

# Effects of artemisinin and TSP-1-human endometrial-derived stem cells on a streptozocin-induced model of Alzheimer's disease and diabetes in Wistar rats

Poorgholam Parvin<sup>1</sup>, Yaghmaei Parichehreh<sup>1\*</sup>, Nouredini Mehdi<sup>2</sup> and Hajebrahimi Zahra<sup>3</sup>

<sup>1</sup>Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran,

<sup>2</sup>Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran,

<sup>3</sup>A&S Research Institute, Ministry of Science Research and Technology, Tehran, Iran,

\*Email: yaghmaei\_p@srbiau.ac.ir

Alzheimer's disease (AD) is an age-associated dementia disorder characterized by A $\beta$  plaques and neurofibrillary tangles. There is a strong link between cerebrovascular angiopathy, oxidative stress, inflammation, and glucose metabolism abnormalities with the development of AD. In this study, we investigated the therapeutic influences of artemisinin and TSP-1-human endometrial-derived stem cells (TSP-1-hEDSCs) on the streptozocin-induced model of AD and diabetes in rats. Hippocampal and intraperitoneal injections of streptozocin were used to induce AD and diabetes in male Wistar rats, followed by intranasal administration of a single dose of TSP-1-hEDSCs and intraperitoneal administration of artemisinin for 4 weeks. Hematoxylin together with eosin staining was performed for demonstrating A $\beta$  plaque formation and for analyzing the influence of treatments on the pyramidal cells in the hippocampus. Biochemical analysis was used to assay the serum levels of glucose, MDA, ROS, and TAC. The expression of *TNF- $\alpha$*  was measured using real-time PCR. Streptozocin induced AD and diabetes *via* A $\beta$  plaque formation and increasing blood glucose levels. It also increased the levels of ROS, MDA, and *TNF- $\alpha$*  and decreased the levels of TAC. Simultaneous or separate administration of artemisinin and TSP-1-hEDSCs ameliorated this influence by considerably reducing A $\beta$  plaque formation in the hippocampus, reducing glucose, MDA, ROS, and *TNF- $\alpha$*  levels, and increasing TAC levels. It appears that artemisinin and TSP-1-hEDSCs improve the adverse features of AD in a rat model of AD and diabetes. Therefore, artemisinin and TSP-1-hEDSCs could be utilized as an adjunct treatment, as well as a protective agent, in AD patients.

**Key words:** Alzheimer's disease, artemisinin, diabetes, human endometrial-derived stem cells, *TSP-1*

## INTRODUCTION

Alzheimer's disease (AD) is one of the most common types of age-related dementia, characterized by irreversible and devastating neuronal degeneration. It slowly destroys memory, cognition, and eventually disrupts the ability to perform the simplest daily activities (Alzheimer's Association, 2014). AD pathology occurs along with the aggregation of  $\beta$ -amyloid pep-

ptide (A $\beta$ ) in the spaces between nerve cells as plaques and the aggregation of twisted fibers of tau-hyperphosphorylation protein (p-tau) inside nerve cells, as tau tangles or neurofibrillary tangles. Furthermore, increasing evidence suggests chronic inflammation in the brain is a fundamental first step in the pathogenesis of AD and increased A $\beta$  and tau structures. Some studies have reported an increase of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in AD patients (Heneka and O'Banion,

2007; Rubio-Perez and Morillas-Ruiz, 2012; Saido, 2013; Belluti et al., 2013). It is a potent pro-inflammatory cytokine that plays a key role in initiating inflammation events. Additionally, the accumulation of plaques leads to ongoing neoangiogenesis, increased vascular permeability, and further hypervascularization (Jefferies et al., 2013), and it plays a considerable role in the pathogenesis of AD and tissue injury.

AD is strongly correlated to diabetes, leading scientists to term it type 3 diabetes. According to the American Diabetes Association, having diabetes is a second risk factor in the later progression of AD in older populations. Although few studies show a link between cognitive dysfunction and type 1 diabetes, the majority of research has concluded that this correlation between diabetes and AD is particular to people with type 2 diabetes (Li et al., 2015). Studies in recent decades have shown that insulin resistance and insulin deficiency occurs in the brain in both type 2 diabetes and AD resulting in cognitive dysfunction and AD. Elevated blood sugar causes inflammation, and this inflammation may damage neurons, deteriorate cognitive function, and facilitate AD development (Hoyer 2002; Liu et al., 2011). Interestingly, some studies indicate that there is a correlation between the reduction of brain insulin signaling and hyperphosphorylation of tau protein (Hoyer 2002, Liu et al., 2011). Also, individuals with hyperglycemia have a dramatic increase in cerebral  $\beta$ -amyloid protein, which is toxic for nerve cells (de la Monte et al., 2012; Chatterjee and Mudher, 2018). Similar to AD, perturbations in vasculature have been identified in diabetes mellitus that lead to retinopathy and nephropathy (Cade, 2008).

Increasing evidence demonstrates that oxidative stress has a crucial role in the expansion and progression of AD (Sharma and Gupta, 2002). Some studies proposed that amyloid-beta induces lipid peroxide, the creation of hydrogen peroxide, inflammatory cytokines, and superoxide in the brain (Esposito et al., 2006; Huang et al., 1999). Oxidative stress, through the generation of reactive oxygen species (ROS), is the main factor in the development of type 2 diabetes mellitus and its related complications (Wright et al., 2006). The above described reports lead to the possibility that using an antioxidant drug may be helpful in the alleviation of symptoms and complications observed in these patients.

In recent years, the use of herbal medicines to treat diseases has received much attention due to fewer side effects and easy accessibility. *Artemisia annua* (*A. annua*), also referred to as Shih, is an annual herb belonging to the Asteraceae family that is native to Asia. Studies have identified that *A. annua* extract has antioxidant, anti-inflammatory, and anti-diabetic ac-

tivity (Ferreira et al., 2010; Helal et al., 2014). The objective of this study was to evaluate the protective effects of artemisinin, the main therapeutic compounds of *A. annua*, in the rat model of diabetes and AD in combination with *TSP-1* gene-transfected human endometrial-derived stem cells (*TSP-1-h-EDSCs*). Human *EDSCs* (*hEDSCs*) are abundant and easily accessible multipotent stem cells that have the ability to differentiate into neuron-like cells and have the potential for use in replacement therapy in the treatment of nervous system degenerative diseases (Wolff et al., 2011). Thrombospondin (*TSP*)-1 is an extracellular glycoprotein first introduced by Good et al. (1990) as a potent inhibitor of angiogenesis. As mentioned above, abnormalities in the vasculature are one of the features in both AD and diabetes mellitus.

In the present work, streptozotocin was used to develop Alzheimer's and diabetes in male Wistar rats. Streptozotocin is a glucosamine-nitrosourea compound that destroys the beta cells of the pancreas and widely utilized to develop animal models of diabetes. Because Alzheimer's is associated with impaired glucose metabolism and diabetes (Virkamäki et al. 1999, Hoyer 2002; Cade 2008; Liu et al. 2011; de la Monte, 2012; Li et al., 2015; Chatterjee and Mudher, 2018) hippocampal injection of STZ has been widely used by researchers for inducing AD model in animals, too. This model is based on brain resistance to insulin that simulates many pathophysiological features of AD, such as loss of cognitive function, glucose metabolism abnormalities, free radical generation, apoptosis, neuroinflammation, aggregation of  $A\beta$  fragments, and hyperphosphorylation of the microtubule protein tau (Peng et al., 2013; Yang et al., 2014). Simultaneously or separately administration of artemisinin and *TSP-1-h-EDSCs* decreased plaque formation, inflammation, and stress oxidative through decreasing  $TNF-\alpha$  expression, malondialdehyde level (MDA), ROS level, and increasing total antioxidant capacity (TAC). The blood sugar levels also decreased following administration of artemisinin and *TSP-1-h-EDSCs*. It seems that artemisinin and *TSP-1-hEDSCs* improve the adverse features of AD in a rat model of Alzheimer's disease and diabetes. Therefore, artemisinin and *TSP-1-hEDSCs* could be utilized as an adjunct treatment and also as a protective agent in Alzheimer's patients.

## METHODS

### Animals and treatments

Male Wistar rats (200–220g) were obtained from the animal laboratory of Islamic Azad University, Sci-

ence and Research Branch, Tehran, and were maintained in the animal house under standard environmental conditions (room temperature: 22°C, humidity: 50±10%, 12-h light and 12-h dark cycles). Animals had free access to food and water throughout the experimental period, under the guidelines for the Care and Use of Laboratory Animals (Committee for the update of the guide for the care and use of laboratory animals, 1996). The included experiments were demonstrated to the Animal Care and Use Committee of Islamic Azad University, Science and Research Branch to minimize animal suffering and the number of animals utilized in the experiments. The animals were familiarized with the environmental conditions of the animal house for 20 days before starting the experiments.

For STZ-induced AD, animals were anesthetized by intraperitoneal (i.p.) injection of urethane (1.5 mg/kg) (HTL, China) and placed in the Stoelting stereotaxic device (USA) as described previously (Poorgholam et al., 2018). Briefly, the stereotaxis measurements were -3.5 mm posterior to the bregma, 2 mm lateral to the sagittal suture and 2.8 mm below the dura, based on a Paxinos and Watson (1986) atlas. Then, 4 µl STZ (3 mg/kg dissolved in saline, Sigma, USA) was slowly injected into the right dorsal hippocampus within 2–3 min by a Hamilton microsyringe (5 µl). Following injection, the needle was kept in place for about 5 min and withdrawn slowly after, ensuring the complete distribution of STZ. The rats were kept in an individual cage, monitored daily, and given postoperative care for 7 days. One week after recovery from stereotaxic surgery, diabetes was induced by a single i.p. injection of STZ (30 mg/kg in a citrate buffer with pH=4). Animals with a blood sugar level of more than 200 mg/dl were considered diabetic. Blood samples were obtained from the animal tail vein. Two days after the induction of diabetes, TSP-1 gene-transfected human endometrial-derived stem cells (TSP-1-hEDSCs) were administered intranasally. The TSP-1-hEDSCs were provided by Dr. Moradian (Department of Applied Cell Sciences, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran) as described previously (Bagheri-Mohammadi et al., 2019). For this purpose, a plastic catheter was linked to a pipette (polyethylene tube; BD, Franklin Lakes, NJ) and left in both nasals of the rat during deep anesthesia. In order to increase the migration of cells to the brain, all rats received 5 µl hyaluronidase (Sigma-Aldrich St. Louis mouse; 100 U hyaluronidase dissolved in 24 ml of sterile PBS) before cell administration. Then, 10 µl of cell-containing solution were administered in two steps (5 µl each time) and one minute apart for each nasal nostril. In order to prevent immune rejection, all animals received cy-

closporine (through daily water consumption) from two days before the stem cell injection to the end of the experimental period. Three days after the induction of diabetes, animals were treated with artemisinin for 4 weeks.

48 rats were divided randomly into six groups (n=8) as follows: control group (C) that received standard diet and distilled water without surgery and treatment; saline treatment (Sal) group which received saline as STZ solvent by stereotaxic surgery; AD+D group; rats with AD and diabetes at the same time, which received i.p. injection of saline (0.3 ml) for 4 weeks; AD+D+art group of AD-diabetes rats that received i.p. injection of artemisinin (50 mg/kg) for 4 weeks; AD+D+SC group of AD-diabetes rats, which received intranasally a single dose of TSP-1-hEDSCs; AD+D+art+SC group: AD-diabetes + TSP-1-hEDSCs rats that obtained i.p. injection of artemisinin (50 mg/kg) for 4 weeks.

### Real-time quantitative PCR

At the end of the fourth week, blood samples were gathered from the heart, and leukocytes were isolated using lysis buffer. Total RNA was isolated from leukocytes utilizing the RNX plus™ kit based on the manufacturer's procedure (Cinnagen, Tehran, Iran). cDNA synthesis was performed by Easy™ cDNA Synthesis Kit (Parstous Biotechnology, Tehran, Iran) following the manufacturer's instruction. Real-time PCR was carried out by a Bio-Rad Real-Time PCR detection system by utilizing SYBR green PCR master mix (Takara, Japan). Real-time PCR was adjusted in different three stages: first, initialization under the temperature of 95°C for 2 min, then denaturation under the temperature of 95°C for 5 s, and finally annealing under the temperature of 60°C for 30 s (total 56 cycles). PCR melting curves were created after real-time PCR by sequential heating of the product to ensure the specificity of PCR products. Change in the fold number was predicted utilizing the  $2^{-\Delta\Delta Ct}$  approach normalized using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene. The primer sequences used were *TNF-α* F: 5' ACTGAACTTCGGGGTGATTG 3', *TNF-α* R: 5' GCTTGGTGGTTTGTACGAC 3', *GAPDH* F: 5' GTATTGGGCGCCTGGTCACC 3', and *GAPDH* R: 5' CGCTCCTGGAAGATGGTGATGG 3'. All primers were synthesized by CinnaGen (Tehran, Iran).

### Tissue preparation

Rats were anesthetized terminally by using 80 mg/kg of ketamine and 10 mg/kg of xylazine and

perfused via the left ventricle of the heart with phosphate-buffered saline (PBS 0.01 M, 200 ml) pH 7.4, followed by 4% paraformaldehyde in 0.01 M PBS, pH 7.4, for fixation. Following perfusion, brains were removed and postfixed in the identical fixative solution for 1 day. Samples were transferred in paraffin and sectioned serially at 6- $\mu$ m thickness after standard tissue processing of clearing and dehydration (from 2.5 mm to 4.5 mm of the hippocampal formation and bregma 2–4 mm of the frontal cortex, as well as from 10 mm to 15 mm of the cerebellar cortex), placed onto glass slides, and covered with hematoxylin and eosin (H & E) as described previously (Poorgholam et al., 2018). Samples were observed under a light microscope. For quantitative analysis, the percentage of plaque area/number of plaques and number of neurons were calculated using the ImageJ analysis program.

### Biochemical analysis

At the end of the fourth week, blood samples were obtained from the heart and left at room temperature for 2 h. Then, serum samples were collected through centrifugation at 2,500 $\times$ g for 5 min and stored at -20°C until usage. The level of total antioxidant capacity (TAC) and malondialdehyde (MDA), as well as reactive oxygen species (ROS) levels, were measured using commercial ELISA kits (Zellbio GmbH, Ulm, Germany) based on the instructions of the manufacturer. The concentration of blood glucose was estimated by commercial spectrophotometric assay kits (Pars Azmun Company, Tehran, Iran) based on the recommendations of the manufacturer.

### Statistical analysis

The data are presented as the means $\pm$ S.E.M. One-way ANOVA with Tukey's *post hoc* test, was used for comparing between groups. All data were analyzed by SPSS software version 17.0. The charts were drawn using Microsoft Excel 2010.  $P < 0.05$  was set as significant.

## RESULTS

Hippocampal and intraperitoneal injections of STZ were used for the experimental model of developing AD and diabetes. In rats, H & E staining was used for demonstrating the formation of A $\beta$  plaques and the development of AD after STZ injection. Comparison of brain tissues from different animal groups revealed apparent histological changes in the rat hippocampus. Table I summarizes the number of A $\beta$  plaques and the number of neurons in all animal groups. No A $\beta$  plaques or neuronal death were observed in the control rats (Fig. 1, C1 and C2), while other animals showed percentages of neuronal death and A $\beta$  plaque formation. Sections of the hippocampus in control rats showed normal brain histology along with regular distribution of clear pyramidal cells that had distinct nuclei as revealed by H & E staining. Extensive histological changes, such as the formation of A $\beta$  plaque (black arrows), and neuronal cell death were observed in the hippocampus in the AD-diabetes group (Fig. 1, AD+D) compared to the control group, which indicated development of AD. Simultaneous or separate administration of artemisinin and TSP-1-hEDSCs ameliorated histological changes in the AD-diabetes-artemisinin (Fig. 1, AD+D+art), AD-diabe-

Table I. Number of plaques and neurons in the CA1 and cortex area of hippocampus.

Group	Region	Number of neurons	Number of plaques
C	CA1	168 $\pm$ 23.56	-
	Cortex	394 $\pm$ 51.36	-
AD+D	CA1	92 $\pm$ 11.25*	5 $\pm$ 2**
	Cortex	213 $\pm$ 21.53*	27 $\pm$ 5***
AD+D+art	CA1	136 $\pm$ 24.35#	2 $\pm$ 1**
	Cortex	321 $\pm$ 41.02#	14 $\pm$ 3**
AD+D+SC	CA1	129 $\pm$ 20.4**	3 $\pm$ 1*
	Cortex	289 $\pm$ 31.63**	17 $\pm$ 4**
AD+D+art+SC	CA1	147 $\pm$ 26.14#	1 $\pm$ 0*#b
	Cortex	341 $\pm$ 39.6#	9 $\pm$ 3*##b

(\*) statistically distinct from the control rats at  $P \leq 0.05$ ; (\*\*) statistically distinct from the control rats at  $P \leq 0.01$ ; (#) statistically distinct from the AD+D group ( $P \leq 0.05$ ); (##) statistically distinct from the AD+D group ( $P \leq 0.01$ ); (b) statistically distinct from the AD+D+SC group ( $P \leq 0.05$ ). C: control rats; AD+D: AD-diabetes rats; AD+D+art: AD-diabetes-artemisinin rats; AD+D+SC: AD-diabetes-TSP-1-hEDSCs rats; AD+D+art+SC: AD-diabetes-artemisinin-TSP-1-hEDSCs rats.

tes-TSP-1-hEDSCs (Fig. 1, AD+D+SC), and AD-diabetes-artemisinin-TSP-1-hEDSCs (Fig. 1, AD+D+art+SC) groups. The number of A $\beta$  plaques, and amount of neuronal cell death were decreased in AD-diabetes-artemisinin (Fig. 1, AD+D+art), AD-diabetes-TSP-1-hEDSCs (Fig. 1,

AD+D+SC), and AD-diabetes-artemisinin-TSP-1-hEDSCs (Fig. 1, AD+art+SC) groups in comparison to the AD-diabetes (AD+D) group. Therefore, simultaneous or separate administration of artemisinin and TSP-1-hEDSCs decreased the adverse histological changes in AD rats.

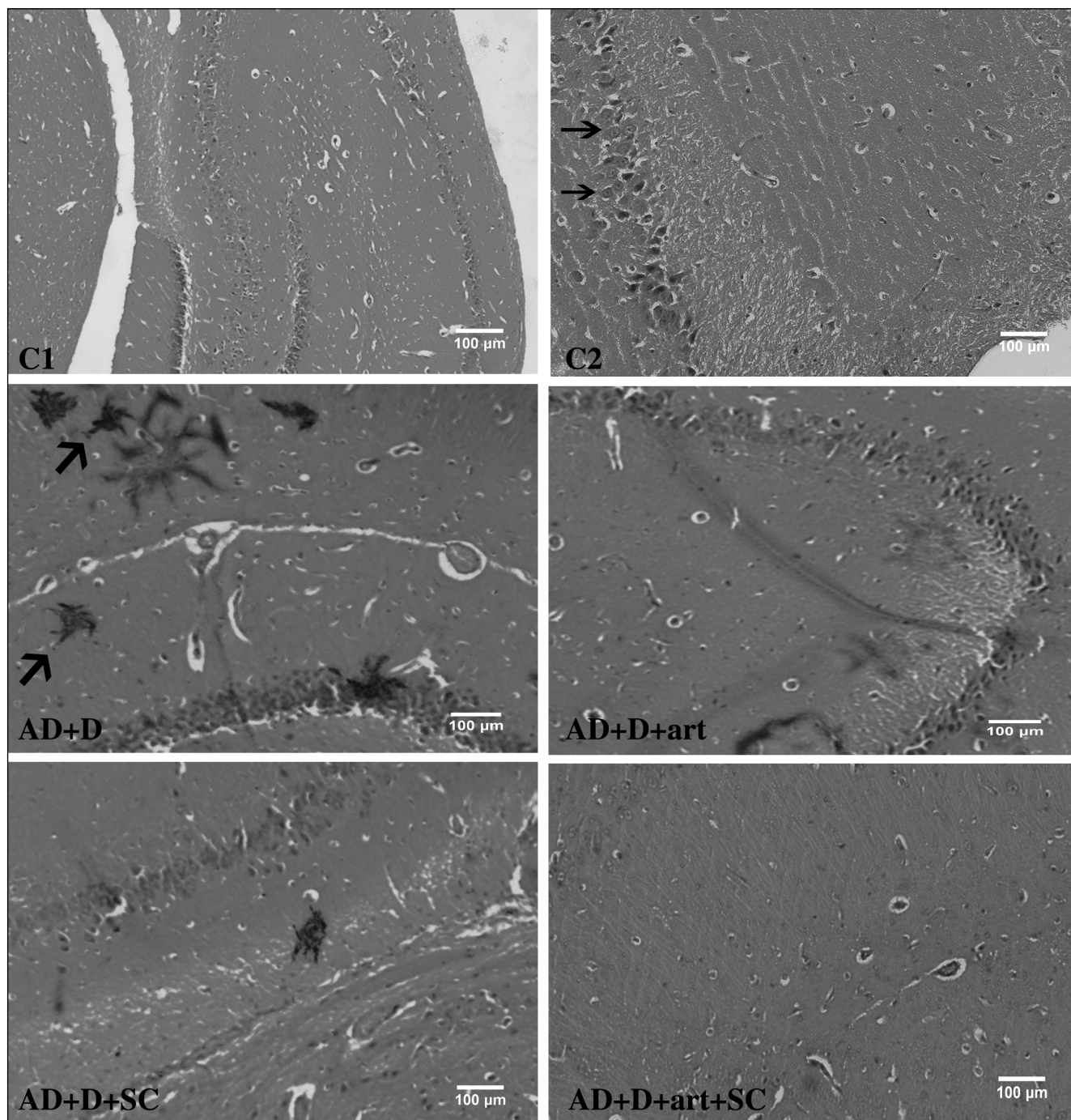


Fig. 1. Photomicrograph of hippocampus of control, 100X (C1); control, 400X (C2); AD-diabetes, 400X (AD+D); AD-diabetes+artemisinin, 400X (AD+D+art); AD-diabetes-TSP-1-hEDSCs, 400X (AD+D+SC); and AD-diabetes-artemisinin-TSP-1-hEDSCs, 400X (AD+D+art+SC) groups. Black arrows show live neurons and A $\beta$  plaques in C2 and AD sections, respectively.

### Effect of artemisinin treatment on serum glucose

In order to evaluate the induction of diabetes and the effect of artemisinin or TSP-1-hEDSCs on diabetes, blood serum glucose was measured at the end of the fourth week for all experimental groups and the results are presented in Fig. 2. Our results revealed a marked increase (2.7 fold) in the level of blood serum glucose in the AD+D group (AD-diabetes rats) compared with controls ( $P \leq 0.01$ ). Artemisinin or TSP-1-hEDSC treatment significantly decreased the blood glucose level of AD+D+art (AD-diabetes-artemisinin rats) and AD+D+SC (AD-diabetes-TSP-1-hEDSCs rats) groups. Simultaneous administration of artemisinin and TSP-1-hEDSCs reduced the blood glucose level in the AD+D+art+SC group (AD-diabetes-artemisinin-TSP-1-hEDSCs rats) to control levels by the end of the experiments. Therefore, simultaneous or separate administration of artemisinin and TSP-1-hEDSCs improved the blood glucose level in AD-diabetes rats.

### Effect of artemisinin treatment on serum levels of MDA, ROS, and TAC

Oxidative stress plays a crucial role in the expansion and progression of AD and diabetes. The serum levels of MDA, ROS, and ROS (Fig. 2) were assayed to evaluate the therapeutic effect of artemisinin and TSP-1-hEDSCs on the prevention of oxidative stress in AD and diabetes animals. The levels of blood serum MDA were assessed at the end of the fourth week for all animal groups. Fig. 2 shows that the levels of MDA in the AD-diabetes rats (AD+D) significantly increased up to 2-fold versus the control (C group) levels ( $P \leq 0.05$ ). Simultaneous or separate administration of artemisinin and TSP-1-hEDSCs significantly decreased MDA levels in the AD-diabetes-artemisinin (AD+D+art), AD-diabetes-TSP-1-hEDSCs (AD+D+SC), and AD-diabetes-artemisinin-TSP-1-hEDSCs (AD+D+art+SC) groups in comparison to the AD+D group. Fig. 2 shows the level of ROS in serum samples of all experimental groups, taken at the end of the fourth week. A marked

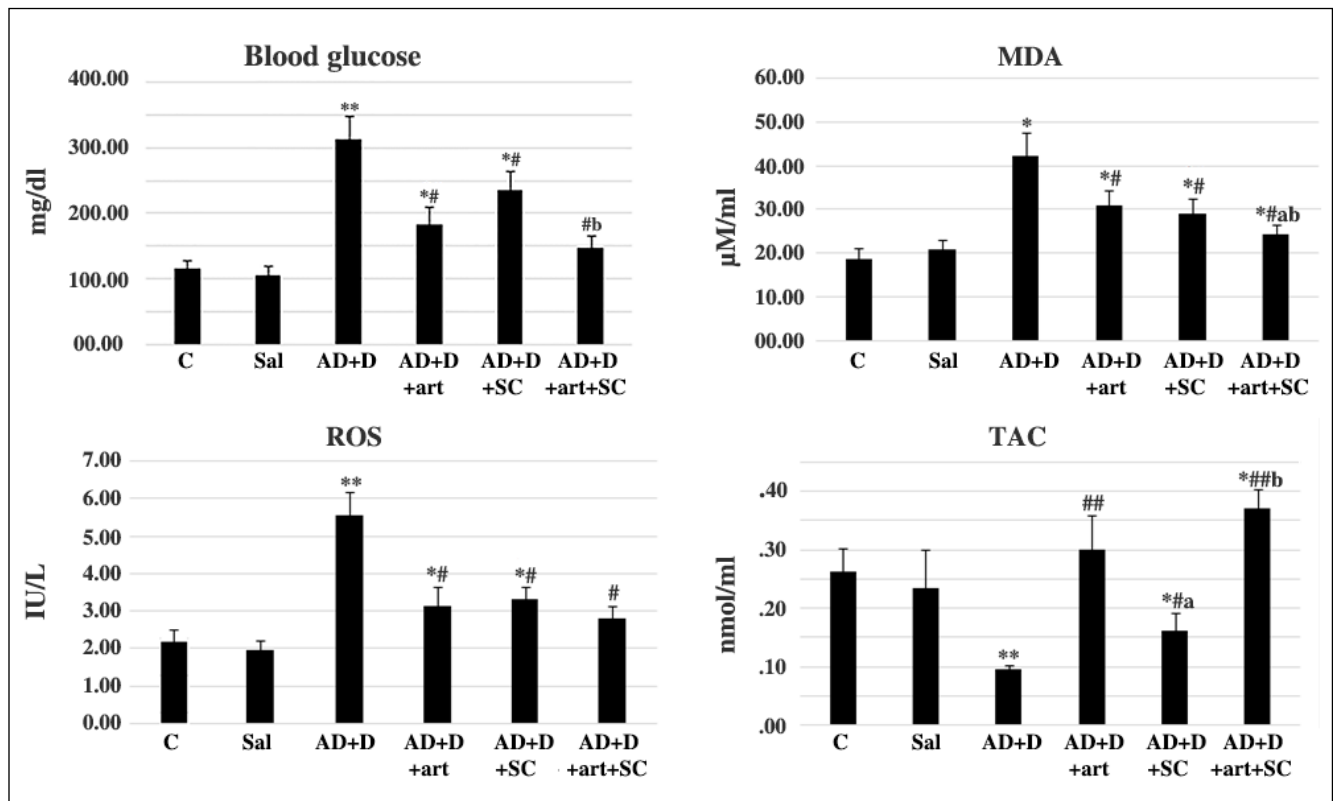


Fig. 2. The serum level of glucose, MD, ROS, and TAC. Values are presented as mean  $\pm$  SE ( $n=8$ /each group). (\*) statistically distinct from the control rats at  $P \leq 0.05$ ; (\*\*) statistically distinct from the control rats at  $P \leq 0.01$ ; (#) statistically distinct from the AD+D group ( $P \leq 0.05$ ); (##) statistically distinct from the AD+D group ( $P \leq 0.01$ ). (°) statistically different from the AD+D+art group ( $P \leq 0.05$ ); (°) statistically different from the AD+SC group ( $P \leq 0.05$ ). C: control rats; Sal: saline treatment group; AD+D: AD-diabetes rats; AD+D+art: AD-diabetes-artemisinin rats; AD+D+SC: AD-diabetes-TSP-1-hEDSCs rats; AD+D+art+SC: AD-diabetes-artemisinin-TSP-1-hEDSCs rats.

increase (up to 2.5 fold) was found in the serum ROS levels for the AD-diabetes (AD+D) group compared with the control (C) group ( $P \leq 0.01$ ). Artemisinin or TSP-1-hEDSC treatment significantly decreased the serum ROS levels of the AD-diabetes-artemisinin (AD+D+art) and AD-diabetes-TSP-1-hEDSCs (AD+D+SC) groups ( $P \leq 0.05$ ). Simultaneous administration of artemisinin and TSP-1-hEDSCs reduced the blood ROS levels of the AD-diabetes-artemisinin+TSP-1-hEDSCs (AD+D+art+SC) group to the control levels by the end of the experiments. As presented in Fig. 2, the TAC in the AD-diabetes rats significantly decreased up to 2.5 fold compared to control (C group) levels ( $P \leq 0.01$ ). Simultaneous or separate administration of artemisinin and TSP-1-hEDSCs significantly increased the TAC of the AD-diabetes-artemisinin (AD+D+art), AD-diabetes-TSP-1-hEDSCs (AD+D+SC), and AD-diabetes-artemisinin-TSP-1-hEDSCs (AD+D+art+SC) groups in comparison to the AD-diabetes (AD+D) group. Administration of artemisinin, alone or in combination with TSP-1-hEDSCs, significantly increased the TAC of the AD-diabetes-artemisinin (AD+D+art) and AD-diabetes-artemisinin-TSP-1-hEDSCs (AD+D+art+SC) groups, respectively. The TAC of AD-diabetes-artemisinin (AD+D+art) and AD-diabetes-artemisinin-TSP-1-hEDSCs (AD+D+art+SC) rats reached the control levels by the end of the experiments. Therefore, simultaneous or separate administration of artemisinin and TSP-1-hEDSCs improved the serum levels of MDA, ROS, and TAC in AD-diabetes rats.

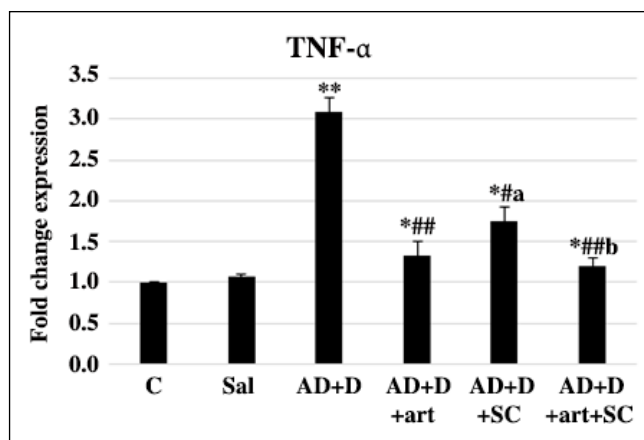


Fig. 3. Gene expression of *TNF-α* using real-time qRT-PCR ( $n=8$ /each group). (\*) statistically different from the control rats ( $P \leq 0.05$ ); (\*\*) statistically different from the control rats ( $P \leq 0.01$ ); (\*\*\*) statistically different from the AD+D group ( $P \leq 0.05$ ); (#) statistically different from the AD+D+art group ( $P \leq 0.05$ ); (°) statistically different from the AD+D+SC group ( $P \leq 0.05$ ). C: control rats; Sal: saline treatment group; AD+D: AD-diabetes rats; AD+D+art: AD-diabetes-artemisinin rats; AD+D+SC: AD-diabetes-TSP-1-hEDSCs rats; AD+D+art+SC: AD-diabetes-artemisinin-TSP-1-hEDSCs rats.

## TNF-α gene expression

Here, we investigated the gene expression for tumor necrosis factor- $\alpha$  (*TNF-α*) using real-time qRT-PCR (Fig. 3). It is a potent pro-inflammatory cytokine that plays a key role in initiating inflammation events. Inflammation has an important role in the pathogenesis of AD and diabetes. The results indicated that expression of *TNF-α* in the AD-diabetes rats (AD+D group) significantly increased up to 3-fold of the control (C group) levels ( $P \leq 0.01$ ), but simultaneous or separate administration of artemisinin and TSP-1-hEDSCs were able to significantly alleviate the effects of AD and diabetes. The expression of *TNF-α* significantly decreased after administration of artemisinin or TSP-1-hEDSCs in the AD-diabetes-artemisinin (Fig. 3, AD+D+art), AD-diabetes-TSP-1-hEDSCs (Fig. 3, AD+D+SC), and AD-diabetes-artemisinin-TSP-1-hEDSCs (Fig. 3, AD+D+art+SC) groups in comparison to the AD-diabetes (AD+D) group. Therefore, simultaneous or separate administration of artemisinin and TSP-1-hEDSCs improved the expression of the *TNF-α* (pro-inflammatory cytokine) gene in AD-diabetes rats.

## DISCUSSION

In this study, we showed that hippocampal and i.p. injections of STZ could induce AD and diabetes in male Wistar rats via  $A\beta$  plaque formation and increased blood glucose levels and neuronal cell death. It increased the levels of ROS, MDA, and *TNF-α* and decreased the TAC. Simultaneous or separate administration of artemisinin and TSP-1-hEDSCs ameliorated these features by a considerably reducing  $A\beta$  plaque formation and neuronal cell death in the hippocampus, reducing glucose, MDA, ROS, and *TNF-α* levels in serum, and increasing TAC.

Hippocampal and i.p. injections of STZ were used for the experimental model of AD and diabetes development. Based on the results, extensive histological changes, such as the formation of  $A\beta$  plaques and neuronal cell death were observed in the hippocampus in the AD-diabetes group (AD+D), which indicated that the AD model was successfully established. The level of blood serum glucose in EXP-1 animals significantly increased and was more than 200 mg/dl, indicating the successful induction of diabetes in rats.

The biochemical findings were in line with the histological data and induction of AD and diabetes in AD-diabetes animals. The level of ROS and MDA in serum samples of EXP-1 animals was significantly higher compared with control rats. MDA and ROS are

known oxidative stress biomarkers. Oxidative stress refers to an increase in ROS levels due to an imbalance between ROS formation and the antioxidant system ability to neutralize them. Oxidative stress has an important role in the development and progression of age-related neurodegenerative disease and cognitive abnormality, such as AD, and it can promote the production of A $\beta$  plaques (Sharma and Gupta, 2002). It has been shown that decreased antioxidant defenses in the brain can lead to memory impairment by affecting synaptic function and neurotransmission in older populations (Tönnies and Trushina, 2017). The brain's structure is largely composed of lipids and its physiology relies highly on glucose metabolism (Hamilton et al., 2007). Therefore, increasing ROS production can easily result in oxidized brain lipids and affect synaptic activity.

In this study, observation of A $\beta$  plaques in the hippocampus of AD-diabetes rats was associated with increased serum levels of oxidative stress biomarkers such as MDA and ROS, which suggests a role for oxidative stress in AD. Oxidative stress was also shown to be the primary factor in the promotion of insulin resistance,  $\beta$ -cell impairment, glucose metabolism disorder, and type 2 diabetes mellitus (Wright et al., 2006). Reports of TAC in AD are contradictory. While Moslemnezhad et al. (2016) observed a decrease in plasma TAC levels in AD patients, no significant differences in plasma TAC levels were detected by Foy et al. (1999) and Sinclair et al. (1999) between AD patients and controls. In the present study, the observed significant decrease in serum TAC levels in the AD+D group (AD-diabetes rats) compared to controls is in line with the results of Moslemnezhad et al. (2016) and re-emphasizes the concept of induced oxidative stress in animals.

AD is recognized by three hallmarks: accumulation of A $\beta$  plaques, accumulation of tau-hyperphosphorylation protein, and chronic inflammation (Heneka and O'Banion, 2007; Rubio-Perez and Morillas-Ruiz, 2012; Saido, 2013; Belluti et al., 2013). TNF- $\alpha$  is an important pro-inflammatory cytokine, which is associated with neurodegenerative disorders like AD. It is the first initiator of immune-mediated inflammation in the brain that induces microglial activation and leads to neuronal death (Janelsins et al., 2008). Studies have shown that the level of TNF- $\alpha$  is higher in plasma and brain of AD patients in comparison to normal individuals (Swardfager et al., 2010). The results of the present study confirmed an increase in TNF- $\alpha$  expression in an AD model. Therefore, the observed significant increase in the mRNA expression of TNF- $\alpha$  in the AD rats (AD+D group) compared to controls is in line with the findings of other studies.

Due to the role of oxidative stress and ROS molecules in the etiology of AD, antioxidant therapies have received a great deal of attention in recent decades. In this study, protective treatment with artemisinin improved histological changes and biochemical parameters. Artemisinin is a primary therapeutic compound of *A. annua* with antioxidant, anti-inflammatory, and anti-diabetic activity (Ferreira et al., 2010; Helal et al., 2014). Administration of artemisinin significantly reduced glucose, MDA, ROS, and TNF- $\alpha$  levels in the AD-diabetes-artemisinin group, indicating the anti-diabetic, antioxidant, and anti-inflammatory effects of artemisinin. Also, TAC levels significantly increased following artemisinin usage in the AD-diabetes-artemisinin group rats and reached the control levels, confirming an antioxidant function of artemisinin.

Brain tissue from the AD-diabetes-artemisinin group showed a decrease in the number of A $\beta$  plaques and neuronal cell death compared to the control rats, positively supporting the idea of treatment of AD with antioxidants. Zhao et al. (2020) found that artemisinin could reduce A $\beta$  plaques and tau protein in a 3xTg AD mouse model. They also showed that artemisinin could reduce apoptosis and neuronal cell death and could stimulate the activation of the ERK/CREB signaling pathway.

In the present study, besides artemisinin, the therapeutic effect of TSP-1-hEDSCs was also investigated. Mesenchymal stem cell (MSC) transplantation has already been used for the treatment of central nervous system disorders, including AD. Cui et al. (2017) reported that human umbilical cord mesenchymal stem cells can improve cognitive ability in a mouse model of AD by reducing oxidative stress and increasing neurogenesis in the hippocampus and enhancing expression of proteins related to neuronal synaptic plasticity. HEDSCs are MSCs that represent a new and valuable source of stem cells in regenerative medicine and clinical application. These cells have comprehensive advantages as opposed to other stem cells due to their high proliferation rate, easy periodic collection in a non-invasive manner, high multi-differentiation potential, low immunogenic properties, reduced inflammatory properties, and low tumorigenicity (Indumathi et al., 2013). In the past decade, *in vitro* differentiation of EDSCs into such neural cells has been shown (Wolff et al., 2011). Zhao et al. (2018) showed that intracerebral transplantation of EDSCs can improve memory and cognitive function in a mouse model of AD. They found that EDSCs can reduce the number of A $\beta$  plaques and tau hyperphosphorylation, increase A $\beta$  degrading enzymes, and regulate pro-inflammatory cytokines in the brain. In another study, Bagheri-Mohammadi et al. (2019) used EDSCs for Parkinson's disease (PD) treatment. They showed that



non-invasive intranasal administration of hEDSCs could improve the behavioral parameters and ameliorate the PD symptoms in a mouse model of PD (Wu and Finley, 2017).

In the present investigation, the impact of hEDSCs transfected with the TSP-1 gene on the treatment of AD was studied. TSP-1 is an extracellular glycoprotein first introduced by Good et al. (1990) as a potent inhibitor of angiogenesis. It is a member of the thrombospondin family that mediates cell-to-cell and cell-to-matrix interactions. It is involved in various biological processes such as angiogenesis and regulation of immune response. This protein can bind to multiple receptors, including CD36 and CD47. It is well known that the anti-angiogenesis activity of TSP-1 is due to it binding to endothelial cells *via* the CD34 receptor. This leads to the expression of FAS ligand, activation of FAS receptor, activation of caspases, and finally induction of endothelial cell apoptosis (Lopez-Dee et al., 2011). One feature of AD is neoangiogenesis and increased vascular permeability due to the accumulation of amyloid plaques (Jefferies et al., 2013). Therefore, pharmacological interventions that target angiogenesis may be beneficial and effective AD therapy.

The results of the present study indicated that intranasal administration of TSP-1-hEDSCs can improve the histological changes in the brain and biochemical changes in the serum in the STZ-induced AD model. A $\beta$  plaque was decreased in the AD+D+SC group (AD-diabetes + TSP-1-hEDSCs) and the levels of neuronal cell death were decreased in these animals in comparison to the AD-diabetes group. Furthermore, the expression of *TNF- $\alpha$*  significantly decreased after administration of TSP-1-hEDSCs in the AD+SC rats in comparison to the AD-diabetes group. These findings are in line with Zhao et al.'s (2018) studies and may confirm the impact of EDSCs in decreasing A $\beta$  plaques and regulating pro-inflammatory cytokines in the brain. Improvement of AD symptoms in AD+D+SC rats may also be caused by TSP-1 administration. Supplementary studies and simultaneously and separately administered EDSCs and TSP-1 are needed to distinguish the impact of EDSCs from TSP-1 for AD therapy. Decreased *TNF- $\alpha$*  expression in AD+D+SC animals may be due to the anti-inflammatory function of TSP-1 protein (Lopez-Dee et al., 2011). Moreover, treatment with TSP-1-hEDSCs also improved the serum levels of ROS, TAC, and MDA. Therefore, it can be suggested that TSP-1-hEDSCs can reduce the pathophysiological features of AD.

Administration of TSP-1-hEDSCs also decreased the level of serum glucose in AD+D+SC groups. As mentioned above, there is a strong correlation between cognitive dysfunction and diabetes. Previous studies

have shown that elevated blood sugar causes inflammation that can lead to neuronal death and development of AD (Hoyer 2002, Liu et al., 2011). Therefore, controlling blood sugar levels may be effective in reducing and improving AD symptoms. These findings again emphasize the therapeutic effect of EDSCs and TSP-1 for AD.

The effect of simultaneous administration of artemisinin and TSP-1-hEDSCs was also studied (AD+D+art+SC group). The data indicated that simultaneous application of artemisinin and TSP-1-hEDSCs further decreased the serum levels of glucose and MDA and the mRNA expression of *TNF- $\alpha$*  in the AD+D+art+SC animals. The data showed that the levels of glucose and ROS in AD+D+art+SC rats reached control levels by the end of the experiments. These findings suggest that successful treatment of AD may not be achieved with just a single pharmacological intervention and it may be better to target two or more pathophysiological features simultaneously.

## CONCLUSION

In this study, hippocampal and i.p. injections of STZ were used to induce AD and diabetes in male Wistar rats, respectively. The model was able to achieve a number of AD features, such as inducing oxidative stress, inflammation, hyperglycemia, aggregation of A $\beta$  plaques, and neuronal cell death and degeneration. Separately or simultaneously administered artemisinin and TSP-1-hEDSCs could prevent adverse features of the disease. The application was able decrease plaque formation, inflammation, and degeneration in the hippocampus. Additionally, it led to an improvement in the levels of serum glucose, a marker of oxidative stress, and mRNA levels of the pro-inflammatory factor, *TNF- $\alpha$* . Therefore, in the case of age-related neurodegenerative diseases like AD and Parkinson's disease, artemisinin, EDSCs, and TSP-1 protein might be utilized as protective agents and/or adjunct treatments. Further studies will be important for distinguishing the impact of EDSCs from TSP-1 and for establishing the clinical application of anti-angiogenic agents for successful AD therapy.

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## REFERENCES

- Alzheimer's Association (2014) Alzheimer's Association Report: 2014 Alzheimer's disease facts and figures. *Alzheimer's Dement* 10: e47–e92.
- Bagheri-Mohammadi S, Alani B, Karimian M, Moradian-Tehrani R, Nouredini M (2019) Intranasal administration of endometrial mesenchymal stem cells as a suitable approach for Parkinson's disease therapy. *Mol Biol Rep* 46: 4293–4302.
- Belluti F, Rampa A, Gobbi S, Bisi A (2013) Small-molecule inhibitors/modulators of amyloid- $\beta$  peptide aggregation and toxicity for the treatment of Alzheimer's disease: a patent review (2010–2012). *Expert Opin Ther Pat* 23: 581–596.
- Cade WT (2008) Diabetes-related microvascular and macrovascular diseases in the physical therapy setting. *Phys Ther* 88: 1322–35.
- Chatterjee S, Mudher A (2018) Alzheimer's Disease and Type 2 Diabetes: A critical assessment of the shared pathological traits. *Front Neurosci* 12: 383.
- Cui YB, Ma SS, Zhang CY, Cao W, Liu M, Li DP, Lv PJ, Xing Q, Qu RN, Yao N, Yang B, Guan FX (2017) Human umbilical cord mesenchymal stem cells transplantation improves cognitive function in Alzheimer's disease mice by decreasing oxidative stress and promoting hippocampal neurogenesis. *Behav Brain Res* 320: 291–301.
- de la Monte SM (2012) Contributions of brain insulin resistance and deficiency in amyloid- related neurodegeneration in Alzheimer's disease. *Drugs* 72: 49–66.
- Esposito L, Raber J, Kekoni L, Yan F, Yu GQ, Bien-Ly N, Puoliväli J, Searce-Levie K, Masliah E, Mucke L (2006) Reduction in mitochondrial superoxide dismutase modulates Alzheimer's disease-like pathology and accelerates the onset of behavioral changes in human amyloid precursor protein transgenic mice. *J Neurosci* 26: 5167–5179.
- Ferreira JF, Luthria DL, Sasaki T, Heyerick A (2010) Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* 15: 3135–3170.
- Foy CJ, Passmore AP, Vahidassr MD, Young IS, Lawson JT (1999) Plasma chain-breaking antioxidants in Alzheimer's disease, vascular dementia and Parkinson's disease. *QJM. Int J Med* 92: 39–45.
- Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP (1990) A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci USA* 87: 6624–6628.
- Hamilton JA, Hillard CJ, Spector AA, Watkins PA (2007) Brain uptake and utilization of fatty acids, lipids and lipoproteins: application to neurological disorders. *J Mol Neurosci* 33: 2–11.
- Helal EGE, Abou- Aouf N, Khattab AM, Zoair MA (2014) Anti-diabetic effect of artemisia annua (kaysom) in alloxan-induced diabetic rats. *Egypt J Hospit Med* 57: 422–430.
- Heneka MT, O'Banion MK (2007) Inflammatory processes in Alzheimer's disease. *J Neuroimmunol* 184: 69–91.
- Hoyer S (2002) The aging brain. Changes in the neuronal insulin/insulin receptor signal transduction cascade trigger late-onset sporadic Alzheimer disease (SAD). A mini-review. *J Neural Transm* 109: 991–1002.
- Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray D N, Lim J, Moir R D, Tanzi RE, Bush AI (1999) The A $\beta$  peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry* 38: 7609–7616.
- Indumathi S, Harikrishnan R, Rajkumar JS, Sudarsanam D, Dhanasekaran M (2013) Prospective biomarkers of stem cells of human endometrium and fallopian tube in comparison to bone marrow. *Cell Tissue Res* 352: 537–549.
- Janelins MC, Mastrangelo MA, Park KM, Sudol KL, Narrow WC, Oddo S, LaFerla FM, Callahan LM, Federoff HJ, Bowers WJ (2008) Chronic neuron-specific tumor necrosis factor- $\alpha$  expression enhances the local inflammatory environment ultimately leading to neuronal death in 3xTg-AD mice. *Am J Pathol* 173: 1768–1782.
- Jefferies WA, Price KA, Biron KE, Fenninger F, Pfeifer CG, Dickstein DL (2013) Adjusting the compass: new insights into the role of angiogenesis in Alzheimer's disease. *Alzheimer's Res Ther* 5: 64.
- Li X, Song D, Leng SX (2015) Link between type 2 diabetes and Alzheimer's disease: from epidemiology to mechanism and treatment. *Clin Interv Aging* 10: 549–560.
- Liu Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong CX (2011) Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. *J Pathol* 225: 54–62.
- Lopez-Dee Z, Pidcock K, Gutierrez LS (2011) Thrombospondin-1: multiple paths to inflammation. *Mediators Inflamm* 2011: 296069.
- Moslemnezhad A, Mahjoub S, Moghadasi M (2016) Altered plasma marker of oxidative DNA damage and total antioxidant capacity in patients with Alzheimer's disease. *Caspian J Intern Med* 7: 88–92.
- Paxinos G, Watson C (1986) *The rat brain in stereotaxic coordinates* (2nd ed.). New York, Academic Press.
- Peng D, Pan X, Cui J, Ren Y, Zhang J (2013) Hyperphosphorylation of tau protein in hippocampus of central insulin-resistant rats is associated with cognitive impairment. *Cell Physiol Biochem* 32: 1417–1425.
- Poorgholam P, Yaghmaei P, Hajebrahimi Z (2018) Thymoquinone recovers learning function in a rat model of Alzheimer's disease. *Avicenna J Phytomed* 8: 188–197.
- Rubio-Perez JM, Morillas-Ruiz JM (2012) A review: inflammatory process in Alzheimer's disease, role of cytokines. *Sci World J* 2012: 756357.
- Saido TC (2013) Metabolism of amyloid  $\beta$  peptide and pathogenesis of Alzheimer's disease. *Proc Jpn Acad Ser B Phys Biol Sci* 89: 321–339.
- Sharma M, Gupta YK (2002) Chronic treatment with trans resveratrol prevents intracerebroventricular streptozotocin induced cognitive impairment and oxidative stress in rats. *Life Sci* 71: 2489–2498.
- Sinclair AJ, Bayer AJ, Johnston J, Warner C, Maxwell SRJ (1999) Altered plasma antioxidant status in subjects with Alzheimer's disease and vascular dementia. *Int J Geriatr Psychiatry* 13: 840–845.
- Swardfager W, Lanctôt K, Rothenburg L, Wong A, Cappell J, Herrmann N (2010) A meta-analysis of cytokines in Alzheimer's disease. *Biol Psychiatry* 68: 930–941.
- Tönnies E, Trushina E (2017) Oxidative stress, synaptic dysfunction, and Alzheimer's disease. *J Alzheimers Dis* 57: 1105–1121.
- Virkamäki A, Ueki K, Kahn CR (1999) Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 103: 931–943.
- Wolff EF, Gao XB, Yao KV, Andrews ZB, Du H, Elsworth JD, Taylor HS (2011) Endometrial stem cell transplantation restores dopamine production in a Parkinson's disease model. *J Cell Mol Med* 15: 747–755.
- Wright EJR, Scism-Bacon JL, Glass LC (2006) Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycaemia. *Int J Clin Pract* 60: 308–314.
- Wu Q, Finley SD (2017) Predictive model identifies strategies to enhance TSP1-mediated apoptosis signaling. *Cell Commun Signal* 15: 53.
- Yang W, Ma J, Liu Z, Lu Y, Hu B, Yu H (2014) Effect of naringenin on brain insulin signaling and cognitive functions in ICV-STZ induced dementia model of rats. *Neurol Sci* 35: 741–751.
- Zhao Y, Chen X, Wu Y, Wang Y, Li Y, Xiang C (2018) Transplantation of human menstrual blood-derived mesenchymal stem cells alleviates Alzheimer's disease-like pathology in APP/PS1 transgenic mice. *Front Mol Neurosci* 11: 140.
- Zhao X, Li S, Gaur U, Zheng W (2020) Artemisinin improved neuronal functions in Alzheimer's disease animal model 3xtg mice and neuronal cells via stimulating the ERK/CREB signaling pathway. *Aging Dis* 11: 801–819.