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Article

Hypolipidemic properties of the extracts of *Belamcanda chinensis* leaves (BCLE) in KK-A^y mice

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The extract of *Belamcanda chinensis* leaves (BCLE) is a traditional Chinese herbal medicine for the treatment of diabetes-related hyperlipidemia in Hainan province, South China. In this study, the lipid-decreasing effects of BCLE on obese diabetes were investigated on KK-A^y mice. The component F2 ameliorated lipid disorder, as indicated by decreased levels of body weight, liver index, levels of TC, TG and LDL-c in the serum and liver. The enhancement effect of F2 on liver SOD and the inhibitory effect of F2 on MDA demonstrated that F2 exhibited significant antioxidant activity on liver injury. F2 also prevented vacuolar degeneration and reduced pathological tissue injury in liver. In addition, the component F1 decreased the levels of TG, LDL-c and MDA in the liver. These findings suggest that F2 may have therapeutic potential in the prevention and therapy of hyperlipemia and liver disease associated with obesity-related diabetes.

Keywords: *Belamcanda chinensis* leaves. Type 2 diabetes. Hypolipidemic effect. SOD. MDA.

INTRODUCTION

Type 2 diabetes is often accompanied by complications such as obesity, insulin resistance, and hyperlipidemia. Hyperlipidemia is a lipid metabolic disorder characterized by elevated serum cholesterol and triglyceride levals. According to the survey, the prevalence of type 2 diabetes in long-term obese people is 5 times that of the general population, and 80% of type 2 diabetes is obese (Jacob, 2015; Dai, Wang, 2011). And the excessive intake of cholesterol and triglycerides is associated with a risk of heart disease, cardiovascular and cerebrovascular diseases. Therefore, it is of great significance to search a natural medicine which cannot only inhibit obesity but also alleviate the disorder of glycolipid metabolism.

Belamcanda Chinensis belongs to the family Iridaceae and blocky root shoots have been widely used to improve human physical and mental performance and treat high-altitude sickness, fatigue, cancer, bacterial infection in China, Japan and Korea. impotence, nervous system disorders and cardiovascular disease (Zhang *et al.*, 2016). At present, there are more than 100 kinds of compounds isolated from the dry shoot, including flavonoids, triterpenoids, anthraquinones, anthraquinones, volatile components, and others.

Our previous research had evaluated the stability, safety and quality control of the dry leaves of *Belamcanda Chinensis* from Hainan (Rosenbaum, Dallongeville, 2013; Benkhalti, 2002). We also found that flavonoids in the leaves could improve glucose and lipid metabolism disorder on STZ induced diabetic rats (Chen *et al.*, 2011; Wu *et al.*, 2011), but the specific effective part is still not sure, its pharmacological function and mechanisms remain to be further investigated. The aim of this study was to investigate the hypolipidemic effect of several extracts from the leaves of *Belamcanda chinensis* in KK-A^y mice, which would provide a scientific basis for the utilization of dry-leaf resources of *Belamcanda chinensis*.

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MATERIAL AND METHODS

Materials

Sodium carboxymethyl cellulose (CMC-Na) were obtained from Sinopharm Chemical Reagent Co. Ltd. Metformin (No. 20071201), as a positive control drug, were purchased from Bristol-Myers Squibb Co. Ltd. (Shanghai, China). Triglycerides (TG), cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), superoxide dismutase (SOD) and malondialdehyde (MDA) biochemical reagent kits were supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TNF-α Elisa kit was purchased from Shanghai enzyme linked Biotechnology Co., Ltd. (Shanghai, China).

Sample preparation

Leaves of *Belamcanda chinensis* (L.) were harvested from Hainan province, South China. Fresh leaves were dried at 50 ºC in an air dryer, then pounded and homogenized in a Waring blender. The powdered leaves were heat reflux extracted thrice in water, then eluted with water on macroporous adsorption resin and finally eluted with 95% ethanol. The combined ethanol extracts were filtered, concentrated, and finally freeze-dried to powder and were designated as ZS.

The extract ZS acquired as above was precipitated by 95% isopropanol. The precipitate was combined which accounted for about 22% of the total weight of ZS. The supernatants were collected, concentrated and freeze-dried to powder for further use. The precipitate contained polysaccharide fraction and saponins fraction of ZS and was named F2. The supernatant included nearly all of flavonoids of ZS and was named F1.

Animals

Male C57BL/6J and KK-A^y mice at the age of nine weeks were obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing, China. The mice were maintained under controlled conditions of 12 h light/12 h dark cycle and 55±5% relative humidity at 22±2 ºC with free access to water and food. The study was carried out according to the "Principles of Laboratory Animal Care" (World Health Organization (WHO) Chronicle, 1985). A standard pellet diet and water were given ad libitum.

Experimental design

In the experiments, seventy-two KK-A^y mice were divided into six groups with similar weights: blank control (BC), model group (MC), positive control (PC), ZS group (ZS), F1 group (F1), F2 group (F2). From 10 weeks of age, the mice in BC group and MC group were gavage once daily with distilled water and the mice in other groups were treated extracts (200 mg dried herbs/kg body weight) for 6 weeks. The body weights of the mice were recorded once a week. At the end of the experiment, the animals were fasted overnight and sacrificed by cervical dislocation, and blood samples were collected to determine serum biomarkers. Livers were removed and immediately stored at -800 ºC after washing.

Blood biochemical measurement

Serum triglycerides (TG), cholesterol (TC), low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) levels were measured by commercial assay kits. Serum TNF-α was determined by using a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit.

Liver biochemical measurement

On day 42, the mice were sacrificed, and their livers were weighed and washed with cold physiological saline. Then a portion of liver was processed into 10% liver tissue homogenate and was stored at - 80 ºC to be measured.

TG、TC、LDL-c、HDL-c、SOD and MDA in the liver were measured by commercial assay kits.

Hepatic histopathology

HE staining: The liver was removed immediately from the killed mice and rinsed in ice-cold saline. The tissue samples were fixed in 10% neutral-buffered formalin, dehydrated in a graded series of ethanol and embedded in paraffin wax. The tissue in paraffin was cut into sections (5 μm thickness) then dewaxed and rehydrated. The sections were stained with hematoxylin-eosin to review the architecture of hepatic tissue and inflammatory cell infiltration for a histological assessment.

Oil red O staining: The liver tissues were put into the cryostat microtome to make the frozen slices of 5-7 micron. Then the sections were fixed by formaldehyde calcium. Samples were stained with oil red O dye solution to review the lipid storage of liver tissues.

Statistical analysis

All data was presented as means ±S.D. Statistical significance was evaluated by one-way ANOVA followed by post-hoc Tukey test using SPSS 17.0 software. A value of $p < 0.05$ was defined statistically significant.

RESULTS

Effect of BCLE on body weight

During the experiment, body weights of all groups of mice were monitored weekly. Body weight is an important morphological indicator for evaluating the effects of BCLE. As shown in Table I, KK-A^y mice had more body weight gain than C57BL/6J mice (BC group). F2 markedly decreased the body weight of KK-A^y mice ($p < 0.05$, $p < 0.01$), while other treatment had no significant difference., which suggested that F2 can attenuate the body weight gain of obese mice.

TABLE I - Effect of BCLE on body weight in KK-A^y mice (n = 12) ($\bar{x} \pm s$)

Group	Body weight/g						
	0 week	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
BC	24.9±0.8	26.7±0.9	27.2±1.0	26.0±1.8	25.8±1.4	26.7±1.6	27.3±1.7
NC	31.8±1.4###	34.1±4.0###	36.1±3.2###	36.6±2.8###	38.6±2.9###	39.6±3.0###	39.4±3.0###
PC	32.0±2.2	33.9±2.0	35.2±1.8	35.7±1.8	36.8±1.8	38.6±2.2	41.0±2.2
ZS	31.8±1.8	33.5±2.2	35.1±3.3	34.5±2.9	34.8±2.8*	36.9±2.9	38.3±2.3
F1	31.7±2.3	34.0±3.4	35.8±2.5	35.6±1.7	36.6±1.9	38.0±2.3	39.5±2.5
F2	31.6±2.0	33.1±1.7	34.7±1.4	35.0±1.1	35.4±1.5*	36.3±1.5**	38.3±1.4

Effect of BCLE on liver and renal index

Liver index (liver weight/body weight) and renal index (renal weight/body weight) are the objective index to measure organ enlargement and hypertrophy, which can reflect the degree of liver and renal damage caused by diabetes. The results (Table II) showed that the liver enlargement and hypertrophy were observed in the early KK-A^y diabetic mice, yet the kidney did not present physiological abnormalities. Treatment with F2 extremely reduced the liver weight and liver index, which suggested that F2 could alleviate the hepatomegaly of KK-A^y mice.

TABLE II - Effects of BCLE on Liver and Renal Index of KK-A^y Mice ($\bar{x}$ ±s, n=12)

Group	Doses (g/kg)	Liver index (%)	Renal index (%)
BC	——	3.33±0.33	1.18±0.05
MC	——	5.92±1.0###	1.04±0.12#
PC	0.20	5.98±0.67	1.02±0.10
ZS	0.20	5.94±0.63	1.11±0.09*
F1	0.15	5.47±0.41	1.06±0.08
F2	0.05	4.77±0.44*	1.06±0.05

BCLE: the extract of *Belamcanda chinensis* leaves.
BC: blank control, MC: negative control, PC: positive control, ZS: 95% ethanol extract, F1: the clear supernatant of 95% isopropanol extract, F2: the precipitate fraction of 95% isopropanol extract.
# $p<0.01$, ## #$p<0.001$ compared with BC group. * $p<0.05$, ** $p<0.01$ compared with MC group.

Effect of BCLE on serum lipid levels

Dyslipidemia is one of the characteristics of KK-A^y mice, thus, the effect of BCLE on serum lipids was studied after 6 weeks. As shown in Figure 1, there was a significant increase in the levels of serum TG, TC ($p<0.01$) and LDL-c ($p<0.05$) of KK-A^y mice in comparison with C57BL/6J mice, and no significant differences in HDL-c were noted. After treatment with F2, serum TC, TG, and LDL-c levels were significantly decreased ($p<0.01$. $p<0.05$, $p<0.001$). ZS and F1 also decreased serum LDL-c level, but showed no significant differences on other indexes.

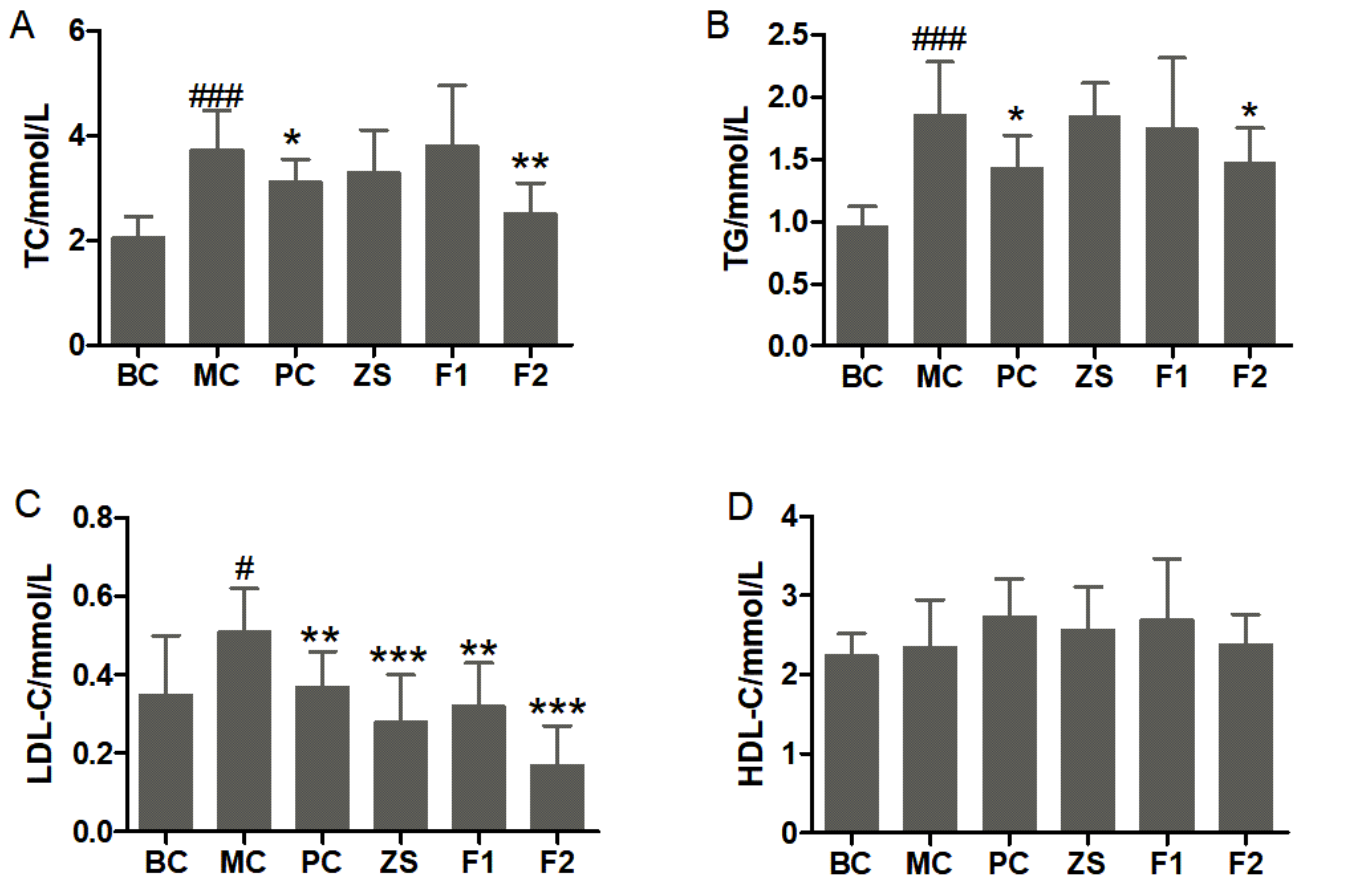

FIGURE 1 - Effect of BCLE on lipid levels in KK-A^y mice (data are expressed as means ± SD)

(A)TC; (B)TG; (C)LDL-c; (D)HDL-c

Effect of BCLE on liver lipid levels of KK-A^y mice

As shown in Figure 2, KK-A^y diabetic mice showed lipid metabolism disorder. Compared with MC group, metformin and F2 significantly reduced the level of TC, TG and LDL-c in liver; F1 decreased the level of TG and LDL-c in liver(p<0.05). F1 and F2 also increased the level of HDL-c in liver (p<0.05). To sum up, F1 and F2 might decrease the liver lipid level and increase the level of HDL-c in KK-A^y mice,

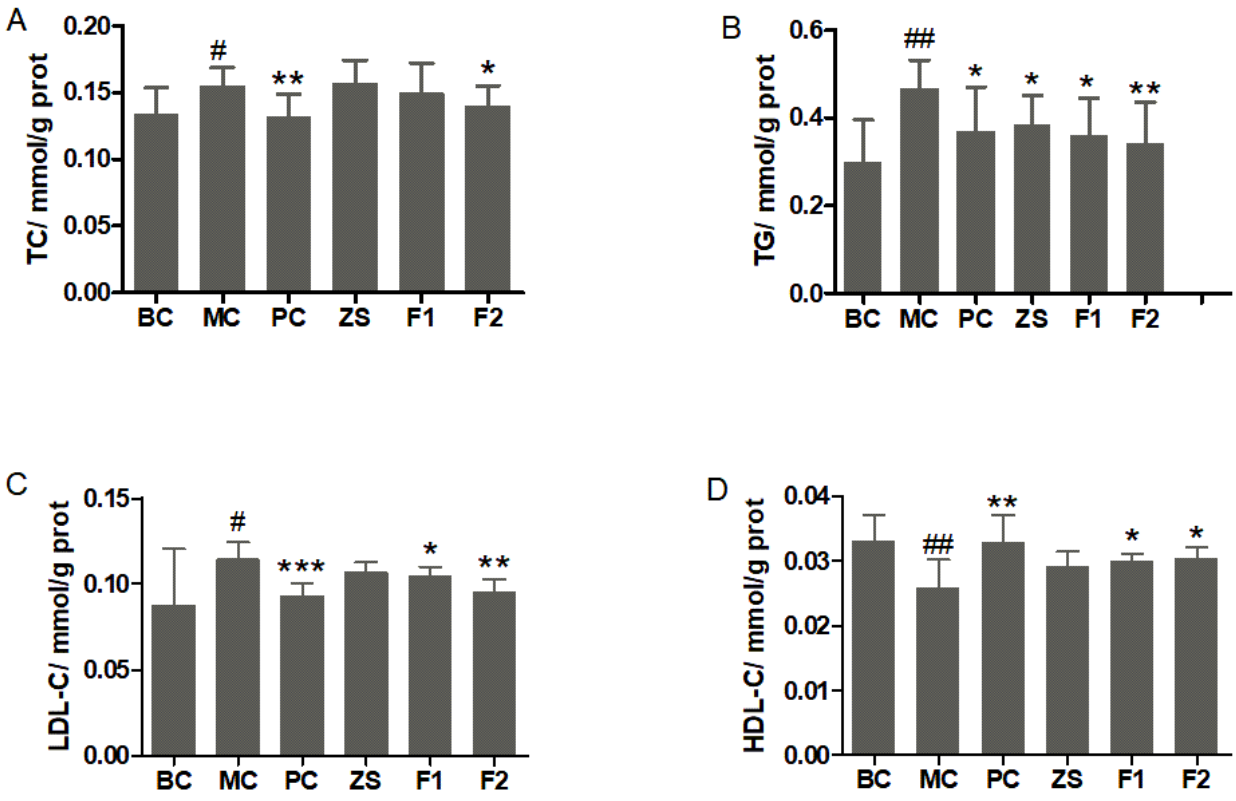

FIGURE 2 - Effect of BCLE on lipid levels in the liver of KK-A^y mice (data are expressed as means ± SD)

(A)TC; (B)TG; (C)LDL-c; (D)HDL-c

Effect of BCLE on TNF-α

TNF-α is one of the key markers of inflammation. The serum levels of TNF-α was measured by an ELISA kit according to the manufacturer' s instructions (Figure 3). The level of TNF-α in serum was increased in the untreated diabetic mice ($p < 0.05$). The administration of metformin and F2 significantly decreased the serum TNF-α level of KK-A^y mice ($p < 0.05$), with TNF-α concentrations returning to control levels

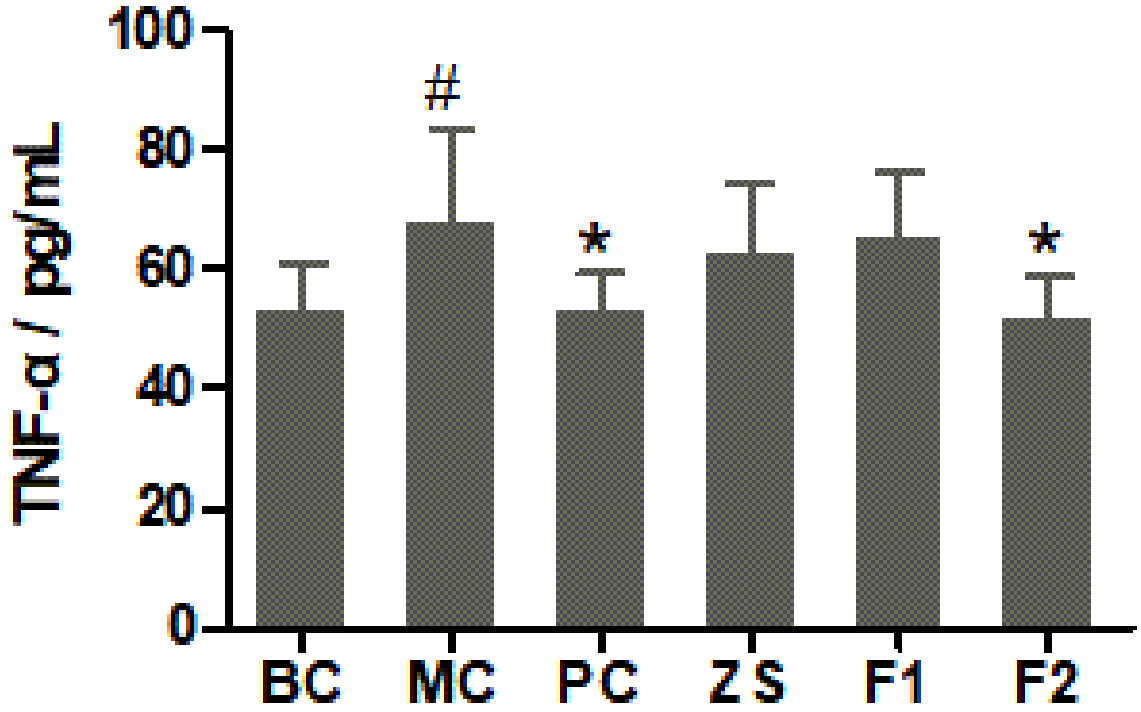

FIGURE 3 - Effect of BCLE on TNF-αof KK-A^y mice (data are expressed as means ± SD)

Effect of BCLE on SOD and MDA in the liver of KK-A^y mice

We detected the level of MDA and SOD in liver to evaluate the antioxidant capacity of BCLE. As shown in Fig. 4, the MDA level was significantly increased ($P < 0.001$) whereas the SOD activity was significantly decreased ($P < 0.05$) in MC group compared with BC group. Metformin, F1 and F2 significantly enhanced ($P < 0.05$) the SOD activity in liver, and decreased ($p < 0.01$, $p < 0.05$) the MDA level at the same time.

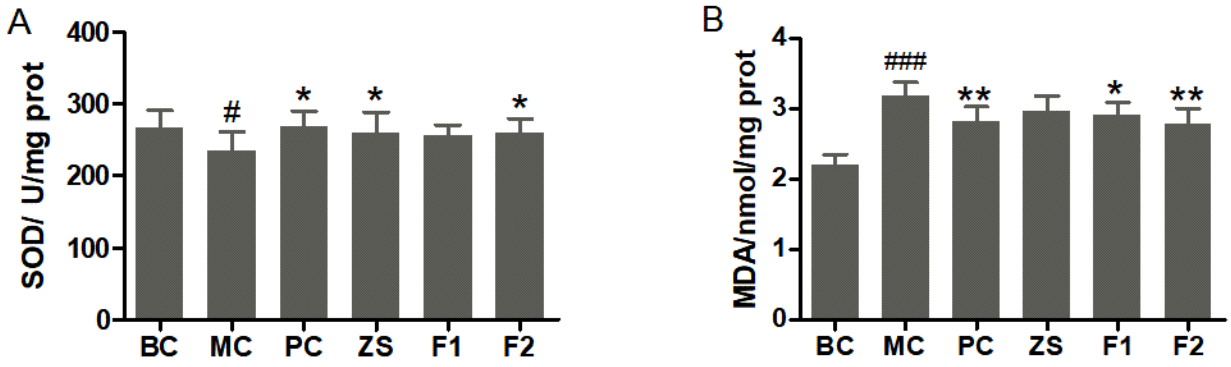

FIGURE 4 - Effect of BCLE on SOD (A) and MDA (B) in KK-A^y mice (data are expressed as means ± SD)

Mice liver histopathological changes

In the study, we performed histological examinations to observe whether BCLE have the capacity of protecting liver in KK-A^y mice. As shown in Figure 5, morphological examination indicated that the liver of KK-A^y mice had high degree of steatosis and cytoplasmic vacuoles. Treatment with F2 was able to attenuate the damages caused by diabetes in KK-A^y mice. Fig. 6 (oil red O staining) also presents lipid accumulation of the liver tissues. The lipid droplet deposition in the liver cells in F2 group was decreased slightly. Yet herbal treatment in the metformin, ZS and F1 group did not relieve liver tissue lesions.

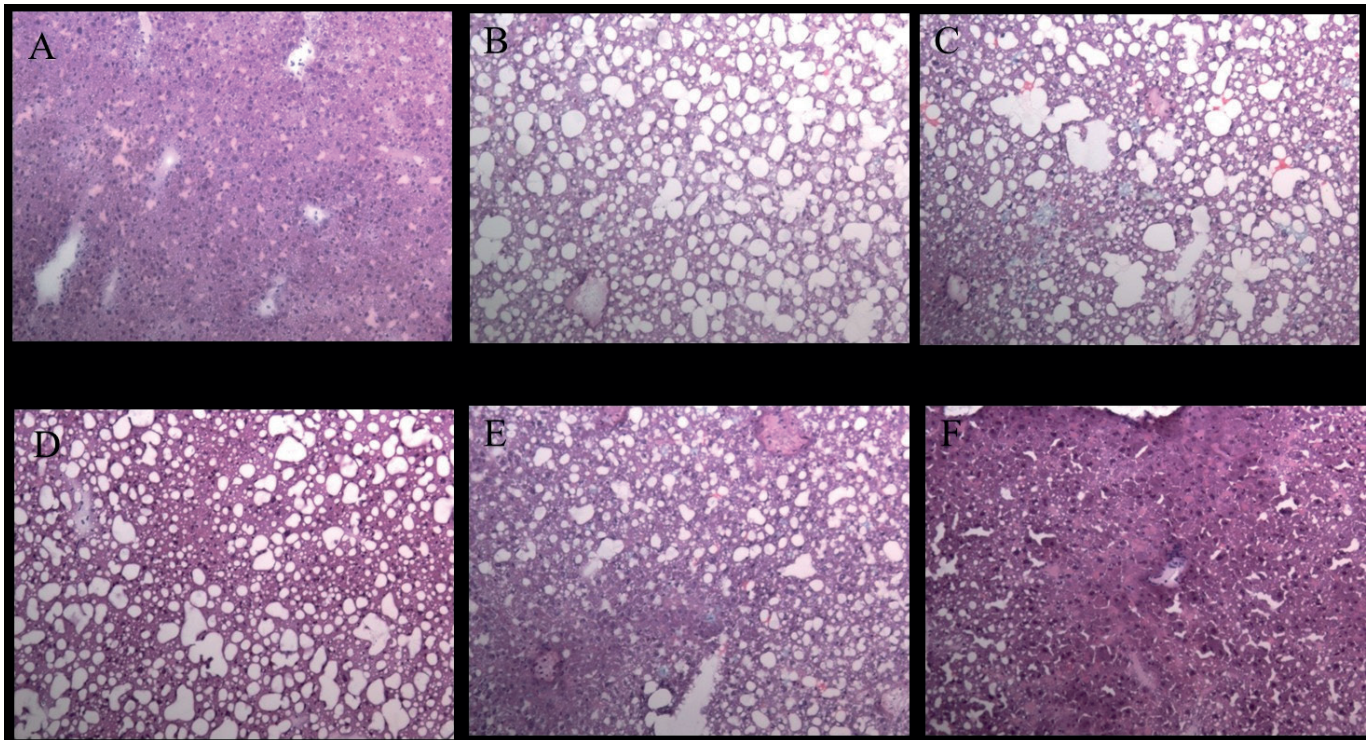

FIGURE 5 - Effects of BCLE on hepatic histopathological changes of KK-A^y mice. Hepar sections by hematoxylin-eosin (HE) staining at a magnification of ×100. A. blank control group, B. Model control, C. positive control, D. ZS group, E. F1 group, F. F2 group.

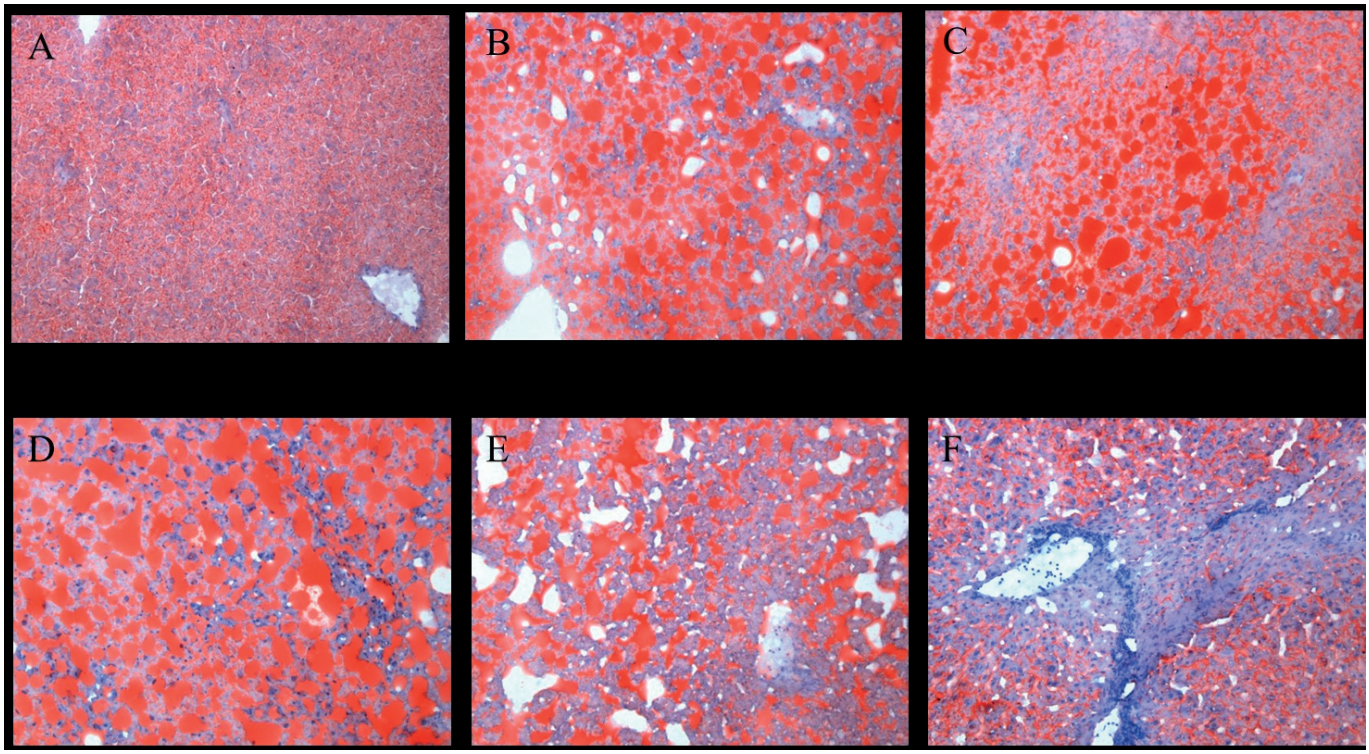

FIGURE 6 - Effects of BCLE on hepatic histopathological changes of KK-A^y mice. Hepar sections by oil red O staining at a magnification of ×100. A. blank control group, B. Model control, C. positive control, D. ZS group, E. F1 group, F. F2 group.

DISCUSSION

KK-A^y mice were introduced from the U.S. Animal Center, their pathogenesis was induced by genetic susceptibility and environmental factors. They are caused by mutations in the yellow gene (Ay) at the Agouti (a) locus on chromosome 2 (Srinivasan, Ramarao, 2012). The homozygotes of the variant all died in the embryonic stage, and the heterozygotes showed severe obesity, hyperglycemia, hyperinsulinemia, lipid metabolism disorders, insulin resistance and other metabolic disorders and yellow fur (Papaioannou, Gardner, 1992). We employed KK-A^y mice as a model to assess the hypolipidemic properties of BCLE on diabetic mice. C57BL/6J mice were black in color and served as a normal control group due to gene homology with KK-A^y mice (Herberg, Coleman, 1977).

Dyslipidemia is manifested by an increase in levels of total cholesterol, triglycerides, low-density lipoprotein, or a decrease in high-density lipoprotein levels in the blood (Li, Zhang, Gu, 2012). HDL-C deficiency and elevated LDL-C are one of the important causes of hyperlipidemia (Miu, Ouyang, Yuan, 2008). TC is a marker of lipid metabolism, while TG is one of the main factors contributing to atherosclerosis. Elevated levels of TG, TC and LDL-c may increase the risk of cardiovascular diseases such as atherosclerosis. In agreement with previous research, we observed an increase in TG, TC and LDL-c levels in MC group. Administration of metformin and F2 resulted in significant decrease in levels of serum TG, TC and LDL-c, but no significant difference was shown in HDL-C level. The decreased serum TG, TC and LDL-c levels may contribute to inhibition of hyperlipidemia. The effect of F2 was a little better than metformin, and F1 and ZS showed no obvious lipid-lowering effect.

Liver is the "chemical plant" of human body, which can express lipoprotein receptor uptake of lipids and regulate blood lipid levels, liver dysfunction seriously affects the transport and transformation of lipids (Perry, Samuel, Petersen, 2014). Excessive accumulation of lipids may cause a certain degree of liver damage, affecting the normal physiological function of the liver. In our study, the increase of liver index and the decrease of liver antioxidant capacity indicate that the liver function of KK-A^y mice is impaired, and the high-

fat diet also promotes the accumulation of lipids in the liver of mice and increases the circulation of lipids in the blood. Administration of metformin and F2 could decrease the content of "bad cholesterol" in liver, increase the content of "good cholesterol", improve lipid metabolism, enhance liver antioxidant capacity and repair liver function. The effect of metformin is similar to F2. Besides, F1 showed improvement on levels of TG, LDL-c and HDL-c.

The body's antioxidant defense system can usually produce enough free radical scavengers such as SOD to prevent reactive oxygen species damage. However, diabetic patients are in a high oxidative stress state due to persistent hyperglycemia, which will result in lower antioxidant capacity and production of reactive oxygen species (Qia *et al.*, 2008). MDA is the end-product of lipid peroxidation induced by free radicals. Its content can reflect the degree of lipid peroxidation in the whole body and indirectly reflect the degree of cell damage (Ou *et al.*, 2013). In our research, the lower SOD activity and higher MDA indicated that the antioxidant capacity of KK-A^y mice was not as good as that of C57BL/6J mice. F2 and metformin significantly enhanced the SOD activity and decrease the MDA content in the liver, but the therapeutic effects of metformin were not equal to the effects of F2. Thereby we presume that F2 could improve the antioxidant capacity of the liver and alleviate the oxidative damage of the liver on KK-A^y mice.

Hepatic steatosis is defined as the presence of visible fat droplets in the cytoplasm of hepatocytes. Obesity or diabetes can increase the workload of liver, leading to massive accumulation of lipids in the liver, followed by hepatocyte vacuolar degeneration and inflammation. From pathological pictures, we believe F2 is able to ameliorate liver vacuolar degeneration and lipid droplet accumulation, but metformin, ZS and F1 showed no improvement in liver lesions.

In summary, our current studies demonstrated that F2 has significant hypolipidemic activity and liver peroxidation injury-repairing effects in diabetic KK-A^y mice and pointed out that F2 may serve as a potential hypolipidemic agent in treating diabetic patients accompanied by hyperlipidemia. In addition, the deeper mechanism of hypolipidemic effect of F2 is necessary to be further studied in both vivo and vitro.

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