International Food Research Journal 29(1): 23 - 31 (February 2022)
Journal homepage: *http://www.ifrj.upm.edu.my*

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

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Article history

Received: 24 August 2020
Received in revised form: 26 March 2021
Accepted: 1 June 2021

Keywords

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

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-induced 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.

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

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DOI: https://doi.org/10.47836/ifrj.29.1.03

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% CO_2 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:

Scavenging activity (%) = [1 – (absorbance of the extract-treated group / absorbance of the untreated group)] × 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 1×10^5 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% CO_2 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 CO_2, 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).

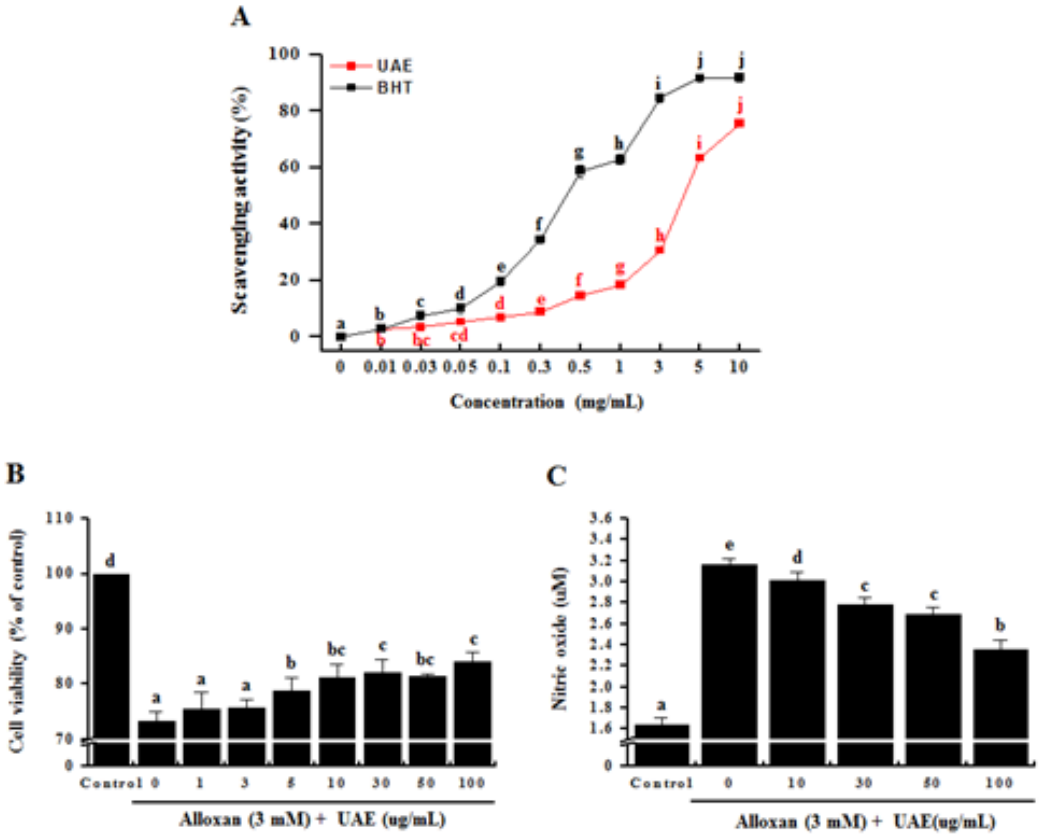

Figure 1. Effect of UAE on DPPH free radical-scavenging activity, alloxan-mediated cytotoxicity, and nitric oxide production in RIN-m5F. (A) Cells were treated with various concentrations (0, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 1, 3, 5, and 10 mg/mL) of UAE. DPPH free radical-scavenging activity was measured 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 ($n = 3$).

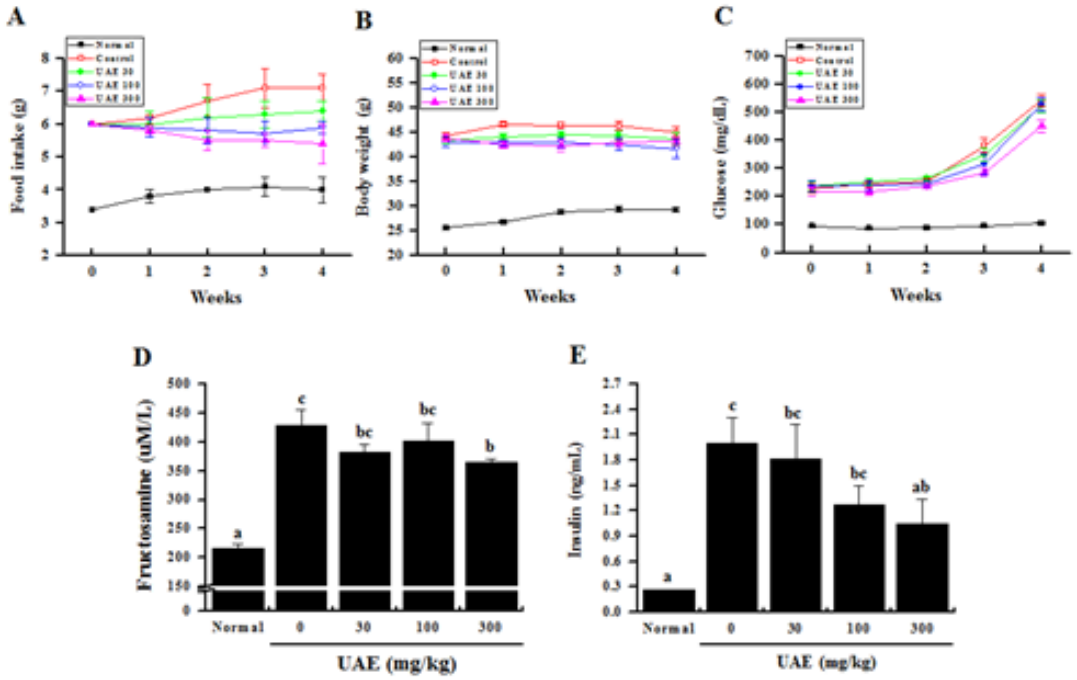

Figure 2. UAE improves glycaemic regulation in *db/db* mice. Mice were orally administered with PBS or UAE at doses of 30, 100, and 300 mg/kg once daily for four weeks. (A) Food intake, (B) body weight changes, (C) fasting blood glucose levels, (D) fructosamine, and (E) insulin levels. Different lowercase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

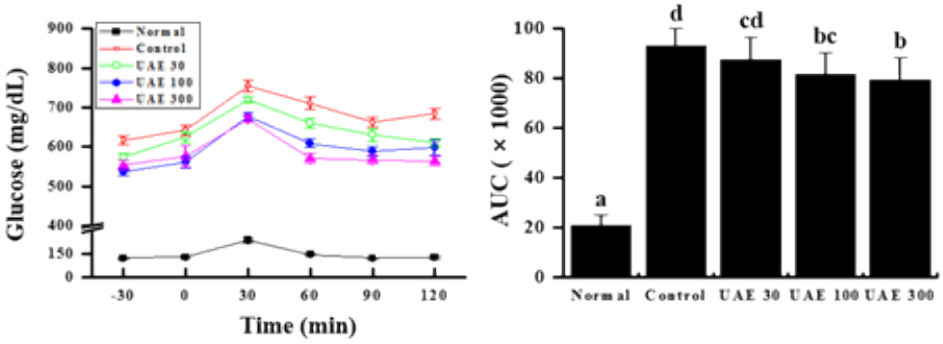

Figure 3. Oral glucose tolerance test. After four weeks of treatment, blood glucose concentrations and areas under the curve (AUCs) were determined in overnight-fasted mice. Different lowercase letters indicate significant differences ($p < 0.05$). Values represent the mean ± standard error of the mean ($n = 7$).

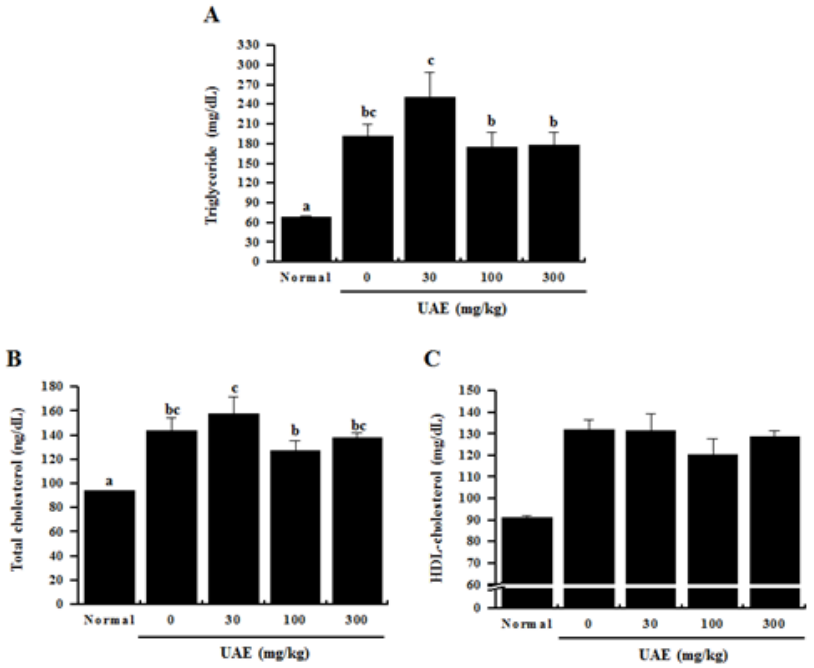

Figure 4. UAE improves plasma lipid profiles in *db/db* mice. Mice were orally supplemented with UAE doses of 30, 100, and 300 mg/kg once daily for four weeks, and plasma levels of (A) triglycerides, (B) total cholesterol, and (C) high-density lipoprotein (HDL) cholesterol were determined. Different 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

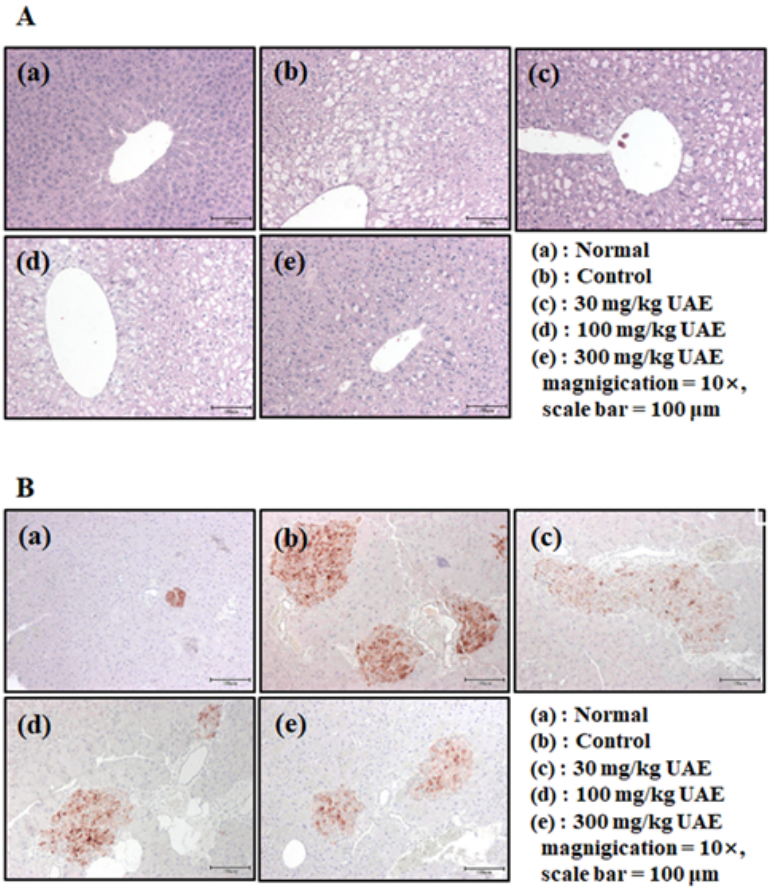

Figure 5. Effect of UAE on lipid accumulation at the liver and pancreatic islet destruction in *db/db* mice after four weeks of oral administration of UAE. (A) Liver sections and (B) pancreas sections (H&E staining; 10× magnification).

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.

Acknowledgement

The present work was financially supported by the Food Functionality Evaluation Program under the Ministry of Agriculture, Food and Rural Affairs and the Korea Food Research Institute (grant no.: E150302-05).

References

Akiyama, H., Sakushima, J., Taniuchi, S., Kanda, T., Yanagida, A., Kojima, T., … and Toyoda, M. 2000. Antiallergic effect of apple polyphenols on the allergic model mouse. Biological and Pharmaceutical Bulletin 23(11): 1370-1373.

Akiyama, H., Sato, Y., Watanabe, T., Nagaoka, M. H., Yoshioka, Y., Shoji, T., … and Maitani, T. 2005. Dietary unripe apple polyphenol inhibits the development of food allergies in murine models. FEBS Letters 579(20): 4485-4491.

Alberti, K. G. and Zimmet, P. Z. 1998. New diagnostic criteria and classification of diabetes--again? Diabetic Medicine 15(7): 535-536.

Bai, X. L., Yue, T. L., Yuan, Y. H. and Zhang, H. W. 2010. Optimization of microwave-assisted extraction of polyphenols from apple pomace using response surface methodology and HPLC analysis. Journal of Separation Science 33(23-24): 3751-3758.

Bouché, C., Serdy, S., Kahn, C. R. and Goldfine, A. B. 2004. The cellular fate of glucose and its relevance in type 2 diabetes. Endocrine Reviews 25(5): 807-830.

Ceriello, A. 2003. New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. Diabetes Care 26(5): 1589-1596.

Corbett, J. A. and McDaniel, M. L. 1992. Does nitric oxide mediate autoimmune destruction of beta-cells? Possible therapeutic interventions in IDDM. Diabetes 41(8): 897-903.

Coughlan, K. A., Valentine, R. J., Ruderman, N. B. and Saha, A. K. 2014. AMPK activation: a therapeutic target for type 2 diabetes? Diabetes, Metabolic Syndrome and Obesity - Targets and Therapy 7: 241-253.

D'Angelo, S., Cimmino, A., Raimo, M., Salvatore, A., Zappia, V. and Galletti, P. 2007. Effect of reddening-ripening on the antioxidant activity of polyphenol extracts from cv. 'Annurca' apple fruits. Journal of Agricultural and Food

Chemistry 55(24): 9977-9985.

DeFronzo, R. A., Ferrannini, E., Groop, L., Henry, R. R., Herman, W. H., Holst, J. J., … and Weiss, R. 2015. Type 2 diabetes mellitus. Nature Reviews Disease Primers 1: article ID 15019.

Dinis, T. C., Maderia, V. M. and Almeida, L. M. 1994. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Archives of Biochemistry and Biophysics 315(1): 161-169.

du Toit, R., Volsteedt, Y. and Apostolides, Z. 2001. Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. Toxicology 166(1-2): 63-69.

Gao, T., Ma, S., Song, J., Bi, H. and Tao, Y. 2011. Antioxidant and immunological activities of water-soluble polysaccharides from *Aconitum kusnezoffii* Reichb. International Journal of Biological Macromolecules 49(4): 580-586.

Goldstein, B. J. 2002. Insulin resistance as the core defect in type 2 diabetes mellitus. The American Journal of Cardiology 90(5A): 3G-10G.

Gurzov, E. N., Tran, M., Fernandez-Rojo, M. A., Merry, T. L., Zhang, X., Xu, Y., … and Tiganis, T. 2014. Hepatic oxidative stress promotes insulin-STAT-5 signaling and obesity by inactivating protein tyrosine phosphatase N2. Cell Metabolism 20(1): 85-102.

Howard, B. V. 1987. Lipoprotein metabolism in diabetes mellitus. Journal of Lipid Research 28(6): 613-628.

Ihara, Y., Toyokuni, S., Uchida, K., Odaka, H., Tanaka, T., Ikeda, H., … and Yamada, Y. 1999. Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes. Diabetes 48(4): 927-932.

Isomaa, B., Almgren, P., Tuomi, T., Forsén, B., Lahti, K., Nissén, M., … and Groop, L. 2001. Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care 24(4): 683-689.

Kaneto, H., Fujii, J., Seo, H. G., Suzuki, K., Matsuoka, T., … and Taniguchi, N. 1995. Apoptotic cell death triggered by nitric oxide in pancreatic beta-cells. Diabetes 44(7): 733-738.

Kang, Y. R., Lee, H. Y., Kim, J. H., Moon, D. I., Seo, M. Y., Park, S. H., … and Oh, H. G. 2012. Anti-obesity and anti-diabetic effects of Yerba Mate (*Ilex paraguariensis*) in C57BL/6J mice fed a high-fat diet. Laboratory Animal Research 28(1): 23-29.

Kodama, H., Fujita, M. and Yamaguchi, I. 1994. Development of hyperglycaemia and insulin resistance in conscious genetically diabetic (C57BL/KsJ-db/db) mice. Diabetologia 37(8): 739-744.

Lee, H. Y., Park, Y. M., Lee, Y. H., Kang, Y. G., Lee, H. M., Park, D. S., … and Lee, Y. R. 2018. Immunostimulatory effect of *Zanthoxylum schinifolium*-based complex oil prepared by supercritical fluid extraction in splenocytes and cyclophosphamide-induced immunosuppressed rats. Evidence-Based Complementary and Alternative Medicine 2018: article ID 8107326.

Lenzen, S. 2008. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia 51(2): 216-226.

Liu, F., Kim, J., Li, Y., Liu, X., Li, J. and Chen, X. 2001. An extract of *Lagerstroemia speciosa* L. has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. The Journal of Nutrition 131(9): 2242-2247.

Matsui, T., Korematsu, S., Byun, E. B., Nishizuka, T., Ohshima, S. and Kanda, T. 2009. Apple procyanidins induced vascular relaxation in isolated rat aorta through NO/cGMP pathway in combination with hyperpolarization by multiple K+ channel activations. Bioscience, Biotechnology, and Biochemistry 73(10): 2246-2251.

Moncada, S., Palmer, R. M. and Higgs, E. A. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacological Reviews 43(2): 109-142.

Nowotny, K., Jung, T., Höhn, A., Weber, D. and Grune, T. 2015. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. Biomolecules 5(1): 194-222.

Renard, C. M., Dupont, N. and Guillermin, P. 2007. Concentrations and characteristics of procyanidins and other phenolics in apples during fruit growth. Phytochemistry 68(8): 1128-1138.

Rother, K. I. 2007. Diabetes treatment--bridging the divide. The New England Journal of Medicine 356(15): 1499-1501.

Schieber, A., Keller, P. and Carle, R. 2001. Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. Journal of Chromatography A 910(2): 265-273.

Stadler, J., Billiar, T. R., Curran, R. D., Stuehr, D. J., Ochoa, J. B. and Simmons, R. L. 1991. Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. The American Journal of Physiology 260(5 Pt 1):

C910-C916.

Takasu, N., Asawa, T., Komiya, I., Nagasawa, Y. and Yamada, T. 1991. Alloxan-induced DNA strand breaks in pancreatic islets. Evidence for H_2O_2 as an intermediate. The Journal of Biological Chemistry 266(4): 2112-2114.

The Emerging Risk Factors Collaboration. 2010. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. The Lancet 375(9733): 2215-2222.

Wang, X., Zhang, W., Chen, H., Liao, N., Wang, Z., Zhang, X. and Hai, C. 2014. High selenium impairs hepatic insulin sensitivity through opposite regulation of ROS. Toxicology Letters 224(1): 16-23.

Wu, J., Gao, H., Zhao, L., Liao, X., Chen, F., Wang, Z. and Hu, X. 2007. Chemical compositional characterization of some apple cultivars. Food Chemistry 103(1): 88-93.

Yamagishi, S. and Imaizumi, T. 2005. Diabetic vascular complications: pathophysiology, biochemical basis and potential therapeutic strategy. Current Pharmaceutical Design 11(18): 2279-2299.

Yanagida, A., Kanda, T., Tanabe, M., Matsudaira, F. and Oliveira Cordeiro, J. G. 2000. Inhibitory effects of apple polyphenols and related compounds on cariogenic factors of mutans streptococci. Journal of Agricultural and Food Chemistry 48(11): 5666-5671.

Yoshioka, Y., Akiyama, H., Nakano, M., Shoji, T., Kanda, T., Ohtake, Y., … and Maitani, T. 2008. Orally administered apple procyanidins protect against experimental inflammatory bowel disease in mice. International Immunopharmacology 8(13-14): 1802-1807.

Yue, T., Shao, D., Yuan, Y., Wang, Z. and Qiang, C. 2012. Ultrasound-assisted extraction, HPLC analysis, and antioxidant activity of polyphenols from unripe apple. Journal of Separation Science 35(16): 2138-2145.

Zheng, H. Z., Hwang, I. W. and Chung, S. K. 2009. Enhancing polyphenol extraction from unripe apples by carbohydrate-hydrolyzing enzymes. Journal of Zhejiang University Science B 10(12): 912-919.