

MiR-204-5p dysregulation is associated with FOXO1 expression and LC3II/I ratio in a streptozotocin-induced rat model of Alzheimer's disease

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ABSTRACT

Objective(s): Growing evidence suggests that dysregulated autophagy contributes to Alzheimer's disease (AD) pathogenesis. MicroRNAs have emerged as important post-transcriptional regulators in neurodegenerative diseases. The present study investigated the expression of miR-204-5p and Forkhead box transcription factor O1 (FOXO1), as well as alterations in the LC3II/I ratio in a Streptozotocin (STZ)-induced rat model of AD.

Materials and Methods: Sixteen male Wistar rats (200-250 g) were randomly divided into control and Alzheimer's model groups. An experimental model was induced by intracerebroventricular injection of STZ (3 mg/kg). Spatial and avoidance memory were assessed using the Y-maze and passive avoidance test. The expression levels of miR204-5p and FOXO1 were determined by real-time PCR, while the LC3II/I ratio was assessed by Western blotting.

Results: Behavioral assessments revealed significant memory deficits in STZ-treated rats, as indicated by reduced alternation percentage in the Y-maze and decreased step-through latency, accompanied by increased time spent in the dark compartment in the passive avoidance test ($P < 0.0001$). Molecular analyses demonstrated that miR-204-5p expression was significantly decreased in the brains of STZ-treated rats ($P < 0.05$). Conversely, FOXO1 levels ($P < 0.01$) and the LC3-II/LC3-I ratio ($P < 0.05$) were significantly elevated compared to controls.

Conclusion: These findings suggest that altered miR-204-5p expression may be associated with increased FOXO1 levels and potential autophagy-related changes in AD. These molecular alterations may contribute to autophagy-related changes associated with AD pathology and highlight the miR-204-5p/FOXO1 axis as a potential target for future mechanistic investigations.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and also the most common cause of dementia, characterized by progressive memory loss and cognitive decline. Pathological features include extracellular amyloid (A β) plaques, intracellular tau formation and accumulation, synaptic dysfunction, and neuronal loss (1). The intracerebroventricular streptozotocin (ICV-STZ) model is a widely used and well-established animal model of sporadic AD, providing a reproducible approach to investigate AD-related cognitive and neuropathological alterations (2). Despite extensive research, the precise molecular mechanisms underlying AD pathogenesis remain incompletely understood.

Among the cellular processes implicated in AD, autophagy has gained considerable attention as a critical mechanism involved in protein quality control and neural homeostasis. Autophagy is a lysosome-dependent self-degradation pathway and a self-repairing biological process involved in cellular growth, metabolism, and defense against oxidative stress. Autophagy plays a crucial role in removing misfolded proteins, lipid droplets, and defective organelles, thereby helping maintain intracellular homeostasis (3). Dysregulated autophagy has been associated with AD pathogenesis. Impaired autophagosome clearance and altered expression of autophagy-related genes (Atgs) have been reported in both patients and experimental models, highlighting the involvement of defective autophagy in

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disease progression (4, 5).

Microtubule-associated protein light chain 3 (LC3) is a central component of the autophagy machinery and a widely used marker of autophagosome formation. During formation, LC3-I is conjugated with phosphatidylethanolamine to form LC3-II, which associates with autophagosomal membranes. Therefore, the LC3-II/LC3-I ratio is commonly employed as an indicator of autophagic activity. Changes in LC3 expression and autophagic flux have been observed in AD models (6); however, the direction and magnitude of these changes may differ depending on disease stage and experimental conditions. These findings suggest that autophagy modulation in AD is complex and highly controlled.

MicroRNAs (miRNAs) are short non-coding RNAs that control gene expression post-transcriptionally. miRNAs play pivotal roles in neural development, synaptic plasticity, and neurodegenerative processes (7). Dysregulation of miRNA expression has been more closely associated with AD, where miRNAs are implicated in A β production, tau phosphorylation, neuroinflammation, apoptosis, and autophagy. Because they are stable and may control other molecules, miRNAs have also been suggested as possible biomarkers and treatment targets for neurodegenerative diseases (8, 9).

Among autophagy-related miRNAs, miR-204-5p has emerged as an important regulator of neural function, apoptosis, and autophagy. miR-204 is abundantly expressed in the cerebral cortex and has been shown to influence neural stem cell differentiation and cortical development (10, 11). Previous studies have reported that miR-204-5p levels are increased in serum samples from Parkinson's disease (PD) patients, leading to dopaminergic cell death by inducing an apoptotic signaling cascade (12). Notably, miR-204 has been identified as a negative regulator of Forkhead box O1 transcription factor (FOXO1), a member of the FOXO family.

FOXO1 is a key transcriptional regulator that plays an important role in many cellular processes, including apoptosis, oxidative stress response, metabolism, and autophagy (13). When cells are stressed by reactive oxygen species or energy deprivation, the adenosine monophosphate-activated protein kinase (AMPK) pathway can activate FOXO1. This promotes autophagy, which removes damaged organelles and unwanted proteins (14).

Recent research shows that increasing FOXO1 levels can reduce A β generation by inhibiting amyloidogenic processing of amyloid precursor protein (APP) through changes in the activity of β -secretase (BACE1) and presenilin-1 (PS1). FOXO1 has also been shown to reduce tau phosphorylation, which could protect neurons from damage in AD (15).

Despite increasing evidence supporting the involvement of autophagy and miRNA-mediated regulation in AD (16, 17), the precise interplay between miR-204-5p, FOXO1, and autophagy-related markers remains insufficiently characterized, particularly in *in vivo* models of AD.

While previous studies have examined changes in miR-204 expression or FOXO1-mediated changes in amyloidogenic pathways on their own, little is known about how these two processes occur simultaneously and how they relate to autophagic activity in the diseased brain. Additionally, variations in the LC3II/I ratio have been

observed in AD models; however, the correlation between these changes and dysregulation of upstream miRNA-transcription factor networks remains poorly investigated.

Therefore, investigating the coordinated expression of miR-204-5p, FOXO1, and LC3 in an established Streptozotocin-induced rat model may provide further insight into autophagy-related molecular alterations associated with cognitive impairment. This approach may help clarify the potential association between miRNA-mediated regulation, transcriptional control, and autophagy-related markers in the context of AD pathology.

Materials and Methods

Animals

Sixteen adult male Wistar rats (200-250 g) were obtained from the laboratory animal breeding facility at Guilan University of Medical Sciences. Rats were housed under standard conditions with a 12-hour light/dark cycle, a controlled temperature of 22 $\pm$ 2 °C, and free access to food and water. All animal experiments followed the guidelines of the "Institutional Guide for the Care and Use of Laboratory Animals." Further, our experiments were accepted by the local ethics committee (Ethical code: IR.GUMS.AEC.1401.006). Rats were randomly divided into two groups (n=8 each): control and STZ group.

Stereotaxic surgery and treatments

The rats were handled and acclimated for five days before surgery. Anesthesia was induced with ketamine (80 mg/kg) and xylazine (20 mg/kg, IP). Bilateral cannulation into the lateral ventricles was performed using 22-gauge cannulae according to Paxinos and Watson coordinates (DV=3 mm, AP=0.8 mm, L=1.6 mm) (18). Seven days following surgery, microinjections were administered via a 27-gauge needle connected to a 10 μ l Hamilton syringe via short polyethylene tubing. Streptozotocin (STZ; 3 mg/kg, Sigma, USA) was dissolved in sterile saline (0.9%) and bilaterally injected ICV in a 10 μ l volume on days 1 and 3 to establish the AD model. Control rats received intracerebroventricular (ICV) injections of 10 μ l normal saline (0.9%) (19).

Behavioral test

The Y-maze device was used to test the rats' memory on the 14th day following surgery, and the passive avoidance device was used on the 15th and 16th days. Training and testing took place from 9 a.m. to 2 p.m. every day. To help the animals acclimate to the work environment and reduce stress, the cages were transported to the behavioral lab 30 min before the experiment started (Figure 1).

Y-maze (YM)

The Y-maze test is a widely used paradigm for assessing spatial memory in animals and focuses on rodents'

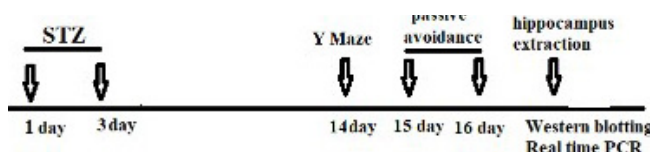


Figure 1. Experimental design showing the time course of drug administration and behavioral and molecular tests in rats
STZ: Streptozotocin

innate exploratory behavior. The apparatus consists of a Y-shaped maze with three similar arms (40 × 15 × 35 cm) at 120° angles. The arms are designated A, B, and C and are connected centrally by a triangular platform with 15 cm sides. The test was carried out in a softly lit, quiet room to minimize stress for the rats during the examination. Rats were placed at the center and allowed to explore for 8 min. Rodents typically prefer to explore the least recently visited arm, resulting in spontaneous alternation between the three arms. We recorded an arm entry when all four paws entered the arm. An alternation was defined as consecutive entries into three different arms (20).

The total number of arm entries was recorded for each subject, and the percentage of alternation was calculated using the following formula:

$$\left[\frac{\text{Actual Alternation}}{\text{Total Number Of Entrance} - 2} \right] \times 100$$

Step-through passive avoidance task

A shuttle box device was used to examine passive avoidance learning. The apparatus had two light and dark partitions (20×20×40 cm, each). Their floors were composed of stainless-steel rods (3 mm in diameter) spaced 1 cm apart. The dark chamber floor was connected to an electric shock generator. For habituation (5 min), the rats were placed in the device, the valve (8×8 cm) between the light and dark chambers was opened, and the animals were free to move and explore without shocks. After 30 min, the habituation phase was repeated. For acquisition, the rat was placed in the light chamber; step-through latency was recorded (STL1); then, upon entry into the dark compartment, an electric shock (1 mA, 50 Hz, 1.5 sec) was administered. After a two-minute interval, the rats were returned to the light chamber. If a rat did not enter the dark compartment within 120 sec, the trial ended; if it did, an additional shock was administered.

Memory retrieval assessment was conducted one hour and 24 hr later. During this test, each animal was initially put in the light chamber for 10 sec before the gate was unlocked. Subsequently, both the step-through latency (STL) and the duration spent in the dark compartment (TDC) were recorded over 10 min (600 sec)(21).

Western blotting

Immediately after the behavioral assessments, the rats were euthanized, and hippocampi were rapidly extracted, rinsed in ice-cold saline, and promptly immersed in liquid nitrogen for 24 hr. Tissue was homogenized in lysis buffer (Tris-HCl, NaCl, SDS, EDTA, Triton X-100, protease inhibitors) and centrifuged at 4000 rpm for 10 min. Protein concentration was measured by Bradford assay.

We separated 50 µg of protein by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred it to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked and incubated with primary antibodies against LC3B (Cell Signaling Technology, USA; 1:1000) and β-actin (a mouse monoclonal antibody, Santa Cruz Biotechnology, Inc.; 1:500) overnight at 4 °C. HRP-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG-HRP, Santa Cruz Biotechnology, Inc.; 1: 10,000) were used, and bands were visualized with ECL (Amersham Biosciences, USA). Densitometry was

performed using ImageJ software. LC3-II/LC3-I ratio was calculated to assess autophagic alteration(22).

RNA extraction and real-time PCR

RNA was extracted from 100 mg of tissue using the FavorPrep™ miRNA Isolation Kit (Cat: FAMK001). The extracted RNA was quantified, and the purity was measured using a NanoDrop spectrophotometer. For miR-204 measurements, a poly (A) sequence was added to the extracted miRNA collection using *E. coli* poly (A) polymerase (NEB#M0276). cDNA synthesis was performed using the Easy cDNA Synthesis Kit (cat. No. A101161; Parstons). Real-time PCR was performed in a 10 µl reaction using the kit (YTA SYBR Green qPCR Master Mix 2X) based on SYBR Green. β-actin was used as a reference gene for FOXO1, and U6 was used as a reference gene for miR-204-5p (17). All sequences of primers were listed as follows: miRNA-204-5p (F, 5'- TTCCCTTTGTCATCCTATGCCT-3' and R, 5'- GTGCGTGTCTGTCGAGAGTCG-3), FOXO1 (F, 5'-CACCTCTGCTGCCAAGATG-3' and R, 5'-GGCGAG GACTGGGTGAC-3), β-actin (F, 5'-ACCACAGTCCATG CCATCAC-3' and R, 5'-TCCACCACCCTGTTGCTGTA-3), U6 (F, 5'-CTCGCTTCGGCAGCACA-3', and R, 5'-AACGC TTCACGAATTTGCGT-3). The relative expression of FOXO1 and miR-204 was calculated and quantified using the comparative 2^{-ΔΔCt} method. It is worth noting that all samples were run in triplicate.

Statistical analysis

Data normality was assessed with the Shapiro-Wilk test. Normally distributed data were analyzed using an independent t-test. *P*<0.05 was considered significant. Data are expressed as mean ± SEM and analyzed using Prism 9.

Results

Y-maze test

The Y-maze test results (Figure 2) showed a significant reduction in spontaneous alternation behavior in STZ-treated rats compared with controls. Given the data's normality, we used an independent t-test to compare the groups. The percentage of alternation (AP%) decreased

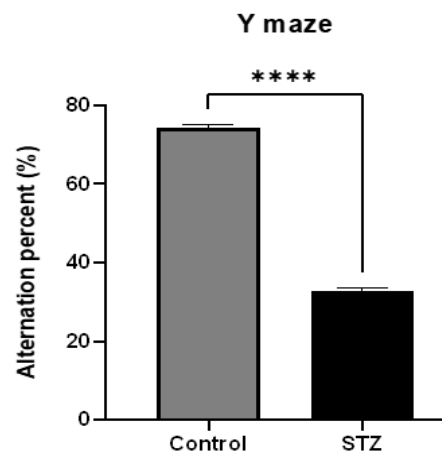


Figure 2. Spatial memory performance in the Y-maze test in the control and experimental rat groups

Spatial memory was assessed using the Y-maze behavioral test by evaluating the percentage of spontaneous alternation (AP). The data values are expressed as mean±SEM (n=8; *****P*<0.0001 compared to control; t-test) STZ: Streptozotocin

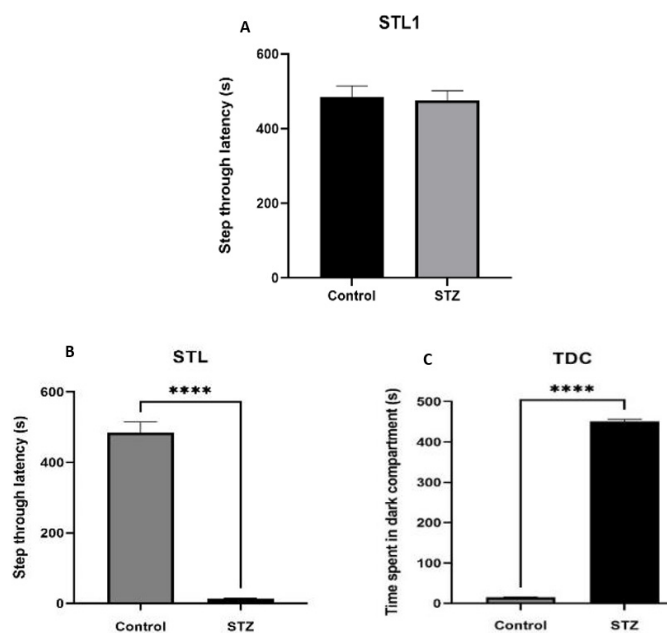


Figure 3. Passive avoidance memory in the control and experimental rat groups

Evaluated using the step-through passive avoidance test. (A) Step-through latency (STL1): the latency to enter the dark compartment before the electrical shock. (B) Step-through latency (STL): the latency to enter the dark compartment during the retention trial. (C) Time spent in the dark compartment (TDC): the duration spent in the dark compartment during the retention trial. The data values are expressed as mean $\pm$ SEM (n=8; ****P<0.0001 compared to control; t-test)

from 74.3 $\pm$ 0.7 in controls to 32.8 $\pm$ 0.7 in the STZ group (P<0.0001), indicating impaired spatial memory.

Passive avoidance memory

The t-test showed no significant difference in step-through latency before the electrical shock (P=0.81; Figure 3A). However, after STZ administration, the latency to enter the dark compartment (STL) decreased significantly from 484.5 $\pm$ 29.6 in controls to 14.1 $\pm$ 0.9 s in the STZ group (P<0.0001; Figure 3B). Also, the total time spent in the dark chamber (TDC) increased from 15 $\pm$ 1.2 s in controls to 451.4 $\pm$ 3.8 s in the STZ-treated group (P<0.0001; Figure 3C), confirming memory impairment.

Real-Time PCR assessment of miR-204-5p and FOXO1 expression

As shown in Figure 4, real-time PCR revealed significant differences between the studied groups in miR-204-5p and FOXO1 expression levels. T-test analysis showed that miR-204-5p expression in STZ-treated rats decreased significantly from 1.09 $\pm$ 0.1 to 0.4 $\pm$ 0.2 compared with the control group (P<0.05; Figure 3A). Conversely, the level of FOXO1 in STZ-treated rats increased significantly from 0.8 $\pm$ 0 to 1.6 $\pm$ 0.2 compared to the control group (P<0.05; Figure 3 B).

Western blot analysis of hippocampal LC3 levels

Western blot analysis showed that the LC3II/LC3I expression ratio differed significantly between groups. Western blotting revealed a significant increase in LC3II expression in STZ-treated rats compared to controls (4.7 $\pm$ 1.1 vs. 1 $\pm$ 0; P<0.01; Figure 4). Furthermore, the LC3II/I ratio was significantly elevated in the STZ group (7.3 $\pm$ 2.7) relative to controls (1 $\pm$ 0; P<0.05), indicating

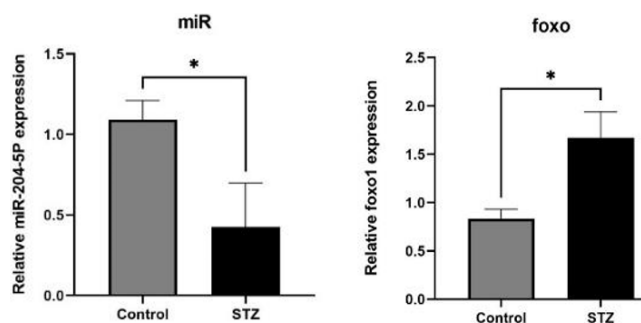


Figure 4. Relative expression levels of hippocampal miR-204-5p and FOXO1 in the control and experimental rat groups as determined by quantitative real-time PCR (qRT-PCR)

(A) Relative expression of hippocampal miR-204-5p. (B) Relative mRNA expression of hippocampal FOXO1. Gene expression levels were quantified using qRT-PCR and calculated using the comparative 2^{-ΔΔCt} method following normalization to the appropriate endogenous reference control. The data values are expressed as mean $\pm$ SEM (n=8; *P<0.05 compared to control; t-test)

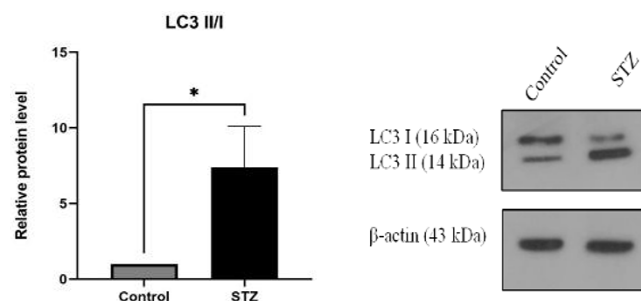


Figure 5. Relative protein expression of LC3-II/LC3-I in the hippocampus of the control and experimental rat groups

(A) Densitometric quantification of the LC3-II/LC3-I protein ratio in the hippocampal tissue samples. (B) Representative western blot bands showing the expression of LC3-I and LC3-II proteins in the hippocampus of the two groups. Protein expression was normalized to the appropriate loading control. The data values are expressed as mean $\pm$ SEM (n=8; *P<0.05 compared to control; t-test)

altered autophagy-related activity (Figure 5).

Discussion

The results showed that ICV administration of STZ significantly altered the expression levels of miR-204-5p, FOXO1, and the LC3II/I ratio, as well as memory loss, in the STZ group.

In neurodegenerative impairments such as AD, brain miRNA profiles are often dysregulated (23). Around 70% of known miRNAs are expressed in the brain, and growing evidence suggests that miRNAs contribute to AD pathogenesis (8). miR-204 is an autophagy- and apoptosis-related gene that is highly expressed in the cerebral cortex. In the current study, miR-204-5p expression was reduced in the brains of STZ-treated rats. Previous research indicates that specific miRNAs can modulate AD-related processes, including A β metabolism, tau expression, apoptosis, and inflammation (24, 25). For instance, Tasdalen *et al.* reported decreased miR-204 levels in AD models (26), and Tan *et al.* (2021) observed down-regulation of cerebrospinal fluid (CSF) exosomal miR-204-5p in Alzheimer's and patients with frontotemporal dementia (FTD) (27). Similarly, Tao *et al.* demonstrated that miR-204-3p overexpression improved synaptic function and memory in APP/PS1 mice (28). These results support the hypothesis that modified miR-204-5p

expression may correlate with cognitive impairments in AD models, while the nature of the alteration may differ among various neurodegenerative disorders, including PD (12).

FOXO transcription factors regulate many biological processes, such as autophagy and apoptosis. FOXO1 is highly expressed in the hippocampus and cortex and is associated with neurodegenerative diseases (29). In this study, STZ-treated rats had higher FOXO1 levels and a higher LC3-II/LC3-I ratio. This suggests that autophagy-related pathways may have changed. Nuclear FOXO proteins can regulate genes involved in autophagosome formation, while cytosolic FOXO proteins can interact with the autophagy machinery, potentially altering autophagic flux (29). Consistent with our results, Liu *et al.* reported elevated FOXO1 levels in AD, indicating its possible involvement in disease progression (14). Additionally, Zhang *et al.* showed that FOXO1 overexpression can reduce A β accumulation and tau phosphorylation, suggesting a complex role in AD pathology (15).

LC3-II is a widely used marker of autophagosome formation, and the LC3-II/LC3-I ratio reflects autophagic activity (30). In this study, the ratio increased in STZ-treated rats, in line with reports from A β -treated cells and transgenic mice (31-33). However, some studies have reported reduced autophagic markers in AD models (34).

AD progression appears to affect autophagic flux significantly. Pomilio *et al.* reported *in vivo* and *in vitro* evidence that microglial autophagy is influenced throughout AD progression. Specifically, microglial BV-2 cells showed an increased LC3-II-to-LC3-I ratio and increased autophagic flux after 2 hr of exposure to fibrillar A β . However, this effect diminished after 24 hr of exposure, resembling the chronic A β exposure observed in human pathology (35). This variability may reflect differences in experimental models, disease stages, and A β exposure duration. Therefore, while LC3-II/LC3-I changes suggest alterations in autophagy, they do not conclusively indicate whether autophagy is beneficial or detrimental in this context.

Overall, the findings suggest that alterations in autophagy-related signaling may be associated with cognitive impairment in a streptozotocin-induced rat model of AD. For instance, lower miR-204-5p levels and higher FOXO1 levels, along with a higher LC3-II/LC3-I ratio, suggest that the miR-204-5p/FOXO1/LC3 axis may not function properly in the brains of people with AD. These results support previous studies showing that miRNAs, FOXO transcription factors, and autophagy markers play a role in neurodegenerative processes. Nonetheless, autophagy is highly dynamic and context-dependent, and emerging evidence suggests that autophagic activity may vary between the early and advanced stages of AD. Thus, the rise in the LC3-II/I ratio seen in this model may reflect stage-specific or compensatory autophagic responses rather than a steady autophagic flux.

This study has several limitations. First, we assessed autophagy only by the LC3-II/LC3-I ratio and did not evaluate other key autophagy markers. Second, due to funding limitations, we could not use pharmacological therapies or pathway-specific inhibitors to clarify the causative mechanisms better. Third, the intracerebroventricular STZ model, although well established, may not fully replicate all clinical characteristics of human AD. Finally, molecular analysis was only done on brain tissue, and biomarkers in plasma or cerebrospinal fluid were not examined.

Conclusion

Despite these limitations, this study primarily provides correlative evidence supporting an association between miR-204-5p, FOXO1, and the LC3 II/I ratio in an experimental model of AD. These findings may contribute to a better understanding of the miR204-5p/FOXO1/LC3 axis and its potential relevance in AD pathology and therapeutic targeting.

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Authors' Contributions

Z M conducted the investigation and wrote the original draft. L A was responsible for conceptualization, methodology, analysis, and reviewed and edited the writing. A J handled conceptualization, supervision, analysis, and contributed to both original writing and review/editing. KR contributed to methodology and reviewed and edited the writing. B K supervised and reviewed the writing. M R conducted the investigation and reviewed the writing.

Conflicts of Interest

All contributing authors declare no conflicts of interest. They are solely responsible for the content and authorship of the paper.

Declaration

During the preparation of this manuscript, the authors used ChatGPT, a generative AI tool, to assist with grammar and language refinement. The authors generated and validated all scientific content, including concepts, data analysis, interpretations, and conclusions, without using AI for content creation.

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