Sini decoction intervention on atherosclerosis via PPARγ-LXRα-ABCA1 pathway in rabbits

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Abstract: AIM: Sini decoction (SND) is a commonly used herbal formula showing lipid-lowering effects and is applied in traditional Chinese medicine (TCM) for the treatment of cardiovascular disease (CVD) and atherosclerosis (AS). However, the mechanisms behind its anti-atherosclerotic effects are still unknown, and will be investigated in this study. METHODS: AS was induced in rabbits by high fat diet (HFD) and treated with solvent (HFD group), atorvastatin (Ator group), or SND (SND group) for 12 weeks. Healthy rabbits (Chow group) were used as control. Serum and liver homogenates were collected, and lipid profiles as well as serum ApoA-I and ApoB were examined. Histopathological changes and lipid deposition in the proximal aorta and liver were detected by Oil red O staining. Western blot was used to detect the expression of ABCA1, ApoA-I, ApoB, PPARγ, and LXRα in liver, peritoneal macrophages, peripheral mononuclear cells (PMC), and adipose tissues. RESULTS: SND significantly attenuated the levels of total cholesterol (TC), triglyceride (TG), and low density lipoprotein cholesterol (LDL-C) in serum and liver. However, high density lipoprotein cholesterol (HDL-C) dramatically increased. SND treatment also decreased lipid deposition and improved the structure of the liver and aorta. Furthermore, SND enhanced the expression levels of ABCA1, PPARγ, and LXRα in liver, adipose tissues, PMC, and peritoneal macrophages. It also upregulated hepatic and serum ApoA-I expression and serum ApoA-I/ApoB ratio. CONCLUSIONS: SND treatment relieved AS, improved lipid profiles, and increased serum HDL-C level. The potential mechanism behind this might be the improvement of reverse cholesterol transport (RCT) involved with enhanced expression of ABCA1, ApoA-I, PPARγ, and LXRα.

Keywords: Sini decoction, High fat diet, Atherosclerosis, Lipid deposition, PPARγ-LXRα-ABCA1 signaling pathway

1 Introduction

Atherosclerosis (AS) is a major disorder resulting in cardiovascular disease (CVD), which contributes to morbidity and mortality globally [1]. The main pathologic feature of AS is focal lipid accumulation in atherosclerotic plaques that injure the arterial wall. This process leads to thickening of the vascular wall and stenosis of the blood vessel lumen, which then induce cardiovascular and cerebrovascular disease. Foam cell formation from mononuclear macrophages is the earliest histologic hallmark of AS and interacts with lipid accumulation closely in the development and complications of AS. Inflammation-induced mononuclear cells migrate from blood circulation into the intima of blood vessel walls to form macrophages. Macrophages internalize lipoprotein cholesterol and release free cholesterol outside of the cell by reverse cholesterol transport (RCT). The harmful imbalance between the uptake and release of cholesterol is a major cause contributing to foam cell formation and is also an important pathological contributor to AS development. Foam cells afterwards release inflammatory cytokines to mediate a non-specific immune response and initiate the occurrence and development of AS plaque. Because RCT is the most classical and major mechanism preventing AS, it is considered an important therapeutic drug target for the prevention and treatment of AS [2].
The counterparts of AS in traditional Chinese medicine (TCM) are exhibited as symptoms such as phlegm syndrome [3], obesity, dizziness, chest discomfort, apoplexy, and amnesia [4]. AS may be caused by Qi-Yang deficiency, phlegm, and blood-stasis according to TCM theory. Qi-Yang deficiency is the initial status resulting in AS and causes endothelial dysfunction. Endothelial dysfunction then brings out pathological phlegm and blood-stasis, which are counterparts of AS plaque, or excessive lipids in body according to TCM theory. Furthermore, phlegm and blood-stasis would be able to interact and deteriorate each other. Sini decoction (SND) is a classic and famous herbal formula, first documented in an ancient Chinese medical book named “Treatise on Cold-Induced Febrile Disease” (Shanghan Lun) written by Zhang Zhongjing in the Han Dynasty. The medicinal history of SND usage can be dated back more than 2000 years. In treating Yang deficiency, SND is effective and affirmative; in treating Qi deficiency, it is partially effective. In another Chinese medical book named “Shishi Milu” written by Chen Shiduo in the Qing Dynasty, it documented that tonifying Qi is the initial, basic, and fundamental treatment for the combined syndrome of Qi-Yang deficiency with phlegm. These books inspired ideas that drugs tonifying Qi and Yang might be used to treat disorders of lipids metabolism and also offered a reliable rationale for SND in AS treatment. Furthermore, long-term clinical application shows that SND can prevent CVD [5-8] as well as AS. Using SND and AS as search keywords, a literature survey of nationwide databases including CNKI (http://cnki.net/), VIP (http://qikan.cqvip.com/) and Wanfang (http://www.wanfangdata.com.cn), respectively reveals 36, 27, and 34 Chinese publications on SND usage in treating AS in both clinical and basic research. The total number of publications combined is 35. Additionally, in international databases, Liu et al [9] has identified that SND could alleviate intimal hyperplasia and thrombus formation in rabbits undergoing balloon injury of the iliac artery.

The main components of SND include Aconiti lateralis radix (Fuzi, ranunculaceae), Glycyrrhizae radix et rhizome praeparata cum melle (Zhigancao, Fabaceae), and Zingiberis rhizome (Ganjiang, Zingibaceae) [10]. Chen et al [10] delineated the chemical profile of five types of SND, identified 83 total medicinal compounds using HPLC/MS, and furtherey revealed that 55 compounds from SND aqueous extracts could be absorbed into the circulatory system of rats. Compounds from SND extracts include alkaloids, flavonoids, ginsenosides, triterpenoids, and bile acids. Alkaloids from Aconiti lateralis radix are major compounds [11] in SND and are particularly effective in preventing inflammation and oxidation damage [12, 13] as well as antagonizing coagulation and thrombosis. Flavonoids and triterpenoids are cardioprotective [14], hepatoprotective [15], and antioxidative compounds [16] from Glycyrrhiza radix and Rhizome praeparata cum melle [17]. Ginsenosides [18] and bile acids [19, 20] are also common active ingredients and exhibit cardiovascular protective effects due to their anti-inflammatory activity. These active compounds might constitute the chemical basis of SND in treating a large number of different human diseases, including AS.

Although SND is widely used clinically and possesses multiple ingredients involved in anti-inflammation, cardioprotection, anti-coagulation, and anti-thrombotic activity, it still needs further study to fully understand the fundamental mechanism in preventing CVD and AS by SND. In this study, it was determined that SND could enhance the RCT process, and the related indexes and possible mechanism in RCT were detected.

2 Materials and methods

2.1 SND extract preparation

Aconiti lateralis radix, Glycyrrhizae radix et rhizome praeparata cum melle, and Zingiberis rhizome were sliced, dried and mixed with a biomass ratio of 3:2:4. Then the materials were soaked in water with a biomass to volume ratio of 1:10 for 30 min, followed by boiling for 1 h. After repeating 2 times, the supernatant was collected by centrifugation and evaporated to a small volume (2.5 g raw herbs per ml) in vacuo.

2.2 AS rabbit model and treatment

24 New Zealand white rabbits (3 months old, male, weighing 1.8 kg – 2.2 kg) were purchased from Hunan Dongchuang Laboratory Animal Center (Changsha, China) and were fed separately.

After a one-week acclimation period, 24 rabbits were randomly divided into 4 groups. “Chow group” was fed a normal diet (n=6). 18 rabbits were fed 120 g/day high fat diet (HFD) supplemented with 1% cholesterol, 8% lard oil, and 0.05% cholate for 12 continuous weeks. “HFD group” was fed HFD (n=6), “Ator group” was fed HFD and treated with atorvastatin at a dose of 0.94mg/kg(n=6, q.d.), and “SND group” was fed HFD and treated with SND extract at a dose of 0.904ml/kg(n=6, q.d.). Atorvastatin powder and SND extract were mixed into HFD and fed first daily.
2.6 Western blot assay

Frozen tissues (adipose or part of the liver), peritoneal macrophages, and PMC were separately homogenized and lysed by lysis buffer, then proteins were detected by western blot for ABCA1 (ATP-binding cassette transporter A1), ApoA-I (apolipoprotein AI), Apo-B (apolipoprotein B), PPARγ (peroxisome proliferator-activated receptor γ), and LXRα (liver X receptor α). Briefly, the total protein content in lysates was measured by Coomassie brilliant blue assay (Beyotime, Shanghai, China). Equal amounts of protein was electrophoresed on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk, subsequently incubated 48 h with primary antibodies at 4°C, and then incubated with secondary antibodies (Boster, Wuhan, China) for 2 h. The blots were visualized using ECL detection reagents (Beyotime). Primary antibodies to ABCA1 and Apo-B were purchased from Abcam (MA, USA); PPARγ antibody and LXRα antibody were purchased from Proteintech (IL, USA); and ApoA-I antibody was purchased from MyBioSource (CA, USA).

2.7 Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed by one-way ANOVA, followed by Student’s two-tailed t-test for comparison between two groups. P < 0.05 was considered statistically significant.

3 Results

3.1 SND downregulated TC, TG, LDL-C, and upregulated HDL-C

Before HFD feeding (Fig.1A), the serum levels of TC, TG, LDL-C, and HDL-C were not different (P>0.05), indicating the comparability of the four groups. After 12weeks of HFD feeding for hypercholesterolemia and AS induction, TC, TG, and LDL-C in HFD rabbits were dramatically elevated in the serum and liver with significant difference compared to Chow rabbits (Fig.1B and 1C). The results indicated the successful establishment of an AS rabbit model. During treatment with atorvastatin or SND, TC, LDL-C, and TG levels in serum and liver tissues were notably decreased compared to HFD rabbits, and SND significantly increased HDL-C level, which was much higher than that achieved...
3.2 Improvement of lipid accumulation by SND

Due to the improvement against dyslipidemia by SND, we further explored the location of accumulated lipid in the proximal aorta and liver with Oil red O staining. As shown in Fig. 2A and 2B, it presented in normal structures of the proximal aorta and liver in the Chow group, rather than damaged structures in the HFD group. Arteries developed a bubble-shaped structure with lipid accumulation, and hepatic lobule damage with excess lipid in the liver was also observed. However, after SND treatment, lipid droplet clusters stained with Oil red O decreased remarkably in both proximal aorta and liver, compared to the HFD group. Abnormal structure in proximal aorta and liver was also notably decreased.

Figure 1. Levels of TC, TG, LDL-C, and HDL-C in serum and liver. Before HFD treatment, TC, TG, LDL-C, and HDL-C levels in serum were analyzed (A). After HFD feeding for 12 weeks with or without treatment with SND or atorvastatin, TC, TG, LDL-C, and HDL-C levels in serum (B) and liver lysates (C) were analyzed. Data shown as mean ± SEM (n = 6). # P<0.05, compared with Chow group; *P<0.05, compared with HFD group.
Increased ABCA1 protein levels in PMC, peritoneal macrophages, liver and adipose tissue by SND treatment

ABCA1 is broadly expressed in a variety of tissues and cells. We detected the effects of SND on ABCA1 expression in liver, macrophages, PMC, and adipose tissue. The liver is the site of cholesterol decomposition, and high levels of ABCA1 are reported in the liver. As shown in Fig. 3A, atorvastatin slightly increased ABCA1 level with statistical significance (P<0.05), whereas SND dramatically enhanced the liver ABCA1 level, nearly achieving the normal level found in the Chow group. Macrophages mainly export free cholesterol out of the cell (cholesterol efflux) through ABCA1 in the RCT process. Consistent with the central role of ABCA1 in cholesterol efflux, increased ABCA1 protein levels in were found in peritoneal macrophages and PMC (the cell that macrophages derive from) in Fig.3B and 3C, respectively. Increased ABCA1 level was also detected in adipose tissue in the SND group, indicating that SND had an RCT effect in adipose tissue, which is a type of tissue that stores cholesterol (Fig.3D). Furthermore, it was interesting that atorvastatin did not increase ABCA1 expression in peritoneal macrophages (Fig.3B), PMC (Fig.3C), or adipose tissue (Fig.3D), compared to the HFD group.

3.5 Enhancement of ApoA-I/Apo-B ratio by SND in RCT

As shown in Fig.3E, serum ApoA-I rose significantly in the HFD group compared with the Chow group (P<0.05) and continuously increased with SND treatment compared with the HFD group (P<0.05). with the HFD group (P<0.05). The serum Apo-B (Fig.3F) concentration remarkably increased in the HFD group and concentration remarkably increased in the HFD group and effectively decreased after SND treatment (P<0.05). Since the ratio of ApoA-I/Apo-B has been suggested to be highly predictive in evaluating cardiac risk [23], this ratio was also measured. Fig.3G shows that, regardless of the elevated trends of ApoA-I and Apo-B in the HFD or drug treatment groups, compared to the Chow group, the ApoA-I/Apo-B ratio in the HFD group decreased significantly, and a notably increased ratio was observed in both the Ator and SND group, indicating that the ApoA-I/Apo-B ratio is a sensitive index.
Induction of PPARγ and LXRα by SND

**Figure 3.** Regulation of ABCA1 expression and ApoA-I/Apo-B ratio by SND. After 12 weeks' HFD feeding and SND treatment, ABCA1 expression in liver (A), peritoneal macrophages (B), PMC (C), and adipose tissue (D) were analyzed by western blotting. Serum levels of ApoA-I (E), Apo-B (F), and ApoA-I/Apo-B ratio (G) were analyzed by biochemical methods. The protein levels of ApoA-I (H) and Apo-B (I) in livers were measured by western blot assay. Data shown as mean ± SEM (n = 6). # P<0.05, compared with Chow group; *P<0.05, compared with HFD group.

Since the liver is the major tissue synthesizing ApoA-I and Apo-B, levels of ApoA-I and Apo-B in the liver were measured. Although increasing trends in ApoA-I were found in the HFD and Ator groups, there was still no statistical significance in either group compared to the Chow group (Fig.3H). However, the SND group had a statistically significant increase in ApoA-I protein level even compared with the HFD group. For Apo-B (Fig.3I), HFD, Ator, and SND treatment all elevated Apo-B levels, but there was not significant difference between any two groups.

### 3.6 Induction of PPARγ and LXRα by SND

PPARγ can indirectly upregulate ABCA1 gene transcription by activating LXRα, so the expression of PPARγ and LXRα were measured. As shown in Fig.4A, hepatic PPARγ was significantly elevated in the HFD group. However, atorvastatin and SND could further boost the increase of PPARγ higher than that in the HFD group. Similar trends were also observed in peritoneal macrophages (Fig.4B) and PMC (Fig.4C) in that SND significantly increased PPARγ.
expression in HFD rabbits. In adipose tissue (Fig.4D), there was not a statistically significant increase of PPARγ level in the SND group compared with the HFD group. Moreover, although there was an increase in PPARγ in the HFD group compared with the Chow group, statistical significance was not observed in peritoneal macrophages (Fig.4B) and PMC (Fig.4C).

Both atorvastatin and SND treatment tended to induce LXRα expression in liver (Fig.4E), peritoneal macrophages (Fig.4F), and PMC (Fig.4G). In adipose tissue (Fig.4H), LXRα upregulation was more effective by SND than by atorvastatin.

4 Discussion

Using in vivo assays, the present study explored the following effects and mechanism against AS by SND: 1) relief of deteriorated lipid profiles and increased HDL-C levels both in serum and liver; 2) improvement of structural abnormality and reduced lipid deposition in artery and liver; 3) activation of the ABCA1/Apo-AI signaling pathway; and 4) increased PPARγ and LXRα protein levels.

Rabbits as a species are capable of both high cholesterol absorption and low lipid clearance so that plasma cholesterol can be dramatically elevated by dietary cholesterol [24]. Long-term intake of excessive cholesterol can induce rabbit hyperlipidemia and AS. In this study, compared to the Chow group, a variety of blood lipids increased significantly in the HFD group, successfully establishing an AS model with hyperlipidemia. Statins drugs, including atorvastatin, have fully confirmed anti-atherosclerotic effects [25]. In this study, the observed effect of atorvastatin on lipid profiles is consistent with the results of previous reports. That is, compared with the HFD group, a very significant lipid-lowering effect of TC, TG, and LDL-C in serum is observed. SND treatment presented a similar efficacy as atorvastatin in regard to preventing high cholesterol levels induced by HFD, which resulted in significantly lower levels of TC, TG, and LDL-C in serum. Moreover, it was interesting that SND upregulated HDL-C in liver and serum more effectively than atorvastatin, suggesting that an anti-AS-correlated

![Figure 4. Expression profiling of PPARγ-LXRα signaling to confirm the mechanism of treating AS by SND. The PPARγ (A-D) and LXRα (E-H) proteins levels in liver, peritoneal macrophages, PMC, and adipose tissue were measured by western blotting. The relative quantities were analyzed by ImageJ software. Data shown as mean ± SEM (n = 6). # P<0.05, compared with Chow group; *P<0.05, compared with HFD group.](image-url)
RCT mechanism should be explored, and RCT might be a superior mechanism in treating AS by SND. In addition, pathological section stained with Oil red O confirmed that SND reversed lipid accumulation and structural damage both in liver tissue and the proximal aorta. The results on the structural protection of the proximal aorta were consistent with Liu's report [9]. In regard to liver protection, Luo [26] has reported that modified SND could preserve liver function and prolong the survival time of acute liver failure rats.

Due to liver protection by SND, as well as the close relationship between the liver and RCT, key molecules in RCT were investigated to seek the possible mechanism involved in treating AS by SND. HDL-C-mediated RCT plays an important protective role against hyperlipidemia and AS development [27]. In the RCT process, outside HDL-C loads and transports cholesterol into the liver for cholesterol decomposition. As a result, the process protects peripheral tissues from excessive lipid accumulation and the development of atherosclerotic plaques. ABCA1 belongs to the superfamily of ATP-binding cassette (ABC) transporters and mediates the efflux of intracellular cholesterol to ApoA-I to produce HDL-C, which subsequently controls AS formation and lipid metabolism. ApoA-I is the most abundant protein in the HDL-C particle and plays the carrier role by incorporating cholesterol and phospholipids to form the HDL-C particle. Apo-B is correlated with LDL-C concentration and clearance [28] because Apo-B, as the main component of LDL-C, can remove LDL-C from lipid circulation by binding with the LDL-C receptor. In this study, ABCA1 expression was elevated by SND in PMC of blood, and it was consistent with the increased trend of Apo-A-I in the serum of the SND group, which indicated a relationship between ABCA1 and ApoA-I. Since the liver synthesizes ABCA1 and ApoA-I, their expression levels in the liver were also investigated, and a dramatic increase in the two proteins could be detected in the SND group. This proved that SND could upregulate the synthesis of ABCA1 and ApoA-I in the liver, potentially accounting for its protective effect in the circulatory system. Although levels of ApoA-I and Apo-B in serum and liver both rose in HFD rabbits after SND treatment, the Apo-A-I/Apo-B ratio was significantly elevated, which indicates that this index is very sensitive. Huang et al [29] found that the Apo-A-I/Apo-B ratio was strongly and positively associated with carotid intima-media thickness in a study with a large sample size (6069 participants). It indicated that an elevated ApoA-I/Apo-B ratio resulting from SND treatment might explain the structure protection in the liver and proximal aorta seen in pathological results.

PPARγ can indirectly upregulate ABCA1 gene transcription [30-32] through LXRs activation. The dimer of PPARγ and retinoic acid X receptor (RXR) binds to the promoter region of target genes for transcription activation. Furthermore, PPARγ agonists can remove cholesterol from human macrophage foam cells through the upregulation of ABCA1 and HDL-C, which decreases the ratio of cholesterol ester to free cholesterol [33]. The LXRs gene is a target of PPARγ, and ABCA1 promoters are regulated by the LXRs/RXR heterodimer [37, 38]. As a consequence of this transcriptional cascade, PPARγ and LXRs cooperate to promote ABCA1 expression and cholesterol efflux from macrophages. In this present study, SND significantly enhanced protein levels of PPARγ and LXRs in multiple tissues, indicating that SND could activate PPARγ and enhance LXRs both intracellularly and on an organ level. The results might explain increased ABCA1 expression in various tissues, since PPARγ and LXRs activate ABCA1 gene transcription.

In addition, we also found that the expression of PPARγ and LXRs in the HFD group were slightly upregulated in the liver (P<0.05) and remained unchanged in peritoneal macrophages, PMC, and adipose tissue, whereas the ABCA1 level in the HFD group decreased significantly in the liver, peritoneal macrophages, PMC, and adipose tissue. This might be explained by the proposal that ABCA1 is not only regulated by PPARγ and LXRs on the transcriptional level in the HFD rabbits but also was affected by other signaling pathways, such as the microRNA pathway. Goedeke [39] has summarized the microRNA regulation of ABCA1. Considering the abnormal decrease of ABCA1 in the HFD group of this study and the transcriptional cascade of PPARγ-LXRs signaling, it was speculated that SND perhaps not only upregulates ABCA1 expression through PPARγ-LXRs signaling but also might regulate and increase ABCA1 through other mechanisms.

5 Conclusion

Overall, the study showed that Sini decoction could reduce atherosclerosis in rabbits fed with a high fat diet by regulating the PPARγ, LXRs, and ABCA1-ApoA-I pathways and could reverse the transport of blood lipids from peripheral blood and adipose tissue to liver tissue to achieve a protective effect against atherosclerosis. However, the mechanism is very complex. Further studies are needed.
ABBREVIATIONS

ABCA1: ATP-binding cassette transporter A1; ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; AS: atherosclerosis; CVD: cardiovascular disease; HDL-C: high-density lipoprotein cholesterol; HFD: high fat diet; LDL-C: low-density lipoprotein cholesterol; LXRα: liver X receptor α; PMC: peripheral mononuclear cells; PPARγ: peroxisome proliferator-activated receptor γ; RCT: reverse cholesterol transport; SND: Sini decoction; TC: total cholesterol; TCM: traditional Chinese medicine; TG: triglyceride.

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