Original Paper

Protective Effects of Total Extracts of *Averrhoa carambola* L. (Oxalidaceae) Roots on Streptozotocin-Induced Diabetic Mice

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Key Words

Extracts of *Averrhoa carambola* L. root • Blood glucose • Pancreas • Apoptosis

Abstract

Background: In Chinese culture, the roots of *Averrhoa carambola* L. have long been used for medical purposes due to their potent pharmaceutical activities, such as improving digestive function and treating diabetes. **Methods:** Recently, we prepared extracts of *Averrhoa carambola* L. root (EACR), which were isolated from *Averrhoa carambola* L. roots using ethanol or water. This study was designed to investigate the potential effects of EACR on streptozotocin (STZ) diabetic mice and to explore the underlying mechanism of these effects. Male mice were injected with STZ through the tail vein (120 mg/kg body weight) and were identified as a diabetic mouse model when the level of blood glucose was ≥ 11.1 mmol/L. Subsequently, the mice were administered EACR (150, 300, 600, 1200 mg/kg body weight/d) and metformin (320 mg/kg body weight/d) via intragastric gavage for three weeks. **Results:** The results indicated that EACR significantly decreased the serum levels of blood glucose, total cholesterol (TC), triglycerides (TGs) and free fatty acids (FFAs), whereas the content of serum insulin was elevated. In addition, the expressions of apoptosis-related regulators (including caspase-3, caspase-8 and caspase-9) and the apoptosis-induced protein Bax were markedly down-regulated by EACR, whereas the expression of the anti-apoptotic Bcl-2 protein was notably increased. Furthermore, EACR could protect the diabetic mice against the STZ-induced apoptosis of pancreatic β cells. **Conclusion:** Taken together, these findings indicate that EACR plays an effective hyperglycemic role that is associated with ameliorating metabolic functions and with inhibiting apoptosis in pancreas tissue.

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Introduction

Diabetic mellitus (DM) is a chronic metabolic disease related to the etiology of pancreatic β cell damage, which results in a relative deficiency of insulin and in type 1 DM development [1]. As we know, insulin is secreted by pancreatic β cells and plays a key role in the progression of diabetes. In addition, pancreas dysfunction or low insulin sensitivity is the mainspring of type 1 DM [2, 3]. Commonly, DM therapy includes diet treatment combined with exercise and hypoglycemic drugs, such as the thiazolidinedione class of insulin sensitizers and biguanide, which is specialized for obesity-diabetes, for increasing the secretion of insulin and ameliorating insulin sensitivity [4, 5].

Increasing studies have suggested that diabetes in animals is accompanied by the elevation of triglycerides (TGs), total cholesterol (TC) and free fatty acids (FFAs) [6, 7]. Previous studies have demonstrated that FFAs are critical for the formation and development of DM [8]. FFAs are considered to inactivate the insulin receptor in target cells and to inhibit the combination between insulin and its receptor [9]. The caspase family is involved in the molecular mechanism of apoptosis induction [10, 11]. This family contains two types of caspases. Initiator caspases, which include caspase-2, caspase-8, caspase-9 and caspase-10, can be self-activated with the participation of an assistant protein factor and activate the downstream caspase. The second type of caspases are executioner caspases, consisting of caspase-3, caspase-6, and caspase-7, which work to alter the biochemistry and morphology of cells, finally resulting in cell apoptosis [12, 13]. The mechanisms of the caspase family in apoptosis are of two types: mitochondria-dependent and mitochondria-independent. Caspase-8 activation of caspase-3 is directly mediated by the death receptor in the mitochondria-independent pathway; caspase-8 can also cleave Bid, which is a precursor of Bcl-2, and then the apoptotic signal is transferred to the mitochondria-dependent pathway, which amplifies the Fas/TNF cell apoptotic signal [14]. In addition, the apoptosis-related Bcl-2 protein and the Bax family play crucial roles in apoptosis. Potential negative effect of the apoptosis-specific pancreas, which the consequence between apoptosis and loss of pancreatic β cells leads to a high risk of worse diabetogenic progression, is particularly dangerous for people's health [15]. Therefore, selecting the key factor may improve the chance of treating or controlling diabetes. Our previous basic research confirmed that the roots of *Averrhoa carambola* L. have a beneficial anti-hyperglycemic effect [16, 17], and effective components were extracted from the roots of *Averrhoa carambola* L., which have a therapeutic potential in diabetic nephropathy and an inhibitory effect on the kidneys of diabetic mice [18, 19]. Thus, we initiated efforts to explore the mechanism of the anti-hypoglycemic effects of EACR.

Diabetes mellitus, which is also named *Xiaoke Zheng* in ancient China, was first documented over two thousand years ago. Thus far, it has been popular to use traditional Chinese medicine theory, in which some curative effects have been identified, to treat diabetes in China. Chinese herbal medicine (CHM) has been proven to protect Chinese people against disease for thousand years, which suggests that CHM may be the potent agent for further investigation. The root of *Averrhoa carambola* L., which is called yangtaogen in Chinese, has been reported to have many pharmacological activities, including an anti-oxidative effect and aid DM management [20-22]. Combined with our previous studies that have shown its hypoglycemic effect, we employed STZ-diabetic mice to investigate the molecular mechanism of EACR-mediated blood glucose reduction further.

Materials and Methods

Plant material and preparation of EACR

Averrhoa carambola L. plants were obtained from Lingshan County, Guangxi Province, China, batch number: 20121016 (identified by Professor Maoxiang Lai at the Traditional Chinese Medicine Research Institute of Guangxi).

The powder from air-dried roots of *Averrhoa carambola* L. (93 kg) was soaked for 30 minutes with 60% aq. EtOH (2×744 L, 1 h for each), then extracted under reflux 2 times. Then, the remnant powder was

Fig. 1. The HPLC chromatogram of the EACR solution.

refluxed in 744 L distilled water for 1 h. The ethanol and water extracts were combined, then the extracts were filtrated and concentrated at $65 \pm 5^\circ\text{C}$, vacuum dried ($70\text{--}80^\circ\text{C}$), and ground into powder (7.3 kg). The EACR was dissolved in distilled water before administration to the mice.

Determinations of the amount of MNDD and DMDD in the EACR

2-Methoxy-6-nonylcyclohexa-2,5-diene-1,4-dione (MNDD) and 2-dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione (DMDD) were separated from the roots of *Averrhoa carambola* L., and the purity of both was $\geq 95.0\%$ [23]. Approximately 7.5 mg each of MNDD and DMDD was accurately weighed and dissolved in methanol. HPLC was run on an Agilent 1100 (Agilent Technologies, America) that was equipped with a VWD detector and with a Thermo ODS-2HYPERASIL column (250×4.6 mm, $5\ \mu\text{m}$, America) under the following chromatographic conditions: column temperature, 30°C ; methanol (Merck, Germany) and H_2O (88:12) as the mobile phase; 1.0 mL/min flow rate; with a detection wavelength of 276 nm.

EACR (0.2 g) was accurately weighed and refluxed in 25 mL methanol for 45 minutes. Then, the solution was filtrated (Jinteng filter, $0.45\ \mu\text{m}$). Next, $5\ \mu\text{L}$ each of MNDD, DMDD and EACR solutions were injected into the HPLC column and eluted under the abovementioned chromatography conditions. The amount of MNDD and DMDD in the EACR was 8.72 mg/g and 3.98 mg/g, respectively (Fig. 1).

Animals

Healthy male Kunming (KM) mice, which were each approximately 18–22 g, were housed in individual cages under controlled temperature ($25 \pm 1^\circ\text{C}$) and humidity ($60 \pm 5\%$) on a 12:12 h light–dark cycle and were fed with standard rodent food (Beijing Vital River Laboratories, China) and with free access to water. The mice were injected with streptozotocin (STZ) via the tail vein (120 mg/kg body weight) after 12 hours fasting. Seventy-two hours later, the fasting blood glucose (FBG) testing was conducted, and the mice with $\text{FBG} \geq 11.1$ mmol/L were selected as diabetic mice. All the experimental procedures and protocols were approved by the Ethical Committee of the Experimental Use of Animals at Guangxi Medical University (Guangxi, China).

Experimental design

The mice were assigned to the following groups:

Group I (n = 10): healthy mice administered distilled water: normal control.

Group II (n = 10): diabetic mice administered distilled water: model control.

Group III (n = 10): diabetic mice administered metformin (320 mg/kg body weight) by gastric perfusion once a day for 21 days: positive control. The concentration of the metformin was adjusted to 16 mg/ml before administration to the mice.

Group IV (n = 10): diabetic mice administered EACR (150 mg/kg body weight) by gastric perfusion once a day for 21 days.

Group V (n = 10): diabetic mice administered EACR (300 mg/kg body weight) by gastric perfusion once a day for 21 days.

Group VI (n = 10): diabetic mice administered EACR (600 mg/kg body weight) by gastric perfusion once a day for 21 days.

Group VII (n = 10): diabetic mice administered EACR (1200 mg/kg body weight) by gastric perfusion once a day for 21 days.

Collection of the blood and tissues

The blood and tissues were collected, and the biochemical indexes were detected after 21 days of treatments. Blood samples were collected from the retro-orbital venous plexus and centrifuged for 10 minutes at 3500 rpm to immediately separate the serum. After the mice were executed under intraperitoneal anesthesia with 0.7% pentobarbital sodium, the pancreas tissues were removed and washed with cold saline. The serum and tissues were stored at -80 °C for further determination.

Biochemical index assays

The collected blood was used for FBG testing with an accurate blood glucose meter (Accu-check Performa, Roche, Germany). The serum levels of TC, TGs and FFAs were measured using an automatic biochemical analyzer (Hitachi Model 7100 Automatic Analyzer). The concentration of serum fasting blood insulin (FINS) was analyzed using an Iodine [125I] Insulin Radioimmunoassay Kit (Beijing North Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions. The insulin sensitivity index (ISI) was calculated using the following formula [24]: $ISI = \ln (FBG \times FINS)^{-1}$

Pathological screening

The pancreatic samples were fixed with 10% formaldehyde over 24 hours and then embedded in paraffin. The slices were prepared using regular hematoxylin-eosin (HE) staining and were observed under a light microscope.

Pancreatic B cell apoptosis assay

The pancreatic sections were processed using routine terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) procedures with an In Situ Cell Death Detection Kit (Roche, Germany). Apoptotic cells were exactly located under light microscope observation through a color reaction.

Immunohistochemical exploration of Caspase-3, Caspase-8, Caspase-9, Bcl-2 and Bax

The expression levels of Caspase-3, Caspase-8, Caspase-9, Bcl-2 and Bax were analyzed by immunohistochemistry [19]. Pancreas samples were treated following the manufacturers' procedures noted in the descriptions of the commercial kits.

Mouse monoclonal antibodies: Caspase-3 (abcam, London, UK); caspase-8 (Santa Cruz Biotechnology, Inc., California, USA); caspase-9 (Cell Signaling Technology, Boston, USA); and Bcl-2 (Santa Cruz Biotechnology, Inc., California, USA). Rabbit polyclonal antibodies: Bax (Santa Cruz Biotechnology, Inc., California, USA); anti-mouse/rabbit (HRP, Shanghai Long Island Biotech. Co., LTD, Shanghai, China).

Observation of pancreas ultrastructure

The pancreases from the tested mice were removed, then cut into small pieces (approximately 2 mm×2 mm×2 mm), and fixed in 2.5% pre-cooling glutaraldehyde immediately for 2 h under low temperature 0 °C. All the samples were fixed in 1% osmium tetroxide for 2 h again, dehydrated with a series of ethanol and acetone, embedded, made into sections, and stained with uranyl acetate and lead citric acid. Finally, the samples were observed under a transmission electron microscope (HITACHI H-7650).

Statistical analysis

All experiment data were statistically processed using SPSS 16.0 software (SPSS Inc., USA). The values were represented as the mean ± S.E. after testing the homogeneity of variance; the significance of the data was analyzed using a one-way analysis of variance (ANOVA). Differences between groups were considered statistically significance at P<0.05.

Fig. 2. Effect of EACR on body weight. I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV-VII: 150, 300, 600, 1200 mg/kg of EACR; Results were presented as the means $\pm$ S.E.. * P <0.05 compared with the normal control group; # P <0.05 compared with the model group.

Fig. 3. Hypoglycemic effect of EACR. I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV-VII: 150, 300, 600, 1200 mg/kg of EACR; Results were presented as the means $\pm$ S.E.. * P <0.05 compared with the normal control group; # P <0.05 compared with the model group.

Results

Effect of EACR on body weight and on the serum levels of blood glucose and insulin parameters

As shown in the behaviouristic observations, the mice in the normal control group exhibited a good mental condition, activity, smooth fur, and stably increasing body weight. In contrast, the mice in the model control group exhibited a poor mental condition, rare fur, and slowly reducing body weight. After the 21 days of treatments, in both the metformin and EACR administered groups, the vital signs of mice were effectively improved and the body weight gradually increased (Fig. 2).

Accordingly, the basic FBG level accompanied with insulin sensitivity index maintained a normal stable level in the mice of the normal control group during the experiment, and the serum insulin level was at the physiological threshold. In contrast, STZ-diabetic mice had a significantly elevated FBG level, decreased insulin sensitivity index and a reduction in the serum insulin level. Interestingly, the groups that were administered metformin and EACR

Fig. 4. Effect of EACR on fasting insulin in the serum (FINS). I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV-VII: 150, 300, 600, 1200 mg/kg of EACR; Results were presented as the means $\pm$ S.E.. * $P < 0.05$ compared with the normal control group; # $P < 0.05$ compared with the model group.

Fig. 5. Effect of EACR on insulin sensitivity index (ISI). I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV-VII: 150, 300, 600, 1200 mg/kg of EACR; Results were presented as the means $\pm$ S.E.. * $P < 0.05$ compared with the normal control group; # $P < 0.05$ compared with the model group.

Fig. 6. Effect of EACR on serum contents of TC, TG and FFA. I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV-VII: 150, 300, 600, 1200 mg/kg of EACR; Results were presented as the means $\pm$ S.E.. * $P < 0.05$ compared with the normal control group; # $P < 0.05$ compared with the model group.

showed effective reductions in the FBG level and up-regulation of the serum insulin level, and enhanced insulin sensitivity (Fig. 3, Fig. 4, Fig. 5).

Fig. 7. Histological observations of pancreas tissue. I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV: 150mg.kg⁻¹.d⁻¹ of EACR; V: 300 mg/kg of EACR; VI: 600mg.kg⁻¹.d⁻¹ of EACR; VII: 1200mg.kg⁻¹.d⁻¹ of EACR. Note: Arrows point to islet contained certain pancreatic β cells.

Fig. 8. Effect of EACR on ultrastructure changes in mouse pancreas. I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV: 150 mg/kg of EACR; V: 300 mg/kg of EACR; VI: 600 mg/kg of EACR; VII: 1200 mg/kg of EACR. Note: Nu: cell nucleus, sg: secretory granules.

Fig. 9. Effect of EACR on apoptosis in mouse pancreas tissue (TUNEL assay, scale bar: 200 μm). I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV: 150 mg/kg of EACR; V: 300 mg/kg of EACR; VI: 600 mg/kg of EACR; VII: 1200 mg/kg of EACR. Note: Arrows represent islet included TUNEL-positive pancreatic β cells (brown).

Effect of EACR on lipid metabolism

The serum contents of TC, TGs and FFAs of EACR-treated mice were clearly lower when compared with those values in the model control group. Notably, the abnormal changes in STZ-diabetic mice were higher than those changes in normal mice (Fig. 6).

Fig. 10. Effect of EACR on the expression of Caspase-3, -8, -9 in mouse pancreas tissue (Immunohistochemical analysis, scale bar: 200 μ m). I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV: 150 mg/kg of EACR; V: 300 mg/kg of EACR; VI: 600 mg/kg of EACR; VII: 1200 mg/kg of EACR. Note: Arrows represent islet included immunity-positive pancreatic β cells (brown).

Fig. 11. Effect of EACR on the expression of Bax and Bcl-2 in mouse pancreas tissue. (Immunohistochemical analysis, scale bar: 200 μ m). I: Normal control; II: Model control; III: 320 mg/kg of metformin; IV: 150 mg/kg of EACR; V: 300 mg/kg of EACR; VI: 600 mg/kg of EACR; VII: 1200 mg/kg of EACR. Note: Arrows represent islet included immunity-positive pancreatic β cells (brown).

Histological and ultrastructural observations

The normal mice showed an integrated cell structure in the pancreas tissue, along with numerous pancreatic β cells. Conversely, injured islet β cells occurred in STZ-diabetic mice, including a decrease in β cells, the morphological signs of cell damage or apoptosis, and cytokine infiltration. After the treatments with metformin and with EACR, the pancreatic β cells gradually increased, and the cytoarchitecture effectively recovered (Fig. 7).

As observed by transmission electron microscope, the cell ultrastructure of normal mouse pancreatic endochylema showed many small secretory granules and mitochondria, the complete cytoarchitecture. Notably, severe injuries occurred in STZ-diabetic mice, including the destruction of mitochondria cristae, dilatation and degranulation of the endoplasmic reticulum, and deformation of the nuclear membrane. After the metformin and EACR treatments, the mitochondrial damage in the pancreas was reduced, the endoplasmic reticulum improved, and β cells and secretory granules increased (Fig. 8).

Evaluation of pancreatic β cell apoptosis

To reflect the therapeutic efficacy of the extracts under different dosages during the same period, TUNEL staining was used to determine the number of positive cells in pancreas samples. As a result, the number of TUNEL-positive cells in STZ-diabetic mice markedly increased compared with that in the normal control group. DM mice through the post-treatment showed a significant decrease in cells positively stained with TUNEL when compared with the model control group (Fig. 9).

Effect of EACR on apoptosis-related regulators in pancreas samples

The results from the immunohistochemistry assay indicated that the number of brown positive cells (including Caspase-3, Caspase-8 and Caspase-9) in pancreas samples of STZ-diabetic mice increased compared with that in normal mice. After the metformin and EACR treatments, these abnormal protein expression levels were down-regulated, resulting in the reduction of positive cells (Fig. 10).

Meanwhile, the pancreas-injured mice, which were induced by STZ, led to the decrease in Bcl-2 positive cells, whereas the Bax protein level was significantly elevated. As a result, the mice treated by metformin and by EACR showed an effectively increasing number of Bcl-2 positive cells and a gradually decreasing level of Bax protein expression. Moreover, these anti-apoptosis benefits of EACR on pancreas tissue showed a dose-dependent manner, in which the bio-efficacy of EACR initiated from relatively lower designed 150 mg/kg dosage (Fig. 11).

Discussion

Many experiments have demonstrated that excessive glucose and fat contents in the body can cause glucotoxicity and lipotoxicity, in which FFAs and adipocyte factors are critical to the formation and development of DM [25, 26]. The over-concentration of free fatty acids (FFAs) inhibits peripheral glucose utilization and promotes gluconeogenesis, which eventually causes insulin secretion disorders [27]. In addition, unregulated FFA release is responsible for inducing the apoptosis of pancreatic β cells. Therefore, the strategy of reducing the FFA level can significantly eliminate the lipotoxicity and effectively lower the blood sugar. In contrast, STZ is a classical toxicant that is used through fasting injection to induce diabetes in animals for scientific research [28]. In this experiment, STZ-diabetic mice were used to evaluate the drug reaction against DM. In the present study, we found that the extracts from the roots of *Averrhoa carambola* L. clearly decreased the level of FBG in diabetic mice after 21 days of treatments.

Furthermore, the results from Fig. 4 show that the serum concentrations of FFAs, TC and TGs in the metformin and EACR-treated (600, 1200 mg/kg/d) groups were lower than those concentrations in STZ-diabetic mice. These beneficial phenomena illustrated that the EACR-mediated hypoglycemic function was related to attenuating lipotoxicity or to reducing lipogenesis. Clinically, metformin acting as one of the antidiabetic drugs, has been shown to effectively prevent against diabetes and related complications. The pharmacological action of metformin is primarily involved in enhancing insulin sensitivity and increasing peripheral glucose uptake, thereby reducing hyperglycemia and metabolic disturbance [29]. Therefore, this study indicated that for diabetes patients, metformin treatment and EACR supplement may contribute to diabetes management, and may yield additional benefits through combined administration.

Pathologically, pancreatic damage, particularly β cell injuries, is one of the most common causes of DM occurrence; the decrease in pancreatic β cells is considered to occur by cell apoptosis, and this result may be due to decrease in the blood glucose concentration. Thus, the attenuation of pancreas injury is an effective approach to manage DM [30, 31]. Insulin dysfunction is caused by pancreatic β cell apoptosis, which is associated with regulatory proteins such as Caspase-3, Caspase-8, Caspase-9, Bcl-2 and Bax. Cell apoptosis is elicited by the activation of irreversible cascade events, in which the Caspase family is the final pathway for inducing apoptosis [32]. Meanwhile, the expression levels of Bcl-2 and Bax also can reflect apoptotic conditions; apoptosis is involved to a certain extent in pancreas injury [33]. In this study, HE pathological stains, TUNEL stains and immunohistochemical assays were used to assess the pancreas damage situation using both histology and molecular biology. The morphology of the pancreas in STZ-diabetic mice resulted in scathing injuries when compared with the normal mice with integrated cyto-architecture of pancreas tissues. However, the abnormal changes were reversed in the groups treated with EACR, particularly

in the treated groups with 600 mg/kg/d and 1200 mg/kg/d. In addition, the number of TUNEL-positive cells in the pancreas tissue of STZ-diabetic mice was significantly elevated compared with that in normal pancreas slices. Apoptosis in pancreas samples treated with EACR gradually reduced. The underlying mechanism of regulatory proteins was shown as follows: the beneficial effect of EACR on DM may be implicated in the inhibition of the apoptosis-related mitochondria-dependent pathway to rescue pancreas cells against STZ-toxicity, thereby improving the metabolic function in the body.

Conclusions

In summary, our study demonstrates that EACR from the roots of *Averrhoa carambola* L. has an effective hypoglycemic effect by regulating the apoptosis-specific effectors as well as modulators for reducing apoptosis in pancreas tissue. Furthermore, EACR can serve as a potential agent for the treatment of diabetic mellitus.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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