High fat diet induces brain injury and neuronal apoptosis via down-regulating 3-β hydroxycholesterol 24 reductase (DHCR24)

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Abstract

Hyperlipidemia (HLP) plays an important role in the pathogenesis of Alzheimer's disease (AD). However, its pathological molecular mechanism remained unclear. 3β-hydroxysterol Δ24- reductase (DHCR24), a key enzyme in the last step of cholesterol synthesis, has been reported to decrease in the affected areas in the brain of AD patients. In this study, hyperlipidemic mouse model was established to study the effect of high blood lipid on brain. The data obtained from HPLC analysis demonstrated that the cholesterol level in brain of mice with hyperlipidemia was noticeably elevated compared to the control conditions. While the pathological damages were observed in both cerebral cortex and hippocampus in the brain of hyperlipidemic mice. Furthermore, the protein level of DHCR24 was downregulated accompanied by elevated ubiquitination level in the hyperlipidemic mice brain. The mouse neuroblastoma cells N2a were exposed to the excess cholesterol loading, the cells underwent apoptosis and the gene and protein of DHCR24 in cholesterol-loaded N2a cells were significantly reduced. In addition, the golden indexes of endoplasmic reticulum stress, BOP and ROP, increased significantly in response to the cholesterol loading. More importantly, overexpression of DHCR24 in N2a reversed neuronal apoptosis induced by the cholesterol loading. Conclusively, these findings suggested that hyperlipidemia could cause brain tissue injuries via down-regulating DHCR24, and overexpression of DHCR24 may alleviate hyperlipidemia-induced neuronal cells damage by reversing the endoplasmic reticulum stress-mediated apoptosis.

Introduction

Cholesterol participates in a variety of basic physiological processes in the human body and plays an important component of cell barrier formation and signal transduction. Transcriptional controls play a significant role in the long-term regulation of cholesterol synthesis, whereas acute regulation occurs during the post-translational control. This is mainly achieved by degrading and inhibiting the key rate control enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) [1–2]. One study demonstrated flux control by degradation of squalene monooxygenase, a downstream enzyme of the synthetic pathway [3]. Previous research showed that the flow was controlled by degrading squalene monooxygenase (an enzyme downstream of the synthesis pathway). DHCR24 is the enzyme in the final step of cholesterol synthesis pathway, so it may be the target of feedback regulation, because it will provide the most specific means to turn on or off cholesterol synthesis. Recently, we found that DHCR24 is also regulated after translation [4, 5]. Cholesterol imbalance is closely related to the occurrence and development of related diseases caused by a variety of circumstances, such as fatty liver, diabetes, and gallstones, and can also induce dyslipidemia. Hyperlipidemia refers to systemic lipid metabolism disorder caused by high blood lipid level (total cholesterol, TC; low density lipoprotein cholesterol, LDL-C and elevated triglyceride, TG) and/or decreased high density lipoprotein cholesterol (HDL-C) [6, 7]. Hypercholesterolemia is one of the biggest risks and closely related to Hyperlipidemia (HLP) and has been strongly linked to Alzheimer's disease (AD).

Alzheimer's disease (AD) is a neurodegenerative disease with hidden onset and progressive development, and one of the most important public health problems in the world with high incidence rate and mortality.

There are many causes and pathogenic factors, which lead to the dysfunction of neurotransmitter system and the decline of cognitive ability that accounts for the mechanism of the pathogenesis of AD. AD has been widely studied in the past thirty years, but its pathological mechanism is still unclear. Among them, the two most classical hypotheses are amyloid β-protein (Aβ) cascade and tau protein hyperphosphorylation.

A major role is currently played by cholesterol metabolism in the development of Alzheimer's disease, which has been repeatedly confirmed as the hypothesis of cholesterol pathogenesis. In addition to participating in the formation and degradation of Aβ in the brain tissue of AD patients, cholesterol also regulates its function and aggregation and affects its neurotoxic effects [8]. On the other hand, oxidized cholesterol (a metabolite of oxysterol) can enter the brain through the blood-brain barrier from the blood circulation, and vice versa. It has been found that oxysterol and the associated catalytic enzymes are changed in the AD brain and have been proven to be the influencing factors of disease progression [9]. Many years of research suggests that patients taking cholesterol lowering drugs can reduce the probability of secondary AD. However, it is worth noting that the mechanism of brain cholesterol metabolism is different from that of the body, and it remains to be determined if there is a correlation between brain cholesterol and serum cholesterol levels. DHCR24, also known as Seladin-1, is one of the selective detection indicators of AD. DHCR24 is a key enzyme involved in cholesterol biosynthesis at its final step, which is found in almost all neurons and neuroglia cells. It is not only involved in the de novo synthesis of cholesterol, but also involved in the neuroprotection of oxidative stress, endoplasmic reticulum stress, Aβ toxicity and apoptosis [10]. Down regulation of DHCR24 expression was observed in brain lesions of AD patients [11]. It is also found that *DHCR24* gene polymorphism can regulate the risk of AD, also suggesting that DHCR24 may be related to AD [12]. In addition, the results of in vitro experiments showed that the decrease of DHCR24 level led to the increase of stability of BACE1, thus promoting the hydrolysis of Aβ by APP. Therefore, increasing the expression level of DHCR24 in the affected area of brain may provide a potential therapy for the intervention of the pathogenesis of AD.

The increase of lipid intake is the main reason for the increase of the proportion of patients with dyslipidemia. However, it is still controversial whether peripheral hyperlipidemia can cause the increase of cholesterol concentration in the brain, and whether the DHCR24 level in the brain would be affected in response to the elevated serum cholesterol level. We speculate that the peripheral hyperlipidemia will induce the brain damage and increase the cholesterol level of brain thus downregulating the expression of DHCR24. Therefore, in the present study, we examined the effect of excess cholesterol loading on the neuron damage and expression level of DHCR24 at both animal and cellular levels.

Materials And Methods

Ethical statement

The present study was conducted in accordance with Laboratory Animal-Guideline for ethical review of animal welfare (GB/T 35,892–2018, National Standards of the People's Republic of China).

Reagents and chemicals

The blood lipid test kit was purchased from Nanjing Jiancheng Biotechnology Research Institute (Nanjing, China). Eosin, and haematoxylin dye was purchased from Nanjing Chemical Reagent Factory (Nanjing, China). The recombinant adenovirus Ad-DHCR24 was purchased from Obio Technology (Shanghai) Corp., Ltd. 4, 6- Dianmidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). PI Assay Kit was purchased from Beijing Sizhengbai Biotechnology Co. Ltd. (Beijing, China).

Antibodies used in the experiment, such as anti DHCR24, Akt, chop and bid were purchased from cell signaling technology company (Danvers, Massachusetts, USA). Secondary antibodies such as anti-b-actin Rabbit mAb, and HRP-labeled Goat Anti-Rabbit IgG were obtained from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China).

Animals and feeding

Animal management procedures and handling methods strictly abide by the "Guidelines for the Management and Use of Laboratory Animal Feeding". Male C57BL/6 mouse were purchased from the Liaoning Changsheng Biological Company. C57BL/6 mouse were separated randomly into two groups with 12 mice in each group. The normal diet group was the control group and the high-fat diet group was the high-fat group. The animals were fed for 4 weeks and monitored every week.

Preparation of CLCD (Cholesterol-loaded Cyclodextrin)

Cholesterol (200 mg, Sigma) dissolved in 1mL chloroform. β-Cyclodextrin (CD, 1 g) dissolved in 2 mL methanol. 0.45 mL aliquot of sterol solution was combined to CD solution, stirred, and dried under N2 gas. Resultant crystals dried for 24 h and stored in glass at room temperature. CLCD working solution: 50 mg crystals/1 mL serum free medium: warm to 37 °C and vortexed briefly.

Cell culture

N2a cells were obtained American Type Culture Collection, Manassas, VA, USA. The cells were nourished in 37 °C 10% FBS/DMEM in 5% CO_2 atmosphere in an incubator. In cholesterol load experiment, CLCD working solution were incubated for 48 h with N2a cells.

Transfection and analysis

N2a cells were seeded onto 6-well plates at a density of 5×10^5 cells per well for 24 h until the confluence reached 60-80%. Transfection method andefficiency have been reported in our former research [13]. The control and Ad-lacZ group were used as negative controls.

Measurement of blood indexes in serum

The serum samples of mice in each group were collected, and the four serum indexes (TG, TC, LDL-C and HDL-C) were detected and calculated according to the instruction of the kit.

Lipid extraction and total cholesterol assay

To extract lipids for the total cholesterol measurement, the brain of mouse was homogenized in chloroform: methanol (2:1, 0.1 g brain/2 ml solvent) mix and centrifuged for 5 min at 2000×g. After collecting the supernatant and added ddH_2O, mix and centrifuged for 10 min at 2000×g. Collect the lipid-containing lower layer liquid (chloroform layer) and dry the liquid with N2 gas to obtain the lipid. High Performance Liquid Chromatography (HPLC) was used to measure the total cholesterol levels in the brain of mouse. In brief, make a standard curve of cholesterol (range 0-10 mg/mL). Dissolve the obtained total lipids in 1 mL of chromatographic grade absolute ethanol and filter the sample using a 0.22 μM filter. Set the sample detection conditions and methods, use the treated chromatographic grade methanol as the mobile phase, and the C18 column as the stationary phase, and detect at UV 210 nm.

Histopathological examination

Histopathological examination was carried out as described earlier with some modifications [14]. Briefly, brain samples were fixed in 10% formalin overnight, dehydrated in a series of alcohol solution and embedded in wax. After embedding, samples were cut into sections (5 μm), stained with H&E and observed under light microscope (Nikon E100, 40X magnification, Tokyo, Japan).

TUNEL assays

The cells were seeded onto a six-well plate for 12 h. Then, the cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Subsequently, apoptosis of cells was determined with TUNEL kit. TUNEL assays were conducted using a TUNEL fluorescence FITC kit (Vazyme Nanjing, Jiangsu, China) according to the manufacturer's instructions. Finally, the slides were counterstained with hematoxylin, examined under a fluorescence microscope (Olympus, Tokyo, Japan), and the percentage of positive cells to the total cells was calculated in each sample.

Duolink^{TM} assay system

Duolink^{TM} fluorescence method was employed as per manufacturer's recommendations (O link Biosciences). N2a cells (1×10^4 cells) were seeded and differentiated in chamber slides. N2a cells were serum-starved overnight (DMEM and 2% BSA) and treated in microvesicle-free DMEM with 0.5% BSA for 24 hours. Mouse DHCR24 antibody (Danvers, MA, USA) and Rabbit MDM2 antibody (Santa Cruz Technology, Inc) were used to assess DHCR24/MDM2 interactions. Images were taken with Zeiss LSM 780 confocal microscope.

Filipin staining assays

The cholesterol level of cells was detected by Filipin (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, cells were fixed with 3% paraformaldehyde for 30 min and three-time washed in TBST. Cells were incubated with Glycine solution (1.5 mg/mL) for 10 min. 1 mL of 5 mg/mL Filipin reagent was added in six-well plate. After incubation for 2 h at 37°C in the dark, invert the cell slides on the glass slide with tablet containing PI. The sample fluorescence was measured.

Detection of gene expression level

The Quantitative real-time RT-PCR analysis was performed in order to confirm the efficiency of DHCR24 expression on mRNA- level in the brain of mouse and N2a cells. Total RNA was extracted from the brain of mouse after feeding high-fat diet and normal diet for 4 weeks. Besides, total RNA was extracted from N2a cells after loading CLCD working solution for 48 h. The specific methods of gene extraction and RT-PCR have been reported in our former research [13]. Applied Biosystems 7500 Fast Real-Time PCR System, using the SYBR® Premix Ex Taq™ II (catalog no. RR820A). All reagents were purchased from TAKARA Biotechnology. All the procedures were carried out according to manufacturer's protocol. The comparative $^{\Delta\Delta}$Ct method was used to calculate GAPDH-normalized expression levels of DHCR24 mRNAs.

The primers used in the RT–PCR were:

DHCR24 sense 5' -GCCGCTCTCGCTTATCTTCG-3',

DHCR24 antisense 5' -GTCTTGCTACCCTGCTCCTT-3',

GAPDH sense 5'-GCACCGTCAAGGCTCAGAAC-3',

GAPDH antisense 5'-TGGTGAAGACGCCAGTGGA-3'.

Detection of protein expression level

The western blotting analysis was performed in order to confirm the efficiency of DHCR24 expression on protein level in the brain of mouse and N2a cells. The specific methods of protein extraction and Western Blot have been reported in our former research [13]. The level of immunoreactivity was assessed as a peak intensity using an image capture and analysis system (GeneGnome, Syngene, UK). The anti-β-actin antibody was used to control the protein quality and ensure equal loading.

Immunoprecipitation assays

Immunoprecipitation assays were performed in order to confirm the expression of ubiquitinated-DHCR24 in the brain of mouse. In brief, total protein was extracted from the brain of mouse. DHCR24 was immunoprecipitated with anti-DHCR24 monoclonal antibody (Santa). Immunoprecipitation assays were conducted using a BeaverBeads™ Protein A/G Immunoprecipitation Kit (Beaver Suzhou, Jiangsu, China) according to the manufacturer's instructions. Immunoprecipitates were separated by electrophoresis and

transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in TBST for 1.5 h at room temperature. The protein blots were incubated with primary anti-DHCR24 or anti-ubiquitin antibodies (Med Chem Express, Monmouth Junction, NJ, USA) overnight at 4 °C, then probed with HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature. The membrane was then washed and used via enhanced chemiluminescence (ECL) detection.

Statistical analysis

All the data were expressed as the mean ± SD. Statistical significance was determined using a student t-test or one-way analysis of variance (ANOVA) in the case of comparisons among more than two groups following Dunnett's T3 test. A value of $p < 0.05$ were considered as statistically significant. All the graphs were made by GraphPad Prism (Version 8.0.1).

Results

Establishment of hyperlipidemia model of mouse

To explore the effect of hypercholesterolemia on brain tissue, the hyperlipidemia mouse model was successfully established by feeding high-fat diet for 4 weeks. The levels of blood lipid, including LDL-C, TC, TG and HDL-C were examined in peripheral blood samples of mouse for control (Normal diet, ND) and Hyperlipidemic model (High-fat diet, HFD) groups. Figure 1 shows that in the HFD group, blood lipid levels (TC, TG, and HDL-C) increased ($p = 0.036$). Meanwhile, LDL-C level was remarkably increased ($p = 0.00067$). The finding showed that Hyperlipidemia Model of mouse was successfully constructed. Therefore, the model was used for subsequent experiments.

Hyperlipidemia increases the cholesterol level and causes tissue damage of mice brains

To determine whether hyperlipidemia caused damage to the brain of mouse, a hyperlipidemia mouse model was established. The Body weight was recorded and monitored for four weeks, and brain tissue weight was recorded after dissection. Results are shown in Figure 2A, the body weight of the two groups of mice monitored had significantly lower at different time points, indicating that four weeks of high-fat diet did not cause an apparent increase in the body and brain tissue weight of the mice. However, it can be observed that the fur of the model group is shiny, greasy and the oil secretion was significantly increased (Fig. 2B). HPLC technology was performed to assess the cholesterol concentration in the brain tissues of mice. The brain cholesterol concentration in HFD group was significantly higher relative to ND group (Fig. 2C). Compared to the ND group, HE staining results showed that the pathological changes of the brain caused by hyperlipidemia was observed in HFD group. The nuclear chromatin in the DG area in HFD group was markedly concentrated in the Hippocampus DG mouse, and the structure of nerve cells in the cerebral cortex was loose and disordered (Fig. 2D). TUNEL assays of mouse brain slices showed that the HFD group significantly increased apoptosis in the hippocampus (Fig. 2E).

Hyperlipidemia downregulated the protein level of DHCR24 while increased the ubiquitination levels of DHCR24 protein in mice brain

To elucidate the relationship between hyperlipidemia-induced brain damage in mouse and DHCR24 expression, we detected the changes of DHCR24 genes and protein expression levels, respectively. Compared with ND group, the RT-PCR results showed that DHCR24 mRNA levels in the hippocampus region of the brain in HFD group had no significantly lower (Fig. 3A). However, DHCR24 protein levels in the hippocampus region of the brain was significantly down-regulated (Fig. 3B; $p = 0.0074$).

In order to further study the molecular mechanism of DHCR24 protein down-regulation, we performed co-immunoprecipitation to evaluate the modification of DHCR24 by ubiquitination. The results showed that ubiquitin modified DHCR24 of HFD treated group was significantly greater compared to ND group in the IP-treated cell lysates while total levels of DHCR24 were significantly reduced of HFD group in both input and IP -treated cells (Fig. 3C). This result suggested that elevated cholesterol level in brain induced by hyperlipidemia might down-regulated DHCR24 expression via increasing its ubiquitination-modified level.

To this end, this study further verified the ubiquitination of DHCR24 at the neuronal level using the mouse neuroblastoma cell line N2a cells. A previous study demonstrated that DHCR24 could interact with MDM2, an E3 ligase [15]. Here we first performed the interaction between DHCR24 and MDM2 in our experimental system by a very powerful tool for detecting in situ protein-protein interaction by Duolink PLA assay. The results showed that increasing DHCR24/MDM2 interactions, characterized by red punctate dots in N2a cells (Fig. 3D). Furthermore, after treated with proteasome inhibitor MG132 in N2a cells, the diffuse bands in the experimental group treated with 20 um MG132 were significantly increased, indicating that the ubiquitination level of DHCR24 was enhanced, supporting that DHCR24 is also modified by ubiquitination and therefore undergoes post-translational regulation at neuronal level (Fig. 3E). To further confirm the ubiquitin-modification of DHCR24, we overexpressed DHCR24 by adenovirus driving and performed directly Western Blotting analysis to study the effect of MG132 on the DHCR24 protein level. N2a cells were transfected with Ad-lacZ/ad-DHCR24 recombinant adenovirus for 48 hours, and one group of cells transfected with ad-DHCR24 recombinant adenovirus was treated with MG132 for 6 h. Western blotting results showed that the protein level of DHCR24 was significantly increased after MG132 stimulation (Fig. 3F), compared with the control group without MG132 stimulation. To summarize, these results fully proved that DHCR24 could undergo the post-translational regulation through proteasome degradation pathway of DHCR24.

Mouse neuroblastoma cells N2a is also sensitive to apoptosis induced by excess cholesterol loading

In order to study the effects of cholesterol loading directly on neuronal cells, we performed the subsequent experiments utilizing the N2a cells. To obtain an appropriate concentration of CLCD (Cholesterol-loaded Cyclodextrin, CLCD) overload in nerve cells, N2a cells was stimulated for 24 h and 48 h at a range of CLCD (0-1 mg/mL). As shown in Figure 4A, the numbers of adherent cells were remarkably decreased in a dose and time-dependent manner in response to the different treatment of CLCD. The

significant loss of adherent cells was observed from CLCD-loaded with 0.4 mg/mL for 48 h compared to the control conditions. Hence, CLCD (0.4 mg/mL) was employed in the subsequent experiments.

To explore whether the CLCD-loading in culture medium could increase the cellular cholesterol level, we performed the free cholesterol fluorescent staining by utilizing the cholesterol fluorescent probe called Filipin. As shown in Figure 4B, the intensity of blue-fluorescent signal in CLCD-loading groups (Cholesterol group) was significantly stronger compared to control. This data demonstrated that the CLCD-loading in the culture medium could increase the cellular cholesterol level in N2a cells.

Excess cholesterol loading down-regulates both mRNA and protein expressions of DHCR24 in N2a cells

To further verify the effect of cholesterol overloading on DHCR24 mRNA and protein expression of N2a at the cellular level, we used Western blot and RT-PCR analysis to detect the expression level of DHCR24 after CLCD overloading at 0 h, 24 h, and 48 h (Fig. 5A and 5B). The results obtained from RT-PCR showed that the mRNA expression of DHCR24 was markedly reduced at 24 h (p = 0.00032) and 48 h (p = 0.0066) by CLCD overload compared to the control group (at 0 h). Meanwhile, DHCR24 proteins were also significantly decreased after 48 h of CLCD-overload (Fig. 5C).

Endoplasmic reticulum stress may mediate apoptosis upon cholesterol overload in N2a cells

In order to examine whether CLCD overload induces apoptosis in N2a cells, the TUNEL assay was performed to assess the apoptosis in CLCD-overloaded N2a cells. Compared with the control group, the green-fluorescent signals representing TUNEL-positive cell were markedly increased in CLCD-overloading group (Fig. 6A), suggesting that the CLCD-overloading induced the apoptosis of N2a cells.

Several studies have shown that the excessive activated endoplasmic reticulum stress signaling pathway may not only contribute to the occurrence of apoptosis but induce protein ubiquitination and degradation [16-18]. We therefore used western blot to detect the endoplasmic reticulum stress (ERS) response iconic protein (Bip and Chop) and apoptosis iconic protein (Caspase-3). As illustrated in Figure 6B, Bip, Chop and cleaved-Caspase-3 expression were up-regulated after CLCD load, suggesting that CLCD load could induce ERS and apoptosis.

Ad-DHCR24 reversed cholesterol overloaded induced apoptosis in N2a cells

Finally, we used the method of manipulated genetic to gain further insight into the neuron-protective function of DHCR24 during cholesterol overloading-induced apoptosis in N2a cells. In the present study, the overexpression of DHCR24 in N2a was achieved by infection of the recombinant adenovirus vector Ad-DHCR24, and the adenovirus Ad-lacZ as a control group. As shown in Figure 7A, the Western blotting analysis conformed the overexpression of DHCR24 induced by Ad-DHCR24. A significant increase in the number of adherent cells was observed in the Ad-DHCR24-infected group following CLCD overloading (Fig. 7B). TUNEL assays showed that the number of green fluorescence dots reduced in CLCD overloaded N2a cells infected by Ad-DHCR24 in comparison to that tin Ad-lacZ-infected group (Fig. 7C). This data

strongly confirmed that the DHCR24 overexpression could rescue neuronal cells from excess cholesterol loading-induced apoptosis.

Discussion

Hyperlipidemia is a systemic disorder of lipid metabolism caused by various reasons. As a chronic disease, it has caused a worldwide health crisis. Relevant experimental studies showed that high fat diet can promote the expression in the brain of transgenic mice Aβ deposition and loss of memory [19]. A 13-year follow-up study confirmed that total cholesterol and low-density lipoprotein cholesterol levels were positively correlated with the risk of AD [20]. Therefore, cholesterol metabolism disorder has become a high-risk factor for AD. At present, the drugs for treating AD are mainly based on the removal of Aβ or Tau protein deposits, but only have a certain role in alleviating the clinical symptoms of the disease. There is no effective cure for AD. It is worth noting that statins have a protective effect on the early onset of AD, especially it could reduce the decline rate of apoE ε 4 gene carriers and cognitive function in Hyperlipidemia [21]. It may be that statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase to regulate the function of various cellular pathways, such as reducing cholesterol synthesis [22]. Although the specific molecular mechanism is still unclear, but this study suggests that the treatment of AD from the perspective of improving the disorder of cholesterol metabolism may be a new idea for the prevention and therapy of AD.

In previous studies, it has been demonstrated that brain cholesterol is synthesized in situ in the adult central nervous system; primarily by astrocytes. Plasma cholesterol cannot cross the blood-brain barrier under normal conditions [23]. Recent study suggests that cholesterol accumulation was observed in cortex, striatum, hippocampus and substantia nigra of hypercholesterolemia chronic mouse model, and found that hypercholesterolemia seriously damaged the blood-brain barrier of mice, which may be the root cause in the increase of brain cholesterol level in hypercholesterolemic mice [24]. It has been suggested that a high-cholesterol diet may alter the permeability of the blood-brain barrier, which could lead to peripheral cholesterol entering the brain and aggravating the disorder of cholesterol metabolism in the brain [25]. However, there is no research on its specific molecular mechanism, including whether hypercholesterolemia accompanied by hyperlipidemia can penetrate the blood-brain barrier and cause pathological changes of brain tissue. This study confirmed that hyperlipidemic mouse were accompanied by the increase of brain cholesterol level (Fig. 2A), consistent with the above reports and Paul et al [26]. In addition, we observed the pathological changes of hippocampal formation in mice brain by HE staining and TUNEL apoptosis detection, and the apoptosis phenomenon was obvious (Fig. 2B and 2C), suggesting that hyperlipidemia accompanied by hypercholesterolemia that could cause the brain damage.

Some studies have found that DHCR24 is the last enzyme for cholesterol synthesis, is down-regulated DHCR24 expression level in the brain injury area of AD patients [11]. According to Greeves et al., down-regulation of DHCR24 in the brain area affected by hydrogen peroxide and Aβ may protect neurons against apoptosis [27]. At the same time, several studies and previous experiments of our research group

have confirmed that DHCR24 functions as a neuroprotective factor by opposing apoptosis induced by oxidative and endoplasmic reticulum stress, or by maintaining cell survival signaling pathway that was related to growth factor receptor in caveolae [28]. However, whether the increase of peripheral blood cholesterol caused by hyperlipidemia affects the content of DHCR24 in the brain becomes the second problem to be solved. The DHCR24 gene and protein expression levels were detected in the brain tissue of mice. It was found that the high-fat diet had no influence on the mRNA expression of DHCR24, but the protein expression was significantly down regulated (Fig. 3A and B). It has been reported that excessive cholesterol can form oxysterol or lanosterol, which can induce insulin-induced gene protein-1 (insig-1) to promote the recruitment of HMGCR membrane domain by ubiquitin ligase AMFR/gp78, which makes HMGCR ubiquitinate and dissociate from the membrane, and then degrade through endoplasmic reticulum related degradation pathway, leading to the rapid reduction of cholesterol content and suppression of cholesterol synthesis [5, 29]. This post transcriptional regulation is an important and rapid cholesterol feedback regulation mechanism besides the downregulation of cholesterol synthesis related enzymes at transcriptional level. It is reported that DHCR24 can interact with MDM2, the E3 ligase so that it might be ubiquitinated [15]. We also confirmed that DHCR24 could interact with E3 ubiquitin ligase MDM2 through Duolink experiment (Fig. 3D), while DHCR24 can be modified by ubiquitination at both animal and cellular level (Fig. 3C, 3E and 3F). As a consequence, we speculate that the mechanisms for DHCR24 protein downregulation may be related to the increased levels of cerebral cholesterol in the present study, IP experiment was used to elucidate the hypothesis that the increased ubiquitination of DHCR24 protein led to the downregulation of DHCR24 protein expression in the brain tissue of mouse with hyperlipidemia (Fig. 3C). The above data suggested that hypercholesterolemia caused by high-fat diet promotes the ubiquitination and degradation of DHCR24 protein, which weakens its protective effect on the brain neurons. Moreover, in our follow-up studies, we used recombinant adenovirus Ad-DHCR24 to drive the overexpression of DHCR24 protein in N2a cells, confirming that up-regulation of DHCR24 protein expression can reverse the apoptosis of N2a nerve cells induced by cholesterol loading (Fig. 7C). Therefore, the downregulation of DHCR24 may also be involved in the pathogenesis of Alzheimer's disease, at least for the AD patients with hyperlipidemia. Our data also proved for the first time that overexpression of DHCR24 could protect neuronal cells by inhibiting cholesterol-loading-induced apoptosis, which indicated that targeted overexpressing of DHCR24 is a promising strategy for develop new drugs against AD, especially against AD caused by hyperlipidemia.

For the CNS to function properly, a balance of cholesterol metabolism must be maintained, since cholesterol is essential for a few neurobiological processes [23]. From previous studies, brain cholesterol homeostasis is crucial to the formation of synapses and normal behavior of the brain, and it decreased during both AD and normal aging [30]. It is well known that DHCR24 is one of the key enzymes in de novo cholesterol synthesis. Recent research has demonstrated that DHCR24 can protect neuronal cells under a variety of stress conditions [15, 28, 30]. Moreover, based on in vivo experiments, we here have clarified the protective mechanism of DHCR24 on neuronal damage induced by hypercholesterolemia through cell-level experiments. In our study, the effect of cholesterol loading on DHCR24 protein expression and the survival of nerve cells, we first confirmed that cholesterol loading induced the increase of

intracellular cholesterol level in nerve cells (Fig. 4B), and found that the expression of DHCR24 protein was down regulated after cholesterol loading (Fig. 5B), which was consistent with the experimental results in vivo. It is noteworthy that the apoptosis increased significantly in N2a cells after cholesterol-loading (Fig. 6A and B), and the endoplasmic reticulum stress response iconic protein (Bip and chop) was significantly upregulated (Fig. 6B), suggesting that cholesterol-loading could cause activation of ER stress pathway. We speculate that this result may be related to the increased ubiquitination modification of DHCR24 and the downregulation of protein levels, and induces the occurrence of apoptosis. RT-PCR results indicated that the DHCR24 gene was down regulated 24 h and 48 h after cholesterol loading (Fig. 5A). This may be because extracellular cholesterol-loading may destroy cholesterol homeostasis, which leads to the negative feedback regulation of DHCR24 and the decrease of mRNA expression at the transcriptional level at cellular level. Sterol regulatory element binding proteins (SREBPs) function to modulate the sterol regulatory elements in the proximal promoter of DHCR24 in HepG2 cells with LDLR as the sole source of cholesterol. Thus, reducing promoter activity of DHCR24 resulting in the decrease of DHCR24 mRNA expression [31]. The present study may suggest that similar regulatory mechanisms may also exist in N2a nerve cells, of course further studies are needed to confirm. Cholesterol in brain tissue is mainly synthesized by glial cells, and apolipoprotein E is responsible for transporting it to neurons. Therefore, the downregulation of DHCR24 protein expression associated with increased cholesterol may be mainly due to protein degradation mediated by ubiquitin proteasome pathway both in vitro and in vivo, while the regulation of mRNA expression of DHCR24 on the transcription level in response to cholesterol loading at cellular level was also observed although its mechanism needs to be further studied.

The results of this study are summarized in Figure 8. Peripheral hyperlipidemia could be accompanied by the increase of cholesterol content in the brain of mouse, and the increase of ubiquitination modification of DHCR24 leads to the down regulation of protein expression, thus weakening its neuroprotective effect in vivo. While, extracellular cholesterol-loading increased the intracellular cholesterol level in vitro, and the protein and mRNA levels of DHCR24 were both down-regulated, leading to apoptosis. The down regulation of DHCR24 protein expression may be associated to the activation of endoplasmic reticulum stress and the degradation of ubiquitinated proteasome pathway of DHCR24. Overexpression of DHCR24 could protect the nerve cells from cholesterol-overloading.

Conclusion

AD has more than 30 possible pathogenic factors and hypotheses as a neurodegenerative disease with stealthy onset. Many studies have proposed that hyperlipidemia is an important factor in inducing the onset of AD and has been widely concerned over recent year. This study suggests that the increase in cholesterol levels in brain accompanied by hyperlipidemia may lead to endoplasmic reticulum stress, resulting in increased ubiquitination of DHCR24 protein and down-regulated expression, thus weakening the neuroprotective effect. The recombinant adenovirus driven DHCR24 overexpression could reverse the damage of cholesterol loaded neurons. In summary, our research provides a possible molecular explanation for the increase in the risk of AD with hyperlipidemia, and suggests that up-regulating the

expression of DHCR24 protein may become a new way of preventing and treating hyperlipidemia-induced AD.

Declarations

Ethics approval and consent to participate

The present study was conducted in accordance with Laboratory Animal-Guideline for ethical review of animal welfare (GB/T 35,892–2018, National Standards of the People's Republic of China).

Consent for publication

Not applicable

Availability of data and materials

Please contact author for data requests.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

Credit author statement

Xiuli Lu and **Bing Gao** supervised the whole experiments. **Ziyin Lu** and **Haozhen Wang**, designed this study and contributed to the paper writing. **Xiujin Zhang, Xiuting Huang**, **Shan Jiang**, **Chen Lu**, **Yang Li**, and **Ting Liu** performed the practical work and completed the experiments. All authors have read and approved the final version of this manuscript to be published.

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Figures

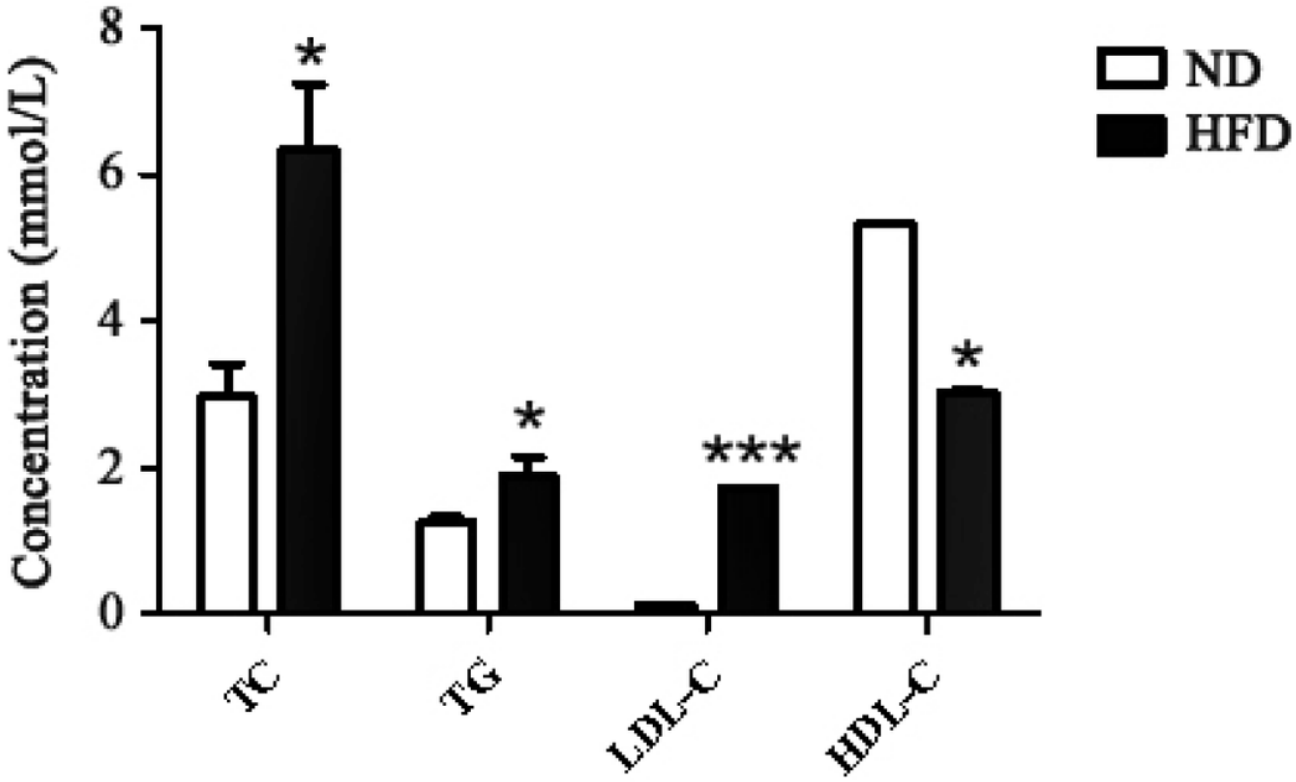

Figure 1

Peripheral blood lipid is displayed in this figure. The experimental groups included the ND (Normal diet) and HFD group (Hight-fat diet). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the ND group. The results are expressed as the mean ± SD, n = 5.

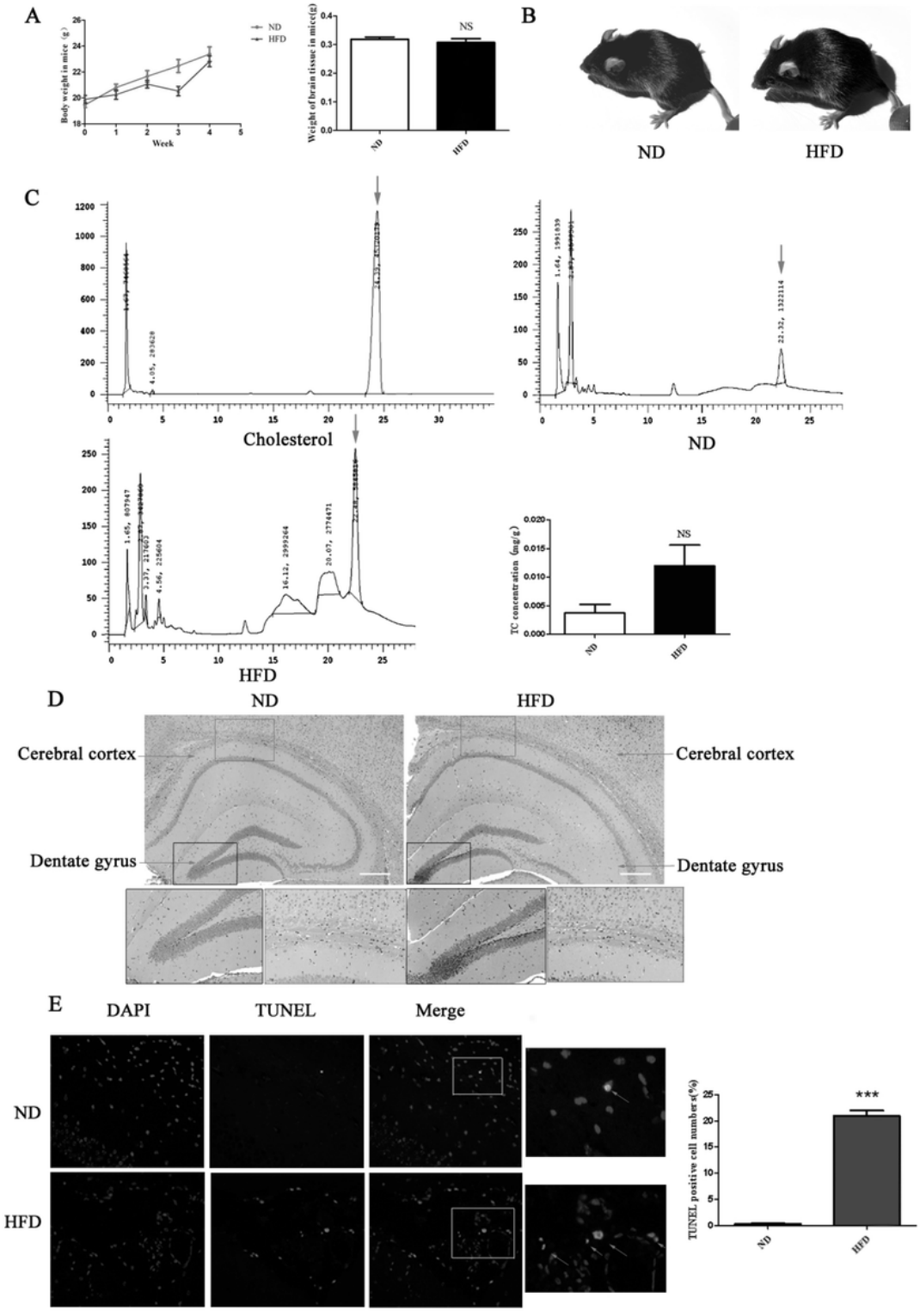

Figure 2

Hyperlipidemia causes brain damage in mice. A. and B. The body weight, brain tissue weight and appearance change of mice were recorded within 4 weeks. **C.** HPLC was used to detect the cholesterol content in the brain of the mice and calculate the cholesterol concentration per unit tissue. **D.** The photomicrographs by HE staining showed chromatin concentration (red arrows), loose structure and chaotic (red arrows). Scale bars, 250 μm. **E.** TUNEL assays were performed on mice brain slices. Nuclei

are stained with DAPI. Green fluorescence indicates TUNEL positive cells. Bar graph represents mean ± SD, n = 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the ND group.

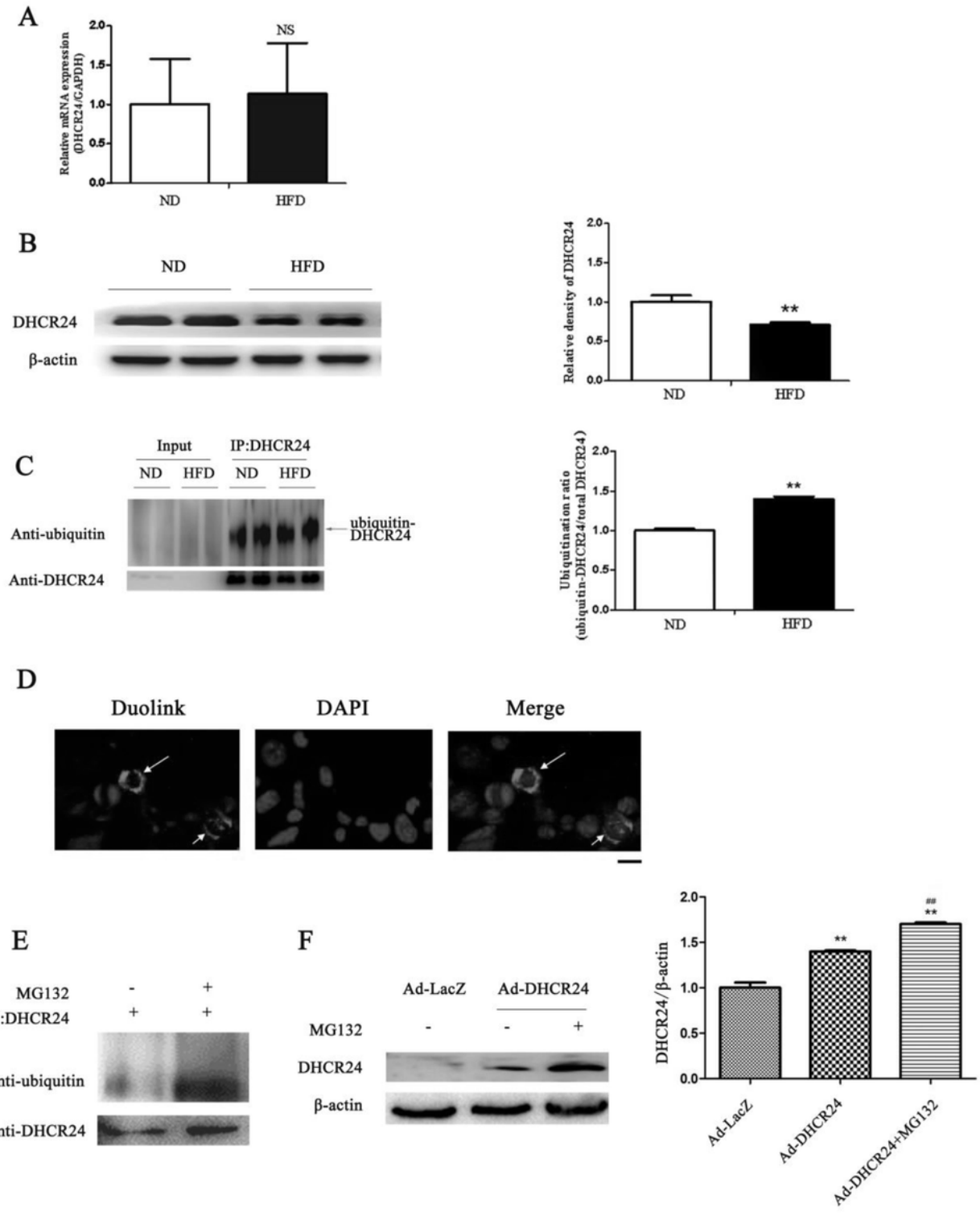

Figure 3

Effect of hyperlipidemia on the regulation of DHCR24 expression in the brain of mice. A. The expression levels of DHCR24 gene were determined by RT-PCR. The experimental groups included the ND group and

HFD group. B**.** Hyperlipidemia induces down-regulation of DHCR24 protein expression in the brain of mice. The protein of brain was extracted, and the concentration was measured. Western blot assaying on the levels of DHCR24. The β-actin level was used as the internal standard. **C.** The ubiquitination level of DHCR24 was analyzed by co-immunoprecipitation. **D.** DHCR24/MDM2 interactions were assessed by Duolink assay in N2a cells (yellow arrows). **E.** N2a cells were treated with the absence and presence of MG132 (20 μm) for 6 h, respectively. The interaction of DHCR24 with MDM2 was assessed by co-immunoprecipitation. **F.** N2a cells transfected with Ad-lacZ/Ad-DHCR24 adenoviruses, untreated or treated MG132 for 6 h and the levels of DHCR24 were quantified by western blotting. The antibody was anti-DHCR24 and anti-ubiquitin body. Bar graph represents mean ± SD, n =5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the ND group.

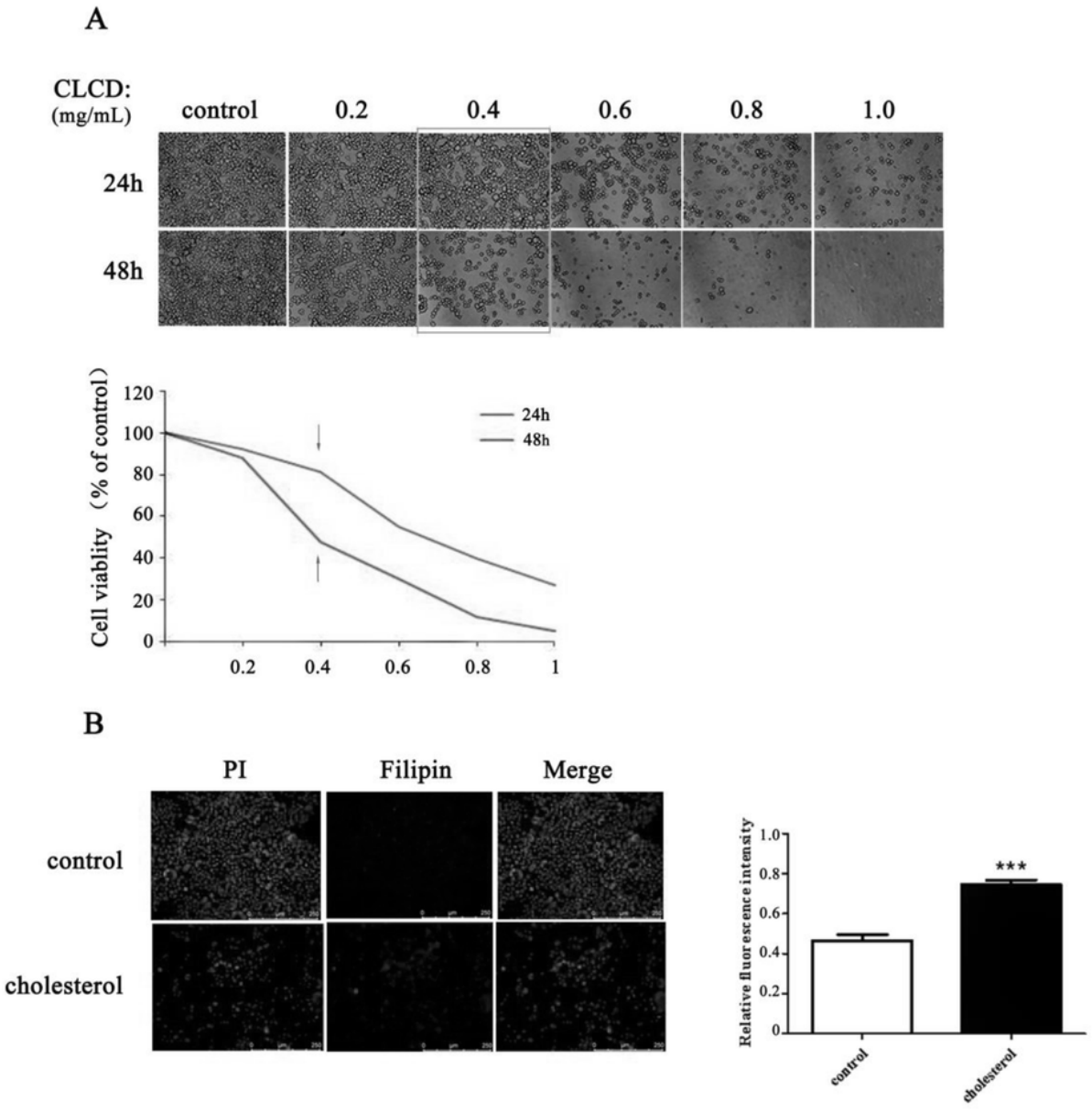

Figure 4

Simulation of hypercholesterolemia in vitro by cholesterol loading. A. Determination of cell viability in N2a cells following CLCD overload by inverted microscope. Different concentrations of CLCD (0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) were applied to N2a cells after 24 h and 48 h. Scale bars, 100 μm. Values are expressed as mean ± SD (n = 5). * $p < 0.05$ as compared with control group; ** $p < 0.01$ as compared with

control group. **B.** After loading N2a with 0.4 mg/mL CLCD for 48 h, Filipin fluorescent staining was performed. Nuclei are stained with PI. Blue fluorescence indicates intracellular cholesterol levels.

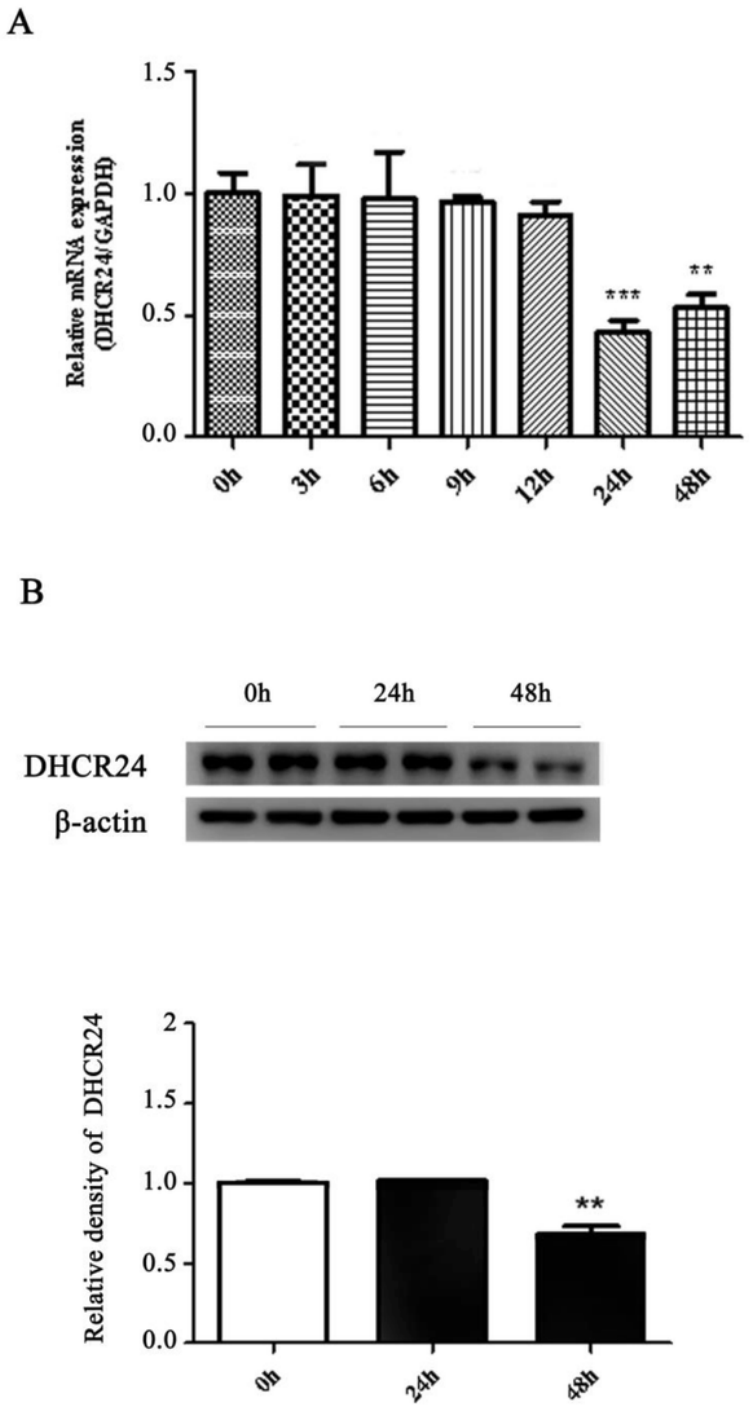

Figure 5

Effect of cholesterol overload on DHCR24 expression in N2a cells. This figure shows **A** mRNA expression and **B** protein expression of DHCR24 in CLCD overload N2a cells of difference time groups. **A.** The N2a

cells was applied with 0.4 mg/mL CLCD load for up to 48 h by RT-PCR. **B.** CLCD overload induced ERS in N2a cells by western blotting. Bar graph represents mean ± SD, n = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group.

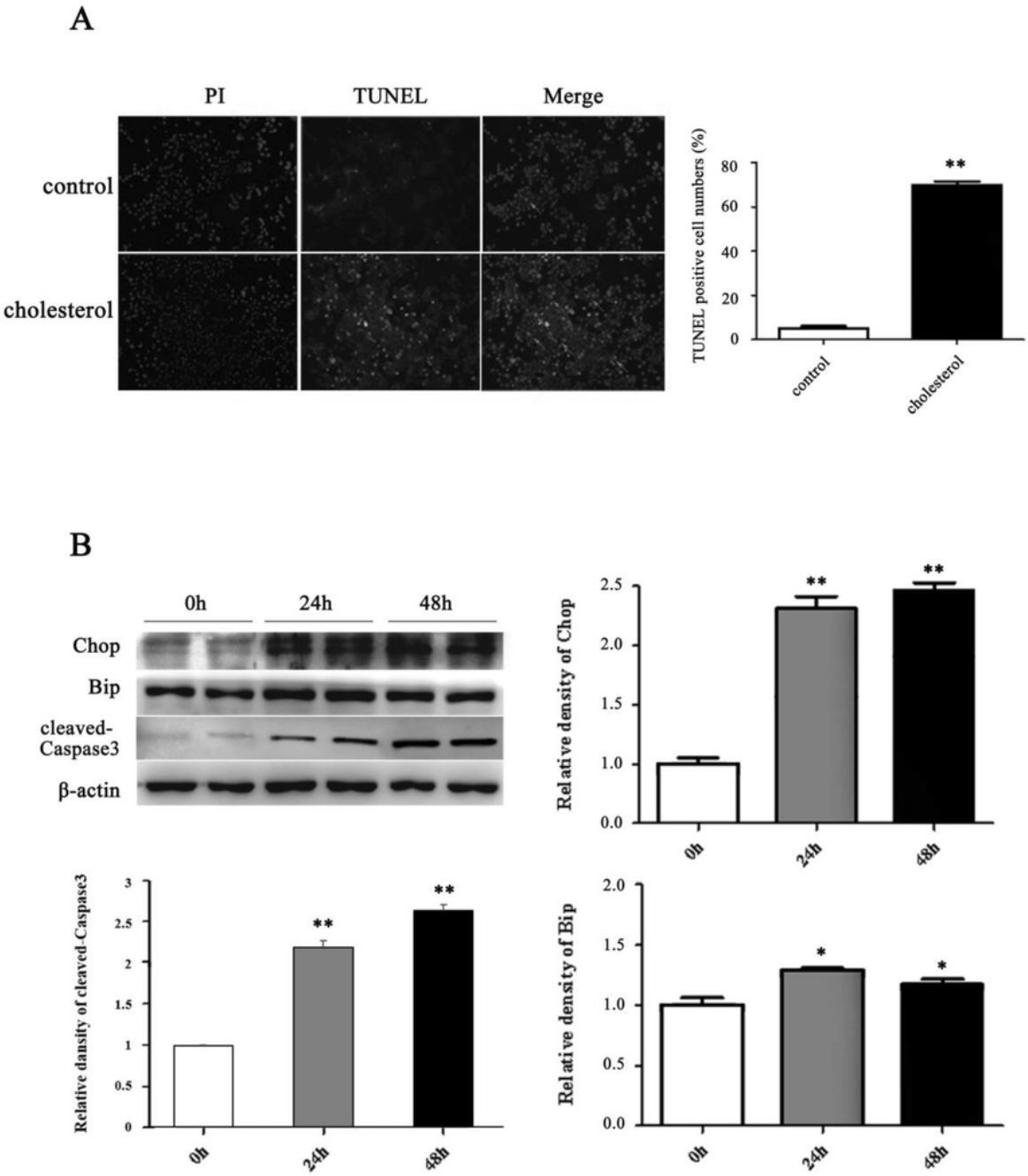

Figure 6

Endoplasmic reticulum stress may mediate apoptosis upon cholesterol overload in N2a cells. A. TUNEL assays of the control N2a cells and N2a overloaded with CLCD for 48 h. Nuclei are stained with DAPI. Green fluorescence indicates TUNEL positive cells (White arrows). **B.** Expression analysis of chop, Bip in N2a cells overloaded with CLCD for 0, 24 and 48 h by western blotting. Bar graph represents mean ± SD, n = 3. * $p < 0.05$, ** $p < 0.01$ versus the control group.

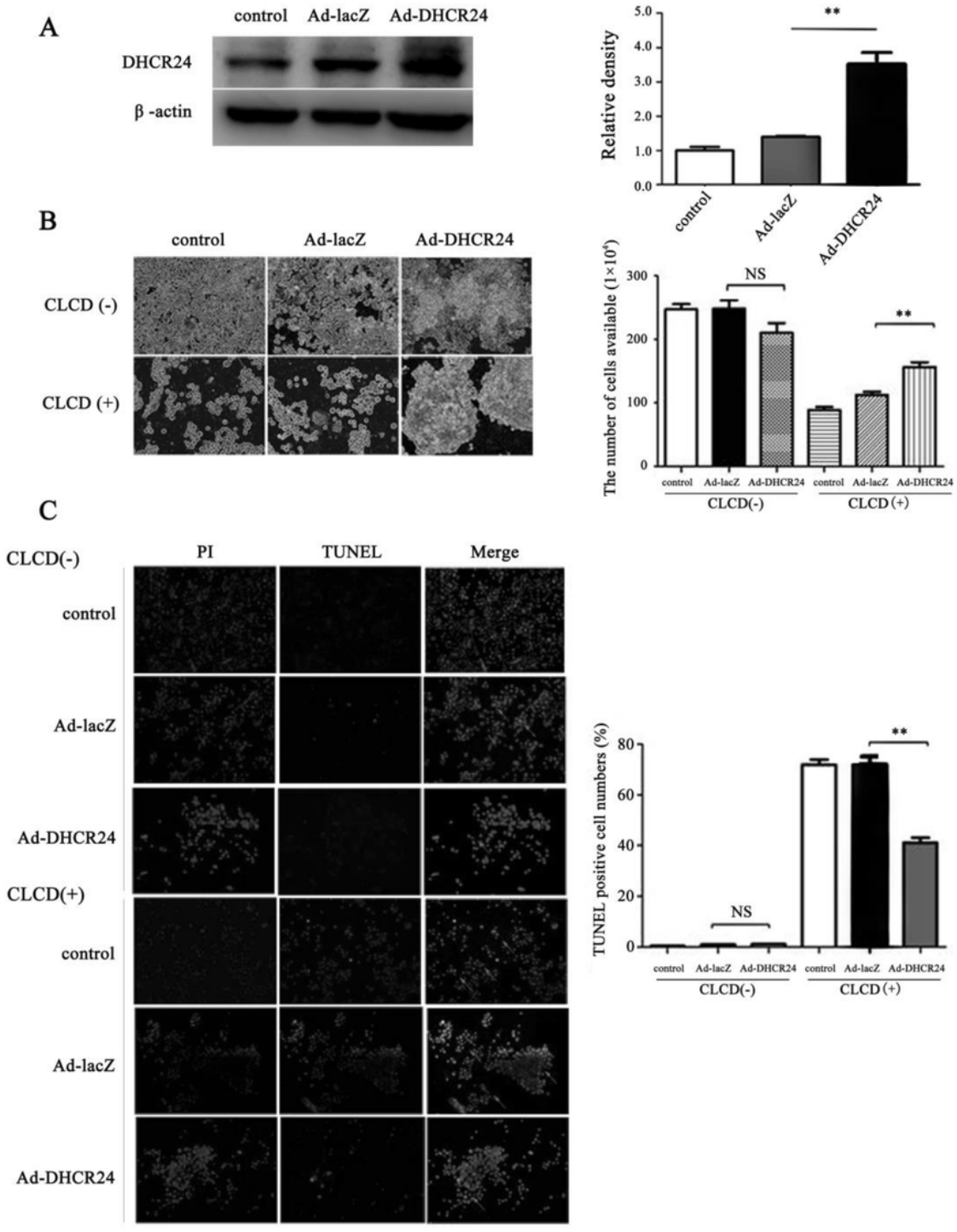

Figure 7

CLCD-overload-induced apoptosis was reversed by overexpression of DHCR24 in N2a cells. A. Ad-DHCR24 up-regulates DHCR24 expression in N2a cells. N2a cells transfected with Ad-lacZ /Ad-DHCR24 adenoviruses, and the levels of DHCR24 were quantified by western blotting. Quantification of the protein levels following transfection with Ad-DHCR24, ** $p < 0.01$ versus Ad-lacZ group. Gels were representative of three different experiments. **B.** N2a-Ad-DHCR24 cells were overloaded with CLCD for 48 h, then these were observed by morphology light microscope. Scale bars, 100 μm. **C.** Adenovirus Ad-DHCR24 reverses CLCD load induced apoptosis of N2a cells. N2a cells were infected with adenovirus for 48 h and then loaded with CLCD for 48 h. N2a cells were infected with Ad-DHCR24 adenovirus for 48 h and then loaded with CLCD for 48 h by TUNEL assays. Nuclei are stained with DAPI. Green fluorescence indicates TUNEL positive cells. Scale bars, 100 μm.

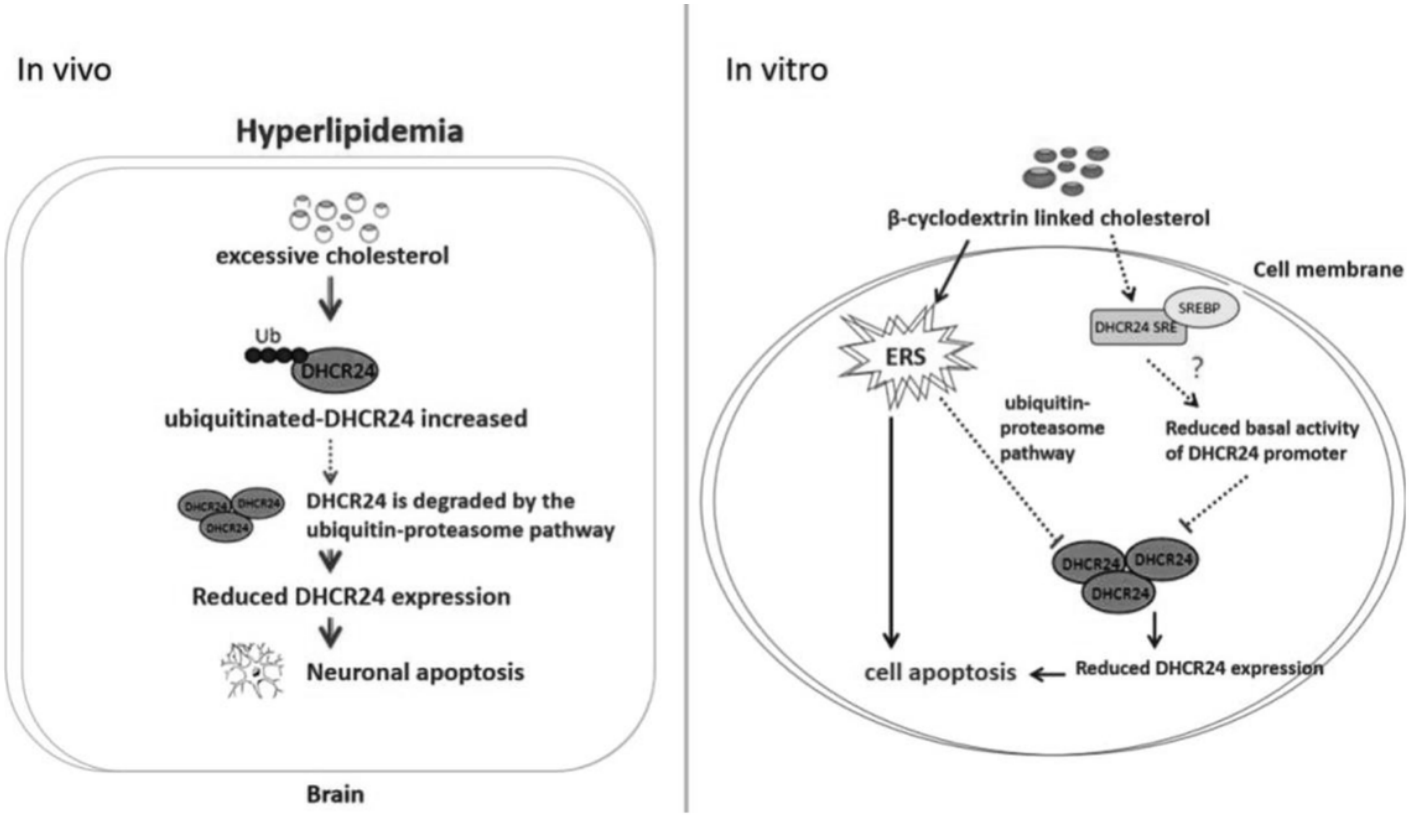

Figure 8

The underlying mechanism of hyperlipidemia induced in brain tissue and DHCR24 expression changed in mouse. Importantly, peripheral hyperlipidemia could be accompanied by the increase of cholesterol content in the brain of mouse, and the increase of ubiquitination modification of DHCR24 leads to the down regulation of protein expression, thus weakening its neuroprotective effect. While in vitro results showed that extracellular cholesterol-loading could increase the intracellular cholesterol level, and the protein and mRNA levels of DHCR24 were down-regulated that could lead to apoptosis.