammation (8) are involved. Meanwhile, peptides such as amyloid beta (Aβ) are one of the most important pathological causes of AD (9). In recent years, the loss of the normal activity of tau protein as a result of hyperphosphorylation and the breakdown of neural pathways is also considered one of the main factors in the development of AD (10). When hyperphosphorylated tau protein accumulates, it forms neurofibrillary tangles (NFTs), which have been shown in studies to correlate with cognitive function loss and AD disease severity (11). There are two conformations of hyperphosphorylated tau peptide at the Thr-Pro domain: cis and trans (12). cis conformation has been found to have toxic effects on neurons and is mostly deposited in neurons, leading to cystinosis (13). This phenomenon refers to the loss of function of microtubules in neurons and cell apoptosis. Thus, this conformation of tau protein (cis) can be considered one of the biomarkers of tauopathy in neurodegenerative diseases including AD (14, 15).

As mentioned, no effective and suitable treatment for AD has been found so far. In recent years, medicinal plants have generated a lot of research interest in the treatment of this neurodegenerative disease. Ginkgo biloba belongs to the Ginkgoaceae family, and its extract has shown extensive pharmacological properties. The extract of this plant has shown neuroprotective effects in AD conditions. Recently, the effects of EGb761 extract on AD rat model induced by Aβ in Tau phosphorylation and the activity of GSK-3β and PP2A were studied and the results showed that this extract reduces Tau phosphorylation (1). It also improved spatial memory and reduced the expressions of GSK-1B and PP2A (1). Reduced Tau phosphorylation by EGb761 extract appears to be associated with decreased activity of GSK-3β and PP2A. In another study, the EGb761 extract decreased Zn-induced Tau phosphorylation at Ser262 by reducing GSK-3β activity. The extract also reduced ROS and reduced nerve cell death (2). In human P301S tau mutant-transgenic mice treated with EGb761 extract for 5 months, inhibition of p38-MAPK and GSK-3β activities was seen, resulting in decreased tau phosphorylation (3). Activation of PI3K/Akt pathway by Ginkgolide A isolated from Ginkgo biloba also reduced Tau phosphorylation (4).

Rosa sp. are shrubs that have red fruits (hips), with strong antioxidant activity. These medicinal plants have antioxidant, anti-inflammatory and antimicrobial properties and have shown therapeutic effects in some metabolic diseases (1). Interestingly, some in vitro studies have shown inhibitory effects on acetylcholinesterase (2, 3). Therefore, this plant can have the potential in inhibiting cognitive impairment in AD. However, it seems that knowing the mechanisms of improving cognitive performance by these plants needs more studies.

The current study aimed to investigate the neuroprotective effects of both Ginkgo biloba and Rose hip extracts in vitro and in vivo and identify their action mechanism by evaluating tau phosphorylation status in both extract-treated cell lines and mice brains.

2. Material and Methods

2.1. In vitro

2.1.1 Preparation of Aqueous extract of Rosa damascene and Ginkgo biloba

Rosa damascene hips and Ginkgo biloba leaves were obtained from Shahid Beheshti Medical Science University and then dried and ground. 1g of them was dissolved in 10 ml of distilled water and placed in a dark place for 72 hours. The resulting extract was filtered using Whatman paper, and its distillation was done with the help of a vacuum distillation device at a temperature of 40 °C. The extract was stored at 4°C.

2.1.2 Cell culture and treatment

The SH-SY5Y cell line was prepared from the Royan- Research Institute cell bank Tehran-Iran and cultured in a DMEM culture medium. Also, 10% fetal bovine serum and 100 units/mL of penicillin/streptomycin antibiotics were added to the culture medium. Retinoic acid (RA) was used to induce tau protein hyperphosphorylation (10). For this purpose, cells were exposed to 10 μM RA in the culture medium for 1 week before performing the experiments. Then, they were transferred to a serum-free culture medium. Finally, the cells (5 105 cells/mL) were treated with concentrations of 500 and 1000 μg/ml of each of the aqueous extracts of the Rosa damascene hips and Ginkgo biloba leaves for 72 hours.

2.1.3 Cell viability tests

The cells were treated with the extracts for 72 hours. Cell viability was examined using an MTT assay kit (Abcam, USA) according to the manufacturer. Briefly, an MTT solution was added to the media in a 1/6 ratio in the dark. Then the cells were incubated at 37°C for 4 hours and subjected to spectrophotometry at 490 nm.

2.1.4 Western blot

RPA buffer containing 1% PMSF was used for total protein extraction. Then, the extracted protein was run on SDS-PAGE, and the separated proteins on the gel were transferred to the PVDF membrane and kept at 24°C for 90 minutes. The primary antibodies were cis pT231-tau (a generous gift from KP Lu, Harvard), anti-Pin1 (Abcam), and β-actin (Sigma, St Louis, MO). Finally, the membrane was incubated with primary antibodies for 24 hours and then treated with horseradish peroxidase (HRP) secondary antibody.

2.6. Immunohistochemical assay

To stain the cells, first, the SH-SY5Y cell line was harvested at different time intervals. For fixation, 4% paraffin was used and then the cells were incubated for 20 min in the dark. Then, these cell lines were treated with the aforementioned primary antibodies. To ensure the staining, the permeability of the cells was augmented by exposure to 0.2% TritonX for 20 minutes and then treated with the primary antibody and incubated for 24 hours at 4°C. Finally, they were treated with Alexa Fluor 488 or 568 conjugated secondary antibodies for 60 seconds at 25°C. After treatment, the cells were observed under a confocal microscope (LSM 800, Zeiss).

2.2 In vivo

2.2.1 Preparation of animals and induction of Traumatic brain injury

24 adult Balb/C male mice (mean age: 24 weeks; MW: 24 -28 g) were obtained from the Pasteur Institute of Iran and were placed in 12-hour light and dark conditions. All animals had free access to food and water. To induce traumatic brain injury (TBI),

Induction animals were initially anesthetized with 2% isoflurane. Then, their heads were placed in a stereotaxic machine, and their scalps were incised. A pneumatic cylinder was used to induce TBI with a speed of 6 m/s, a depth of 0.6 mm, and a time of 150 ms. Then, the scalp was sutured and the animals were transferred to the cage (1). The treatment of aqueous extracts continued immediately after surgery in the form of gavage every day for 14 days. The treatment of animals was by ethical principles.

2.2.2 Beam balance and walk tests

Beam balance and beam walk tests were used before the induction of TBI and on days 1, 3, 7, 10, and 14 after the surgery to check the gross motor and fine locomotor abilities, respectively. In the first test, each animal was placed on a beam with a width of 1.4 cm and the time to maintain balance on the beam was recorded. In the Beam walk test, a beam with a width of 2.4 cm and a length of 110 cm was placed in a dark box that ended with a bright light, and the time to reach the end of the beam was recorded (2).

2.2.3 Immunohistochemistry.

Brain samples of mice were prepared for analysis after perfusion with 4% paraformaldehyde at different time intervals. For this purpose, coronal sections of 8 microns were prepared and placed on the slides. The analysis was performed according to the previously described method (9). For this purpose, RIPA buffer was used to lyse brain sections and cells, containing proteinase and phosphatase inhibitors. Polyacrylamide gel electrophoresis was used to separate proteins. Then, it was transferred to the PVDF membrane and blocked in TBST for 1 hour. Next, PVDF was treated with primary antibodies and placed at 4°C for 24 hours. After that, it was incubated with horseradish peroxidase secondary antibody. Chemiluminescence reagent (Perkin Elmer, San Jose, CA) and Quantity One from BioRad were used to detect signals and measure immunoblotting results, respectively.

The previously described method (9) was used to perform an immunostaining analysis. For this purpose, the previously prepared slides were first treated with 0.3% H_2O_2 and then placed in 10 mM sodium citrate. Later, slides were incubated with primary antibodies for 24 hours at 4°C. After overnight, these slides were treated with the secondary antibody mentioned above for 60 minutes at 25°C. Finally, the slides were observed under a confocal microscope (LSM 800, Zeiss).

2.11. Statistical analysis.

The data were analyzed using a two-way analysis of variance after ensuring the normal distribution of the data by the Kolmogorov-Smirnov test. Data were reported as mean ± standard deviation. Means were compared using Tukey's method ($P<0.05$), and GraphPad Prism V.8 software was used for data analysis.

3. Results

3.1 In vitro

3.1.1 Cell viability

Exposure of SH-SY5Y line cells with RA for 72 h led to a significant decrease in cell viability compared to the control, indicating neurodegeneration (~20%, $P=0.007$). However, 1000 µg/ml of each of Rosa damascena hips and Ginkgo biloba leaves aqueous extracts prevented an RA-induced decrease in cell viability (~14%, $P<0.01$, Figure 1). Therefore, both aqueous extracts have neuroprotective effects in vitro.

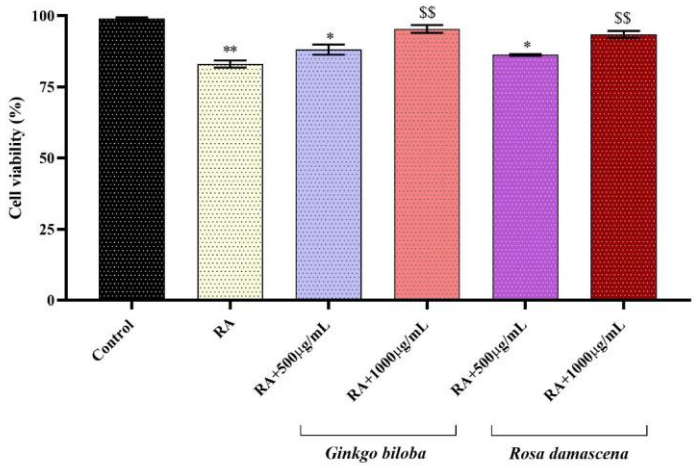

Figure 1. The viability of the SH-SY5Y cell line was exposed with retinoic acid (RA) and treated with 500 and 1000 µg/mL Rosa damascena hips and Ginkgo biloba leaves aqueous extracts after 72 h incubation. *P<0.05 and **P<0.01 show significant differences compared with control, $$P<0.01 shows significant differences compared with RA cells.

3.1.2 Phosphorylation of tau protein

In the present study, we used two techniques, western blotting, and immunostaining, to investigate tau protein phosphorylation (Figures 2, 3). The results of both techniques indicated the up-regulation of hyperphosphorylated tau protein expression in cells exposed to RA. Nevertheless, 1000 µg/L of Rosa damascena hips and Ginkgo biloba leaves aqueous extracts were able to inhibit the phosphorylation of tau protein, so that no significant difference was observed in the expression of phosphorylated tau in the cells exposed to these extracts compared to the control.

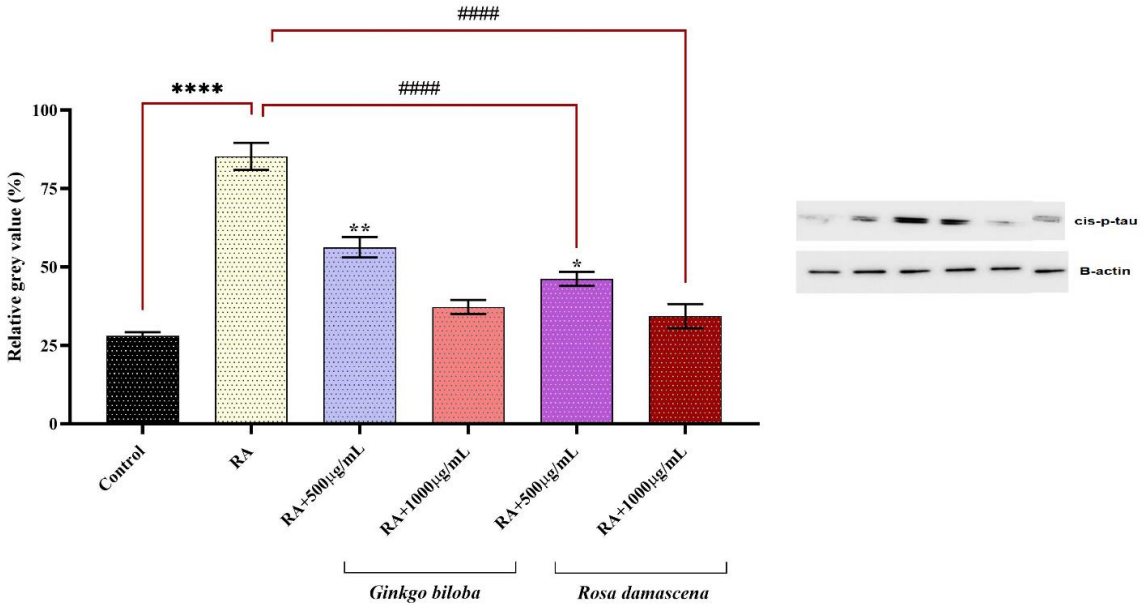

Figure 2. The western blotting of p-Tau protein of SH-SY5Y cell line exposed with retinoic acid (RA) and treated with 500 and 1000 µg/mL Rosa damascena hips and Ginkgo biloba leaves aqueous extracts after 72 h incubation. *P<0.05,**P<0.01 and ****P<0.0001 show significant differences compared with control, ####P<0.0001 shows significant differences compared with RA cells.

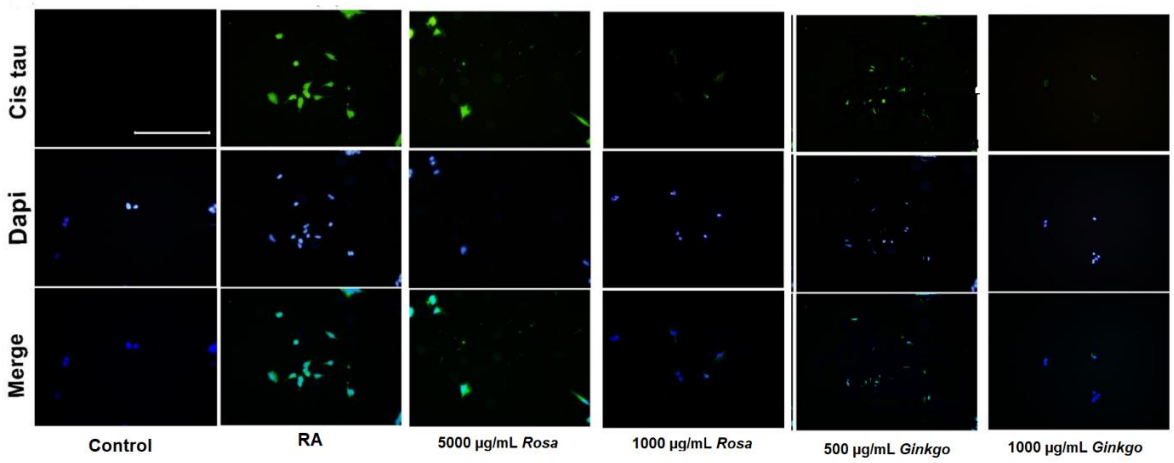

Figure 3. Anti-cis-p-Tau antibody Immunostained cultured SH-SY5Y cell line exposed with retinoic acid (RA) and treated with 500 and 1000 µg/mL Rosa damascena hips and Ginkgo biloba leaves aqueous extracts after 72 h incubation.

3.2. In vivo

3.2.1. Beam balance and walk tests

Beam balance latency was increased by TBI induction I n mice up to 100%, and treatments of TBI mice with 500 and 1000 µg/mL Rosa damascena hips and Ginkgo biloba leaves aqueous extracts resulted in improved performance in this test. 1000 µg/mL Rosa damascena hips and Ginkgo biloba leaves aqueous extracts led to the lowest beam balance latency compared TBI group (Figure 4a).

TBI led to a significant reduction in beam walk latency compared sham group. However, the TBI mice treated 1000 µg/mL Rosa damascena hips and Ginkgo biloba leaves aqueous extracts had significant decreases in beam walk latency compared to TBI mice (Figure 4b, P<0.001).

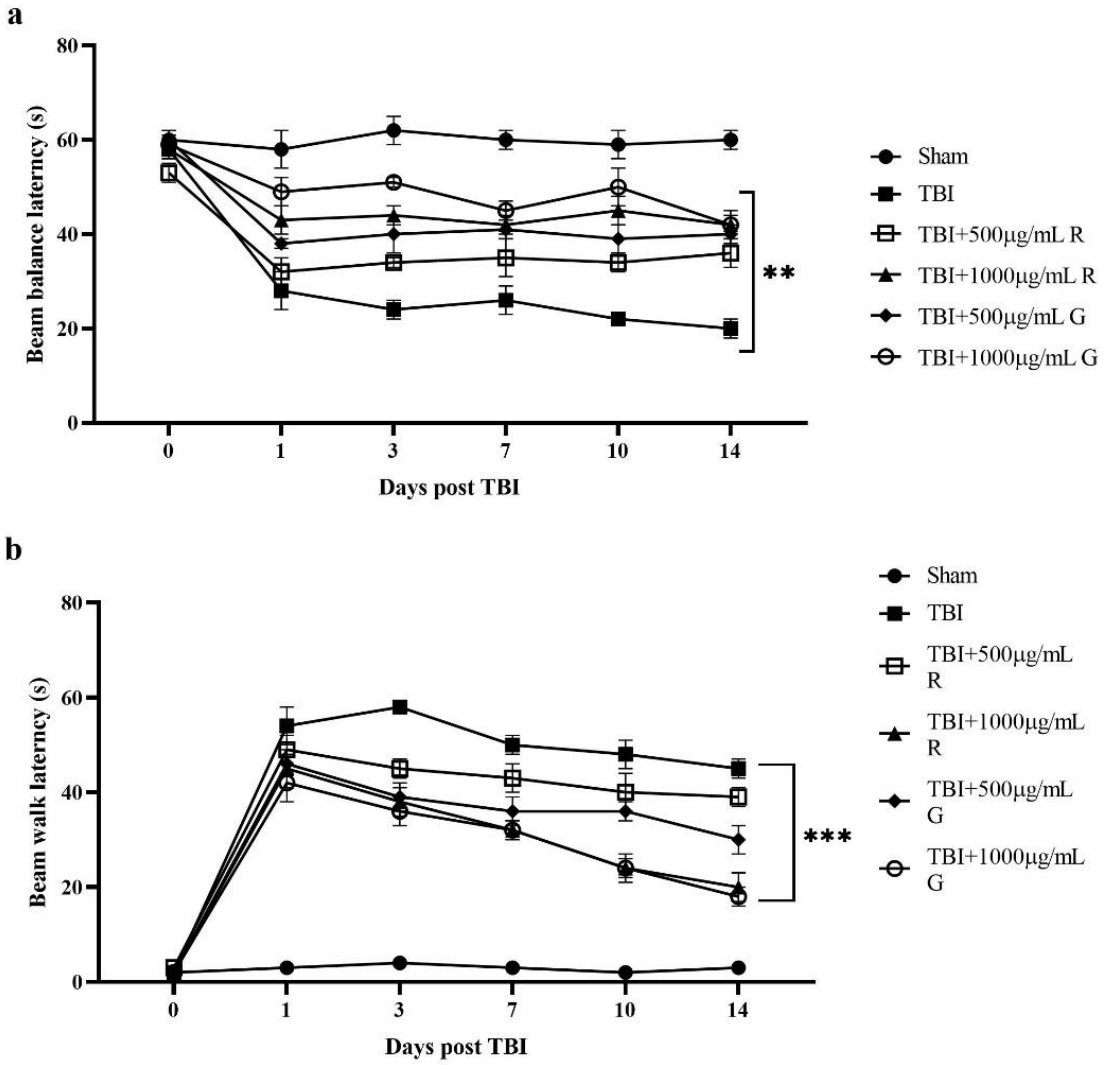

Figure 4. The beam balance (a) and walk (b) tests of traumatic brain injury (TBI) mice treated with 500 and 1000 µg/mL Rosa damascena hips and Ginkgo biloba leaves aqueous extracts before and post-TBI (n=4). **P<0.01 and ***P<0.001 show significant differences compared with sham.

3.2.2 Tau phosphorylation

Because 1000 µg/ml of both aqueous extracts showed better results in behavioral tests, therefore this dose was used in the study of tau phosphorylation. While TBI profoundly induced pathogenic cis p-tau, treatment by either 1000 µg/mL Rosa damascena hips or Ginkgo biloba leaves aqueous extracts decreased tau pathology in the mouse brains (Figure 5).

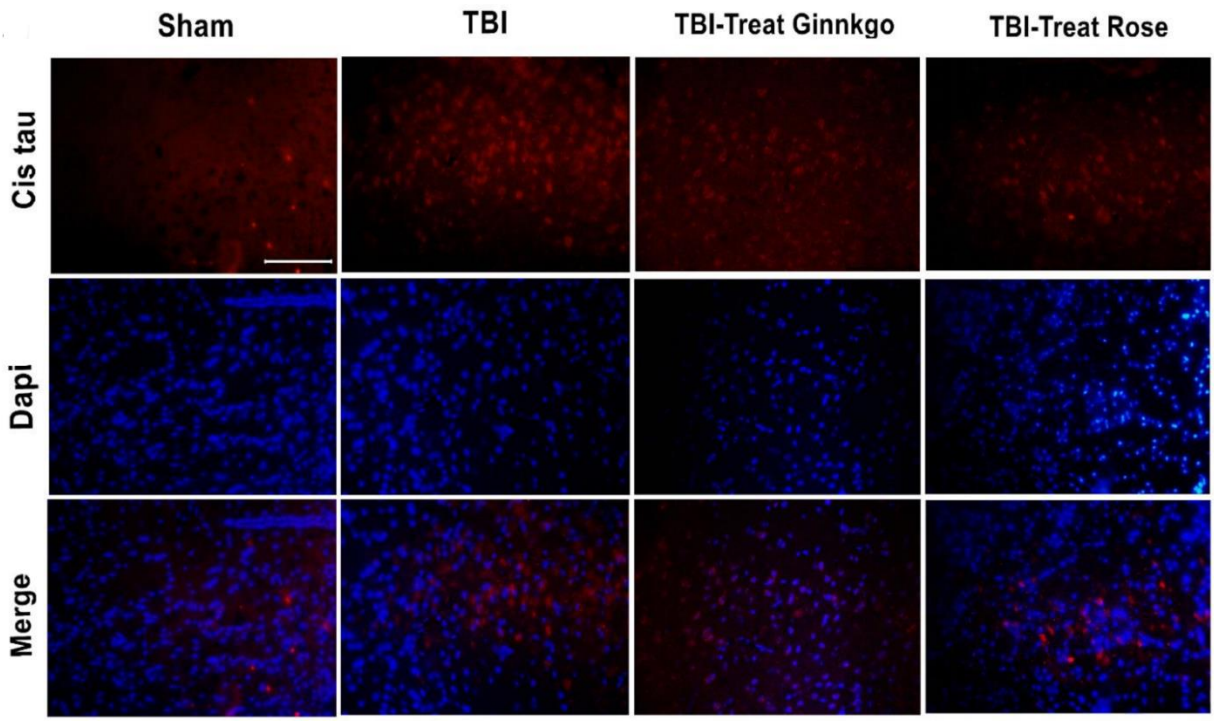

Figure 5. . Immunostained mouse brains with anti cis p-tau antibody treated with 1000 µg/mL Rosa damascena hips or Ginkgo biloba leaves aqueous extracts every day for 14 days (n=3).

4. Discussion:

Alzheimer's disease is a neurodegenerative disorder, that leads to memory loss and cognitive decline in the patients (1). It may be triggered by Environmental stimulators, such as TBI, nutritional starvation, hypoxia (2). or Ca2+ signaling disruption (3). Although several factors may lead to the disease development, however, amyloidopathy and tauopathy have been the most probable mechanisms of neurodegeneration upon AD (4). There have been extensive efforts to heal the patients but there is no effective therapeutic thus far. Although immunotherapy against pathogenic factors has been promising there is yet a long way to the clinic as immune reaction risks (5, 6). As Nanosciences and Nanomaterials are developing, there is intense interest in their clinical implications for neurodegenerative disorders (7). However, they showed severe side effects, making them less impressive for therapeutic purposes (8).

Recently, stem cell therapy has attracted more attention for AD treatment. Despite some promising results, the stem cells showed severe side effects of trauma and even have an extremely expensive cost of treatment for patients (1, 2). The other strategy has been acetylcholinesterase inhibitors (ACHEIs) but the medication has some symptomatic treatment only (3). Taking these together, many researchers have focused on herbal medicine (HM) (4). HM can be a single compound or a multi-herbal extract (5). Some reports have demonstrated that Ginkgo biloba extract is an antioxidant and memory enhancer (4, 6), Panax Ginseng is anti-aging (7), and Salvia Officinalis shows therapeutic effects on AD patients (8), but there are some addictive side effects raised by Salvia (9). Ptychopetalum olacoides root shows an ACHEI effect on the cortex (10). Also, many polyphenolic extracts may interfere with Aβ aggregation in the AD brain and prevent its neurotoxicity (11). The other widespread herbal extracts are Rosa canina & Ginkgo biloba-rich flavonoid; which can suppress Aβ neurotoxic effects upon AD (12-14). Rosa canina almost affects the CA1 area of the hippocampus (13). It has been claimed that a mixture extract of Rosa canina (R. canina), Tanacetum Vulgare (T. vulgare), and Urtica dioica (U. dioica) has a therapeutic benefit in aging; as an anti-inflammatory and anti-oxidative agent (15). It normally improves synaptic function through synaptophysin (SYP) expression (16, 17). Ginkgo biloba belongs to the Ginkgoaceae family and is the only living representative of the Ginkgoales. Ginkgo biloba possesses antioxidant, anti-inflammatory, and anti-apoptotic, and it has mitochondria-protecting effects, preventing the accumulation of Aβ, and the improvement of cognitive function by preventing the activation of platelets (18, 19). However, it remains uncertain how the herbal extracts suppress neurodegeneration upon AD.

We herein demonstrated that Rose & Ginkgo biloba extracts have a suppressive effect on neurotoxic cis p-tau formation. We found both extracts could block cis p-tau accumulation as well as neurodegeneration in those stressed-out SH-SY5Y cells. Moreover, we found the same results in TBI mouse models (1).

5. Conclusion

In general, it can be concluded that both Rose & Ginkgo biloba extracts have neuroprotective effects in traumatic brain injury conditions, and inhibition of Tau phosphorylation seems to be one of the action mechanisms of both extracts. Clinical studies investigating the effects of aqueous extracts of both of these plants in TBI patients are needed. Also, considering the inhibitory effects of Tau phosphorylation by Rose & Ginkgo biloba extracts, these two plants can be studied in animal models of Alzheimer's disease.

Declaration of competing interest

The authors stated that there is no conflict of interest.

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