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Betulinic Acid Ameliorates Postprandial Hyperglycemia in Diabetic Mice

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The objective of this study was to investigate whether betulinic acid can inhibit the activities of carbohydrate-digesting enzymes and reduce postprandial hyperglycemia in mice with streptozotocin-induced diabetes. Our results revealed that betulinic acid has potent inhibitory effects on α-glucosidase and α-amylase activities. The half-maximal inhibitory concentrations (IC_{50}) of betulinic acid were 12.83±6.81 and 18.32±3.24 μM for α-glucosidase and α-amylase, respectively. This result indicates lower IC_{50} values and higher inhibitory activities than those of acarbose, an oral hypoglycemic drug. The increase in postprandial blood glucose levels was significantly suppressed in the betulinic acid group than in the control group of diabetic and normal mice. Postprandial blood glucose levels were 23.22 ±1.1, 24.38±1.31, and 21.05±1.36 μM in the betulinic acid group compared to 24.64±1.7, 27.22±1.58, and 26.36±1.40 μM in the control group of diabetic mice at 30, 60 and 120 min, respectively. The area under the curve also significantly decreased with the administration of betulinic acid in diabetic mice, however, it did not decrease more than that after acarbose administration. Our results showed that betulinic acid may be a potent inhibitor of carbohydrate-digesting enzymes and ameliorate postprandial hyperglycemia in diabetic mice.

Key words : Betulinic acid, diabetic mice, postprandial hyperglycemia, α-glucosidase, α-amylase

Introduction

Globally, the number of individuals diagnosed with diabetes mellitus has quadrupled over the last 30 years. Ten percent of adults worldwide currently have diabetes, 90% of whom have type 2 diabetes mellitus [28]. Diabetes mellitus is defined as a disorder characterized by postprandial hyperglycemia resulting from absolute or relative insulin deficiency or resistance [25]. Postprandial blood glucose levels are more predictive of diabetic complications and mortality than fasting blood glucose levels. Therefore, controlling postprandial hyperglycemia is vital for preventing diabetes-related cardiovascular complications [9].

Prevalent anti-diabetic agents, such as insulin and sulfonylureas, are less effective at reducing postprandial hyperglycemia in patients with low fasting blood glucose levels. Effective pharmacological agents include acarbose and voglibose. To lower postprandial hyperglycemia, the activity of carbohydrate-digesting enzymes in the intestine needs to be inhibited. α-glucosidase inhibitors, such as acarbose, block the activity of α-glucosidase, thereby slowing the rate of carbohydrate digestion [18]. However, these agents can cause flatulence, soft stool, and abdominal discomfort [21]. Due to these side effects, many natural alternatives that inhibit glucose production in the gut or glucose absorption in the intestine have been investigated [26].

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic triterpenoid that is derived from various plants [31]. It is usually isolated from medicinal plants such as birch trees (*Betula sp*., Betulaceae), but it has also been found in various species of the genera *Ziziphus* (Rhamnaceae), *Syzygium* (Myrtaceae), *Diospyros* (Ebenaceae), and *Paeonia* (Paeoniaceae) [20]. Betulinic acid has a variety of pharmacological effects, including antiviral [1], antitumor [11], and anti-inflammatory effects [19]. Betulinic acid is particularly effective under anti-diabetic conditions as it can inhibit α-glucosidase activity [10], improve insulin sensitivity [4], and affect glucose metabolic regulation [24]. Although a previous study reported that betulinic acid had high α-glucosidase inhibitory activity, there were no reports on whether it reduced postprandial hyperglycemia by inhibiting carbohydrate-digesting enzymes *in vivo*. Therefore, this study investigated whether betulinic acid ameliorates postprandial hyperglycemia

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in a diabetic mouse model.

Materials and Methods

Materials

Betulinic acid, α-glucosidase, and α-amylase were purchased from Sigma–Aldrich (St. Louis, MO, USA). The chemicals and reagents were of analytical grade and were used without any further purification.

Inhibition of α-glucosidase activity by betulinic acid *in vitro*

The α-glucosidase inhibition assay was conducted using the chromogenic method with a readily available yeast enzyme, as described by Watanabe et al. [29]. α-glucosidase yeast (0.7 U; Sigma–Aldrich Co.) was briefly dissolved in the enzyme solution, which consisted of 100 mM phosphate buffer (pH 7.0), 2 g/l bovine serum albumin, and 0.2 g/l NaN_3. The substrate also consisted of 100 mM phosphate buffer (pH 7.0), but with 5 mM p-nitrophenyl α-D-glucopyranoside (pNGP). Next, 50 μl of the enzyme solution and 10 μl of the sample dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10–100 μM were mixed in the wells of a microtiter plate. The titer was measured by determining the absorption at 405 nm at time 0 using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). After incubation for 5 min, the substrate solution (50 μl) was added and incubated for another 5 min at room temperature, and the absorbance was measured again. Inhibitory activity was expressed as 100 minus the absorbance difference (%) of the test compounds relative to the absorbance change of the control (DMSO with sample).

Inhibition of α-amylase activity by betulinic acid *in vitro*

The inhibitory activity of α-amylase was assayed as described for α-glucosidase inhibition, except that porcine pancreatic amylase (100 U) and p-nitrophenyl α-D-maltopentoglycoside were used as the enzyme and substrate, respectively.

Experimental animals

Male ICR (Institute of Cancer Research) mice (4-week-old) were purchased from Joongang Laboratory Animal Co. (Seoul, Korea). All animals were housed individually in a light- (24 hr light/dark cycle) and temperature-controlled room at 23±1℃, with pelleted food and tap water available *ad libitum*. After an adjustment period of approximately 2 weeks, diabetes was induced by an intraperitoneal injection of streptozotocin (STZ) (60 mg/kg) dissolved in freshly prepared citrate buffer (0.1 M, pH 4.5). After 7 days, tail bleeds were performed, and animals with blood glucose concentrations above 13.89 mM were considered diabetic. The procedures for the handling and care of animals adhered to the guidelines of the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals) and were approved by the animal ethics committee at our university (PNU-2018-1797).

Measurement of blood glucose levels

Normal and STZ-induced diabetic mice were fasted overnight and randomly divided into three groups (n=7). Fasted animals were deprived of food for at least 12 hr but allowed free access to water. After overnight fasting, the mice were orally administered either soluble starch (2 g/kg body weight [bw]), betulinic acid (10 mg/kg bw), or acarbose (10 mg/kg bw). Blood samples were collected from the tail vein after 0, 30, 60, and 120 min. Blood glucose levels were measured using a glucometer (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). The area under the curve (AUC) was calculated using the trapezoidal rule.

Data and statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Student's *t*-test was used to compare the control and sample groups. Differences were evaluated by one-way analysis of variance (ANOVA), followed by a post-hoc Tukey test ($p<0.05$).

Results

Inhibitory effects of betulinic acid on α-glucosidase and α-amylase *in vitro*

The inhibitory effect of betulinic acid on α-glucosidase was determined using p-nitrophenyl α-D-glucopyranoside as a substrate and compared to the inhibitory effect of acarbose. The activity of α-glucosidase was inhibited by betulinic acid in a concentration-dependent manner, with an inhibition of 37.24±1.66%, 42.03±3.61%, 55.97±3.20%, and 60.33±1.71% at concentrations of 5, 10, 15, and 20 μM, respectively (Fig. 1). Acarbose inhibited α-glucosidase activity by 46.76% at a concentration of 100 μM. These results show that betulinic acid has significantly higher α-glucosidase inhibitory activity than acarbose. The inhibitory effects of betulinic acid on α-

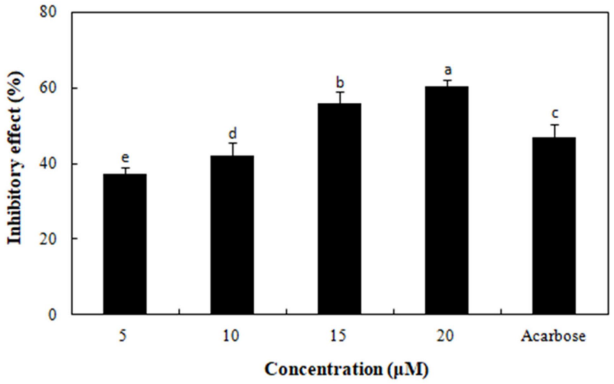

Fig. 1. Inhibitory activity of betulinic acid against α-glucosidase. Each value is expressed as a mean ± SD of triplicate experiments. Values with different letters indicate significantly difference (p<0.05 in Duncan's multiple range tests). The concentration of acarbose, which was used as positive control, was 100 μM.

amylase activity were determined in a similar manner. Betulinic acid inhibited α-amylase in a concentration-dependent manner with an inhibition of 20.11±2.45%, 32.47±1.46%, 38.97±1.53%, and 55.24±2.64% at concentrations of 5, 10, 15, and 20 μM, respectively (Fig. 2). The IC_{50} values of betulinic acid for α-glucosidase and α-amylase were 12.83±6.81 and 18.32±3.24 μM, respectively (Table 1). These results indicate that betulinic acid could be useful as a natural anti-hyperglycemic agent for patients with diabetes.

Effects of betulinic acid on blood glucose levels

The effects of betulinic acid on hyperglycemia following a meal were investigated in normal and STZ-induced diabetic

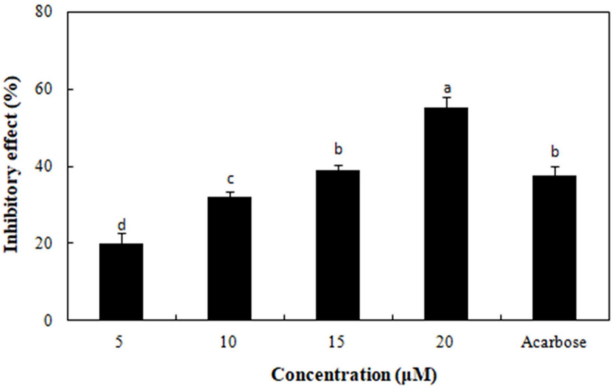

Fig. 2. Inhibitory activity of betulinic acid against α-amylase. Each value is expressed as a mean ± SD of triplicate experiments. Values with different letters are significantly different at p<0.05 (as per Duncan's multiple range tests). The concentration of acarbose, which was used as positive control, was 100 μM.

Table 1. IC_{50} values of betulinic acid for inhibiting α-glucosidase and α-amylase

Sample	IC_{50} (μM)	
	α-glucosidase	α-amylase
Betulinic acid	12.83±6.81*	18.32±3.24*
Acarbose	130.91±4.23	179.52±5.67

IC_{50} value is 1/2 the maximal inhibitory concentration. Each value is expressed as the mean ± SD of triplicate experiments. *Values are significantly different at p<0.05, as analyzed using Student's t-test.

mice. Postprandial blood glucose levels in diabetic mice that ingested betulinic acid were significantly lower than those in the diabetic control mice (Fig. 3). The blood glucose levels in the diabetic control mice increased to 24.64±1.73 mM at 30 min and to 27.22±1.58 mM at 60 min. Blood glucose levels were then decreased to 26.36 ± 1.40 mM at 120 min. However, when betulinic acid was administered with a meal, the increase in postprandial blood glucose levels was significantly alleviated (23.22±1.1, 24.38±1.31, and 21.05±1.36 mM at 30, 60, and 120 min, respectively; p<0.05). Postprandial blood glucose levels were also significantly decreased in normal mice who were administered betulinic acid with their meals compared to those in normal control mice. (Fig. 4; p<0.05). The AUC for the glucose response in diabetic mice that were administered betulinic acid (2,677.33±148.74 mmol min/l) was significantly lower than that in diabetic control mice (3,006.94±178.07 mmol min/l; Table 2).

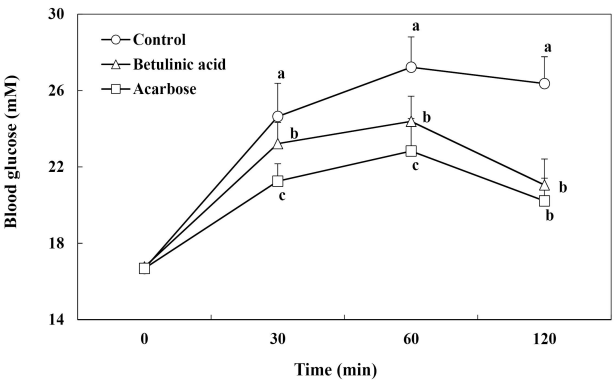

Fig. 3. Blood glucose levels after administering betulinic acid in mice with streptozotocin-induced diabetes. In the control group, distilled water, betulinic acid (10 mg/kg), and acarbose (10 mg/kg) were orally co-administered with starch (2 g/kg). Each value is expressed as mean ± SD. Values with different letters are significantly different at p<0.05 (according to Duncan's multiple range tests).

Table 2. Area under curve of blood glucose levels in normal and STZ-induced diabetic mice

Group[1)]	AUC (mmol min/l)	
	Normal mice	Diabetic mice
Control	1,300.00±114.39[a]	3,006.94±178.07[a]
Betulinic acid	827.67±45.98[b]	2,677.33±148.74[b]
Acarbose	761.11±65.68[c]	2,320.69±140.12[c]

The control group was orally administered starch (2 g/kg), distilled water, betulinic acid (10 mg/kg), or acarbose (10 g/kg). Each value is expressed as the mean ± SD. Values with different letters differ significantly at $p<0.05$ in Duncan multiple range tests.

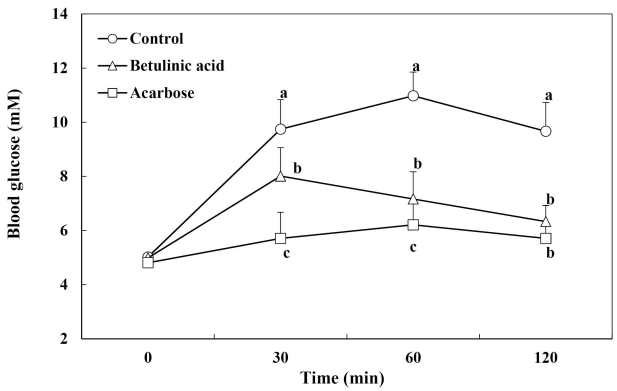

Fig. 4. Blood glucose levels after administering betulinic acid in normal mice. In the control group, distilled water, betulinic acid (10 mg/kg), and acarbose (10 mg/kg) were orally co-administered with starch (2 g/kg). Each value is expressed as mean ± SD. Values with different letters are significantly different at $p<0.05$ (according to Duncan's multiple range tests).

Discussion

Fasting and postprandial blood glucose levels are widely used as predictors of diabetic complications and mortality [12]. Abnormal postprandial blood glucose levels are an important contributing factor in the development of diabetes mellitus and diabetic complications [7]. Postprandial hyperglycemia can cause endothelial dysfunction, oxidative stress, and inflammation [8], which can lead to progression of atherosclerosis and cardiovascular events [17]. Thus, the alleviation of postprandial hyperglycemia is critical for the treatment of type 2 diabetes. One therapeutic approach for reducing postprandial hyperglycemia is to prevent the absorption and digestion of carbohydrates after food [23].

Carbohydrate digestion in the intestine was performed using α-amylase and α-glucosidase. α-amylase catalyzes the hydrolysis of the α-1,4-glycosidic bonds in starch, glycogen, and other oligosaccharides. α-glucosidase, a significant catalyst for carbohydrate digestion, located in the brush border of the small intestine, converts oligosaccharides and disaccharides to monosaccharides, which are required for gastrointestinal absorption. An α-glucosidase inhibitor can block the action of the α-glucosidase enzyme, which limits the rate of conversion required for gastrointestinal absorption [13]. Hydrolyzed dietary carbohydrates are a leading cause of increased blood glucose levels. After the hydrolysis of dietary carbohydrates by α-amylase, intestinal absorption is achieved after it is broken down into monosaccharides by α-glucosidase [3]. Inhibitors of these enzymes can effectively delay carbohydrate digestion and glucose absorption and thus suppress postprandial hyperglycemia [27].

This study investigated the role of betulinic acid in inhibiting the activities of carbohydrate-hydrolyzed enzymes and alleviating postprandial hyperglycemia. Betulinic acid showed more significant inhibitory effects on both α-glucosidase and α-amylase than acarbose. The IC_{50} values of betulinic acid against α-glucosidase and α-amylase were much lower than that of acarbose. Betulinic acid is a pentacyclic triterpenoid which has a structure containing a hydroxyl group and has the ability of inhibiting carbohydrate digestive enzymes through hydrogen bonds [32]. When betulinic acid bonds with α-glucosidase to form a complex, the α-helices increase and the β-sheet content decreases in the polypeptide structure of α-glucosidase. When these alterations occur, the substrate is blocked from entering α-glucosidase, thereby reducing α-glucosidase activity [10]. Therefore, the inhibitory effect of betulinic acid on α-glucosidase activity observed in this study was due to the hydrogen bonding between the hydroxyl group of betulinic acid and α-glucosidase. This result are similar to other studies that showed the inhibitory effect of α-glucosidase by forming hydrogen bonds between amino acids surrounding the catalytic site of α-glucosidase and the hydroxyl group of various other pentacyclic triterpenoids, such as corosolic acid, maslinic acid, and oleanolic acid [16]. In particular, it is consistent with the results of previous studies that betukinic acid binds to the active site of α-glucosidase and then decreases the activity of glucosidase by changing the microenvironment and secondary structure of α-glucosidase [22].

The *in vitro* test results for the inhibitory effect of betulinic acid on α-glucosidase allowed us to evaluate whether it could reduce postprandial blood glucose levels *in vivo*. Betulinic acid significantly reduced postprandial hyperglycemia and blood glucose levels at the peak time points in diabetic mice.

Postprandial blood glucose levels were also significantly reduced in normal mice administered betulinic acid with meals compared to normal control mice. In diabetic mice administered betulinic acid, the AUC for the glucose response was significantly lower than that in control mice. Normal mice administered betulinic acid also had a lower AUC than the normal control mice, demonstrating the effect of betulinic acid in alleviating the increase in postprandial blood glucose levels. Our results suggest that betulinic acid can delay carbohydrate digestion by inhibiting carbohydrate digestive enzymes and reducing postprandial hyperglycemia *in vivo*. Coronary artery disease due to atherosclerosis is a major cause of morbidity and mortality in patients with diabetes [15]. For this reason, the management of postprandial hyperglycemia to prevent diabetic cardiovascular complications and alleviate diabetes is essential. This study suggests that betulinic acid could have a significant effect on the prevention of diabetic complications and progression of diabetes.

The long-term use of synthetic diabetic agents can lead to a variety of complications, such as cardiovascular disease, liver damage, kidney failure, and weight gain [14]. Compounds derived from natural products can be used as alternatives to alleviate diabetes and its complications, without the same side effects. Many plants have already been shown to be effective in the treatment of diabetes [5], and pentacyclic triterpenes have also been used to alleviate diabetes and its complications [2]. Ursolic acid, for example, inhibits α-glucosidase *in vitro* and decreases postprandial blood glucose levels *in vivo* [30]. Oleanolic acid not only lowers blood glucose levels and improves the body's response to insulin, but also controls hyperlipidemia and prevents diabetic complications [6]. This study showed that betulinic acid may exhibit anti-diabetic activity by inhibiting α-glucosidase and α-amylase, delaying carbohydrate digestion and absorption, and suppressing the increase in postprandial blood glucose levels. Therefore, we suggest that betulinic acid may be considered a potential natural agent for inhibiting postprandial hyperglycemia in patients with diabetes. However, more research is needed for the clinical use of betulinic acid as a natural oral hypoglycemic agent.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : STZ에 의한 당뇨 유발 마우스에서 betulinic acid의 식후 고혈당 개선 효과

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이 연구에서 베툴린산이 STZ에 의해 유발된 고혈당 쥐에서 탄수화물 소화 효소의 활성을 억제하고 식후 고혈당을 감소시킬 수 있는지 여부를 알아보았다. 그 결과, 베툴린산이 α-글루코시다아제와 α-아밀라아제 활성에 강력한 억제 효과를 보여주었다. α-글루코시다아제와 α-아밀라아제에 대한 베툴린산의 IC_{50}는 각각 12.83± 6.81 및 18.32±3.24 μM으로, 이는 경구 혈당강하제인 acarbose의 IC_{50} 보다 값이 낮아 베툴린산의 탄수화물 소화효소의 억제 활성이 높다는 것을 나타낸다. 당뇨쥐와 정상쥐에서 증가된 식후 혈당은 베툴린산을 투입한 고혈당군, 정상군 모두 대조군보다 유의하게 식후 혈당이 억제되었습니다. 30, 60, 120분에 각각 혈당을 측정하였을 때, 당뇨쥐에서 베툴린산을 투여한 군의 혈당은 23.22±1.1, 24.38±1.31, and 21.05±1.36 μM 으로 대조군의 혈당인 24.64± 1.7, 27.22±1.58, and 26.36±1.40 μM 보다 유의하게 감소하였다. 당뇨쥐에서 베툴린산 투여한 군의 AUC도 대조군 쥐에 비해 유의하게 감소하였지만, acarbose를 투여한 군에 비해서는 더 많이 감소하지 않았다. 이러한 연구 결과는 베툴린산이 탄수화물 소화 효소의 강력한 억제제로의 가능성을 보여주어, 당뇨병 쥐의 식후 고혈당증을 개선할 수 있을 것이라 사료된다.