Original Paper

Effect of the Total Extract of *Averrhoacarambola*(Oxalidaceae) Root on the Expression Levels of TLR4 and NF- κ B in Streptozotocin-Induced Diabetic Mice

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Key Words

Extracts of *Averrhoacarambola* L. root • Interleukin-6 • Tumor necrosis factor- α • Toll-like receptor 4 • Nuclear factor- κ B

Abstract

Background: *Averrhoacarambola* L., which is a folk medicine used in diabetes mellitus (DM) in ancient China, has been reported to have anti-diabetic efficacy. **Aims:** The aim of this study was to evaluate the hypoglycemic effect of the extract of *Averrhoacarambola* L. root (EACR) on the regulation of the Toll-like receptor 4 (TLR4)-Nuclear-factor kappa B (NF- κ B) pathway in streptozotocin (STZ)-induced diabetic mice. **Methods:** the mice were injected with STZ (120 mg/kg body weight) via a tail vein. After 72 h, the mice with FBG $\geq$ 11.1 mmol/L were confirmed as having diabetes. Subsequently, the mice were treated intragastrically with EACR (300, 600, 1200 mg/kg body weight/d) and metformin (320 mg/kg body weight/d) for 14 days. **Results:** As a result the serum fasting blood glucose (FBG), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) levels were decreased following EACR administration. Immunohistochemical analysis revealed that the pancreatic tissue expression levels of TLR4 and NF- κ B were downregulated after EACR administration. EACR suppressed pancreatic mRNA expression level of TLR4 and blocked the downstream NF- κ B pathway in the pancreas. According to Western blot analysis EACR suppressed pancreatic TLR4 and NF- κ B protein expression levels. Histopathological examination of the pancreas showed that STZ-induced pancreas lesions were alleviated by the EACR treatment. **Conclusion:** These findings suggest that the modulation of the IL-6 and TNF- α inflammatory cytokines and the suppression of the TLR4-NF- κ B pathway are most likely involved in the anti-hyperglycemic effect of EACR in STZ-induced diabetic mice.

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Introduction

Many complications result from the metabolic disorders of diabetes mellitus (DM) [1]. Oxidative stress and inflammation play crucial roles in the progression of DM [2]. The study conducted by Fumei Tang revealed that the cytokine levels of 160 patients with type 2 diabetic mellitus (T2DM) indicated a low level of inflammation, and the level of inflammation was associated with the control of blood glucose [3]. Many studies have demonstrated that decreases in pro-inflammatory cytokines that contribute to β -cell dysfunction and apoptosis, such as IL-6, TNF- α and interferon (IFN)- γ have a beneficial effect on DM [4-6]. TLR4 is an important molecular receptor in innate immunity and inflammatory responses. TLR4 is directly activated by pro-inflammatory factor kinases and active oxygen such as $O_2^{\cdot-}$ and OH $\cdot$ or indirectly activated by the cytokine signaling cascade and pro-inflammatory cytokines, resulting in the release of insulin desensitization factor, which inhibits the effect of insulin [7].

An abnormal increase in FFAs is one cause of lipotoxicity, which could stimulate the excessive release of IL-6 and TNF- α inflammatory cytokines and monocyte chemoattractant protein-1 (MCP-1) [8]. These cytokines interfere with glucose and lipid metabolism. Inflammatory cytokines also combine with classical cytokine receptors and pattern recognition receptors in adipose tissues. The interaction of FFAs and inflammatory cytokines could cause long-term low-level inflammation in the major metabolic organs and result in metabolic syndrome (MS), which is characterized by glucose and lipid metabolism disorders. DM and MS are closely linked with insulin resistance, and a higher incidence of diabetes related complications such as cardiovascular disease and central adiposity is associated with MS. MS control and higher insulin doses are necessary in the therapy of T1DM [9-11].

Averrhoacarambola L. root (ACLR) refers to the fresh and dried root of the genus *Oxalis*. Historically, ACLR has been a folk medicine used in DM, and the blood glucose of patients with diabetes has been observed to remain stable after ACLR treatment. Our preliminary results suggest that the administration of an EACR medication to STZ-induced diabetic mice could down-regulate the secretion of inflammatory factors and improve islet cell apoptosis by reducing the serum content of FFAs [12]. This finding suggests that EACR can potentially improve inflammation in diabetic mice. In this study, a STZ-induced diabetes model was used to investigate the therapeutic effect of regulating the expression levels of inflammatory cytokines in pancreatic tissue.

Materials and Methods

Chemicals

EACR was extracted from ACLR by the Department of Pharmacology of Guangxi Medical University (Guangxi, China) [12]. The levels of the effective components MNDD and DMDD in EACR were determined by HPLC [13]. The other chemicals that were used were purchased from local commercial sources.

Animals and drug administration

Healthy male SPF Kunming (KM) mice weighing 18-22 g were provided by the Experimental Animal Center of Guangxi Medical University (registration number SCXK 2009-0002). The animals were housed at $25 \pm 1^\circ\text{C}$, a relative humidity of $60 \pm 5\%$ through air exchange in the room and a 12h light/12h dark cycle. All of the animal protocols were approved by the institutional ethics committee of Guangxi Medical University (approval No.: 2012011121).

Briefly, the mice were fasted for 12 h before the treatment with STZ at a dose of 120 mg/kg of the body weight via a tail vein injection. After 72 h, the mice with FBG ≥ 11.1 mmol/L were confirmed as having diabetes. The diabetic mice were randomly divided into the following five groups with 10 animals per group: the STZ group, the metformin control group and the groups administered low, moderate, and high doses of EACR. And 10 healthy mice were given EACR ($1200 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) as EACR control group. The normal control group was composed of ten healthy mice. The mice belonging to the normal and STZ groups were

administered saline. The metformin group was treated intragastrically with 320 mg·kg⁻¹·d⁻¹ metformin. The groups administered low, moderate, and high doses of EACR were treated intragastrically with 300, 600, and 1200 mg·kg⁻¹·d⁻¹ EACR, respectively.

After 14 days, the mice were sacrificed. The blood was collected and centrifuged for 10 min at 3500 rpm to separate the serum, and the pancreases were removed. The serum and tissues were stored at -80 °C until further analysis.

Biochemical detection of FBG and the IL-6 and TNF-α serum cytokines

Tail vein blood samples were used for FBG detection using Roche ACCU-CHEK® Performa (Strip lot: 470664, Switzerland). IL-6 (Mouse Interleukin 6 (IL-6) ELISA Kit, Lot: 031015173, CUSABIO BIOTECH, Ltd.) and TNF-α (Mouse TNF-α ELISA Kit, Lot: L18016432, CUSABIO BIOTECH, Ltd.) serum cytokines were measured by ELISA according to the manufacturer's instructions. The final data are presented in units of pgml⁻¹. The descent rate of EACR on FBG (%) was calculated using the following formula: [(FBG_{d0} - FBG_{d14}) / FBG_{d0}] × 100%.

Histopathological examination

The 4μm pancreas samples (n=10, per group) were stained with hematoxylin and eosin (HE) and observed under a light microscope. The pathological changes in the structure of the pancreas and damage to the islet cells of each group were evaluated.

Immunohistochemical analysis

The procedures were performed in accordance with the manufacturer's instructions. The paraffin blocks were dewaxed and rehydrated, and the slides were stained by SP immunohistochemistry after retrieval of the antigens with antibodies for TLR4 (Santa Cruz Biotechnology, USA), NF-κappa B (E498, Lot: 1, rabbit ab, Cell Signaling Technology, Boston, MA, USA), insulin (Santa Cruz Biotechnology, USA) and anti-mouse/rabbit (HRP, Shanghai Long Island Biotec Co., Ltd., Shanghai, China).

RNA extraction and PCR analysis

The total RNA from 30 mg of pancreatic tissue was extracted using Trizol reagent (Aidlab Biotechnologies Co., Ltd., Beijing, China) according to the manufacturer's instructions. The RNA concentrations were measured using a spectrophotometer (Bio-Rad, USA). The RNA integrity was determined via agarose gel electrophoresis, and the RNA quality was determined by the A₂₆₀/A₂₈₀ ratio. Five micrograms of RNA was converted to cDNA using a Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, USA). The PCR primers were synthesized by Takara Biotechnology (Dalian) Co., Ltd., and the sequences of the three primers were the following: GAPDH forward primer, 5'-TGTGTCCGTCGTGGATCTGA-3', and reverse primer, 5'-TTGCTGTTGAAGTCGAGGAG-3' (150 bp); TLR4 forward primer, 5'-CATGGATCAGAACTCAGCAAAGTC-3', and reverse primer, 5'-CATGCCATGCCTTGTCTCA-3' (179 bp); and NF-κB forward primer, 5'-GAACGATAACCTTTGCAGGC-3', and reverse primer, 5'-TTTCGATTCGCTATGTGTG-3' (130 bp). The PCR reactions were performed in a total volume of 20 μL containing 2.0 μL of the cDNA template, 1.0 μL of the forward and reverse primers, 10 μL of the Taq Mix and 6 μL of water. The PCR reaction was conducted at 94 °C for 10 min, 30 cycles of 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 60 s, and a final extension at 72 °C for 10 min. GAPDH was used as a housekeeping gene, and the PCR products were tested by gel electrophoresis and scanned using the Step One Software.

Western blot analysis

The pancreas samples were homogenized in lysis buffer (pH 7.5) (20 mM Tris-HCl, 137 mM NaCl, 1% Tween-20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture in DMSO), and the total protein concentration was obtained. The protein concentration was estimated using a protein assay reagent (Bio-Rad). The proteins were separated by 10% SDS-PAGE. The proteins were transferred to a PVDF membrane, and the membrane was blocked with PBST buffer (20% Tween-20, PBS) and incubated with the anti-TLR4 antibody and anti-NF-κB antibody (1:1000 Santa Cruz, USA) overnight at 4 °C. The blots were washed three times with PBST and incubated with a goat anti-rabbit and/or goat anti-mouse horseradish peroxidase-conjugated secondary IgG (Boster Biotechnology) for 2 h at room temperature. The protein signals were detected using a gel image analysis system (UVP) after staining with diaminobenzidine.

The band densities were analyzed using Scion image software (Scion Corp., Frederick, MD, USA). GAPDH was employed to ensure that the western blotting was successful.

Statistical analysis

The statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., USA). The experimental data are expressed as the mean $\pm$ S.E. One-way ANOVA was used for the comparison of multiple groups. A difference with a P value less than 0.05 was considered significant.

Results

Effects of EACR on the general physical condition of diabetic mice

The mice in the normal and EACR control groups were in good condition in terms of their disposition and activities, and their body weight increased in a stable manner. The mice in the STZ group showed listlessness and weight loss. The improvement in the disposition and body weight of the mice was more pronounced in the animals that received EACR or metformin for 14 days than the STZ group (Fig. 1).

Effects of EACR on the FBG of diabetic mice

As shown in Fig. 2, the FBG levels in the STZ group were significantly higher than those observed in the normal control group ($P < 0.05$). Compared with the STZ group, the groups administered either metformin or EACR medication showed an effective reduction in the FBG levels. The FBG levels decreased as the EACR doses increased, indicating a dose-dependent inverse correlation between FBG and EACR. However, EACR did not affect the FBG level of the healthy mice (Fig. 3).

Fig. 1. Effect of EACR on body weight ($n=10$). A: Normal control; B: EACR control; C: STZ control; D: 320 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of metformin; E: 300 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of EACR; F: 600 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of EACR; G: 1200 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of EACR. The results were presented as the means $\pm$ S. * $P < 0.05$ compared with the normal control group; # $P < 0.05$ compared with the STZ group.

Fig. 2. Hypoglycemic effect of EACR ($n=10$). A: Normal control; B: EACR control; C: STZ control; D: 320 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of metformin; E: 300 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of EACR; F: 600 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of EACR; G: 1200 $\text{mg.kg}^{-1}.\text{d}^{-1}$ of EACR. The results were presented as the means $\pm$ S. * $P < 0.05$ compared with the normal control group; # $P < 0.05$ compared with the STZ group.

Fig. 3. Descent rate of EACR on FBG (%) (n=10). A: Normal control; B: EACR control; C: STZ control; D: 320 mg.kg⁻¹.d⁻¹ of metformin; E: 300 mg.kg⁻¹.d⁻¹ of EACR; F: 600 mg.kg⁻¹.d⁻¹ of EACR; G: 1200 mg.kg⁻¹.d⁻¹ of EACR. The results were presented as the means±S. *P<0.05 compared with the normal control group; #P<0.05 compared with the STZ group.

Fig. 4. Effect of EACR on the serum contents of IL-6 and TNF-α (n=10). A: Normal control; B: EACR control; C: STZ control; D: 320 mg.kg⁻¹.d⁻¹ of metformin; E: 300 mg.kg⁻¹.d⁻¹ of EACR; F: 600 mg.kg⁻¹.d⁻¹ of EACR; G: 1200 mg.kg⁻¹.d⁻¹ of EACR. The results were presented as the means±S. *P<0.05 compared with the normal control group; #P<0.05 compared with the STZ group.

Effects of EACR on IL-6 and TNF-α in the serum of diabetic mice

The serum IL-6 and TNF-α levels in the STZ-induced diabetic mice were higher than those found in the normal control group (P < 0.05). These cytokine levels were decreased by EACR, but it had no effect on basal cytokine levels (Fig. 4).

Histopathological observation

As shown in Fig. 5, the pathological examination of the pancreas using HE staining revealed an integrated cellular structure in the normal and EACR control groups. The islet cells in STZ group were damaged. This pathological phenomenon in the STZ group was ameliorated in the mice treated with metformin. In the low-dose ERAC group, the structure of the pancreas was improved to a lesser degree. The integrity of the cell structure in the high-dose ERAC group was markedly improved compared with the model control group, and the cell damage was also mitigated.

Effect of EACR on TLR4, NF-κB and insulin in the pancreas samples

As shown in Fig. 6, the TLR4-immunopositive islet area was significantly increased in the STZ group compared with the normal control group; the number of TLR4-immunopositive cells was reduced following treatment with metformin and EACR. The number of NF-κB-positive cells was decreased in the groups administered metformin and EACR compared with the STZ group. In particular, the high-dose EACR group (1200 mg.kg⁻¹.d⁻¹ EACR) had a significant decrease in the number of positive cells. In addition, the insulin-immunopositive cells in the STZ group were remarkably decreased compared with the normal control group, while the number of positive cells in the EACR groups was significantly increased. The numbers of TLR4 and NF-κB-immunopositive cells in EACR control group were similar to normal control group. And the expression of insulin in pancreas tissue was as high as normal control group.

Fig. 5. Histological observations of pancreas tissue. A: Normal control; B: EACR control; C: STZ control; D: 320 mg.kg⁻¹.d⁻¹ of metformin; E: 300 mg.kg⁻¹.d⁻¹ of EACR; F: 600 mg.kg⁻¹.d⁻¹ of EACR; G: 1200 mg.kg⁻¹.d⁻¹ of EACR.

Fig. 6. Effect of EACR on the expression of TLR4, NF-κB and insulin in mouse pancreas tissue (n=10). (Immunohistochemical analysis, scale bar: 200 μm). A: Normal control; B: EACR control; C: STZ control; D: 320 mg.kg⁻¹.d⁻¹ of metformin; E: 300 mg.kg⁻¹.d⁻¹ of EACR; F: 600 mg.kg⁻¹.d⁻¹ of EACR; G: 1200 mg.kg⁻¹.d⁻¹ of EACR.

Fig. 7. Effect of EACR on the expressions of TLR4 mRNA in mouse pancreas tissue (n=10). A: Normal control; B: EACR control; C: STZ control; D: 320 mg.kg⁻¹.d⁻¹ of metformin; E: 300 mg.kg⁻¹.d⁻¹ of EACR; F: 600 mg.kg⁻¹.d⁻¹ of EACR; G: 1200 mg.kg⁻¹.d⁻¹ of EACR. The results were presented as the means±S. *P<0.05 compared with the normal control group; #P<0.05 compared with the STZ group.

Effects of EACR on the mRNA expression levels of TLR4 and NF-κB

PCR analysis revealed that the mRNA expression levels of TLR4 and NF-κB were upregulated in the pancreas of the STZ group compared with the levels observed in the

Fig. 8. Effect of EACR on the expressions of NF-κB mRNA in mouse pancreas tissue (n=10). A: Normal control; B: EACR control; C: STZ control; D: 320 mg.kg⁻¹.d⁻¹ of metformin; E: 300 mg.kg⁻¹.d⁻¹ of EACR; F: 600 mg.kg⁻¹.d⁻¹ of EACR; G: 1200 mg.kg⁻¹.d⁻¹ of EACR. The results were presented as the means±S. *P<0.05 compared with normal control group; [#]P<0.05 compared with the STZ group.

Fig. 9. Effect of EACR on the expression of TLR4 and NF-κB proteins in mouse pancreas tissue (n=10). A: Normal control; B: EACR control; C: STZ control; D: 320 mg.kg⁻¹.d⁻¹ of metformin; E: 300 mg.kg⁻¹.d⁻¹ of EACR; F: 600 mg.kg⁻¹.d⁻¹ of EACR; G: 1200 mg.kg⁻¹.d⁻¹ of EACR. Results were presented as the means±S. *P<0.05 compared with the normal control group; [#]P<0.05 compared with the STZ group.

normal control group. Following the EACR treatment, the mRNA expression levels of TLR4 and NF-κB were reduced, particularly in the mice administered 1200 mg.kg⁻¹.d⁻¹ EACR. Whereas the mRNA levels in the EACR treated healthy mice were not significantly different from the Normal controls (Fig. 7, 8).

Western blotting for the analysis of the TLR4 and NF-κB proteins

The western blot analysis showed that the protein expression levels of TLR4 and NF-κB were markedly downregulated by metformin or EACR in a dose-dependent manner compared with the levels observed in the STZ group. According to the band densities, the protein expression levels in the EACR group were higher than those in the normal control group. There were no significant differences in the protein levels of TLR4 and NF-κB between EACR control group and normal control group (Fig. 9).

Discussion

Increasing numbers of studies have demonstrated that various adipokines and cytokines are involved in the development of DM [14, 15]. The inflammatory pathway is activated by FFAs, TNF-α, and IL-6 and 8 through the TLR4 signal transduction pathway, and the tyrosine kinase of the insulin receptor is activated by JNK and IKK/NF-κB [16, 17].

Our recent study on diabetic mice found that the extract of *Averrhoacarambola* L. root has beneficial effects by reducing the serum levels of triglycerides (TG), total cholesterol (TC) and free fatty acids (FFAs) and regulating apoptosis-related proteins, resulting in improvements in metabolic function and inhibition of apoptosis. We performed this study using diabetic mice to investigate the probable molecular mechanisms underlying the hypoglycemic effect of EACR that may be associated with a reduction in the levels of inflammatory cytokines and the inhibition of TLR4 expression to restrain the inflammatory response downstream of the NF- κ B pathway.

The STZ-induced diabetes mouse model is characterized by abnormal blood glucose levels caused by damage to the structure of the pancreas [18]. Our results suggested that EACR has a hypoglycemic effect on STZ-induced diabetic mice, and a marked effect was observed in the mice that were administered 1200 mg·kg⁻¹·d⁻¹ EACR.

Tumor necrosis factor- α (TNF- α) and IL-6 are important components of the proinflammatory cytokines secreted by a variety of cell types involved in inflammatory responses and energy metabolism, such as macrophages and adipocytes [19-21]. It is hypothesized that high levels of TNF- α can cause diabetic nephropathy, retinopathy and other complications of DM [22, 23]. The immune system is activated by TNF- α , and leukotrienes and platelet-activating factor are then synthesized and mobilized, which results in an inflammatory response. An extended duration of hyperglycemia produces glycation products that cause excess IL-6 synthesis and initiate β -cell apoptosis [24]. Our data demonstrate that EACR can reduce serum TNF- α and IL-6 activities in diabetic mice and that EACR inhibits inflammatory cytokine secretion, whereas it did not affect the cytokine activities in healthy mice.

HE staining was used to observe the histopathological changes in the pancreatic tissues. The integrity of the pancreatic tissue structure is reflected by the morphology of islet cells. The HE and insulin immunohistochemical observation results showed that a high dose of EACR has an obvious effect on ameliorating the damage to the islet cell structure.

The TLR family plays a crucial role in innate immunity and inflammation because of their wide range of microbiological ligands. There are 11 types of TLRs. Previous studies have shown that TLR4 expression is detected in many insulin-targeted tissues, such as adipose, liver, muscle and pancreas tissues [17, 25-27]. A previous study revealed that TLR4 plays a negative role on anxiety and cognition in type 1 diabetes models [28]. Increased TLR2 and TLR4 expression levels are found in type 1 diabetic NOD mice, and these increases are correlated with the activation of NF- κ B and an increase in the levels of pro-inflammatory cytokines. We hypothesized that the effects of EACR on DM involve the TLR4/NF- κ B pathway. The TLR4-immunopositive islet area was markedly downregulated in the groups administered EACR compared with the levels detected in the STZ group. The number of NF- κ B-positive cells in the EACR group was decreased compared with the number detected in the STZ group. In our diabetic mouse model, the administration of EACR decreased the expression levels of TLR4 and NF- κ B and may have facilitated the alleviation of inflammation. The expression of TLR4 mRNA was at a baseline level in the normal control group. The TLR4 mRNA level in the STZ group was markedly higher than that detected in the normal group; however, the TLR4 mRNA level was significantly decreased in a dose-dependent manner in the mice treated with EACR compared with that found in the STZ group. The expression of the NF- κ B downstream transduction pathway mRNA corresponded to the TLR4 mRNA levels. The pancreatic TLR4/NF- κ B pathway activity was confirmed at the protein level by western blot analysis. And EACR had no significant effect on the basal parameters of healthy mice. These findings suggest that the beneficial effect of EACR in diabetes may involve the reduction of the inflammatory response linked to TLR4 and the downstream NF- κ B transduction pathway. This may indicated that EACR works well on improving inflammatory response in general diabetes.

Our results indicate that EACR serves as a potential DM management agent through its efficacy in reducing the FBG level in STZ-induced diabetic mice. The mechanism may be associated with an anti-inflammatory response in pancreas tissues resulting from the

regulation of proinflammatory serum cytokines and the blocking of the activation of TLR4/NF- κ B pathway.

Abbreviations

EACR (Extract of *Averrhoacarambola* L. root); TLR4 (Toll-like receptor 4); NF- κ B (Nuclear factor kappa B); STZ (Streptozotocin); FBG (Fasting blood glucose); IL-6 (Interleukin-6); TNF- α (Tumor necrosis factor- α); DM (Diabetes mellitus); T2DM (Type 2 diabetic mellitus); IFN- γ (interferon- γ); FFAs (Free fatty acids); MCP-1 (Monocyte chemoattractant protein-1); MS (Metabolic syndrome); ACLR (*Averrhoacarambola* L. root); MNDD (2-methoxy-6-nonylcyclohexa-2,5-diene-1,4-dione); DMDD (2-dodecyl-6-methoxy- cyclohexa-2,5-diene-1,4-dione).

Disclosure Statement

The authors have no conflicts of interest.

Acknowledgments

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