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Research Article

Lipid Metabolism Effects of Freeze-dried Powder of Bamboo Juice on Liver Cell Line L02 with Steatosis

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Abstract: Freeze-Dried Powder of Bamboo Juice (FDPBJ) has high edible and medicinal values for abundant polyphenols, terpenoids and other biologically active ingredients. They can lower cholesterol level, regulate fat influent metabolism, prevent and cure Cardiovascular Diseases (CVDs) and so on. The aims of study are to research the lipid metabolism effects of FDPBJ and to develop a new type of raw material for the treatment of fat metabolism diseases. In this research, an accurate, sensitive and rapid *in vitro* model, steatosis hepatic L02 cell, was applied to assay the activities of FDPBJ on lipid metabolism. The steatosis L02 cells were obtained after being cultured with 1% fat emulsion-10% fetal bovine serum (FBS)-RPMI 1640 medium for 48 h. Contents of triglyceride (TG), Total Cholesterol (TC) and Free Fatty Acid (FFA) in L02 cells were determined after exposure. The results showed that the intracellular TG content was increased from 33.83±2.32 mmol/L to 40.67±2.73 mmol/L in steatosis L02 cells, while the intracellular TC content was increased from 3.48±0.57 mmol/L to 8.83±0.72 mmol/L. At the same time, the exposure of FDPBJ could reduce the TG, TC and FFA contents in steatosis L02 cells significantly. FDPBJ displayed a distinct TG regulation activity and TC regulation activity. In terms of morphology, FDPBJ can clearly reduce the accumulation of lipid droplets in the steatosis liver cell line L02. On account of these *in vitro* results, FDPBJ might have satisfactory effects in clinic treatment of fat metabolism disease characterized by the elevation of TG and TC.

Keywords: Bamboo juice, fatty liver disease, human liver cell line L02, lipid metabolism, polyphenols, terpenoids

INTRODUCTION

Bamboos are perennial plants of the *Gramineae* family and widely distributed in China that is the largest bamboo producing country (Zhang *et al*., 2006). *Dendrocalamus hamiltonii* (*Dendrocalamus hamiltonii* Nees et Arn. ex Munro) is widely distributed in the south of China region and it belongs to the family *Gramineae*, *Bambusoideae*, *Dendrocalamus* (Shan *et al*., 2013). Bamboos have a long history of being used as a source of both food and medicine in China and Southeast Asia and it is an excellent source of polyphenols, terpenoids and other natural active ingredients, which have antioxidant, antiinflammatory (Hu *et al*., 2000), antimicrobial and antifungal, antiviral activities (Fujimura *et al*., 2005) and can help to lower cholesterol level, prevent and cure Cardiovascular Diseases (CVDs) (Jiao *et al*., 2007). Bamboos juice in our research, which was regarded as "a kind of magic water", is extracted from the stems of *Dendrocalamus hamiltonii*. It contains phenolic compounds, terpenoids, essential amino acids (Yang *et al*., 2009), polysaccharides (Wang and Zhan, 1998) and so on.

The term of fatty liver is defined as a liver in which lipids account for more than 5% of liver wet weight. It is recognized as a feature of the metabolic syndrome and has relationship with the imbalance between the hepatic influx and the oxidation or excretion (Bradbury, 2006) (Fig. 1A). Based on the cause of formation, fatty liver has two types. One is alcoholic steatosis and the other is Non-Alcoholic Fatty Liver Disease (NAFLD). In the fatty liver cells, lipids are primarily stored in the cytoplasm as triglycerides (neutral lipids); leading to micro-and macro-vesicular steatosis, balloon cell degeneration (Mulhall *et al*., 2002) and fibrosis (Serfaty and Lemoine, 2008). The progress of NAFLD is usually characterized by the morphologic changes of hepatocytes and the hepatic

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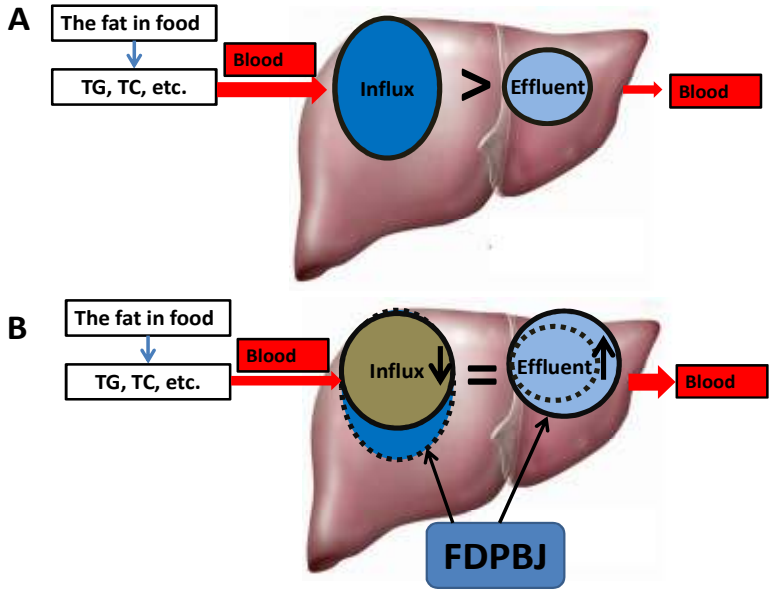

Fig. 1: Schematic representation of the fatty liver formation and lipid-lowering effect of FDPBJ; (A): Schematic representation of the fatty liver formation; (B): Schematic representation of the lipid-lowering effect of FDPBJ

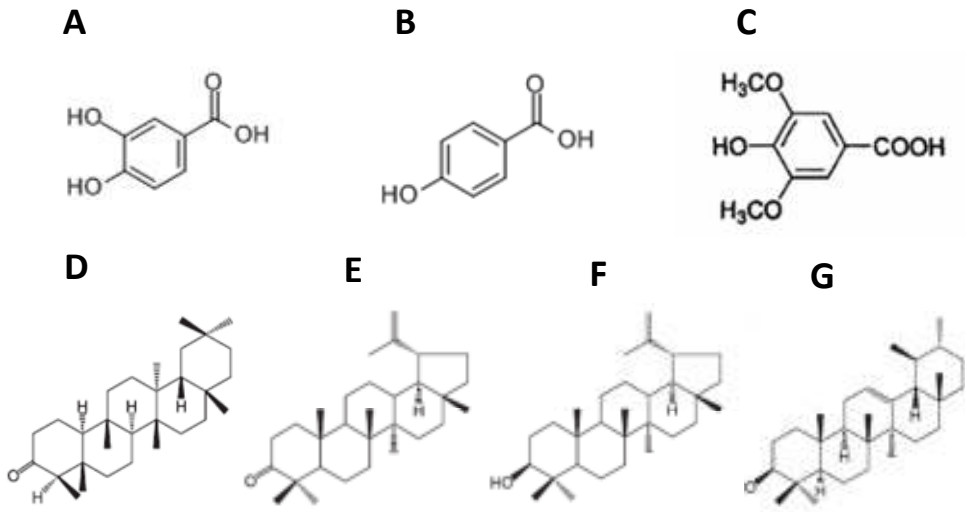

Fig. 2: The active ingredient in bamboo juice; (A): Protocatechuic acid; (B): p-Hydroxybenzoic acid; (C): Syringic Acid; (D): friedelan-3-one; (E): lup-20(29)-en-3-one; (F): lup-20(29)-en-3-ol; (G): α-amyrin

triglyceride contents. NAFLD is prevalent in Western countries and it is the same to China. Since accumulation of lipids in hepatocytes is a pathologic hallmark, more *in vitro* models of steatosis have been used to study the hepatocellular consequences of lipid accumulation in hepatic cells of a human origin (Feldstein *et al*., 2003). The liver cell plays an important role in lipid metabolism, for example, the distribution, biosynthesis, transferring and removal of TG, TC and other related lipoproteins. Liver cell line L02 could maintain characteristics and ultrastructure of normal liver cells after continuous passage (Ye *et al*., 1980). L02 cell line has been shown to express many specific liver cell functions and could be used in many fields such as activity (Ye *et al*., 2011) and toxicity (Yuan *et al*., 2009). The steatosis L02 cells are widely used in the lipid metabolism regulation researches (Gomez-Lechon *et al*., 2007; Zhang *et al*., 2011).

Some studies have reported that some natural active substances, which can decrease blood lipid levels, may have a role in the prevention and treatment of NAFLD and hyperlipidemia. Present study showed that theabrownin, which is a kind of phenolic pigment in puerh tea has a significant blood lipid-lowering effect in hyperlipidemic rats (Gong *et al*., 2010a). In some tests, it was shown that triterpenoids were the main active components in bamboo shavings (Zhang *et al*., 2004). Triterpenoids, which are made up of 30 carbons,

are considered as a condensation compound of six isoprenes (Zhang *et al*., 2006). A triterpenoid-rich extract from bamboo shavings and outer skin of a bamboo culm is also an excellent source of antihyperlipidemic, antifatigue and antihypertensive and the extract was found to have low toxicity indicating its potential use in functional food development (Gong *et al*., 2010b). Some studies have showed that bamboos juice from bamboo culm also had high polyphenol content and triterpenoid content (Lu *et al*., 2006). The most important phenolic compounds in bamboo are protocatechuic acid, p-Hydroxybenzoic acid and syringic acid and the structures of them were listed in Fig. 2 (Nirmala *et al*., 2011). Structures of triterpenoids in banmboo were also listed in Fig. 2 (Zhang *et al*., 2006). However, only a few studies have reported the intervention effects of FDPBJ on lipid metabolism.

In this research, bamboo juice was extracted by the living body extraction method and the freeze-dried powder was obtained by vacuum freeze drying method. On the other hand, we developed an accurate, sensitive and rapid *in vitro* model, hepatic L02 cell with steatosis, to evaluate the effects of FDPBJ on lipid metabolism regulation.

EXPERIMENTAL

Chemicals: L02 cells were purchased from Cell Bank, Kunming Institute of Zoology, Chinese Academic of Science. RPMI-1640 was purchased from Gibco Invitrogen Corporation, USA (Cat.No.31800-014). Hyclone FBS was purchased from Shanghai Luwenkeji Biological Technology Co., Ltd, China. Fat emulsion for human use was purchased from Sichuan Guorui Pharmaceutical Co., Ltd, China. The fat emulsion (each 250 mL) contained 50 g of refined soybean oil, 3 g of refined lecithin and 5.5 g of glycerol (Batch No. 0912042). TG (Batch No.201105110521) and TC (Batch No.201105110781) assay kits were purchased from the Biosino Biotechnology Co., Ltd., Beijing, China. FFA (Batch No. 20111213) assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute Co., Ltd., Beijing, China. Chemicals and reagents were of analytical grade obtained from Sigma Chemical (St. Louis, MO, USA) and Sinopharm (Shanghai, China). Water used in the experiments was ultrapure deionized water. All reagents were of analytical grade.

Acquisition of live bamboo juice and preparation of freeze-dried powder: The bamboos juice used in this experiment was from live *Dendrocalamus giganteus* grown in Xishuangbanna, Yunnan Province, China. The natural bamboo juice was collected in the spring of 2014 and the schematic diagram of the live bamboo juice extraction device was shown in Fig. 3. A film was penetrated into the bamboo intracavity and the insert position was lower than the section of bamboo juice (Zou *et al*., 2006). Natural bamboo juice was extracted through the juice extractor, filter tube, container, pump and piping. The live bamboo juice extraction method without heating will maintain the active components of natural fresh bamboo juice, as well as it will not destroy forest resources. For the convenience of storage and transport, FDPBJ was obtained by freeze-drying method with the cold trap temperature of -30~-40°C, lyophilizer temperature of -69~-82°C and the vacuum degree of 5.5~2.5Pa. The FDPBJ contained proteins of 10.08%, total flavonoids of 77.36 mg/100 g, polysaccharides of 46.95 mg/100 g, polyphenols of 38.70 mg/100 g, dietary fiber of 10.4%, calcium of 1.27%, magnesium of 0.70%, germanium of 0.65 mg/kg and total amino acids amount of 6.48%, respectively (Shan *et al*., 2013).

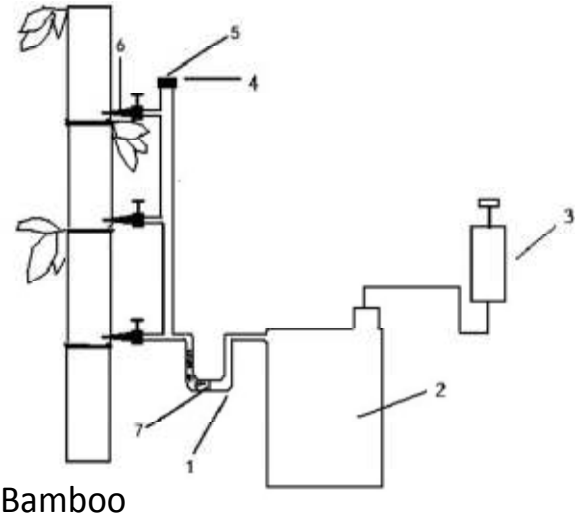

Fig. 3: Schematic diagram of the live bamboo juice extraction device. The extraction device includes; (1): filter tubes; (2): container; (3): pump; (4): pipe; (5): Sealing cover; (6): juice extractor; (7): filter

Liver cell line L02 culture and the preparation of hepatocyte steatosis models: Liver cell line L02 was grown in RPMI-1640 medium with 10% FBS. The culture was maintained in a humidified Series II water jacketed CO_2 incubator (Model 3111, Thermo Electron Corporation, USA) with 5% carbon dioxide and 95% air at 37°C. Then 0.25% Trypsin was used for digestion after the rate of cell fusion rose to 80-90% in the incubator to get control group cell.

Fat emulsion is a high energy and essential fatty acids provider, which could provide the biosynthesis material for the steatosis procedure of hepatic cells in *in vivo* and *in vitro* studies (Zou *et al*., 2006; Meisel *et al*., 2011). Thus, the hepatocyte steatosis models were

established by treating the normal liver cell line L02 with fat emulsion (Zhou and Yang, 2007). L02 cells were seeded in 6-well plates with a cell density of 3×10^5 in 2 mL/well, then incubated in the medium of RPMI-1640 medium with 10% FBS for 48 h to obtain the model group.

Lipid metabolism effect: FDPBJ was dissolved in dimethylsulfoxide (DMSO) to get a stock solution at a concentration of 10 mg/mL. Then it was further diluted in G_0 medium (0.2% FBS-RPMI 1640 medium) to get sample solutions with FDPBJ concentrations of 10 μg/mL, 100 μg/mL and 300 μg/mL. And then all of the cells were transferred in 6-well plates to evaluate their lipid metabolism regulation activity. The cells of the control group (normal liver cell line L02) and the model group (steatosis liver cell line L02) were incubated in G_0 medium for cell synchronization; the cells of three treatment groups were incubated in FDPBJ solutions (10 μg/mL, 100 μg/mL and 300 μg/mL FDPBJ). Twenty-four hours later, the cells were washed with 12 mL PBS for 2 times. Then, the cells were trypsinized, collected, resuspended with PBS and centrifuged for removing PBS. Cell debris was lysed in 0.01% Triton X-100 solution and were centrifuged at 12,000 rpm for 10 min in 4°C. Hepatic TG, TC and FFA contents in the supernatant were determined using corresponding assay kits by AB-1020 automatic biochemical analyzer (Sunostik Medical Technology Co., Ltd, China) and morphological observations were completed by fluorescent inverted phase contrast microscope (ECLIPSE TS 100, Nikon, Japan).

Statistical analysis: Experiments were performed in triplicate and all data were expressed using the mean± standard deviation (n = 3). Degree of variation and significance of difference were analyzed using Analysis of Variance (ANOVA) that was made with the General Linear Model procedure to determine the treatment effects. Significant ($p<0.05$) differences between means were identified by the Least Significant Difference (LSD) procedure.

RESULTS AND DISCUSSION

Establishment of steatosis liver cell line L02: Fat emulsion and high concentration of FBS were reported to be used in the steatosis procedure of liver cell line L02 (Zhang *et al*., 2011; Zhou and Yang, 2007). In this study, both fat emulsion and FBS were chosen together to prepare steatosis liver cell line L02. The steatosis L02 cell model was also estimated by the intracellular lipid contents. Morphological observations showed that the lipid drops were accumulated in the liver cell line L02 cleartly (Fig. 4A and B). TG, TC and FFA contents in the hepatic cells after being cultured by fat emulsion

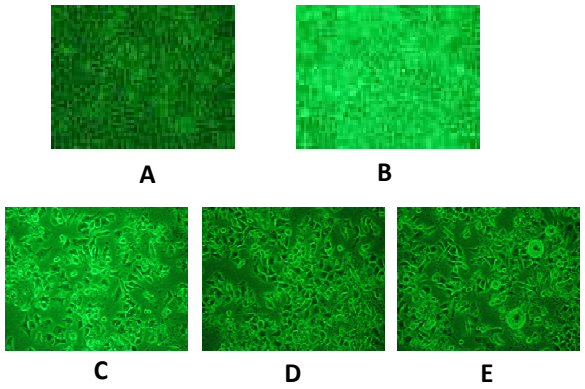

Fig. 4: Cell morphology of liver cell line L02 (10×10); (A): Control group (normal); (B): Model group (Steatosis liver cell line L02); (C): Treatment group I (FDPBJ 10 μg/mL); (D): Treatment group II(FDPBJ 100 μg/mL); (E): Treatment group III (FDPBJ 300 μg/mL)

Table 1: Contents of TG, TC and FFA in FDPBJ treated steatosis L02 cells (mmol/L)

Category	Contents of TG	Contents of TC	Contents of FFA
Control group	33.83±2.32^{c}	3.48±0.57^{c}	0.14±0.02^{b}
Model group	40.67±2.73^{a}	8.83±0.72^{a}	0.24±0.01^{a}
Treatment group I (10μg/mL FDPBJ)	38.50±3.15^{b}	4.81±0.65^{bc}	0.13±0.01^{b}
Treatment group II (100μg/mL FDPBJ)	35.00±2.83^{c}	4.27±0.78^{c}	0.08±0.01^{c}
Treatment group III (300μg/mL FDPBJ)	32.67±1.97^{c}	3.55±0.63^{c}	0.06±0.01^{c}

$^{a-c}$: Means (n = 6) ±standard deviations within a column with different superscripts differ significantly ($p<0.01$)

(model group) were listed in Table 1. Model group had significantly higher contents of TG, TC and FFA than that of control group ($p<0.05$). Compared with the control group, model group could increase 20.2% of TG content, 153.7% of TC content and 71.4% of FFA content. Therefore, fat emulsion and FBS are effective in establish the steatosis L02 cells model.

Effect of FDPBJ on lipid metabolism: Excess amounts of TG and TC indicated the disorder of lipid metabolism, so they were typical indicators in diagnosis and were considered as crucial indexes in the prevention and treatment of diseases associated with lipid metabolism, for example, NAFLD and hyperlipidemia (Targher and Arcaro, 2007). FFA is the decomposed product of TG and it is helpful to analyze lipid metabolism process. Herein, the contents of TG, TC and FFA were selected as the key indexes to analyze the lipid-regulation activities of FDPBJ on steatosis liver L-02 cells in this researh. The steatosis liver L-02 cells were treated by different contents of FDPBJ and the TG, TC and FFA lowering effects of FDPBJ were displayed in Table 1. FDPBJ lowered the TG, TC and FFA contents of model group significantly ($p<0.05$). Treatment group I (100 μg/mL FDPBJ) could reduce about 14% TG content, about 52% TC content and about 67% FFA content after exposure for 24 h. However, treatment group III (300 μg/mL FDPBJ)

could reduce about 20% TG content, about 60% TC content and about 75% FFA content after exposure for 24 h. Thus it can be seen that FDPBJ showed the high TG-regulation, TC-regulation activity and FFA-regulation activity. In conclusion, FDPBJ displayed a clear lipid lowering effect on steatosis L02 cells.

There are many types of hyperlipidemia, while the increasing of TG and TC is a common characteristic of various kinds of hyperlipidemia. Some hyperlipidemia symptoms are TG-rising, some are TC-rising. TG content is important to the onset and progression of NAFLD and an excess amount of TG is definitely associated with NAFLD. On the other hand, TC is closely related to the progression of the atherosclerosis, because cholesterol is a major component of arterial thrombosis and hypercholesterolemia is the major risk factor of cardiovascular and cerebrovascular diseases (Targher and Arcaro, 2007). According to the result of our study, an original hypothesis on the lipid-lowering mechanism of FDPBJ was shown in Fig. 1B and we can assume that FDPBJ would regulate the activity of key enzymes involved in lipid metabolism and accelerate the excretion and the transformation of TG and exogenous cholesterol in steatosis liver cell line L02. For example, TG may be broken down into fatty acid, which can be oxidized and excreted. So the effluent lipid content is increased. The second possible mechanism is that FDPBJ may regulate lipid influent metabolism in steatosis liver cell line L02. As a result, the influent lipid content is decreased.

FFA is the metabolite of TG and its content change is associated with the initial content of TG. If the initial content of TG is reduced, the decomposition product FFA will reduce. In our research, the FFA content reduced with the TG lowering significantly, even the FFA content of treatment group III (0.06±0.01 mmol/L) was lower than that of the control group (0.14±0.02 mmol/L). It may be due to FDPBJ significantly reduces the amount of the TG inflow, so FDPBJ group had less decomposition product (FFA) than control group. Another possible cause is that FDPBJ can promote the oxidation and excretion of FFA. So the content of FFA is surprisingly low.

Effect of FDPBJ on liver cellular morphology: Figure 4 shows morphological changes in hepatocytes. The liver cells line L02 in normal control group (Fig. 4A) were present as single cells. The nucleolus was easily visible and the cytoplasm had plenty of substances. The edges of the cell were clear and lipid droplets were not visible under fluorescent inverted phase contrast microscope (ECLIPSE TS 100, Nikon, Japan). The cells were essentially normal without fatty degeneration or other pathological changes. Figure 4B shows that the structure of the steatosis human liver cell line L02. Appearance of lipid droplets in the cytoplasm of these cells and increasing in the number of lipid droplets indicates fatty degeneration. Some of these droplets had combined into large droplets and covered the entire cytoplasm. Figure 4C to E, less lipid droplets can be seen in the hepatocytes than that of model group (Fig. 4B). Compared with model group (Fig. 4B), treatment group III (Fig. 4E) shows that there are fewer lipid droplets in the hepatocytes and only a few lipid droplets have combined into large droplets.

CONCLUSION

In the present study, steatosis human liver cell L02 was applied as a sensitive and rapid *in vitro* model to investigate the lipid regulation effects of FDPBJ for the first time. FDPBJ had significant TG-lowering and TC-lowering effects with a dose dependent relationship and bamboo juice is a readily available material for natural lipid-lowing compositions. Considering the high concentration of polyphenols and terpenoids in FDPBJ and their excellent activities in TG-lowering and TC-lowering, we should speculate that polyphenols and terpenoids might play the most important role in the lipid-lowering effect of FDPBJ. Synergistic effects of multi-constituents may play crucial role in the lipid-regulation effects of FDPBJ. On account of these *in vitro* results, FDPBJ was reasonably speculated to have satisfactory effects in clinic treatment of NAFLD or hyperlipidemia characterized by the elevation of TG and TC, because of its influence on lipid metabolism. However, *in vivo* assays results were required to confirm these suspects. Moreover, the type and content of the active ingredients in FDPBJ needs further research.

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