Asian acid mitigates hyperglycemia and reduces islet fibrosis in Goto-Kakizaki rat, a spontaneous type 2 diabetic animal model

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[ABSTRACT] The Goto-Kakizaki (GK) rat is a spontaneous type 2 diabetic animal model, which is characterized by a progressive loss of beta islet cells with fibrosis. In the present study, the hypoglycemic effect of asiatic acid (AA) in GK rats was examined. GK rats receiving AA at a daily dose of 25 mg·kg⁻¹ for four weeks showed a significant reduction in blood glucose levels. Age-matched normal Wistar rats were given 0.5% sodium carboxymethyl cellulose (CMC-Na) solution for the same periods and used as control. Compared to the normal Wistar rats, GK rats treated with AA showed improvement in insulin resistance partially through decreasing glucose level (P < 0.01) and insulin level (P < 0.05). Furthermore, the results of immunohistochemistry indicate that AA treatment reduced islet fibrosis in GK rats. Fibronectin, a key protein related to islet fibrosis, was over-expressed in GK rats, which was reversed significantly by AA treatment (P < 0.05). These findings suggest that AA has a beneficial effect on lowering blood glucose levels in GK rats and improves fibrosis of islets in diabetes, which may play a role in the prevention of islets dysfunction.

[KEY WORDS] Asiatic acid; Hyperglycemia; Islet fibrosis; Goto-Kakizaki (GK) rats; Diabetes mellitus; Pancreas

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Introduction

Type 2 diabetes is a hyperglycemic disorder mediated mainly by insulin resistance and impaired insulin secretion [1-2]. The Goto-Kakizaki (GK) rat is a spontaneous animal model for type 2 diabetes without obesity [3]. The diabetic model was developed by the Japanese scientists Goto and Kakizaki in 1975 [3]. With the progression of the disease, GK rats develop typical characteristics of tissue damage in the peripheral nerves, as well as kidneys, recapitulating the systemic manifestations encountered in human type 2 diabetes [4]. Histopathological examinations show that islets in GK rats are changed considerably compared with the normal age-matched Wistar rats. The changes include irregular cellular shape, fibrosis, and progressive reduction of pancreatic beta cells commencing from eight weeks of age in the Stockholm colony [5]. Previous reports have indicated that islet fibrosis is a vital pathological complication for diabetes development. With the depletion of beta cells, GK rats lose the islet function, resulting in a decrease in insulin secretion. Therefore, it is well-established that preventing beta cell loss and curbing fibrosis are an efficient strategy for type 2 diabetes therapy.

Asiatic Acid (AA, Fig. 1) is one of the constituent triterpenes found in the plant Centella asiatica (L.) Urb. (Apiaceae). This ancient medicinal plant is known in both traditional Chinese medicine and in Indian Ayurvedic medicine [6]. Notably, AA is the major effective ingredient of a wound-healing drug (commercial name: Madecassol) that can accelerate nerve regeneration [6]. Earlier studies have demonstrated that the aqueous extract of the whole plant of C. asiatica is used by traditional healers in the Haya tribe in Bukoba Region in Tanzania, for the treatment of both insulin and non-insulin dependent diabetes mellitus [7]. Recently, our laboratory reported that AA can preserve the beta mass and mitigate hyperglycemia in streptozotocin-induced diabetic rats [8].
In the present study, we demonstrated that AA improved insulin resistance and mitigated hyperglycemia in the GK rats. Immunohistochemical staining revealed a marked preservation of insulin-producing beta cells by AA in the pancreatic islets of the diabetic rats. The results of immunohistochemistry also revealed that AA could improve the hyperplasia of the beta cells, proportional to the degree of the fibrosis. RT-PCR results showed that AA could reverse the over-expression of fibronectin in the GK rats. These findings suggested that AA could prevent beta cells from destructive depletion and improve the islet fibrosis in GK rats which might indicate a promising effect on diabetic treatment.

Materials and Methods

Chemicals

AA was obtained from Guangxi Changzhou Natural Products Development Co. Ltd. (Guangxi, China; purity > 95%). Glibenclamide was purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of reagent grade. All drugs used in the present study were solubilized in a 0.5% solution of carboxymethylcellulose sodium salt.

Animals and treatments

Male GK rats, aged four months, were purchased from Shanghai Laboratory Animal Center (Shanghai, China). The rats were housed in the animal facility of our University with free access to food and tap water. The rats were handled according to university and institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals. Age-matched, male, non-diabetic Wistar rats (Shanghai Laboratory Animal Center) were used as the controls. Twenty-four rats (six Wistar rats and eighteen GK rats) were divided into four groups (six animals in each group): (a) Wistar rat control group (treated with 0.5% CMC-Na solution); (b) Untreated GK rat model group (treated with 0.5% CMC-Na solution); (c) Glibenclamide (10 mg·kg\(^{-1}\)) treated group; and (d) AA (25 mg·kg\(^{-1}\)) treated group. The drug treatments lasted for 4 weeks. On the last day of treatment, the animals were fasted for 12 h, then the blood samples were collected from the abdominal aorta, and blood glucose and insulin levels were measured. After exsanguination by serving the abdominal aorta, the pancreases were excised and weighed after removing the fat and lymph nodes. Pancreatic slides were prepared and immunostained for insulin, fibronectin, collagen I, collagen II, and von Willebrand factor (vWF), respectively.

Blood glucose measurement

The blood samples were collected from the abdominal aorta. Blood glucose levels were determined using a colorimetric glucose assay kit, according the procedures specified by the manufacturer (Whitman Biotech Co. Ltd., Nanjing, China).

Determination of insulin level

Rat serum insulin levels were determined using an ultrasensitive, insulin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem Inc., Chicago, IL, USA). Briefly, the serum samples were collected two weeks after drug administration. Five-microliter samples or different concentrations of rat insulin standard solutions and 95 μL of sample diluents were added to the 96-well microtiter plates pre-coated with the guinea pig anti-rat insulin antibody. After incubation for 2 h at 4 °C and extensive washing, a 100 μL aliquot of horseradish peroxidase-conjugated anti-rat insulin antibody was added, and the plates were incubated for 30 min at room temperature. Following washing, the plates were incubated with 100 μL/well of enzyme substrate solution and kept for 40 min at room temperature, and the reaction was stopped by the addition of the enzyme reaction stopping solution. The absorbance was read at 405 nm using a SAFIRE2 Microplate Reader (Tecan, Männedorf, Switzerland). The concentration of serum insulin was expressed as ng mL\(^{-1}\).

Immunohistochemical staining

The rat pancreatic sections of 4-μm thickness were prepared by a routine procedure. The sections were deparaffinized and rehydrated through a graded series of alcohol to distilled H\(_2\)O. Endogenous peroxidase was blocked in a solution of 3% H\(_2\)O\(_2\) in methanol for 10 min at room temperature. For insulin labeling, the pancreata from Wistar and GK rats were excised, fixed in aqueous Bouin solution, and embedded in paraplast, according to standard procedures. Histological sections of the pancreas were prepared and mounted on glass microscope slides. The slides were incubated overnight at 4 °C in a humidified chamber with guinea pig anti-porcine insulin. For insulin detection, a peroxidase-conjugated rabbit anti-guinea pig IgG was used. After washing, the peptide immunoreactivity was localized with 3, 3′-diaminobenzidine -tetra-hydrochloride using a peroxidase substrate kit (DAB; Vector Laboratories, Burlingame, CA, USA). The tissue sections were counterstained with hematoxylin and mounted under glass coverslips. The slides were viewed with a Nikon Eclipse E600 microscope equipped with a Nikon DXM200 digital camera (Melville, NY, USA).

For fibronectin, Collagen I, Collagen III, and vWF labeling, the pancreata were removed and stored at −80 °C until analysis. The sections were fixed in acetone for 10 min. After washing with PBS containing 0.05% Tween 20 (Merck, Paris, France) (PBS/Tween), the slides were incubated with primary antibodies for 30 min at room temperature in a moist
chamber. The slides were then washed twice with PBS/Tween and incubated with appropriate peroxidase-coupled secondary antibodies for 30 min at room temperature. Following further PBS/Tween washing, the slides were incubated with 3- amino-9-ethylcarbazole (Sigma) as the substrate in 50 mmol·L⁻¹ of sodium acetate and 0.02% H₂O₂ and washed in water after 3 min. Finally, the slides were counterstained with Harris hematoxylin (Merck) for 3 min, dehydrated in serially-graded ethanol baths, and mounted. For each series of pancreas sections, one slide was stained only with the second antibody as a control for endogenous peroxidase activity and nonspecific antibody binding.

**Islet isolation**

The rat pancreatic islets were isolated by collagenase digestion, followed by a density gradient separation according to the procedure described previously [9]. Briefly, the pancreata were digested with collagenase according to standard procedures. Then, the islets were purified using a continuous Histopaque (Sigma, St. Louis, MO, USA) gradient. The purified islet fractions were collected, and extracted by hand under a stereomicroscope and lysed. The homogenized lysate was kept at −80 °C until RNA extraction.

**Quantitative RT-PCR**

The total RNA was isolated using RNeasy Mini kit (Qiagen). cDNA was synthesized with Superscript II (Invitrogen, Basel, Switzerland), using 1 µg of total RNA in a 20-µL reaction volume. The dsDNA-specific dye SYBR Green I (Eurogentech, Brussels, Belgium) and fluorescein (Biorad, Hercules, CA, USA) were incorporated into the PCR buffer (qPCR core kit, Eurogentech, Brussels, Belgium) to allow for quantitative detection of the PCR product. The results were analyzed using the iCycler iQ System (Biorad, Hercules, CA, USA). The primers for fibronectin were: forward 5′-CCTAGCTCATGCTTT-3′, reverse 5′-CAGAT AACCGCTC CCATCC-3′; the primers for GAPDH were: forward 5′-ACC ACAGTACATGCCATC AC-3′ and reverse 5′-TCC ACCAC CCTGTTGCTG TA-3′. The house-keeping gene GAPDH was used as internal control. The data were normalized to GAPDH. The relative expression was calculated as 2⁻ΔΔct × 100%, where ΔΔct was the difference in the ct value between the target gene and GAPDH. All quantifications were performed in triplicate in three separate experiments.

**Statistical analysis**

All results were expressed as mean ± SD. One-way ANOVA and Student’s t-test were used to analyze the differences between different treatments. Statistic analyses were performed using GraphPad Pro. P < 0.05 was considered statistically significant.

**Results**

**Effects of AA on serum blood glucose and insulin levels**

The blood glucose levels and serum insulin levels were significantly reduced in GK rats treated with AA (Figs. 2A and 2B). The data indicated that AA exerted hypoglycemic effect, in part, through improving insulin resistance in GK rats.
four months \cite{10}. Additionally, islet fibrosis progresses rapidly in GK rats \cite{11}. In the present study, the insulin labeling demonstrated the concomitant presence of large fibrotic islets in a 4-month-old GK rat pancreas (Fig. 3A), compared with an age-matched control Wistar rat pancreas (Fig. 3B). At 4 months of age, the fibrosis was extensive in large GK rat islets (Figs. 3C and 3D). This was in agreement with insulin labeling of the GK rat islets (Fig. 3A). AA preserved the insulin secretion and islet mass, and prevented islet fibrosis as revealed by immunohistochemical staining for insulin (Fig. 3E) and fibronectin (Fig. 3F).

**Discussion**

Type 2 diabetes is a hyperglycemic disorder mediated mainly by insulin resistance and impaired insulin secretion. Pancreatic beta cell failure plays a primary role in the development and progression of type 2 diabetes \cite{14}. Islet fibrosis has been observed in type 2 diabetes in both rodent models and patients, which is recognized as a critical pathological factor of diabetes \cite{15}. Currently, animal models are often used to investigate the mechanisms of diabetes mellitus and to develop therapeutic drugs. In the field of type 2 diabetes, a variety of animal models, such as high-fat diet and streptozotocin-induced diabetic rats \cite{16}, spontaneous diabetes, and knockout mouse models \cite{17} have become available. The GK rat is a spontaneous animal model of type 2 diabetes without obesity, in which diabetes is displayed by the loss of islet functions and the decrease of insulin secretion\cite{3}. Islets fibrosis can be important.

![Fig. 3](image1)

**Fig. 3** Insulin labeling for the concomitant presence of large fibrotic islets in 4-month-old GK rat pancreas (A), compared with age-matched control Wistar rat pancreas (B) (×160). At 4 months of age, fibrosis was extensive in large GK rat islets (×160) (C versus D). This was in agreement with insulin labeling of GK rat islets (A). AA preserved islet mass and prevented islet fibrosis as evidenced by immunohistochemical staining for insulin (E) and fibronectin (F).

Furthermore, the antibodies against three proteins in GKIIslets were selected: collagen I, collagen III, and vonWillebrand factor (vWF); all of which are known to be produced by vascular endothelial and/or smooth muscle cells \cite{12-13}. These antibodies were used for a immunohistochemical analysis of the development of islet fibrosis in GK rats. As shown in Figs. 4A, 4E, and 4I, the fine labeling for collagen I, collagen III, and vWF was present with intra- and peri-islet localization in the Wistar islets. However, peri- and intra-islet thickening was observed in the GK islets (Figs. 4B, 4F, and 4G), which was in agreement with the insulin labeling of GK islets (Fig. 3A). After receiving glibenclamide (Figs. 4C, 4G, and 4K) and AA (Figs. 4D, 4H, and 4L), the islet fibrosis was reduced, compared with the GK rats.

To gain more insights into the effects of AA on islet fibrosis, the fibronectin mRNA expression was examined in the GK rats. As shown in Fig. 5, the relative mRNA level of fibronectin was increased significantly compared to that of the Wistar control rats. However, after receiving AA for four weeks, the gene expression levels of fibronectin were decreased significantly.

![Fig. 4](image2)

**Fig. 4** Effects of AA on islet fibrosis in the GK rats. Compared with Wistar islets, GK islets show thickening of vascularization as demonstrated by labeling for collagen I (A versus B), collagen III (E versus F), or vWF (I versus J) (400 ×). Glibenclamide can affect the islet fibrosis as shown by collagen I (C), collagen III (J), and vWF (K) labelings. AA could improve islet fibrosis as shown by collagen I (D), collagen III (H), and vWF (L) labelings.
The present study demonstrated that AA at 25 mg·kg\(^{-1}\) accompanied with increased insulin in patients suffering from progression of islet fibrosis.

These results indicated that AA improved hyperinsulinemia in patients with type 2 diabetes [23-25]. In line with these observations, our results from the present study demonstrated that AA at 25 mg·kg\(^{-1}\) reduced the plasma glucose and insulin levels, which showed significant anti-diabetic activity after oral administration for four weeks in GK rats. AA reduced spontaneous hyperglycemia to a normal level, as displayed in the Wistar rat group, and decrease plasma insulin levels to 70% as compared with the GK rat group. These results indicated that AA improved hyperinsulinemia in GK rats with a similar efficacy to glibenclamide, through decreasing glucose and insulin levels.

Furthermore, antibodies against fibronectin and insulin were selected to investigate the effects of AA on the development of islet fibrosis. In the 4-month-old GK rats, the largest islets showed a massive fibrosis, as illustrated by the high fibronectin level. After treated with AA, the labeling for fibronectin was reduced significantly, which was in agreement with the decrease of insulin labeling in the GK islets. The islets in the GK rats were irregular in shape with spots of heterogeneously insulin-stained cells intermingled with fibrosis. There was a significant difference between the islets of GK rats and age-matched control Wistar rats; which showed a weak labeling of beta cells covered almost homogeneously in the islet area. The islet vascularization was also altered in the 4-month-old GK rats. The labeling for Collagen I, Collagen III, and vonWillebrand factor (vWF), a specific endothelial cell marker, is increased in the blood of type 2 diabetic patients [26-27]. As expected, the Wistar islets were characterized by the regular labeling of endothelial cells. Islet vascularization differed remarkably in the GK rats as assessed by vWF labeling. From the immunohistochemistry results, AA improved the islet vascularization significantly. The overexpression of fibronectin in GK rats was observed, compared to the Wistar rats. AA could significantly reverse the gene expression level of fibronectin in GK rats. Although AA and glibenclamide reduced hyperglycemia and improved insulin resistance, leading to antidiabetic activity, glibenclamide seemed to have no significant effect on islet fibrosis. These results indicated that AA and glibenclamide can both influence the pancreatic beta cells. However, their underlying molecular mechanisms may be different.

In summary, the natural and low-toxic compound AA exhibited significant glucose-lowering and hypoinsulnemic effects in the GK rats. In addition to reducing blood glucose, AA exhibited preventative effects against islet fibrosis. Additionally, AA has been shown to have other therapeutic benefits, such as anti-inflammatory and antioxidant activities [28-30]. Our laboratory has previously reported that maslinic acid, asiatic acid, and related pentacyclic triterpenes represent a new class of inhibitors of glycogen phosphorylase, suggesting that their glucose-lowering activity in diabetic mice induced by adrenaline be due to the modulation of hepatic glycogen metabolism [31].

As a part of this project, aimed at drug discovery based on pharmacological intervention on glucose metabolism, efforts have been focused on research and the development of pentacyclic triterpenes as natural and low-toxic anti-diabetic agents with preventive and therapeutic effects against ischemic diabetic complications. In the present study, the data demonstrated for the first time that AA could improve islet fibrosis. The immunohistochemistry proved that AA also improved the islet fibrosis formation in GK rats. In conclusion, AA may hold great promise as a natural therapeutic agent for the treatment of type 2 diabetes, and further research regarding the mechanism of action will be carried out.

![Fig. 5 The levels of fibronectin mRNA determined by q-PCR.](image-url)
References


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