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Hesperidin ameliorates pancreatic β-cell dysfunction and apoptosis in streptozotocin-induced diabetic rat model

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ABSTRACT

Aims: The current study was conducted to investigate the potential protective effects of hesperidin and its possible mechanisms of action on pancreatic β-cells in diabetes.

Main methods: Male Sprague Dawley rats were made diabetic using 65 mg/kg intraperitoneal injection of streptozotocin, and then administered daily with 100 mg/kg of hesperidin over 4 weeks. On conclusion of the experiment, blood and pancreatic tissue were collected to determine the function of β-cells, apoptosis, oxidative stress, ER stress, and inflammation.

Key findings: Treatment of diabetic rats with hesperidin, significantly decreased fasting blood glucose and food intake, along with increased body weight, serum and pancreatic insulin levels, and pancreatic-duodenal homeobox-1 (PDX-1) protein expression. The beneficial roles of hesperidin on diabetic pancreatic β-cells exhibited an increment in antioxidant SOD and GPx activities, and a decrement in nitrotyrosine as well as malondialdehyde (MDA) levels. Additionally, the elevated concentration of TNF-α and expressions of ER stress maker GRP78 and CHOP proteins in the pancreas of diabetic rats were significantly diminished by hesperidin treatment. Furthermore, hesperidin effectively modulated expressions of apoptosis-regulatory proteins in diabetic rat pancreas, as revealed by upregulating anti-apoptotic Bcl-xL; with a concomitant downregulating pro-apoptotic Bax, cleaved caspase-3, and inhibiting the activation of DNA repair protein poly (ADP-ribose) polymerase (PARP).

Significance: Collectively, these findings suggest that hesperidin may have the potential to protect pancreatic β-cells and improve their function by suppressing oxidative and ER stress, along with activating its antioxidant, anti-inflammatory, and anti-apoptotic effects.

1. Introduction

Diabetes mellitus (DM) is a multifactorial chronic metabolic disorder with a global prevalence in 2017 of 451 million people. This statistic is expected to increase to 693 million by 2045 [1]. DM, which is characterized by hyperglycemia, arises due to an impairment in insulin synthesis and secretion from pancreatic β-cells and/or resistance to insulin action on target tissues [2]. Persistent hyperglycemia causes an increase in reactive oxygen species (ROS) generation. This in turn induces the apoptosis of pancreatic β-cells, resulting in a defect in insulin production and secretion, and eventually hyperglycemia [3]. Additionally, in hyperglycemic state, excess ROS production could be due to the reduction of antioxidant enzymatic defenses [4]. Thus, an imbalance between increased ROS formation and decreased antioxidant enzymes, leads to enhanced oxidative stress in pancreatic β-cells; resulting in cell apoptosis. Along with oxidative stress, growing evidence shows that endoplasmic reticulum (ER) stress contributes to pancreatic β-cell apoptosis and dysfunction in diabetes [5]. Perturbation of β-cell ER homeostasis occurs due to ER Ca^{2+} depletion, and excessive misfolded and unfolded proteins by affecting high glucose and lipid loads, cytokines, and oxidative stress [6]. ER stress can activate cell apoptosis by activating transcription factor C/EBP homologous protein (CHOP),

Abbreviations: BSA, Bovine serum albumin; b.w., Body weight; CHOP, CCAAT-enhancer-binding protein homologous protein; DM, Diabetes mellitus; ELISA, Enzyme-linked immunosorbent assay; ER, Endoplasmic reticulum; FBG, Fasting blood glucose; JNK, c-Jun N-terminal kinases; GCK, Glucokinase; Glut-2, Glucose transporter-2; GPx, Glutathione peroxidase; GRP78, 78-kDa glucose regulated protein; Hsp, Hesperidin; IFN-γ, Interferon-gamma; IL-1β, Interleukin-1β; IL-6, Interleukin-6; MDA, Malondialdehyde; NF-κB, Nuclear factor kappa-B; PARP, Poly (ADP-ribose) polymerase; PDX-1, Pancreatic-duodenal homeobox-1; PKC, Protein kinase C; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; SOD, Superoxide dismutase; STZ, Streptozotocin; TBARS, Thiobarbituric acid reactive substances; TNF-α, Tumor necrosis factor-alpha

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c-Jun N-terminal kinases (JNK) pathway, as well as pro-apoptotic Bcl-2 proteins, and decreasing anti-apoptotic Bcl-2 proteins [6,7]. Additionally, mounting evidence reveals that pro-inflammatory cytokines have an important role in the regulation of pancreatic β-cell death and function [8]. Pro-inflammatory cytokines such as interleukins, tumor necrosis factor–alpha (TNF-α), and interferon-gamma (IFN-γ) are up-regulated in both the pancreas and serum of diabetic animals [9]. These cytokines generate the formations of ROS and ER stress, and in turn, oxidative and ER stress can enhance the production of pro-inflammatory cytokines, thereby causing pancreatic β-cell apoptosis and dysfunction [10,11]. The crosstalk between these signal pathways is associated with the apoptosis of β-cells in diabetes. Thus, disrupting the cellular stress-mediated pancreatic β-cell death could be a valuable therapeutic approach to prevent and cure diabetes.

Conclusive evidence shows the protective effects of flavonoids on pancreatic β-cells in diabetic models [12]. For instance, naringenin increases the survival and function of diabetic rat β-cells by inhibiting oxidative stress [13]. Similarly, quercetin prevents β-cells from STZ-induced apoptosis and oxidative damage, and improves the capacity of β-cells to secret insulin [14,15]. Kaempferol has also been shown to reduce high glucose-induced β-cell apoptosis via the suppression of caspase-3 protein and up-regulation of Bcl-2 protein [16]. Hesperidin (Hsp, 3′,5,7-trihydroxy-4′-methoxy-flavanone-7-rhamnoglucoside) is a natural flavanone glycoside widely found in Citrus fruits [17]. Hesperidin has been found to possess a wide range of pharmacological activities, including anti-oxidative, anti-hyperglycemic, anti-hypercholesterolemic, anti-inflammatory, anti-hypertensive, and anti-carcinogenic properties [18–22]. Previously, hesperidin was shown to attenuate blood glucose and cholesterol levels and increase insulin level in both type 1 and 2 diabetic models [18,23]. In addition, it inhibited nuclear factor-kappa B (NF-κB) and oxidative stress in liver and kidney of diabetic animals [20]. In STZ-induced diabetes, hesperidin can decrease serum levels of pro-inflammatory cytokines and oxidative stress markers [24]. Furthermore, hesperidin protected against high glucose-induced oxidative stress and apoptosis in retinal ganglion cells [25]. Nevertheless, the exact mechanisms which hesperidin exerts its protective effect on pancreatic β-cells in diabetes remain largely unknown. This study was designed to investigate the possibility of the beneficial roles of hesperidin on pancreatic β-cell protection in STZ-induced diabetic model.

2. Materials and methods

2.1. Chemicals

Hesperidin (CAS Number: 520-26-3, Product Number: H5254), and streptozotocin (STZ) (CAS Number: 18883-66-4, Product Number: S0130) were purchased from Sigma-Aldrich Chemicals (St Louis, Mo, USA); stored at 2–4 °C and protected from sunlight. Antibodies for PDX-1, CHOP, GRP78, cleaved caspase-3, cleaved PARP, Bax, Bcl-xL, and β-actin, and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Cell signaling Technology (Danvers, MA, USA). All other chemicals were of analytical grade, high purity, and were obtained from Merck Millipore, USA.

2.2. Animals

Male Sprague Dawley rats weighing approximately 180-200 g, were used as experimental animals in this study. The animals were obtained from National Laboratory Animal Center, Mahidol University. All rats were housed under controlled temperature at 22 ± 1 °C with 12/12-h light/dark cycle and were fed a standard rodent chow and water ad *libium*. This work has been approved by the Institutional Animal Care and Use Committee of Naresuan University, Thailand (Ethics number: NU-AE570926 and NU-AEE601011).

2.3. Diabetic induction

Following an acclimatization period of 1 week, diabetes was induced in the overnight fasted rats, with a single intraperitoneal injection of STZ dissolved in 0.1 M sodium citrate buffer, pH 4.5 at a dose of 65 mg/kg body weight (b.w.). After three days of STZ injection, blood was collected from the tail vein, and fasting blood glucose (FBG) level was determined using a digital glucometer (Roche Diagnostics, USA). The rats with FBG higher than 250 mg/dl were considered to be diabetic and were used in this study.

2.4. Experimental animal groups and treatments

Hesperidin was dissolved in distilled water, and the dose of 100 mg/kg hesperidin used in this study was based on previous studies [20,21]. The rats were randomly divided into three groups, each group comprising 8 rats and detailed as follows:

Group 1: Normal control rats were intragastrically administered with 1 ml/kg b.w. of distilled water.

Group 2: Diabetic control rats were intragastrically administered with 1 ml/kg b.w. of distilled water.

Group 3: Diabetic rats were intragastrically administered with 100 mg/kg b.w. of hesperidin in aqueous suspension.

Supplementation of hesperidin was initiated after 3-days of STZ injection and continued for 28 days. The dosage was adjusted every week, according to any change in body weight, to maintain similar dose per kilogram body weight of rat over the entire period of study. During this treatment period, the daily food intake, and weekly FBG and body weight were measured.

2.5. Collection of blood sample and tissue

At the end of the experimental period, after 12 h fasting, the rats were euthanized with intraperitoneal injection of overdose sodium pentobarbital (100 mg/kg b.w.). Blood samples were collected via cardiac puncture and then centrifuged at 4000*g*, 4 °C for 15 min to obtain the serum. The pancreas was rapidly removed, weighed, and processed for biochemical studies and protein expressions.

2.6. Assessment of serum and pancreatic insulin levels

Pancreatic tissue was homogenized in acid ethanol (0.18 M HCL in 70% ethanol), overnight at −20 °C, and then centrifuged at 14000*g*, 4 °C for 30 min to collect the supernatant for the assay of insulin content. Both pancreatic and serum insulin levels were measured using mouse/rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Cat. number EZRMI-13K, Merck Millipore, Germany) following the instructions of the commercial kit.

2.7. Determination of pancreatic antioxidant and oxidative status

Pancreatic superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured using SOD assay kit (Cat. number 574601, Merck Millipore, USA) and GPx assay kit (Cat. number 703102, Cayman Chemical, USA), respectively. Nitrotyrosine level in the pancreas was determined by nitrotyrosine assay kit (Cat. number 17-10006, Merck Millipore, USA). The assays were performed according to the manufacturer's instructions.

2.8. Determination of lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) method. Briefly, the reaction mixture, which consisted of 25 µl of the sample or malondialdehyde (MDA) standard, 50 µl of 8.1% (w/v) sodium dodecyl sulfate (SDS), 375 µl of 0.8% (w/v) TBA, and 375 µl of 20% (w/v) acetic acid solution (pH 3.5) was heated at

95 °C for 45 min. After cooling, the reaction was centrifuged at 4000 rpm for 10 min at 4 °C and then the supernatant was measured colorimetrically for the formation of MDA and TBA at a wavelength of 530 nm. The MDA content in pancreas was expressed as nmol/mg protein.

2.9. Measurement of pro-inflammatory cytokine TNF-α

The concentration of TNF-α in pancreatic tissue was assayed using commercially rat TNF-α ELISA kit (Cat. number EZRTNFA, Merck Millipore, USA) according to the manufacturer's protocol.

2.10. Western blot analysis

Total pancreatic proteins were extracted with cold Pierce RIPA buffer containing 1% halt protease inhibitor and then centrifuged at 14000 *g*, 4 °C for 15 min. The protein concentrations were quantified by micro bicinchoninic acid (BCA) protein assay kit (Cat. number 71285, Merck Millipore, USA). Equal amounts of pancreatic proteins (50 μg) were separated on 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked with 5% (w/v) bovine serum albumin (BSA) followed by incubation either with primary anti-PDX-1 (1:1000), anti-CHOP (1:500), anti-GRP78 (1:500), anti-cleaved caspase-3 (1:500), anti-cleaved PARP (1:1000), anti-Bax (1:500), anti-Bcl-xL (1:1000), or anti-β-actin (1:1000) antibody at 4 °C overnight. After that, the membranes were washed and incubated with HRP-conjugated secondary antibodies (1:3000–1:5000). The protein bands were then detected with enhanced chemiluminescence (Bio-Rad, USA). Band intensities were quantified using Image Lab software (Bio-Rad, USA).

2.11. Statistical analysis

All values were expressed as mean ± standard error of the mean (S.E.M.). Differences between the groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of hesperidin on FBG, food intake, and body weight in diabetic rats

Increment in blood glucose and food consumption, and reduced body weight are considered common signs of diabetes. In this study, STZ-induced diabetic rats showed a significant increase in the levels of FBG compared with those of normal rats, and the elevation in FBG levels were maintained throughout the entire experiment (Fig. 1A). Additionally, STZ-exposed diabetic rats demonstrated a marked increase in food intake but a decrease in body weight as compared to those in the normal rats (Fig. 1 B−C). Importantly, treatment of diabetic rats with 100 mg/kg b.w. of hesperidin exhibited a gradual reduction in the FBG levels compared with untreated diabetic rats (Fig. 1A). Administration of hesperidin 100 mg/kg b.w. for 28 days also significantly diminished the food intake and increased the body weight in the diabetic rats as shown in Fig. 1B-C.

3.2. Effect of hesperidin on serum and pancreatic insulin levels, and PDX-1 protein expression in the pancreas of diabetic rats

As shown in Fig. 2A-B, serum and pancreatic insulin levels in STZ-induced diabetic rats were dramatically reduced when compared with those in the control rats, which indicates pancreatic β-cell dysfunction. As expected, treatment with 100 mg/kg b.w. of hesperidin to diabetic rats significantly elevated the levels of insulin in both serum and pancreatic tissue as compared to untreated diabetic rats.

Transcription factor PDX-1 plays a major role in the regulation of pancreatic β-cell function [26]. Thus, the expression level of PDX-1 protein in the pancreas was measured using Western blotting. The result showed a downregulation in the pancreatic PDX-1 protein expression of diabetic rats; however, it was significantly upregulated by hesperidin treatment in diabetic rats (Fig. 3A). Taken together, these results indicated that hesperidin improves PDX-1 protein expression and the functions of pancreatic β-cells to synthesize and secrete insulin.

3.3. Effect of hesperidin on pancreatic TNF-α level in diabetic rats

The induction of pro-inflammatory cytokines is involved with pancreatic β-cell death in diabetes [27]. Thus, the level of TNF-α has been determined in this study. As demonstrated in Fig. 3B, diabetic rats had significantly higher pancreatic TNF-α level than the control rats. Importantly, hesperidin treatment resulted in a significant reduction of pancreatic TNF-α level in diabetic rats.

3.4. Effect of hesperidin on oxidative status in the pancreas of diabetic rats

Table 1 depicted the levels of antioxidant activities and oxidative stress markers in the pancreas of normal and experimental rats. In diabetic rats, the activities of antioxidant SOD and GPx in pancreatic tissues were obviously diminished, while treatment of hesperidin to diabetic rats significantly enhanced these antioxidant activities in the pancreas as compared to untreated diabetic rats. In contrast, the pancreatic levels of nitotyrosine and MDA were significantly up-regulated in diabetic rats; however, these effects were reversed by administration of 100 mg/kg b.w. of hesperidin to diabetic rats.

3.5. Effect of hesperidin on apoptosis-related protein expressions in the pancreas of diabetic rats

To further investigate the protective effect of hesperidin on apoptotic cell death in the pancreas of diabetic rats, we measured the expressions of Bax, Bcl-xL, cleaved caspase 3, and cleaved PARP proteins. The results showed an evident upregulation in Bax, cleaved caspase 3, and cleaved PARP proteins in the pancreas of diabetic rats, whereas these proteins were markedly downregulation in hesperidin-treated diabetic rats (Fig. 4A-B, and D-E). Additionally, a significant decrease in the pancreatic Bcl-xL protein expression was detected in diabetic rats. Treatment of hesperidin significantly increased the expression of Bcl-xL protein in the pancreas of diabetic rats as shown in Fig. 4A and C.

3.6. Effect of hesperidin on ER stress markers in the pancreas of diabetic rats

ER stress is known to regulate cell apoptosis [6]. In present study, ER stress marker GRP78 and CHOP proteins were measured to indicate the activation of ER stress in the pancreas. As demonstrated in Fig. 5A-C, the diabetic rats had significantly higher pancreatic GRP78 and CHOP protein expressions than the normal rats. By contrast, treatment with 100 mg/kg b.w. of hesperidin significantly reduced the expression proteins of GRP78 and CHOP in the pancreas of diabetic rats.

4. Discussion

An impairment in pancreatic β-cell mass and function with excessive pancreatic β-cell apoptosis is a contributor to the development of diabetes [28]. In this study, we investigated the beneficial effects of hesperidin to counteract rat pancreatic β-cell damage caused by STZ. From the results we obtained, hesperidin exhibited an antidiabetic property by improving pancreatic β-cell function, and inhibiting oxidative and ER stress, as well as inflammatory cytokine-mediated pancreatic β-cell death in diabetic rats. STZ enters pancreatic β-cell through

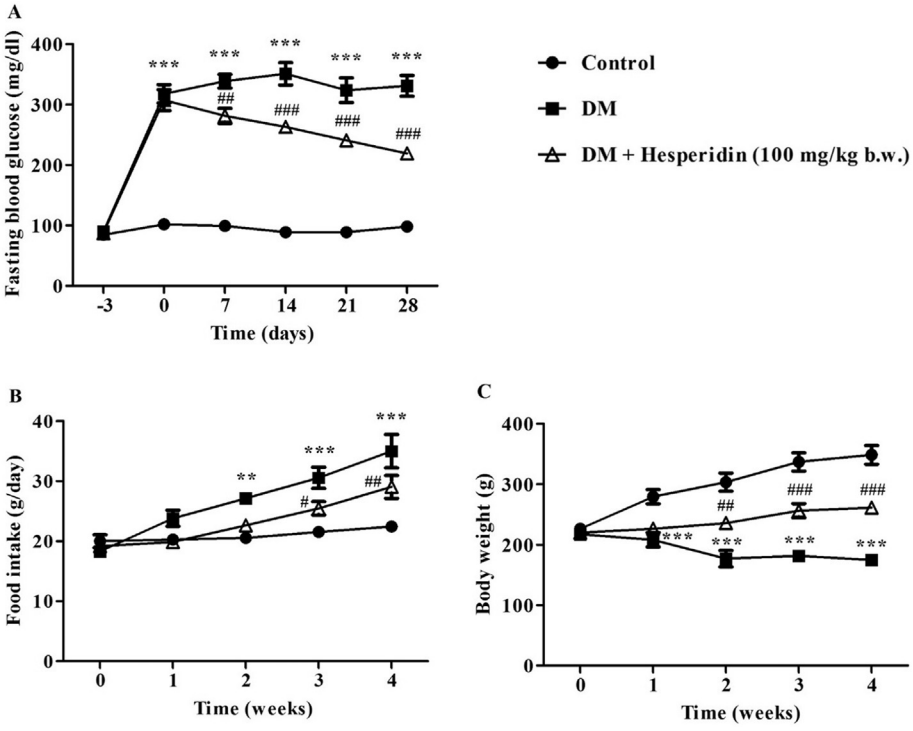

Fig. 1. Effect of hesperidin on fasting blood glucose (FBG) levels in diabetic rats before (−3 day) and after STZ injection at 0,7,14, 21, and 28 days of treatment (A). Food consumption (B) and body weight change (C) in STZ-induced diabetic rats before (0 week) and after 1–4 weeks of hesperidin treatment. Data are presented as mean ± S.E.M. of 8 rats per each group. $^{***}P < 0.001$ vs control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs DM group. DM: diabetic rats; DM + Hesperidin: diabetic rats treated with hesperidin (100 mg/kg b.w.).

glucose transporter-2 (Glut-2), causing abundant generation of ROS and nitric oxide (NO) which leads to oxidative stress, and further results in inflammation by enhancing inflammatory cytokines [29]. In turn, the production of inflammatory cytokines can activate ER stress, which leads to oxidative stress [30]. Rats that were made diabetic using STZ, presented with an elevated blood glucose, excess food consumption, and loss in body weight due to the depletion of insulin-producing pancreatic β-cells [31]. Our findings are similar to these characteristics of diabetes. STZ induces pancreatic β-cell destruction and causes a decrease in insulin synthesis and secretion; which finally manifests as hyperglycemia. Reduced insulin secretion also leads to weight loss due to the lack of carbohydrate utilization as an energy source, thus enhancing the breakdown of protein and fat [32]. Treatment with 100 mg/kg of hesperidin significantly reverses these changes in diabetic rats induced by STZ, indicating its antidiabetic property. The effects might be derived from improving pancreatic β-cell function.

Pancreatic-duodenal homeobox-1 (PDX-1) is a transcription factor in pancreatic β-cells that regulates pancreatic β-cell differentiation and function [26]. Glucose stimulates the translocation of PDX-1 into the nucleus that further promotes the transcription of insulin gene, and insulin secretion-associated genes such as glucokinase (GCK) and Glut-2 [33]. With a diabetes condition, sustained high glucose suppresses the PDX-1 gene, and activates PDX-1 translocation to the cytosol, which results in reducing the binding of PDX-1 and insulin gene promotor, thereby inhibiting insulin synthesis and secretion [34]. Numerous studies report that PDX-1, GCK, and Glut-2 protein expressions were downregulated in diabetic rats, causing impaired expression and secretion of insulin [35,36]. Similarly, our study showed a decrease in pancreatic PDX-1 protein expression in diabetic rats caused by STZ, followed by reduced levels of pancreatic and serum insulin. Treatment with hesperidin to diabetic rats resulted in a significant increase in pancreatic PDX-1 protein, and insulin levels in both serum and pancreas. These findings indicate that hesperidin might improve pancreatic β-cell function to synthesize and release insulin by upregulation of PDX-1 expression in diabetic rats.

In addition to impaired pancreatic β-cell secretory capacity, the deficit in pancreatic β-cell mass by enhanced apoptosis plays a pivotal role in the pathophysiology of diabetes. There is a clear line of evidence showing that the rate of apoptosis is increased in both human and animal diabetic islets [28,37–39]. The decrement in anti-apoptotic Bcl-2 proteins and increment in pro-apoptotic Bad, Bid, Bik, and Bax as well as caspase 3 activity, have been observed in diabetic models

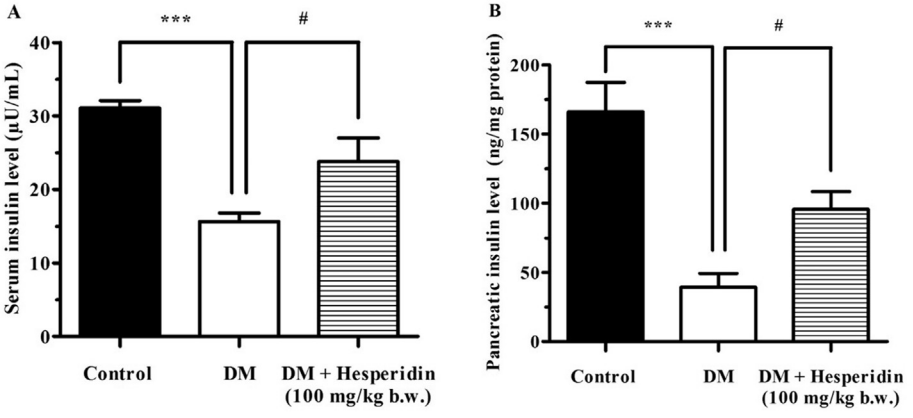

Fig. 2. Effect of hesperidin on the levels of insulin in serum (A) and pancreas (B) of STZ-induced diabetic rats. Serum and pancreatic insulin levels were measured using the ELISA method. Data are expressed as mean ± S.E.M. of 5–6 rats per group. $^{***}P < 0.001$ vs control group, $^{\#}P < 0.05$ vs DM group. DM: diabetic rats; DM + Hesperidin: diabetic rats treated with hesperidin (100 mg/kg b.w.).

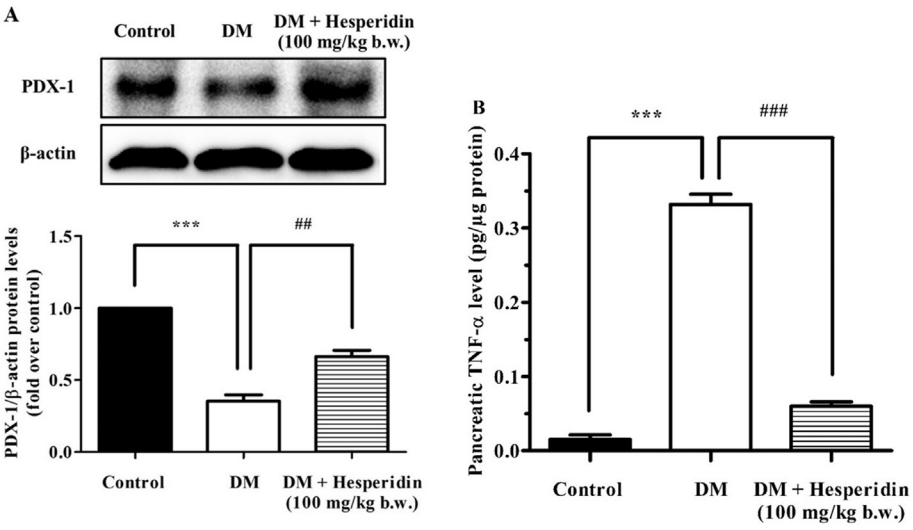

Fig. 3. Analysis of pancreatic PDX-1 protein expression in normal, diabetic, and hesperidin-treated diabetic rats (A). A; A representative Western blot analysis of PDX-1 and β-actin from each group. The bar graph below is the fold change of PDX-1 protein level normalized to β-actin protein. Values are presented as mean ± S.E.M. of 3 rats in each group from independent experiments. B: The levels of TNF-α in normal, diabetic, and hesperidin-treated diabetic rats. Pancreatic TNF-α levels were analyzed by TNF-α ELISA kit. Values are expressed as mean ± S.E.M. of 6 rats in each group. $^{***}P < 0.001$ vs control group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs DM group. DM: diabetic rats; DM + Hesperidin: diabetic rats treated with hesperidin (100 mg/kg b.w.).

[30,35,40,41]. In our study, STZ-induced diabetic rats consistently exhibited a pancreatic β-cell apoptosis with a marked increase in cleaved caspase-3, Bax protein expressions, and a decrease in anti-apoptotic Bcl-xL protein. However, hesperidin treatment was able to reverse these effects in the pancreas of diabetic rats. Caspase 3 can cleave poly (ADP-ribose) polymerase (PARP), which eventually induces DNA damage and cell apoptosis [42–44]. This study showed that overexpression of cleaved PARP protein was presented in diabetic rat pancreas, which was significantly diminished by hesperidin. In support of our results, it has been reported that hesperidin suppresses high glucose-induced retinal ganglia cell apoptosis by downregulating caspase-9, and −3, as well as the ratio of Bax/Bcl-2 proteins [25]. Furthermore, hesperidin was shown to prevent renal and hepatic apoptosis induced by sodium arsenic-induced in rats [45]. The findings described above suggest that hesperidin has an anti-apoptotic activity to protect pancreatic β-cells against death in diabetic rats via modulating Bcl-2 family proteins and inhibiting the activation of caspase cascade pathway.

Chronic hyperglycemia enhances the formation of both ROS and reactive nitrogen species (RNS), and reduces antioxidant enzyme defenses, causing the induction of oxidative stress, which is one of the important causes of pancreatic β-cell apoptosis [46]. Overproduction of ROS/RNS also leads to increased lipid and protein oxidation, resulting in cellular dysfunction and death [47]. The MDA levels, a biomarker of lipid peroxidation, are shown to obviously increase in the pancreas of diabetes, indicating increased oxidative damage to cells [48]. In addition, superoxide anion can react with NO to produce peroxynitrite anion ($ONOO^-$), which reacts with cellular tyrosine to form nitrotyrosine production [49]. In pancreatic β-cell line, high glucose induced the formation of nitrotyrosine, thereby causing β-cell apoptosis [50]. Several studies have also shown an elevation in reactive oxygen and nitrogen species levels, which are related with apoptosis of pancreatic β-cells in the animal models of diabetes [30,46,51]. Consistent with previous works [30,36], experimental animals exposed with STZ had increased levels of MDA and nitrothyrosine in pancreatic tissues, and led to β-cell damage. Furthermore, it is well established that the activities and expressions of these antioxidant enzymes SOD, CAT, and GPx were attenuated in the pancreatic tissue of STZ exposed diabetic rats [30,52]. In this study, we found a decline in pancreatic SOD and GPx activities of diabetic rats caused by STZ. Hesperidin administration improved the activities of antioxidant SOD and GPx, and also decreased the productions of MDA and nitrotyrosine in pancreases of this model. This is the first study to demonstrate the antioxidant property of hesperidin in rat diabetic pancreas by scavenging free radicals and activating endogenous antioxidant enzymes. However, previous studies clearly established that hesperidin increased antioxidant enzymes, and reduced free oxygen and nitrogen species as well as lipid peroxidation in other tissues of diabetic rats, including kidney, liver, and brain [17,20].

Pro-inflammatory cytokines are involved in the inflammation process that activates pancreatic β-cell apoptosis and dysfunction in diabetes [27]. In diabetes, significantly increased levels of pro-inflammatory cytokine markers e.g. interleukin-1β (IL-1β), interleukin-6 (IL-6), and TNF-α have been present in both animals and patients with diabetes [10,52–54]. Excessive glucose concentrations are shown to increase pro-inflammatory cytokines by inducing oxidative mechanism-mediated NF-κB activation. In turn, these cytokines produce NADPH oxidase-generated ROS formation, leading to oxidative stress which eventually results in the destruction of pancreatic β-cells [55]. Pro-

Table 1
Effect of hesperidin (100 mg/kg b.w.) on pancreatic GPx, SOD, nitrotyrosine, and MDA levels.

Parameters	Groups		
	Control	DM	DM + Hesperidin (100 mg/kg b.w.)
GPx (nmol/min/mg protein)	111.60 ± 7.24	28.88 ± 3.98 ***	68.44 ± 6.28 ###
SOD (U/mg protein)	3.73 ± 0.27	1.48 ± 0.12 ***	2.56 ± 0.21 ##
Nitrotyrosine level (μM)	0.27 ± 0.01	1.55 ± 0.07 ***	0.79 ± 0.06 ###
MDA level (nmol/mg protein)	30.26 ± 5.62	60.90 ± 2.88 ***	33.04 ± 1.88 ###

Each value represents the mean ± SEM of 5–6 rats in each group.
Statistical significant:
*** $P < 0.001$ compared with control rats.
$P < 0.01$ compared with diabetic rats.
$P < 0.001$ compared with diabetic rats.

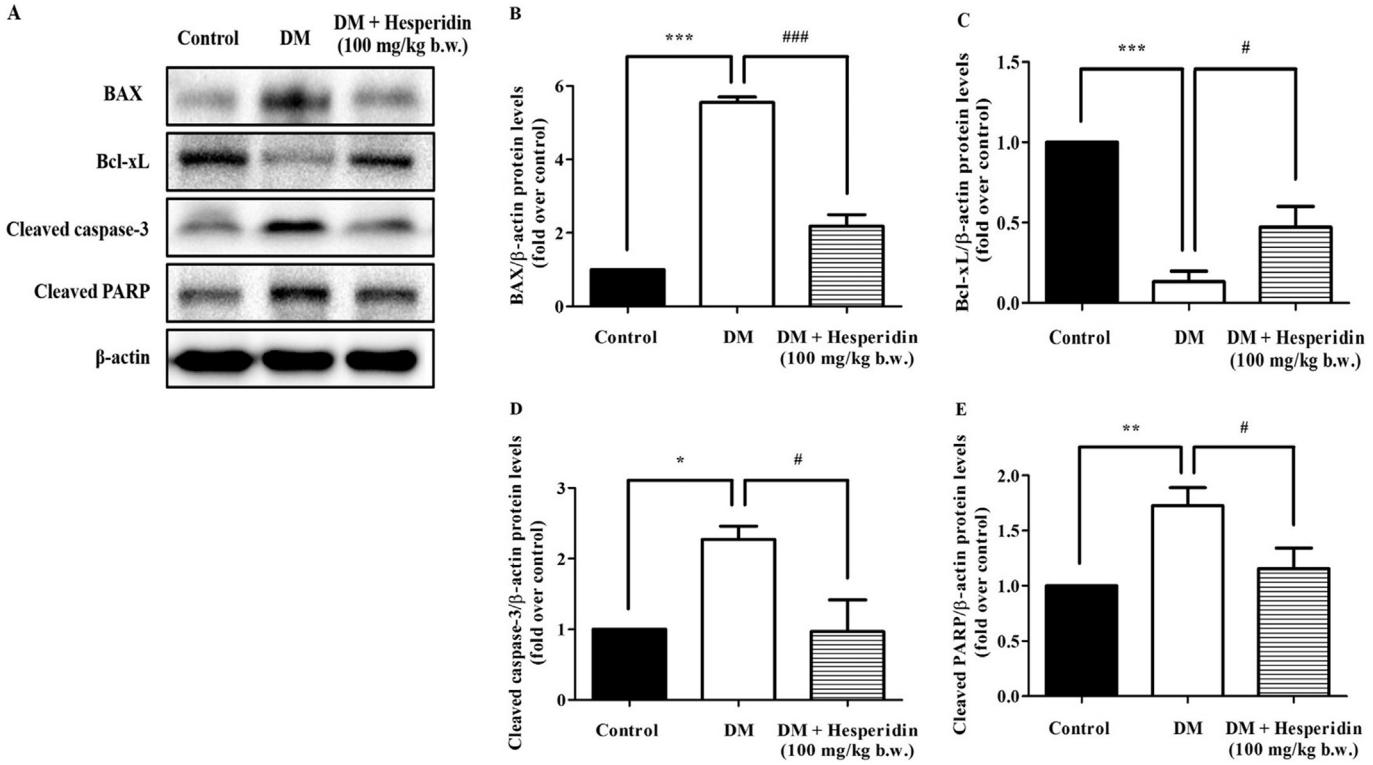

Fig. 4. Effect of hesperidin on the expressions of apoptosis-related proteins in the pancreas of diabetic rats. Analysis of Bax, Bcl-xL, cleaved caspase 3, and cleaved PARP proteins from the pancreatic tissues in each group were performed by Western blotting. A: A representative Western blot analysis of Bax, Bcl-xL, cleaved caspase 3, cleaved PARP, and β-actin proteins. The bar graphs are the fold change of Bax (B), Bcl-xL (C), cleaved caspase 3 (D), and cleaved PARP (E) protein levels normalized to β-actin protein. Values are expressed as mean ± S.E.M. of 3–6 rats in each group from independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs DM group. DM: diabetic rats; DM + Hesperidin: diabetic rats treated with hesperidin (100 mg/kg b.w.).

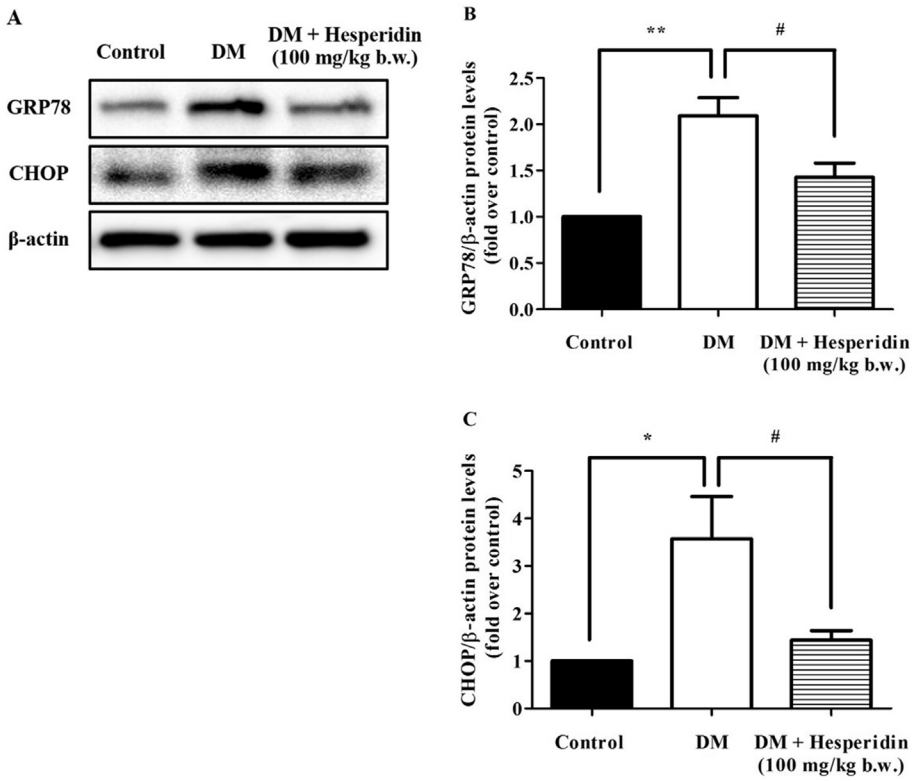

Fig. 5. Effect of hesperidin on the expressions of ER stress marker GRP78 and CHOP proteins in the pancreas of diabetic rats. Western blot analysis of GRP78 and CHOP protein expressions performed in the pancreatic tissues of each group. A: A representative Western blot analysis of GRP78, CHOP, and β actin proteins. B: The bar graph shows fold change of GRP 78 protein normalized to β-actin. C: The bar graph shows fold change of CHOP protein normalized to β-actin. Data are presented as mean ± S.E.M. of 4 rats in each group from independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group, $^{\#}P < 0.05$ vs DM group. DM: diabetic rats; DM + Hesperidin: diabetic rats treated with hesperidin (100 mg/kg b.w.).

inflammatory cytokine TNF-α has been demonstrated to activate NF-κB expression and NO production. This process induces the intrinsic mitochondrial apoptosis pathway, and promotes β-cell dysfunction by reducing insulin production [56]. In line with earlier studies [30,57], we found a significantly elevated TNF-α level in the pancreas of STZ-exposed diabetic rats; whereas administration of hesperidin dramatically ameliorated the pancreatic TNF-α level in diabetic rats, which reflects its anti-inflammatory activity; and in this way may prevent damage to pancreatic β-cells. Many studies with experimental diabetic models showed that hesperidin ameliorated pro-inflammatory cytokine TNF-α and IL-6 levels in rat serum [24], and also attenuated renal IL-1β, IL-6, and TNF-α concentrations in mice [58]. In another study, Iskender H, et al. (2017) reported significantly suppressed renal and hepatic NF-κB levels in diabetic rats caused by STZ, thereby inhibiting renal and hepatic inflammation [20].

Increased cytokines and ROS productions produced by high glucose are able to deplete the ER Ca^{2+} store, causing ER stress; further culminating in the activation of apoptosis [11,59]. Evidence also confirmed that ER stress disrupts cellular redox balance and causes oxidative stress, which in turn enhances inflammatory cytokines, resulting in cell apoptosis [60,61]. In animal models of diabetes, ER stress-associated proteins were upregulated and related to the activation of apoptosis in pancreatic β-cells [59,62–64]. In islets of diabetic patients, they demonstrated an increase in ER chaperone GRP78 and CHOP proteins, along with elevated pancreatic β-cell dysfunction and apoptosis [59,65,66]. GRP78 protein is upregulated in ER stress condition to degrade misfolded proteins and enhance protein folding. CHOP is highly expressed during ER stress which mainly promotes cell apoptosis [61]. Numerous studies demonstrate that CHOP activates pro-apoptotic Bcl-2 proteins but reduces anti-apoptotic Bcl-2 proteins, and also stimulates ROS formation, which in turn leads to amplifying the apoptotic pathway [67,68]. Furthermore, this notion is proven by demonstrating that depletion of CHOP inhibits β-cell death and improves β-cell function, as well as glucose tolerance in multiple mouse models of diabetes [69]. In our experiment, we observed that levels of CHOP and GRP78 proteins were induced in the pancreas of diabetic rats, indicating ER stress is present in pancreatic β cells of this experimental setting. Previously [63], diabetic rats exposed with STZ showed markedly induced protein expressions of ER stress markers in the pancreas, and their expressions were involved in downregulation of Bcl-2 protein and upregulation of caspase-3 protein, which further may trigger apoptosis of β-cells. However, our findings showed that administration of hesperidin results in an obvious decrease in pancreatic CHOP and GRP78 protein expressions in diabetic rats. These results provide that hesperidin might antagonize β-cell apoptosis and improve β-cell function in diabetic rats by diminishing ER stress pathway.

4.1. Research limitation

It is important to note that our study determined the protein expressions of pro- and anti-apoptotic Bcl-2 family and the effector caspases, and thus was limited to only the regulator of cell apoptosis. DNA fragmentation, a major hallmark of apoptosis, was not detected in this study. Although, this work demonstrated that hesperidin treatment upregulated Bcl-xL, and downregulated Bax, cleaved caspase-3, as well as cleaved PARP proteins in the pancreas of diabetic rat; apoptosis-specific parameters could be investigated to confirm the anti-apoptotic role of hesperidin in future studies. However, from the results obtained, we can say that by regulating Bcl-2 family proteins and the caspase cascade, hesperidin may have a preventive effect on diabetic rat pancreas.

5. Conclusion

Our results provide evidence that hesperidin has a potential protective function against STZ-induced pancreatic β-cell apoptosis and dysfunction in diabetic rats. These effects are associated with properties relating to the activation of antioxidant enzymes, and modulation of Bcl-2 family proteins, along with the inhibition of caspases activation, oxidative stress, and ER stress, as well as inflammatory responses. The actual molecular mechanisms underlying antidiabetic effect of hesperidin and its anti-apoptotic activity from other diabetic stimuli need to be clarified in further studies. Before the clinical application, more comprehensive studies are worthwhile to validate the antidiabetic action of hesperidin.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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