Effect of tetramethylpyrazine and hyperlipidemia on hepcidin homeostasis in mice

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Abstract. Iron homeostasis is strictly regulated in mammals, and disordered iron metabolism is recognized as a risk factor for various diseases, including cardiovascular disease. The hepcidin-ferroportin axis is the key signaling mechanism that controls systemic iron homeostasis. Increased serum hepcidin is associated with multiple types of cancer and atherosclerosis (AS), and therapeutics that decrease hepcidin levels have been proposed to treat these diseases. However, the effects of abnormal circulating hepcidin on hyperlipidemia remain unexploited. The natural compound tetramethylpyrazine (TMP) has been reported to have therapeutic effects on cardiovascular diseases, whereas the mechanisms involved remain incompletely understood. Thus, the effects of TMP on the expression of hepcidin in hyperlipidemic mice were investigated and the mechanisms involved were explored. Hyperlipidemia increased serum hepcidin, which was inhibited by TMP intervention. The results also indicated that TMP may decrease hepcidin expression via inhibition of Stat3 signaling. These findings suggest a promising rationale to prevent and hyperlidemia by targeting hepcidin or its upstream regulators, and highlight the potential application of natural compounds in treating hepcidin disorder-associated diseases.

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Abbreviations: FPN, ferroportin; AS, atherosclerosis; TMP, tetramethylpyrazine; TC, total cholesterol; TG, triglycerides; BMP, bone morphogenetic protein; IL-6, interleukin-6; HJV, hemojuvelin; HH, hereditary hemochromatosis; H&E, hematoxylin and eosin

Key words: hepcidin, atherosclerosis, tetramethylpyrazine, signal transducer and activator of transcription 3, Smad1/5/8

Introduction

Iron homeostasis is tightly regulated under normal settings, where the hepcidin-ferroportin axis is fundamentally responsible for the regulation of iron supply, utilization, recycling and storage. Hepcidin, a peptide hormone, is prominently synthesized by the liver and secreted into serum followed by tissue localization through circulation (1). Hepcidin inhibits iron absorption from the duodenum, and iron export from macrophages and hepatocytes is reduced by via its hepcidin-induced degradation of iron exporter ferroportin (FPN) (2). The expression of hepcidin is regulated by multiple signals directly or indirectly, including iron levels, anemia, hypoxia, inflammation, pathological conditions and cytokines, and changes in hepcidin expression are associated with various diseases (3). Deregulated hepcidin-FPN signaling is implicated iron-associated diseases, such as hereditary hemochromatosis and anemia of inflammation (4-6), and also in atherosclerosis (AS) (7-9).

AS is a chronic vascular disease and it is the pathological basis of various cardiovascular pathologies. There are different theories regarding the pathogenesis of AS, including lipid infiltration theory (10). With increasing studies on the metabolism of AS, the role of iron in the progression of AS has been established (11). A previous study reported that high expression of hepcidin in AS plaques can increase the iron deposition in macrophages and promote the instability of plaques (4). Tetramethylpyrazine (TMP), a natural compound extracted from the Chinese herb Chuanxiong rhizome (12), has been reported to have an anti-AS effect by inhibiting aggregation of platelet, proliferation of smooth muscle cells and reduced lipid peroxidation and protecting endothelial cell function (12,13). Thus, in the current study, the potential effect of TMP on hepcidin expression was investigated.

At present, two signaling pathways are recognized to predominantly control hepcidin expression under normal conditions, hemojuvelin (HJV)-bone morphogenetic proteins (BMPs)/Smad and Janus kinase (JAK)/signal transducer and activator of transcription (Stat) signaling pathways (14,15). BMPs are a class of multifunctional transforming growth factors, which have an important role in cell proliferation, differentiation, apoptosis and tissue development (16). Among

them, BMP6 also has an important role in iron absorption and storage via modulation of hepcidin expression. HJV, a co-receptor of BMP, promotes the phosphorylation of Smad1/5/8 and, thus, stimulates the expression of hepcidin (17). The JAK/Stat signaling pathway regulates the expression of hepcidin in response to inflammatory factors, and interleukin 6 (IL-6) is the major factor that regulates hepcidin expression in inflammatory responses mediated by Stat3 (18). Furthermore, it has been reported that JAK/Stat signaling regulates hepcidin via SMAD4 (19). Therefore, to fully understand the underlying mechanism of the potential effects of TMP on hepcidin expression regulation, changes of the phosphorylation of Stat3 and Smad1/5/8 protein were also investigated in this study.

Materials and methods

Drug, reagents and diets. TMP (molecular formula $C_8H_{12}N_2 \cdot HCl \cdot 2H_2O$) extracted from Chinese medicinal plants was purchased from the National Institutes for Food and Drug Control of China (Beijing, China). Total cholesterol (TC) reagent and triglyceride (TG) reagent were purchased from Beijing Wantaiderui Diagnostic Technology Company (Beijing, China). Serum hepcidin ELISA kit (cat. no. AE92047Mu) was purchased from AMEKO (Lianshuo Biotechnology Co., Ltd., Shanghai, China). Ordinary diet was purchased from Beijing Keao Xieli Feed Co., Ltd. (Beijing, China). High-fat diet were purchased from Beijing NuoKangYuan Biotechnology Company (Beijing, China), and the ingredients were as follows: 2% cholesterol, 0.5% bile salt, 10% lard, 10% egg yolk powder, 5% sugar and 72.5% basic feed.

Animal experiments. Specific pathogen-free C57BL/6 mice (16 male and 16 female; 4 weeks old; 18-20 g) were purchased from Beijing Weitong Lihua Experimental Animal Technology Company (Beijing, China), and housed in the animal breeding room of Xiyuan Hospital, China Academy of Chinese Medical Sciences (Beijing, China). Mice were selected randomly for the following experiments. All mice were divided into the following groups: Control group fed with normal diets (group N; n=8); high-fat diets (group M; n=8), mice fed with normal diets plus TMP treatment (group NT; n=8) or high-fat diets plus TMP treatment (group MT; n=8). The high-fat model was generated by feeding with high-fat diets for 4 weeks. The control group mice were administered 2 ml/(kg/day) normal saline via intraperitoneal injections for 7 days consecutively, and TMP treatment was performed by daily intraperitoneal injection of 40 mg/kg body weight for 7 days.

Following anesthesia of mice, serum and liver samples were collected. Liver samples were fixed in 4% paraformaldehyde or stored at -80˚C for further analysis. All animal feeding and experiments were in compliance with and approved by the ethics committee of the Gansu University of Traditional Chinese Medicine (Lanzhou, China).

Assay of serum indices. Serum TG and TC were analyzed using a T300 automatic biochemical analyzer (DIRUI Industrial Co., Ltd, Changchun, China), including TG and TC, the methods and procedures were determined according to the manufacturer's instructions. The serum levels of hepcidin were detected using ELISA kits according to the manufacturer's instructions.

Western blot analysis. Following treatment with TMP, liver tissues were snap-frozen in liquid nitrogen and homogenized in the radioimmunoprecipitation (RIPA) assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and total proteins were then extracted using RIPA supplemented with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The concentration of liver tissue protein was measured using a bicinchoninic acid protein assay kit according to the manufacturer's protocol. Equal amount of protein lysates (30-50 μg/sample) were subjected to SDS-PAGE on 8-12% gels and transferred onto nitrocellulose membranes, which were blocked with 7% nonfat milk/Tris-buffered saline-Tween or 5% bovine serum albumin and hybridized with specific primary antibodies overnight at 4˚C, followed by 1 h of hybridization with specific secondary antibody (anti-mouse antibody, 1:8,000; anti-rabbit antibody 1:8,000) at 37˚C as described previously (20,21). The primary antibodies used were anti-phospho (p)-Stat3 antibody (1:1,000; cat. no. 9145; Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-Stat3 antibody (1:1,000; cat. no. 9139; Cell Signaling Technology, Inc.), anti-p-Smad1/5/8 antibody (1:1,000; cat. no. sc-12353; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Smad1 antibody (1:1,000; cat. no. sc-7965; Santa Cruz Biotechnology, Inc.), and anti-GAPDH (1:2,000; cat. no. G8795; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). GAPDH was used as a loading control. Densitometric measurements of immunoblotted bands were determined using ImageJ digitalized software (National Institutes of Health, Bethesda, MA, USA).

Histological examination. The liver tissues were fixed in 10% buffered formaldehyde at 4˚C for 7 days, then embedded in paraffin. Tissue sections (5-8 μm) were stained with hematoxylin and eosin to examine the morphology of the liver following a standard protocol as previously described (21-23).

Statistical analysis. SPSS Statistics 19.0 package (IBM Corp., Armonk, NY, USA) was used to analyze the experimental data; statistical differences were analyzed by independent t-test or one-way analysis of variance (ANOVA) test. Alternatively, post hoc test (Student-Newman-Keuls) used following ANOVA and non-parametric test (Kruskal-Wallis H) was employed for non-normal distributions. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of high-fat diet and TMP intervention on lipid metabolism. Initially, the effects of high-fat diet and TMP intervention on lipid metabolism were investigated. As shown in Fig. 1A, the level of serum TC in groups M and MT was significantly increased compared with group N ($P<0.05$), demonstrating the successful establishment of a hyperlipidemia mouse model. However, there was no significant change in serum TC among the groups N and FN, and groups M and FM ($P>0.1$), demonstrating that TMP intervention does not attenuate hyperlipidemia. Furthermore, no significant change in serum TG was observed among all groups ($P>0.1$; Fig. 1B).

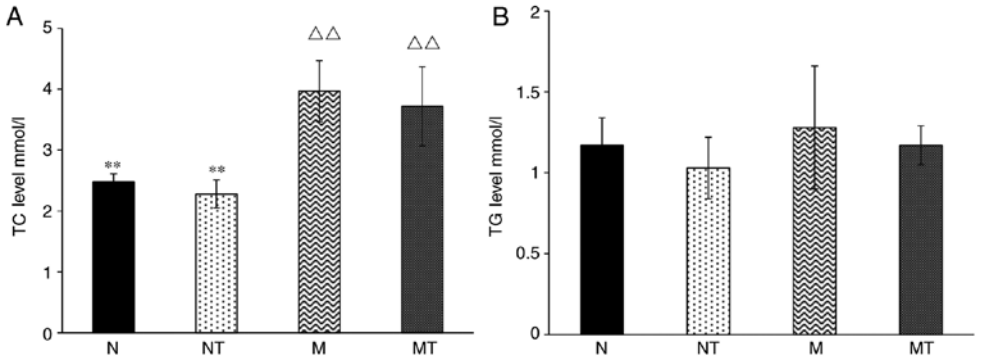

Figure 1. Levels of serum (A) TC and (B) TG. Mouse serum samples was collected for measuring the blood lipid indices (TG and TC) using automatic biochemical analyzer (n=8). $^{\Delta\Delta}$P<0.01 vs. group N; **P<0.01 vs. group M. TMP, tetramethylpyrazine; TC, total cholesterol; TG, triglyceride; N, normal diet; NT, normal diets plus TMP treatment; M, high-fat diet; MT, high-fat diets plus TMP treatment.

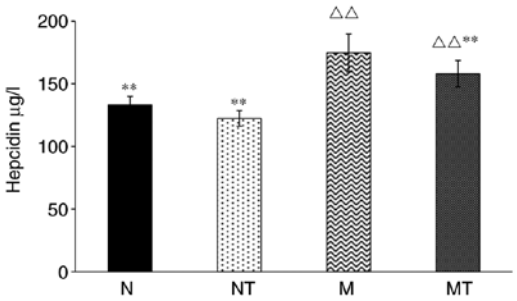

Figure 2. Level of serum hepcidin. Serum hepcidin concentration was determined by ELISA (n=8). $^{\Delta\Delta}$P<0.01 vs. group N; **P<0.01 vs. group M. TMP, tetramethylpyrazine; N, normal diet; NT, normal diets plus TMP treatment; M, high-fat diet; MT, high-fat diets plus TMP treatment.

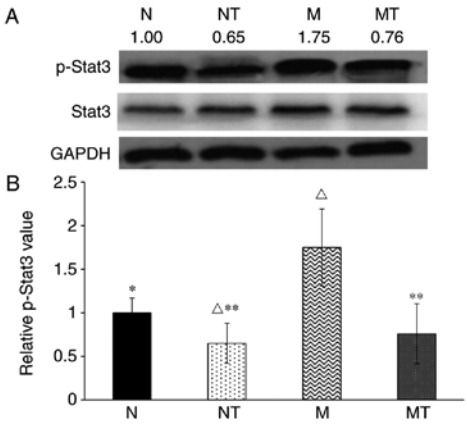

Figure 3. Changes in p-Stat3 protein levels in liver upon TMP treatment. (A) Mice were treated with TMP at 40 mg/(kg/day) for 7 days. Livers were then collected for total protein extraction. The changes of phosphorylated Stat3 concentrations were determined via western blot analysis. Total Stat3 was also assessed for normalization. (B) Ratio of p-Stat3 and total Stat3 protein contents (n=3 replicates). Total Stat3 was determined for normalization. GAPDH was used as an internal control. $^{\Delta}$P<0.05 vs. group N; *P<0.05, **P<0.01 vs. group M. TMP, tetramethylpyrazine; p-, phospho-; Stat3, signal transducer and activator of transcription; N, normal diet; NT, normal diets plus TMP treatment; M, high-fat diet; MT, high-fat diets plus TMP treatment.

These results demonstrated that TMP intervention had no effect on high-fat diet-induced hyperlipidemia.

Effect of hyperlipidemia and TMP intervention on serum hepcidin. As the main regulator of systemic iron homeostasis, the effects of hyperlipidemia and TMP intervention on hepcidin expression was also investigated. As shown in Fig. 2, the levels of serum hepcidin in groups M and MT were increased dramatically compared with group N (P<0.01), whereas there was no significant difference between group NT and group N. Compared with group M, the level of hepcidin in group MT was significantly decreased (P<0.01). Therefore, these results demonstrated that the high-fat diet resulted in an increase of serum hepcidin concentration, which was partially inhibited by TMP treatment.

Effect of hyperlipidemia and TMP intervention on the phosphorylation of p-Stat3 protein. In order to understand the molecular mechanism involved in these effects, the signaling pathways that regulate hepcidin expression regulation were investigated. Although the expression of hepcidin is altered by a variety of stimuli, two signaling pathways are recognized to predominantly control hepcidin expression: IL-6-JAK-Stat3 and BMP-Smad signaling (14,15). As shown in Fig. 3, the level of p-Stat3 was significantly increased in group M compared with group N (P<0.05). Following TMP intervention, p-Stat3 protein contents the TMP-treated groups were significantly decreased compared with their respective control groups. However, as shown in Fig. 4, there was no significant

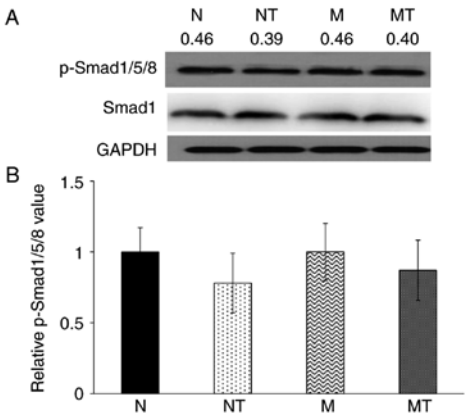

Figure 4. Alteration of p-Smad1/5/8 levels in liver upon TMP treatment. (A) Mice were treated with TMP at 40 mg/(kg/day) for 7 days. Livers were collected for total protein extraction. p-Smad1/5/8 concentrations were determined by western blot analysis. Total Smad1 was determined for normalization. (B) The ratio of p-Smad1/5/8 and total Smad1 (n=3 replicates). Total Smad1 was determined for normalization and GAPDH was used as an internal control. TMP, tetramethylpyrazine; p-, phospho-; N, normal diet; NT, normal diets plus TMP treatment; M, high-fat diet; MT, high-fat diets plus TMP treatment.

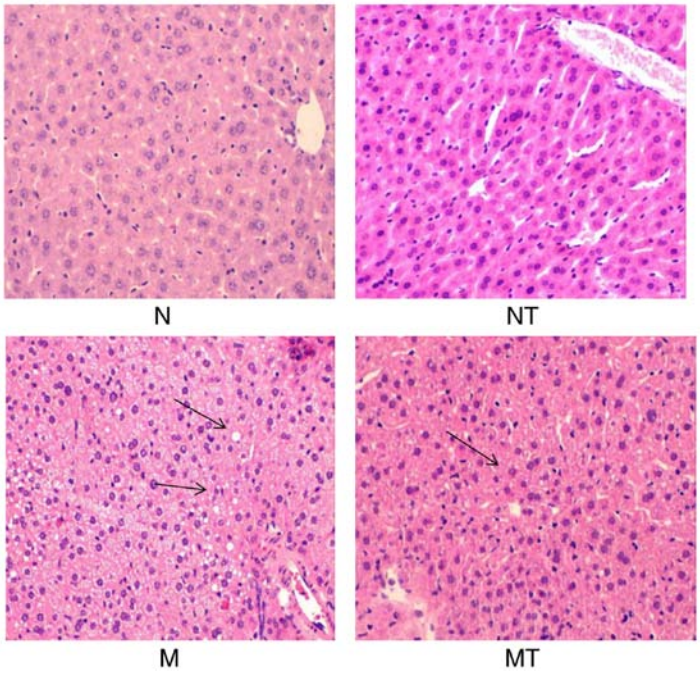

Figure 5. Histological examination of liver specimens. Liver specimens were fixed for histological examination. The representative images of liver sections were stained with hematoxylin and eosin. Original magnification, x200. TMP, tetramethylpyrazine; N, normal diet; NT, normal diets plus TMP treatment; M, high-fat diet; MT, high-fat diets plus TMP treatment.

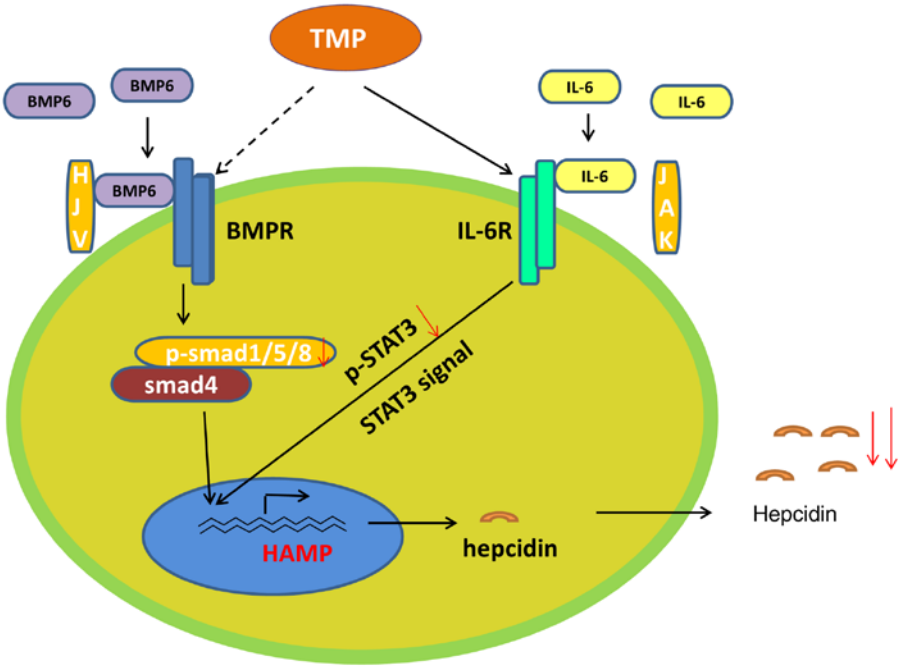

Figure 6. Potential effect of TMP on hepcidin expression. Two signaling pathways predominantly control hepcidin expression: Stat3 and Smad1/5/8 pathways. TMP, tetramethylpyrazine; BMP, bone morphogenic protein; IL, interleukin; JAK, Janus kinase; BMPR, BMP receptor; IL-6R, IL-6 receptor; p-, phospho-; Stat3, signal transducer and activator of transcription; HAMP, hepcidin antimicrobial peptide.

difference in the levels of p-Smad1/5/8 in liver samples among all groups (P>0.1). These results demonstrated that hyperlipidemia upregulated hepatic hepcidin expression by enhancing the phosphorylation of Stat3, but not Smad1/5/8, while TMP intervention inhibited this effect.

Pathological changes in the liver. As shown in Fig. 5, clear structures of hepatic lobules and hepatic cords were observed in group N and NT with large and round nuclei in the center of the cells, which were arranged orderly. However, liver lobule structures in group M were not clear, as shown by the circular fat vacuoles with different sizes, and the hepatocytes with degeneration, inflammatory cell infiltration and apoptosis were also observed. However, significant improvements in pathological features were observed in group MT compared with group M (Fig. 5). No noticeable alterations were observed

in liver sections from group NT compared with group N. These results demonstrated that TMP intervention attenuated hyperlipidemia-induced hepatic structural abnormalities, which may be associated with regulation of hepcidin expression in the liver.

Discussion

Although iron metabolism has been investigated in numerous previous studies, the molecular mechanisms of systemic iron homeostasis have been only been recently identified, with the characterization of the hepcidin-FPN axis (4,24). Disorder of iron metabolism is associated with various diseases, including iron-deficiency anemia and iron overload diseases, and the deregulation of hepcidin expression is also involved in these iron disorder diseases (15). It has been previously reported that hyperlipidemia can induce iron deposition in the aorta of mice (25). Compared with the use of proteins and synthesized chemicals as drugs, natural compounds from traditional Chinese medicinal plants have important properties, including abundant natural availability, high stability and low toxicity (26,27). Previous studies have suggested that certain medicinal plant extracts and natural compounds are able to regulate hepcidin expression (28,29). Among 16 medicinal plant extracts tested in a previous study (28), Caulis Spatholobi exhibited the greatest inhibitory effect on hepcidin expression. Another study testing 12 natural compounds reported that icariin enhanced the expression of hepcidin (29). TMP has been reported to have various therapeutic effects on inflammation and cardiovascular diseases, and to prevent cells from oxidative damage *in vitro* and in experimental animal models (12). TMP also been reported to improve clinical manifestations and liver function, which may be mediated by reduced expression of tissue factor via downregulated expression of the transcription factors, early growth response factor-1 and nuclear factor-κB p65 (30). In our previous study, TMP was shown to reverse the effect of iron deposition on endothelial functions and reduce IL-6 levels in hyperlipidemic FPN1 knockout mice (25). In the present study, the association between hyperlipidemia and serum hepcidin, and the effect of TMP were investigated. The results demonstrated that hyperlipidemia increased the serum hepcidin level, which was reversed by TMP treatment.

Additionally, the molecular mechanisms underlying the stimulating effect of hyperlipidemia and inhibitory effect of TMP intervention on hepcidin expression were investigated. The JAK/Stat and HJV-BMP/Smad pathways are two fundamental signaling pathways that regulate for hepcidin expression (13,14,31). Inflammatory factors, including IL-1, IL-6 and TNF-α, regulate the expression of hepcidin via the JAK/STAT pathway, and the role of IL-6 is the most established (32). IL-6 and IL-6 receptor binding can activate the JAKs and Stat3; Stat3 is then phosphorylated, moves into the nucleus and binds the hepcidin gene promoter to regulate hepcidin expression. HJV is a glycosyl phosphatidylinositol-linked cell surface protein expressed in skeletal muscle, liver cells and myocardial cells. HJV acts on BMPs to stimulate the growth of neurons. BMPs activate BMP receptor signal transduction, which promotes Smad1/5/8 phosphorylation. Phosphorylated Smad1/5/8 and SMAD4 form a complex to stimulate hepcidin expression (33,34). The results of the present study revealed that the phosphorylation of Stat3 was significantly enhanced mice with hyperlipidemia, and subsequently inhibited by TMP intervention. However, phosphorylation of Smad1/5/8 protein was not significantly altered by hyperlipidemia or TMP. Furthermore, the disordered liver structure may be associated with the upregulation of hepcidin expression in mice with hyperlipidemia, which was also be attenuated by TMP intervention. These results indicate that TMP may activate IL-6R and then induce Stat3 signaling (Fig. 6).

Notably, TMP had no significant inhibitory effect on hepcidin expression in normal mice, which may be associated with the normal expression of inflammatory factors (IL-6 and TNF-α among others) in these mice. In order to validate a direct causal relationship, RNA interference/overexpression experiments should be performed. There are limitations in the present study research will be undertaken, including setting multiple time points to observe the differences in the degree of elevated blood lipids, which may have different effects on the JAK/STAT and HJV-BMP/SMAD pathways, and effects of different doses of TMP on the expression of hepcidin and the pathways. *In vivo* experiments will be designed as the basis for supporting the *in vitro* findings.

In conclusion, TMP intervention blocked the simulation of hepatic hepcidin expression induced by hyperlipidemia, which decreased serum hepcidin levels, potentially by inhibiting Stat3 phosphorylation. The results may provide a promising novel strategy for the treatment of hyperlipidemia and AS using natural compounds, particularly extracts from traditional Chinese medicine plants, by targeting hepcidin expression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HJY conceived and designed the study. MZ, MYS, CYG and JSW performed the experiments and analyzed the data. MYS, MZ, CYG and JSW contributed reagents and materials. MZ, MYS, FQX and HJY designed the study, wrote and revised the manuscript.

Ethics approval and consent to participate

All animal feeding and experiments were in compliance with and approved by the ethics committee of the Gansu University of Traditional Chinese Medicine (Lanzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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