Protection against vascular endothelial dysfunction by polyphenols in sea buckthorn berries in rats with hyperlipidemia

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Summary
Chronic hyperlipemia increases the incidence of vascular endothelial dysfunction and can even induce cardiovascular disease. Sea buckthorn contains a host of bioactives such as flavonoids and polyphenols that can prevent the development of cardiovascular disease. The current study isolated active ingredients, polyphenols, from sea buckthorn berries (SVP) and orally administered SVP at a dose of 7-28 mg/kg. This treatment significantly reduced serum lipids, it enhanced the activity of antioxidant enzymes, and it decreased the level of serum TNF-α and IL-6. SVP also alleviated vascular impairment by decreasing the expression of eNOS, ICAM-1, and LOX-1 mRNA and proteins in aortas of rats with hyperlipidemia. Based on these findings, SVP has antioxidant action and it protects endothelium.

Keywords: Vascular endothelial dysfunction, polyphenol, sea buckthorn berries, hyperlipidemia, protection

1. Introduction

Hyperlipidemia, hypercholesterolemia, and obesity are major risk factors for the development of cardiovascular diseases (CVDs), such as hypertension, atherosclerosis, coronary artery disease, and diabetes mellitus (1-3). One of the features of CVDs is vascular dysfunction, which involves an elaborate interplay between modified plasma lipoproteins (Lp), vascular endothelial cells (ECs) and smooth muscle cells (SMCs), migratory cells such as monocytes, T-lymphocytes, and platelets, and molecules produced by these cells (4). Furthermore, vascular endothelial dysfunction (VED) plays a key role in the development of many CVDs that are associated with a state of chronic, low-grade inflammation characterized by abnormal cytokine production and macrophage infiltration into tissues (5). A reasonable assumption is that these situations could be targeted to protect the vascular endothelium and maintain plasma homeostasis.

Many studies have indicated that the main etiology of VED involves a decrease in NO generation and bioavailability, leading to an increase in the generation of reactive oxygen species (ROS) (6). Oxidative stress may play a significant role in the development of metabolic syndrome (7). Accumulating evidence suggests that mitochondrial dysfunction might be associated with the response to inflammation. The excessive production of ROS and the release of oxidized mitochondrial DNA have been noted in inflammasomes (8,9). Vascular dysfunction is characterized by leukocyte recruitment, foam cell (FC) formation, endothelium dysfunction, and vascular inflammation. Inflammation is considered to be a hallmark of vascular dysfunction (10,11).

Some phytochemicals in certain plants have excellent pharmacological effects that have been studied extensively, and these effects are evident particularly in the antioxidant action of teas, spices, and herbs. Polyphenols are important additives in the food and pharmaceutical industries and these compounds have been widely studied because of their unique activities,
such as anti-tumor activity, anti-diabetic activity, anti-inflammatory activity, hypolipidemic activity, and antioxidant activity, as well as immune regulation (12,13). Flavonoids are polyphenol compounds, and an epidemiological study has reported noting an inverse correlation between flavonoid consumption and the risk of CVD (14). Sea buckthorn (*Hippophae rhamnoides* L.), is a traditional Chinese medicine and Tibetan medicine and is thus one of the most important shrubs grown widely in China. In folk medicine, the fresh berries are used to relieve coughing, reduce phlegm, cure indigestion, stimulate the circulation of blood, and decrease hyperlipidemia (15). Since sea buckthorn is an excellent source of natural flavonoids such as isorhamnetin, quercetin, and aglycones (Table 1), many researchers have examined whether these components can effectively prevent and treat CVD. A study has examined the bioavailability and absorption of these components and their effect on emerging risk factors for CVD (21). Previous studies have reported that sea buckthorn mainly contains amino acids (22), triterpene derivatives, phytosteroids, and phenolic compounds such as oligomeric catechins and quercetin derivatives (23-26). According to vitro and vivo analyses of antioxidant activity, these active compounds possess anti-oxidative properties that can reduce the levels of free radicals and provide anti-diabetic activity, anti-inflammatory activity, hypolipidemic activity, and antioxidant activity (27). However, the role of polyphenols in vascular protection has not fully studied. In order to further investigate the pharmacological effects of polyphenols on vascular dysfunction, the current study evaluated the protection that polyphenols from sea buckthorn berries (SVP) provide against hyperlipidemia-induced VED in rats.

### 2. Materials and Methods

#### 2.1. Reagents and chemicals

Sea buckthorn (*Hippophae rhamnoides* L.) berries were used to yield SVP, a compound containing phenolic actives such as flavonoids. Briefly, approximately 5 kg of sea buckthorn juice was dried with lyophilized, and then the powder was extracted 3 times with 1 L of 70% ethanol at a temperature of 60°C. Each extraction took 2 h. The supernatant was concentrated into a rough extract of about 1 kg. The rough extract was dissolved in 1 L of distilled water, and the resulting solution was subjected to column chromatography over D101 macro porous resin and eluted with 50-70% ethanol. Thirty-five g of refined SVP was prepared. The purity of SVP was determined using ultraviolet spectrophotometry. All reagents and chemicals used were of analytical grade.

#### 2.2. Animals and experimental design

Four week-old male Sprague Dawley (SD) rats (n = 72) (specific pathogen-free grade, certification no. 62001000000134) weighing 90-100 g were purchased from the Animal Experimentation Center of Gansu University of Chinese Medicine. All animals were housed in groups of 12 per cage on a 12-h light/dark cycle. Rats were fed a basic diet in the experimental environment before the experiment was conducted. After one week of adaptation, all rats were randomly divided into 6 groups. Three of these groups were a normal control group (NC), a model control group (MC), and a positive control group (PC) receiving 2 mg/kg simvastatin (a dose corresponding to that administered to patients; simvastatin was from Lukang Drugs Group Co. Ltd., Lukang, Shandong, China). The remaining 3 groups received a low dose of SVP (7 mg/kg, equal of 1 g of herbs, SVPL), a medium dose of SVP (14 mg/kg, SVPM), or a high dose of SVP (28 mg/kg, SVPH). Each group consisted of 12 rats, and the chosen drug dose was based on the Chinese Pharmacopoeia (15). All groups except for the NC group were fed a high-fat diet (made with standard diet supplemented with 12 % lard, 10% yolk, 5% cholesterol, 1% bile salt, and 0.2% propylthiouracil) from Keaoxieli Co. Ltd. (Keaoxieli, Beijing, China). Every other day, rats were fed a high-fat emulsion preparation (10 mL/kg) as previously described (28). Body weight was recorded and blood was collected from the ocular fundus of two rats on a high-fat diet every two weeks to determine if
the model of hyperlipidemia was established. After 6 weeks, the NC and MC groups were orally administered the same volume of water while the other groups were administered the corresponding treatment once daily. After 5 weeks, animals were sacrificed, and the blood and tissues were collected for biochemical assays and structural analysis.

This experiment was carried out in accordance with local guidelines for the care of laboratory animals of Animal Experimentation Center and was approved by this institution's ethics committee for research on laboratory animal use.

2.3. Morphology of the thoracic aorta

The thoracic aorta was harvested and adherent connective tissue was removed. The thoracic aorta was initially fixed with a solution of 3% glutaraldehyde and then fixed in 1% osmium tetroxide, dehydrated with serial acetone solutions, and embedded in Epox 812. Semi-thin sections were stained with methylene blue. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (TEM; HITACHI, H-600IV, Japan).

2.4. Biochemical analysis

Rats were anesthetized with urethane. Blood samples were collected from the celiac artery and centrifuged at 4000 r/min for 10 min to obtain serum. All of the biochemical parameters were determined with colorimetry using commercially available kits (Jiancheng Biotech, Nanjing, China). Parameters related to antioxidant activity such as superoxide dismutase (SOD) activity in serum and the liver were measured using WAT-1 as a superoxide detector, the level of malondialdehyde (MDA) in serum and the liver was measured using the TBA method, and glutathione peroxidase (GSH-Px) in hepatic tissue was measured in a coupled system by measuring the decrease in NADPH at 340 nm. Biochemical parameters also included the levels of serum total cholesterol (TC), triglycerides (TG), high-density lipoproteins (HDL) and low-density lipoproteins (LDL).

Serum levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were determined using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (Jiancheng Biotech, Nanjing, China).

2.5. Quantitative real-time PCR analysis of relative mRNA

Aortic tissue was homogenized and total RNA was isolated using Trizol (Invitrogen, CA, USA). One microgram of total RNA from each rat sample was reverse-transcribed into cDNA using oligo (dt) 18 and reverse transcriptase (Fermentas, MA, USA). The cDNA of endothelial nitric oxide synthase (eNOS), lectin-like oxLDL receptor-1 (LOX-1), and intercellular adhesion molecule-1 (ICAM-1) was amplified using SYBR Green Mix (Thermo Fisher Scientific, MA, USA). PCR was performed with cycles of denaturation at 95°C for 15 s, annealing at 60°C for 45 s, and extension at 72°C for 30 s. The oligonucleotide primers used are listed in Table 2.

2.6. Western blotting analysis of relative protein

Frozen aortas were homogenized with a Polytron homogenizer on ice in PIPA lysis buffer (Xinfan Biotech, Shanghai, China). After aortic tissue was fully lysed, the samples were centrifuged at 12000 g for 5 min at 4°C. Supernatants were collected and protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, MA, USA). The supernatants were then denatured at 90°C for 10 min. Proteins were examined with 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then protein was transferred to polyvinylidene fluoride (PVDF) membranes using Bio-Rad Trans-Blot SD transfer cells (Bio-Rad Laboratories, CA, USA). The membranes were washed in TBST and blocked with 5% nonfat milk in TBST for 2 h at room temperature. Membranes were incubated overnight at 4°C with the primary antibodies (Polyclonal rabbit LOX-1 1:800, ICAM-1 1:500, eNOS 1:800 GAPDH 1:1500). The membranes were washed and incubated with horseradish peroxidase goat anti rabbit secondary antibody (Beyotime Institute of Biotechnology, Haimen, China) for 1 h at 37°C. The membranes were washed in TBST and then incubated in chemiluminescent substrate (ECL Western Blotting) for 30 s. Protein bands were detected on X-ray film. The software Image J was used to measure the integral optical density of protein bands from the scanned film and to calculate the integral optical density of the relative ratio of eNOS, LOX-1, ICAM-1, and GAPDH bands.

2.7. Statistical analysis

Data are presented as the mean ± SD. The statistical

<table>
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<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Amplicon size (bp)</th>
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<tbody>
<tr>
<td>eNOS</td>
<td>F: 5'-CTTTTGGAAGCCGGTTCGAC-3'</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AACTTCGGTGCTGCTATTGC-3'</td>
<td></td>
</tr>
<tr>
<td>LOX-1</td>
<td>F: 5'-CAACCCAGCTTACGCTATC-3'</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ATGCGCTTCTTGACTTCC-3'</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>F: 5'-CCCCCTCCTACTACATCCCTACC-3'</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TTCGCTTCAGCTTCTTG-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-GTCAATGGTGAAAGGATTTG-3'</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TCCACATCCAGCTTGAC-3'</td>
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analysis package SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA) was used for one-way analysis of variance (ANOVA) followed by a post-hoc test for multiple comparisons with a pre-determined significance level of $p < 0.05$.

3. Results

3.1. The purity of SVP

The main component of SVP was a flavonoid, and its activity significantly increased after column chromatography over D101 macroporous resin. The purity of the SVP was determined at 760 nm using UV with gallic acid as the standard control. The linear equation was $Y = 0.0504X + 0.0723$, $R^2 = 0.9998$, and the yield was $89.98 \pm 0.032\%$.

3.2. Effects of SVP on body weight

All of the groups had a similar increase in body weight before receiving the corresponding treatment. Body weight was $299 \pm 4.92$ (g), and it began to significantly change once treatment with SVP and simvastatin began. The MC group had a significant increase in body weight compared to other groups, but there was no significant weight gain in the SVPM and SVPH groups in comparison to the MC group ($p < 0.05$). The rate of weight gain in rats in the SVPM and SVPH groups was similar to that in the PC group (Figure 1).

3.3. Effects of SVP on TNF-α and IL-6 in serum

The MC group had significantly higher levels of serum TNF-α and IL-6 in comparison to those in the NC group ($p < 0.05$). However, SVP decreased the levels of serum TNF-α and IL-6 in a dose-dependent manner (Figure 2). The level of serum TNF-α in the SVPH group was significantly lower than that in the PC group and that level was similar to that in normal rats (Figure 2A). However, the serum IL-6 level did not differ in the SVPH group and the PC group (Figure 2B).

3.4. Effects of SVP on antioxidant enzyme activity in serum and the liver

Results indicated that the activity of SOD in serum and the liver of rats with hyperlipidemia decreased ($p < 0.05$), and the level of SOD in serum was markedly lower than that in the liver. The activity of SOD in serum and the liver was markedly higher in the SVPL, SVPM, and SVPH groups in comparison to that in the MC group ($p < 0.05$) (Figure 3A). Similarly, a high-fat diet induced higher MDA levels, and the MDA levels in the SVPL, SVPM, and SVPH groups were distinctly lower than those in the MC group ($p < 0.05$). MDA levels in the SVPL, SVPM, and SVPH groups were similar to those in the PC group and slightly elevated in comparison to those in normal rats (Figure 3B). The level of GSH-Px in the liver decreased significantly in the MC group while the GSH-Px level increased significantly in the SVPH, SVPM, and PC groups ($p < 0.05$). The level of GSH-Px in the MC group was similar to that in the PC group (Figure 3C). In addition, the SVPM and SVPH groups had improved levels of serum SOD in comparison to the PC group. The SVPH group had improved levels of liver SOD in comparison to the PC group. The SVPH group had improved levels of liver MDA in comparison to the PC group. The SVPM and SVPH groups had improved levels of liver GSH-Px in comparison to the PC group. However, none of these differences were significant.

3.5. The effect of treatment on blood lipids

As shown in Figure 4, the serum levels of TC, TG, and LDL levels increased significantly in the MC group but

![Figure 1. Effect of SVP on the body weight of rats. Data are presented as the mean ± SD ($n = 10$). Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. $p < 0.05$ (Turkey's test).](image)

![Figure 2. Effect of SVP on the level of serum TNF-α (A) and IL-6 (B) in rats. Data are presented as the mean ± SD ($n = 7$). Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. $p < 0.05$ (Turkey's test).](image)
Figure 3. Effect of SVP on antioxidant activity in serum and the liver. Superoxide dismutase (SOD) (A) in serum and the liver, malondialdehyde (MDA) (B) in serum and the liver, and glutathione peroxidase (GSH-Px) (C) in serum from rats. Data are presented as the mean ± SD (n = 8). Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. p < 0.05 (Turkey's test).

Figure 4. Effect of SVP on blood lipids in rats. Data are presented as the means ± SD (n = 8). (A) indicates TC, (B) indicates TG, (C) indicates HDL, and (D) indicates LDL. Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. p < 0.05 (Turkey's test).

Figure 5. Effects of SVP treatment on histopathology in rats with hyperlipidemia. A indicates endothelial cells (ECs) in each group. B indicates smooth muscle cells (SMCs) in each group. FC indicates foam cells in proportion to ECs and SMCs in each group. Swollen mitochondria with an indistinct structure were noted. All images were taken at ×8000.
the level of HDL decreased significantly. In contrast, levels of TC, TG, and LDL were lower in the SVPL, SVPM, and SVPH groups than in model rats \( (p<0.05) \). Levels of TC, TG, and LDL were considerably lower than those in positive control rats. The SVPL, SVPM, and SVPH groups had significantly increased HDL levels in comparison to the MC group. Effects were dose-dependent in the SVPL, SVPM, and SVPH groups (Figure 4).

3.6. Histological changes in the aorta

The histological appearance of the transverse section of the aorta is shown in Figure 5. In ECs from the MC group, abnormalities like abnormal chromatin assembly in the cell nucleus and an increased number of FCs were noted; moreover, organelles lacked a distinct structure and ECs were sloughed off. FCs were also noted among the SMCs. In comparison, these changes were not noted in the ECs and SMCs from the NC group. Cells were intact and had a normal cytoarchitecture, and organelles had a distinct structure. The arterial structure in the SVPL, SVPM, and SVPH groups had significantly fewer FCs, and slight chondriosome swelling in ECs and SMCs was noted in the SVPL, SVPM, and PC groups. However, cytoarchitecture was intact in the SVPH group.

3.7. Effect of SVP on aortic cytokines

Hyperlipidemia induced expression of LOX-1, ICAM-1, and eNOS, and RT-PCR results indicating how SVP affected that expression are shown in Figure 6A. The expression of LOX-1, ICAM-1, and eNOS mRNA was barely detected in aortic tissue from the NC group while expression of these mRNAs increased significantly \( (p<0.05) \) in the MC group. After treatment with SVP, the expression of these mRNAs was much lower than that in the MC group \( (p<0.05) \). The regulating actions of SVP were found to proportionally decrease in a dose-dependent manner. Moreover, as shown in Figure 6B and 6C, the expression of ICAM-1, LOX-1, and eNOS proteins in aortic tissue from the MC group increased approximately 2.0-fold in comparison to that in the NC group. After treatment with SVP, the expression of these proteins decreased significantly \( (p<0.05) \).

4. Discussion

The purpose of this study was to investigate the effects of SVP on vascular endothelial function in rats with hyperlipidemia. Results indicated that the vascular structure of the aorta was severely damaged in the MC group, such as sloughing off of ECs, formation of FCs, swollen mitochondria in ECs and SMCs, and destruction or disappearance of mitochondrial cristae. These phenomena were significantly abated in the SVPL, SVPM, SVPH, and PC groups in comparison to the MC group (Figure 5). Changes in vascular morphology coincided with diminished vascular dysfunction. eNOS expressed in ECs is the major source of endothelial NO \( (29) \). Early disruption of eNOS expression may have contributed to the reduced level of NO and even VED. In the current study, the levels of aortic eNOS expression (levels of both mRNA and protein) increased significantly in the MC group. This seems contradict the mechanism whereby VED is induced by eNOS/NO, and there is some evidence that uncoupling of endothelial NO synthase can decrease the bioavailability of NO, aggravating the development of atherosclerosis and vascular disease \( (30,31) \). According to the current findings, all 3 doses of SVP decreased expression of eNOS mRNA and protein (Figure 6A, 6B, and 6C). This suggests that SVP can increase the bioavailability of endothelium-derived NO and it can also protect ECs, ameliorating VED induced by eNOS/NO disorders.

Previous studies have indicated that high cholesterol (hypercholesterolemia) induces the generation of ROS, thereby decreasing the generation and bioavailability of NO \( (29) \). ROS may result in the instability of critical macromolecules and represent the molecular basis of many diseases including inflammation processes, cardiovascular alterations, and cancer \( (32-34) \). Antioxidant enzymes have an essential physiological
function, serving as the first line of defense against ROS by coordinating to reduce the generation of active oxygen radicals and by preventing lipid peroxidation and intermediate products of metabolism from undermining the body (35). The MDA level is closely associated with several components of metabolic syndrome (36). The current study examined the activity of antioxidant enzymes (SOD and GSH-Px) and the levels of the oxidation product MDA. As shown in Figure 3, SVP increased the activity of SOD in serum and the liver but it significantly decreased the levels of the oxide metabolite MDA in serum and the liver in comparison to levels in normal rats. However, SVP significantly increased the activity of the antioxidant enzyme GSH-PX in the liver. A previous study indicated that flavonoids have the ability to effectively reduce oxidative stress (37). Therefore, the current results suggest that SVP could improve the efficiency of antioxidant enzymatic systems and that SVP might be a compensatory way to counteract the possible detrimental effects associated with oxidative stress induced by exhaustive exercise.

In addition to the ROS and oxidative stress mentioned earlier, the inflammatory response is considered to be a common pathogenic factor in vascular impairment (38). A higher level of IL-6 may cause insulin resistance and metabolic disorders in patients with metabolic syndrome (36). TNF-α, another pro-inflammatory cytokine, is also closely associated with the pathogenesis of oxidant stress and vascular dysfunction (39). In order to investigate the possible involvement of the molecular mechanism of SVP in endothelial dysfunction induced by hyperlipidemia, the current study evaluated the expression of the inflammatory cytokines IL-6 and TNF-α in serum. Levels of serum TNF-α, and IL-6 in the MC group were significantly higher than those in the NC group (p < 0.05). However, SVP reduced the levels of serum TNF-α and IL-6 in a dose-independent manner (Figure 4). This suggests that vascular protection by SVP may result from its anti-inflammatory activity since it reduces the levels of TNF-α and IL-6 in rats with hyperlipidemia. These results coincide with the pathologic changes mentioned earlier.

Elevated serum LDL levels are thought to be a compensatory way to counteract the possible oxidative stress and inflammation. Moreover, a previous study on an artichoke extract indicated that flavonoids retard LDL oxidation (43) and reduce oxidative stress in ECs stimulated with TNF-α and oxidized LDL (44). Furthermore, flavonoids have a positive cardiovascular effect, including decreased leukocyte adhesion and inflammation as well as vasodilatory properties (45). According to the current results, the modulating effects of SVP were evident as a decrease in LDL in a dose-dependent manner. Moreover, the expression of ICAM-1 and LOX-1 proteins in aortic tissue from the MC group was approximately 2.0-fold more than that in the NC group, as shown in Figure 6B. After SVP treatment, the expression of these proteins decreased significantly (p < 0.05). Therefore, findings indicate a molecular mechanism underlying the therapeutic effects of SVP and they highlight a new therapeutic intervention for the prevention of endothelial dysfunction induced by hyperlipidemia.

In conclusion, results indicated that oral administration of SVP at a dose of 7-28 mg/kg significantly improved the tolerance of hyperlipidemia in rats given a high-fat diet. More importantly, SVP prevented endothelial dysfunction of the aorta by enhancing the activity of antioxidant enzymes, attenuating the levels of inflammatory cytokines such as TNF-α and IL-6, and decreasing the level of eNOS, ICAM-1, and LOX-1 expression. These findings suggest that SVP has significant preventive and therapeutic action that could be explored as a promising food additive to prevent chronic CVD.

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