Investigation into the Properties of L-5-Methyltetrahydrofolate and Seal Oil as a Potential Atherosclerosis Intervention in Rats

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(Received August 21, 2021)

Summary Atherosclerosis is a chronic inflammatory disease that leads to tissue ischemia. As the biologically active form of folic acid, L-5-methyltetrahydrofolate (L-5-MTHF) can improve endothelial function. And seal oil plays a beneficial role in the progression of atherosclerosis. The study aims to evaluate beneficial effects of L-5-MTHF alone or in combination with seal oil on atherosclerosis. Seventy-two male Wistar rats were randomly divided into six groups: control (normal diet), atherosclerosis (high-fat diet), folic acid (high-fat + 3 mg/kg folic acid), low-dose L-5-MTHF (high-fat + 3 mg/kg L-5-MTHF), low-dose L-5-MTHF + seal oil (high-fat + 3 mg/kg L-5-MTHF + 0.5 g/kg seal oil), high-dose L-5-MTHF (high-fat + 10 mg/kg L-5-MTHF). After 13 wk, rats were sacrificed. Rats exhibiting atherosclerosis had dyslipidemia and serious aortic lesions. Supplementation with low-dose L-5-MTHF + seal oil or use of high-dose L-5-MTHF increased serum folate concentrations, decreased homocysteine levels, improved the serum lipid profile, up-regulated expression of NO and NOS, enhancement of the antioxidant properties of GSH-Px activity and reduction in the concentration of MDA, levels of Olr1 and RelA mRNA decreased in aortic tissues, and expression of inflammatory factors, TNF-α, IL-6, IL-1β and endothelial cell injury factors ET-1 and sICAM-1, were also down-regulated. In addition, HD-L-5-MTHF increased the antioxidant activity of serum SOD. We conclude that L-5-MTHF has obvious anti-inflammatory and antioxidant effects on diseased blood vessels. The intervention of L-5-MTHF alone or in combination with seal oil can improve atherosclerosis in rats and reduce the occurrence of aortic lesions. The anti-atherosclerotic mechanism may be related to down-regulation of Olr1 and RelA expression.

Key Words L-5-MTHF, seal oil, atherosclerosis, inflammation, oxidative stress

Atherosclerosis (AS) is a chronic inflammatory vascular disease in which the affected arteries begin to develop from the intima. It is the main cause of coronary heart disease, cerebral infarction and peripheral vascular disease resulting in major global mortality (1). Currently, approximately 7.2 million people die each year and the prevalence is expected to increase by 18% by 2030 (2). It is known that the pathogenesis of AS is related to vascular endothelial dysfunction, oxidative stress and chronic inflammation (3). Clinical approaches to AS include the use of statins and beta blockers but such treatments may have toxic side-effects on liver and muscle (4). Thus, there is an urgent need to identify further nutritional and drug interventions for AS treatment.

Many studies have established that dietary supplementation with folic acid (FA) is beneficial in AS prevention, and its effects include enhancement of antioxidiant capacity, improvement of lipid disorders and reduction of serum homocysteine (Hcy) levels (5). Epidemiological studies have indicated that hyperhomocysteinemia (HHcy) is an independent risk factor for AS (6). However, FA, itself, lacks coenzyme activity and must be converted into the biologically active form of L-5-methyltetrahydrofolate (L-5-MTHF) by dihydrofolate reductase (DHFR) and methylenetetrahydrofolate reductase (MTHFR) (7). The L-5-MTHF product is stable in the blood and able to cross the blood-brain barrier. During catalysis by methionine synthetic reductase (MTRR), the L-5-MTHF molecule assists the metabolism of Hcy through methylation and keeps Hcy at low levels. Statistical analyses indicate that 26% of the Chinese population have disorders of FA metabolism due to mutations in the MTHFR and MTRR genes (8), rendering them unable to directly use FA. Furthermore, excessive FA has been shown to saturate DHFR, which normally reduces FA, resulting in the presence of potentially harmful levels of unmetabolized FA in circulation (9). However, L-5-MTHF may have advantages over FA as a dietary supplement (10), it is well absorbed even when gastrointestinal pH changes, its bioavailability is unaffected by metabolic deficiencies and the potential negative effects of unmetabolized FA are avoided (11). Moreover, FA fortification can mask the hematological indicators of vitamin B12 deficiency, an effect which is unlikely to be
reproduced by L-5-MTHF (12). To date, L-5-MTHF has only been approved as a “nutrition fortifier” in China and there is an urgent need for intervention studies to evaluate any impact on AS in order to boost the development of further formula foods for medicinal purposes.

Seal oil is a rich source of omega-3 polyunsaturated fatty acids (ω-3 PUFAs), such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). These are all essential fatty acids which cannot be synthesized de novo by the body itself. High dietary intake of ω-3 PUFAs in some economically developed countries, such as Denmark, has been associated with very low rates of cardiovascular disease (13), and Seal oil has been found to play a beneficial role in the progression of AS (14). Combination interventions have been widely applied to the treatment of chronic diseases. Therefore, in addition to exploring the endothelium coenzyme factor, L-5-MTHF, this study also investigated whether combining L-5-MTHF with Seal oil would prove to be more effective in ameliorating AS in rats. To approach these questions, serum lipid profiles, inflammatory mediators, oxidative stress parameters, endothelial cytokines, aortic tissue lesions, oxidized low-density lipoprotein receptor 1 (Olr1) and the RelA subunit of NF-kappaB factor mRNA levels were measured and evaluated. Possible intervention mechanisms were explored. The current study aims to identify new lead compounds for early intervention in AS.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 150–180 g were provided by Spiff Biotechnology Co., Ltd. Laboratory Animal Center (Beijing, China, SCXK2019-0010) and kept on a 12-h light/12-h dark cycle under standard conditions (60±10% humidity and temperature of 22±2°C). The study protocols were approved by the Institutional Animal Care and Use Committee at Shandong University (LL20200802, China) and followed the guidelines.

Model preparation and group administration. Seventy-two healthy male Wistar rats of 150–180 g were fed for 7 d to allow adaptation and then randomly divided into 6 groups of 12 using spss21.0 software: control, atherosclerosis (AS), folic acid (FA), low-dose L-5-methyltetrahydrofolate (LD-L-5-MTHF), low-dose L-5-methyltetrahydrofolate+Seal oil (LD-L-5-MTHF+Seal oil) and high-dose L-5-methyltetrahydrofolate (HD-L-5-MTHF). FA and L-5-MTHF were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China) and Seal oil from Four Generations, Yantai liuzitang International Trade Co., Ltd. (Shandong, China). Ordinary laboratory chow and high-fat chow were purchased from Spiff Biotechnology Co., Ltd.

To establish the model of atherosclerosis, all rats except the control group were given vitamin D2 (Jiangxi Gannan Haixin Pharmaceutical Co., Ltd., batch number: 200311801) at a dose of 300,000 IU/(kg/d) by gavage for 4 consecutive days and were given a 20 g/d high-fat diet (1% cholesterol, 0.5% sodium cholate, 5% sugar, 10% lard, 0.2% propylthiouracil, 5% egg yolk powder, 78.3% ordinary chow) with water ad libitum. The control group was given an equal volume of normal saline by gavage and fed with ordinary chow and water ad libitum. The atherosclerotic regime was maintained for 7 wk. From the 8th week, the treatment conditions for each group were modified as follows: FA received 3 mg/kg/d folic acid; LD-L-5-MTHF received 3 mg/kg/d L-5-MTHF; LD-L-5-MTHF+Seal oil received 3 mg/kg/d L-5-MTHF and 0.5 g/kg/d Seal oil; HD-L-5-MTHF received 10 mg/kg/d L-5-MTHF. Control and AS groups received the same volume of saline. All treatments were given by intragastric administration. Rats otherwise had access to a normal diet with water ad libitum until the end of 13 wk.

Sample collection. After 13 wk, rats were fasted overnight (12 h) before intraperitoneal anesthesia with 10% chloral hydrate. Two rats were randomly selected from each group for cardiac perfusion with PBS solution and 4% paraformaldehyde solution. The intact aorta was separated and immediately fixed with 4% paraformaldehyde for subsequent hematoxylin-eosin (HE) staining. Pathological changes to the aorta were observed under a light microscope. Blood was collected from the abdominal aorta of the remaining rats in each group, centrifuged at 3,500 rpm for 15 min to separate the serum and stored at −20°C. The aorta was stripped and stored in liquid nitrogen for testing of Olr1 and RelA mRNA expression.

Assessment of serum folate and homocysteine (Hcy). Serum folate and Hcy levels were estimated by enzyme-linked immunocompetition using ELISA kits (Quanzhou Ruixin Biotechnology Co., Ltd., Quanzhou, China) according to the manufacturer’s protocols. Absorbance was measured at 450 nm and a standard curve was established by four-parameter logistic curve fitting (4-pl) to calculate the sample concentration. The detection limits for the folate and Hcy kits were 1.5–24 nmol/L and 1–16 μmol/L, respectively.

Assessment of serum lipid profile. Serum total cholesterol (TC) and triglyceride (TG) levels were measured using the glycerol-3-phosphate oxidase (GPO)-phenolaminophenazone (PAP) method. High-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All measurements were made by enzyme-labeled instrument (Tecan Infinite 200pro, Switzerland).

Assays of serum nitric oxide (NO) and nitric oxide synthase (NOS). Serum NO and NOS levels were determined using kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s instructions. NO assay: NO is metabolized to NO$_2^-$ and NO$_3^-$ in vivo. Nitrate reductase was used to reduce NO$_3^-$ to NO$_2^-$ and the concentration determined by color development at 550 nm. NOS assay: NOS catalyzes the reaction of L-arginine (L-Arg) with molecular oxygen to form NO which reacts with nucleophilic substances to form colored compounds. The absorbance is measured at 530 nm allowing calculation of NOS activity.
Determination of serum oxidative stress indices. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) activities were assayed using kits (Nanjing Jiancheng Bioengineering Institute). Reagent was added to 20 μL of a 20-fold dilution of serum before measurement of absorbance at 450 nm and calculation of SOD activity. GSH-Px activity was measured at 405 nm and a 5-fold dilution of serum. MDA was degraded by lipid peroxide with an absorption peak at 532 nm.

Evaluation of inflammatory mediators and endothelial factors. Serum levels of the inflammatory mediators, tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), and endothelial factors, endothelin-1 (ET-1) and soluble intercellular adhesion molecule 1 (sICAM-1) were estimated using ELISA kits (Shanghai Jianglai Industrial Limited ByShare Ltd., Shanghai, China) according to the manufacturer’s protocols. Briefly, the serum samples were removed from −80 °C storage and allowed to thaw naturally at 4 °C for 30 min. The supernatant was centrifuged at 3,000 rpm at 4 °C for 5 min. A 5-fold dilution of the serum was added to the well before chromogenic agent and termination solution. Absorbance was measured at 450 nm.

Histopathological evaluation of thoracic aorta. Sections of thoracic aorta were fixed in 4% paraformaldehyde solution at room temperature for 48 h and then cut longitudinally into 3 mm pieces before dehydration and embedding. Paraffin embedded tissue was cut into 5 μm slices by paraffin microtome (Leica Company, Germany, RM2135), dried for 2 h and stained with hematoxylin-eosin staining (HE). Histology was evaluated by inspection with an optical microscope (Olympus Company, Japan, BX53F).

Measurement of Olr1 and RelA mRNA by RT-qPCR. Total RNA was extracted from 60 mg rat aorta by adding RNAex Pro reagent (Accurate Biotechnology Co., Ltd., Hunan, China) followed by assessment of RNA purity and concentration. Total RNA was reverse-transcribed into cDNA using Evo M-MLV RT Kit with gDNA Clean for qPCR II (Accurate Biotechnology Co., Ltd) as described in the manufacturer’s protocol. The sequences of primers used are given in Table 1. The RT qPCR reaction system was as follows: 2 μL of cDNA preparation; 10 μL 2×SYBR Green Pro Taq HS Premix (Accurate Biotechnology Co., Ltd.); 0.4 μL aliquots of forward and reverse primers for Olr1, RelA or GAPDH genes; 7.2 μL ddH2O giving a total reaction volume of 20 μL. Real time fluorescent quantitative PCR (lightcycler480, Roche Diagnostic Products Co., Ltd., Shanghai, China) conditions were as follows: denaturation at 95°C for 30 s; reaction 40 times at 95°C for 5 s and 60°C for 30 s. The cycle threshold (Ct) was obtained after the reaction and the results analyzed by the comparative Ct (2−ΔΔCt) method.

Statistical analysis. The statistical software SPSS 21.0 (IBM Corp., Armonk, N.Y., USA) was used for data analysis. All data are expressed as means±SD. One-way analysis of variance (ANOVA) was used to compare multiple groups and comparison of two groups was performed by the least significant difference (LSD) test. A value of p<0.05 was considered statistically significant.

Table 1. Primer sequences used for RT-qPCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olr1</td>
<td>Forward</td>
<td>5′-CCCCATTCACCTCCCATTT-3′</td>
</tr>
<tr>
<td>RelA</td>
<td>Forward</td>
<td>5′-GTACTTGCCAGACACAGACG-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5′-ATCGTTCACACCGACCTTC-3′</td>
</tr>
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</table>

Olr1: oxidized low-density lipoprotein receptor 1; RelA: the RelA subunit of NF-κB factor; GAPDH: glyceraldehyde phosphate dehydrogenase.

Fig. 1. Concentration levels of folate and homocysteine (Hcy) in serum of rats. Control (normal diet), atherosclerosis (AS, high-fat diet), folic acid (FA), low-dose (LD-) L-5-MTHF, low-dose (LD-) L-5-MTHF+Seal oil, high-dose (HD-) L-5-MTHF (A) serum folate concentration, (B) serum homocysteine (Hcy) concentration. All data are expressed as means±SD (n=9). #p<0.01 compared with the control group, *p<0.05, **p<0.01 compared with the AS group, ♦p<0.05 compared with the LD-L-5-MTHF group, ♣p<0.05 compared with the LD-L-5-MTHF+Seal oil group.
RESULTS

Effects of L-5-MTHF and Seal oil on folate and Hcy levels in serum

Concentrations of serum folate and Hcy in rats are shown in Fig. 1. There were no significant differences in serum folate levels between control and AS groups. LD-5-MTHF and LD-5-MTHF+Seal oil groups, suggesting that a high-fat diet and Seal oil had no effect on serum folate levels (p>0.05, Fig. 1A). Furthermore, FA and L-5-MTHF increased serum folate levels (p<0.05), and increased with the increase of L-5-MTHF doses (p<0.01).

Compared with controls, the serum Hcy levels of rats fed a high-fat diet increased significantly (p<0.01, Fig. 1B). L-5-MTHF, alone, or combined with Seal oil decreased Hcy levels (p<0.05, p<0.01, respectively) in a dose-dependent manner (p<0.05). Moreover, LD-L-5-MTHF+Seal oil produced a greater reduction in Hcy levels than LD-L-5-MTHF alone (p<0.05).

Effect of L-5-MTHF and Seal oil on lipid profile

Table 2 shows blood lipid levels in the different groups. Concentrations of TC, TG and LDL-C in AS group serum were significantly higher than those of controls, while the concentration of HDL-C was significantly lower (p<0.01). Serum TC, TG and LDL-C concentrations in LD-L-5-MTHF+Seal oil and HD-L-5-MTHF groups were lower than those in the AS group while HDL-C concentration was higher (p<0.05, p<0.01, respectively). The serum TC and LDL-C concentrations in the HD-L-5-MTHF group were lower than those in the LD-L-5-MTHF group (p<0.05). FA and LD-L-5-MTHF had no statistical significance in improving blood lipids (p>0.05). These results suggest that LD-L-5-MTHF+Seal oil and HD-L-5-MTHF improved lipid abnormalities. The HD-L-5-MTHF regime had the more potent effect, especially in reducing the serum cholesterol concentration.

Effects of L-5-MTHF and Seal oil on NO and NOS contents in serum

NO and NOS have played a key role in regulating the function of endothelial cells. It can be seen from Fig. 2 that serum NO content and NOS activity in the AS group were significantly reduced (p<0.05). LD-L-5-MTHF+Seal oil and HD-L-5-MTHF improved serum NO content (p<0.05), especially NOS activity significantly (p<0.01). However, FA and LD-L-5-MTHF did not show this effect (p>0.05). Therefore, LD-L-5-MTHF+Seal oil and HD-L-5-MTHF both improved NO levels released by vascular endothelial cells in AS rats and increased the

Table 2. Comparison of blood lipid levels in different groups (mmol/L).

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC</th>
<th>TG</th>
<th>HDL-C</th>
<th>LDL-C</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.87±0.48</td>
<td>0.62±0.24</td>
<td>2.26±0.45</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>AS</td>
<td>2.73±0.49*</td>
<td>1.44±0.44*</td>
<td>1.76±0.30*</td>
<td>0.28±0.06*</td>
</tr>
<tr>
<td>FA</td>
<td>2.35±0.51</td>
<td>1.20±0.45</td>
<td>2.02±0.37</td>
<td>0.25±0.08</td>
</tr>
<tr>
<td>LD-L-5-MTHF</td>
<td>2.48±0.50</td>
<td>1.25±0.49</td>
<td>2.03±0.28</td>
<td>0.26±0.05</td>
</tr>
<tr>
<td>LD-L-5-MTHF+Seal oil</td>
<td>2.23±0.63*</td>
<td>0.90±0.40*</td>
<td>2.14±0.36*</td>
<td>0.23±0.08*</td>
</tr>
<tr>
<td>HD-L-5-MTHF</td>
<td>1.99±0.43**</td>
<td>0.79±0.31**</td>
<td>2.21±0.30**</td>
<td>0.20±0.04***</td>
</tr>
</tbody>
</table>

Control (normal diet), atherosclerosis (AS, high-fat diet), folic acid (FA), low-dose (LD-) L-5-MTHF, low-dose (LD-) L-5-MTHF+Seal oil, high-dose (HD-) L-5-MTHF. All data are expressed as means±SD (n=9). *p<0.05 compared with the control group, **p<0.01 compared with the AS group, ***p<0.01 compared with the LD-L-5-MTHF group. TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

![Fig. 2. Levels of nitric oxide (NO) and nitric oxide synthase (NOS) in serum different groups. Control (normal diet), atherosclerosis (AS, high-fat diet), folic acid (FA), low-dose (LD-) L-5-MTHF, low-dose (LD-) L-5-MTHF+Seal oil, high-dose (HD-) L-5-MTHF. All data are expressed as means±SD (n=9), #p<0.01 compared with the control group, *p<0.05, **p<0.01 compared with the AS group.](image-url)
Intervention of 5-Methyltetrahydrofolate and Seal Oil on Atherosclerosis

Effects of L-5-MTHF and Seal oil on serum oxidative stress

In order to evaluate levels of oxidative stress, serum activities of antioxidant enzymes, SOD and GSH-Px, as well as the oxidative stress biomarker, MDA, were measured. As shown in Fig. 3, after LD-L-5-MTHF + Seal oil treatment, activities of GSH-Px and MDA were improved and showed a significant difference from the LD-L-5-MTHF only condition (p < 0.05, Fig. 3B and C). HD-L-5-MTHF significantly improved activities of SOD, GSH-Px and MDA (p < 0.01) while FA and LD-L-5-MTHF did not (Fig. 3A–C). Differences between the HD-L-5-MTHF and the LD-L-5-MTHF groups indicated a more potent effect of the former (p < 0.05, Fig. 3B and C). Our results demonstrate that LD-L-5-MTHF + Seal oil improved activity of NOS, thereby improved endothelial dysfunction.

Effects of L-5-MTHF and Seal oil on serum oxidative stress

In order to evaluate levels of oxidative stress, serum activities of antioxidant enzymes, SOD and GSH-Px, as well as the oxidative stress biomarker, MDA, were measured. As shown in Fig. 3, after LD-L-5-MTHF + Seal oil treatment, activities of GSH-Px and MDA were improved and showed a significant difference from the LD-L-5-MTHF only condition (p < 0.05, Fig. 3B and C). HD-L-5-MTHF significantly improved activities of SOD, GSH-Px and MDA (p < 0.01) while FA and LD-L-5-MTHF did not (Fig. 3A–C). Differences between the HD-L-5-MTHF and the LD-L-5-MTHF groups indicated a more potent effect of the former (p < 0.05, Fig. 3B and C). Our results demonstrate that LD-L-5-MTHF + Seal oil improved
activities of GSH-Px and MDA, HD-L-5-MTHF significantly increased the activities of SOD and GSH-Px and down-regulated MDA, thereby improved oxidative stress in atherosclerotic rats.

Effects of L-5-MTHF and Seal oil on the expression of inflammatory mediators and endothelial factors

Expression contents of serum TNF-α, IL-6, IL-1β and ET-1, sICAM-1 are shown in Fig. 4. The contents of inflammatory factors and endothelial factors in serum of atherosclerotic rats all increased (p<0.01, Fig. 4A–E), and all decreased after treatment with LD-L-5-MTHF+Seal oil and HD-L-5-MTHF (LD-L-5-MTHF+Seal oil: p<0.05 and HD-L-5-MTHF: p<0.01, Fig. 4A–E). FA only reduced the serum IL-1β levels (p<0.05, Fig. 4C). In addition, LD-L-5-MTHF inhibited inflammatory response (p<0.05, Fig. 4A–C), and there was no statistical significance in reducing the levels of endothelial factor ET-1 and sICAM-1 (p>0.05, Fig. 4D and E). These results have indicated that LD-L-5-MTHF+Seal oil and HD-L-5-MTHF perform a positive role in preventing vascular endothelial inflammation and cell adhesion.

Effects of L-5-MTHF and Seal oil on pathological changes of thoracic aorta

Inspection of atherosclerotic plaques, cell proliferation and apoptosis in aortic tissue gives information about the development of atherosclerotic lesions. As shown in Fig. 5, vascular endothelial cells of controls were regular, the inner elastic membrane was intact and the smooth muscle cells of the media were arranged neatly (Fig. 5a). By contrast, in the AS group, the intima was obviously thickened, with diffuse lipid plaques, containing cholesterol crystals, necrotic tissue and a small amount of cellulose. Foam cell aggregation, media smooth muscle cell proliferation and irregular arrangement could all be observed (Fig. 5b). For the FA and LD-L-5-MTHF groups, the thoracic aortic lesions were basically the same as for the AS group with visible lipid
plaque and constricted and disordered nuclei of smooth muscle cells in the media (Fig. 5c and d). LD-L-5-MTHF + Seal oil and HD-L-5-MTHF rats had significantly lower plaque thickness than the AS group, foam cells were reduced and there was less proliferation of smooth muscle cells (Fig. 5e and f). These results have suggested that we have successfully established a rat model of AS and that intervention with LD-L-5-MTHF + Seal oil and, especially, with HD-L-5-MTHF improved the pathological changes of the AS aorta.

**Effect on the expression of Olr1 and RelA mRNA in rat thoracic aorta**

Olr1 and RelA are two key genes that mediate vascular endothelial dysfunction and cause atherosclerosis. Their expression levels in rat thoracic aorta tissue are shown in Fig. 6. Comparing with controls, expression of Olr1 and RelA was increased in the AS group (p < 0.01). LD-L-5-MTHF + Seal oil and HD-L-5-MTHF caused down-regulation of expression (p < 0.05, p < 0.01, respectively). HD-L-5-MTHF was more effective than low-dose in down-regulating Olr1 and RelA mRNA (p < 0.05). The above results have showed that intervention with LD-L-5-MTHF + Seal oil or HD-L-5-MTHF can ameliorate the Olr1 and RelA mRNA expression in atherosclerotic vascular tissues of rats.

**DISCUSSION**

Atherosclerosis is a complex chronic process, involving accumulation of lipids, endothelial injury, release of inflammatory factors and overexpression of endothelial factors. These changes lead to lumen stenosis or thrombosis and thereafter to cardiovascular disease (CVD) (15). The current study used a high-fat diet combined with vitamin D2 to establish a rat model of AS. The high-fat diet promoted lipid deposition and vitamin D2 promoted the absorption of blood calcium which damages the vascular endothelium and accelerates the formation of plaques in arterial lesions (16). Levels of serum TC, TG and LDL-C were significantly increased and HDL-C was significantly decreased in the AS rats. There were also pathological signs of atherosclerotic plaques in the aorta, suggesting that we had successfully established an AS rat model.

L-5-MTHF is the active form of FA (vitamin B9), FA supplementation having been evaluated as a potential clinical intervention for the treatment of vascular disease (17). L-5-MTHF itself has been rarely studied. Seal oil is often regarded as a cardiovascular health promoter, due to its high content of ω-3PUFAs. Epidemiological studies have indicated anti-thrombotic, anti-atherosclerotic and anti-inflammatory effects of a high intake of long-chain ω-3PUFAs (18). Bhatt et al. used high doses of EPA (4 g/d) to reduce the incidence of fatal and non-fatal cardiovascular events in patients (19). For atherosclerotic patients with high blood sugar, a dose of 3-4 g of ω-3PUFAs per day has been found to have no adverse effect on glucose tolerance, although an intake exceeding 10 g/d may have a harmful effect (20). Taking into account these previous findings, different doses of L-5-MTHF were used to ameliorate AS in rats, together with an adjuvant therapy of a low dose of Seal oil. The current study thus confirms that a low dose of L-5-MTHF, combined with Seal oil therapy, was beneficial for improving AS, as was a high dose of L-5-MTHF. However, the toxicity of high-dose L-5-MTHF needs further study.

FA is very stable, has high bioavailability and has been shown to improve vascular endothelial function in patients with coronary artery disease (21). The United States Food and Drug Administration (FDA) has authorized FA as a food additive for infant formulae and for foodstuffs intended for medicinal and dietetic purposes (22). However, people with disordered FA metabolism cannot activate this supplement and, moreover, excessive FA intake may mask the hematological symptoms of vitamin B12 deficiency (11). It is well known that FA is transformed into bioactive L-5-MTHF through the DHFR and MTHFR pathways and then combined with MTRR in order to metabolize Hcy to methionine. These reactions serve to maintain the body’s Hcy homeostasis. HHcy is considered to be an independent risk factor for AS and insufficient daily intake of B vitamins and reduced MTHFR activity may be the main factors responsible (6). However, clinical studies have shown that FA intake >200 μg/d can saturate DHFR, leading to a decrease in serum folate concentration and the accumulation of unmetabolized FA (23). A study done by Bailey and Aylng showed that with FA as a substrate, the activity of human liver DHFR is 56 times lower than the average activity of rat DHFR (9). In addition, high concentrations of FA may competitively inhibit the conversion of dihydrofolate to tetrahydrofolate, resulting in a deficiency of intracellular folate (24). However, L-5-MTHF does not require DHFR and MTHFR metabolism in vivo, which directly affects serum folate levels. In conclusion, L-5-MTHF supplementation may be more effective than FA supplementation for human patients. Clinical trials are required to confirm this finding. Christensen et al. found that the consumption of a high FA diet is related to decreased MTHFR expression and activity in mouse liver (25). Venn et al. compared the efficacy of 100 μg/d FA supplementation with equimolar L-5-MTHF in reducing Hcy in healthy volunteers, and found that L-5-MTHF was more effective (12). The current study found an effect of supplementation with equal doses of FA and L-5-MTHF in increasing serum folate levels. L-5-MTHF was more beneficial than FA in reducing Hcy concentration and its effects on both serum folate and Hcy levels were dose dependent. Therefore, it can be concluded that L-5-MTHF is a suitable replacement for FA in regulating serum Hcy. Combined application of Seal oil and L-5-MTHF appears to have a synergistic effect in reducing Hcy levels. Previous studies have shown that dietary supplementation with 600 g/d ω-3PUFAs reduces Hcy, higher levels of DHA up-regulate MTHFR mRNA expression, which is condu­cive to Hcy metabolism (26).

Abnormal lipid metabolism is the primary risk factor for AS (2) and causes damage to vascular endothelial cells, increasing the secretion of vasoactive substances.
The current study found that a high dose of L-5-MTHF improved serum concentrations of TC, TG, LDL-C and HDL-C in AS rats, eliciting an especially large decrease in TC and LDL-C levels, and its possible mechanism is to indirectly inhibit cholesterol synthesis by promoting Hcy metabolism. Previous studies have shown that Hcy activates 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme for cholesterol synthesis, in human vascular endothelial cells, thereby affecting blood lipid metabolism and capturing LDL-C aggregates (27). Mann et al. found that Seal oil improved the blood lipid profile, reducing the risk of thrombus (28), the findings of the current study support this conclusion since we have shown that the combination of Seal oil and low-dose L-5-MTHF also improved dyslipidemia in rats, but more mechanisms need to be further explored.

Endothelial cell dysfunction is a primary driver for the development of AS and is related to decreased production of the endodermal vasodilator, NO, due to low activity of NOS (3). In the vascular endothelium, the substrate l-arginine synthesizes NO under the catalysis of NOS, thereby enhancing vasodilation, inhibiting expression of pro-inflammatory factors and antagonizing LDL-C oxidation. These effects lead to improved endothelial function. The homeostasis around vascular endothelium is a function of the equilibrium between the bioavailability of nitric oxide and oxidizing reactive oxygen species (ROS) (29). ROS play a role in the inflammatory response, apoptosis and LDL-C oxidation (30). It has been shown that reduced NO synthesis plus oxidative stress caused by excessive production of ROS are the key mechanisms leading to AS (31). In fact, FA has an antioxidant capacity, which improves endothelial cell NOS dysfunction (32). The main antioxidant systems in the vascular wall are SOD and GSH-Px, which act to reduce the production of oxidized low-density lipoprotein (ox-LDL). By contrast, excessive MDA causes oxidation of LDL-C to form MDA-LDL, resulting in disorder of the antioxidant system and endothelial dysfunction. Omura et al. found that the EPA derived from ω-3PUFAs increased NO levels in situ endothelial cells and stimulated NO-dependent dilation of bovine coronary arteries (33). The findings of the current study are that high-dose L-5-MTHF or L-5-MTHF in combination with Seal oil increased NOS/NO expression, inhibited superoxide production and reduced oxidative stress in AS rats. Seal oil had the advantage of improving GSH-Px and MDA levels. It is also likely that L-5-MTHF alleviates oxidative stress and inflammation in AS rats by enhancing the NOS/NO pathway, thereby reducing progression of AS.

AS has long been known as a chronic inflammatory disease and reducing inflammation is an appropriate treatment and prevention strategy. The inflammatory cytokines, TNF-α, IL-1β and IL-6, have been considered potential therapeutic targets for AS (34). TNF-α plays a key role in the induction and promotion of inflammation (35), up-regulating expression of other pro-inflammatory cytokines and endothelial adhesion molecules. Local activation of IL-1β in blood vessels is central to mediating the pro-inflammatory response and promotes activation of secondary inflammatory mediators, including IL-6 (36). IL-6 is considered a biomarker of inflammation and has been shown to be associated with endothelial dysfunction and subclinical AS and to be predictive of future cardiovascular events (37). The current study found that serum TNF-α, IL-1β and IL-6 were highly expressed in AS rats and that treatment with L-5-MTHF reduced their levels in a dose-dependent manner, an effect that was strengthened by Seal oil. Further studies are required to confirm the beneficial effects of L-5-MTHF on AS via the proposed anti-inflammatory mechanism. The potent vasoconstrictor, ET-1, has a role in regulation of blood pressure, and previous clinical studies in AS patients have found increases in serum ET-1 (38). SICAM-1 promotes monocyte adhesion to endothelial cells during AS development and apolipoprotein E-deficient mice have been shown to have increased sICAM-1 levels as atherosclerosis progresses (39). Several studies have linked ω-3PUFAs intake to improved serum markers of endothelial dysfunction, such as reduced expression of VCAM-1 and sICAM-1 (40). The current study found that both high dose L-5-MTHF or low dose L-5-MTHF combined with Seal oil inhibited release of ET-1 and sICAM-1, suggesting that the treatment may have a beneficial impact on blood pressure and monocyte adhesion leading to improvements in endothelial dysfunction.

Olr1 is a receptor which binds ox-LDLs, promoting their phagocytosis and degradation. Previous studies have shown that Olr1 is expressed by endothelial cells and when ox-LDLs bind, the endothelium is activated and a state of oxidative stress leads to endothelial dysfunction and promotion of AS (41). Olr1 also activates NF-kappaB, a transcription factor containing P50/RelA, which has roles in the immune and inflammatory responses, cell proliferation and apoptosis as well as other pathophysiological processes (42). Previous studies have demonstrated the presence of activated RelA in the smooth muscle, endothelial cells and macrophages of AS lesions where it promotes transcription of cytokines and inflammatory mediators (43). The present study found elevated Olr1 and RelA mRNA in aortic tissue from AS rats, expression of which was decreased by treatment with high dose L-5-MTHF or low dose L-5-MTHF + Seal oil. Many current AS treatments are aimed at Olr1 inhibition to reduce damage caused by the inflammatory response (44). We suggest that L-5-MTHF may antagonize ox-LDL mediated AS by inhibiting the inflammatory pathway of RelA mediated by Olr1.

L-5-MTHF participates in one-carbon reactions, providing methyl groups for the metabolism of Hcy into methionine (45). The toxic effects of HHcy on endothelial cells include increasing oxidative stress, promoting release of inflammatory factors and reducing bioavailability of NO. These events lead to abnormal lipid metabolism and endothelial cell damage. The high ω-3PUFAs of Seal oil can inhibit the metabolism of arachidonic acid (AA), thereby reducing blood viscosity, inhibiting platelet aggregation, and reducing the formation of
MDA (46). Although L-5-MTHF and Seal oil participate in different metabolic pathways, the combination of their effects ameliorates AS. Moreover, the current study demonstrated that L-5-MTHF and Seal oil have a synergistic effect on reducing serum Hcy and improving dyslipidemia and NO/NO regulation. Doshi et al. investigated the use of L-5-MTHF as a drug to improve endothelial function in vascular patients and speculated that high levels of circulating folate may be responsible (47). A study of ω-3PUFAs intervention in 188 US patients with AS found reductions in macrophage infiltration of plaques. Plaques readily absorbed ω-3PUFAs, especially EPA and DHA, from Seal oil supplements (48). Cell and animal studies have shown that ω-3PUFAs reduce the expression of adhesion molecules on endothelial cells, monocytes, and macrophages and inhibit plaque formation (18). Combined with this study and previous conclusions, it is concluded that L-5-MTHF and Seal oil delay the development of AS plaque formation. Mechanisms responsible may include down-regulation of Ohr1 expression, direct metabolism of Hcy and enhancement of the NOS-mediated NO activity pathway. These events culminate in the inhibition of oxidative stress and the inflammatory cascade, thereby promoting plaque stabilization and regression.

In conclusion, we can consider L-5-MTHF as a substitute for FA. L-5-MTHF alleviated disordered lipid metabolism in rats fed a high-fat diet, improved the body’s antioxidant capacity and down-regulated the expression of the inflammatory and endothelial injury factors, Ohr1 and RelA. These effects combine to improve AS plaque infiltration in the blood vessel intima and ameliorate the conditions of AS in rats. We also found low-dose L-5-MTHF supplemented with 0.5 g/kg Seal oil to be beneficial for vascular endothelial dysfunction in rats. Our findings suggest that L-5-MTHF may have potential as an anti-atherosclerotic agent, appropriately supplemented by Seal oil. However, whether high-dose L-5-MTHF has a toxic effect on the body needs further research, and the applicability of this potential treatment in the clinic needs to be evaluated.

Authorship

Research conception and design: XG, HW, and ZZ; experiments: HW, ZZ, SQ, and YT; statistical analysis of the data: HW, YW, and TZ; interpretation of the data: HW, ZZ, YW, and TZ; writing of the manuscript: HW and XG.

Disclosure of state of COI

The authors state that there is no conflict of interest.

Acknowledgments

This research was financially supported by the Key Technology Research and Development Program of Shandong, China (No. 2019GSF107002). We would like to express our gratitude to EditSprings (https://www.editsprings.com/) for the expert linguistic services provided.

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