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Basic Study

Effect of herbal cake-partitioned moxibustion on Leptin/JAK2/STAT3 in lipid-lowering pathway of hyperlipidemia rabbits

隔药饼灸对高脂血症兔调脂通路Leptin/JAK2/STAT3的影响

Liao Zong-li (廖宗力)[1], Tan Jing (谭静)[1], Zhu Chong-zheng (朱重政)[2], Sun Lu (孙璐)[1], Huang Wen-tao (黄文韬)[3], Yang Ren-da (阳仁达)[1], Chang Xiao-rong (常小荣)[1]

1 Hunan University of Chinese Medicine, Changsha 410208, China
2 Huizhou Hospital of Traditional Chinese Medicine, Guangdong 516000, China
3 The Second Affiliated Hospital of Hunan University of Chinese Medicine, Changsha 410005, China

Abstract

Objective: To observe the lipid-lowering effect of different transdermal absorption enhancers applied to the herbal cake-partitioned moxibustion in hyperlipidemia model rabbits, and to explore the possible mechanism.

Methods: Forty New-Zealand rabbits were randomly divided into 5 groups using the random number table method, with 8 rats in each group. Rabbits in the blank group were fed routinely with normal diet; rabbits in the other groups were fed with high-fat diet for 12 weeks to establish the hyperlipidemia model. Rabbits in the blank and the model groups were not treated. After the model was prepared, rabbits in the non-transdermal absorption enhancer group received herbal cake-partitioned moxibustion without transdermal absorption enhancer; rabbits in the laurocapram group and the borneol group received herbal cake-partitioned moxibustion with laurocapram or borneol respectively. After 4 weeks of treatment, serum was collected for enzyme-linked immunosorbent assay (ELISA), and the liver tissues were isolated for immunohistochemistry, quantitative polymerase chain reaction (qPCR) and Western-blotting (WB) detection.

Results: Serum ELISA results showed that leptin was significantly decreased in the model group compared with the blank group (P<0.05); compared with the model group, leptin was significantly increased in the non-transdermal absorption enhancer, the laurocapram and the borneol groups (all P<0.05); compared with the non-transdermal absorption enhancer group, leptin was significantly increased in the laurocapram group and the borneol group (both P<0.05); there was no significant difference in leptin between the laurocapram and the borneol groups (P>0.05). The qPCR results of rabbit liver tissues showed that the mRNA expressions of leptin, Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) in the model group were significantly lower than those in the blank group (all P<0.05); compared with the model group, the mRNA expressions of leptin, leptin receptor (LR), JAK2 and STAT3 in the non-transdermal absorption enhancer, the laurocapram and the borneol groups were significantly increased (all P<0.05); compared with the non-transdermal absorption enhancer group, the mRNA expressions of leptin, LR, JAK2 and STAT3 in the laurocapram and the borneol groups were significantly increased (all P<0.05); compared with the laurocapram group, the mRNA expressions of leptin, LR, JAK2 and STAT3 in the borneol group were significantly increased (P<0.05). The trend of immunohistochemistry and WB detection results was basically consistent with the qPCR assay results. The immunohistochemistry and WB detection results of phosphorylated JAK2 (phospho-JAK2) and phosphorylated STAT3 (phospho-STAT3) were basically consistent with those of JAK2 and STAT3.

Conclusion: The molecular expression of Leptin/JAK2/STAT3 pathway in the hyperlipidemia model rabbits was decreased. The molecular expression of Leptin/JAK2/STAT3 pathway was significantly increased after the herbal cake-partitioned moxibustion. The application of laurocapram and borneol, as transdermal absorption enhancers, in the herbal cake-partitioned moxibustion could more obviously up-regulate the factors of the Leptin/JAK2/STAT3 lipid-regulating pathway than the herbal cake-partitioned moxibustion alone.

Keywords: Moxibustion Therapy; Indirect Moxibustion; Herbal Cake-partitioned Moxibustion; Hyperlipidemias; Laurocapram; Janus Kinase 2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT 3) Pathway; Leptin; Rabbits

Author: Liao Zong-li, doctoral degree candidate
Corresponding Author: Chang Xiao-rong, professor.
E-mail: xrchang1956@163.com

【摘要】**目的**：观察不同促透剂运用于隔药饼灸对高脂模型兔降脂效果的影响，探讨其可能机制。**方法**：将40只新西兰兔按随机数字表法随机分成5组，每组8只。空白组正常喂养普通饲料；其余组高脂饲料喂养12周，复制高脂模型。空白组和模型组不治疗。无促透剂组成模后采用不加促透剂药饼施灸；氮酮组和冰片组分别将氮酮和冰片用于药饼中，进行隔药饼灸。治疗4周后，分离血清进行酶联免疫吸附测定(ELISA)，分离肝脏组织进行免疫组化、定量聚合酶链式反应(qPCR)和蛋白免疫印迹(WB)检测。**结果**：血清ELISA检测结果显示，与空白组比较，模型组Leptin显著下降($P<0.05$)；与模型组比较，无促透剂组、氮酮组和冰片组Leptin显著升高(均$P<0.05$)；与无促透剂组比较，氮酮组和冰片组Leptin均显著升高(均$P<0.05$)；氮酮组和冰片组Leptin差异无统计学意义($P>0.05$)。兔肝脏组织qPCR结果显示，与空白组比较，模型组Leptin、JAK2和STAT3 mRNA表达显著降低(均$P<0.05$)；与模型组比较，无促透剂组、氮酮组和冰片组Leptin、Leptin受体、JAK2和STAT3 mRNA表达显著升高(均$P<0.05$)；与无促透剂组相比，氮酮组和冰片组的Leptin、Leptin受体、JAK2和STAT3 mRNA表达显著升高(均$P<0.05$)；与氮酮组比较，冰片组的Leptin、Leptin受体、JAK2和STAT3 mRNA表达显著升高(均$P<0.05$)。免疫组化和WB检测趋势与qPCR结果基本一致。磷酸化JAK2 (Phospho-JAK2)和磷酸化STAT3 (phospho-STAT3)免疫组化和WB检测趋势与JAK2和STAT3基本一致。**结论**：高脂模型兔的Leptin/JAK2/STAT3通路分子表达下降，隔药饼灸后Leptin/JAK2/STAT3通路因子表达显著上升，氮酮和冰片作为促透剂运用于隔药饼灸比单纯隔药饼灸对Leptin/JAK2/STAT3调脂通路相关因子的上调作用更明显。

【关键词】灸法；间接灸；药饼灸疗法；高脂血症；月桂氮酮; Janus 激酶 2/STAT3 通路；瘦素；兔
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Hyperlipidemia (HLP) refers to abnormal systemic lipid metabolism and is a significant risk factor for health. The adverse reactions of oral lipid-lowering drugs have been troubling the HLP patients and clinicians. Therefore, it is of great significance to actively carry out the intervention study on HLP for discovering a safe, effective, convenient, economical, and highly reliable lipid-lowering external treatment to promote the health of patients, prolong the life and improve their quality of life (QOL).

Moxibustion is a treasure of traditional Chinese medicine (TCM) culture[1]. Studies have shown that moxibustion can improve local temperature and metabolism, and is an effective non-drug therapy for dyslipidemia[2]. Herbal cake-partitioned moxibustion is to apply a cake of herbs to a certain acupoint, and then apply moxibustion on the herbal cake. In which, moxibustion, drugs and acupoints play a synergistic role. Herbal cake-partitioned moxibustion has been proved effective for lipid-lowering and is easy to operate[2].

In this study, the herbal cake was made of *Dan Shen* (*Radix Salviae Miltiorrhizae*), *Da Huang* (*Radix et Rhizoma Rhei*), *Shan Zha* (*Fructus Crataegi*), *Yu Jin* (*Radix Curcumae*) and *Ze Xie* (*Rhizoma Alismatis*), which had the effect of dredging qi and blood in the meridians, and unblocking stagnation to relieve mass. Related studies have shown that emodin in *Da Huang* (*Radix et Rhizoma Rhei*)[3-4], tanshinone ⅡA in *Dan Shen* (*Radix Salviae Miltiorrhizae*)[5-8], curcumin in *Ze Xie* (*Rhizoma Alismatis*) and *Yu Jin* (*Radix Curcumae*)[9-11], flavonoids, flavans and their polymers, organic acids and other substances in *Shan Zha* (*Fructus Crataegi*)[12-14] can significantly reduce the blood lipid index of HLP rabbits and rats.

Liu CY, *et al*[15] found that dietary-induced obesity led to changes in serum leptin level, while Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) protein expression also changed accordingly. This indicates that the JAK2/STAT3 signaling pathway is involved in lipid metabolism.

In this study, New-Zealand rabbits were fed with high-fat diet to prepare the HLP models; the herbal cake was made of five traditional Chinese drugs: *Dan Shen* (*Radix Salviae Miltiorrhizae*), *Da Huang* (*Radix et Rhizoma Rhei*), *Shan Zha* (*Fructus Crataegi*), *Yu Jin* (*Radix Curcumae*) and *Ze Xie* (*Rhizoma Alismatis*); different transdermal absorption enhancers were used as a drug delivery method during herbal cake-partitioned moxibustion for HLP model rabbits; the effect of different transdermal absorption enhancers in the herbal cake-partitioned moxibustion on the expression of the lipid metabolism pathway (Leptin/JAK2/STAT3) related factors in the HLP model rabbits was observed; and the lipid-lowering mechanism of herbal cake-partitioned moxibustion in HLP model rabbits was further elucidated, thus to investigate whether there was a difference in the effect of herbal cake-partitioned moxibustion on Leptin/JAK2/ STAT3, the lipid-lowering pathway, after adding different transdermal absorption enhancers. These results will provide theoretical basis for the exploration of the safe and effective lipid-lowering external treatment.

1 Experimental Materials

1.1 Experimental animal

Forty clean grade New-Zealand purebred rabbits with the body weight of 1.4-1.9 kg, 3-month old, regardless of male or female, were provided by the Animal Experimental Center of Hunan University of Chinese Medicine [animal certificate number: SYXK (Xiang) 2013-0005]. Each rabbit was housed in an individual cage of the animal laboratory. The indoor temperature was kept at 20-25 ℃ and the humidity was maintained at 50%-70%. The cages, cage standers, feeders and water tumblers were sterilized before use.

1.2 Experimental drugs and reagents

Dan Shen (*Radix Salviae Miltiorrhizae*), *Da Huang* (*Radix et Rhizoma Rhei*), *Shan Zha* (*Fructus Crataegi*), *Yu Jin* (*Radix Curcumae*) and *Ze Xie* (*Rhizoma Alismatis*) were provided by the Traditional Chinese Medicine (TCM) Pharmacy of the First Affiliated Hospital of Hunan University of Chinese Medicine. When used, the herbs were finely powdered, filtered with a 200 mesh sieves, and mixed at equal proportion.

Laurocapram (Cat. No. 59227-89-3, Xinxiang Gaojin Pharmaceutical Co., Ltd., China), precisely measured 5 mL of the liposolubility laurocapram solution and dissolved in 100 mL absolute ethanol solution, mixed for later use; borneol (Cat. number: 207-352-6, Shandong Baiweitang Herbal Pieces Co., Ltd., China), precisely weighed 2 g of borneol and dissolved in 50 mL of absolute ethanol solution, and then added with 35 mL of distilled H_2O to dilute; ulatan (Cat. No.: 51-79-6, Shandong Qilu Xinghua Pharmaceutical Co., Ltd., China); saline (Cat. No.: 65230, Guizhou Tiandi Pharmaceutical Co., Ltd., China); absolute ethanol (Cat. No. E7023, SIGMA, Germany); Shenjiu 300 moxa cone (Oriental Type 1, Suzhou Oriental Moxa Factory, China); propylthiouracil (batch number: XW00515251) and cholesterol (batch number: 69008214), (Sinopharm Pharmaceutical Co., Ltd., China); egg yolk powder (Cat. number: 57583-35-8, Zhangzhou Haichuan Egg Products Co., Ltd., China); rabbit anti-JAK2 antibody (bs-23004R), rabbit anti-STAT3 antibody (bs-1141R), rabbit anti-phospho-JAK2 antibody (bs-2485R), rabbit anti-phospho-STAT3 antibody (bs-1658R), rabbit anti-leptin antibody (bs-0409R), rabbit anti-leptin receptor antibody (bs-0961R) and rabbit anti-GAPDH antibody (bs-0755R), (Beijing Boaosen Biological Technology Co., Ltd., China); rabbit leptin, leptin receptor (LR), JAK2, STAT3 enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Elabscience, China); ReverTra Ace real-time quantitative polymerase chain reaction (RT-qPCR) detection kit (FSQ-101, Toyobo, Japan); SYBR Premix Ex TaqTM (perfect real time) Takara kit (DRR041A, Takara, Japan).

2 Experimental Methods

2.1 Preparation of animal models and grouping

Forty New-Zealand rabbits were numbered by weights, and eight of them were randomly selected as the blank group using the random number table method and fed normally without any intervention.

High-fat diet: each 100 g feed contained 15 g of egg yolk powder, 0.5 g of cholesterol, and 5 g of lard, 10 mg/(kg·bw) propylthiouracil and basic diet. The above ingredients were well mixed in proportion (the lard was pre-heated to be dissolved), then processed into granular high-fat feed. The remaining 32 rabbits were used to prepare models by feeding 100 g of the high-fat feed at 8:00 a.m. every day. The high-fat feed was fed before the normal feed was added. About 100-130 g food in total was consumed per rabbit per day, and rabbits all had free access to drinking water, for a total of 12 weeks.

Successful modeling standards: According to the standard of Dai XJ, *et al*[16], serum total cholesterol (TC) and triglyceride (TG) were significantly increased in rabbits of the model group compared with the blank group (P<0.05).

After successful modeling, serum triglyceride level of each rabbit was numbered from low to high and labeled on the inner side of the right ear with a marker pen. Rabbits were then randomly divided into 4 groups by random number table method (n=8). Plus the blank group, there were 5 groups in total.

Blank group: Rabbits were fed normally without any intervention.

Model group: Rabbits were not treated after successful modeling.

Non-transdermal absorption enhancer group: After successful modeling, H_2O was used as the solvent to make herbal cakes for moxibustion.

Laurocapram group: After successful modeling, laurocapram solution was used as the solvent to make herbal cakes for moxibustion.

Borneol group: After successful modeling, borneol solution was used as the solvent to make herbal cakes for moxibustion.

During the intervention, rabbits were fed with about 60 g of normal feed at 8 a.m., with free access to drinking water.

2.2 Acupoint selection and positioning

Acupoints: Group Ⅰ consisted of Juque (CV 14), bilateral Tianshu (ST 25) and Fenglong (ST 40); group Ⅱ consisted of bilateral Pishu (BL 20), Ganshu (BL 18) and Xinshu (BL 15).

Acupoint positioning: Acupoint location was determined according to the *Experimental Acupuncture Science*[17] and anthropomorphic comparison method. The line from the lower edge of the xiphisternal synchondrosis to the upper edge of the pubic symphysis was divided into 13 equal parts; Juque (CV 14) was at the second equal part below the xiphisternal synchondrosis. Tianshu (ST 25) was 3 cm away from the umbilicus. Fenglong (ST 40) was at the midpoint of the shin and the trailing edge of the fibula. Xinshu (BL 15) was 1.5 cm away from the site between the spinous processes of the 5th and the 6th thoracic vertebrae (the first vertebral spinous process touched in the posterior cervical occipital region was the second cervical spinous process, from which the spinous process descended to the fifth spinous process of the thoracic vertebra). Ganshu (BL 18) was 1.5 cm away from the point between the spinous processes of the 9th and the 10th thoracic vertebrae. Pishu (BL 20) was 1.5 cm away from

the point between the spinous processes of the 11th and the 12th thoracic vertebrae.

2.3 Moxibustion methods

The rabbit was fixed on a rabbit stand. After the acupoints were positioned, hair within 2 cm in diameter around the acupoints was removed with 8% sodium sulfide solution, then the skin was rinsed with saline to prevent the skin from being damaged by the depilatory solution.

Prior to moxibustion, the Chinese medicine powder was made into a paste with different solvents (80 mL solvent was added per 100 g of powder) accordingly for different groups, and pressed into cakes of 3 mm in thickness and about 1 cm in diameter by a self-made mould. Oriental Type 1 Shenjiu 300 moxa cone was used, whose the burning time was about 7.5 min each at room temperature. First put the herbal cake on the acupoint, then put the moxa cone on the herbal cake after removing the stand to ensure the herbal cake was close to the acupoint skin, then ignited the moxa cone for moxibustion. Moxibustion was alternately performed at a group of acupoints each day with 4 moxa cones for each acupoint (about 30 min), once a day, for 4 consecutive weeks.

2.4 Sample collection and processing

After 4 weeks of intervention, 10% urethane solution was injected intravenously from the ear margin at 4 mL/(kg·bw). After anesthesia, 2 mL blood was collected from the heart, and 30 μL 10% EDTA-Na_2 and 40 μL 40 000 U/mL aprotinin were added and slowly inverted up and down 3 times to mix. After centrifugation at 4 ℃ and 3 000 r/min for 10 min, the serum was separated and placed in a small test tube and stored at –80 ℃. During the experiment, a rabbit in the borneol group died of diarrhea and no blood samples were collected.

Cardiac blood collection method: The rabbit was fixed in a supine position on the rabbit table, and the left chest was shaved and disinfected with fortified iodine solution. The heart's beating position touched at 2.0-2.5 cm above the left thoracic-axillary level (3-4 intercostal) was used as the needle insertion point; after the quick insertion, the needle was punctured at 45° to the horizontal plane toward the inner sternum. Blood was collected when the needle (9 gauge needle) was inserted for 3.5 cm.

2.5 ELISA detection

The rabbit serum was isolated for leptin test by ELISA; the TC and TG were determined by enzymatic method; the high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were directly determined by one-step method.

2.6 Quantitative polymerase chain reaction (qPCR) detection

The fluorescent qPCR primer was synthesized by Nanjing Kingsray Biotechnology Co., Ltd., China. The primer sequences are shown in Table 1.

Table 1. The qPCR primer names and sequences

Name	Upstream primer sequence	Downstream primer sequence	Amplification length (bp)
Leptin	5'-CCTGGGAAGGAAAATGCG-3'	5'-TGGAGGAGACCGACTGCG-3'	173
LR	5'-AAATCACGGACAAGGGCAAT-3'	5'-TATGAAGACCCAGGAAGCACA-3'	175
JAK2	5'-AAAGAGTAAAAGTCCACCAG-3'	5'-TCCATCTGGTCTTGGTAATCTCC-3'	124
STAT3	5'-CCGTGACGAGGCAGAAGAT-3'	5'-CGCCTCTTCCAGTCAGCC-3'	157
GAPDH	5'-AAGGTCGGAGTGAACGGATT-3'	5'-CTCAGCCTTGACCGTGCC-3'	177

Fluorescence qPCR test steps: 100 mg liver tissue sample from each rabbit was aseptically weighed and put into a 2 mL centrifuge tube with Trizol, then ground with liquid nitrogen to extract tissue ribose nucleic acid (RNA). ReverTra Ace qPCR RT detection kit was used. RNA was heat-denatured at 65 ℃ for 5 min and immediately cooled on ice, then with other components added into the 20 μL reaction system. The composition of the reaction solution is shown in Table 2.

Reaction conditions: 37 ℃, 15 min; 98 ℃, 5 min. Stored in a refrigerator at –20 ℃ as a template for later real-time qPCR (RT-qPCR) assay.

The SYBR Premix Ex TaqTM (perfect real time) Takara kit was used. Fluorescent qPCR reaction system is shown in Table 3 (the reaction was carried out on ice).

Table 2. Composition of the reaction solution

Composition	Volume
Nuclease-free H_2O	10 μL
5×RT Buffer	4 μL
RT Enzyme Mix	1 μL
Rrimer Mix	1 μL
RNA	2 μL
ddH_2O	2 μL

Table 3. Fluorescent qPCR reaction system

Composition	Volume
SYBR Premix Ex TaqTM (2×)	10.0 μL
F (10 μmol/L)	0.4 μL
R (10 μmol/L)	0.4 μL
Rox Reference Dye (50×)	0.4 μL
cDNA	2.0 μL
ddH_2O	6.8 μL
Total	20.0 μL

Reaction conditions: Pre-denaturation (1 cycle), 95 ℃, 30 s; PCR reaction (40 cycles), 95 ℃, 5 s and 60 ℃, 34 s; melting curve (1 cycle), 60-95 ℃, 560 s.

The ddH_2O was used instead of complementary deoxyribonucleic acid (cDNA) as a negative control, and the housekeeping gene beta-actin was used as an internal reference for fluorescent RT-qPCR reaction. The relative quantitative method was used to obtain the ΔCt value of each sample (the Ct value of the target gene − the Ct value of the internal reference gene), and the gene expression level was calculated by the $2^{-\triangle\triangle ct}$ method.

2.7 Western-blotting (WB) detection

Sterilely weighed 100 mg of liver tissue samples at the same site from each group were placed into 2 mL centrifuge tubes; 200-300 μL RIPA lysate was added into each tube with sample [volume = 1:9 ratio to the lysate, RIPA should be supplied with PMSF, protease inhibitor cocktail (100×); a phosphatase inhibitor (100 mmol/L) should also be added if a phosphorylated protein was to be detected. The tissue sample was homogenized with the Fluka motorized homogenizer until completely mushy. The EP tube was requested to be immersed in ice-water mixture for cooling during homogenization. After homogenization, samples were incubated on ice for 20 min, centrifuged at 4 ℃ and 13 000 r/min for 20 min. After centrifugation, the supernatant was collected, the protein concentration was adjusted with RIPA lysate, and the final concentration of the sample was adjusted to 3 mg/mL by adding 5× reducing protein loading buffer. The samples were boiled in H_2O for 5 min to denature the proteins, then subpackaged and stored at –20 ℃ for later use. A 10 μL sample was subjected to 10% SDS-PAGE electrophoresis, incubated with the primary antibody (1:500 dilution) and secondary antibody (1:3 000 dilution), then developed and photographed with ECL.

2.8 Immunohistochemistry detection

The liver samples from each group were cut into 1 cm pieces, fixed in 4% paraformaldehyde for 24 h, then embedded and sectioned at 6 μm for later or immediate experiments.

Immunohistochemical procedures: The paraffin sections were baked at 65 ℃ for 2 h before the experiment; checked the materials and reagents in each dyeing tank, and opened the fan in the fume hood; the paraffin sections to be analyzed were placed in a copper staining frame in sequence: dewaxed in xylene (Ⅰ) for 10 min; dewaxed in xylene (Ⅱ) for 10 min; and xylene was washed in 100% ethanol (Ⅰ) for 5 min; xylene was washed in 100% ethanol (Ⅱ) for 5 min; hydrated in 95% ethanol for 5 min, in 85% ethanol for 5 min, in 75% ethanol for 5 min, and in distilled H_2O for later use. Blocked for 15 min in 3% H_2O_2 solution; rinsed with phosphate buffer solution (PBS) twice for 5 min each time; antigen retrieval-high pressure heat repair, i.e., according to the required amount, 1 mol/L sodium citrate buffer was diluted 100 times to a final concentration of 0.01 mol/L. The diluted repair solution was added into the beaker with the slice holder, submersed the slice to ensure that the tissues were totally immersed into the repair solution, and then covered the lid; heated the beaker on the electromagnetic oven to boil the repair solution, turned off the power, and naturally cooled it down to room temperature; after heated for another time and cooled down to room temperature, the sections were taken out.

Washed twice with PBS for 5 min each time; added 50-100 μL non-immune normal goat serum and incubated for 15 min at room temperature; removed the blocking solution, added the primary antibody (diluted at 1:150) and incubated overnight at 4 ℃; washed with PBS 4 times for 5 min each time; added 100 μL HRP-labeled goat anti-rat immunoglobulin (Ig) G secondary antibody working solution (dilution ratio 1:500), incubated at 37 ℃ for 30 min, rinsed with PBS thoroughly 4 times for 5 min each time; developed for 10 s with the DAB and the degree of dyeing was mastered under the microscope; slightly washed with the distilled H_2O to stop the color development; the hematoxylin staining was carried out for 10 min and rinsed for 2 min with the running water; differentiated with 1% hydrochloric acid alcohol for 2 s; washed with running water for 15 min; washed with distilled H_2O for 1-2 s; dehydrated with 85% ethanol for 5 min; dehydrated with absolute ethanol for 10 min; transparency was performed with xylene (Ⅰ) for 10 min, and with xylene (Ⅱ) for another 10 min; added appropriate amount of neutral gum in the center of the paraffin section, and covered with a cover slip to mount. Microscopic examination was conducted.

2.9 Statistical methods

All the experimental data were statistically processed by SPSS 18.0 Windows software. The measurement data were first tested for normality, and those conforming to the normal distribution were expressed as mean ± standard deviation ($\bar{x} \pm s$). For comparison between groups, the least significant difference (LSD) method was used if the variance was homogeneity; the

Tamhane method was used if the variance was uneven. The rank-sum test was used when the normal distribution was not satisfied. P<0.05 was considered statistically significant.

3 Results

3.1 Blood lipid test results

Compared with the blank group, the levels of TC, TG and LDL-C in the model group were significantly increased (all P<0.05), and the level of HDL-C was significantly decreased (P<0.05). Compared with the model group, the levels of TC, TG and LDL-C were significantly lower (all P<0.05), and the HDL-C levels were significantly increased (all P<0.05) in the non-transdermal absorption enhancer, the laurocapram and the borneol groups. Compared with the non-transdermal absorption enhancer group, TC and TG levels were significantly decreased in the laurocapram group (both P<0.05), TC, TG and LDL-C levels were significantly decreased in the borneol group (all P<0.05). Compared with the laurocapram group, TC, TG and LDL-C levels were decreased in the borneol group, while the increase of HDL-C was more significant (all P<0.05), (Table 4).

Table 4. Comparison of serum TC, TG, HDL-C and LDL-C levels ($\bar{x}$ ±s, mmol/L)

Group	n	TC	TG	HDL-C	LDL-C
Blank	8	2.56±0.28	0.55±0.19	0.67±0.20	1.14±0.16
Model	8	8.15±0.47[1)]	1.23±0.31[1)]	0.37±0.07[1)]	1.98±0.21[1)]
Non-transdermal absorption enhancer	8	4.38±0.18[2)]	0.73±0.14[2)]	0.56±0.09[2)]	1.51±0.11[2)]
Laurocapram	8	3.76±0.41[2)3)]	0.59±0.06[2)3)]	0.55±0.05[2)]	1.42±0.15[2)]
Borneol	7	3.07±0.26[2)3)4)]	0.50±0.05[2)3)4)]	0.64±0.08[2)4)]	1.25±0.13[2)3)4)]

Note: Compared with the blank group, 1) P<0.05; compared with the model group, 2) P<0.05; compared with the non-transdermal absorption enhancer group, 3) P<0.05; compared with the laurocapram group, 4) P<0.05

3.2 Serum leptin test results

Compared with the blank group, the leptin expression in the model group was significantly lower (P<0.05); compared with the model group, the leptin expressions of the non-transdermal absorption enhancer, the laurocapram and the borneol groups were significantly increased (all P<0.05); compared with the non-transdermal absorption enhancer group, the leptin expressions of the laurocapram and the borneol groups were significantly increased (both P<0.05); compared with the laurocapram group, the expression of leptin was increased in the borneol group, but the difference between the groups was not statistically significant (P>0.05), (Table 5).

Table 5. Comparison of serum leptin level ($\bar{x}$ ±s, ng/mL)

Group	n	Serum leptin
Blank	8	11.06±1.15
Model	8	9.55±0.81[1)]
Non-transdermal absorption enhancer	8	12.42±1.44[2)]
Laurocapram	8	15.33±3.50[2)3)]
Borneol	7	15.91±2.42[2)3)]

Note: Compared with the blank group, 1) P<0.05; compared with the model group, 2) P<0.05; compared with the non-transdermal absorption enhancer group, 3) P<0.05

3.3 Fluorescent qPCR test results

Compared with the blank group, the leptin, JAK2 and STAT3 mRNA expressions in the model group were significantly lower (all P<0.05); compared with the model group, the mRNA expressions of leptin, LR, JAK2 and STAT3 in the non-transdermal absorption enhancer, the laurocapram and the borneol groups were significantly increased (all P<0.05); compared with the non-transdermal absorption enhancer group, the mRNA expressions of leptin, LR, JAK2 and STAT3 in the laurocapram and the borneol groups were significantly increased (all P<0.05); all the above indicators were significantly higher in the borneol group than those in the laurocapram group (all P<0.05), (Figure 1).

3.4 Comparison of WB test results

Compared with the blank group, the expressions of leptin, LR, JAK2, phosphorylated JAK2 (phospho-JAK2), STAT3, and phosphorylated STAT3 (phospho-STAT3) were significantly decreased in the model group (P<0.05). Compared with the model group, the protein expressions of leptin, LR, JAK2, phospho-JAK2, STAT3 and phospho-STAT3 in the non-transdermal absorption enhancer, the laurocapram and the borneol groups were significantly increased (all P<0.05); compared with the non-transdermal absorption enhancer group, the protein expressions of leptin, JAK2, phospho-JAK2, STAT3 and phospho-STAT3 were significantly increased in the laurocapram group and the borneol group (all P<0.05), but the differences in the LR expression were

not statistically significant (P>0.05); compared with the laurocapram group, the protein expressions of leptin, JAK2, phospho-JAK2, STAT3 and phospho-STAT3 were significantly increased in the borneol group (all P<0.05), but there was no significant difference in the LR expression (P>0.05), (Figure 2 and Table 6).

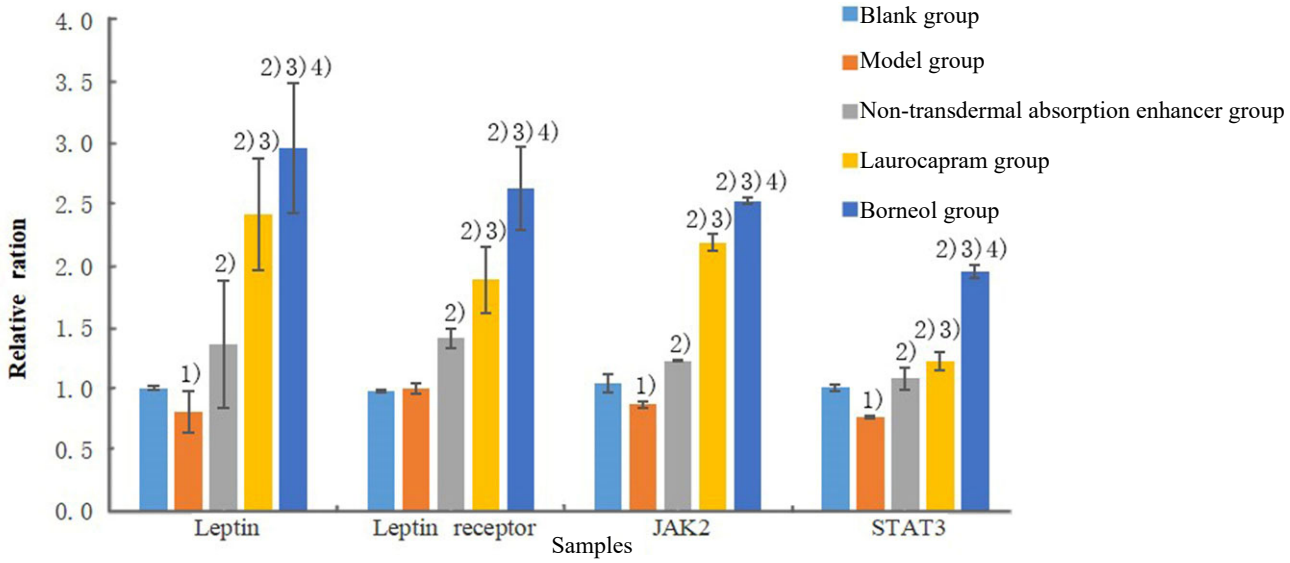


Figure 1. Comparison of fluorescence qPCR results

Note: Compared with the blank group, 1) P<0.05; compared with the model group, 2) P<0.05; compared with the non-transdermal absorption enhancer group, 3) P<0.05; compared with the laurocapram group, 4) P<0.05

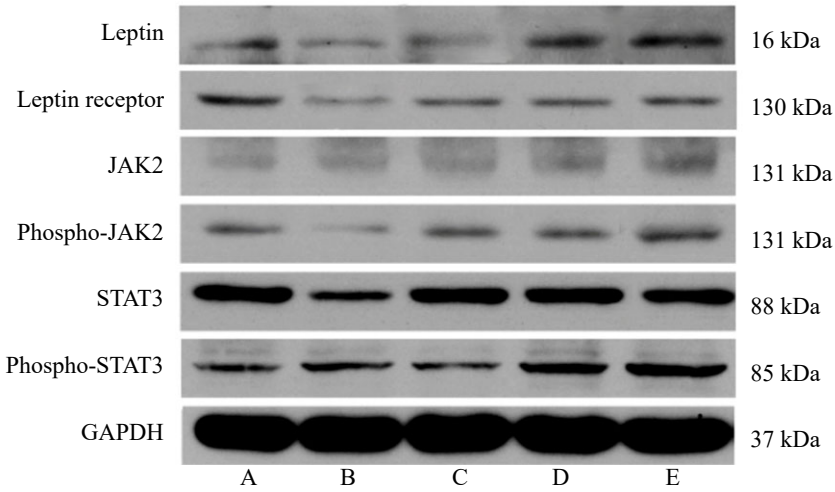


Figure 2. WB test results for each group

Note: A=Blank group; B=Model group; C=Non-transdermal absorption enhancer group; D=Laurocapram group; E=Borneol group

Table 6. Comparison of WB test results ($\bar{x}$ ±s)

Group	n	Leptin	LR	JAK2	Phospho-JAK2	STAT3	Phospho-STAT3
Blank	8	1.00±0.02	1.00±0.03	1.00±0.07	1.00±0.05	1.00±0.03	1.00±0.06
Model	8	0.72±0.17[1)]	0.57±0.04[1)]	0.88±0.06[1)]	0.56±0.13[1)]	0.68±0.09[1)]	0.98±0.12
Non-transdermal absorption enhancer	8	1.27±0.22[2)]	0.98±0.08[2)]	1.44±0.13[2)]	1.03±0.11[2)]	1.09±0.09[2)]	1.16±0.10[2)]
Laurocapram	8	1.48±0.23[2)3)]	0.98±0.34[2)]	2.04±0.11[2)3]	1.22±0.06[2)3)]	1.16±0.17[2)3)]	1.43±0.15[2)3)]
Borneol	8	1.56±0.25[2)3)4)]	0.99±0.27[2)]	2.20±0.16[2)3)4)]	1.55±0.17[2)3)4)]	1.23±0.17[2)3)4)]	1.65±0.08[2)3)4)]

Note: Compared with the blank group, 1) P<0.05; compared with the model group, 2) P<0.05; compared with the non-transdermal absorption enhancer group, 3) P<0.05; compared with the laurocapram group, 4) P<0.05

3.5 Immunohistochemical test results

The positive expression rates (%) of leptin, LR, JAK2, phospho-JAK2, STAT3 and phospho-STAT3 protein were counted by ImageJ software. Compared with the blank group, the expressions of leptin, LR, JAK2, phospho-JAK2, STAT3 and phospho-STAT3 in the model group were significantly lower (all $P<0.05$); compared with the model group, the expressions of leptin, LR, JAK2, phospho-JAK2, STAT3 and phospho-STAT3 in the non-transdermal absorption enhancer, the laurocapram and the borneol groups were significantly increased (all $P<0.05$); compared with the non-transdermal absorption enhancer group, the expressions of leptin, LR, JAK2, phospho-JAK2, STAT3 and phospho-STAT3 in the laurocapram and the borneol groups were significantly increased (all $P<0.05$); compared with the laurocapram group, the expressions of leptin, JAK2, STAT3 and phospho-STAT3 in the borneol group were significantly increased ($P<0.05$), while the difference in the LR and phospho-JAK2 expressions were not statistically significant (both $P>0.05$), (Figure 3-Figure 8).

The results of immunohistochemistry in each group are shown in Figure 9-Figure 14.

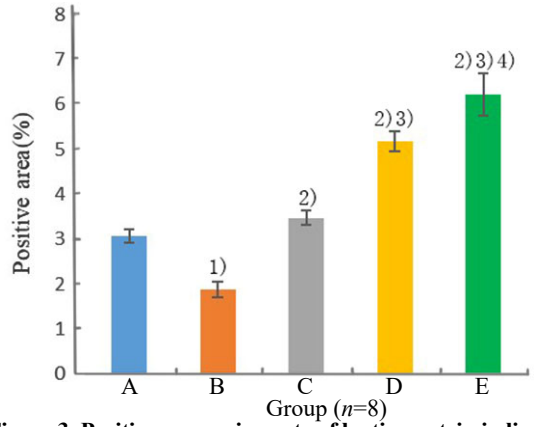


Figure 3. Positive expression rate of leptin protein in liver tissue sections of each group

Note: A=Blank group; B=Model group; C=Non-transdermal absorption enhancer group; D=Laurocapram group; E=Borneol group; compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$; compared with the non-transdermal absorption enhancer group, 3) $P<0.05$; compared with the laurocapram group, 4) $P<0.05$

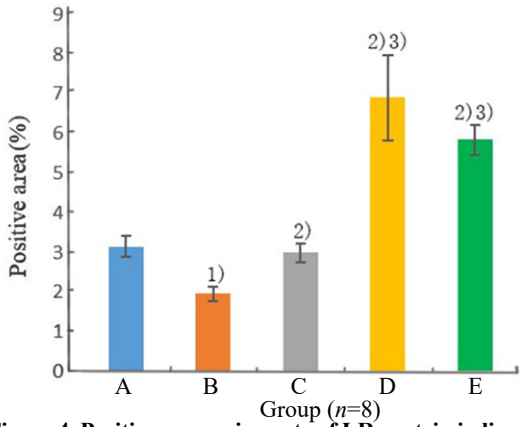


Figure 4. Positive expression rate of LR protein in liver tissue sections of each group

Note: A=Blank group; B=Model group; C=Non-transdermal absorption enhancer group; D=Laurocapram group; E=Borneol group; compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$; compared with the non-transdermal absorption enhancer group, 3) $P<0.05$

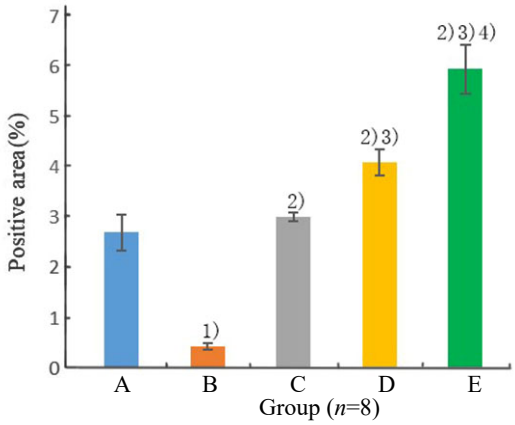


Figure 5. Positive expression rate of JAK2 protein in liver tissue sections of each group

Note: A=Blank group; B=Model group; C=Non-transdermal absorption enhancer group; D=Laurocapram group; E=Borneol group; compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$; compared with the non-transdermal absorption enhancer group, 3) $P<0.05$; compared with the laurocapram group, 4) $P<0.05$

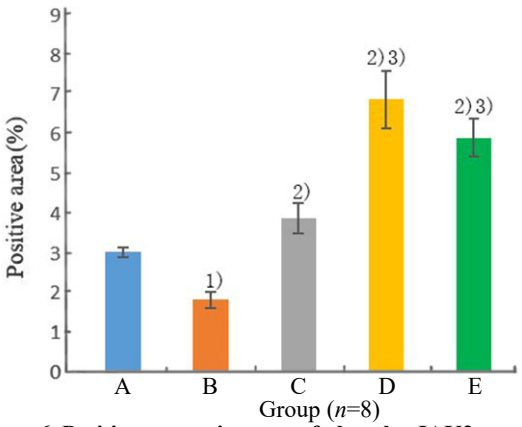


Figure 6. Positive expression rate of phospho-JAK2 protein in liver tissue sections of each group

Note: A=Blank group; B=Model group; C=Non-transdermal absorption enhancer group; D=Laurocapram group; E=Borneol group; compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$; compared with the non-transdermal absorption enhancer group, 3) $P<0.05$

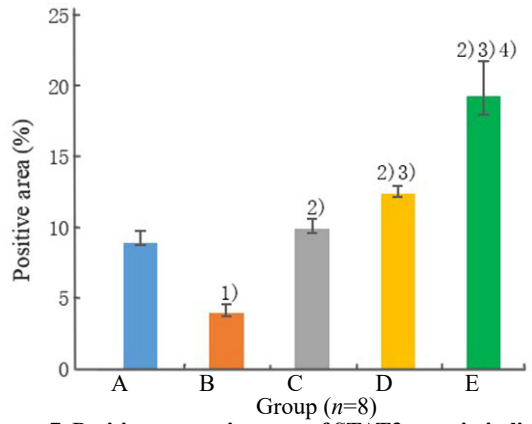


Figure 7. Positive expression rate of STAT3 protein in liver tissue sections of each group

Note: A=Blank group; B=Model group; C=Non-transdermal absorption enhancer group; D=Laurocapram group; E=Borneol group; compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$; compared with the non-transdermal absorption enhancer group, 3) $P<0.05$; compared with the laurocapram group, 4) $P<0.05$

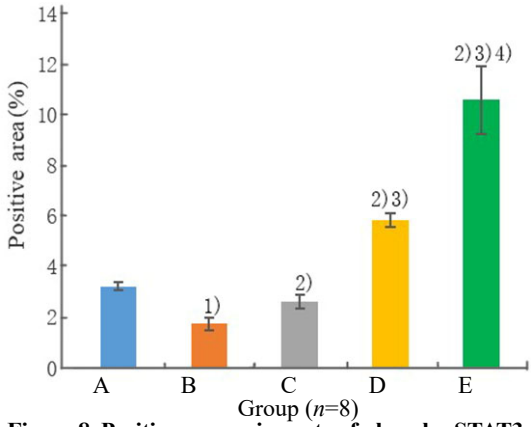
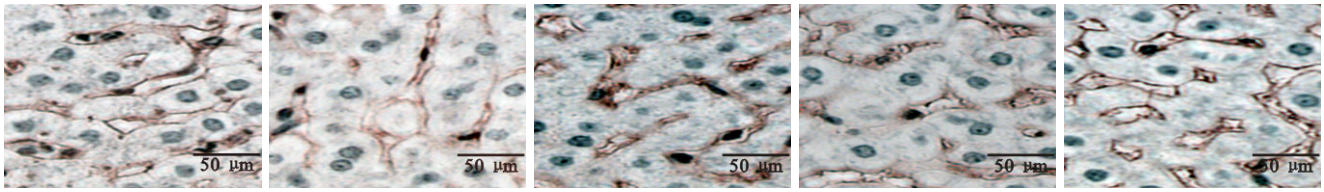


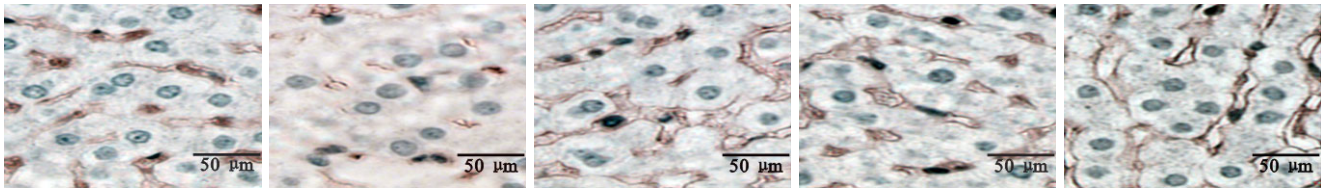
Figure 8. Positive expression rate of phospho-STAT3 protein in liver tissue sections of each group

Note: A=Blank group; B=Model group; C=Non-transdermal absorption enhancer group; D=Laurocapram group; E=Borneol group; compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$; compared with the non-transdermal absorption enhancer group, 3) $P<0.05$; compared with the laurocapram group, 4) $P<0.05$



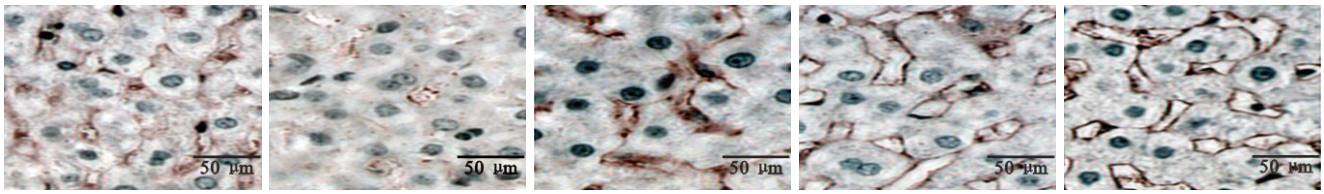
Blank group | Model group | Non-transdermal absorption enhancer group | Laurocapram group | Borneol group

Figure 9. Leptin immunohistochemistry results of rabbit liver tissue sections in each group (n=8, ×200)



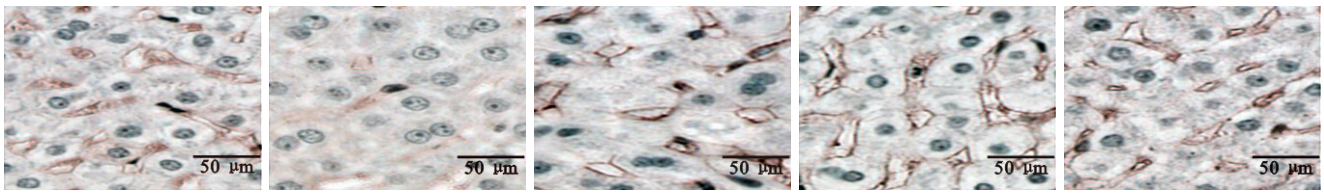
Blank group | Model group | Non-transdermal absorption enhancer group | Laurocapram group | Borneol group

Figure 10. LR immunohistochemistry results of rabbit liver sections in each group (n=8, ×200)



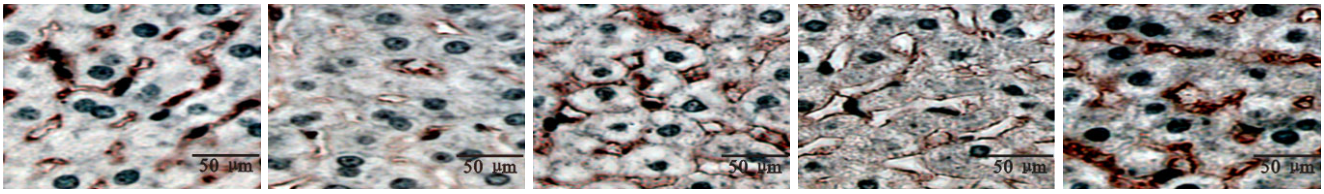
Blank group | Model group | Non-transdermal absorption enhancer group | Laurocapram group | Borneol group

Figure 11. JAK2 immunohistochemistry results of rabbit liver sections in each group (n=8, ×200)



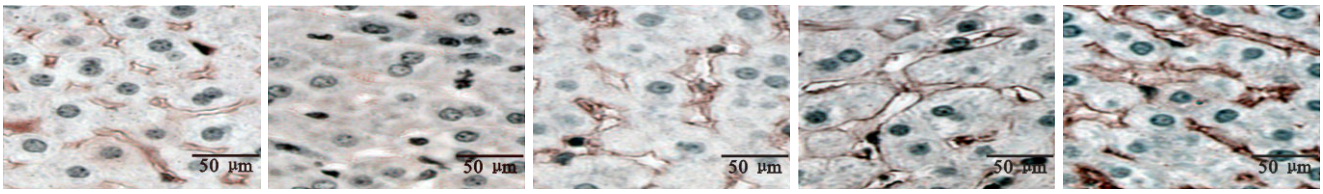
Blank group | Model group | Non-transdermal absorption enhancer group | Laurocapram group | Borneol group

Figure 12. Phospho-JAK2 immunohistochemistry results of rabbit liver sections in each group (*n*=8, ×200)



Blank group | Model group | Non-transdermal absorption enhancer group | Laurocapram group | Borneol group

Figure 13. STAT3 immunohistochemistry results of rabbit liver sections in each group (*n*=8, ×200)



Blank group | Model group | Non-transdermal absorption enhancer group | Laurocapram group | Borneol group

Figure 14. Phospho-STAT3 immunohistochemistry results of rabbit liver sections in each group (*n*=8, ×200)

4 Discussion

HLP refers to aberrant metabolism or transport of lipids in the body due to various factors, leading to abnormally elevated levels of TC, TG, LDL-C, and (or) reduced levels of HDL-C, coupled with a series of clinical symptoms. With the development of social economy, the incidence of this disease is getting higher and higher, especially among the younger population[18]. HLP is the primary risk factor for atherosclerosis. It is also a significant and independent risk factor for cardiovascular and cerebrovascular emergencies (coronary heart disease, stroke, sudden cardiac death, etc.)[19] Therefore, actively exploring effective lipid-lowering therapy has far-reaching significance. The body's lipid metabolism is a complicated process. There are many biologically active molecules involved, such as transcription factors and enzymes, which need to be regulated by a series of precise and complex signaling pathways. The signaling pathway of lipid metabolism has many downstream target genes that can be regulated, and the signal pathway affects each other in many unknown fields. The results of this study showed that after modeling, the serum levels of TC, TG and LDL-C were significantly increased, and the level of HDL-C was significantly decreased, indicating that the HLP model was successfully prepared. Herbal cake-partitioned moxibustion can effectively regulate the serum levels of TG, TC, LDL-C and HDL-C in HLP rabbits, and improve the metabolism of blood lipids. However, the molecular biological mechanism of herbal cake-partitioned moxibustion for lipid-lowering is still not fully understood and worthy of research and exploration.

In recent years, studies have found that the Leptin/JAK2/STAT3 signaling pathway has a wide range of biological effects on energy metabolism, immune regulation, growth and development. The activation of this pathway is involved in the regulation of lipid metabolism and plays an important role in the decomposition of fatty acids[20]. Leptin binds to the leptin functional receptor and activates the JAK/STAT pathway. The activated STAT is transported into the cells and regulates the expression of related genes, such as the peroxisome proliferator-activated receptor-gamma (PPARγ). PPARγ regulates the transcription of hormone sensitive lipase (HSL) and affects the hydrolysis of triglycerides[21]. Studies have shown that activated JAK-STAT pathway inhibits 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) expression by encoding the expression of related genes, thereby regulating the metabolism of TC *in vivo*[22]. Zheng XR, *et al*[23] found

that curcumin drug serum stimulated the proliferation of HSC-T6 by inhibiting leptin, when JAK2-STAT3 pathway was blocked and the decrease of JAK2-STAT3 protein expression may be one of the main mechanisms. Meng XQ, *et al*[24] discovered that *Ci Ji Li* (*Fructus Tribuli*) improved leptin resistance by regulating leptin-mediated JAK2/STAT3 pathway for obese hypertension treatment. This indicates that the decreased expression of JAK2/STAT3 pathway protein is associated with obesity, therefore, elevated protein expression of JAK2/STAT3 pathway has the potential to inhibit obesity.

Lipid metabolism is usually processed mainly in the liver, and abnormal lipid metabolism or lipid transport disorders in the liver can lead to dyslipidemia. Studies have shown that leptin has the effect of regulating energy metabolism in the body, mainly for regulating body fat metabolism and maintaining body weight. By binding to related receptors, leptin can significantly inhibit the synthesis of fatty acid synthase and accelerate the decomposition of TG to reduce blood lipid level[25]. Knockout of the rat leptin gene showed a significant increase in blood lipid-related factors. Leptin binding to the corresponding LR is required for the regulation of lipids. The LR long form is a functional receptor with signal transduction effect, which is affected by high-fat diet and LR gene expression[26]. When leptin binds to its long form receptor, the carboxy terminus of the receptor and JAK2 are phosphorylated, resulting in JAK2 phosphorylation at the Tyr1007/1008, and then the activated JAK2 regulates TC to be transported into liver for metabolism[27]. STAT3 has transcriptional and regulatory signal transduction function. Activation of JAK2 also promotes the phosphorylation of STAT3 at the corresponding carboxy-terminal site Tyr705 to form phospho-STAT3, and transports into the nucleus to initiate transcription of the related genes. Activated STAT3 regulates the transcription of genes involved in lipid metabolism. The activated JAK2/STAT3 pathway triggers downstream target factors to regulate lipid metabolism and plays a key role in regulating lipid metabolism[28].

This study showed that high-fat diet feeding could significantly inhibit the leptin expression in the serum of HLP rabbits. At this time, abnormal lipid metabolism may be related to leptin-mediated inhibition of lipid metabolism pathway. This is consistent with the findings of Wang J[28]. The herbal cake-partitioned moxibustion significantly increased the serum leptin level in HLP rabbits, suggesting that the lipid-lowering mechanism of herbal cake-partitioned moxibustion may be associated with the regulation of leptin expression. At the same time, different test results of liver tissues in this study indicated that leptin expression in rabbit liver tissues was significantly inhibited after fed with high-fat diet.

After 4 weeks of herbal cake-partitioned moxibustion intervention, leptin was significantly higher in the liver tissues of the three herbal cake-partitioned moxibustion groups than in the model group, indicating that herbal cake-partitioned moxibustion has the effect of up-regulating leptin in HLP rabbit liver. And the results of this study suggested that herbal cake-partitioned moxibustion could significantly up-regulate the expression of leptin-mediated JAK2/STAT3 pathway-related factors in liver tissues. This may be related to that the herbal cake-partitioned moxibustion could up-regulate the leptin level in the liver of HLP rabbits, and prompt more leptin to bind with the corresponding LR in the liver, thereby activating the Leptin/JAK2/STAT3 pathway. The activation of this pathway could effectively regulate the level of lipid metabolism-related factors in the downstream, and finally achieve the effect of well regulating blood lipid metabolism. This may be one of the molecular biological mechanisms by which herbal cake-partitioned moxibustion can effectively regulate the lipid metabolism. This study also found that laurocapram and borneol used as transdermal absorption enhancers in herbal cake-partitioned moxibustion could enhance lipid-lowering efficacy, at the same time, the activation of the above pathway was more significant. This may be one of the synergistic mechanisms of moxibustion and regulating lipid metabolism after adding borneol. This provides an experimental basis for improving the curative effect of herbal cake-partitioned moxibustion and deserves further study.

The WB results of this study showed that the leptin expression was significantly increased in the laurocapram and the borneol groups compared with the non-transdermal absorption enhancer group. However, there was no statistically significant difference in the effect on LR. It is speculated that there may be some leptin resistance factors, and the specific reasons need further study and exploration.

Conflict of Interest

There was no potential conflict of interest in this article.

Acknowledgments

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Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria in this experiment.

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Translator: Yang Yan-ping (杨燕萍)

