Effects of Sheep Bone Collagen Peptide on Liver Lipid Deposition in Ovariectomized Rats

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Summary Liver can be directly involved in the synthesis and decomposition of fatty acids. Liver lipid deposition is one of the most common chronic liver diseases. Estrogen deficiency can cause lipid deposition and energy metabolism disorders in the liver. Sheep bone collagen peptide (SBCP) has been shown to have estrogen-like effects in previous studies. And SBCP has high bioavailability, safety and non-toxic side effects. This study aimed to investigate the effect of SBCP on liver lipid deposition (LLD) caused by estrogen deficiency. Female Wistar rats were treated as follows (n=10): sham group: underwent peri-ovary fat removal operations, ovariectomized rats (model group), ovariectomized rats receiving SBCP treatments: SBCP high dose group (SBCP-H), SBCP medium dose group (SBCP-M) and SBCP low dose group (SBCP-L). After 8 wk, the model group demonstrated severe LLD and liver pathological changes, with increased malondialdehyde (MDA) and free fatty acid (FFA) levels (p<0.05). Additionally, the total superoxide dismutase (T-SOD) activity (p<0.05), serum albumin-to-globulin (A/G) ratio (p<0.05), amount of butyric acid-producing bacteria and short-chain fatty acids (SCFAs) content decreased. SBCP intervention could inhibit the occurrence of LLD and alleviate the liver histopathological damage induced by estrogen deficiency by relieving oxidative stress, preventing the loss of butyric acid-producing bacteria, and decreasing the abundance of Lactobacillus reuteri in the gut. The results suggested that SBCP could improve the LLD induced by estrogen deficiency.

Key Words liver lipid deposition, collagen peptide, estrogen deficiency, butyric acid-producing bacteria, lactobacillus reuteri

The liver plays a critical role in regulating lipid metabolism in the body (1). Liver lipid deposition (LLD) is one of the most common chronic liver diseases worldwide (2). Excessive LLD can cause obesity, hepatic adipose infiltration, and other metabolic diseases (3, 4). Multiple factors cause LLD, including oxidative stress, insulin resistance, inflammation, and other metabolic disturbances (5–7). In addition, LLD can be easily induced by estrogen deficiency, which causes lipid metabolism disorders in the liver (8, 9). Accumulated studies have shown that estrogen replacement therapy can greatly reduce the incidence of LLD, suggesting that estrogen has a protective effect on liver lipid metabolism (10, 11). However, estrogen replacement therapy will inevitably induce detrimental effects (12). Thus, scientists are searching for alternative options to inhibit the occurrence of LLD.

Collagen is an extensively distributed protein that is traditionally extracted from the skin of land animals (13). Sheep bone collagen can be obtained from the byproducts of the lamb processing industry, with a yield of approximately 12.5% to 18% (14), and can be processed into sheep bone collagen peptide (SBCP) by enzymatic hydrolysis. SBCP has demonstrated high biological activities such as antihypertensive, antimicrobial, antioxidative, anticancer, and immunomodulatory functions (15–17), with the merits of low immunogenicity and good biocompatibility.

SBCP was also proven to have estrogen-like effects in the prevention and treatment of osteoporosis caused by estrogen deficiency in our previous study (18). The purpose of this study was to investigate whether SBCP could inhibit LLD induced by estrogen deficiency and to unveil the underlying mechanisms. First, the LLD model was established by removing the bilateral ovaries of the rats (19). Then, the ovariectomized rats were treated with SBCP. Finally, histopathological changes, LLD, liver function, oxidative stress levels, and changes in intestinal microflora were examined. All these studies would be useful for identifying a potential and alternative therapeutic agent for LLD and finding a new and high-value application of the byproducts of animal food processing.

MATERIALS AND METHODS

Animals and treatment. Fifty 8-wk-old specific pathogen-free female Sprague-Dawley rats (body weight, 235.2±21.62 g) were purchased from Beijing Changhai Western Mountain Farm (SCXK[Jing]2017-0005). After 1 wk of acclimatization to the new environment, 40 rats were subjected to bilateral ovariectomies and 10

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in the sham group underwent peri-ovary fat removal operations. The sham group was the blank control group. One week later, the ovary-free rats were randomly divided into 1 model group and 3 SBCP groups (n=10). The rats in the SBCP groups were given an intragastric administration of SBCP (Inner Mongolia Taihao Biological Products Co., Ltd., China) daily for 8 consecutive weeks according to body weight (kg bw). SBCP was a small molecular peptide made by enzymolysis of sheep bone. The molecular weight of SBCP was less than 1,000 Dalton. According to the previous administration concentration (18), the SBCP dosages for the high- (SBCP-H), medium- (SBCP-M), and low-dose groups (SBCP-L) were 3.40, 2.05, and 0.68 g/kg bw, respectively. SBCP powder was dissolved in 1 mL of distilled water before administration. In the model and sham groups, the same volume (1 mL) of distilled water was intragastrically administered every day. All the rats were caged individually with free access to food and water and kept in an air-conditioned (22–24°C) and humidity-controlled room (relative humidity, 60–65%), with a 12-h light/dark cycle throughout the experiment. Each rat was weighed once per week. All experimental protocols were reviewed and approved by the ethics committee of Shanxi Agricultural University for the Use of Laboratory Animals (IACUC: SXAU-EAW-2020 SD 0201).

Collection of blood samples and liver tissues. After 8 h on the last day of SBCP administration, all the rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate at 3 mL/kg bw. Blood samples were collected from the orbital cavity. Serum was collected by centrifugation for 10 min at 4°C and stored at −80°C for further analysis. The liver tissues were sampled immediately after exsanguination. For each liver, a portion was fixed in 10% formaldehyde. Other tissues were embedded in optimal cutting temperature (OCT) compound for histopathological examination. The remaining tissues were stored in liquid nitrogen for antioxidative capacity analysis. The liver weight (g) divided by the body weight (g) was defined as the liver index.

Evaluation of liver function and hepatocyte injury. To evaluate the degree of hepatocyte injury, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and free fatty acid (FFA) levels were measured on an A6 semi-automatic biochemical analyzer (Songshan Technology Co., Ltd., Beijing, China). As indicators of liver function, blood albumin (ALB) level, globulin (GLB) level, and albumin-to-globulin (A/GL) ratio were examined using an automatic biochemical analyzer (SMT-120V, Seamaty, Chengdu, China).

Histological changes in liver tissues. After fixation in formaldehyde, liver tissues were embedded in paraffin and then sectioned. The resulting slides were stained with hematoxylin and eosin (H&E). To examine the accumulation of lipid droplets (LD), the frozen hepatic tissues in the OCT compound were cut into 8-μm-thick sections using a cryostat (Leica, CM1850) and stained with Sudan III. Histological images of the liver were observed under optical microscopy (Olympus, Inc., Tokyo, Japan) at ×400 original magnification. The morphological changes observed in the H&E-stained sections were assessed and scored by 3 experienced pathologists according to the following scales: disorder of the hepatic cord arrangement (0–5 points), erythrocyte infiltration (0–5 points), and hepatocyte swelling or steatosis (0–5 points). The Sudan III dyed cryosections were examined to determine the distribution of LD in hepatocytes (0–5 points) and disorder of the hepatic cord arrangement (0–5 points). Results were compared using non-paired statistical analyses.

Antioxidative capacity of liver homogenates. The total superoxide dismutase (T-SOD: A001-1) and malondialdehyde (MDA; A003-1) levels in liver homogenates were detected following the manufacturer’s instructions for the corresponding commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

16S rDNA sequencing. On the last day of the experiment, fresh rat fecal samples (1 g) were collected from the rats in the sham, model, and SBCP-M groups (approximately 0.1 g each). The fecal genomic DNA was extracted, and the V3-V4 region of the 16S rDNA gene of bacterial samples was amplified and sequenced.

Detection of SCFAs content in feces. Short-chain fatty acids (SCFAs) content in feces of rats in the sham group, model group, and SBCP-M group (approximately 0.3 g each) was determined by gas chromatography-mass spectrometry (GC-MS-QP2010 Ultra, Shimadzu, Japan). The specification of Wax capillary column was 30 m×0.25 mm×0.25 μm (Wax, Shimadzu). The interface temperatures of the injector, ion source and GC-MS were set at 220°C, 230°C and 220°C, respectively. The flow rate of helium carrier gas was 1.0 mL/min. Add sample 1 μL, the solvent delay time was 3.5 min, without shunt. The initial column temperature was 40°C and maintained for 3 min, then the temperature was raised to 210°C at a rate of 30°C/min and maintained for 5 min. Ionization took place in electron bombardment mode at a tuned voltage. MS data were collected in sin mode with a collection interval of 0.3 s. The compounds were identified by injecting pure standard compounds and comparing the retention time and corresponding MS spectra.

Statistical analyses. Statistical analysis of the data was carried out using the Tukey method of the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as mean±SD. Values labeled with different lowercase letters mean significant difference (p<
0.05), if same, mean no significant difference (p>0.05).

**RESULTS**

*SBCP administration reduced ovariectomy-induced body weight gain*

After 8 wk of treatment, the mean body weight gains in the model group was significantly higher than those in the SBCP (p<0.05) and sham groups (p<0.05; Fig. 1A). The weight gain was higher in the SBCP groups than in the sham group (p<0.05) and showed no dose-dependent effect (Fig. 1A). No statistically significant difference in liver index was found among all the groups (p>0.05; Fig. 1B).

![Fig. 1. Body weight development and liver index. (A) Body weight; (B) Liver index (×10^{-3}); Data were expressed as mean±SD.](image)

![Fig. 2. Evaluation of liver function and hepatocyte injury. (A) Aspartate aminotransferase (AST) levels; (B) Alanine aminotransferase (ALT) levels; (C) Free fatty acid (FFA) levels; (D) Blood albumin (ALB) levels; (E) Globulin (GLB) levels; (F) Albumin-to-globulin ratios (A/G). Data were expressed as mean±SD. Different lowercase letters above the column mean significant difference (p<0.05).](image)
SBCP attenuated LLD and improved the liver histopathological changes induced by estrogen deficiency

AST and ALT are enzymes normally located inside hepatocytes, and serum AST and ALT levels are commonly used as indicators of hepatocyte injury. Elevated serum FFA levels indicate lipid metabolism disorders and the development of LLD. In the present study, serum AST and ALT levels showed no significant differences among the five groups ($p>0.05$; Figs. 2A and 2B). Compared with the sham group, the model group had a significantly higher FFA level ($p<0.05$), which was significantly reduced with SBCP intervention ($p<0.05$; Fig. 2C). We can deduce that LLD might have occurred in the model group and that the SBCP groups had less LLD than the model group. In comparison with the sham group, the model group had remarkably lower ALB levels and significantly higher GLB levels ($p<0.05$; Figs. 2D and 2E), which resulted in a significantly
decreased A/G ratio \((p<0.05; \text{Fig. 2F})\). The decreased ALB levels, A/G ratios, and increased GLB levels suggested histopathological changes and weakened function of liver protein synthesis in the model group. As shown in Figs. 2D, 2E, and 2F, SBCP administration had no significant effect on the ALB level and A/G ratio \((p>0.05)\) but remarkably decreased the GLB level \((p<0.05)\), which was associated with inflammation.

In the sham group, the structure of the hepatic lobule remained intact and hepatocytes were clearly structured and regularly arranged in the liver cords that were radially distributed around the central vein (CV; Fig. 3A-a). In the model group, the hepatic cords were disorganized. The hepatocytes became swollen and appeared as vacuoles of different sizes (round or irregular) in the cytoplasm (Fig. 3A-b). SBCP intervention improved the above-mentioned histopathological changes. The hepatic cords in all the SBCP groups were maintained. In the SBCP-H group, hepatocytes were maintained normally and clearly (Fig. 3A-c). In the SBCP-M group, a few round or irregular vacuoles of different sizes could still be found inside the liver cells (Fig. 3A-d). In the SBCP-L group, the hepatic cords were arranged regularly and radially, while some hepatocytes were slightly swollen, and red-stained fine particles could be found in the cytoplasm (Fig. 3A-e).

Sudan III was used to visualize LDs in liver cells (i.e., LLD) in frozen sections. In the sham group, LD were scarcely scattered, whereas in the model group, many LD accumulated in the hepatocytes around the CV (Figs. 3B-a and 3B-b). There were fewer LLD in both the SBCP-M and SBCP-H groups than that in the model group (Figs. 3B-c and 3B-d). In the SBCP-L group, hepatocytes containing LD were markedly reduced (Fig. 3B-e). The histopathological score in the model group was significantly higher \((p<0.05)\) than that in the sham group, and the scores in the SBCP groups were significantly lower than that in the model group (Figs. 3A-f and 3B-f).

All these observations indicated that ovariectomy or estrogen deficiency could lead to liver pathological changes and LLD in rats, and SBCP intervention could improve the histological changes and inhibit LLD accumulation. Low-dose SBCP administration exhibited the best effect.

**Enhanced antioxidative capacity of the liver**

The T-SOD activity varied according to the sample concentration. When the inhibition rate of the enzyme is 45–50%, the corresponding concentration of liver homogenates is the best sampling concentration. In the present study, the T-SOD inhibition rate was 50% in 0.1% liver homogenates (Fig. 4A). The T-SOD activity was significantly lower in the model group than in the sham group \((p<0.05; \text{Fig. 4B})\) but significantly higher in all the SBCP groups than in the model group \((p<0.05)\). Furthermore, the T-SOD activity in the SBCP-L group was not significantly different from that in the sham group \((p>0.05; \text{Fig. 4B})\). The MDA level in the model group was significantly higher than that in the sham group \((p<0.05; \text{Fig. 4C})\). SBCP intervention decreased the MDA levels in a dose-dependent manner, and the SBCP-H group showed no statistically significant difference from the sham group in terms of decrease in the MDA level \((p>0.05; \text{Fig. 4C})\).
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Fig. 5. The total profiling of intestinal microbes in three groups.

Fig. 6. Relative abundance of intestinal microflora in each group. (A) The relative abundance of intestinal microflora at the phylum level; (B) The relative abundance of intestinal microflora at the genus level; (C) The relative abundance of intestinal microflora at the species level.
**Changes in intestinal microorganisms**

Firmicutes (52%), Bacteroides (34%), Actinomycetes (8%) and Proteobacteria (4%) were the dominant microflora in the intestinal microorganisms of all groups. Clostridia and Bacilli accounted for the largest proportion in Firmicutes, with 38% and 12%, respectively (Fig. 5). The dominant bacteria in Clostridiales (38%) were Ruminococcaceae and Lachnospiraceae, accounting for 22% and 12%, both of which were butyric acid-producing bacteria (Fig. 5) (20, 21). At the family level, the relative abundance of Ruminococcaceae in each group were 30% in the sham group, 17% in the model group, and 20% in the SBCP-M group. The relative abundance of Lachnospiraceae among the groups was 19%, 7% and 11%. At the genus level, the relative abundance of *Ruminococcus UCG-005* in each group were 8% in the sham group, 1% in the model group, 5% in the SBCP-M group; the relative abundance of *Ruminococcus* 1 among the groups was 3%, 2% and 5%; the relative abundance of *Lachnospiraceae NK4A136 group* among the groups was 3%, 1% and 3%. The relative abundance of *Ruminococcaceae UCG-005*, *Ruminococcaceae*, *Lachnospiraceae*, *Ruminococcus* 1 and *Lachnospiraceae NK4A136 group* in each group followed the order of sham group ≫ SBCP-M group ≫ model group (Figs. 6A and 6B). This indicates that the butyric acid-producing bacteria of the Ruminococcaceae and Lachnospiraceae were missing from the rats’ guts owing to estrogen deficiency, and SBCP could inhibit the decrease of their abundance. Compared with
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the sham group (0.1%), the relative abundance of *Lactobacillus reuteri* increased in the model group (1%), which SBCP intervention (0.5%) effectively reduced (Fig. 6C).

Changes in SCFAs content in feces

According to the GC-MS chromatogram (Fig. 7) and quantitative results of the spectra (Table 1), eight kinds of SCFAs were isolated and identified from rat feces, which were acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and caproic acid. According to the detection results of SCFAs in rat feces (Table 2), the content of butyric acid in model group was significantly lower than that in sham group, while the contents of acetic acid, propionic acid, isobutyric acid, valeric acid, isovaleric acid and caproic acid were significantly increased. The content of SCFAs in SBCP-M group was significantly lower than that in model group.

DISCUSSION

Ovariectomy can cause estrogen deficiency in rats and is often used to develop an LLD model (22–24). After ovariectomy, body weight and FFA content were significantly higher in the model group than in the sham group (p<0.05), and the accumulation of LD in liver cells was apparent, which suggests that the LLD model was successfully established in this study. The liver index in the model group had an upward trend
compared with the sham group, but there is no statistical significance (p > 0.05). This might be the weight gain of rats in the model group was much greater than the increase in liver weight. According to frozen sections, the liver tissues in the model group underwent pathological changes. LLD mainly occurs in hepatocytes around the CV because these cells are easily threatened by metabolic stress when intestinal microbes, microbial products, and FFA enter the liver through portal circulation (25–27). Therefore, during the process of lipid infiltration, the accumulation of LD in hepatocytes occurred earlier than it did in the surrounding cells.

T-SOD can scavenge superoxide anion free radicals and play a vital role in the balance between oxidation and antioxidation. After ovariectomy, more FFA was transported to the liver. This not only increases fatty acid β oxidation and the production of reactive oxygen species (ROS) but also leads to tissue damage and tissue dysfunction (28, 29). ROS can also interact with polyunsaturated fatty acids and trigger intracellular lipid peroxidation. As the product of lipid peroxidation, the MDA level is inversely proportional to the antioxidative capacity (30). In this experiment, the activity of the antioxidant enzyme T-SOD decreased and the MDA level increased in the model group (p < 0.05), causing oxidative stress in the liver (31).

Studies have demonstrated the anti-obesity role of SBCP by inhibiting lipid accumulation and regulating lipid metabolism in the liver (32, 33). In addition, SBCP could increase the expression levels of lipid metabolism-related genes (34). In this study, SBCP intervention significantly enhanced the antioxidative capacity of the liver and reduced lipid accumulation in hepatocytes. However, the AST and ALT levels were not significantly different among the groups (p > 0.05). In conclusion, although the liver tissues underwent histopathological deterioration, the protein synthesis function of the liver was not affected in the model group and even in the SBCP groups, consistent with the views of Pacifico et al. and Stefan et al. (35, 36).

Studies have shown that by regulating the levels of intestinal endocrine and fermenting carbohydrates, the progression of obesity and obesity-associated diseases can be attenuated (37). Short-chain fatty acids (SCFAs) are the most important metabolites of the microbial community, including acetate, propionate, and butyrate (38). Inflammatory bowel disease, type 2 diabetes, and obesity closely correlated with imbalanced intestinal microbiota and reduced amounts of bacterial metabolites such as SCFAs. Among all SCFAs, butyric acid can regulate lipid metabolism and has the strongest anti-inflammatory effect (39, 40).

The results of the present study indicate that the levels of the butyric acid-producing bacteria Ruminococcaceae UCG-005, Ruminococcus 1, and Lachnospiraceae were depleted in the model group and increased in the SBCP-M group. This indicates that SBCP regulated the lipid metabolism disorder in the ovariectomized rats by enriching the relative abundance of butyric acid-producning bacteria in the gut. The results of SCFAs showed that the butyric acid content in the model group was significantly lower than that in the sham group, which further confirmed this speculation. Lactobacillus is a well-known intestinal probiotic. However, some bacteria of the genus Lactobacillus, such as Lactobacillus reuteri, can ferment intestinal carbohydrates into lactic acid, acetate, and ethanol, which could induce liver damage such as liver inflammation and LLD (41). This result is consistent with those of previous studies that revealed that late fibrosis in obese nonalcoholic steatohepatitis patients was associated with an increased number of Lactobacillus (42). Our study also showed an increase in the number of Lactobacillus reuteri bacteria in the model group with serious LLD and a decrease in the number of Lactobacillus reuteri bacteria in the SBCP group with apparently alleviated LLD, providing new evidence that Lactobacillus reuteri is the main Lactobacillus involved in regulating LLD. This can be explained by the inhibitory effect of SBCP on LLD by reducing the abundance of Lactobacillus reuteri and inhibiting the loss of butyric acid-producing bacteria induced by estrogen deficiency.

CONCLUSION

SBCP administration can effectively inhibit the deterioration of liver tissues and alleviate the LLD induced by estrogen deficiency by improving liver antioxidative abilities, increasing the abundance of gut butyric acid-producing bacteria, and decreasing the number of Lactobacillus reuteri bacteria.

Authorship

Research conception and design: DZ and JX; experiments: DZ, JX, MLF, JSX, CJ, and LT; statistical analysis of the data: HKG and GSP; writing of the manuscript: DZ and JX. Funding acquisition and supervision: HNR.

DZ and JX contributed equally to this work.

Authorship declaration

The authors claim the manuscript has not been published or submitted elsewhere. All authors have contributed significantly and all authors agree to submit in this journal.

Disclosure of state of COI

No conflict of interest was declared by the authors.

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