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MicroRNA modulation is a potential molecular mechanism for neuroprotective effects of intranasal insulin administration in amyloid βeta oligomer induced Alzheimer's like rat model

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ABSTRACT

Substantial evidence indicates that imbalance in the expression of miR-132-3p, miR-181b-5p, miR-125b-5p, miR-26a-5p, miR-124-3p, miR-146a-5p, miR-29a-3p, and miR-30a-5p in the AD brain are associated with amyloid-beta (Aβ) aggregation, tau pathology, neuroinflammation, and synaptic dysfunction, the major pathological hallmarks of Alzheimer's disease)AD(. Several studies have reported that intranasal insulin administration ameliorates memory in AD patients and animal models. However, the underlying molecular mechanisms are not yet completely elucidated. Therefore, the aim of this study was to determine whether insulin is involved in regulating the expression of AD-related microRNAs. Pursuing this objective, we first investigated the therapeutic effect of intranasal insulin on Aβ oligomer (AβO)-induced memory impairment in male rats using the Morris water maze task. Then, molecular and histological changes in response to AβO and/or insulin time course were assessed in the extracted hippocampi on days 1, 14, and 21 of the study using congo red staining, western blot and quantitative real-time PCR analyses. We observed memory impairment, Aβ aggregation, tau hyper-phosphorylation, neuroinflammation, insulin signaling dys-regulation, and down-regulation of miR-26a, miR-124, miR-29a, miR-181b, miR-125b, miR-132, and miR-146a in the hippocampus of AβO-exposed rats 21 days after AβO injection. Intranasal insulin treatment ameliorated memory impairment and concomitantly increased miR-132, miR-181b, and miR-125b expression, attenuated tau phosphorylation levels, Aβ aggregation, and neuroinflammation, and regulated the insulin signaling as well. In conclusion, our study suggest that the neuroprotective effects of insulin on memory observed in AD-like rats could be partially due to the restoration of miR-132, miR-181b, and miR-125b expression in the brain.

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder defined clinically by progressive loss of memory and other cognitive functions. (Wang et al., 2021). Complex interactions between genetic, epigenetic, and environmental factors contribute to the occurrence and progression of AD. AD is characterized by extracellular accumulation of senile plaques consisting of the Aβ peptide, intracellular neurofibrillary tangles (NFTs) composed of abnormally hyper-phosphorylated tau (p-tau), mitochondrial dysfunction, neuroinflammation, reduced insulin sensitivity and deregulated brain insulin signaling, dysfunctional synapses, and neuronal degeneration in brain regions related to learning and memory, such as the hippocampus (Ferreira, 2021; Srivastava et al., 2021). The causes of AD are not yet fully understood, but studies over the past few decades indicate that Aβ and tau play a key role in AD pathogenesis (Penke et al., 2020). The amyloid hypothesis assumes that Aβ aggregation triggers a cascade of events, ultimately resulting in AD (Hardy and Higgins, 1992). It has long been assumed that fibrillar Aβ (fAβ) assemblies initiate neurodegenerative cascades and are upstream of other pathological events (Hardy and

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Higgins, 1992). Today, new evidence demonstrates that oligomeric Aβ assemblies, not fAβ assemblies, are the pathogenic forms of Aβ and can instigate multiple features of AD neuropathology (Cline et al., 2018). Due to the multifactorial and complicated pathogenesis of AD, there is currently no modifying therapy for AD.

Genome-wide transcriptome studies demonstrate that many important genes dysregulated in AD are essential for AB/Tau metabolism, regulation of tau phosphorylation, synapse function and formation, inflammatory responses, energy metabolism, mitochondrial function, etc. (Fan et al., 2021; Tan et al., 2010). As a principal group of gene regulators, microRNAs (miRNA/miR) contribute to maintaining transcriptome homeostasis. miRNAs are small non-coding RNA molecules that induce mRNA degradation and/or translational repression by binding to the 3′- UTR region of target mRNA (O'Brien et al., 2018). Substantial evidence indicates that altered expression of certain miRNAs such as miR-132-3p, miR-181b-5p, miR-125b-5p, miR-26a-5p, miR-124-3p, miR-146a-5p, miR-29a-3p, and miR-30a-5p in the AD brain are associated with AD pathogenesis (Table S1) (Kou et al., 2020; Li and Cai, 2021; Wang et al., 2019). It has been demonstrated that these eight miRNAs regulate genes expression associated with tau phosphorylation, inflammatory responses, synaptic plasticity, and Aβ production (Table S1). Taking advantage of the fact that microRNAs affect many biological pathways, the regulation of microRNAs will provide a new perspective for discovering the pathogenesis and treatment of AD that results from abnormal changes in several pathways (Bazrgar et al., 2021; Hussein and Magdy, 2021). Therefore, many researchers have attempted to develop a new therapeutic strategy to modulate the aberrantly expressed miRNAs in the AD brain by introducing miRNA mimics or inhibitors. However, there are several challenges to developing miRNAs-based therapies. The main problem is the lack of an effective and specific delivery system to deliver miRNAs to the brain.

Insulin is described as a critical regulator of gene expression, neuronal differentiation, neurite outgrowth, energy metabolism, mitochondrial function, neuronal survival, neurotransmission and, synaptic plasticity in the brain (Ghasemi et al., 2013). The neuromodulatory actions of insulin are mediated by its receptors which are distributed throughout the brain. One of the main signaling pathways activated by insulin is phosphatidylinositol 3-kinase (PI3K) / protein kinase B)AKT (pathway, in which insulin binds to its receptor, causing autophosphorylation of the insulin receptor (IR) and invoke insulin receptor substrate (IRS). IRS phosphorylation (at the tyrosine residues) leads to PI3K activation, which in turn phosphorylates and activates Akt. Activation of Akt has involved in neuronal protection and learning and memory functions through glycogen synthase kinase 3β (GSK-3β) phosphorylation (at the serine 9 residues) and consequently its inactivation (Akhtar and Sah, 2020; Bedse et al., 2015). Growing evidence indicates that alterations in brain insulin levels and disruption of insulin signaling contribute to the development of AD (Akhtar and Sah, 2020; Nguyen et al., 2020). Of note, several studies have reported that intranasal insulin administration ameliorates memory in healthy individuals, AD patients, and animal models (Craft et al., 2020; Hallschmid, 2021; Lv et al., 2020). However, the underlying molecular mechanisms are not yet completely elucidated. Granjon et al. in 2009 showed that insulin is involved in the regulation of microRNAs expression in human skeletal muscle (Granjon et al., 2009).

Therefore, the aim of this study was to determine whether intranasal insulin improves memory in AD through regulating the expression of miRNAs associated with tau pathology, Aβ aggregation, neuroinflammation, and synaptic dysfunction in the hippocampus of an AβO induced AD-like animal model.

2. Material and methods

2.1. Materials

Amyloid β Protein Fragment 1–42, Phospho-Tau (Thr231) polyclonal antibody (SAB4504563-100UG), and Skimmed milk powder were purchased from Sigma-Aldrich Co (USA). Regular human insulin was obtained from Exir Co, Tehran, Iran. Phospho-GSK3B (Ser9) polyclonal antibody (PA1–4688), Phospho-Tau (Ser404) polyclonal antibody (44-758G) and, Phospho-Tau (Ser396) polyclonal antibody (44-752G) were purchased from Invitrogen Co (USA). Beta-Actin (4970) and Phospho-Tau (Thr205) monoclonal antibody (49561) were purchased from Cell Signaling Technology, Danvers, MA, USA. Horseradish peroxide (HRP) conjugated anti-rabbit secondary antibodies were obtained from Cell Signaling Technology. Salts were purchased from Merck Co (Darmstadt, Germany). Clarity western ECL substrate (170–5060) was purchased from Bio-Rad (USA), polyvinylidene fluoride (PVDF) membrane (IPVH00010) was obtained from Millipore Co (Germany). Protein size marker (10714914) was purchased from Fisher-Scientific. Total RNA purification kit was purchased from GeneAll Co (South Korea). FIREScript RT cDNA Synthesis Kit was obtained from Solis BioDyne Co (Tartu, Estonia). SYBR Green Real-Time PCR Master Mix was purchased from Amplicon Co (Denmark). MicroRNA assay kit was purchased from Ana Cell Co (Tehran, Iran). Congo Red Stain Kit was obtained from Asia Pajhohesh Co (Amol, Iran). Other reagents were acquired from usual commercial sources.

2.2. Animals

A total of 64 adult male Wistar rats with an average age of 12-weeks weighing 230–250 g from the breeding colony of Neuroscience Research Center were housed in Plexiglas cages in groups of 4 per cage. Animals were kept in a standard room with a controlled temperature (23 ± 2 °C), and light on between 7:00 AM and 7.00 PM. Animal and human data indicate that males and females with memory problems respond to intranasal insulin treatment differentially (Claxton et al., 2011). On memory tasks, women may be more sensitive to insulin's beneficial effect on the CNS. studies have shown that males benefitted from both low dose and high dose of intranasal insulin, whereas females only benefitted from the lower dose (Claxton et al., 2011). Cortisol, a hormone related to obesity that is typically higher in females, may interact with insulin to influence cognition. Research has been inconclusive regarding the role that estradiol may have, which may also interact with insulin to influence between-sex differences. In this study, male rats were used to eliminate the effects of hormonal changes. All experimental procedures were approved by the ethics committee of the Shahid Beheshti University of Medical Sciences (IR. SBMU.MSP.REC.1397.556) and were following the National Institutes of Health Guide for the care and use of laboratory animals.

2.3. Soluble amyloid beta oligomers (AβOs) preparation

Soluble AβOs were prepared from synthetic human Aβ1–42 using the procedure of Lambert et al. (Lambert et al., 1998). Briefly, Aβ1–42 was reconstituted with ice-cold hexafluoroisopropanol (HFIP) and allowed to evaporate overnight in microcentrifuge tubes. The next day, the unaggregated Aβ1–42 in tubes as a dried HFIP film was stored at −80 °C until oligomer preparation. On the day prior to testing, the film was dissolved to 5 mM in anhydrous, sterile dimethyl sulfoxide (DMSO), diluted in phosphate buffer saline (PBS) to a final concentration of 100 μM and maintained at 4 o C for 24 h to enhance oligomer formation. Preparation of vehicle control followed the same protocol.

2.4. Drug administration and experimental groups

2.4.1. Intra-hippocampal injection of soluble AβOs

The rats were first intraperitoneally anesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg, respectively) and restrained onto a stereotaxic apparatus; then the following coordinates were used for intra-hippocampal injection based on the Paxinos and Watson atlas: −3.84 mm AP, ±2.2 mm ML to Bregma and − 3.2 mm DV to the dura.

AβOs (2*7.5 μg/2*3 μL/rat) were bilaterally injected into the CA1 area of the hippocampus at a rate of 0.5 μL/min to create an AD-like animal model. The control animals were treated identically but with vehicle (DMSO+PBS).

2.4.2. Intranasal insulin administration

One week after one single intra-hippocampal injection of AβOs, the rats received repeated intranasal insulin (2 IU/day; 1 IU in each nostril) or saline daily for 10 consecutive days (day 7 to day16). Intranasal insulin was delivered as per the previous report (Marks et al., 2009). In brief, rats were anesthetized with very mild inhalation of CO2 and kept in a supine position with neck extended. The dose of intranasal insulin is based on the earlier reported study (Yang et al., 2013). 24 h after the last insulin injection, the MWM test was started to assay spatial learning and memory. Fig. 1 shows the design of this study graphically.

2.4.3. Groups of animals

64 rats were randomly divided into four equal groups with 16 rats in each one, of which three rats on day 1, three rats on day 14 and 10 rats on day 21 were examined. The groups are as follows:

1. Control Group: Rats were injected (Intra-hippocampal) DMSO+PBS (vehicle of AβO) on day 0 and treated with intranasal saline starting from days 7 to 16.
2. AβO Group: Rats were injected (Intra-hippocampal) AβO (3 μL, 7.5 μg) in each hippocampus on day 0 and treated with intranasal saline starting on days 7 to 16.
3. AβO + Insulin Group: Rats were injected (Intra-hippocampal) AβO (3 μL/(7.5 μg) in each hippocampus) on day 0 and treated with intranasal insulin (2 IU/rat/day) starting on days 7 to 16.
4. Insulin Group: Rats were injected (Intra-hippocampal) DMSO+PBS (vehicle of AβO) on day 0 and treated with intranasal insulin (2 IU/rat/day) starting from day 7 to 16.

2.5. Behavioral assessment

2.5.1. Morris water maze (MWM) test

The day after the last insulin/saline administration, spatial learning (acquisition) and memory were assessed with the MWM task from day 17 to 21 of experiments. The maze consisted of a black circular pool (150 cm in diameter, 60 cm in height) filled with water (the temperature at 22 °C). The pool was conceptually divided into four equal quadrants by imaging lines. A black platform (diameter, 10 cm) was located in the center of the target quadrant, and submerged approximately 1.5 cm below the water's surface. The day before the beginning of training (day 17), rats were habituated to swimming in the pool without the platform. During spatial acquisition, all rats were trained to find the platform and underwent four trials per day for 3 consecutive days. All animals were released the water facing the wall of the pool. The starting quadrant was changed each day. If the rat fails to find the hidden platform within 60 s, it was directed to the platform by the experimenter. The rats were allowed to remain on the platform for 20 s. The probe trial was performed 24 h after the last acquisition trial. In this phase, the hidden platform was removed, and rats were given 60 s to swim in the pool. The time to reach the platform (escape latency), the time spent in the target quadrant, and swimming speeds were recorded by a computerized tracking system (Noldus EthoVision, 11.1 versions).

2.6. Tissue preparation

Animals were sacrificed at three time points of the study (on days 1 (n = 3 different sample/group), 14 (n = 3 different sample/group), and 21 (n = 6 different sample/group)) by cervical dislocation after deep CO2 inhalation; thereafter, the hippocampi were rapidly dissected on the ice and then transferred to the liquid nitrogen for 24 h. Finally, all samples were stored in −80 °C until molecular and biochemical analysis. miRNAs expression patterns in response to AβO and/or intranasal insulin time course were measured on days 1, 14, and 21 of the study. The levels of tau protein phosphorylation, pro-inflammatory cytokines, and some component involved in the insulin signaling were assessed on day 21 of the study. Fig. 1 shows the design of this study graphically. In order to histological analysis, **three rats** from each group on day 21 were deeply anesthetized and perfused transcardially using ice-cold PBS (10 mM, pH.7.4), followed by 4% paraformaldehyde (PFA) in PBS. Then, the whole brains were isolated and post-fixed in PFA for at least 72 h at 4 °C. Following the gradient dehydration, the tissues were embedded in paraffin wax at 59 °C and sectioned to a thickness of 5–7 μm.

2.7. Western blot

Hippocampi of rats (n = 3 different sample/group) were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM Sodium orthovanadate, 15 mM Sodium pyrophosphate, 50 mM Sodium fluoride, and 1 mM PMSF. Protein concentrations of the homogenates were measured by the Bradford method. Equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis, and electro-transferred onto a polyvinylidene fluoride membrane. Then, membranes were incubated for 1 h in blocking buffer (Skimmed milk 2.5%) at room temperature. Next, the membranes were incubated with the primary antibody at 4 °C overnight. The next day, the membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 90 min at room temperature. After being washed, membranes were smeared with ECL one by one to assess their immunoreactivity. β-actin expression was analyzed using anti-β-actin rabbit monoclonal antibody as an internal control for the normalization of protein amounts. Finally, the intensities of protein bands were analyzed by Image-j software (from NIH (Bethesda, MD, USA)).

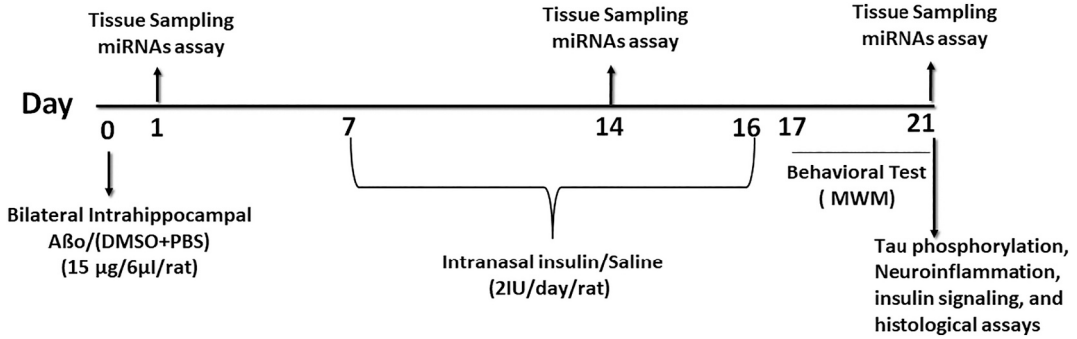

Fig. 1. Schematic illustration of the study protocol.

2.8. Quantitative real-time PCR (qRT-PCR) analysis

Briefly, after hippocampal isolation ($n = 3$ different sample/group) and total RNA extraction using Hybrid-R™ Kit (Gene All) according to the manufacturer's instructions, cDNA for microRNAs was prepared using a microRNA reverse transcription (RT) kit (Ana Cell, Tehran, Iran) and microRNA-specific stem-loop RT primer provided in the kit. cDNA for mRNAs was synthesized using FIREScript RT cDNA Synthesis Kit (Solis BioDyne) according to the manufacturer's instructions. qRT-PCR for microRNAs was performed by the SYBR green MicroRNA assay kit (Ana Cell, Tehran, Iran) and microRNA-specific forward and reverse primers included in the kit. qRT-PCR for mRNAs encoding IL-1β, TNF-α, IR, PI3K, and AKT was performed with RealQ Plus 2× Master Mix Green, High ROX TM (Ampliqon, Denmark) and primers (Metabion, Martinsried, Germany) listed in Table 1, using ABI PRISM 7000 sequence detection system (Ambion-Applied Biosystems, Foster City, CA, USA). The threshold cycles (Ct) were used to quantify the microRNA, and mRNA expression. The relative expression levels were calculated using the $2^{-\triangle\triangle ct}$ method after normalization with U6 and β-actin housekeeping genes. U6 was used as the internal control for 8 selected microRNAs expression analysis, and β-actin was used as the internal control for determining the expression of mRNAs encoding IL-1β, TNF-α, IR, PI3K, and AKT. A primer set for the U6 gene was also included in the MicroRNAs assay kits.

2.9. Congo red staining

Congo red staining of brain sections was employed for identification of congophilic Aβ deposits in the hippocampus. Binding studies have revealed the presence of binding sites for Congo red on both AβOs and Aβ fibril plaques (Maezawa et al., 2008). Congo red stain kit (Asia Pajohesh, Iran) was used for staining of Aβ aggregates. Initially, the brain sections were deparaffinized and rehydrated with xylene and descending graded methanol. Next, the sections were stained with congo red for 60 min at room temperature. Brain sections were then washed in running tap water for 5 min. After this, brain sections were rinsed in lithium carbonate solution and distilled water. In the next step, brain sections were counterstained with a Mayer hematoxylin solution for 2 min and then rinsed in running tap water for 10 min. Brain sections were quickly dipped in 95% methanol (three times) and then dipped twice in 100% methanol. After this, brain sections were clarified with xylene and finally mounted with entellan glue (Merck, Germany). Five to seven Congo red-stained sections of each animal ($n = 3$/group) at a level of 2.9 to 3.8 mm posterior to the bregma, were selected for detection of congophilic Aβ deposits by the light microscope (Nikon). The CA1 and Dentate gyrus (DG) regions were assessed and taken photos at fields of 10×, 20×, and 40× magnification. The amyloid deposits were stained red and the nuclei were stained Purple.

2.10. Statistical analysis

Data were analyzed by GraphPad Prism 9.0.0. The data obtained from the MWM training days were analyzed with repeated measures two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The data of retention test, AUC-escape latency, and molecular and histological studies were analyzed by one-way ANOVA followed by Tukey's post-hoc test. The data of miRNA assay on day 1 of the study were analyzed using an unpaired *t*-test. All data have been reported as mean ± standard error of the mean (SEM). $P < 0.05$ is considered as a significant difference, in all reported statistical comparisons.

Table 1
Primer sequences (5′-3′) used in qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
IR	GGTGTAGTGGCTGTCACATT	GAGCGGAGGAGTCTTCATT
PI3K	ATGTGTATGGACCCGGAAGG	AGCCATCTGCCTCCACGTTAG
Akt	ACGTAGCCATTGTGAAGGAGG	TGCCATCATTCTTGAGGAGGAA
TNFα	ACTGAACTTCGGGGTGATCG	CGCTTGGTGGTTTGCTACG
IL1β	ACCCAAGCACCTTCTTTTCCTTC	GTCGTTGCTTGTCTCTCCTTGTA
β-actin	TCTATCCTGGCCTCACTGTC	AACGCAGCTCAGTAACACTCC

3. Results

3.1. Intranasal insulin ameliorated AβO induced learning and memory impairment in animals

Previous studies have demonstrated that intrahippocampal administration of AβOs impairs learning and memory in animals (Balducci and Forloni, 2014; Pearson-Leary and McNay, 2012). MWM test was used to evaluate the effect of AβO and/or intranasal insulin on spatial reference learning and memory. As shown in Fig. 2A, all animals ($n = 8$–10/group) learned the hidden platform location during the 3-day acquisition phase, as demonstrated by the reduced latency to locate the platform ($F(2, 64) = 34.09$, $P < 0.0001$). Although Two-way repeated measures ANOVA revealed that there was a significant difference in the mean escape latency between groups during the 3-day acquisition ($F(3,32) = 4.81$, $P = 0.0071$), Post-hoc comparison by Tukey's test showed no significant difference in the mean escape latency insulin/ insulin+ AβO/ AβO receiving animals compared to control animals. For further comparison, the areas under the learning curves (AUCs) for escape latency during the 3-day acquisition were calculated for each individual. AUC analysis of the parameter 'escape latency' was performed through one-way ANOVA ($F(3, 32) = 5.430$, $P = 0.0039$) and revealed that AUC for the escape latency was significantly increased in the AβO group compared to control animals ($p < 0.05$), indicating impaired spatial learning in these animals, while it was substantially reduced in animals received both AßO and insulin ($p < 0.05$), indicating intranasal insulin treatment improved AβO induced learning disorders in animals (Fig. 2B).

In the next step, to evaluate the spatial memory retention level of animals, a probe trial was employed 24 h after the last acquisition trial. As presented in Fig. 2C, One- way ANOVA revealed that there was a significant difference in the mean time spent in the target quadrant between groups ($F(3, 32) = 3.881$, $P = 0.0179$). Post-hoc analysis by Tukey's test demonstrated that AβO receiving animals spent significantly less time than the control animals in the target quadrant ($p < 0.05$), indicating impaired spatial memory in these rats. As it is depicted in Fig. 1B, treatment with intranasal insulin for 10 days (day 7 to day 16 (significantly increased the time spent in the target quadrant ($p < 0.05$), indicating intranasal insulin ameliorated AβO induced memory impairment in animals. No significant differences were detected in the mean time spent following only insulin treatment compared with control group.

To assess if AβO and/or intranasal insulin didn't affect motor function, the swimming speed of all animals was measured on probe day. As it is shown by Fig. 2D, one-way ANOVA didn't show a significant difference in the swimming speed of the animals between groups ($F(3, 32) = 1.643$, $P = 0.1990$).

3.2. The effect of intranasal insulin treatment on the number of congophilic Aβ deposits in the hippocampus of AβO receiving animals

The whole-brain sections collected on day 21 were stained with Congo red for detection of Aβ deposits in the hippocampus of different groups. As shown in Fig. 3, congophilic amyloid deposits were observed in the hippocampal CA1, and DG areas of the AβO receiving rats. On the other hand, the congophilic amyloid deposits density were markedly reduced in the hippocampus of the insulin-treated AβO group compared to the untreated AβO group (Fig. 3). Fig. 4 shows the effects of intranasal insulin treatment on the number of congophilic Aβ deposits in the hippocampus of rats after the administration of AβO. AβO-treated rats showed a significant increase in the mean number of congophilic

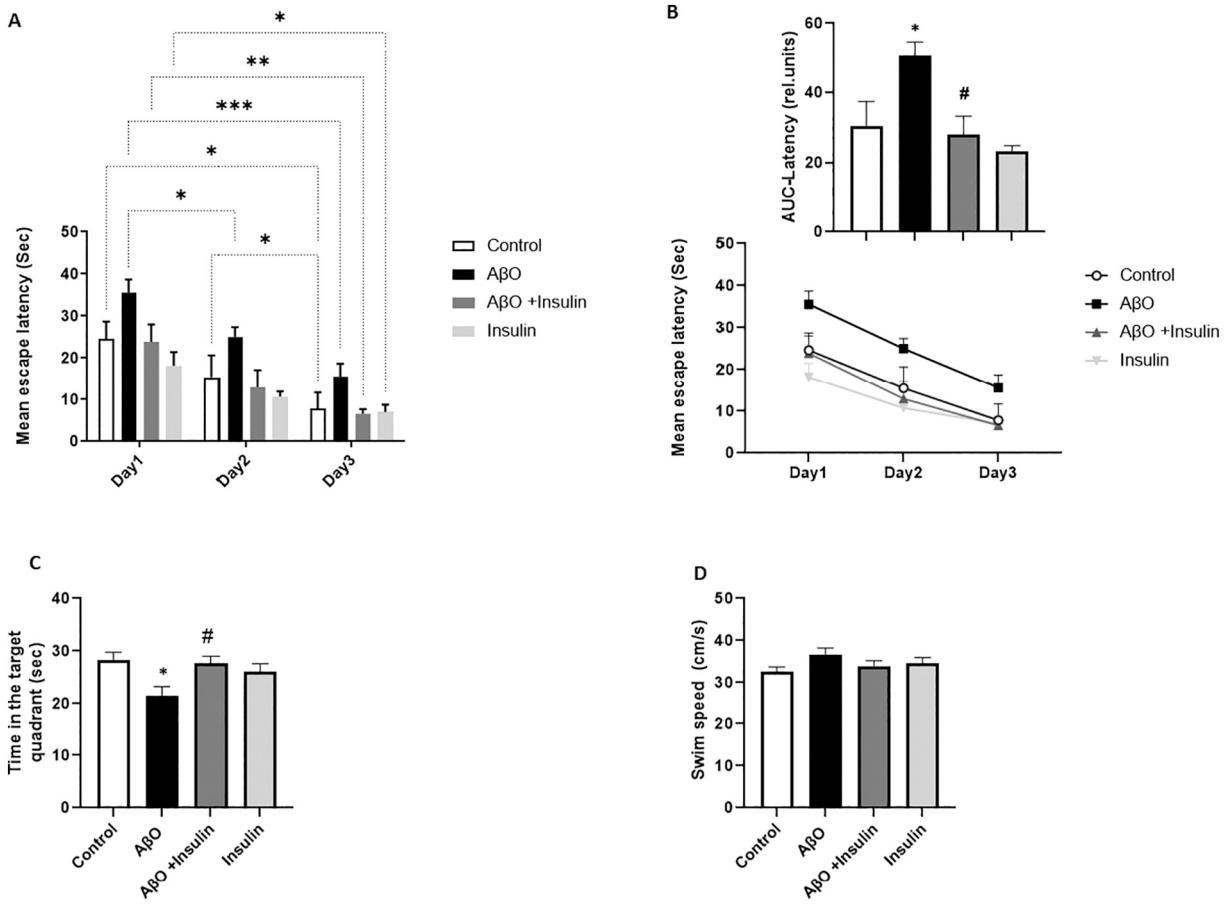

Fig. 2. The effect of AβO and/or intranasal insulin administration on MWM performance. Spatial learning and memory were assessed with the MWM task from day 17 to 21 of the study. During acquisition days, the mean escape latency to find the hidden platform (A) and AUC for it (B) was measured. All animals, regardless of the treatments, learned the hidden platform location during the 3-day acquisition phase, as demonstrated by the reduced latency to locate the platform (A). Although, the escape latencies reported for the AβO group did not have a rapid or robust descending pattern as has been observed for other groups (B). In the probe trial, the time in the target quadrant in 60 s (C) and the mean swim speed (D) were recorded. Intranasal insulin treatment [2 IU/day, for 10 consecutive days (day 7 to day16 of the study)] attenuated long-term memory impairments in AβO (2*7.5 μg/2*3 μL/rat)-induced AD-like rats. Data are represented as the means ± SEM (n = 8–10 per group). *P < 0.05 versus the control group; #*P* < 0.05 versus the AβO group (for fig. B and C).

amyloid deposits in the CA1 and DG regions of the hippocampus as compared with the control group (*P* < 0.0001, Fig. 4A and B). Treatment with intranasal insulin for 10 days resulted in a significant decrease in the mean number of congophilic amyloid deposits in the CA1 and DG regions of the hippocampus, compared with the untreated AβO group (P < 0.0001, Fig. 4A and B).

3.3. Intranasal insulin attenuated tau phosphorylation levels in the hippocampus of AβO receiving animals

Abnormal tau hyper-phosphorylation is one of the most important pathophysiological hallmarks in AD observed in the brains of AβO receiving animals (De Felice et al., 2008; Forny-Germano et al., 2014). We evaluated the effect of AβO and/or intranasal insulin on tau phosphorylation levels at AD- associated four sites (Ser396, Ser404, Thr231 and Thr205) (Bazrgar et al., 2020; Wang and Mandelkow, 2016) in the hippocampus of all animals using western blotting. As it is evident in Fig. 5A, B and C, One-way ANOVA analysis reported a significant difference in tau phosphorylation levels at Ser396 ($F(3, 8) = 9.969$, $P = 0.0044$), Ser404 ($F(3,12) = 6.597$, $P = 0.0070$), and Thr231($F(3, 8) = 8.452$, $P = 0.0073$) between groups. Moreover, Tukey's test showed a significant increase in tau phosphorylation levels at Ser396 ($P < 0.01$), Ser404 ($P < 0.05$), and Thr231 ($P < 0.05$) in the hippocampus of AβO receiving animals compared to control animals. Interestingly, intranasal administration of insulin for 10 days to the AβO receiving animals significantly attenuated the hyper-phosphorylation of tau at these three sites in the hippocampus ($P < 0.05$). No significant differences were detected in tau phosphorylation levels following only insulin treatment compared with control group. We did not observe any significant differences in the tau phosphorylation levels at Thr205 among groups ($F(3, 8) = 0.3350$, $P = 0.8007$). The bands for all four tau protein phosphorylation sites were detected at 55 kDa (Fig. 5A, B, C, and D). These findings illustrate that treatment with intranasal insulin can effectively decrease tau hyper-phosphorylation in the hippocampus of AβO receiving animals.

3.4. Intranasal insulin attenuated neuroinflammation in the hippocampus of AβO receiving animals

To evaluate the effect of AβO and/or intranasal insulin on neuroinflammation in the hippocampus of animals, we assessed the expression levels of IL-1β and TNF-α genes, as pro-inflammatory cytokines, using qRT-PCR. One-way ANOVA analysis demonstrated a significant difference in the gene expression of IL-1β and TNF-α between groups ([$F(3, 8) = 8.894$, $P = 0.0063$], [$F(3, 8) = 27.27$, $P = 0.0001$] respectively) (Fig. 6A and B). As expected, the post-verification analysis showed that IL-1β and TNF-α genes expression was markedly increased in the hippocampus of AβO-treated rats compared with control rats ($P < 0.05$ and $P < 0.001$, respectively). Intranasal insulin treatment for 10 days could significantly attenuate IL-1β and TNF-α genes expression in the

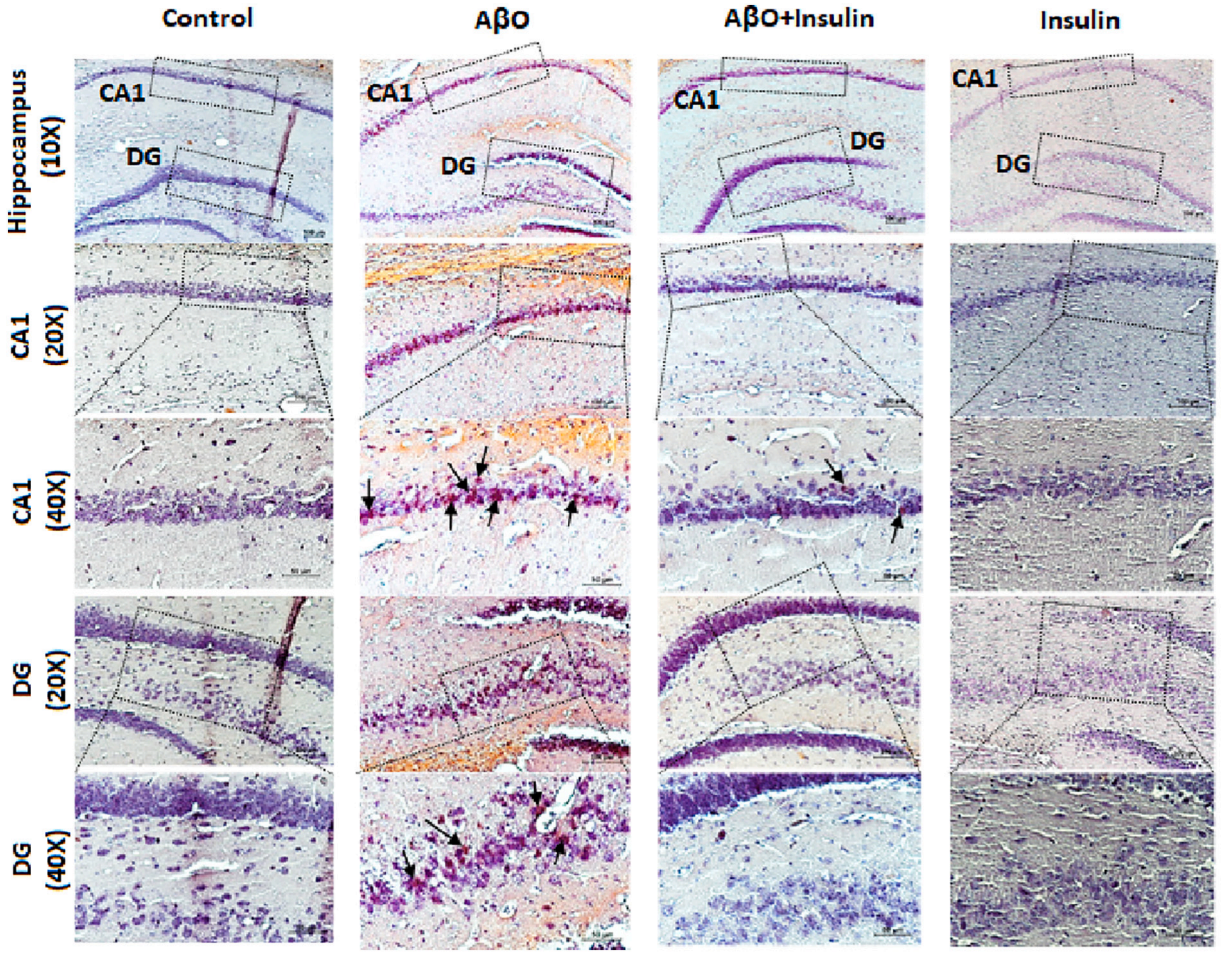

Fig. 3. Photomicrographs of Congo red staining in the hippocampal CA1, and DG regions. Extracellular amyloid deposits were visualized in red; the nucleus was counterstained with hematoxylin (blue). No morphologic changes were observed in control rats hippocampus. Scale bar =100 μm and 50 μm. Black arrows demarcate congophilic amyloid deposits in the hippocampal CA1 and DG regions of AβO and AβO + insulin groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

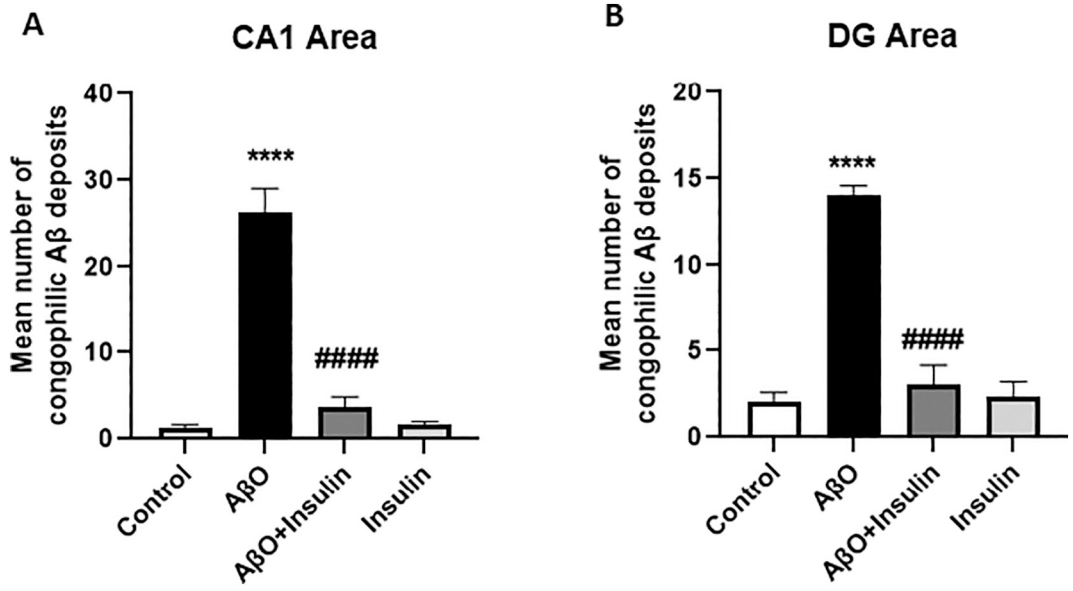

Fig. 4. The mean number of congophilic amyloid deposits after administration of intranasal insulin in the hippocampal CA1 (A) and DG (B) areas of the of AβO-induced AD model rats. Data are expressed as the mean ± SEM ($n = 3$ per group). **** $P < 0.0001$ as compared to the control group; #### $P < 0.0001$ as compared to the AβO group.

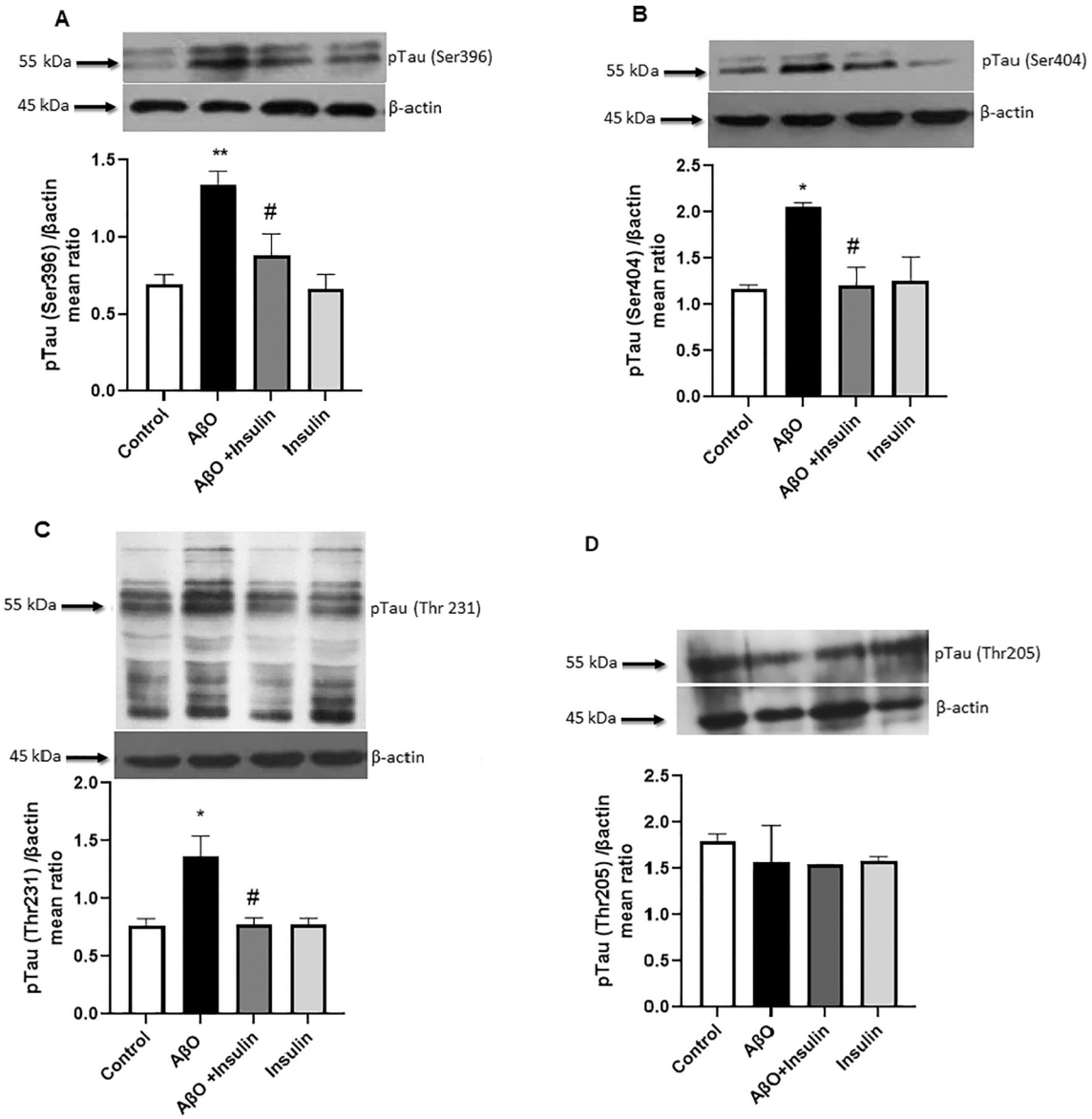

Fig. 5. Western blot analysis showing the effect of AβO and/or intranasal insulin on the tau phosphorylation levels at Ser396 (A), Ser404 (B), Thr231 (C) and Thr205 residues (D) in the hippocampus of animals. The data obtained from densitometric analysis of scanned X-ray films normalized to corresponding immunoblots of β-actin. Intranasal insulin treatment [2 IU/day, for 10 consecutive days (day 7 to day16 of the study)] attenuated tau hyper-phosphorylation induced by AβO administration (2*7.5 μg/6 μL/rat) in the hippocampus of rats. Data are represented as the means ± SEM ($n = 3$ per group). *$P < 0.05$ and **$P < 0.01$ versus the control group; #$P < 0.05$ versus the AβO group.

hippocampus of AβO-treated rats ($P < 0.01$ and $P < 0.001$, respectively). No significant differences were detected in the expression of IL-1β and TNF-α following only insulin treatment compared with the control group. Taken together, these findings indicate that intranasal insulin administration can successfully reverse neuroinflammation to the level of insignificancy to control animals significantly.

3.5. Intranasal insulin treatment restored insulin signaling pathway in the hippocampus of AβO receiving animals

To explore the effects of AβO and/or intranasal insulin on the insulin signaling pathway, we assessed the expression of some components involved in this pathway, including insulin receptor (IR), phosphorylated-IR substrate-1 at Ser-612 (p-IRS1), phosphatidylinositide 3-kinases (PI3K), protein kinase B (Akt), phosphorylated Akt at Ser473 (pAkt) and phosphorylated glycogen synthase kinase-3 beta at Ser-9 (p-GSK-3 β) in the animal's hippocampus in each group using qRT-PCR and western blot analyses. One-way ANOVA analysis indicated there was no significant difference in the mRNA levels of IR between the experimental groups ($F(3, 8) = 1.193$, $P = 0.3724$) (Fig. 7A). On the other hand, a significant change was observed in the protein levels of p-IRS (Ser612) ($F(3, 8) = 12.09$, $P = 0.0024$) and mRNA levels of PI3K ($F(3, 8) = 9.346$, $P = 0.0054$), and AKT ($F(3, 8) = 14.26$, $P = 0.0014$) between groups. Further, Tukey's post hoc test revealed a significant increase in the mRNA levels of PI3K ($P < 0.05$) and Akt ($P < 0.01$), as well as a significant increase in the protein levels of inactivated IRS1 (pIRS1 (Ser612)) ($P < 0.01$) in the AβO-treated animals compared to the control group. Intranasal insulin treatment for 10 days restored the levels of p-IRS1, PI3K and Akt in the hippocampus of AβO–treated animals ([$P < 0.01$], [$p < 0.05$], [$p < 0.01$], respectively ((Fig. 7B, C and D). No significant differences were detected in the expression of p-IRS1, PI3K and AKT following only insulin treatment compared with the control group. In addition, One-way ANOVA did not show any significant differences in the protein levels of pAkt and p-GSK-3 β between the

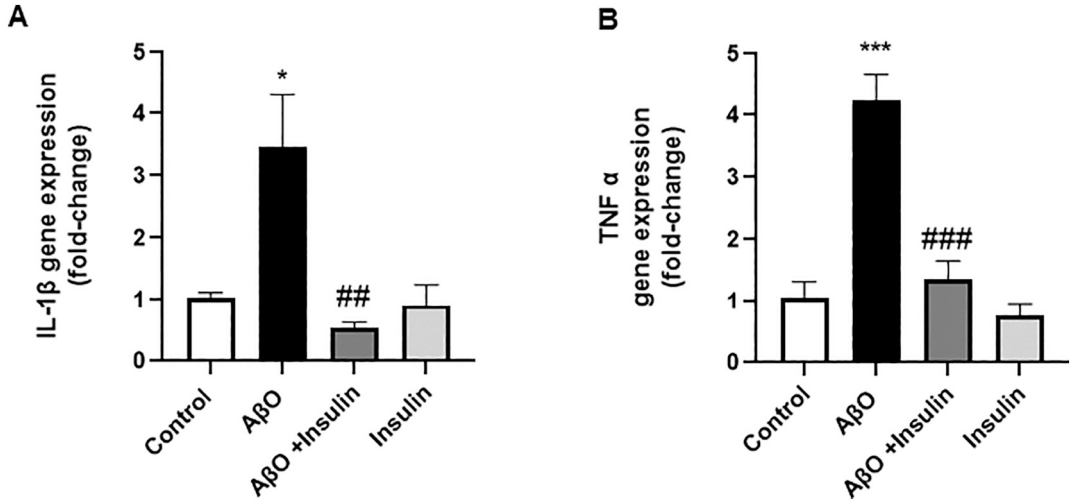

Fig. 6. qRT-PCR analysis showing the effect of AβO and/or intranasal insulin on neuroinflammation markers in the hippocampus of animals. Intranasal insulin treatment [2 IU/day, for 10 consecutive days (day 7 to day16 of the study)] attenuated neuroinflammation induced by AβO administration (2*7.5 μg/6 μL/rat) in the hippocampus of rats, as was supported by decreased levels of IL-1β (A) and TNF-α (B) expression. Data are represented as the means ± SEM ($n = 3$ per group). *$P <$ 0.05 and ***$P < 0.001$ versus the control group; ##P < 0.01 and ###P < 0.001 versus the AβO group.

groups ([F (3, 8) = 0.6879, $P = 0.5843$], [F (3, 8) = 2.434, $P = 0.1399$], respectively) (Fig. 7E and F). These results suggest that intranasal insulin treatment can regulate the insulin signaling pathway in the hippocampus of AβO–treated animals.

3.6. The effect of AβO and/or intranasal insulin on the expression of AD-related miRNAs in the hippocampus

In the next part of the study, we investigated the effect of AβO and/or intranasal insulin on the expression of 8 miRNAs that target genes involved in AD-related pathways using qRT-PCR. Table S1 summarized the known facts of these miRNAs chosen for this study. We analyzed the expression patterns of these eight miRNAs in the hippocampus of rats (*Rattus norvegicus*/rno) on days 1, 14 and, 21 of the study. Heatmaps for 8 rat miRNAs expression alteration in four experimental groups on days, 1, 14, and 21 of the study are shown in Fig. 8. The significantly deregulated miRNAs are also rearranged in Table 2. We observed down-regulation of miR-26a, miR-124, miR-29a, miR-181b, miR-125b, miR-132, and miR-146a in the hippocampus of AβO-exposed rats 21 days after AβO injection. Intranasal insulin treatment increased miR-132, miR-181b, and miR-125b expression, and partially restorated the expression of miR-26a and miR-124.

3.6.1. Expression of three miRNA's was responding to intranasal insulin treatment: rno-miR-132, rno-miR-181b, and rno-miR-125b

3.6.1.1. rno-miR-132-3p. As it is depicted in Fig. 9A1, the expression levels of miR-132 gradually decreased with time in the AβO group compared to control group, which was significant on day 21. *t*-test analysis on day 1 and One-way ANOVA analysis on day 14 of the study showed no significant change in miR-132 expression in the hippocampus between groups ([$P = 0.2217$] and [F (3, 8) = 3.195, $P = 0.0839$] respectively). Instead, on day 21 of the study, a significant difference was observed in miR-132 expression between groups (F (3, 8) = 10.19, $P = 0.0042$). Further, the post-verification analysis revealed that miR-132 expression was significantly down-regulated in the hippocampus of AβO-exposed animals compared with control animals 21 days after AβO injection ($P < 0.05$). Interestingly, intranasal insulin treatment for 10 days (day 7 to day 16 of the study) was able to increase miR-132 expression in AβO-treated animals dramatically ($P < 0.01$). No significant differences were detected in the expression of miR-132 following only insulin treatment compared with the control group. These findings indicate that intranasal insulin treatment could successfully reverse AβO mediated miR-132 down-regulation to the level of insignificancy to control animals significantly.

3.6.1.2. rno-miR-181b-5p. Both increased and decreased miR-181b expression in the AD brain has been associated with AD pathogenesis (Lu et al., 2019; Rodriguez-Ortiz et al., 2014). *t*-test analysis on day 1 of the study showed that the expression levels of miR-181b significantly decreased in the hippocampus of AβO-exposed animals than control animals 1 day after AβO injection ($P < 0.05$) (Fig. 9A2). On day 14 of the study, One-way ANOVA analysis reported a significant difference in miR-181b expression levels between groups (F (3, 8) = 37.11, $P <$ 0.0001). Further, Tukey's test indicated that miR-181b expression was markedly increased in the AβO group than control group 14 days after AβO injection (P < 0.05). There was no significant difference in the expression of miR-181b in the AβO + Insulin group compared to the AβO group after exposure to insulin for 7 days (on day 14 of the study) (Fig. 9A2). On day 21 of the study, a significant change was also observed in the expression levels of miR-181b between groups (F (3, 8) = 9.202, $P = 0.0057$). The post-verification analysis showed that miR-181b expression was markedly decreased in the AβO group than control group 21 days after AβO injection ($p < 0.01$), and intranasal insulin treatment for 10 days could successfully reverse this effect to the level of insignificancy to control animals ($p < 0.05$) (Fig. 9A2). No significant differences were detected in the expression of miR-181b following only insulin treatment compared with the control group. These findings indicate that chronic insulin treatment can regulate miR-181b expression changes in AβO- exposed animals.

3.6.1.3. rno-miR-125b-5p. As it is evident in Fig. 9A3, no significant difference was observed in miR-125b expression between the experimental groups on days 1 and 14 of the study ($p = 0.5464$, F (3, 8) = 2.021, $P = 0.1896$, respectively). On day 21 of the study, One-way ANOVA analysis demonstrated a significant difference in miR-125b expression levels between groups (F (3, 8) = 12.02, $P = 0.0025$). Tukey's post hoc test indicated that although AβO decreased miR-125b expression compared to control ($P < 0.01$), intranasal insulin treatment significantly enhanced its expression compared to AβO groups ($P < 0.05$). Moreover, miR-125b expression was significantly reduced in the insulin group compared to the control group. The different effects of insulin in healthy rats and the ABO group may be due to the involvement of indirect pathways modulating the upregulation of miR-125b in

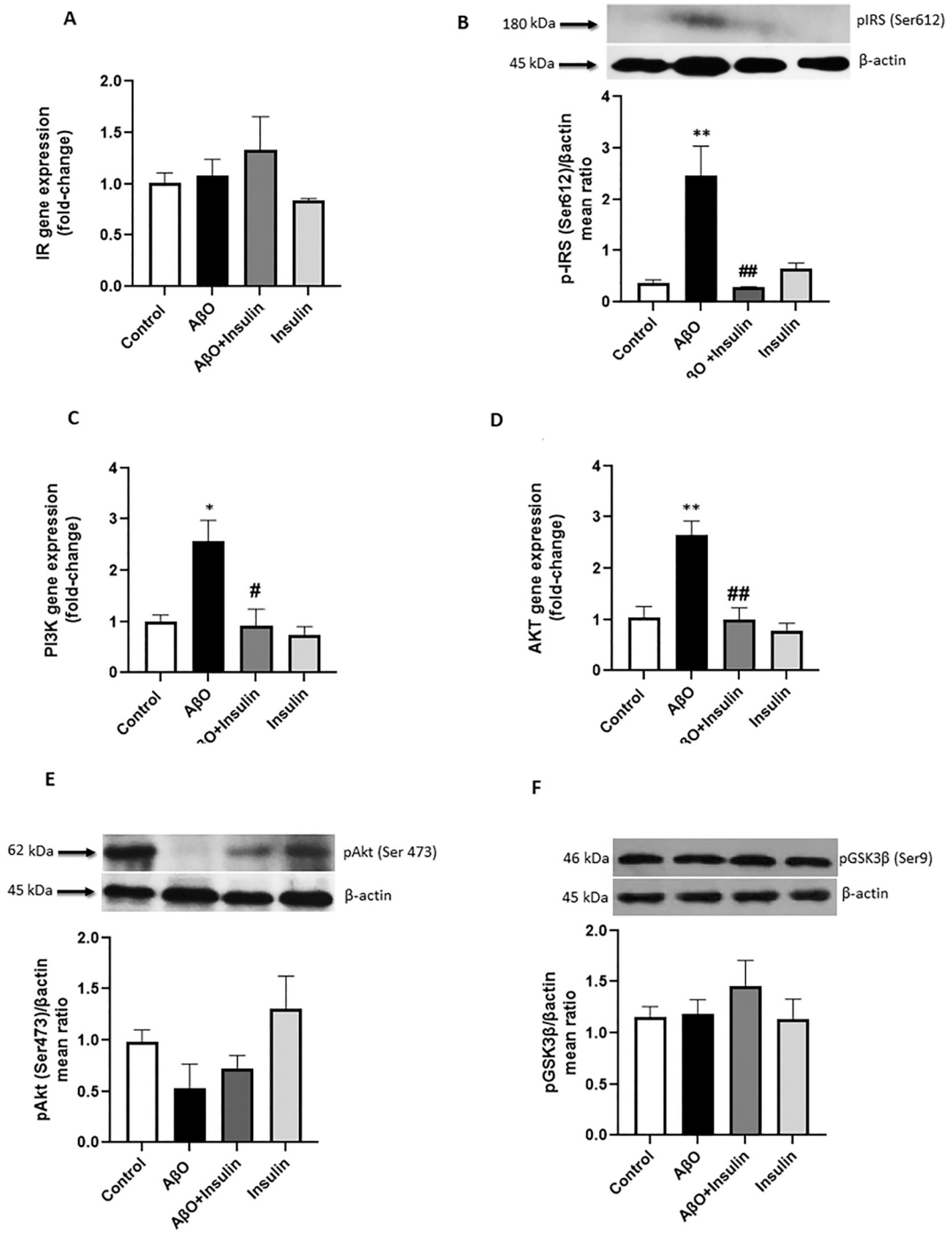

Fig. 7. Effect of AβO and/or intranasal insulin on the brain insulin signaling pathway. qRT-PCR data analysis showed that AβO and/or intranasal insulin had no effect on the mRNA levels of IR (A). Western blot analysis indicated that AβO (2*7.5 μg/6 μL/rat) significantly increased protein levels of pIRS (B). In addition, qRT-PCR data analysis also showed that AβO markedly increased the mRNA levels of PI3K (C) and AKT (D). But intranasal insulin treatment [2 IU/day, for 10 consecutive days (day 7 to day16 of the study)] significantly decreased the protein levels of pIRS, as well as the mRNA levels of PI3K (C) and AKT (D). Western blot analysis indicated that there are no significant differences in the protein levels of pAkt (E) and p-GSK-3 β (F) between the groups. Data are represented as the means ± SEM (n = 3 per group). *$P < 0.05$ and **$p < 0.01$ versus the control group; #P < 0.05 and ##p < 0.01 versus the AβO group.

insulin- AβO group. However, a clear answer requires further experiments such as promoter analysis in cell culture or transgenic animals. These findings suggest that long-term exposure to AβO reduces miR-125b expression and this could be compensated by insulin therapy.

3.6.2. Expression of two miRNA's was partially restored after intranasal insulin treatment: rno-miR-124 and rno-miR-26a

3.6.2.1. rno-miR-124-3p. T-test analysis on day 1 and One-way ANOVA analysis on days 14 and 21 showed that there was a significant difference in miR-124 expression between the groups ([$p = 0.0441$], [F (3, 8) = 66.52, $P < 0.0001$], [F (3, 8) = 5.185, $P = 0.0279$], respectively) (Fig. 9B1). AβO reduced miR-124 expression in AβO-receiving animals

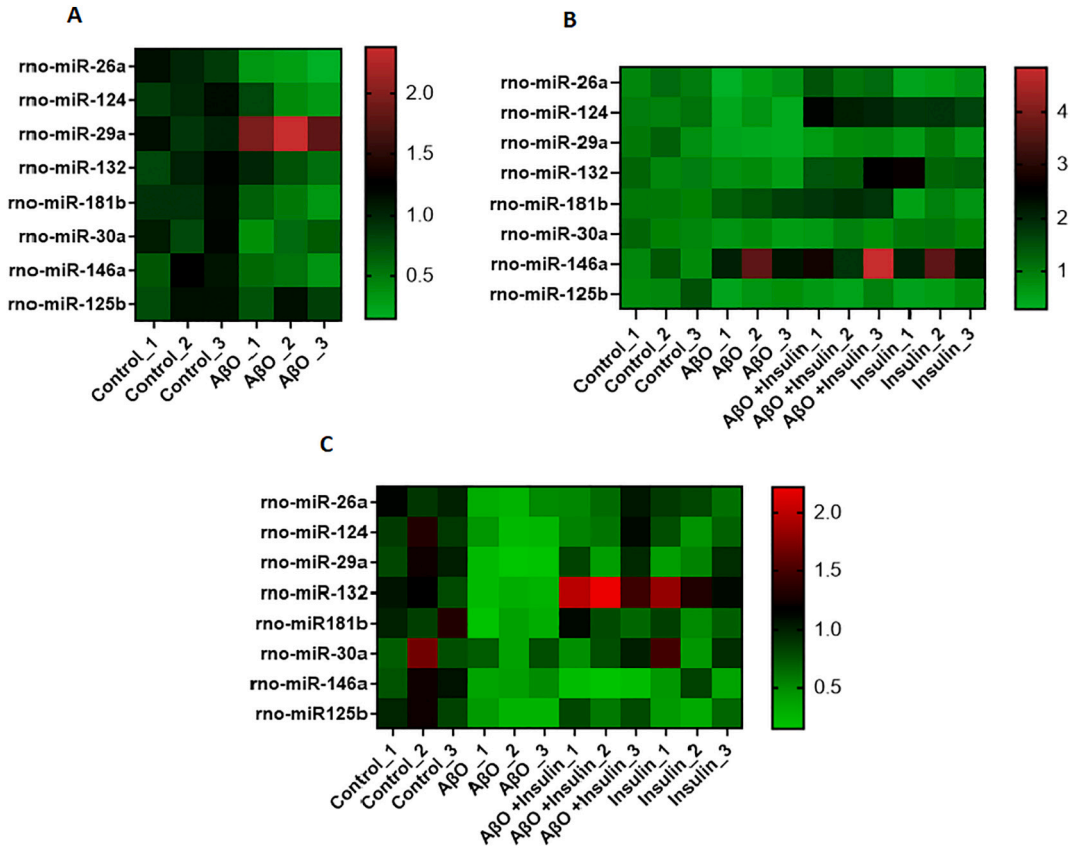

Fig. 8. Heatmaps for 8 miRNAs expression alteration in four experimental groups on days 1 (A), 14 (B) and, 21 (C) of the study. Each column represents samples and each row represents miRNAs. The high (red), average (black) and low (green) expression levels are depicted by the colourgram (n = 3/group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The significantly deregulated miRNAs in the AβO, AβO + Insulin and Insulin rats hippocampus on days 1, 14 and, 21 of the study.

miRNAs	Regulation in AβO group vs the control group			Regulation in AβO + Insulin group vs the AβO group		Regulation in Insulin group vs the control group	
	Day1	Day14	Day21	Day14	Day21	Day14	Day21
miR-132	–	–	down	–	up	–	–
miR-181b	down	up	down	–	up	–	–
miR-125b	–	–	down	–	up	–	down
miR-26a	down	down	down	up	–	–	–
miR-124	down	down	down	up	–	up	–
miR-146a	down	–	down	–	–	–	down
MiR-29a	up	down	down	–	–	–	–
miR-30a	down	–	–	–	–	–	–

compared to control animals 1, 14, and 21 days after AβO injection ($p < 0.05$). Although, intranasal insulin treatment for 7 days significantly enhanced miR-124 expression in the AβO + Insulin group compared to both control and AβO groups ($p < 0.0001$), insulin treatment for 10 days could not increase miR-124 expression in AβO + Insulin group than AβO groups. These findings suggest that intranasal insulin treatment may partially regulate miR-124 expression.

3.6.2.2. rno-miR-26a-5p. A significant change was observed in the expression of miR-26a between groups on all three days 1, 14, and 21 of the study ([$p = 0.0014$], [F (3, 8) = 11.51, $P = 0.0028$], [F (3, 8) = 6.584, $P = 0.0149$], respectively) (Fig. 9B2). The expression of miR-26a was significantly down-regulated in the AβO group compared to the control group on all three days 1, 14 and 21 ($p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively). Although, intranasal insulin treatment for 7 days significantly elevated miR-26a expression in the AβO + Insulin group compared to AβO groups ($p < 0.01$), insulin treatment for 10 days could not increase miR-26a expression in AβO + Insulin group than AβO groups. These findings indicate that intranasal insulin treatment can partially restore miR-26a expression.

3.6.3. Expression of three miRNA-s was not altered after intranasal insulin treatment: rno-miR-29a, rno-miR-146a, and rno-miR-30a

3.6.3.1. rno-miR-29a. As shown in Fig. 9C1, there is a significant difference in miR-29a expression on all three days 1, 14, and 21 of the study ([$p = 0.0059$], [F (3, 8) = 5.836, $P = 0.0206$], [F (3, 8) = 7.094, $P = 0.0121$], respectively). The expression of miR-29a was significantly up-regulated in the AβO group compared to the control group 1 day after injection of AβO ($p > 0.01$). In contrast, miR-29a expression was significantly down-regulated 14 and 21 days after AβO injection ($p < 0.05$ and $p < 0.01$, respectively). Intranasal insulin treatment had no significant effect on miR-29a expression.

3.6.3.2. rno-miR-146a-5p. T-test analysis on day 1 of the study showed that the expression levels of miR-146a significantly decreased in the hippocampus of AβO-exposed animals than control animals 1 day after injection of AβO ($P < 0.05$) (Fig. 9C2). One-way ANOVA analysis on day 14 of the study showed no significant change in miR-146a expression in the hippocampus between groups (F (3, 8) = 2.561, $P = 0.1279$). The miR-146a expression significantly decreased in AβO-exposed animals compared to controls animals 21 days after AβO injection ($p < 0.05$). Although, intranasal insulin treatment had no effect on miR-146a

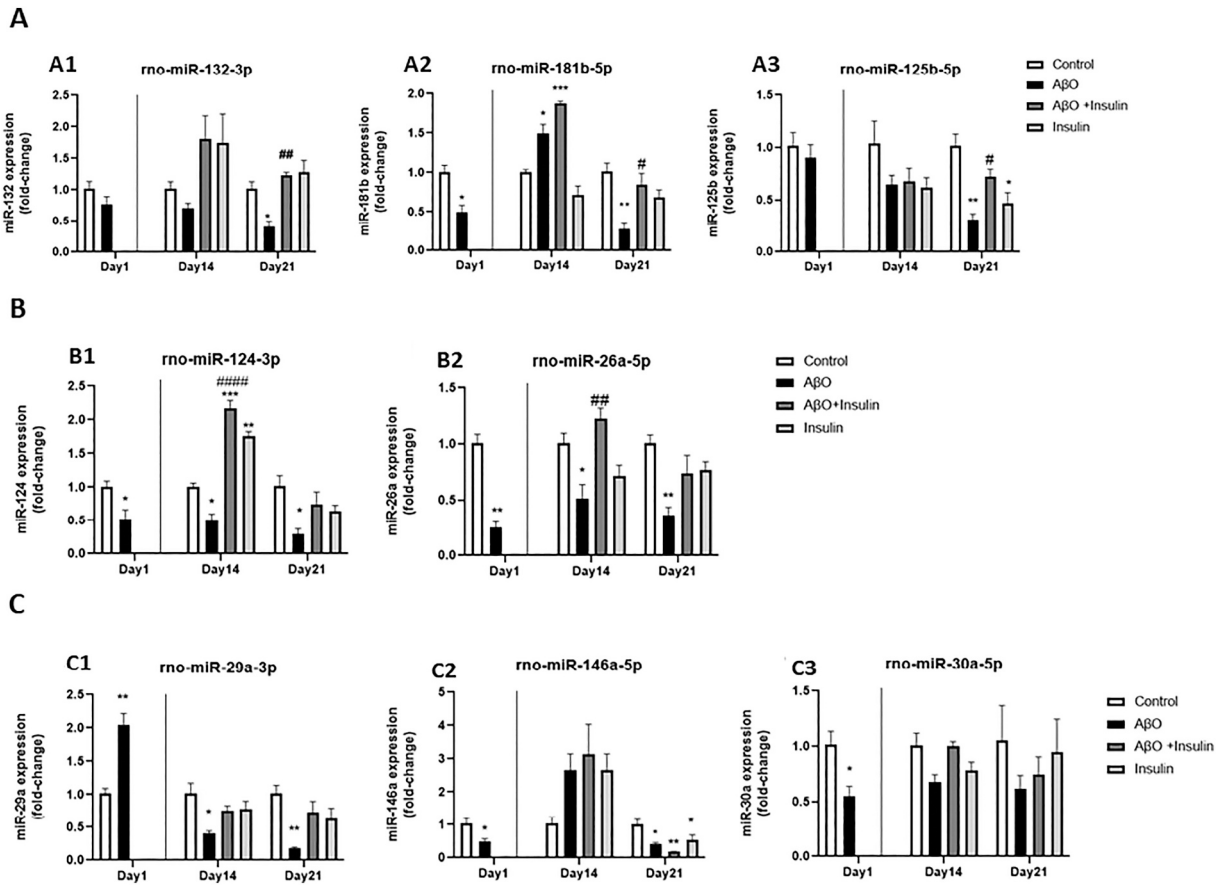

Fig. 9. AD-related miRNAs expression patterns in response to AβO and/or intranasal insulin time course in the hippocampus of animals were assessed using qRT-PCR.

A: Expression of three miRNA's was responding to intranasal insulin treatment: rno-miR-132 (A1), rno-miR-181b (A2), and rno-miR-125b (A3).

B: Expression of two miRNA's was partially restored after intranasal insulin treatment: rno-miR-124 (B1) and rno-miR-26a (B2).

C: Expression of three miRNA-s was not altered after intranasal insulin treatment: rno-miR-29a (C1), rno-miR-146a (C2), and rno-miR-30a (C3).

Data are represented as the means ± SEM (n = 3 per group). *P < 0.05, **$P < 0.01$ and ***$P < 0.001$ versus the control group; #$p < 0.05$, ##p < 0.01 and ###$p <$ 0.001 versus the AβO group.

expression in the AβO + Insulin group compared to the AβO group, it decreased miR-146a expression in both the AβO + Insulin group and Insulin group compared to the control group ($p < 0.01$ and $p < 0.05$ respectively).

3.6.3.3. rno-miR-30a-5p. T-test analysis on day 1 of the study demonstrated that the expression levels of miR-30a were markedly decreased in the hippocampus of AβO-exposed animals than control animals 1 day after injection of AβO ($p < 0.05$) (Fig. 9C3). Although One-way ANOVA analysis on day 14 of the study demonstrated a significant difference in miR-30a expression between groups ($F(3, 8) = 4.694$, $P = 0.0357$), Tukey's post hoc test did not show a significant difference between groups. There was no significant difference in miR-30a expression in the hippocampus between groups on day 21 of the study ($F(3, 8) = 0.6983$, $P = 0.5789$) (Fig. 9C3).

4. Discussion

The current study investigated the effects of intranasal insulin administration on memory impairment, and its possible molecular mechanisms of the neuroprotective effect concerning Aβ aggregation, tau phosphorylation levels, neuroinflammation, insulin signaling pathway, and the expression of 8 miRNAs associated with Aβ aggregation, tau pathology, neuroinflammation, and synaptic dysfunction in AβO induced AD-like rats.

Our results showed that AβOs intra-hippocampal administration impairs spatial learning and memory in animals, instigate Aβ aggregation, tau hyper-phosphorylation, and neuroinflammation in the hippocampus, which is consistent with previous studies (Cline et al., 2018; Forny-Germano et al., 2014; Pearson-Leary and McNay, 2012; Xu et al., 2014). AβOs exert their toxicity through direct interactions with neuronal membranes and receptor-dependent mechanisms (Cline et al., 2018). It has been reported that AβOs can cause IR dysfunction/loss and subsequently impairs kinases activity related to cognitive functions and neuronal survival (Bedse et al., 2015; Chatterjee and Mudher, 2018). In addition, it has been observed that accumulation of AβOs in the brain through the release and subsequent activation of pro-inflammatory cytokines leads to the activation of stress-activated kinases like c-Jun NH3-terminal kinase (JNK), resulting in inhibitory serine phosphorylation of IRS-1 instead of normal Tyr-phosphorylation. This makes the IRS-1 mediated insulin signaling dysfunctional, thereby hindering the insulin actions (Bedse et al., 2015). In agreement with these studies, we also observed that AβOs increased pIRS1 (Ser612) levels, which play a central role in insulin resistance. Although, many previous studies have reported that AβOs through phosphorylation of IRS-1 at serine residues can reduce PI3K/Akt pathway activity and subsequently increase GSK-3β activity, which is responsible for increased tau phosphorylation, Aβ production and accumulation, and neuroinflammation (Akhtar and Sah, 2020; Bedse et al., 2015; Jimenez et al., 2011), surprisingly, we observed that AβOs increased the mRNA expression of PI3K and Akt,

relatively decreased Akt activity (pAkt (Ser473) and had no effect on the protein expression of p-GSK3β (GSK3β inactive form) in the hippocampus of rats. In support of our results, some studies have reported that Akt and GSK3β activity do not change in AD animal models (Guo et al., 2017a; Pei et al., 1997). Moreover, inactivation of GSK-3β have been demonstrated in the AD brain (Griffin et al., 2005; Morroni et al., 2016). Therefore, we assume that GSK-3β may not be the major downstream kinase of the insulin signaling associated with AD pathogenesis. In future, evaluation of another downstream kinase of the insulin signaling including MAPK/ERK, PKA, and JNK will be very informative and helpful in this model. Interestingly, we observed that intranasal insulin treatment for 10 days could ameliorate cognitive deficits in AβO induced AD-like rats, attenuates Aβ aggregation, tau hyper-phosphorylation and neuroinflammation in the hippocampus of these rats, as were supported by reduced density of Aβ accumulations, decreased levels of tau phosphorylation at Ser396, Thr231, and Ser404 as well as decreased levels of IL-1β and TNF-α expression. In addition, this drug restored mRNA levels of AKT and PI3K and protein levels of pAkt (Ser473) in the hippocampus of AD-like rats to the level of insignificancy to control animals. In agreement with our results, Guo et al., demonstrated that long-term treatment with intranasal insulin ameliorates cognitive impairment, tau hyper-phosphorylation, and microglial activation in a streptozotocin-induced AD rat model (Guo et al., 2017b). Another study showed that pretreatment with intranasal insulin for 7 days attenuates propofol-induced tau hyper-phosphorylation in 3 × Tg-AD mice (Chen et al., 2014). In summary, our study together with others has displayed that intranasal insulin treatment plays an important role in improving memory, reducing inflammatory cytokine expression, reducing tau phosphorylation, regulating brain insulin signaling, and regulating amyloid precursor protein metabolism and AB production (Chen et al., 2018; Gabbouj et al., 2019; Kellar et al., 2022; Rajasekar et al., 2017). However, how insulin exerted these beneficial effects were not clearly described.

Substantial evidence have revealed that imbalance in the expression of miR-132-3p, miR-181b-5p, miR-125b-5p, miR-26a-5p, miR-124-3p, miR-146a-5p, miR-29a-3p, and miR-30a-5p in the AD brain are associated with AD pathogenesis (Table S1). In this study, we illustrate that intranasal insulin treatment for 10 days regulates the expression of miR-132-3p, miR-181b-5p, and miR-125b-5p in the hippocampus, providing evidence that regulation of miRNAs expression may be a novel molecular pathway of insulin beneficial effects in the AD brain. Our results showed that in the early stage of AβO induced neurotoxicity (1 day after AβO injection into the hippocampus of rats), the expression of miR-26a, mi181b, miR-124, and miR-146a were significantly decreased, and miR-29a expression was significantly increased in the hippocampus of AβO exposed rats when compared with control rats. In the middle stage of AβO induced neurotoxicity (14 days after AβO injection), the expression of miR-26a, miR-124, and miR-29a was significantly down-regulated, and miR-181b expression was significantly up-regulated. Intranasal insulin treatment for 7 days (day 7 to day 13 of the study) significantly increased miR-26a and miR-124 expression in the hippocampus of the AβO + Insulin group compared to the AβO group at this stage of neurotoxicity. Finally, in the late stage of AβO induced neurotoxicity (21 days after AβO injection), the expression of miR-26a, miR-124, miR-29a, miR-181b, miR-125b, miR-132, and miR-146a were significantly down-regulated and intranasal insulin treatment for 10 days (day 7 to day16 of the study) dramatically elevated miR-132, miR-181b, and miR-125b expression in the hippocampus of AβO + Insulin group compared to AβO group.

The miR-132 has been described as an essential regulator of neuronal differentiation, neurite outgrowth, synaptic plasticity, inflammatory responses, tau phosphorylation, neuronal survival, and cognitive functions in the central nervous system (Salta and De Strooper, 2017). In agreement with our results, extensive studies have shown that miR-132 expression is down-regulated in the AD brain (Li and Cai, 2021; Salta and De Strooper, 2017). In addition, there are bodies of evidence showing downregulation of miR-132 in the brain related to several aspects of AD pathogenesis and cognitive decline (Table S1) (Deng et al., 2020; El Fatimy et al., 2018; Salta et al., 2016). On the other hand, several studies have shown that miR-132 overexpression in the brain of AD mice/rats improves cognitive deficits, attenuates Aβ accumulation, abnormal tau phosphorylation, and neuronal death (Cong et al., 2021; Smith et al., 2015; Su et al., 2020; Walgrave et al., 2021; Wang et al., 2017). Our results showed that intranasal insulin treatment can reverse AβO mediated miR-132 down-regulation.

miR-181b has been reported to be a negative regulator of the inflammatory response (an anti-inflammatory miRNA) by regulating the expression of IL-1β and TNF-α(Lu et al., 2019). Cogswell et al. reported that miR-181b was down-regulated in the hippocampus of the AD brain (Richardson et al., 2008). Similarly, we also observed downregulation of miR-181b in the hippocampus of AβO exposed rats compared with control rats in the early and late stages of AβO induced neurotoxicity. Lu et al. showed that miR-181b overexpression in the hippocampus attenuates early postoperative cognitive impairment by suppressing hippocampal neuroinflammation in a mouse model of postoperative cognitive dysfunction (Lu et al., 2019). Our results showed that intranasal insulin treatment in the AβO-exposed group was efficacious enough to reverse the decrease in miR-181b expression.

miR-125b is extensively expressed in the brain and plays pivotal regulatory roles in the diverse biological processes (Le et al., 2009). Numerous studies have reported that miR-125b is elevated in different brain regions of AD patients compared to healthy individuals (Lukiw and Alexandrov, 2012; Richardson et al., 2008). Moreover, it has been found that overexpression of miR-125b in both neuronal cells and in mice could induce abnormal tau hyper- phosphorylation, synaptic deficits, apoptosis of neuronal cells, and cognitive deficits (Banzhaf-Strathmann et al., 2014; Ma et al., 2017). Surprisingly, we found that the expression of miR-125b was down-regulated in the hippocampus of AβO exposed animals, and intranasal insulin treatment increased the expression of miR-125b. In support of our results, one study reported that miR-125b decreased in the Aβ -induced AD cell model and was also found that miR-125b overexpression ameliorates oxidative stress and apoptosis induced by Aβ through targeting and down-regulating β-site amyloid precursor protein cleaving enzyme 1 (BACE1) (Li et al., 2020).

miR-26a is a physiological regulator of tau phosphorylation, inflammatory responses, and synaptic plasticity by targeting and controlling the expression of DYRK1A, GSK3β, Activating transcription factor 2 (ATF2), Phosphatase and tensin homolog (PTEN), and ribosomal S6 kinase 3 (RSK3) (Gu et al., 2015; Jiang et al., 2015; Kumar et al., 2015; Li and Sun, 2013; Liu et al., 2020). In support of our study, several studies have reported that miR-26a was downregulated in the brains of AD patients and AD animal models (Richardson et al., 2008; Schonrock et al., 2010; Wang et al., 2011b). In addition, it has been shown that overexpression of miR-26a improves memory impairment, attenuates tau hyperphosphorylation, and Aβ accumulation in the AD mice by targeting DYRK1A (Liu et al., 2020). Our results showed that intranasal insulin treatment partially regulates miR-26a expression.

miR-124 is specifically expressed in the CNS and is one of the most abundant miRNAs in the brain (Sun et al., 2015). Both increased and decreased miR-124 expression in the AD brain has been associated with AD pathogenesis. Down-regulation of miR-124 is associated with tau hyperphosphorylation, Aβ accumulation, and neuroinflammation through increased Caveolin-1, BACE1, IL-6, and TNF-α, expression (Fang et al., 2012; Kang et al., 2017; Slota and Booth, 2019). On the other hand, it has been reported that overexpression of miR-124 can also cause tau hyperphosphorylation and synaptic dysfunction through targeting and downregulating PTPN1 (Hou et al., 2020). Our results showed that miR-124 was downregulated in the hippocampus of AβO exposed animals, and intranasal insulin treatment could partially regulate miR-124 expression.

The role of miR-146a in the pathogenesis and progression of AD is summarized in Table S1. Interestingly, the relative expression of miR-

146a is dynamic during the progression of AD. It has been reported that the miR-146a level is increased in the hippocampi of patients with Braak stage III and IV AD but decreased in patients with Braak stage VI AD (Müller et al., 2014). In our study, down-regulation of miR-146a was observed in the both AβO and insulin groups compared to control group. In support of our results, one study reported that miR-146a is decreased in the Aβ -induced AD cell model (Schonrock et al., 2010). Intranasal insulin treatment did not alter miR-146a expression in the hippocampus of the AβO + insulin group compared with the AβO group.

Mounting evidence has shown that miR-29a plays a crucial role in regulating the expression of BACE1, which restricts the production of Aβ (Hébert et al., 2008). Moreover, it was validated that decreased levels of miR-29a could promote the production of Aβ and contribute to the pathogenesis of AD. Down-regulation of miR-29a was observed in the AD patients and AD animal models compared to age-matched controls (Hébert et al., 2008; Wang et al., 2009). Our results showed that in the early stage of Aβ neurotoxicity, the expression of miR-29a increases, and in the middle and late stages of neurotoxicity, its expression decreases. Intranasal insulin treatment could not compensate for the decrease in miR-29a expression.

miR-30a is involved in regulating mitochondrial dynamics through direct targeting of tumor suppressor p53 and thus suppressing the expression of dynamin-related protein 1 (Drp1) (Li et al., 2010; Wang et al., 2011a). Moreover, miR-30a can play an essential role in neuronal survival and neural plasticity associated with memory formation and consolidation by targeting and regulating brain-derived neurotrophic factor (BDNF) expression. Dysregulation of miR-30a was found in AD (Croce et al., 2013; Wang et al., 2011b). Our results showed that in the early stage of AβO induced neurotoxicity, miR-30a expression was significantly decreased in the hippocampus of AβO exposed rats when compared with control rats. In the middle and late stages of AβO induced neurotoxicity had no effect on miR-30a expression. Intranasal insulin treatment also had no effect on miR-30a expression.

The present study was a pilot study to examine whether insulin could affect miRNAs expression. Further studies are needed to understand better the regulatory role of miRNAs in the beneficial effects of intranasal insulin treatment in this model and other models of AD such as transgenic and sporadic models. It should be investigated whether inhibition of miR-132, miR-181b, and miR-125b curb the neuroprotective effect of intranasal insulin treatment. In addition, it would be more informative and precise to investigate whether administration of miR-132, miR-181b, and miR-125b mimic also reduces Aβ aggregation, tau pathology, neuroinflammation, and cognitive impairment, although several studies have examined this, as mentioned above, it would be very helpful to examine the effect of concurrent treatment of these three miRNAs. We will further investigate these aspects in the future to provide a more comprehensive understanding of the action of intranasal insulin in the brain.

5. Conclusions

Taken together, these findings demonstrated that intranasal insulin treatment ameliorates spatial learning and memory impairment in AβO-induced AD-like rats, and this improvement was accompanied with modulation of miR-132, miR-181b, and miR-125b expression, partial restoration of miR-26a and miR-124, attenuation of Aβ aggregation, tau hyper-phosphorylation levels, and neuroinflammation. Due to the fact that dysregulation of these miRNAs is associated with AD pathogenesis and cognitive impairment, our findings suggest that the neuroprotective effects of intranasal insulin on cognition observed in AD-like animals could be partially due to the regulation of miRNAs in the brain. These results also provide experimental and mechanistic evidence to support intranasal insulin for AD patients in clinical trials.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2022.111812.

Declaration of competing interest

There are no conflicts of interest.

Data availability

The authors do not have permission to share data.

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CRediT authorship contribution statement

All authors contributed to the study conception and design. All authors read and approved the final manuscript.

Maryam Bazrgar: contributed in Methodology, material preparation, data collection, analysis and interpretation of data, and Writing - Original Draft.

Pariya Khodabakhsh: contributed in Methodology, material preparation, data collection, analysis and interpretation of data.

Leila Dargahi and Fatemeh Mohagheghi: contributed in Supervision, Review & Editing.

Abolhassan Ahmadiani: Validation Methodology, Review & Editing, Project administration, and Funding acquisition.

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