Triiodothyronine improves morphology and upregulates seladin-1 of neurospheres extracted from subventricular zone in streptozotocin-induced rat model of Alzheimer's disease

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Objectives In this study, the effects of triiodothyronine (T3) on neurospheres isolated from subventricular zone (SVZ) of Alzheimer's disease (AD) induced rats were examined.

Methods Eighteen male Wistar rats were classified into two groups: Sham (Sh) and STZ (streptozotocin injected, 1.5 mg/kg in each lateral ventricle on days 1 and 3 after recovery). On day 21, the SVZ was extracted and neurospheres were cultured. T3 (50 nM) was added to the culture medium (STZ+T3 group) and then, the morphology and seladin-1 gene expression of neurospheres were evaluated.

Results The diameter and the number of neurospheres along with the gene expression of seladin-1 were significantly decreased in the STZ group compared to Sh group (P < 0.05) while the administration of T3 significantly (P < 0.05) increased all these parameters in the STZ group.

Conclusion STZ decreases the proliferation of stem cells extracted from SVZ and administration of T3 to the culture media improves the morphology and upregulates the gene expression of seladin-1 of neurospheres.

Keywords Alzheimer's disease, subventricular zone, neurospheres, seladin-1.

Introduction

Alzheimer's disease (AD) is the most common progressive disorder in central nervous system (CNS) with effects on awareness contents and cognitive abilities.[1,2] *Alois Alzheimer* introduced AD for the first time by dissecting the brain of a middle-aged woman who had suffered from memory deficits and lack of progressive abilities.[1,3] Three obvious symptoms of AD are: (1) Atrophy of brain in which its weight drops, gyri narrow, sulci widen and ventricles dilate. (2) Acceleration of extracellular beta-amyloid plaques (Aβ, also called neuritic or senile plaques) formed by the amyloid precursor protein (APP) assembling. (3) Neurofibrillary tangles attributed neuronal death: Abnormalities in the cellular skeleton upset the axonal transport due to disorganization of microtubules involved there-in.[4] In terms of prevalence, 1/8th or, in other words, about 10% of people over the age of 65 are afflicted with AD.[5] AD is divided into two main types: (1) Familial Alzheimer's disease (FAD) or the inherited type that occurs prematurely and (2) Sporadic Alzheimer disease (SAD) that occurs individually without genetic mutations, often in the 80's.[5]

In this study, intracerebroventricular (ICV) injection of streptozotocin (STZ), as a diabetogenic factor, was used to suppress glucose metabolism. The STZ disrupts the regulating role of insulin and its receptor resulting in destroyed glucose metabolism in brain, lack of energy, lack of cholinergic system, and nerve growth along with learning, memory, general, and local anomalies.[6] All of these disorders are attributed to STZ that mediates glucose metabolism by means of insulin signaling cascade.[4,7]

To induce progressive deterioration, like the one seen in SAD type, STZ is injected which causes a significant reduction of energy in hippocampal and cerebral cortical tissue and therefore, a disruption in the energy associated processes including eating, sorting, and packing of proteins in the Golgi organelles. The transmission of neurotransmitters, learning behaviors, memory, and energy-rich compounds are progressively affected.[8] The progressive decline in short- and long-term memory in the post-administration phase of intravenous injection and the resultant disorders in behavior and glucose metabolism make STZ suitable for SAD model. Morris water maze (MVM) and passive avoidance memory behavioral tests have confirmed that STZ produces AD.[9]

It was believed for many years that the CNS has no neuroregenerative ability at adulthood. Nearly a century ago, as the technology progressed, cells that can replicate and differentiate into neurons and glia were identified in some areas of the brain and spinal cord. And until now, active neuronal activity in the subventricular zone (SVZ) in lateral wall of lateral ventricles, Subgranular zone of dentate gyrus, and hypothalamic subependymal zone has been documented.[10–12] In the early part of the century, Allen identified mitotic cells in the SVZ of lateral ventricles in mature rats. Ventricular region forms during embryonic development near the

ventricular area and is very noticeable in the ganglionprotu-berance region.[13] It is composed of a number of cells, possibly, B1 cells, which are the same as astrocytes. They are stem cells in the ventricular region, and these cells divide and make type C-cells that are capable of rapid proliferation. C-type cells, in turn, increase and convert to type A cells that are migratory neuroblasts. A-cells are surrounded by cells of type B1 and C.[14,15]

Preconditioning of stem cells with growth factors can improve their ability of proliferation and development and also promotes neuronal regeneration and survival.[16] Among these factors, thyroid hormones (THs) such as triiodothy-ronine (T3) are critical for brain development.[17] Although, T3 is less than thyroxine [T4], it is the most active form of THs.[18] Furthermore, the neuroprotective impacts of THs are related to enhancement of neurotrophic factors (NFs) and their anti-apoptotic and anti-inflammatory mechanisms have been proven.[19]

In this study, we decided to evaluate the effects of this hor-mone on the level of seladin-1 expression in the neurospheres isolated from SVZ in AD-induced rats.

Methods and Materials

Study Design and Animals

A total number of 18 male Wistar albino rats (100–150 g) were purchased from Shefa Neuroscience Research Center, Khatam Alanbia Hospital, Tehran, Iran. All animals were maintained in a clean and hygienic environment, on a 12-h light and dark cycle and 23 ± 2 °C temperature, and had access to food and water *ad libitum*. All procedures were carried out in accordance with the guidelines of the Iranian Council for use and care of animals and approved by Ethical Committee of Tehran University of Medical Sciences. This study was granted by Iran National Science Foundation (Grant No. 93009595). Subjects were randomly divided into two different groups: sham (Sh) group which received 5 mL normal saline in each lateral ventricle, STZ group which received 1.5 mg/kg STZ in 5 mL normal saline in each lateral ventricle on days 1 and 3. All subjects were housed in keeping cages in the resting time.

Stereotaxic Surgery

Rats were deeply anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg) (both Razi Co., Iran) intraperitoneally. For chronic implantation of cannula (27-Gauge) into lateral cerebral ventricles (ICV) under stereotactic guidance, appro-priate narcosis was verified by reflex testing such as the lack of ocular reflex and the absence of a pedal withdrawal response to a hard pinch. Therefore, animals were fixed in a stereotac-tic apparatus, and a midline incision was made on the cranial skin. Then, a small hole was induced in the cranial region and the guide cannula was implanted and fixed. Coordinates were AP-0.8 mm, L ± 1.5 mm (midline) and 3.6 mm deep from the dura mater. Animals were allowed 1 day to recover before STZ injection. STZ and its vehicle were administered on days 1 and 3 after recovery with a 10 μL Hamilton syringe (5 μL in each ventricle at a rate of 1 μL/min) connected via a Teflon tube to an injector that exceeded by 2 mm the length of the guide cannula.[4,7]

Morris Water Maze

At the end of the experiment, to confirm the defects in learn-ing and memory of animals, their behaviors were assessed by MWM test based on the previously described methods.[20] It consisted of a circular water tank (160 cm diameter, 60 cm height) filled with water (25 ± 1 °C) to a depth of 25 cm. A non-toxic water dispersible emulsion was used to render the water opaque. Four equally spaced locations around the edge of the pool (North, South, East, and West) were used as start points which divided the pool into four quadrants. An escape platform (10 cm in diameter) was placed in the pool 2 cm below the surface of water. The escape platform was placed in the middle of one of the randomly selected quadrants of the pool and kept in the same position throughout the entire experiment (north-east for this study). Animals received a training session consisting of four trials per session (once from each starting point) for 4 days (days 16, 17, 18, and 19), each trial having a ceiling time of 90 s and a trial interval of approximately 30 s. After climbing onto the hidden platform, animals remained there for 30 s before commencement of the next trial. If the rat failed to locate the hidden platform within the maximum time of 90 s, it was gently placed on the platform and allowed to remain there for the same interval of time. The time taken to locate the hidden platform (latency in s) was measured. Twenty-four hours after the acquisition phase, a probe test (day 20) was conducted by removing the platform. Rats were allowed to swim freely in the pool for 90 s and the time spent in target quadrant, which had previously contained the hidden platform, was recorded. The time and distance spent in the target quadrant indicated the degree of memory consolidation which had taken place after learning.

Cell Culture

On day 21, animals were sacrificed from both the groups (Sh and STZ) and the SVZ was isolated. The tissue samples were digested after the mechanical digestion with 250 μL trypsin 0.05% for 4 min and centrifuged by 810 rpm for 5 min after adding trypsin inhibitor. Cell deposition in neurosphere cell culture, that was consist of DMEM + F12 (gibco) glutamine 1% (gibco), B27 supplement 1% (gibco) and epidermal growth factor 20 ng/mL (sigma), was cultivated in incubator with 37 °C temperature, 95% humidity, and 5% CO_2. Within 15 days, nerve/protozoal stem cells were proliferated as a series of spherical cells called neurospheres. By increasing the volume of the neurospheres, the cell passage by collecting the neu-rons in the Falcon tube and centrifuging for 5 min at 810 rpm was performed. To test the effects of T3 on neurospheres, T3 (50 nM) was added to the medium (STZ + T3 group). Medium was renewed every 2–3 days.

Morphometric Studies

In order to evaluate cell proliferation, both the numbers and diameters of neurospheres were considered. On the seventh day of cultivation, five microscopic images were taken randomly from each container. Cell counting was performed by using Trypan blue and Neobar lam and after the second passage, the diameter and number of neurons were measured. Number of neurospheres was counted by infinity software. The neuro-spheres diameter were measured by using the infinity software

and the results were described as the mean of two perpendicular diameters to the diameter of the neurospheres. The number and diameter of the neurospheres and cells were compared.

Real-Time PCR

Expression of Seladin-1 in neurospheres was measured by Real-time PCR. Total RNA was isolated from hemispheres, then mRNA (1 μg) was converted to cDNA via reverse transcription using First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Specific primers along with cDNA and PCR reagents (polymerase, dNTP, magnesium and buffer; 5× HOT FIREPol® EvaGreen® qPCR Mix Plus [ROX] 1 mL, Solis Bio Dyne, Estonia) were placed on the three-color real-time PCR machine (Applied Biosystems Step One, USA) for further analysis. At first, incubation of samples was performed at 95 °C for 15 min for initial polymerase activation. Then, samples underwent the three subsequent phases as follows: (1) denaturation (at 95 °C for 15 s); (2) annealing (at 60 °C for 20 s); and (3) elongation (at 72 °C for 20 s). Finally, quantification of data, its normalization to GAPDH and fold change comparison to the control were performed.[21] In Table 1, the nucleotide sequences of primers are shown.

Table 1.

Primer		Sequence
Seladin-1	Forward	5'-GGGTGTTTGTGTGCCTCTTCC-3'
	Reverse	5'-GCTCCTTCCACTCCCGTACC-3'
b-actin	Forward	'-CTGTCTGGCGGCACCACCAT-3'
	Reverse	5'-GCAACTAAGTCATAGTCCGC-3'

Statistical analysis

All data were analyzed through one-way analysis of variance (ANOVA) and the post-hoc Tukey's, and two-way ANOVA and the Bonferroni's multiple comparison tests were applied for behavioral data belonging to learning and memory skills using Graph-Pad PRISM version 6 Software (San Diego, California, USA). Results were represented as mean ± SEM and $p < 0.05$ was considered significant.

Results

Effects of ICV injection of STZ on learning and memory in the rat model of AD

Injection of STZ induced defect in spatial learning and memory of STZ group. According to Fig. 1, the mean latency time to reach the hidden platform (P < 0.05, Fig. 1a) and distance traveled in the MVM (P < 0.05, Fig. 1b) were significantly increased in STZ group compared to Sh group. Moreover, STZ-treated groups spent significantly less time (P < 0.05, Fig. 1c) and distances in the platform quadrant (P < 0.05, Fig. 1d) compared to Sh group.

Effects of T3 on the number of neurospheres extracted from SVZ in the rat model of AD

The number of neurospheres decreased in the AD model of rat compared with Sh group (p < 0.05, Fig. 1). Culturing

with medium contained T3 increased the number of neurospheres in the STZ + T3 neurospheres compared to STZ group (p < 0.05, Fig. 1).

Effects of T3 on diameter of neurospheres extracted from SVZ in the rat model of AD

Based on the results, the diameter of neurospheres decreased in the AD model group (STZ group) compared with Sh group (p < 0.05, Fig. 2). The diameter of neurospheres in the STZ + T3 increased compared to STZ group ($p < 0.05$, Fig. 1).

Effects of T3 on seladin-1 gene expression of neurospheres extracted from SVZ in the rat model of AD

Based on the results, the seladin-1 gene expression decreased in the AD model group (STZ group) compared to Sh group (p < 0.05, Fig. 2). The seladin-1 gene expression in T3 group increased compared to STZ group (p < 0.05, Fig. 1).

Discussion

In this study, the effects of T3 on morphology and seladin-1 gene expression of neurospheres extracted from SVZ were investigated in rat model of AD. In this study, STZ was used to induce AD in animals. AD is the most common cause of dementia diagnosed after the age of 60, and it is characterized by neuronal loss as a consequence of neurofibrillary tangles and senile plaques. There are different theories which explain the accumulation of beta amyloid plaques, but abnormal levels of oxidative stress have been reported in both brain and blood stream in AD.[22,23] An enhanced oxidative stress induced modified proteins and mitochondrial dysfunction in AD brain have been reported,[24,25] and it has also been suggested that oxidative stress is a key for the progression of AD.[26] The mechanism of AD development in the brain is unknown. However, different studies have proved that chronic ICV injections of STZ can reduce uptake of cerebral glucose and induce multiple other effects that resemble behavioral, molecular, pathological characteristics of AD.[27]

The results of present study revealed that the diameter and the number of neurospheres were significantly decreased in the STZ treatment group compared to Sh group.

AD, the widespread cause of dementia, is characterized by progressive neurodegeneration and the appearance of specific histopathological markers represented by focal extracellular deposits of fibrillar Aβ in the brain parenchyma and in the wall of blood vessels, and by the intraneuronal accumulation of neurofibrillary tangles formed as a result of the abnormal hyperphosphorylation of cytoskeletal Tau filaments.[28] The initial neurodegenerative events of AD appear in the transentorhinal cortex, and subsequently spread to the entorhinal cortex and to the hippocampus.[29,30] SVZ, as a neurogenic niche, contains multipotent neural stem cells (NSCs).[31,32] The NSCs that exhibit slow self-renewal produce neural progenitor cells with a faster dividing cell cycle, which ultimately differentiate into neurons or neuroglia; the differentiation process is regulated by numerous trophic factors.[33,34] The majority of studies performed on transgenic animals expressing the mutant APP demonstrated decreased neurogenesis either in the Dentate Gyrus (DG) or in both the DG and the SVZ.[35–38] In a previous similar study, Mozhdeh et al. demonstrated that the ICV
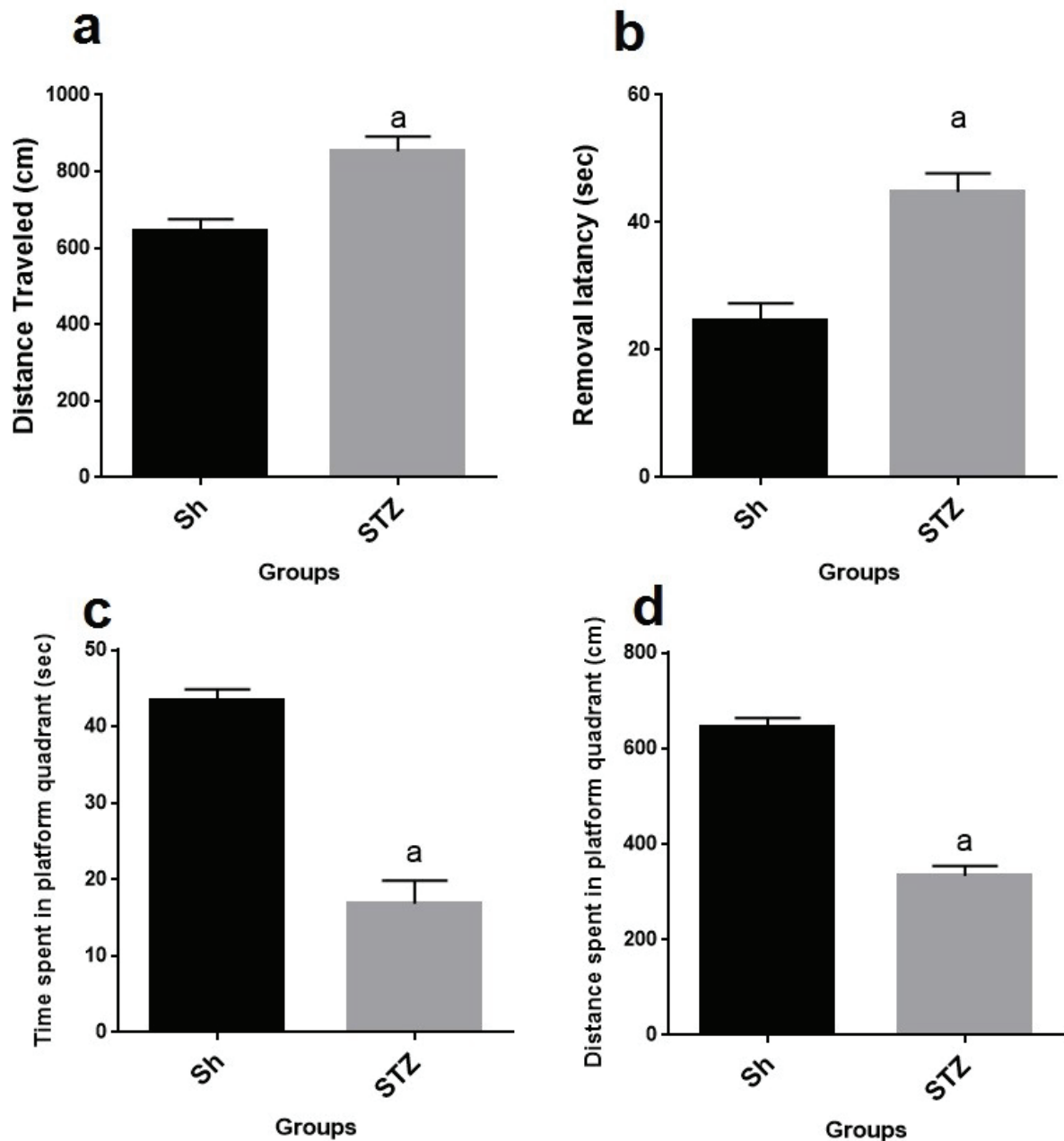
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T3 improved the neurospheres from SVZ in model of Alzheimer's disease

Fig. 1 **Effects of ICV injection of STZ on spatial memory and learning in the rat model of AD.** (**a**) Latency to platform (s), (**b**) sistance traveled to platform, (**c**) time, and (**d**) distance spent in the platform quarter. p < 0.05 compared to Sh group. Sh: sham group with ICV injection of normal saline; STZ: animals with ICV injection of STZ

injection of STZ impaired the proliferation feature of SVZ extracted neurospheres in a rat model of AD[9] which confirmed the results of present study.

The results of present study showed that T3 could increase the seladin-1 gene expression in the stem cells extracted from SVZ in the rat model of AD.

The mechanisms of TH levels alteration as a factor to increase the mobility towards AD in older people is unclear. Numerous researches tried to find a relation between AD and TH levels. In a study by Ceresini et al.,[39] 1171 participants from Italy were analyzed and it was reported that dysfunction of thyroid gland tends to be more frequent in older people than in youngsters, with subclinical hyperthyroidism being

the most prevalent condition. Their findings demonstrated an independent association between cognitive impairment and subclinical hyperthyroidism. Their findings were similar to those obtained from Kalmijn et al. investigation.[40]

Some studies have demonstrated a very high prevalence of autoimmune thyroid disease in familial AD.[41,42] Similarly, Ganguli et al. reported that the subclinical hypothyroidism state correlates with cognitive disorders in patients aged ≥65.[43]

An alternative mechanism of TH to improve AD has been described. The increase of the seladin-1 gene and protein expression of seladin-1 (selective AD indicator 1) gene cause decrease neuronal death,[31,44] promotes cholesterol synthesis inside the neuron,[45] and reduce Aβ accumulation by inhibition
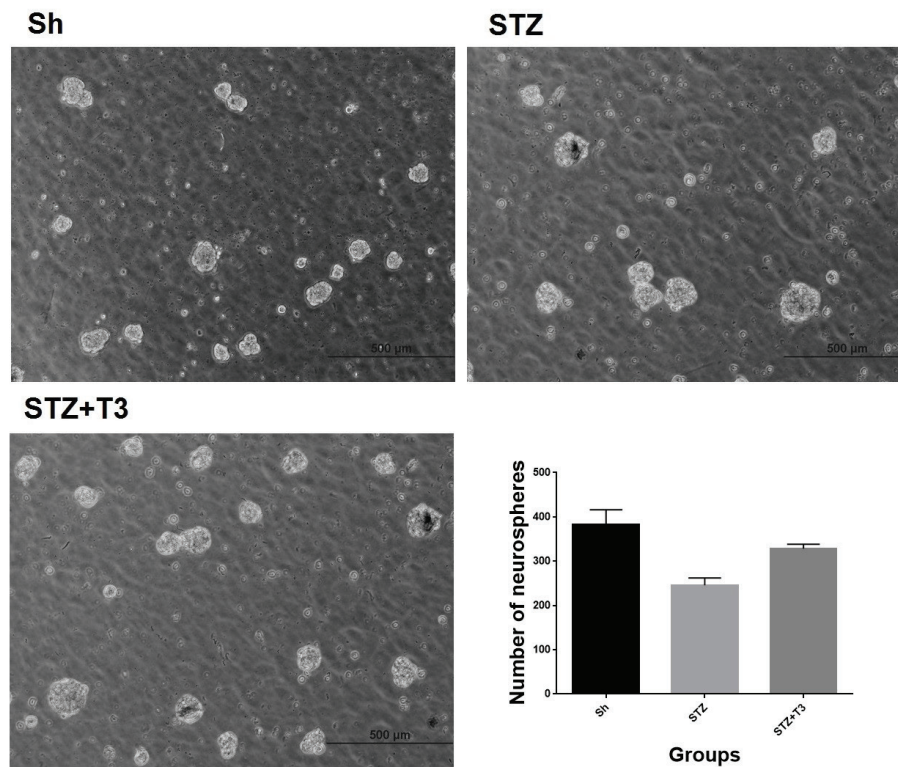
Fig. 2 **Effects of T3 on the number of neurospheres extracted from SVZ in the rat model of AD. (a)** p < 0.05 compared to Sh group, **(b)** p < 0.05 compared to STZ group. Sh: neurospheres isolated from rats received ICV injection of normal saline; STZ: neurospheres isolated from rats received ICV injection of STZ, STZ + T3: neurospheres isolated from rats received ICV injection of STZ and cultured with T3.
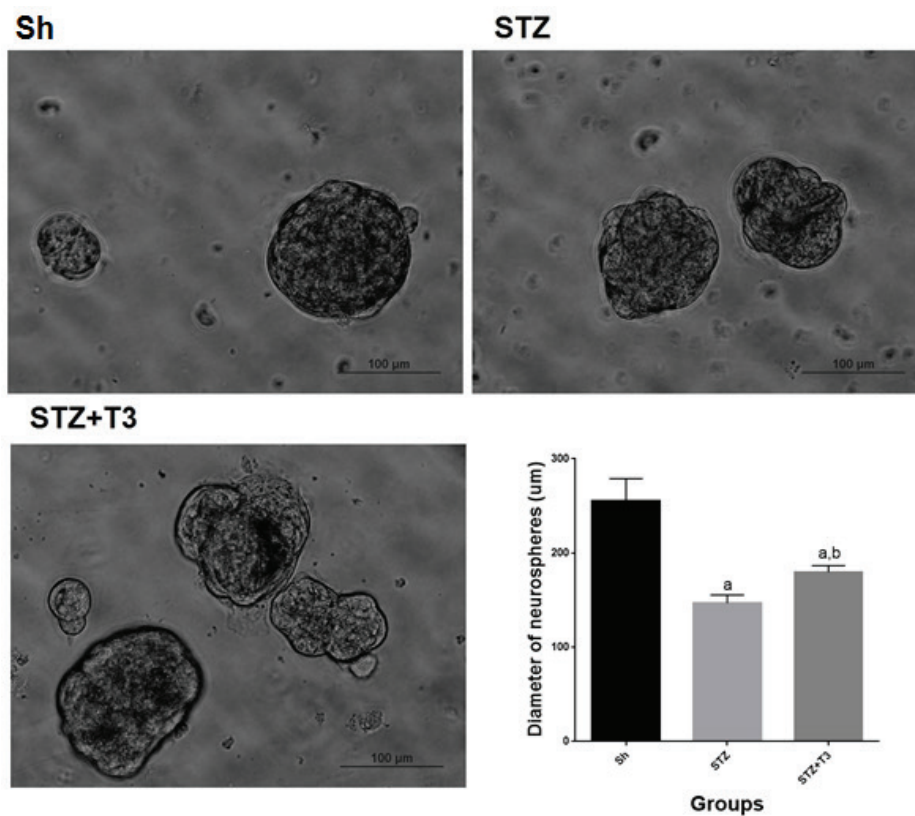

Fig. 3 **Effects of T3 on seladin-1 gene expression of neurospheres extracted from SVZ in the rat model of AD. The gene expression of seladin-1 was investigated using real-time PCR. (a)** p < 0.05 compared to Sh group, **(b)** p < 0.05 compared to STZ group. Sh: neurospheres isolated from rats received ICV injection of normal saline; STZ: neurospheres isolated from rats received ICV injection of STZ; STZ + T3: neurospheres isolated from rats received ICV injection of STZ and cultured with T3.
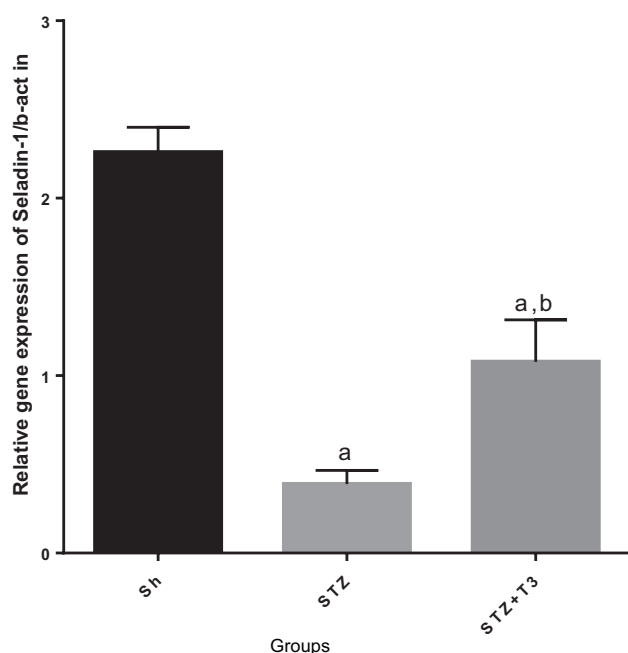
Fig. 3 **Effects of T3 on the diameter of neurospheres extracted from SVZ in the rat model of AD. (a)** $p < 0.05$ compared to Sh group, **(b)** $p < 0.05$ compared to STZ group. Sh: neurospheres isolated from rats received ICV injection of normal saline; STZ: neurospheres isolated from rats received ICV injection of STZ; STZ +T3: neurospheres isolated from rats received ICV injection of STZ and cultured with T3.

of co-localization of Aβ precursor protein.[46] TH exerts an essential effect on the maintenance and development of CNS.[47]

The effectiveness of TH on seladin-1 gene expression was investigated. Ishida et al. reported that TH upregulated the expression of seladin-1, a gene related to AD in whole cell extract from mouse forebrain.[44] Benvenuti et al. reported that TH increased TR-α1, TR-β1, and TR-β2 and seladin-1 gene expression in an *in vitro* model of human neuronal precursor cell lines.[48] Likewise, the beneficial effects of T3 on neurodegenerative diseases have been presented in a study by Mokhtari et al., it was proven that injection of T3 improved memory and learning by upregulation of brain-derived neurotropic factor and Glial cell-derived neurotropic factor in CA1 hippocampal region in rat model of ischemic brain stroke.[49]

Conclusion

The results obtained from this study showed that the ICV injection of STZ decreases the number and diameter of neurospheres and seladin-1 gene expression in rats (model of AD). Additionally, adding T3 to the culture medium of these neurospheres improves their morphological characteristics and increases seladin-1 gene expression.

Acknowledgement

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