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High Cholesterol Diet Increases Expression of Cholesterol 24-Hydroxylase and BACE1 in Rat Hippocampi: Implications for the Effect of Diet Cholesterol on Memory

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Abstract

Background: Abnormal cholesterol homeostasis is associated with the pathogenesis of neurodegenerative disease and cognitive impairment.
Objectives: Our objective was to evaluate changes in the expression of proteins related to cognition and cholesterol homeostasis in the hippocampi of rats as well as behavioral modifications following the administration of a cholesterol-rich diet.
Methods: In this experimental study, lasting 16 weeks, 20 male Wistar rats (aged 8 weeks) were randomly divided into two groups. One group was fed with a normal diet (ND; n = 10) and the second with a high cholesterol diet (HD; n = 10). The expression of the cognition-related proteins N-methyl-D-aspartate receptor (NMDAR) and beta-secretase 1 (BACE1) and cholesterol 24-hydroxylase (CYP46A1), the key cholesterol hemostasis protein, were determined by an immunoblotting assay in the hippocampus homogenate. The Morris water maze (MWM) test was used to examine cognitive performance. Plasma lipidic parameters, including total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglycerides (TG), as well as brain TC were measured by colorimetric assay.
Results: After a high cholesterol diet had been administered for a period of 16 weeks, a significant increase in TC, LDL-C and TG was observed in the HD group in comparison with the ND group ($P < 0.05$). Neither the mean of brain wet weight nor brain TC showed significant change in the HD versus the ND group ($P = 0.114$, $P = 0.84$, respectively). Despite this fixity, differences in the expression of BACE1 and CYP46A1 were significant ($P < 0.05$) between the two groups, with high levels of BACE1 and CYP46A1 in the HD group compared with the ND group. These biochemical changes were associated with a significant decrease in the time traveled on a platform quadrant in the HD versus the ND group ($P < 0.05$) during a spatial memory probe test administered at the same time.
Conclusions: The findings show that irregularities in cognitive performance as a result of a high cholesterol diet can be partially mediated by distortion in brain cholesterol homeostasis and processing of the amyloid precursor protein (APP).

Keywords: Alzheimer's Disease, BACE1 Protein, Cholesterol, Cholesterol 24-Hydroxylase, NMDAR

1. Background

Distorted cholesterol homeostasis and augmented levels of LDL-C play an essential role in the pathogenesis of sporadic Alzheimer's disease (AD) (1). Despite restrictions to the entrance of LDL, the main transporter of cholesterol in plasma from the blood brain barrier (BBB) to the brain parenchyma (2), an association between elevated levels of plasma cholesterol and the risk of dementia or other cognition-related diseases (3) has been demonstrated frequently (1, 4).

LDL-C helps amyloid-beta (Aβ), derived from the internalization of APP processing, to disrupt the neuronal endolysosome function and raises Aβ accumulation in neurons (5). Recent studies have also shown that APP processing by BACE1 is sensitive to cholesterol levels. Aβ deposits trigger several pathologic pathways involved in neuronal cell death (6). As the uptake of cholesterol from the circulating blood is prevented by an intact BBB, the brain is dependent on in situ synthesis of cholesterol (7-9). However, this does not mean that there is no link between these two features of cholesterol metabolism. Many in vitro and in vivo studies (10-12), investigating the mechanism by which hypercholesterolemia on the brain leads to AD pathology, have revealed that plasma and the brain exchange a number of oxysterols such as 24(S)-hydroxycholesterol (24S-

OHC), the major brain cholesterol metabolite, and 27(S)-hydroxycholesterol, one of the plasma cholesterol metabolites that cross the BBB in equal amounts. The enzyme responsible for the production of 24S-OHC is CYP46A1, which belongs to the cytochrome P450 super family, almost exclusively expressed in neuronal cells. Burlot et al. (13) have shown that the amounts of CYP46A1 and 24S-OHC in the hippocampus were higher in a model of AD-like tau pathology than in control mice. A study by Milagre et al. (11) showed that neurodegeneration leads to a change in the CYP46A1-dependent pathway. In addition, Kuo et al. (14) have shown that a high cholesterol diet induces significant cognitive deficits, along with a significant increase in the mRNA expression of BACE1, Cyp46A1, and 24S-OHC levels in the hippocampus and brain cortex. 24S-OHC is a potent oxysterol with positive allosteric effects on NMDARs, which have been shown to trigger cognitive impairment and behavioral deficit in the presence of channel blockers (15). However, there are considerable differences in previous studies investigating the effect of hypercholestrolemia on the expression of CYP46A1. Based on the evidence cited above, we hypothesized that the effect of a high cholesterol diet on the promotion of AD-associated degeneration may be partially mediated by alteration in the protein expression of CYP46A1, which is involved in brain cholesterol homeostasis.

2. Objectives

To test this idea, the present study was designed to consider the effect of a high cholesterol diet on the expression of brain cholesterol homeostasis, cerebral neurodegeneration, and cognition-related proteins after receiving a long-term high cholesterol diet (16 weeks). We took the opportunity to evaluate cognitive performance in the study population at the same time.

3. Methods

Cholesterol was purchased from Sigma-Aldrich. Polyclonal rabbit anti-BACE1, anti-CYP46A1, anti-GAPDH, anti-NMDAε2, and HRP-conjugated goat anti-rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All biochemical analysis kits for TC, HDL-C and TG were obtained from Pars Azmoon Inc. (Tehran, Iran). Antioxidant assay kits were purchased from Randox (Randox Laboratories-US).

3.1. Animals

In the current experimental study, animals were obtained from our local breeding colony (animal laboratory of neurosciences research center (NSRC), Tabriz University of Medical Sciences, Tabriz, Iran). All experimental steps were carried out in the NSRC at Tabriz University of Medical Sciences in 2014. The entire experimental approach complied with the principles of the latest revised guidelines of the national Institute of health for the care and use of laboratory animals (NIH Publications No.80-23) and was approved by the ethical committee of the Higher Academic Education Institute of Rabe Rashid (approval number: 11/5/1721). At the start, 20 male Wistar rats (body weight: 250 $\pm$ 30 g) were kept under the following controlled conditions: light (12 hours day/night), temperature (22 - 24°C) and humidity (55% - 65%), with free access to water and a normal diet (20). The rats were randomly divided into two groups: normal (ND; n=10) or high cholesterol diet (HD; n = 10). The control group was fed with a normal chow diet whereas the HD group received the chow diet plus 2% cholesterol (Sigma-Aldrich, NO; C8667). All animals had daily access to food and water ad libitum throughout the experiment.

3.2. Sample Collection and Storage

After 16 weeks of treatment and a complete performance of the behavioral tests, the rats were anesthetized using Xylazine (4 mg/kg; sigma) and ketamine hydrochloride 10% (40 mg/kg; sigma) through intraperitoneal (IP) injection. Blood samples were loaded directly from the heart into the syringe. The entire blood sample was divided into two tubes containing ethylenediaminetetraacetic acid (EDTA) and serum separator tube, to prepare plasma and serum, respectively. After clot formation over 2 hours at room temperature, serum was collected after centrifuging at 2000 $\times$ g for 15 minutes (Beckman model L centrifuge). All samples were aliquoted and stored at -20°C to avoid repeated freeze-thaw. Following the blood sample collection, the rats were subjected to transcardiac perfusion with normal saline and then decapitated, with the brains removed from the skull. The right hemisphere of the brain and the hippocampus of the left hemisphere were placed separately for each rat into liquid nitrogen and then kept at -70°C for biochemical and immunoblotting analysis, respectively. The estimated bregma levels were between -3.60 and -4.30 mm for the hippocampus (16).

3.3. Biochemical Measurements

The plasma levels of TG, TC and HDL-C were determined photometrically in a VITROS 5600 Autoanalyser (Ortho-Clinical Diagnostics Inc. USA) by the enzymatic endpoint method, using Pars Azmoon kits (Tehran, Iran). Serum levels of LDL-C were calculated using Friedewald's formula (17), as follows:

LDL-C (in mg/dL) = TC (in mg/dL) - (TG (in mg/dL)/5) - HDL-C (in mg/dL).

In addition, we measured TC in the brain extract, which was obtained by homogenizing 100mg of brain tissue in 1 mL of 95% ethanol. The supernatant was collected after separating at 7000 × g at room temperature for 10 minutes. The pellet was repeatedly suspended in 1 mL of ethanol dichloromethane (Sigma, USA) (1: 1; v/v), sonicated (Ban Delin Sonoplus, Germany) at 40 W for 8 minutes, and then centrifuged as in previous conditions. All supernatant was pooled and air dried. The dried remnant was rehydrated by16μL of 95% ethanol and 484μl of phosphate-buffered saline (PBS) to fully solubilize the sterols content. Subsequently, the brain extract was subjected to TC analysis.

3.4. Western Blotting

We used an immunoblotting assay to measure the expression of the BACE1, NMDAε2 and CYP46A1 proteins, following the Santa Cruz manufacturer's instructions (http://www.scbt.com/protocols.html?protocol = western_immuno_blotting). Briefly, 100 mg of hippocampal tissue in 1 mL RIPA lysis buffer (sigma) containing 1 M Tris-HCl; PH 7.4, 25% Triton-x100, 5M NaCl, 10 % sodium deoxycholate, and 20% SDS with 1% protease inhibitor cocktail (Roche, USA) were minced using a homemade Dounce homogenizer. The homogenate was centrifuged (SW14R, Froilabo, France) for 20 minutes at 12000 × g at 4°C. The amount of protein in the supernatant was quantified using the Bradford assay with commercial reagents (Bio-Rad Laboratories, CA, USA) and spectrophotometric (Jenway 6305 spectrophotometer, Bibby Scientific Ltd, UK) measurements at 595 nm. Next, electrophoresis of the total proteins was carried out in 4% -10% SDS/polyacrylamide gel and 200v in 2 hours using Pars Azmoon apparatus (Mashhad, Iran). The proteins were then transferred to the polyvinylidene fluoride (PVDF) membrane (Bio Rad). Bovine serum albumin (BSA) (1%) in BPS plus 0.05% Tween 20 polybutylene succinate-co-butylene terephthalate (PBST) was used to cover the empty spaces on the membrane. Subsequently, the membrane was incubated overnight at 4°C with the rabbit polyclonal anti-BACE1 (1: 1000; abcam®, Catalog Number ab10716, Cambridge, MA, USA), anti-NMDAε2 (1:500; Santa Cruz Biotechnology Inc., Catalog Number sc-9057, Santa Cruz, CA, USA), or anti-CYP46A1 (1: 500; Novus Biologicals, Catalog Number NB400-140, Littleton, Colorado, USA). After being washed three times with PBST, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1: 5000; sc-2030Santa Cruz Biotechnology Inc., Sweden) for 2 hours at 4°C. The ECL-plus kit (GE Healthcare, USA) was used to visualize the protein bands. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression was applied as a loading control. Target to reference protein ratios were presented as a percentage of control.

3.5. Morris Water Maze (MWM)

We used the classic behavioral MWM test to investigate memory performance. A black circular maze (diameter 135 cm and height 70 cm), filled with up to 50 cm water (20 ± 2°C), was used in this experiment. Four landmarks, spaced along the wall of the pool, were designated north (N), south (S), west (W) and east (E) to create NE, SE, NW and SW quadrants.

One transparent platform (diameter 10 cm) was positioned in the target quadrant. All movements and times were monitored by a computer equipped with a camera and smart software (Panlab, Barcelona, Spain). The rats underwent two series of experiments: the hidden platform and the probe test. The experiment began with the hidden platform tests, with two sessions per day, followed by one day with the spatial probe test. In the hidden platform test, the rats were allowed to swim for up to 60 seconds. If the rats could not find the platform in 60 seconds, they were placed on it for 20 seconds. On the spatial probe test day, after removing the platform, the rats were allowed to swim for 60 seconds from a randomly selected zone. The time traveled in the target zone, the rate at which they crossed the platform, the ability to arrive at the platform in the hidden phase, the whole distance swum, and the time spent in the target quadrant were recorded by the software. The rats were also subjected to visual training to detect possible differences in motor control and visual discrimination.

3.6. Statistical Analysis

As our sample sizes were the same as those used in a previous paper, no statistical methods were used to determine the required sample sizes (18). All data were reported as mean ± SD. The mean values of variables and the difference of mean values between groups were evaluated by using the Mann-Whitney U Test, in which $p < 0.05$ and a confidence interval of 95% were considered as significant differences across the study. Correlations between variables were tested using the Spearman test.

4. Results

4.1. A Cholesterol-Rich Diet Has an Effect on Body Weight but Not on Brain Wet Weight

The rats were weighed on day 0 (the initial day), day 30 and day 60 of the experiment using digital scales (0.01 gram accuracy). No significant difference was observed

in initial body weight between the HD and ND groups (P = 0.85). The weight and exact amount of food consumed daily was measured (complete data not shown). The amount of food consumed and respective weight gains were significantly (P < 0.05) higher in the HD group than in the ND group. After adjusting for the food consumed, the weight gain remained significant (P< 0.05), with lower levels in the control group (The The brain wet weight was significantly higher (P < 0.05) in the HD group than in the ND group. After adjusting for weight control, the mean of the wet weight of brain did not show a significant change in the HD group versus the ND group (P = 0.114).

4.2. Diet Cholesterol Has an Effect on Plasma Lipid Parameters but Not on Brain Cholesterol Levels

We aimed to identify perturbations in the blood analyses of rats fed on a high cholesterol diet after 16 weeks treatment. As shown in Table 2, the levels of serum TC in the HD group were approximately three times higher than in the ND group, whereas the levels of serum LDL-C in the HD group were more than ten times higher than in the ND group (Table 2). Based on the results, a significant increase in TG levels was observed in the HD group when compared to the ND group (P< 0.05). A slight and non-significant decrease was also observed in HDL-C (P = 0 .36). As shown in Table 2, no significant change was observed in the TC brain levels, even after adjusting for weight as a controlling factor (P = 0.84).

4.3. Diet Cholesterol Leads to Irregularities in Memory Recall

The MWM examination was applied to test spatial learning and memory performance, which are indicators of hippocampal function. None of the rats in any of the groups had problems in learning to pass the visible platform (time latency, P = 0.90; traveled distance, P = 0.27). In addition, no significant difference was observed between the two groups with regard to time latency (P = 0.80) and distance traveled (P = 0.46) to find the hidden platform. However, there was a decrease in time swum in the target zone as a percentage of the total probe test time (P < 0.05) as well as in the distance swum in the target zone (P < 0.05) (Figure 1) for the HD group in comparison with the ND group.

4.4. Diet Cholesterol Up Regulated the Protein Expression of CYP46A1 and BACE1

The results showed that 16 weeks of treatment with high cholesterol could lead to a significant increase in the expression of CYP46A1 (P < 0.05) (Figure 2B) and BACE1 (P < 0.05) (Figure 2C) in the hippocampi of the HD group in comparison with the ND group. However, no significant change was observed in the expression of the NMDAR protein (P = 0.38) (Figure 2D).

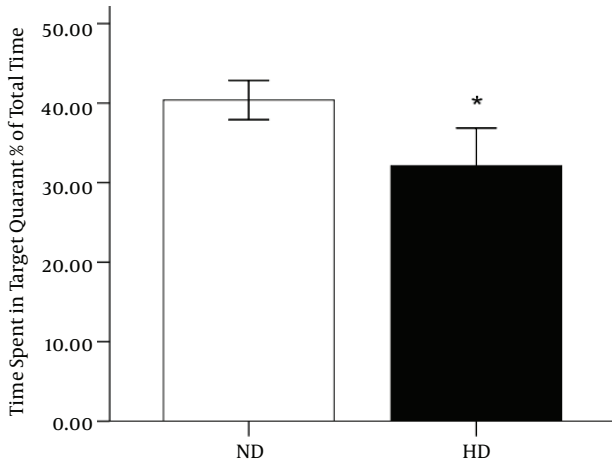


Figure 1. Decreased Time Spent in the Target Quadrant for High Cholesterol Diet (HD) vs. Normal Diet (ND) Rats at the End of the Experimental Period *P < 0.05

5. Discussion

To the best of our knowledge, this is the first time that any study has evaluated the effects of a high cholesterol diet over such a long period of time (4 months), which is approximately equal to 12 years in the human lifespan, while also evaluating cholesterol homeostasis in both the blood and brain, and measuring cognitional modifications by behavioral tests. In the current study, the most striking findings are that diet cholesterol increases body weight without any change in brain weight. It modifies plasma (19) protein expression as a cholesterol homeostasis-related protein (11) and BACE1 as a protein related to APP processing (12) in the hippocampi of Wistar rats, while also inducing irregularities in memory recall. Based on our data, body weight gain in the test group (hypercholestrolemic) rats was significantly higher than weight gain in the control rats fed with a normal diet. These findings confirm earlier reports on the use of a low cholesterol diet for controlling weight (20). High cholesterol resulted in down regulation of both peroxisome proliferator-activated receptors (PPAR)-α and PPAR-γ in the adipose tissue. Decreased PPAR expression in the adipose tissue (20) of hypercholesterolemic mice was related to a modified expression of the genes that explain decreased insulin sensitivity and glucose transport, elevated hypertriglyceridemia, inflammation, and increased oxidative stress. The association of insulin sensitivity (21), glucose transport (22), elevated hypertriglyceridemia (23),

Table 1. Body and Brain Wet Weight of the Normal Diet (ND) and High Cholesterol Diet (HD) Groups at the End of the Experimental Period[a]

	ND (n = 10)	HD (n = 10)
Food consumed daily, g/day	34.37± 5.01	43.93± 5.92[b]
Weight 0 (initial weight), g	249.01± 15.6	251.12± 11.63
Weight 60 (end point weight), g	321.02± 19.12	349.4±8.81[b]
Brain wet weight, g	1.95± .05	2.41± .11[b]

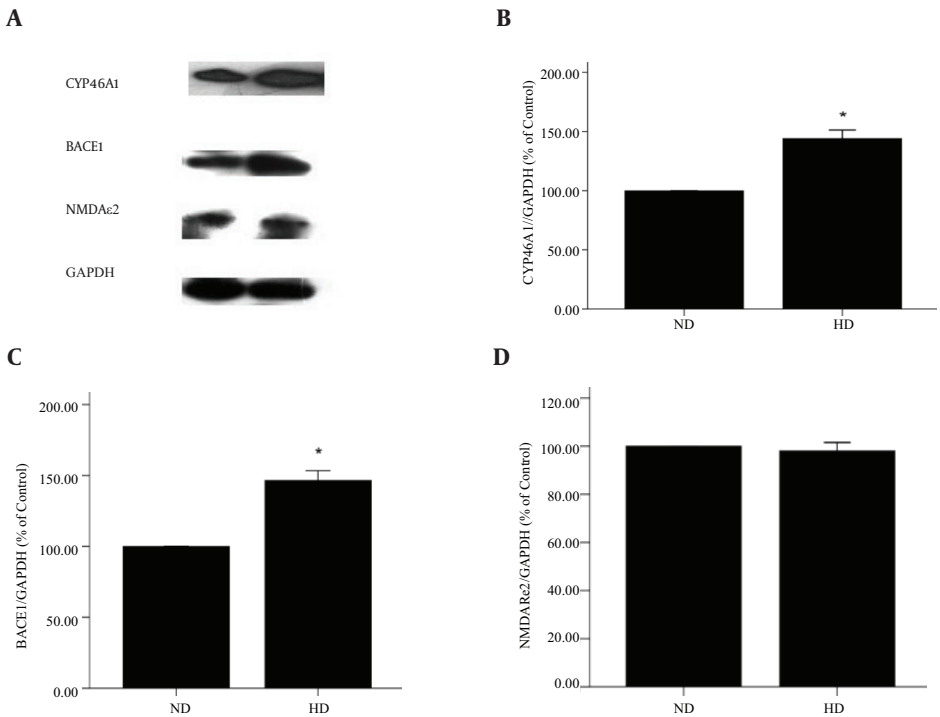
[a]Values are expressed as the mean ± SD, P < 0.05.
[b]P < 0.01 vs ND.

Table 2. Brain and Plasma Lipid Parameters of the Normal Diet (ND) and High-Cholesterol Diet (HD) Groups at the End of the Experimental Period[a]

	Serum TC, mg/dL	Brain TC, mg	LDL-C, mg/dL	HDL-C, mg/dL	TG, mg/dL
ND (n = 10)	67.89 ± 5.14	27.18 ± 1.47	15.20 ± 2.34	33.66 ± 2.90	50.12 ± 7.16
HD (n = 10)	229.35 ± 13.26[b]	28.58 ± 1.73	177.39 ± 10.38[b]	31.27 ± 4.69	65.41 ± 10.66[c]

[a]Values are expressed as the mean ± SD.
[b]P < 0.01 vs. ND.
[c]P < 0.05.

Figure 2. Overexpression of CYP46A1 and BACE1 in the Hippocampi of High Cholesterol diet Rats but not NMDAε2



A, immunoblotting of CYP46A1, BACE1, NMDAε2, and GAPDH in the ND and HD groups; B, quantitation of immunoblotting of CYP46A1; C, quantitation of immunoblotting of BACE1; and D, quantitation of immunoblotting of NMDAε2; values are shown as the mean ± SD of 6 animals in each group *P < 0.05.

inflammation (17, 24), and increased oxidative stress (25) with AD has already been established. In the current study, measurement of brain weight in the test group showed no change in brain wet weight, which supported findings in another study (11). The loss of brain mass is a clear morphologic change in neurodegenerative disease, particularly in

AD (26), but there is no clear evidence pointing to the effect of hypercholesterolemia on brain wet weight. As expected, analysis of the lipidic parameter for the HD group revealed a significant modification of the plasma lipidic profile, with an increase of 1000% in LDL-C, 400% in TC, and 130% in TG levels. Many epidemiological (27) and in vivo studies using wild type and transgenic animals such as mice (1, 28-30) have supported a strong association between the pathogenesis of AD and circulating LDL-C. Chen et al. have shown that increased levels of LDL-C can lead to disturbances in the endolysosome structure in neurons and, following APP internalization, can promote the accumulation of Aβ deposits (31).

In our experiment, despite an approximately four-fold increase in TC plasma levels, we observed no marked change in brain TC. These findings are in contrast to another study (14), which showed that cholesterol concentrations in both blood and brain were significantly higher in the HC group than in the control group. It should be noted that in that study 5% cholesterol was administered in comparison with the 2% cholesterol dose in our study. A severe increase in plasma cholesterol is often accompanied with elevated levels of oxidized LDL that increase BBB permeability and facilitate entrance of LDL to the brain parenchyma (28). On the other hand, our findings were supported by another study on hypercholestrolemic rats fed with 2% cholesterol (28). However, in that experiment, de novo cholesterol synthesis in the brain appeared to be up regulated, as indicated by higher absolute levels of lathosterol (a cholesterol precursor) compared to the normal control group. This part of their findings was confirmed in our study by increased expression of CYP46A1 as a key enzyme in the excretion of extra cholesterol from the brain toward the plasma (32). This result suggests that plasma cholesterol cannot directly increase brain cholesterol due to the BBB restriction in LDL transport into the postcapillary compartment of brain parenchyma. However, this does not mean that it cannot affect brain cholesterol homeostasis. As mentioned earlier, 24S-OHC produced by CYP46A1 (11) as a final brain cholesterol metabolite, flows from the BBB into the blood circulation (33). In contrast (12), 27(S)-hydroxycholesterol, another cholesterol metabolite in the blood circulation is taken up by the brain to a significant degree. This uptake is of the same amount as the flux of 24S-OHC in the opposite direction (11). Both oxysterols are potent modulators of cholesterol synthesis and ligands for liver X receptor (LXR) a and b, members of the nuclear receptor family of transcription factors, which play an important role in lipid homeostasis in the central nervous system. In the current study, we tried to find out whether hypercholesterolemia can affect the expression of CYP46A1in the hippocampi of rats and we observed a clear increase in its expression, indicative of disturbed cholesterol homeostasis in the hippocampus (10).

Disturbance in cholesterol homeostasis is believed to be involved in the pathogenesis of cognition-related disorders (34). The evaluation of the effect of 24S-OHC on cell viability using the assay showed that cell death happened in a concentration-dependent manner (33).

A polymorphism in the CYP46A1 gene, which causes an increase in 24S-OHC levels, has been reported to be associated with AD (35). In addition, 24S-OHC at physiological concentrations, suppresses APP trafficking through the improvement of the complex formation of APP with endoplasmic reticulum chaperones (3, 29). Based on this mechanism, 24S-OHC suppressed amyloid β production. In contrast, high concentrations of 24S-OHC induced apoptosis in the presence of caspase-8, with a form of programmed necrosis in the absence of caspase-8 (33). An epidemiological study has revealed that brain 24S-OHC decreases in the late stages of neurodegenerative diseases such as AD, vascular dementia (VD), and multiple sclerosis (MS), indicating the degeneration rate of active neurons, and the degree of cell death (33). To elucidate the effect of a high cholesterol diet on APP processing, we assayed the expression of BACE1 in the hippocampus homogenates of rats and found a significant increase in the hippocampal expression of BACE1. In support of our findings, Kuo et al. have also reported a significant increase in the mRNA expression of CYP46A1, BACE1 and 24S-OHC levels in the hippocampus and brain cortex (14). In another study, Ghribi et al. (36) demonstrated that feeding rabbits with 1% cholesterol for 7 months led to increased levels of BACE1. Up regulation of 24S-OHC has an indirect effect on BACE1 activities (37). A clear result of BACE1 up regulation is the high rate of APP processing and Aβ deposition (36).

Cholesterol is known as a negative regulator of NMDARs, which are positively involved in synaptic plasticity and cognition (38). We therefore assayed the NMDAR expression in hippocampus tissue using anti NMDAε2 by immunoblotting assay. Not only did we observe no change in the content of this protein in the hippocampus, our data also showed overexpression of CYP46A1. Its production is a direct, positive and very potent allosteric modulator of NMDARs (15). As this finding contradicts our hypothesis, additional research should be carried out, such as electrophysiological recordings and measurements of 24S-OHC levels in the brain. We were unable to do this in the current experiment due to time limitations. On the other hand, we found a statistically significant difference in the probe test part of the MWM test. However, this may not be enough to conclude that the HD group had a cognitive deficit. Similar and more conclusive results confirmed our hypothesis in a study on both LDL receptor (LDLr) rodents and C57BL/6

mice that were fed with a high-cholesterol diet (1). Many studies have demonstrated that the LDLr mice exhibit cognitive impairment and irregularities in the MWM test (1, 36). In addition, recent studies have demonstrated an association between the severity of cognitive impairment and elevated levels of cholesterol (29). It seems that other behavioral tests of learning and memory such as fear conditioning, passive avoidance, or object location can better clarify the effects of cholesterol administration on cognitive behaviors.

5.1. Conclusion

The findings, including irregularities in memory recall and increased expression of BACE1 and CYP46A1, suggest that a distortion in cognition can be at least partially mediated by disturbances in brain cholesterol homeostasis. This basic knowledge identifies the CYP46A1 gene as a new drug target for preventing the cognition-related effects of cholesterol.

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Footnote

Authors' Contribution: Mitra Nikasa and Soltanali Mahboob contributed to the design of the study. Hosnieh Rajavand, Fatemeh Afshari and Pouran Karimi contributed to the analysis and interpretation of data. Mahdi Jafarlou contributed to the drafting of the article.

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