Effects of Theabrownin from Pu-erh Tea on the Metabolism of Serum Lipids in Rats: Mechanism of Action

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Abstract: Theabrownin (TB), one of the main bioactive components in pu-erh tea, has a significant blood lipid-lowering effect in hyperlipidemic rats. Therefore, it was hypothesized that TB would regulate the activity of key enzymes involved in lipid metabolism and accelerate the catabolism of exogenous cholesterol in rats fed a high fat diet. A total of 90 Sprague–Dawley rats were randomly divided into a normal control group (Group I), a high fat diet group (Group II), and high-fat diet plus TB group (Group III). A total of 10 rats were selected from each group and killed at 15, 30, or 45 d after starting the study for analysis. After feeding 45 d, the contents of TC, TG, and LDL-C levels in Group II were increased by 54.9%, 93.1%, and 134.3% compared with those in Group III, respectively, and the content of HDL-C in Group II was decreased by 55.7%. These effects were inhibited in the rats in Group III, which exhibited no significant differences in these levels compared with Group I, indicating that TB can prevent hyperlipidemia in rats fed a high fat diet. TB enhanced the activity of hepatic lipase and hormone-sensitive triglyceride lipase (HSL) and increased the HSL mRNA expression in liver tissue and epididymis tissue. The HL activity in serum of Group III was increased by 147.6% compared with that in Group II. The content of cholesterol and bile acid in the feces of rats was increased by 21.11- and 4.08-fold by TB. It suggested that TB could promote the transformation and excretion of dietary cholesterol of rats *in vivo*.

Keywords: hepatic lipase (HL), hormone-sensitive triglyceride lipase (HSL), hyperlipidemia, reaction mechanism, theabrownin

Introduction

Pu-erh tea, produced mainly in the Yunnan Province of China, is a traditional beverage in Hong Kong, Taiwan, and areas of southeast Asia. Sano and others (1986) noted that pu-erh tea significantly reduced the plasma levels of cholesterol ester and triglyceride in rats (Sano and others 1986). Although pu-erh tea possesses lower levels of catechins than green, oolong, and black teas, it had remarkable effects, suppressing genotoxicity induced by nitroarenes (Ohe and others 2001), lowering the atherogenic index and increasing the high density lipoprotein (HDL)-total cholesterol ratio (Yang and Koo 1989). Wu and others (2007) reported that the water extract of pu-erh tea had a potential inhibitory effect on a direct-acting mutagen, NQNO, and on an indirect-acting mutagen, AFB_1, which requires metabolic activation (Wu and others 2007). However, what is the main bioactive component in pu-erh tea? Unfortunately, few reports have investigated the components in pu-erh tea (Duh and others 2004). Our previous study showed that the rust-brown pigment in pu-erh tea, which can be dissolved in water but is not soluble in ethyl acetate, n-butyl alcohol or other organic solvents, had a significant cholesterol-lowering effect (Gong and others 2007). The features of this pigment are very similar to theabrownin (TB) in black tea (Roberts and others 1957; Ruan and Cheng 1983; Bailey and others 1991); therefore, it is also called pu-erh tea TB. The TB in black tea liquor was first named by Ruan (1983). It was considered to be a rust-brown pigment, which can be dissolved in water but not soluble in ethyl acetate, n-butyl alcohol or other organic solvents, its chemical components are not clear. The total TB content in black tea ranges from 4% to 9%, with an average of 6.5% (Ruan and Cheng 1983). By contrast, the TB content in pu-erh tea is higher than that of in black tea, and ranges from 10% to 14%, with an average of 12% (Liang and others 2005). This suggests that TB is the main bioactive component in pu-erh tea.

Our recent studies demonstrated that TB fractionated from pu-erh tea has a significant blood lipid-lowering effect, and reduced the serum triglyceride, total cholesterol (TC) and low density lipoprotein (LDL) levels in experimental animals and increased the level of HDL, but had no significant effect on the metabolism of blood lipids in normal rats (Chen 2009). Therefore, it is necessary to explore the mechanism of TB in hyperlipidemic rats. In this study, we hypothesized that the mechanism of TB in rats with hyperlipidemia is via the regulation of key enzymes involved in lipid metabolism. Thus, the objectives of this study were to investigate the effects of TB on the activity of key enzymes and their mRNA expression levels, in addition to the conversion of exogenous cholesterol in rats fed a high-fat diet. This research is important in understanding the benefits of TB from pu-erh tea on the treatment of hyperlipidemia because one of the most manageable ways of dealing with cardiovascular disease in the human population is to prevent its development through dietary management.

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Materials and Methods

Materials

Typical pu-erh tea (Dayi brand, produced in 2008) was obtained from the Menghai Tea Factory (Xishuan Banna, Yunnan Province of China). Assay kits for free cholesterol and total bile acid were obtained from Shanghai Mingdian Bioengineering Limited Co. Assay kits for TC, triglyceride (TG), HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were obtained from Yantai Ausbio Biochemical Engineering Co., Ltd. Kits for total RNA extraction, RT-PCR, reverse transcriptase M-MLV, TaqDNA polymerase, and DNA Marker-1 were obtained from Beijing Quanshijin Biotechnology Co., Ltd. β-actin gene primers and HSL gene primers were obtained from Beijing Bomaide Biotechnology Co., Ltd. Heparin sodium was obtained from Shanghai Chemical Regent Co. Cholesterol and methanol were obtained from Fisher Scientific Co. The total esterase detection kit was obtained from Nanjing Jiancheng Bioengineering Inst. Freshly redistilled water was prepared in our laboratory. All of the other reagents used were of analytical grade.

TB fractionated from pu-erh tea

Pu-erh tea (2000 g) was extracted 3 times with ethanol (1 : 4, w/v) for 12 h at 35 °C, after which the ethanol extraction was discarded. The tea residues were dissolved in distilled water (1 : 5, w/v) for 3 h at 85 °C and filtered 3 times. The resulting aqueous extract was extracted with chloroform (1 : 1, v/v), ethyl acetate (1 : 1, v/v), and n-butanol (1 : 1, v/v) at 25 °C, and the extract was discarded. The residual tea aqueous extract was precipitated by anhydrous ethanol (1 : 4, v/v) for 12 h at 25 °C and the precipitation (TB) (192 g) was collected by centrifuge method. The TB fraction consists of TB 68.67% ± 5.92%, TR 7.96% ± 1.42%, TF 0.13% ± 0.02%, conjugated protein 12.1% ± 1.64%, and conjugated polysaccharide 3.54% ± 0.32%, respectively.

Animal studies

Selection of experimental animals. Healthy adult male Sprague–Dawley rats (weighing 180 to 200 g; 7 to 8 wk old) were obtained from the Animal Experimental Center of Kunming Medical College (license nr: SCXK [Dian 2005–0008]). They had good nutrition with a normal diet before the study, and exhibited healthy bowel movements, rapid activity, and sensitive reactions (Shi 1980; Miao 1997). All animal procedures were approved by the Natl. Natural Science Fund of China, in accordance with guidelines from the China Council for Healthy Food.

Feeding method and management. The rats were fed ad libitum. The padding and cages were replaced once daily, and the drinking bottle was cleaned. We measured the quantity of food and water ingested. Regular ventilation was provided everyday, and the temperature was maintained at 20 ± 2 °C and humidity between 45% and 60%.

Experimental groups. Basal feed (Table 1) was obtained from the Animal Experimental Center of Kunming Medical College. The formulation of the high fat diet (Table 1) was based on the *Technical Standards for Determination and Assessment of Health Foods* issued by China Ministry of Health in February 2003. All of the rats were fed with the basal feed, with *ad libitum* access to food and water for 1 wk. The rats were then randomly allocated into 3 groups ($n = 30$ per group) based on the blood TC level. Group I, the normal control group, was fed the basal feed. Group II, the high fat model group, was fed the high fat diet. Group III was fed the high fat diet and TB, which was administered by gavage daily at 18:00 at a dose of 0.15 g/kg body weight dissolved in 2 mL distilled water. The rats in Groups I and Group II were given an equivalent volume of distilled water. On d 15, 30 and 45, 10 rats were selected from each group and killed by cervical dislocation and analyzed as described subsequently.

Selection of dosage. Gong and others (2007) reported that the LD_{50} of TB was >10 g/kg body weight and TB is a nontoxic substance (Gong and others 2007). According to the requirements of *Technical Standards for Determination and Assessment of Health Foods* issued by China Ministry of Health in February 2003, the dose at which compounds should be administered in animal experiments should be equivalent to 5 to 30 times the recommended dose for humans (converted to the dose per kilogram). According to Jing and others (2007), the recommended dose of TB for human is 2.7 g/60 kg·body weight (Jing and others 2007). Therefore, we selected the dosage of 675 mg/kg·body weight, which is 15 times the recommended dose for humans.

Preparation of serum, feces, and liver and adipose tissue. *Preparation of serum*. The rats were fasted for 12 h before collecting blood. Rats were anesthetized with ether and blood was collected from the orbital cavity. The blood samples were placed at room temperature for 1 h, and centrifuged for 15 min at 2500 revolutions/min. Then, 0.5 mL of the serum was collected using a pipette and stored in a centrifuge tube for later use.

Preparation of feces. The feces of 10 rats excreted within 72-h periods before adaptation period, feed 15-d, feed 30-d, and feed 45-d were collected and baked at 60 °C until the weight was constant.

Preparation of liver and epididymal adipose tissue. Rats were killed by cervical dislocation. The liver and epididymal adipose tissue were collected and rapidly frozen in liquid nitrogen for HSL mRNA expression.

Preparation of pathological liver sections. Rats were killed by removing their cervical vertebrae. The fresh liver tissue was then removed and weighed. Then, liver tissue, approximately 5 mm away from the edge with the maximum width, was removed and fixed in 10% formaldehyde solution.

Determination of serum lipid levels. The serum levels of TC, TG, LDL-C, and HDL-C were determined using a Toshiba

Table 1–Composition of basal feed diet, high fat diet, and high fat diet containing TB.

Ingredient	Amount in basal feed diet[a] (g/kg)	Amount in high fat feed diet [b](g/kg)	Amount in TB diet (g/kg)
Corn	350	350	350
Wheat bran	250	250	250
Bean pulp	250	250	250
Fish meal	80	80	80
Yeast	20	20	20
Bone meal	20	20	20
Whey powder	10	10	10
Salt sodium	5	5	5
Rape oil	5	5	5
Mineral mix	1	1	1
Vitamin mix	0.3	0.3	0.3
Methionine	1.3	1.3	1.3
Lysine	0.7	0.7	0.7
Cod liver oil	0.5	0.5	0.5
Lard oil	0	100	100
Yolk powder	0	100	100
Cholesterol	0	10	10
Bile salt	0	2	2
TB	0	0	675 mg/kg·BW

[a]Basal feed diet was purchased from the Animal Experimental Center of Kunming Medical College.
[b]The formulation of high fat feed consists of basal feed 78.8%, lard oil 10.0%, yolk powder 10.0%, cholesterol 0.1%, and bile salt 0.2%.

TBA-40FR full-automatic biochemical analyzer (Japanese Sanko Medical Systems Co. Ltd.) using appropriate assay kits specific for each molecule.

Determination of the activity of key enzymes involved in lipid metabolism. *Hormone-sensitive triglyceride lipase (HSL).* HSL was determined using a previously described method (Jin 1995). Briefly, the definition of a lipase activity unit in adipose tissue represents the hydrolysis of 1 μmol of matrix achieved by reacting with 100 g of fat in a water bath at 37 °C for 1 min. Lipase activity was calculated as the μmol quantity of matrix hydrolyzed by enzyme/0.005 × 20 × 100.

Serum lipoprotein lipase (LPL) and hepatic lipase (HL). Heparin sodium (130 U/kg) was injected into the body of animals through the caudal vein. After 15 min, we collected heparinized venous blood and separated the serum to determine the activity of LPL and HL. Serum LPL and HL activity was determined using a color development method with a 755B ultraviolet and visible spectrophotometer (Shanghai Jinghua Instrument Co. Ltd.) at the wavelength of 550 nm, with a total lipase assay kit (Nanjing Jiancheng Bioengineering Inst.).

Serum lecithin cholesterol acyltransferase (LCAT).

Determination of the serum free cholesterol (FC) content. FC was determined using a colorimetric procedure with a 755B ultraviolet and visible spectrophotometer at 500 nm, with a FC assay kit (Shanghai Mingdian Bioengineering Co. Ltd.).

Determination of serum LCAT activity. For the serum LCAT activity, the auto-matrix method was used to transfer 15 μL of serum into Tubes A and B. Tube A was placed in an ice bath and Tube B was placed at 37 °C. The FC content in both tubes was measured 60 min later. LCAT activity was then calculated according to the decrement in non-esterified cholesterol by comparing the FC content in both tubes. LCAT was calculated using the following formula:

$$\text{LCAT activity (kat/L)} = \frac{OD_A - OD_B}{OD_S} \times C_S \times \frac{V_R}{V_S \times HoldingTime}$$

Note: OD_A and OD_B are optical densities for Tubes A and B; OD_S is the absorbency of the cholesterol standard solution provided in the assay kit; C_S is the concentration of the cholesterol standard solution; V_R is the total volume of the reaction solution; V_S is the volume of the serum sample.

Determination of HSL mRNA in rat liver and epididymal adipose tissue. *Extraction of total RNA.* The epididymal adipose tissue and the liver was removed from liquid nitrogen, and ground under liquid nitrogen, using Trizol reagent to extract total RNA. The absorbance at the wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}) was determined using an ultraviolet spectrophotometer. The ratio A_{260}/A_{280} was used to determine the quality of total RNA in the samples.

Primer design. Primers for the rat β-actin (BC138614) and HSL (X51415) genes were designed using oligo6.0 software. The β-actin gene primers, sense 5'-TCT ACA ATG AGC TGC GTG TG-3', and antisense 5'-GGT CAG GAT CTT CAT GAG GT-3' amplified a 341-bp product. The HSL gene primers, sense 5'-CGC CTT ACG GAG TCT ATG C-3', and antisense 5'-GAG GAC ACC TTG GCT TGA-3', amplified a 331-bp product.

RT-PCR system. The total volume of the RT reaction was 25 μL. The concentration of total RNA was used as the basis to calculate the amount of RNA in the different organs. In accordance with the recommended amplification procedures, oligo (d)T was used as the reverse transcription primer, and reverse transcriptase M-MLV (100 U) was added and incubated at 42 °C in a water bath for 1 h to form the first cDNA chain. For the PCR reaction, 4 and 2 μL of the reverse transcription product from liver and epididymis adipose tissue, respectively, was used as the template. Then, 0.5 μL of primers (25 pmol/μL) and 0.25 μL of TaqDNA polymerase were added to a total reaction volume of 25 μL. The PCR amplification conditions were as follows. β-actin was denatured for 30 s at 94 °C, annealed for 60 s at 57 °C, and extended for 45 s at 72 °C for 35 cycles. HSL was denatured for 30 s at 94 °C, annealed for 60 s at 60 °C and extended for 45 s at 72 °C for 35 cycles. The PCR products were subjected to 2% agarose gel electrophoresis, and the gray value of the PCR product was scanned and analyzed using the gel imaging system and BIO-CAPT MW software.

Analysis of fecal bile acid content. Samples of feces were extracted 3 times with anhydrous alcohol at 70 °C. The extracts were evaporated to dryness at 80 °C. Fat and neutral sterols were removed by petroleum ether. The residual sediment was dissolved in alcohol containing 2% Triton X-100, evaporated to dryness at 80 °C and the sedimentation was finally dissolved in distilled water. This solution contains the extracted bile acid solution of feces (Shi and others 2006). Colorimetric analysis of the total bile acids was performed using a 755B ultraviolet and visible spectrophotometer at 405 nm with assay kits manufactured by Shanghai Mingdian Bioengineering Co. Ltd.

Analysis of the fecal cholesterol content by HPLC. *Saponification.* Samples (2 g) were placed in a 250-mL flat-bottomed flask, and 30 mL of anhydrous alcohol and 10 mL of potassium hydroxide was added to a concentration of 50%. The samples were placed in a water bath at 100 °C for saponification and refluxed for 1 h. The flask was mixed regularly to prevent the samples adhering to the side of the flask. After the saponification was completed, the inside of the flask was rinsed with 5 mL of anhydrous alcohol from the top of the cooling pipe. The flask was then cooled to room temperature.

Extraction. The saponified mixture was transferred to a 250-mL separating funnel by rinsing the rinse the flat bottomed flask 2 to 3 times with 30 mL distilled water, which was collected in the separating funnel. Then, 40 mL of petroleum ether/ether (1 : 1, V/V) was used to rinse the flat bottom flask 2 to 3 times and collected in the separating funnel. The flask was covered and air removed under a vacuum. After mixing for 2 min, the flask was placed statically, and delaminated in this context. The water phase was transferred to another separating funnel and extracted twice with 30 mL of petroleum ether/ether (1 : 1, V/V). The water phase was discarded and the 3 organic phases were collected together. The extract was rinsed with 100 mL of distilled water until it was neutral. The flask was gently mixed during the first rinse to prevent emulsification. The extract was dehydrated using anhydrous sodium sulfate and transferred to a 150-mL flat-bottomed flask.

Extract concentration. The extract was concentrated in the flat-bottomed flask in a water bath at 50 °C to dryness under a vacuum. The residue was dissolved in 5 mL methanol and filtrated through a 0.45-μm filtration membrane. The filtrate was collected and analyzed by a high-performance liquid chromatograph (HPLC1100, Agilent Technologies Co. Ltd.) (Li and others 2007).

Chromatographic conditions. Chromatographic column: ZORBAXSB-C18 (5 μm × 4.6 × 150 mm); mobile phase: 100% methanol (V/V); wavelength: 205 nm; flow velocity: 1 mL/min; column temperature: 38 °C; sample volume: 10 μL.

Pathological changes of liver. Fixed liver tissue from rats fed for 45 d was dehydrated and the paraffin-embedded tissue was sectioned. The sections were stained with hematoxylin/eosin and observed under a microscope (Tian and others 1992).

Statistical analysis

Analysis of variance (ANOVA) was performed using SPSS 13.0 software (SPSS Inc., Chicago, Ill., U.S.A.).

Results

Effect of TB on lipid metabolism in rats

During the feeding time, the body weights of rats in group I, II, and III all increased, the differences in body weights between rats in Groups I (control diet) and III (high fat diet plus TB) were no significant, but the body weights were significantly different between the rats in Group I, Group III, and Group II ($P < 0.05$) (as shown in Table 2).

As shown in Table 3, there were no significant differences in the serum levels of TC, TG, LDL-C, and HDL-C between each group after 7 d adaptation period. By contrast, at 30 d after the adaptation period, the differences in TC, TG, and HDL-C levels between rats in Groups I (control diet) and II (high fat diet) were significant ($P < 0.05$). At 45 d, the LDL-C level was significantly different between the rats in Group I and Group II ($P < 0.05$). Furthermore, TG and HDL-C levels were also significantly different between the rats in Group I and Group II ($P < 0.01$), indicating that the high fat diet successfully induced hyperlipidemia after dietary feeding for more than 30 d. During the entire feeding period, the serum TC, TG, and LDL-C levels in the rats in Group III (high fat diet and TB) were lower than that in Group II, while the HDL-C level was higher than that in Group II. After 30 d, the TC, TG, and HDL-C levels in Group III were significantly different compared with those in Group II ($P < 0.05$), but not Group I. The contents of TC and TG levels in Group II were increased by 55% and 73.2% compared with those in Group III, respectively, but the content of HDL-C in Group II was decreased by 41% compared with that in Group III. The differences in serum TC and LDL-C levels between Group III and Group II remained at 45 d ($P < 0.05$), and the TG and HDL-C levels in Group III were significantly different compared with those in Group II ($P < 0.01$), but not against Group I. The contents of TC, TG, and LDL-C levels in Group II were increased by 54.9%, 93.1%, and 134.3% compared with those in Group III, respectively, but the content of HDL-C in Group II was decreased by 55.7% compared with that in Group III. This means that the administration of TB during high fat feeding can effectively prevent the increase in TC, TG, and LDL-C levels and the decrease in HDL-C level caused by the high-fat diet.

Mechanism of the effects of TB effect on lipid metabolism in rats

Effect of TB on the activity of lipid metabolic enzyme. *Changes in HSL activity and mRNA expression in rat liver and epididymis adipose tissue.* As shown in Table 4, the HSL activity in the liver tissue from rats in Group III at 45 d was significantly higher than that in Group I ($P < 0.05$). The HSL activity in the epididymal adipose tissue in Group III at 30 d was significantly higher than that in Group I ($P < 0.05$), and that as 45 d was significantly higher than that in Groups I ($P < 0.01$) and II ($P < 0.01$). The HSL activity in the epididymal adipose tissue in

Table 2–The effect of TB on the body weight of rats.

Group ($n = 10$)	Adaptation period (g)	Feed 15-d (g)	Feed 30-d (g)	Feed 45-d (g)
(Group I)	214.45 ± 16.46^{A}	240.58 ± 18.97^{A}	272.88 ± 25.99^{A}	283.20 ± 25.58^{A}
(Group II)	206.85 ± 12.61^{A}	247.50 ± 12.38^{A}	289.93 ± 24.12^{A}	325.95 ± 24.55^{B}
(Group III)	201.95 ± 9.68^{A}	227.94 ± 12.41^{A}	259.26 ± 20.58^{A}	268.49 ± 23.53^{A}

$^{A}P < 0.05$, $^{B}P < 0.05$, compared with Group I and II; all values are expressed as $\bar{x} \pm$ SD.

Table 3–Contents of TC, TG, LDL-C, and HDL-C in serum of rats.

Lipid content in serum	Group ($n = 10$)	Adaptation period (mmol/L)	Feed 15-d (mmol/L)	Feed 30-d (mmol/L)	Feed 45-d (mmol/L)
TC	(Group I)	1.02 ± 0.17^{A}	0.99 ± 0.28^{A}	0.99 ± 0.21^{A}	1.00 ± 0.19^{A}
	(Group II)	1.00 ± 0.13^{A}	1.29 ± 0.36^{A}	1.55 ± 0.21^{B}	1.58 ± 0.27^{B}
	(Group III)	1.01 ± 0.14^{A}	0.99 ± 0.25^{A}	1.00 ± 0.21^{A}	1.02 ± 0.21^{A}
TG	(Group I)	0.56 ± 0.22^{A}	0.57 ± 0.14^{A}	0.60 ± 0.11^{A}	0.58 ± 0.08^{a}
	(Group II)	0.57 ± 0.17^{A}	0.80 ± 0.25^{A}	0.97 ± 0.21^{B}	1.12 ± 0.23^{b}
	(Group III)	0.58 ± 0.17^{A}	0.57 ± 0.07^{A}	0.56 ± 0.16^{A}	0.58 ± 0.17^{a}
LDL-C	(Group I)	0.37 ± 0.12^{A}	0.37 ± 0.11^{A}	0.35 ± 0.17^{A}	0.36 ± 0.22^{A}
	(Group II)	0.35 ± 0.15^{A}	0.58 ± 0.20^{A}	0.71 ± 0.29^{A}	0.82 ± 0.13^{B}
	(Group III)	0.38 ± 0.11^{A}	0.34 ± 0.14^{A}	0.34 ± 0.12^{A}	0.35 ± 0.16^{A}
HDL-C	(Group I)	0.99 ± 0.16^{A}	1.02 ± 0.14^{A}	1.00 ± 0.19^{A}	1.01 ± 0.09^{a}
	(Group II)	1.00 ± 0.30^{A}	0.78 ± 0.07^{A}	0.56 ± 0.20^{B}	0.43 ± 0.18^{b}
	(Group III)	0.98 ± 0.20^{A}	0.96 ± 0.14^{A}	0.95 ± 0.16^{A}	0.97 ± 0.11^{a}

$^{A}P < 0.05$, $^{B}P < 0.05$, compared with Group I and II; $^{a}P < 0.05$, $^{b}P < 0.01$, compared with Group I and II, all values are expressed as $\bar{x} \pm$ SD.

Table 4–Variation of HSL activity in rat liver and epididymis adipose tissue during feeding process.

	HSL activity in liver (U)			HSL activity in epididymis adipose tissue (U)		
Group ($n = 10$)	Feed 15-d	Feed 30-d	Feed 45-d	Feed 15-d	Feed 30-d	Feed 45-d
Group I	2.70 ± 0.23	2.69 ± 0.11	2.63 ± 0.04	3.20 ± 0.23	3.29 ± 0.33	3.33 ± 0.08
Group II	2.75 ± 0.39	2.99 ± 0.32	3.13 ± 0.51	3.75 ± 0.26	3.77 ± 0.23	4.11 ± 0.21^{a}
Group III	2.77 ± 0.67	3.33 ± 0.53	3.79 ± 0.61^{a}	3.72 ± 0.67	3.83 ± 0.58^{a}	8.64 ± 0.13^{bc}

$^{a}P < 0.05$, $^{b}P < 0.01$, compared with Group I, $^{c}P < 0.01$, compared with Group II, all values are expressed as $\bar{x} \pm$ SD.

Group III at 45 d was increased by 110.2% and 159.4% compared with those in Group II and Group I, respectively.

Bands corresponding to the HSL gene and the internal reference β-actin gene products were located between 300 and 400 bp, and the size of the fragments was consistent with the expected value (as shown in Figure 1). To eliminate systematic error, we recorded an image of the electrophoresis strip using a gel imaging analyzer, and conducted semi-quantitative analysis for each electrophoretic band using the BIO-CAPT MW analysis software. The results were expressed as the relative change in HSL mRNA expression, which was defined as the ratio between the RT-PCR products of the HSL gene and the β-actin gene. As shown in Table 5, after 30 d of the respective diets, the HSL gene expression in epididymal adipose tissue in Group III was significantly higher than that in Groups I ($P < 0.01$) and II ($P < 0.01$). After 45 d, the HSL gene expression in the liver and epididymal adipose tissue in Group III was significantly higher than that in Group I ($P < 0.05$ and $P < 0.01$, respectively), respectively, and that in Group II (both: $P < 0.01$). The densitometric value of HSL expression/β-actin expression in the liver and epididymal adipose tissue in Group III was increased by 15.2% and 163.1% compared with those in Group II, respectively.

Serum LCAT, LPL, and HL activity in rats. In terms of lipoprotein metabolism, improvements in LCAT activity are advantageous for the removal of cholesterol (Wang and Zhu 1993). As shown in Table 6, the activity of LCAT and LPL in rats in Group II showed an obvious downward trend at each time point, but there was no significant difference between each group. However, at 45 d, the HL activity in Group III was significantly higher than that in Groups I and II ($P < 0.05$) (Table 7). The HL activity in

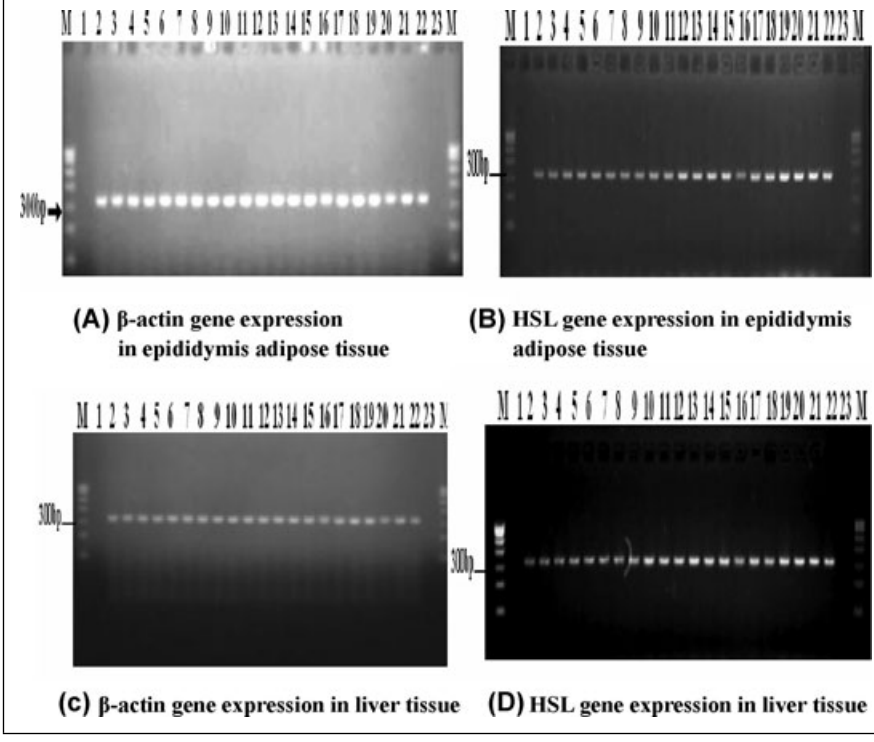

Figure 1–HSL gene and β-actin expression in epididymis adipose and liver tissue. (M: marker; 1,23: control; 2: Group I [15 d]; 3 and 4: Group II [15 d]; 5 to 8: Group III [15 d]; 9: Group I [30 d]; 10 and 11: Group II [30 d]; 12 to 15: Group III [30 d]; 16: Group I [45 d]; 17 and 18: Group II [45 d]; 19 to 22: Group III [45 d]).

Table 5–Densitometric value of HSL expression/β-actin expression calculated by 545 BIO-CAPT MW software during feeding process.

	In rats liver tissue			In rats epididymis adipose tissue		
Group ($n = 10$)	Feed 15-d	Feed 30-d	Feed 45-d	Feed 15-d	Feed 30-d	Feed 45-d
Group I	1.12 ± 0.23	1.20 ± 0.35	1.16 ± 0.28	1.22 ± 0.18	1.19 ± 0.31	1.36 ± 0.35
Group II	1.22 ± 0.29	1.28 ± 0.27	1.38 ± 0.32	1.28 ± 0.25	1.75 ± 0.43[a]	1.68 ± 0.37[a]
Group III	1.18 ± 0.31	1.21 ± 0.42	1.59 ± 0.30[a]	1.27 ± 0.30	3.15 ± 0.38[bc]	4.42 ± 0.78[bc]

[a] $P < 0.05$, [b] $P < 0.01$, compared with Group I, [c] $P < 0.01$, compared with Group II, all values are expressed as $\overline{x} \pm$ SD.

Table 6–Activity of LCAT and LPL enzyme in blood serum of rats.

Enzyme	Group ($n = 10$)	Feed 15-d ($nmol.mL^{-1}.h^{-1}$)	Feed 30-d ($nmol.mL^{-1}.h^{-1}$)	Feed 45-d ($nmol.mL^{-1}.h^{-1}$)
LCAT activity	Group I	2.69 ± 0.31[A]	2.65 ± 0.28[A]	2.68 ± 0.30[A]
	Group II	2.70 ± 0.35[A]	2.54 ± 0.31[A]	2.44 ± 0.37[A]
	Group III	2.60 ± 0.29[A]	2.57 ± 0.32[A]	2.61 ± 0.19[A]
LPL activity	Group I	0.27 ± 0.05	0.26 ± 0.07	0.26 ± 0.05[A]
	Group II	0.23 ± 0.07	0.22 ± 0.05	0.19 ± 0.04[A]
	Group III	0.27 ± 0.06	0.25 ± 0.08	0.22 ± 0.08[A]

[A] $P < 0.05$, compared with Group I and II, all values are expressed as $\overline{x} \pm$ SD.

Group III was increased by 147.6% compared with that in Group II. The HL activity in Group II showed a downward trend, indicating that the administration of TB during high fat feeding can significantly improve the activity of HL in rats.

Effect of TB on fecal bile acid content in rats. As shown in Table 8, at the end of the 7 d adaptation period, the bile acid content was not significantly different between each group. However, the fecal bile acid content in the feces of rats in Group III increased over time, and its content at 45 d was 5.08-fold higher than that at the end of the adaptation period. Furthermore, the fecal bile acid content was significantly higher in Group III than in Group I and II at day 30 ($P < 0.05$) and day 45 ($P < 0.01$). The bile acid in the feces of rats in Group II also showed an increasing trend, and its content after 45 d was 2.14-fold higher than that at the end of the adaptation period. However, Group II was not significant different from that in Group I during the intervention.

Effect of TB on fecal cholesterol content. As shown in Table 9, at the end of the 7 d adaptation period the fecal cholesterol content was not significantly different between each group. In Group II, the fecal cholesterol content showed an increasing trend over time, and its content after 45 d was 4.5-fold higher than that at the end of the adaptation period. However, it was not significant different from that in Group I during the entire intervention period. The fecal cholesterol content in Group III also showed a increasing trend, and its content after 45 d was 22.11-fold higher than that at the end of the adaptation period. The difference in the fecal cholesterol content between rats in Group III versus that in Groups I and II was significant ($P < 0.01$) during the study period. The results suggested that theabrownin could promote the transformation and excretion of dietary cholesterol of rats *in vivo*.

Pathological changes in the liver. The liver tissue sections from rats in normal control Group I (as shown in Figure 2A) showed an obvious liver sinusoid, and the hepatocytes were hexagonal in shape with a radial distribution around the central veins. The cells were basophilic and pale. The cells were present as monocytes or double cells. The nucleolus was easily visible and the cytoplasm was basophilic and had plenty of substances. The edges of the cell were clear and, under normal circumstances, lipid droplets in other cells, excluding lipocytes, were not visible under light microscopy. Appearance of lipid droplets in the cytoplasm of these cells and increases in the number of lipid droplets indicates fatty degeneration. Figure 2B and 2C show that the structure of liver tissue in rats fed for 15 d was essentially normal without fatty degeneration or other pathological changes. In Figure 2D and 2E, many lipid droplets can be seen in the hepatocytes, and were mostly scattered around the nucleus, while the distribution of hepatic cords was also radial after fed for 30 d. Figure 2F shows many lipid droplets in hepatocytes and these lipid droplets were scattered throughout the cytoplasm after fed for 45 d. Some of these droplets had combined into large droplets and covered the entire cytoplasm, pushing the cell nucleus towards the edge of the cell. The large droplets resembled lipocytes and the edges of the hepatocyte were ambiguous. The hepatocyte had developed cellular edema and its distribution was disordered. The cytoplasm was pale and sparsely scattered. Compared with Figure 2F and 2G shows that there were fewer lipid droplets in the hepatocytes, and only a few lipid droplets had combined into large droplets. The edges of the hepatocyte were distinguishable, and the distribution of hepatocyte was ordered.

Table 7–Activity of HL enzyme in serum of rats.

	HL activity (μmol.mL^{-1}.h^{-1})		
Group ($n = 10$)	Feed 15-d	Feed 30-d	Feed 45-d
Group I	0.32 ± 0.09	0.34 ± 0.12	0.33 ± 0.07^{A}
Group II	0.28 ± 0.21	0.25 ± 0.18	0.21 ± 0.09^{A}
Group III	0.37 ± 0.17	0.45 ± 0.14	0.52 ± 0.08^{B}

$^{A}P < 0.05$, $^{B}P < 0.05$ compared with Group I and II, all values are expressed as $\overline{x} \pm$ SD.

Table 8–Fecal bile acid contents in feces of rats.

Group ($n = 10$)	Adaptation period (μmol/g)	Feed 15-d (μmol/g)	Feed 30-d (μmol/g)	Feed 45-d (μmol/g)
Group I	1.35 ± 0.32^{A}	1.37 ± 0.24^{A}	1.36 ± 0.19^{A}	1.33 ± 0.30^{a}
Group II	1.41 ± 0.46^{A}	1.60 ± 0.31^{A}	2.54 ± 0.79^{A}	3.02 ± 1.15^{a}
Group III	1.46 ± 0.69^{A}	2.72 ± 0.06^{B}	5.38 ± 1.61^{B}	7.41 ± 2.17^{b}

$^{A}P < 0.05$, $^{B}P < 0.05$, compared with Group I and II. $^{a}P < 0.05$, $^{b}P < 0.01$, compared with Group I and II, all values are expressed as $\overline{x} \pm$ SD.

Table 9–Fecal cholesterol contents in feces of rats.

Group ($n = 10$)	Adaptation period (mg/g)	Feed 15-d (mg/g)	Feed 30-d (mg/g)	Feed 45-d (mg/g)
Group I	0.16 ± 0.11^{A}	0.18 ± 0.08^{a}	0.21 ± 0.12^{a}	0.19 ± 0.10^{a}
Group II	0.20 ± 0.13^{A}	0.35 ± 0.13^{a}	0.79 ± 0.36^{a}	0.90 ± 0.34^{a}
Group III	0.18 ± 0.14^{A}	0.97 ± 0.22^{b}	3.39 ± 0.94^{b}	3.98 ± 0.92^{b}

$^{A}P < 0.05$, compared among the different groups, all values are expressed as $\overline{x} \pm$ SD.
$^{a}P < 0.05$, $^{b}P < 0.01$, compared among the different groups, all values are expressed as $\overline{x} \pm$ SD.

Discussion

The main finding of this study was that the TB effectively prevented high-fat diet-induced increases in serum TC, TG, and LDL-C levels and decreases in HDL-C level, and enhanced HSL activity and mRNA expression in adipose tissue. Meanwhile, TB also enhanced serum HL activity. The effect of TB on serum lipid metabolism is closely associated with the activity of HSL and HL, while its association with LCAT and LPL is weaker. HSL was the first triglyceride lipase to be discovered and cloned, and plays a role in many aspects of lipid metabolism. HSL is involved in the metabolism of triglycerides in lipocytes and is the catabolic rate-limiting enzyme of steatolysis in animals (Holm and others 1988; Yeaman 2004). The above results indicate that TB significantly enhances the activity and mRNA expression of HSL in rat epididymal adipose tissue and liver, and promotes lipid metabolism in the body, particularly the degradation of triglycerides; thus exerting a significant blood lipid-lowering effect. HL is a lipolytic enzyme involved in the catabolism of plasma lipoproteins, and is an important determinant of the HDL concentration and distribution of LDL subclasses. Accordingly, HL activity may influence the body's susceptibility to coronary artery disease. Our findings suggest that TB has the potential to enhance HL activity and prevent hyperlipidemia in rats fed a high-fat diet (Su and others 2002).

In addition, the findings of this study also indicate that the content of cholesterol and bile acid in the feces of rats can be increased by TB administration (21.11- and 4.08-fold, respectively, compared with the end of the adaptation period). This suggests that TB may inhibit the absorption of and accelerate the excretion of exogenous cholesterol *in vivo*, and thus reduce the serum cholesterol content. Pathological changes of liver tissue from rats fed for 45 d and stained with hematoxylin-eosin showed that TB alleviated and slowed the rate of fatty degeneration in the liver and played an important role in the prevention of hyperlipidemia, although the fatty degeneration of liver induced by high fat

feeding is irreversible. Thus, it appears that the rat model used in the present study experiences systemic oxidative stress in addition to severe hyperlipidemia.

The original hypothesis of our study was that TB would regulate the activity of key enzymes involved in lipid metabolism and accelerate the excretion of exogenous cholesterol in rats fed a with high fat diet. The findings in the present study suggest that the increases in HSL activity and mRNA expression in liver and adipose tissue in rats comprise one of the important mechanisms involved in the blood lipid lowering effects of TB extracted from pu-erh tea.

Another possible mechanism is that TB may promote the transformation and excretion of dietary cholesterol of rat *in vivo*, thus reducing the serum cholesterol content. It was found that the content of "fecal TB" in Group I was higher than that of Group III (as shown in Table 10), this means that TB could be absorbed and utilized by rat *in vivo*, and there is the off chance for direct excretion of mixtures of TB and exogenous cholesterol. In general, the cholesterol catabolism is mainly conducted in liver, the main part of dietary cholesterol could be directly transformed into fecal bile acid (as shown in Table 8), another part could be transformed into fecal cholesterol by microbial in the intestinal and output through fecal (as shown in Table 9). On the one hand, the cholesterol catabolism activity of liver was enhanced because the liver was protected by TB (as shown in Figure 2). On the other hand, a partial increase of the fecal cholesterol may be caused by microbial in the intestinal. It presumes from chemical components of TB fractioned from pu-erh tea that the conjugated polysaccharide (3.54%) and conjugated protein (12.10%) are the main factors for the reproduction of microbial. But the real function mechanism needs to further investigate.

In this study, using rat to establish a high blood lipids and atherosclerosis models was kept in a convenient breeding, good reproducibility, strong resistance, and similar diet with human. The formation of pathological changes of rat was similar to earlier people with high blood lipids and atherosclerosis, and easy to form thrombosis. However, when compared with the rabbit model, its rate of absorption of exogenous cholesterol is not high, only 40%, while the rabbit up to 75% to 95%. To increase the amount of cholesterol in the feed were unlikely to cause the serum cholesterol level, therefore, to help rat to absorb the exogenous cholesterol and form hypercholesterolemia, the biliary acid or bile salt must be added in the feed at the same time. The diseases formed by experimental artificial or induced animal models which have some limitations have the difference compared with naturally occurring diseases. In future studies, it is necessary to further increase the rabbits or other animal experiments for comprehensive understanding the effects of TB on the regulation of blood lipid. Other limitations are the relatively short duration of feeding and we did not evaluate various doses. Ongoing studies are being conducted to confirm and expand the results of this study.

Table 10–"Fecal TB" contents in feces of rats.

Group ($n = 10$)	Breeding time	Content of "fecal TB" (%)
Group I	Mixture of fecal	5.00 ± 0.12^{A}
Group III	15-d	4.41 ± 0.07^{AB}
	30-d	4.22 ± 0.05^{AB}
	45-d	3.49 ± 0.02^{B}

$^{A}P < 0.05$, compared among the different groups, all values are expressed as $\overline{x} \pm$ SD.

Conclusions

It was found that TB can prevent hyperlipidemia in rats fed a high fat diet via enhanced the activity of hepatic lipase and hormone-sensitive triglyceride lipase (HSL) and increased the HSL mRNA expression in liver tissue and epididymis tissue. TB also had potential to prevent the absorption of exogenous cholesterol in diet of rats *in vivo*. Our results also indicate that TB is one of the most important bioactive ingredients in pu-erh tea that lowers serum lipid levels.

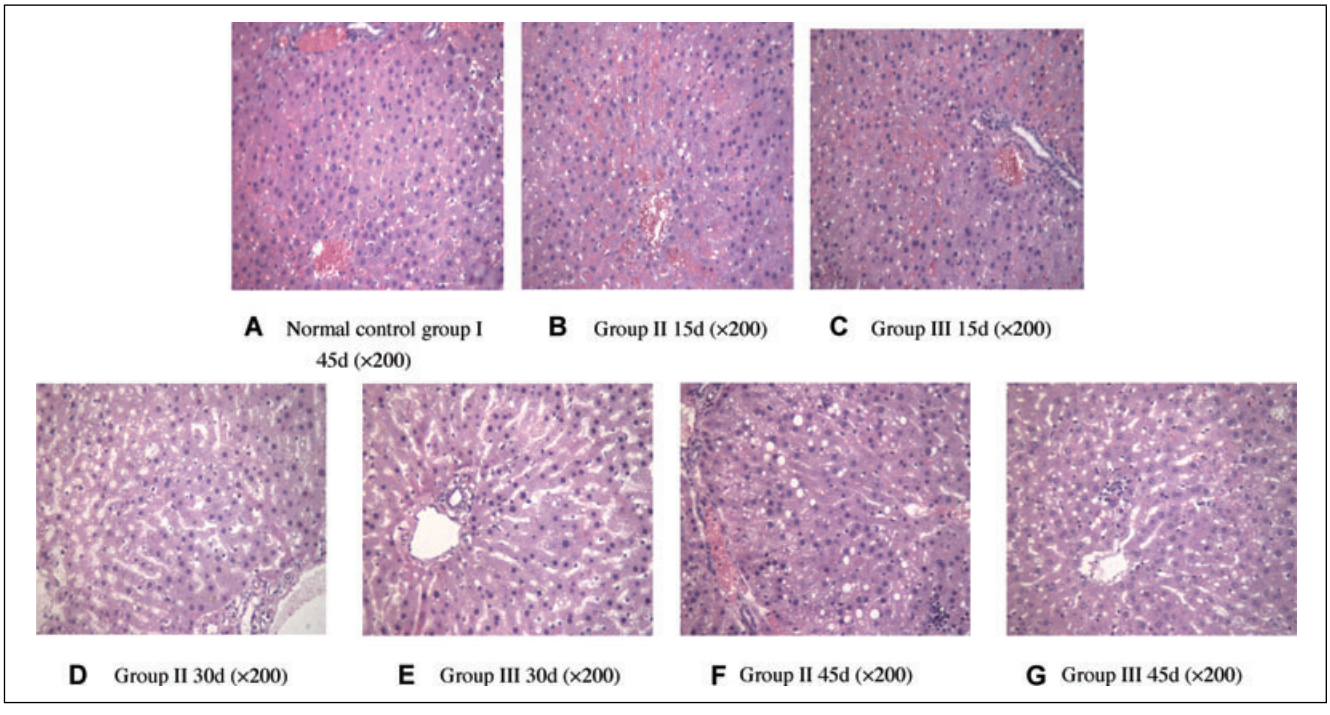

Figure 2–Pathological slices of liver tissue for Group I, Group II, and Group III.

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