Hypoglycaemic and hypolipidemic effects of unripe apple extract in a murine diabetic model


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Abstract
The increasing incidence of diabetes is a global concern. Current treatment modalities involving lifestyle modifications and pharmacotherapy are not effective in 50% of patients. Unripe apples contain polyphenols which are known to have antidiabetic effects. To verify the effects of unripe apple extract (UAE), the antioxidant activity of UAE was confirmed using a cell-free DPPH assay; and nitric oxide production and cytotoxicity were investigated in RIN-m5F cells. Biochemical analysis, oral glucose tolerance test, and histological analyses of the pancreas and liver were also conducted. UAE treatment induced hypoglycaemic conditions in alloxan-treated RIN-m5F cells. Further, UAE treatment showed antidiabetic effects in db/db mice by reducing plasma glucose and fructosamine levels without affecting the food intake and body weight. Additionally, UAE improved glucose tolerance and lipid parameters, and restored the pancreatic tissue in db/db mice. These results demonstrated the antidiabetic effects of UAE on alloxan-treated pancreatic islet-derived cells and in db/db mice, and suggested that UAE can be potentially used as a medicine and dietary supplement or functional food in antidiabetic therapy.

Keywords
unripe apple, diabetes, hyperglycaemia, hyperlipidaemia, RIN-m5F cells, db/db mouse

Introduction
Diabetes is the most prevalent chronic metabolic disease, accounting for 3% of deaths worldwide, and increasing type 2 diabetes incidence rates is a global concern (DeFronzo et al., 2015). Type 2 diabetes is a metabolic disorder characterised by hyperglycaemia due to insulin resistance (Rother, 2007). In the absence of appropriate treatment, diabetes leads to long-term complications that ultimately reduce the quality of life and increase the treatment costs and mortality (The Emerging Risk Factors Collaboration, 2010). In general, diabetes is treated with various diets and drugs, but approximately 50% of patients with diabetes do not meet the treatment goals, as recommended by the 2015 Standards of Medical Care in Diabetes of the American Diabetes Association (Yamagishi and Imaizumi, 2005). Therefore, the development of new natural compounds for treating diabetes without side effects has received considerable attention.

Hyperglycaemia is a major cause of the production of reactive oxygen species (ROS) (Nowotny et al., 2015). Increased ROS levels due to hyperglycaemia can stimulate the development of insulin resistance by inhibiting the insulin signalling pathway (Wang et al., 2014). In addition, ROS increase nitric oxide (NO) production via increased activation of endothelial nitric oxide synthase and expression of inducible nitric oxide synthase (Ceriello, 2003). Additionally, the energy-related enzyme AMP-activated protein kinase diverts fatty acid oxidation, and blocks ATP consumption pathways such as gluconeogenesis and lipogenesis (Coughlan et al., 2014).

Alloxan is currently the most commonly...
used agent to induce experimental diabetes owing to its selective ability to destroy insulin-producing pancreatic beta islets (Lenzen, 2008). Alloxan is thought to produce ROS, such as NO, during its metabolism (Takasu et al., 1991).

Apples contain dietary fibre, minerals, vitamin C, and phenolic compounds such as flavonoids, chlorogenic acid, catechin, rutin, epicatechin, phlorizin, and tannins (Schieber et al., 2001). These phenolic compounds show important pharmacological activities such as antioxidant (Bai et al., 2010), anti-inflammatory (Yoshioka et al., 2008), anti-allergic (Akiyama et al., 2000), anti-arteriosclerosis (D’Angelo et al., 2007), and anticancer effects (Yanagida et al., 2000), and improve cardiovascular diseases (Matsui et al., 2009). Interestingly, the diversity and maturity of apples are strongly associated with the concentrations of phenolic compounds (Wu et al., 2007; Renard et al., 2007). Unripe apples account for 20 - 30% of the total apple production, and are discarded after falling from tree or when trees are thinned out (Zheng et al., 2009). In previous reports, the polyphenol content was 10-fold higher in unripe apples than in ripe apples (Akiyama et al., 2005; Yue et al., 2012). Due to this abundance of polyphenols, unripe apples may have economic value, and their utilisation rate may be improved by identifying new pharmacological effects. Unripe apples also share some of the diverse biological activities of ripe apples. However, there are no studies of the antidiabetic effects of unripe apples. Therefore, we investigated the antidiabetic effects of unripe apple extract (UAE) on alloxan treated pancreatic islet-derived cells and in db/db mice.

Materials and methods

Extract preparation

Unripe apples were provided by the Chung-ju Apple Producer Council Farming Association Corporation (Chungju-si, Chungcheongbuk-do, Korea). An extract of unripe apples (300 g) was prepared in an electric pot by boiling apples in 372 mL of distilled water for 3 h, centrifuged, and filtered through a 0.45-µm syringe filter. The extract was evaporated at 45°C and low pressure, and subjected to freeze-drying (ILShin BioBase Co., Ltd., Dongducheon, Korea) before use in experiments.

Cell culture

The rat RIN-m5F cell line was obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) containing a high-glucose concentration at 37°C in a 5% CO₂ incubator. The medium was supplemented with 10% foetal bovine serum and 1% antibiotics.

DPPH free radical-scavenging activity

The scavenging of DPPH free radicals was assayed as previously described (Gao et al., 2011). Briefly, 100 µL of the sample and 100 µL of 300 mM DPPH were mixed and reacted at 37°C for 30 min, followed by measuring the absorbance at 517 nm. The antioxidant activity of the extract at different concentrations was calculated using Eq. 1:

\[ \text{Scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of the extract-treated group}}{\text{absorbance of the untreated group}}\right) \times 100 \]  

(Eq. 1)

Cell viability

The cell viability was assayed as previously described (Lee et al., 2018) using a water-soluble tetrazolium salt-1 assay kit (ITSBio, Seoul, Korea) in accordance with the manufacturer’s instructions. RIN-m5F cells were plated at 1 × 10⁵ cells/well, and treated with UAE (0, 10, 30, 50, and 100 µg/mL) alone or in the presence of alloxan (3 mM) for 24 h in a 5% CO₂ incubator. Absorbance was read using a multimode plate reader (Infinite 200; Tecan Group, Ltd., Switzerland).

NO assay

The NO is rapidly oxidised to nitrate and nitrite in aqueous solutions (Moncada et al., 1991). Following the treatment with UAE and alloxan for 24 h, 100 µL of each cell culture supernatant was reacted with 100 µL of the Griess reagent for 5 min. To determine the NO concentration, the absorbance was read at 540 nm using sodium nitrite as a standard.

Animals and experimental design

Healthy male C57BL/KsJ db/db (db/db) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Following one week of acclimation, the mice that developed diabetes were treated with UAE (30, 100, or 300 mg/kg) by oral gavage daily for four weeks. The body weight was measured weekly during the feeding period. The animal study was performed using protocols approved by the Committee on Care and Use of Laboratory Animals of Wonkwang University (Iksan, Jeollabuk-do, Korea; approval no.: WKU18-16).
Biochemical analyses

Biochemical analyses were performed as previously described (Kang et al., 2012). Whole-blood glucose levels were determined using an Accu-Chek Aviva glucose monitoring system (Roche Diagnostics, Basel, Switzerland); and plasma insulin was measured using a mouse insulin quantitation kit (ALPCO, Salem, NH, USA). In addition, the plasma concentrations of fructosamine, triglycerides, total cholesterol, and high-density lipoprotein (HDL) cholesterol were measured using commercially available kits (Asan Pharmaceutical, Seoul, Korea).

Oral glucose tolerance test

For the oral glucose tolerance test (OGTT), the animals were fasted overnight, and then, 25% glucose (1 g/kg body weight) was administered orally. This test was performed at a 3-day interval, with the sampling times of 30 min before, and 0, 30, 60, 90, and 120 min after the glucose challenge. Blood samples were collected from the tail vein to measure the glucose levels.

Histological analysis

Histological analysis was performed as previously described (Kang et al., 2012). The mice were anesthetised with CO₂, and sacrificed by decapitation. The liver and pancreatic islets were collected and placed in a fixation solution (10% neutral buffered formalin). Tissues were then embedded in paraffin, and cut into 4-7 µm sections. The sectioned tissues were stained with haematoxylin and eosin (H&E), and examined under an optical microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using the SAS software (version 9.3; SAS Institute, Inc., Cary, NC, USA). Data were compared using one-way analysis of variance and Duncan’s multiple range test. *p*-values < 0.05 were considered statistically significant.

Results

Free radical-scavenging activity of UAE

The DPPH radical is widely used for the assessment of radical scavenging (Dinis et al., 1994). The soluble free radical DPPH is known to be a good hydrogen abstractor that yields DPPH-H as a by-product (du Toit et al., 2001). The antioxidant activity of UAE and BTH (a positive control) was measured based on their scavenging activities against a stable DPPH radical. Results showed that the DPPH radical-scavenging activity of UAE increased in a concentration-dependent manner (Figure 1A).

Effect of UAE on cell viability

Treatment with UAE at concentrations less than 1 mg/mL did not cause any changes in the cell viability. Therefore, subsequent experiments were carried out at UAE concentrations less than 1 mg/mL.

Effects of UAE on alloxan-mediated cytotoxicity and NO production in RIN-m5F cells

Next, we evaluated whether UAE could protect against the cytotoxic effects of alloxan by measuring the viability of RIN-m5F cells. Results showed that alloxan-mediated cytotoxicity significantly decreased the number of viable cells when compared with that in the untreated (control) group. However, the cell viability increased in the UAE-treated group in a concentration-dependent manner (Figure 1B). These results suggested that UAE inhibited the damage to pancreatic cells under diabetic conditions. Furthermore, the inhibitory effect of UAE on alloxan-mediated NO production was confirmed by measuring NO production in alloxan-treated cells (Figure 1C).

Effects of UAE on hyperglycaemia and dyslipidaemia

To investigate the antidiabetic effects of UAE, we orally administered UAE for four weeks to db/db mice. The weight gain was not significantly different between the normal and UA-treated groups (Figures 2A and 2B). Glucose-lowering effects were not observed with low-dose UAE (30 mg/kg) treatment, whereas high-dose UAE (300 mg/kg) treatment significantly decreased glucose levels (Figure 2C). Additionally, the levels of fructosamine and insulin decreased in the UAE-treated mice (Figures 2D and 2E). After four weeks of UAE treatment, an OGTT was performed to determine the effects of UAE on glucose tolerance (Figure 3). The area under the curve for the glucose response dose-dependently decreased in the UAE-treated mice. In addition, supplementation with moderate- and high-dose UAE significantly decreased triglyceride and cholesterol levels in db/db mice (Figures 4A and 4B). The levels of plasma HDL cholesterol were not significantly different among the groups (Figure 4C).
Figure 1. Effect of UAE on DPPH free radical-scavenging activity, alloxan-mediated cytotoxicity, and nitric oxide production in RIN-m5F. (A) Cells were treated with various concentrations (0, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 1, 3, 5, and 10 mg/mL) of UAE. DPPH free radical-scavenging activity was measured as described in “Materials and Methods”. BHT was used as a positive control. (B) Cell viability and (C) nitric oxide production in cells pre-treated with the indicated concentrations of UAE, followed by the addition of alloxan and incubation for 24 h. Different lowercase letters indicate significant differences ($p < 0.05$). Values represent the mean ± standard error of the mean ($n = 3$).

Figure 2. UAE improves glycaemic regulation in $db/db$ mice. Mice were orally administered with PBS or UAE at doses of 30, 100, and 300 mg/kg once daily for four weeks. (A) Food intake, (B) body weight changes, (C) fasting blood glucose levels, (D) fructosamine, and (E) insulin levels. Different lowercase letters indicate significant differences ($p < 0.05$). Values represent the mean ± standard error of the mean ($n = 7$).
Effects of UAE on fatty liver development and pancreatic β-cell damage

As diabetes can cause hepatic steatosis, we assessed the degree of fat accumulation in the liver of db/db mice. As shown in Figure 5, the liver sections stained with H&E showed significant macrovesicular steatosis, whereas hepatic steatosis was remarkably alleviated in the UAE-treated group (Figure 5A). Islets of H&E-stained pancreatic tissue showed poorly defined margins and degeneration, whereas the UAE-treated group showed nearly normal islets (Figure 5B).

Discussion

The present work investigated the potential antidiabetic effects of UAE in db/db mice. Recently, UAE has been shown to significantly improve antihyperglycemic and antilipidemic activities in the liver, and prevent fatty liver development. Of the two types of diabetes, type 1 diabetes is the main cause of damage to pancreatic islets, while type 2 diabetes is known to be induced by obesity, and is related to insulin resistance. Type 2 diabetes is a metabolic syndrome affecting approximately 3% of
the world population, and its common symptoms include hypertension, hyperlipidaemia, and abdominal obesity (Isomaa et al., 2001; Goldstein, 2002). However, the general pharmacological treatment for type 2 diabetes has many limitations due to side effects and high failure rates (Liu et al., 2001). Recently, research has focused on developing agents that inhibit an increase in blood glucose levels using nontoxic natural products and food components. Hyperglycaemia promotes the development of insulin resistance by inhibiting insulin signalling, and the main purpose of diabetic treatment is to reduce insulin resistance. Therefore, our results confirmed the inhibitory effect of UAE on various signal transduction molecules associated with glucose control.

Previous studies have shown that oxidative stress resulting from increased ROS production plays an important role in inducing hyperglycaemia, decreasing glucose-stimulated insulin secretion, and increasing the death of pancreatic β cells (Ihara et al., 1999). High ROS levels lead to increase NO production via various signal transduction mechanisms (Ceriello, 2003). Additionally, increased ROS levels due to hyperglycaemia can stimulate the development of insulin resistance by inhibiting the insulin signalling pathway (Gurzov et al., 2014; Wang et al., 2014). In previous studies, inhibitors of NO production were found to protect against the destruction of cytokine-mediated insulin-secretory pancreatic β cells (Stadler et al., 1991; Corbett and McDaniel, 1992; Kaneto et al., 1995). Therefore, NO production is an important factor in cytokine-induced cytotoxicity. We confirmed the inhibitory effects of UAE on NO production and oxidative stress. Alloxan is a diabetes-inducing drug that is widely used in diabetes research because it is particularly toxic to pancreatic β cells by increasing NO production (Takasu et al., 1991; Corbett and McDaniel, 1992). In our study, alloxan increased, and UAE prevented oxidative stress and NO production in RIN-m5F cells, respectively (Figure 1A and 1C). Additionally, we confirmed that UAE inhibited alloxan-induced cytotoxicity (Figure 1B). These antioxidant effects may be the basis for the beneficial effects of UAE in diabetic management.

In this study of the antidiabetic effects and
the underlying mechanism of UAE action, the total weight of db/db mice was not changed by UAE treatment. However, UAE significantly inhibited the increase in the levels of postprandial glucose and fructosamine when compared with those in the control group (Figures 2C and 2D), and plasma insulin levels were decreased in db/db mice supplemented with UAE than in PBS-treated db/db mice (Figure 4E). Insulin plays a pivotal role in glycogen synthesis, enhances glucose tolerance, and inhibits glucose production to maintain normal postprandial blood glucose levels (Bouche et al., 2004). In general, db/db mice showed early stages of hyperinsulinemia to compensate for insulin resistance, and progressively showed insulinemia with age, which is commonly observed in late-stage type 2 diabetes (Kodama et al., 1994). Also, we performed OGTT to determine the effect of UAE on glucose tolerance after UAE treatment. The OGTT is one of the important methods for assessing the effects of hypoglycaemic agents (Alberti and Zimmet, 1998). The OGTT results indicated that UAE improved glucose utilisation by significantly decreasing blood glucose levels in db/db mice (Figure 3). In addition, UAE treatment inhibited pancreatic β-cell damage in these mice (Figure 5B). In these results, despite the improvement of blood glucose levels and recovery of pancreatic function by the UAE, a decrease in fasting insulin levels indicated an increase in insulin sensitivity.

It has been reported that serum lipid levels increased in patients with diabetes (Howard, 1987). Therefore, the diabetes-improving effect may improve serum lipid levels in UAE-treated db/db mice. In the present work, liver fat accumulation and triglyceride, total cholesterol, and HDL cholesterol levels were significantly decreased in the UAE-supplemented mice (Figures 4 and 5A). These results confirmed that UAE improved lipid parameters and inhibited fat accumulation in the liver.

Conclusion

The results of our study support the antihyperglycemic effect of unripe apples in alloxan-treated RIN-m5F cells and db/db mice. When compared with normal db/db mice, the UAE-treated db/db mice showed enhanced glucose tolerance, inhibited pancreatic β-cell damage, improved lipid parameters, and suppressed fat accumulation in the liver. Therefore, the present work suggests that the supplementation with unripe apples may be effective for maintaining blood glucose levels within normal ranges in patients with prediabetes or diabetes.

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References


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