SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 17: 1235-1240, 2018

High-cholesterol diet results in elevated amyloid-β and oxysterols in rats

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Received May 31, 2016; Accepted March 30, 2017

DOI: 10.3892/mmr.2017.8003

Abstract. The aim of the present study was to investigate the effects of diet cholesterol on oxysterol levels and amyloid-β (Aβ) production in the peripheral blood and the brains of Sprague-Dawley (SD) rats. SD rats were randomly divided into five groups and fed 0.015, 0.05, 0.2, 0.5 and 1.6% cholesterol-containing diets for 8 weeks. The effect of the different diets on the levels of cholesterol, oxysterols [including 27-hydroxycholesterol (OHC), 24S-OHC, 7α-OHC and 7β-OHC], and the Aβ1-40 and Aβ1-42 peptides were examined in the plasma and the brain of the rats. The results demonstrated that diet cholesterol increased the levels of plasma cholesterol in a dose-dependent manner. The plasma levels of 27-OHC, 7α-OHC and 7β-OHC significantly increased in the 0.5 and 1.6% cholesterol diet groups and the brain levels of 27-OHC significantly increased in the 1.6% cholesterol diet group. Increased concentration of cholesterol in the diet had no significant influence on plasma and brain levels of 24S-OHC in the rats. In addition, Aβ1-40 and Aβ1-42 levels in plasma and brain were significantly elevated following administration of 0.5 and 1.6% diet cholesterol. The present study revealed that high diet cholesterol contributed to increased level of oxysterols, especially 27-OHC, in the peripheral blood and the brain, which may be the link between increased peripheral cholesterol and brain Aβ production.

Introduction

Hypercholesterolemia is implicated as a risk factor for various health problems (1). To date, the association between cholesterol and dementia, cognitive decline or impairment is not fully understood (2). In the last decade, there is growing evidence that hypercholesterolemia and dietary cholesterol are linked to an increased risk of cognitive decline or impairment that do not meet diagnostic criteria and dementias with different etiologies (3,4). Studies examining the potential mechanisms linking cholesterol to neurotoxicity have identified amyloidogenic proteins to be involved in neurodegenerative diseases (5-7). However, clinical studies using statins to lower cholesterol for therapeutic management of neurodegeneration revealed contradictory results (8). In addition, the role of cholesterol on the regulation of amyloid-β (Aβ) generation and aggregation remains elusive. To gain insight into the association between cholesterol and cognitive decline, the present study focused on the oxidized derivatives of cholesterol, including 27-hydroxycholesterol (OHC), 24S-OHC, 7α-OHC and 7β-OHC. It has been reported that elevated oxysterols in the plasma have cytotoxic and pro-apoptotic effects on neurons (9,10). Furthermore, oxysterols can pass through the blood brain barrier into the central nervous system (11) and increase Aβ production via upregulation of the amyloidogenic pathway (12). A previous study from our group has also demonstrated that 27-OHC serves a negative role on learning and memory abilities (13). However, it remains unknown whether cholesterol affects the learning and memory ability as well as related biomarkers via oxysterols. The aim of the present study was, therefore, to investigate the effects of dietary cholesterol on plasma levels of cholesterol and oxysterols and their potential effect on Aβ production in rats, to further elucidate the role of dietary and blood cholesterol in learning and memory abilities and to provide scientific evidence and new ideas for therapeutic and preventive strategies of cognitive disorders.

Materials and methods

Animals and experimental design. A total of 35 10-month old male Sprague-Dawley rats (SPF class, 450-600 g) were provided by the Academy of Military Medical Sciences (Beijing, China) and housed 1 per cage in a room of controlled illumination (12-h light/dark cycle), humidity (30-50%), and temperature (18-22°C). A standard rodent diet and water were accessed *ad libitum*. Following 1 week's acclimation, the rats were randomly divided into 5 groups (n=7 in each group) which were respectively fed 0.015 (control diet group), 0.05, 0.2, 0.5 and 1.6% cholesterol-containing diets for 8 weeks.

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Key words: diet cholesterol, oxysterols, 27-hydroxycholesterol, amyloid β 1-40, amyloid β 1-42, Alzheimer's disease

Table I. Ingredients of the experimental diets.

	Dietary cholesterol				
Ingredient (g)	0.02%	0.05%	0.20%	0.50%	1.60%
Sucrose	530	530	530	530	530
Skimmed milk	40	40	40	40	40
Casein	230	230	230	230	230
Cystine	2	2	2	2	2
Lard	92	92	92	92	92
Nut oils	8	8	8	8	8
Salt-mixture	50	50	50	50	50
Vitamin mix	2	2	2	2	2
Cellulose	23	23	23	23	23
Yeast	23	23	23	23	23
Cholesterol	0.08	0.43	1.93	4.93	15.93
Total	1,000.08	1,000.43	1,001.93	1,004.93	1,015.93

The detailed composition of the diets is listed in Table I. Experiments were designed and conducted in accordance with the Chinese Committee of Experimental Animal Supervision and the guidelines of Animal Ethics Committee of Capital Medical University (Beijing, China).

Plasma and tissue collection. Body weight was measured and tail vein blood was collected once every two weeks to evaluate levels of cholesterol, Aβ1-40, Aβ1-42 and oxysterols (27-OHC, 24S-OHC, 7α-OHC and 7β-OHC). Following 8-week dietary intervention and 24 h of fasting from the last feeding, all of the rats were weighed, deeply anesthetized with 5% chloral hydrate (400 mg/kg) and dissected. Blood samples were collected and fresh tissue, including brain, was removed, weighed and subsequently frozen at -80˚C until use. Serum and plasma were obtained by centrifugation of blood samples at 3,000 x g for 10 min at 4˚C and stored at -80˚C until use.

Biochemical analysis of blood and brain samples. Cholesterol, Aβ1-40 and Aβ1-42 levels in blood and brain samples were determined using commercial kits (Kexin Biotech Co., Ltd. Shanghai, China) on a Hitachi 7250 automatic clinical analyzer (Hitachi, Ltd., Tokyo, Japan), following the manufacturers' instructions. All samples were analyzed in duplicate.

Measurement of oxysterols in blood and brain samples. Plasma and brain levels of oxysterols were measured using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) as described by Burkard *et al* (14), with slight modifications. The C-7 position of cholesterol is liable to autoxidize. In order to prevent potential autoxidation during sample preparation, 50 μg butylated hydroxytoluene (BHT) was added per ml plasma. During the collection and preparation of blood samples, standard procedures were conducted to avoid repeated freeze-thaw cycles to minimize the impact of the plasma concentration of 7α-OHC and 7β-OHC. Briefly, 0.1 ml of plasma sample was transferred to a screw-capped vial. Then, 100 ng 19-OHC and 1.5 ml of 1 M ethanolic sodium hydroxide were added to the vial, serving as internal standard and alkaline hydrolysis, respectively. Alkaline hydrolysis was performed in a water bath at 50˚C for 2 h. Phosphoric acid (50%) and 1 ml of phosphate buffer were added to the samples for pH adjustment to 7. The supernatant was harvested following centrifugation at 1,000 x g for 5 min at 4˚C and then applied to the C18 cartridges for solid-phase extraction. The eluted substances were dried at 30˚C and dissolved in 100 μl of methanol for future testing. Total lipid was extracted from approximately 20-70 mg of the brain on ice by homogenation in 2 ml of ice-cold chloroform:methanol (2:1 v/v), containing 0.005% (v/v) BHT as an antioxidant and 19-OHC as an internal standard. The extract was ultrasonicated for 15 min at room temperature, centrifuged at 5,000 x g for 5 min at 4˚C and the supernatant collected. The supernatant was evaporated under nitrogen and the residue was redissolved in 1 ml of

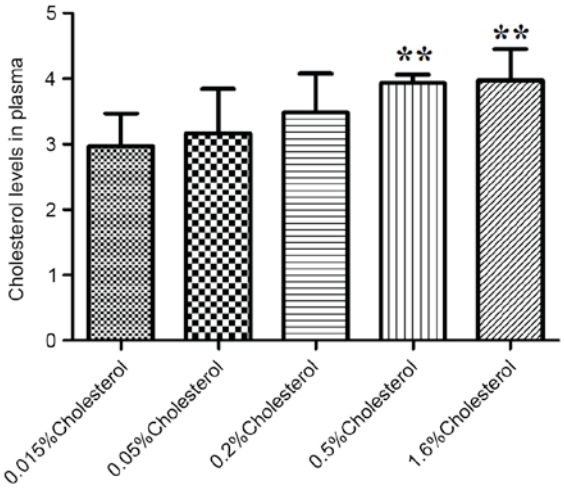

Figure 1. Levels of cholesterol in the plasma of rats following 8 weeks of 0.015, 0.05, 0.2, 0.5 and 1.6% cholesterol-containing diets. Data are presented as mean ± standard deviation. **P<0.01 compared with the 0.015% group.

Table II. Body weight and plasma cholesterol levels in rats pre- and post-intervention.

	Dietary cholesterol					
Variable	0.015% (n=7)	0.05% (n=7)	0.20% (n=7)	0.50% (n=7)	1.60% (n=7)	P-value
Pre-intervention						
Body Weight (g)	629.0±45.9	630.3±34.3	631.7±48.8	606.9±49.8	614.9±43.9	0.79
Cholesterol (mmol/l)	3.09±0.45	3.20±0.69	3.21±0.45	3.10±0.49	3.13±0.22	0.98
Post-intervention						
Body Weight (g)	795.1±45.4	772.9±59.7	726.3±51.9	728.0±44.5	734.7±97.4	0.18
Cholesterol (mmol/l)	2.96±0.51	3.17±0.68	3.48±0.59	3.93±0.14	3.97±0.48	<0.01

Data are presented as mean ± standard deviation.

Table III. Plasma and brain levels of oxysterols in rats pre- and post-intervention.

	Dietary cholesterol					
Variable	0.015% (n=7)	0.05% (n=7)	0.20% (n=7)	0.50% (n=7)	1.60% (n=7)	P-value
Pre-intervention						
Plasma						
27-OHC (ng/ml)	80.6±42.6	73.7±38.3	73.2±44.8	66.0±30.5	59.8±30.7	0.87
24S-OHC (ng/ml)	48.4±25.5	44.2±23.0	43.9±26.9	39.6±18.3	35.9±18.4	0.86
7β-OHC (ng/ml)	63.6±20.0	59.1±21.8	43.4±15.6	60.0±31.3	43.6±16.9	0.27
7α-OHC (ng/ml	53.9±12.3	44.4±6.7	52.5±19.0	52.7±18.8	47.4±12.2	0.70
Post-intervention						
Plasma						
27-OHC (ng/ml)	59.8±20.0	65.9±31.7	82.7±38.2	99.7±26.0	118.2±35.1	<0.01
24S-OHC (ng/ml)	55.5±18.4	51.4±10.7	51.9±8.6	59.1±14.4	60.6±7.6	0.56
7β-OHC (ng/ml)	78.7±21.2	85.6±54.9	75.0±19.7	233.4±144.8	195.6±96.5	<0.01
7α-OHC (ng/ml)	108.9±35.4	120.5±91.6	102.8±32.9	366.9±241.3	303.8±160.8	<0.01
Brain						
27-OHC (ng/ml)	0.96±0.44	0.78±0.15	0.76±0.08	1.31±0.39	1.37±0.42	<0.01
24S-OHC (ng/ml)	14.28±1.54	15.28±3.37	15.23±1.87	16.11±1.90	14.43±3.20	0.66

Data are presented as mean ± standard deviation. OHC, hydroxycholesterol.

hydrolyzate (10% KOH and methanol) overnight. The hydrolyzate was extracted with 0.5 ml of water and 2 ml of ether and then washed twice with distilled water. The extracts were taken to dryness under nitrogen at room temperature and then 1 ml of methanol:water (9:1 v/v) was added. The mixture was centrifuged at 2,400 x g at 4˚C for 15 min, and the supernatant was collected (15). A total of 40 μl sample was injected into the HPLC-MS system. HPLC with an Agilent G1312B HPLC pump and an Agilent C18 column (0.35 μm bead size; 4.6x250 mm; Agilent Technologies, Inc., Santa Clara, CA, USA) was used for the measurement of oxysterols. For the first 10 min, the mobile phase consisted of water:acetonitrile (90:10 v/v) with a corresponding flow rate of 0.25 ml/min. The eluents were then linearly changed in a gradient system to water:acetonitrile (10:90 v/v) within 5 min. Afterwards, the eluents were again changed to the previous ratio for 2 min. Quantification of oxysterols was performed using the multiple reaction monitoring mode. The ionization mode was positive atmospheric pressure chemical ionization. The gas temperature and the nebulizer were maintained at 325˚C and 4,000 pounds per square inch (psi) respectively. The discharge current was fixed at 5 μA, and the capillary voltage was set at 4,000 V. The vaporizer temperature was held at 450˚C, and the sheath gas pressure was maintained at 35 psi.

Statistical analysis. Results were expressed as means ± standard deviation. One-way analysis of variance followed by the least significance difference post hoc test was used to evaluate the significance of differences between groups. P<0.05 was considered to indicate a statistically significant difference.

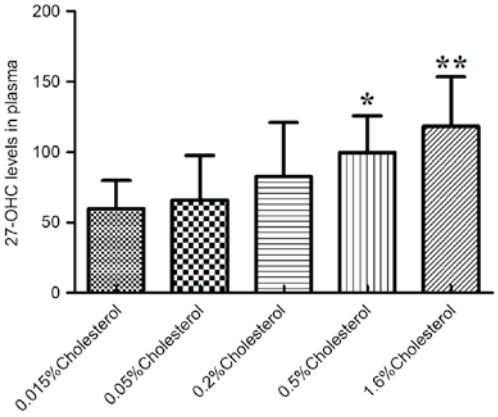

Figure 2. Levels of 27-OHC in the plasma of rats following 8 weeks of 0.015, 0.05, 0.2, 0.5 and 1.6% cholesterol-containing diets. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01 compared with the 0.015% group. OHC, hydroxycholesterol.

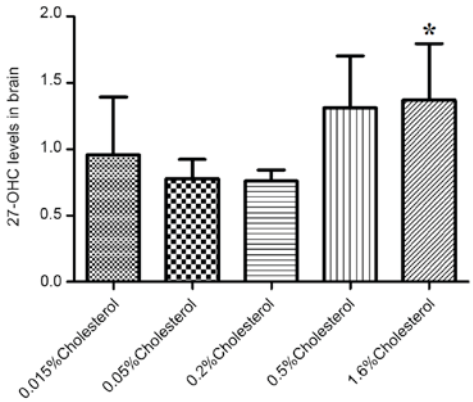

Figure 3. Levels of 27-OHC in the brain of rats following 8 weeks of 0.015, 0.05, 0.2, 0.5 and 1.6% cholesterol-containing diets. Data are presented as mean ± standard deviation. *P<0.05 compared with the 0.015% group. OHC, hydroxycholesterol.

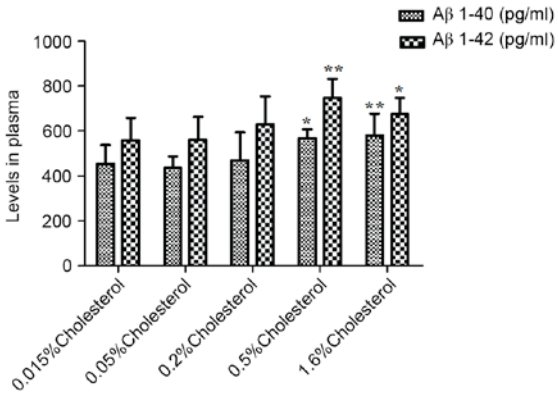

Figure 4. Levels of Aβ1-40 and Aβ1-42 in the plasma of rats following 8 weeks of 0.015, 0.05, 0.2, 0.5 and 1.6% cholesterol-containing diets. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01 compared with the 0.015% group. Aβ, amyloid-β.

Results

Total cholesterol and body weight. Body weight and plasma levels of cholesterol pre- and post-intervention are summarized in Table II. Dietary intervention for 8 weeks induced a diet-dependent increase in plasma cholesterol levels (P<0.01; Table II), but no significant change was observed in body weight (P>0.05; Table II). Plasma cholesterol levels were significantly elevated following 8 weeks of dietary intervention in the 0.5 and 1.6% cholesterol-containing diet groups compared with the control diet group (Fig. 1).

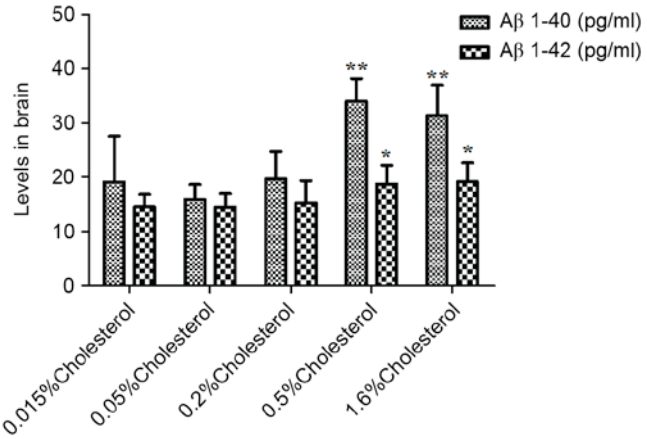

Figure 5. Levels of Aβ1-40 and Aβ1-42 in the brain of rats following 8 weeks of 0.015, 0.05, 0.2, 0.5 and 1.6% cholesterol-containing diets. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01 compared with the 0.015% group. Aβ, amyloid-β.

Oxysterol levels in plasma and brain. No significant differences were observed in oxysterol levels among the five diet groups prior to intervention, as illustrated in Table III. Following 8 weeks of diet intervention, analysis of oxysterol levels in the plasma of rats revealed significant differences in the levels of 7α-OHC (P<0.01; Table III), 7β-OHC (P<0.01; Table III) and 27-OHC (P<0.01; Table III), but no significant changes in 24S-OHC levels (P=0.56; Table III). At week 8 post-intervention, the plasma levels of 27-OHC in the diet groups containing 0.5 and 1.6% cholesterol were significantly elevated compared with the control diet group (Fig. 2). Similarly, the brain levels of 27-OHC in the 1.6% cholesterol-containing diet group were significantly elevated compared with the control diet group (Fig. 3).

Aβ levels in plasma and brain. As illustrated in Table IV, the plasma levels of peptides Aβ1-40 and Aβ1-40 in the five groups were not significantly different prior to dietary intervention. However, at week 8 post-intervention, both plasma (Table IV and Fig. 4) and brain (Table IV and Fig. 5) levels were significantly elevated in the 0.5 and 1.6% cholesterol-containing diet groups compared with the control group.

Discussion

The main finding of the present study is that the levels of cholesterol and 27-OHC in the peripheral blood were increased by dietary cholesterol intervention in a dose-dependent manner. In addition, 7α-OHC and 7β-OHC levels in the blood and 27-OHC in the brain were elevated following high-cholesterol diets compared with control diet, while 24S-OHC levels were not affected by dietary cholesterol in SD rats. Furthermore,

Table IV. Plasma and brain levels of Ab1-40 and Ab1-42 in rats pre- and post-intervention.

	Dietary cholesterol					
Variable	0.015% (n=7)	0.05% (n=7)	0.20% (n=7)	0.50% (n=7)	1.60% (n=7)	P-value
Pre-intervention						
Plasma						
Aβ1-40 (pg/ml)	449.9±67.0	502.1±51.7	480.1±41.7	467.9±38.0	461.5±44.8	0.36
Aβ1-42 (pg/ml)	431.9±49.2	448.5±52.5	415.9±58.7	412.4±46.3	412.6±59.5	0.66
Post-intervention						
Plasma						
Aβ1-40 (pg/ml)	455.0±80.5	434.6±52.2	469.1±123.6	565.9±40.9	579.8±96.9	<0.01
Aβ1-42 (pg/ml)	558.9±99.2	560.4±101.7	629.0±123.5	746.9±84.2	674.9±72.5	<0.01
Brain						
Aβ1-40 (pg/ml)	19.06±8.53	15.90±2.69	19.65±5.06	34.01±4.18	31.36±5.58	<0.01
Aβ1-42 (pg/ml)	14.49±2.37	14.38±2.58	15.29±4.06	18.69±3.55	19.13±3.49	0.02

Data are presented as mean ± standard deviation. Aβ, amyloid-β.

blood Aβ1-40 and Aβ1-42 levels, commonly involved in Alzheimer's disease (AD) pathogenesis, were also upregulated as a result of accumulated cholesterol. Finally, high-cholesterol diet resulted in elevated blood 27-OHC levels as well as elevated Aβ1-40 and Aβ1-42 levels, suggesting that 27-OHC may be linked to Aβ accumulation in the brain.

A previous study has also investigated the effect of dietary cholesterol on plasma cholesterol levels, and reported a dose-dependent increase of plasma cholesterol concentrations following 6 months of high cholesterol-containing diet (15). However, the effect of cholesterol intake on production and distribution of oxysterols remains unknown and the present study for the first time investigated these effects. 27-OHC is catalyzed by the cytochrome P450 family 27 subfamily A member 1 (CYP27A1) enzyme, which is expressed in the liver (16). Unlike 27-OHC, 7β-OHC is generated by non-enzymatic oxidation, whereas 7α-OHC is generated by both non-enzymatic and enzymatic oxidation, which is catalyzed by cytochrome P450 family 7 subfamily A member 1 (CYP7A1). It is hypothesized that increased cholesterol levels contribute to 27-OHC, 7α-OHC and 7β-OHC production (17). 24S-OHC is converted from cholesterol via the cytochrome P450 family 46 subfamily A member 1 (CYP46A1) enzyme, which is primarily expressed in neurons (18). Cholesterol in the central nervous system (CNS) is not directly influenced by cholesterol in the peripheral blood. Consequently, 24S-OHC levels in brain and blood are less likely affected by dietary cholesterol, as demonstrated in the present results.

There are accumulating studies regarding the association of cholesterol and Aβ production and aggregation with controversial results. Some studies from several animal species suggest that Aβ accumulation in the brain is promoted by high-cholesterol diet (19). By contrast, another study reported that Aβ1-42 levels are negatively correlated with plasma cholesterol in APP/PS1 transgenic mice receiving a cholesterol diet (20). The age of the animals examined is considered as the main cause of these controversies, because cholesterol may serve different roles during the development and maturation of the brain. Therefore, in the present study, 10-month-old SD rats instead of young rats were selected for experiments, in order to avoid confounding effects of cholesterol on brain development. The present results demonstrated that Aβ1-40 and Aβ1-42 levels, in both plasma and brain, were significantly affected by a cholesterol-rich diet in rats.

Cholesterol cannot freely cross into the brain, owing to the impermeability of the blood-brain barrier (BBB). Subsequently, brain cholesterol metabolism is separated from the peripheral metabolism (21). The question as to how peripheral cholesterol influences Aβ generation and accumulation in the brain remains to be answered. A previous study from our group focused on the impact of cholesterol metabolism on the rats' brains and revealed that 27-OHC was involved in learning and memory disorders (13). Of note, the amyloidogenic pathway of amyloid precursor protein processing is upregulated in the human neuroblastoma cell line SH-SY5Y following treatment with 27-OHC (12). Based on the ability of 27-OHC to cross the BBB, the present results indicated that accumulating 27-OHC in the peripheral blood, induced by cholesterol-enriched diets, may contribute to increased 27-OHC and Aβ generation in the CNS, and may subsequently be involved in the pathogenesis of AD or cognitive decline.

In conclusion, the present study suggested that cholesterol-enriched diets induced increased Aβ production in the plasma and the brain and that this effect may be mediated via oxysterols, especially 27-OHC. The present study indicated that it may be possible to prevent the generation of Aβ by targeting 27-OHC. Deciphering the molecular mechanism involving oxysterols may lead to novel therapeutic strategies for neurodegenerative diseases in the future.

Acknowledgements

The present study was supported by the State Key Program of the National Natural Science Foundation of China (grant no. 81330065).

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