Amyloid Beta Alters the Expression of microRNAs Regulating HMGCR and ABCA1 Genes in Astrocytes of C57BL/6J Mice

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Article type:	**ABSTRACT**
Original Article	Dysregulation of brain cholesterol homeostasis causes the accumulation of extracellular protein deposits called amyloid plaques in the hippocampus which eventually leads to neuronal death, memory and learning deficits. The aim of the present study was to investigate the effect of beta amyloid on miRNAs regulating HMGCR and ABCA1 as cholesterol synthesis and homeostasis genes. Primary astrocytes were isolated from C57BL/6J mice, and were treated with 0.5 μM amyloid beta (Aβ). Expression levels of genes and miRNAs were measured by real-time PCR. In comparison to control, Aβ treatment resulted in a significant decrease in miR-96-5p expression as a positive and negative regulator of HMGCR and ABCA1, respectively. There was no significant increase in miR-27a-3p expression as a negative regulator of HMGCR. miR-106b-5p and miR-143-3p expressions were also dramatically decreased as ABCA1 negative regulators. Amyloid beta can alter the expression of major genes in the cholesterol homeostasis pathway via their regulatory miRNAs.
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Introduction

An adult human brain stores about 20-25% of the body's total cholesterol, which is the highest level of cholesterol accumulation in one area of the human body (1, 2). Normal cholesterol levels are critical for optimal brain function (3). Most of the cholesterol in the brain is produced by astrocytes, microglia, and oligodendrocytes. During the embryonic phase, the cholesterol required by neurons is often synthesized by the neurons and, to a lesser extent, by astrocytes. However, after birth, astrocytes take over the capacity to supply the cholesterol needed by neurons, and the neurons become less involved in cholesterol production (4). In healthy individuals, cholesterol is produced by the enzyme HMGCR in astrocytes and loaded on apolipoprotein E (ApoE) via the ABCA1 transporter; thus, the cholesterol needed by the neurons reaches them from astrocytes in the form of apolipoprotein E-high-density lipoprotein (ApoE-HDL), which neurons absorb through receptors on the plasma membrane of their cell body (5). Disruption of cholesterol homeostasis results in central nervous system (CNS) abnormalities and chronic neurological disorders, including Alzheimer's disease. Impaired brain cholesterol homeostasis ultimately leads to the formation of extracellular protein deposits known as amyloid plaques in the hippocampus (6). Cholesterol serves as a fundamental constituent of cellular membranes, engaging in a bidirectional interaction with Aβ. Aβ exerts influence over the equilibrium of cholesterol homeostasis, and alterations in cholesterol concentrations lead to modifications in Aβ formation (7). Amyloid plaques are caused by defects in the processing of amyloid beta precursor proteins and eventually lead to neuronal death, memory and learning impairments, and Alzheimer's disease (8). Considering the reciprocal influence of cholesterol and beta-amyloid on each other and their significant roles in the pathogenesis of certain neurodegenerative diseases, the discovery of intermediary molecules capable of directly regulating gene expression and cellular responses can shed light on the disease mechanisms. MicroRNAs are among these intermediary molecules, directly impacting the expression of genes involved in cholesterol homeostasis regulation and beta-amyloid cleaving enzymes, resulting in cellular response (9).

MicroRNAs are a group of non-coding RNAs that have been extensively studied in recent years in the normal and pathological processes of cells and molecules as well as the pattern of the nervous system of living organisms. The use of these molecular biomarkers in the post-transcriptional phase leads to the inhibition or degradation of mRNAs by acting on the 3'UTR sequence, which eventually inhibits the translation of specific genes (10, 11). MicroRNAs are regulatory molecules that can be modified to influence cholesterol production by regulating the HMGCR and ABCA1 genes, both of which play a critical role in cholesterol synthesis and homeostasis. In other words, microRNAs serve as the link between amyloid beta and cholesterol metabolism. In the present study, the effect of beta-amyloid on the expression of cholesterol homeostasis-regulating genes through microRNAs has been investigated.

Materials and methods

Culture and isolation of astrocytes from the brain of C57BL/6J mice

In order to grow and isolate astrocytes, one-day-old C57BL/6 mice were acquired from the Laboratory Animal Reproduction and Maintenance Center at Ahvaz Jundishapur University of Medical Sciences. Since the mice were one day old, we could not use chemicals to anesthetize them. Therefore, CO_2 gas was used

for 10 seconds to sacrifice them with the least pain and in the shortest time. After ensuring that the mice were sacrificed, they were sterilized using 70% ethanol. The brain tissue was carefully removed from the cranial cavity and the meninges were separated from the brain fragments using forceps. The brain lobes were then cut into smaller pieces and incubated in 5% trypsin solution at 37°C for 3 min to obtain separate cells. After neutralizing the effect of trypsin, the cells were centrifuged at 1000 rpm for 3 min. Following the suspension of cell precipitate in culture media containing 10% fetal bovine serum (FBS 10%), the cells were transferred to a 25-cm2 flask and incubated for one week in an incubator at 37°C and 5% CO2. To remove dead cells, the cells were washed with a PBS buffer every three days and their culture medium was changed to ensure optimum growth conditions (12). All the procedures of working with animals were carried out according to the guidelines of the ethics committee and the center of research on laboratory animals at Ahvaz University of Medical Sciences. (IR.AJUMS.ABHC.REC.1399.029).

Cell characterization

In order to characterization of astrocytes, the cells were immunostained using anti-glial fibrillary acidic protein (GFAP) antibody and visualized with fluorescein-conjugated horse anti-mouse IgG antibody and then, observed by confocal microscopy.

Preparation of amyloid beta 1-42

To prepare 100 mM stock solution of amyloid beta 1-42, 22 μL of dimethyl sulfoxide (DMSO) solution was added to 100 μg of amyloid beta 1-42 (Sigma Co.), and after shaking for 15 s, the solution was aliquoted and stored at -20°C (in the freezer) until use (13).

Treatment of mice astrocytes with amyloid beta

Astrocytes were isolated by trypsin after reaching a density of about 80%, and 1×10^6 cells were cultured in each well of a 6-well plate. The first group was the control group treated with DMSO, and the second group was treated with 0.5 μM amyloid beta soluble in DMSO. The cells were incubated for 24 h at 37°C and 5% CO_2. It is worth mentioning that all stages of cell culture and treatment were repeated three times.

Interaction of miRNAs with target genes

In order to perform a silico analysis of miR-27a, miR-96, miR-106b and miR-143 target genes, miRBase and RNAhybrid databases were used (Table 1).
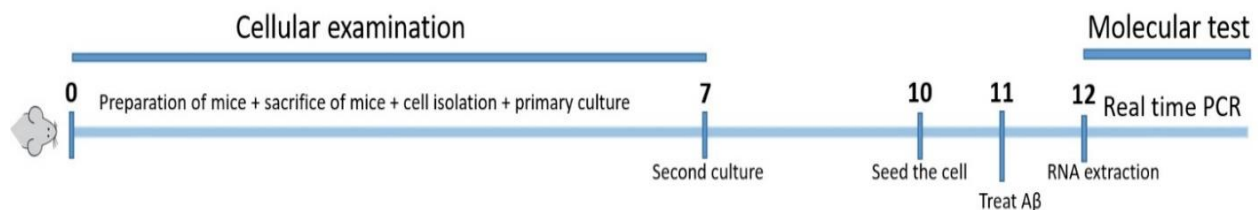
RNA extraction

The TRIzol kit was used to extract total RNA and miRNA. Briefly, after removing the medium and washing with PBS, 600 μL of TRIzol was added to each well. After separating the cells with a cell scraper, 130 μL of chloroform was added to the microtube containing the cells and TRIzol, and it was incubated at room temperature for 5 to 10 min. After centrifugation for 15 min at 12000 rpm in a refrigerated centrifuge, the supernatant containing RNA was collected, transferred to a 1.5-mL microtube with 300 μL of cold isopropanol, and gently pipetted. Then, after incubation for 24 h at -20°C, the cell suspension was centrifuged for 40 min at 12000 rpm and 4°C. Finally, the supernatant was removed and after rinsing the cell plate with 70% ethanol, 20 to 40 μL of RNase-free water was added to the cell plate, which was then kept in the freezer for the next steps. A Thermo Scientific 2000 NanoDrop was used to evaluate the extracted RNA.

Table 1. In silico analysis of miR-27a, miR-96, miR-106b and miR-143 target genes using miRBase and RNAhybrid databases

miRNA transcript	Name/ Gene ID	Position of predicted target site	Folding energy (in -Kcal/mol)	Heteroduplex
mir-27a-3p	Mus musculus Hmgcr transcript variant 3 NM_001360166.1	*1057*	-25.8	target 5' C GUGU UUGUUC C 3' UGGAGCU GCC CUGUGG GCCUUGA CGG GACACU miRNA 3' C AU U U 5'
mir-96-5p	Mus musculus Hmgcr transcript variant 3 NM_001360166.1	*516*	-27.7	target 5' A AU AAGU U 3' CAGA AAUGUGCU AGUGCUAAA GUUU UUACACGA UCACGGUUU miRNA 3' UC 5'
mir-106b-5p	Mus musculus ABCA1 member 1 NM_013454.3	*1025*	-28.3	target 5' U A 3' GUUUGCAUUGUUAGUAUU UAGACGUGACAGUCGUGA miRNA 3' AAU 5'
mir-143-3p	Mus musculus ABCA1 member 1 NM_013454.3	*483*	-24.6	target 5' A U CAAGUUG G 3' GAGUUAC AGUGC CUCA CUCGAUG UCACG GAGU miRNA 3' AAGUA 5'

cDNA synthesis and real-time PCR

For cDNA synthesis, the Yekta Tajhiz Azma cDNA kit was used with random hexamer and oligo-dT primers. In addition, to synthesize cDNA from miRNA, the BONmiR 1 st-strand cDNA synthesis kit (Bon Yakhteh Technology Research Center, Iran) was used. According to the kit protocol, the polyadenylation reaction followed by reverse transcription was conducted, and the polyadenylated RNA was converted to the corresponding cDNA. Finally, using the high-specificity miRNA qPCR Core BONmiR reagent kit with a specific forward primer for each miRNA. The levels of mRNAs and miRNAs were quantified using a real-time PCR kit (Ampliqon, Denmark) and the QuantStudioTM 3 Real-Time PCR System (ABI Applied Biosystems). Figure 1 shows the flow chart of experiments.

Fig.1. Flowchart of the study design.
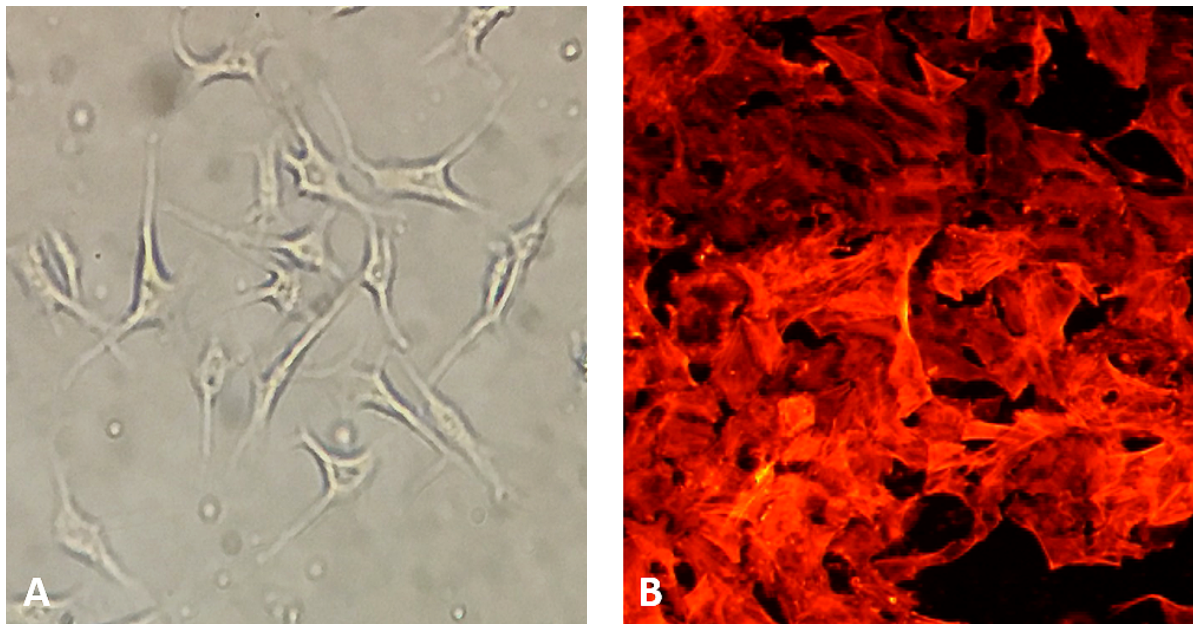
Statistical analysis

Data analysis was conducted using Excel and SPSS software. The rate of gene expression change compared to the control group was estimated by calculating $2^{-\Delta\Delta Ct}$ using the Cts obtained from the reference

and target genes in the control group (untreated) and the group treated with amyloid beta. The data are reported as mean ± standard deviation (SD). The results of each test were related to at least three separate experiments. Statistical analysis was performed using the t-test, and $P < 0.05$ was considered the significance level.

Results

Characterization of astrocytes

In our previous studies (13) and in this work, we initially conducted an immunohistochemical characterization of isolated astrocytes using a specific anti-glial fibrillary acidic protein (GFAP) antibody. The findings demonstrated that approximately 95 to 97% of the cellular population consisted of GFAP-positive cells, which serve as a marker for astrocyte identification (Figure 2).
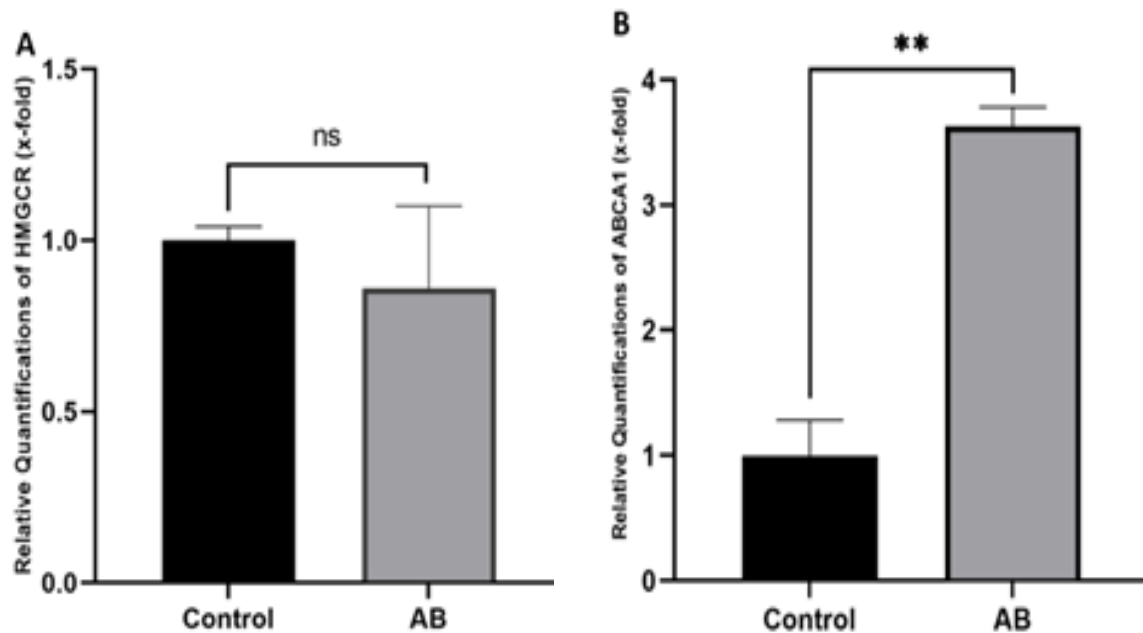

Fig. 2. Validation of Primary Mouse Astrocyte Culture Purity through GFAP Immunostaining. Astrocyte culture after one week (A), the results represent 95-97% of cells stained positive for GFAP (B). Scale bars represent 50 μm, and the images were captured at 20x magnification using a confocal microscope.

Effect of amyloid beta on the expression level of HMGCR and ABCA1 genes in mice astrocytes

To determine which of the genes involved in cholesterol metabolism are affected in Alzheimer's disease, astrocytes as brain tissue-supporting cells were treated with amyloid beta at a concentration of 0.5 μM to evaluate its effect on the gene expression of major proteins involved in brain cholesterol homeostasis. Mice astrocytes were then collected, and following the extraction of total RNA, HMGCR mRNA was evaluated in both amyloid beta-treated and control groups by real-time PCR. The results showed a small and negligible reduction ($p = 0.5$) in the HMGCR gene expression in the amyloid beta-treated group compared to that in the control group (Figure 3A). In addition, the results of the t-test revealed that the expression of the ABCA1 gene in astrocytes treated with amyloid beta was more than threefold higher than that in the control group (Figure 3B) (** $p = 0.007$).
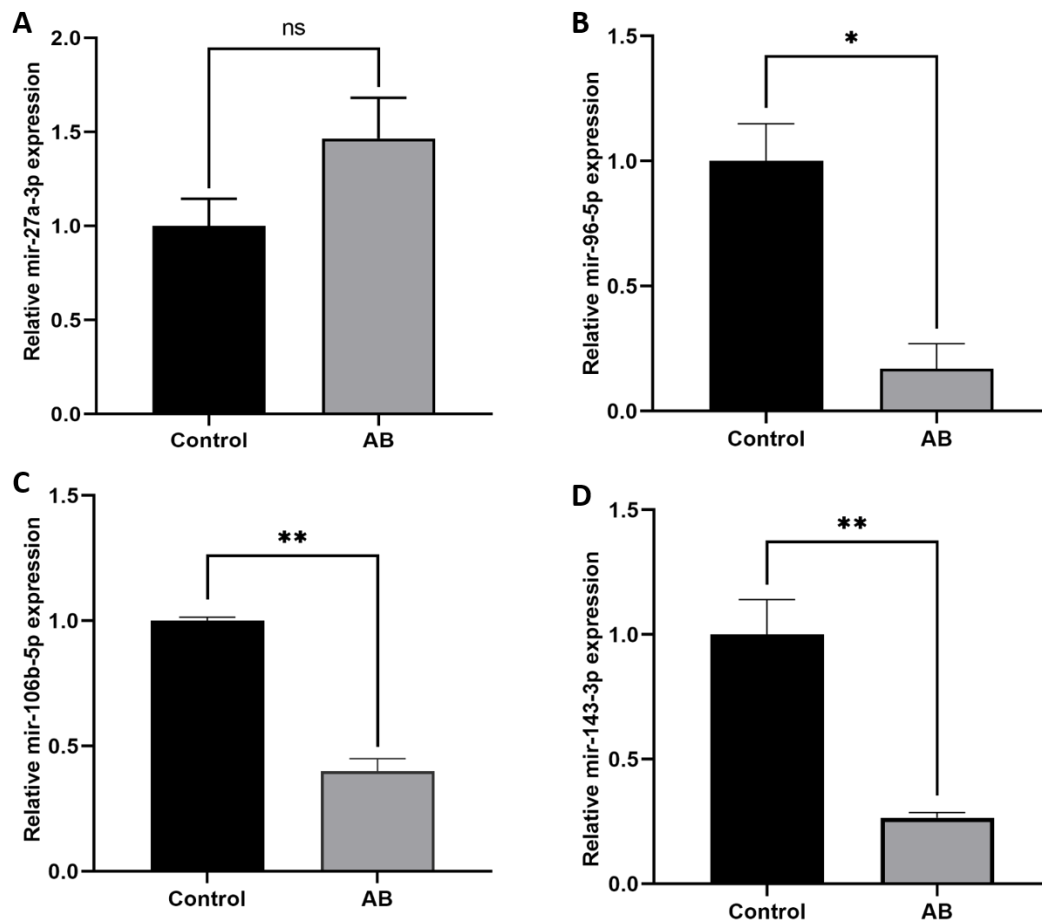
Fig. 3. Effect of amyloid beta (Aβ) on HMGCR and ABCA1 gene expressions in primary culture of astrocytes. Mouse astrocytes were incubated with or without 0.5 μM of Aβ. After 24 hours of incubation, relative expression of HMGCR (A) and ABCA1 (B) were measured by real time-PCR. Data was analyzed using SPSS and is shown as Mean ± SD of triplicate samples. *P value < 0.05 indicates a significant difference.

Effect of amyloid beta on the expression level of miRNAs regulating major proteins involved in cholesterol homeostasis

Isolated astrocytes were treated with amyloid beta at a concentration of 0.5 μM, and the expression of 27a-3p, 96-5p, 106b-5p, and 143-3p miRNAs assessed. Insilico approaches using TargetScan, IRNdb, RNAhybrid softwares, considered that these miRNAs can directly or indirectly affect the HMGCR and ABCA1 genes, it was hypothesized that changes in the expression of these miRNAs might help elucidate the molecular mechanism of Alzheimer's disease. In this study, the real-time PCR technique was employed to evaluate the expression levels of miRNAs 27a-3p, 96-5p, 106b-5p, and 143-3p. U6 was used as the internal control. The results showed a non-significant increase (p = 0.1) in the expression level of mir-27a-3p as a negative regulator of the HMGCR gene in amyloid beta-treated cells compared with the control group (Figure 4A). The expression of mir-96-5p as a positive regulator of the HMGCR gene and a negative regulator of the ABCA1 gene showed a significant decrease in the treated group compared to the control group (*P < 0.02) (Figure 4B). Moreover, the expression of mir-106b-5p as a negative regulator of the ABCA1 gene was significantly reduced in amyloid beta-treated cells compared to the control group (** P < 0.003) (Figure 4C). In addition, mir-143-3p expression as a negative regulator of the ABCA1 protein, which plays an important role in intracranial cholesterol homeostasis, was shown to be fourfold lower (** P < 0.004) in the amyloid beta group compared to the control group (Figure 4D).

Fig. 4. Effect of amyloid beta (Aβ) on miR-27a-3p, miR-96-5p, miR-106b-5p, and miR-143-3p gene expressions in primary culture of astrocytes. Mouse neurons were incubated with or without 0.5 µM of Aβ. After 24 hours of incubation, relative expression of miR-27a-3p (A), miR-96-5p (B), miR-106b-5p (C), and miR-143-3p (D) genes were measured by real time-PCR. Data was analyzed using SPSS and is shown as Mean ± SD of triplicate samples. *P value < 0.05 indicates a significant difference.

Discussion

This study aimed to assess the impact of amyloid beta on the gene expression levels of HMGCR and ABCA1, two pivotal proteins central to maintaining cholesterol homeostasis, along with their respective regulatory microRNAs (miRNAs). The results indicate that treatment with amyloid beta led to a non-significant reduction in the expression of the HMGCR gene compared to the control group. Concurrently, mir-27a-3p exhibited a non-significant increase, whereas mir-96-5p demonstrated a statistically significant decrease. Additionally, exposure to amyloid beta prompted a notable increase in the expression of the ABCA1 gene. However, miRNAs mir-106b-5p and mir-143-3p, known as negative regulators of ABCA1, exhibited significant reductions in expression.

Maintaining cholesterol homeostasis holds significant importance for optimal brain function, particularly concerning memory and cognitive processes. The data from our study reveal a non-significant decrease in the HMGCR enzyme's expression, which could indicate the cells' effort to maintain cholesterol

balance despite the presence of beta-amyloid. This trend aligns with previous studies that have shown that the transcription of the HMGCR enzyme in the brain of Alzheimer cases does not change compared to the control group (11).

Our previous research, using western blotting, demonstrated a notable increase in enzyme protein levels following beta-amyloid treatment. Interestingly, this increase occurred without any noticeable changes in RNA level. This rise might be due to the stabilization of proteins and reduced degradation within cells, rather than increased gene expression. Consequently, cells seem to regulate expression levels to ensure steady cholesterol balance (12).

The transcription factor SREBP plays a crucial role in controlling cholesterol production, and miR-96 regulates its downstream targets like HMGCR (14). In our study, we examined miR-96-5p as a positive regulator HMGCR expression, which showed decreased expression. In a parallel context, the study by Abrar Khan et al. revealed that miR-27a is involved in post-transcriptional control of HMGCR and other enzymes in the cholesterol production pathway (15). This control applies under both normal and abnormal conditions, exerting a probable inhibitory effect on HMGCR expression. In our analysis, miR-27a was investigated as a negative controller of HMGCR, showing a slight increase. The data obtained from the expression of two miRNAs suggests a cellular response to the presence of beta-amyloid, aiming to maintain consistent HMGCR gene expression. Our results imply that the stability of HMGCR mRNA levels might be influenced by miRNA molecules, restricting HMGCR protein levels within cells exposed to amyloid beta.

ABCA1, a member of the ATP binding cassette family, is another gene involved in cholesterol homeostasis (16). It can reduce amyloid beta production by preventing the cleavage of amyloid beta precursors by beta- and gamma-secretases and also enhancing the outflow of cholesterol to ApoE on astrocyte membranes (17). The findings of this study revealed a significant increase in ABCA1 gene expression levels following beta-amyloid treatment in mouse astrocytes, as compared to the control group. Given the multifaceted role of this protein in amyloid beta reduction and cholesterol efflux enhancement from astrocyte cells, it can be inferred that cells orchestrate a concerted effort to uphold cholesterol levels while concurrently diminishing amyloid beta concentration. This coordinated response contributes to the overall stability of the cell's internal environment.

Furthermore, the data derived from the miRNA analysis targeting ABCE1 in this study delineated a downregulation in the expression of mir-96-5p, mir-106b-5p and mir-143-3p. Several investigations have explored the impact of these three miRNAs on ABCA1 expression. An inquiry into the effect of mir-96-5p on ABCA1 expression within Alzheimer's disease models revealed that mir-96-5p suppression not only decreased the Aβ42/Aβ40 ratio but also led to heightened ABCA1 mRNA and protein level (18). In a similar vein, a study focusing on the role of mir-143-3p in ABCA1 expression unveiled an inverse correlation (19). Similarly, an exploration of ABCA1 expression in astrocytes disclosed that elevated mir-106-5p expression led to diminished ABCA1 levels in both astrocytes and neurons, while concurrently increasing β-amyloid secretion (20).Recognizing the critical role of ABCA1 in maintaining cholesterol balance, it is plausible that the presence of beta-amyloid triggers a cellular response to enhance ABCA1 expression by modulating these regulatory miRNAs.

Amyloid beta modulates the expression of mir-27a, mir-96-5p, mir-106b-5p, and mir-143-3p, which in turn regulate the expression of HMGCR and ABCA1 genes in mouse astrocytes .The current study's findings underscore the significance of cellular cholesterol regulation within astrocytes, the principal cholesterol-synthesizing cells within the brain. These cells employ a multitude of mechanisms to uphold cholesterol homeostasis, reflecting the paramount importance of this process. The prime objective of these regulatory mechanisms is to intricately modulate cholesterol levels and curtail the accumulation of beta-amyloid.

Acknowledgement

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