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Stevia aquatic extract protects the pancreas from streptozocin (STZ) induced damage: A stereological study

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In recent years, among antidiabetic medicinal herbs, *Stevia* received a lot of attention due to its diverse therapeutic applications. Despite extensive reports on the effects of *Stevia* on the pancreas, its molecular mechanism is not clear yet. In this study, we investigated the protective and preventive effects of oral extracts of *Stevia* on the pancreas through the stereological methods in streptozocin (STZ) induced diabetic rats. Thus, 66 adult male rats were assigned to six groups (n = 11) *viz*., healthy control, healthy *Stevia* (400 mg /kg), diabetic-control, diabetic-metformin (500 mg/kg), diabetic-*Stevia* and pre-*Stevia*-diabetic group. Treatment with *Stevia* significantly reduced fasting blood sugar (FBS) and MDA compared to the diabetic control group (P <0.05). The results indicated that the weight and the volume of the pancreas increased significantly in all our treated groups compared to the diabetic one (P <0.05). The volume density of the pancreatic islands and the number of beta cells increased in healthy and diabetic groups treated with *Stevia* (P <0.05). However, the pre-treated diabetic rats with *Stevia* did not show significant preventive effects on the volume and number of beta cells as well as the volume of islets against destructive effects of STZ. More specifically, our results confirmed the protective effects of *Stevia* through restoring pancreatic cells and repairing the stereological damage induced by STZ.

Keywords: Blood glucose, Candyleaf, Diabetes mellitus, Oxidative stress

Diabetes mellitus is caused by a not only deficiency in insulin secretion but also decreased responsiveness of body organs to insulin, referred to as insulin resistance[1]. According to the latest WHO estimate, there are 536.6 million people with diabetes aged 20-79 years worldwide, which is likely to increase to 783.2 million by 2040[2].

Nowadays, there is increasing interest in plant-based medicine and modulating the physiological effects of functional foods on the prevention and cure of diabetes[3,4]. *Stevia rebaudiana* Bertoni (Fam. Asteraceae), commonly called the candyleaf, mainly grows in north-eastern Paraguay as well as parts of Brazil and Argentina. This bushy shrub is now cultivated in parts of Canada, Europe and Asia. In Iran, the plant is cultivated in the Northern region of the country. i.e., Rasht[5,6].

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The therapeutic and preventive use of *Stevia* is not limited to hypertension, diabetes and obesity[7]. *Stevia* and its derivatives may have therapeutic use in the treatment of inflammation, oxidative stress, dental caries, microbial infections, and some types of tumors[8] No associations have been found between *Stevia* and mutagenic, teratogeny, carcinogenic or allergy[9]. In diabetic rats, *Stevia* has been shown to have anti-inflammatory and antihyperglycemic effects, as well as regulating blood glucose[10,11].

We have already established that it is through the pancreatic tissue that *Stevia* elevates the insulin level. Similarly, it is through the PPARγ-dependent mechanism that it shows antihyperglycaemic and antioxidant effects[10]. Also, *Stevia* could diminish the reproductive system problems and improve infertility in diabetic male rats[11]. Our recent literature also suggests that an aquatic extract of *Stevia* improves diabetes metabolic disorders in rat muscles and kidneys through antioxidant properties and it increases glucose transporters and aquaporin-2 in the mentioned tissues[12].

Since preventive or/and protective effects of *Stevia* on the pancreatic tissue are unknown yet, in this study, we employed an unbiased stereological method to evaluate the antidiabetic effects of aquatic extracts of *Stevia* with focus on the pancreatic beta cells in diabetic rats induced by STZ-NA.

Materials and Methods

Stevia aqueous extract preparation

Stevia leaves (Eupatorieae, Asteraceae) harvested in September were from a local herbarium (HMS-536, Traditional Medicine and Materia Medica Research Center, Iran) with complete cultivation analysis of plant[10,13]. After being washed, the leaves were dried at <50°C, which was then powdered. Soxhlet instrument was used to extract the material. A vacuum rotator was used to evaporate the material, which was then air-dried. Thereafter, *Stevia* leaves (100 g) were immersed in distilled water (1200 mL) and kept in a cabinet for 24 h. The filtration process was carried out at 40-50°C. A vacuum desiccator was used to remove the humidity of the material. Finally, we prepared 35 g of extract from 100 g of Stevia leaves and used HPLC to determine the concentration of stevioside, the most abundant and effective diterpene glycoside in Stevia leaves[10].

Animals and tissue preparation

The study was carried out on 60 matured normoglycemic male Sprague–Dawley rats, weighing 200-250 g with free access to water and a standard rodent diet. Animals were housed with a fixed 12 h artificial light period and temperature (23±2°C) conditions and the air was adequately recycled. This study was ratified and approved by the Ethics Committee of Shiraz University of Medical Sciences (Approval number: 95-01-01-12484\03-07-2016).

A total of 66 rats in six groups, each with 11 members, were housed in five cages and had access to a row chow diet obtained from Parsdam Inc., Tehran, Iran, and water. The rats were injected with nicotinic amide (110 mg/kg) and STZ (60 mg/kg) intraperitoneally (IP) with an interval of 15 min[12]. To determine fasting blood sugar (FBS), the blood sample was taken from the experimental rats seven days later. Diabetic symptoms, namely hyperplasia, polyuria, polydipsia and weight loss were observed among the rats with FBS level of >300 mg/dL.

Rats were seperated into following 6 groups; Group A; healthy group (without treatment); Group B: healthy group treated with 400 mg/kg of *Stevia;* Group C: diabetic control group (without treatment); Group D: diabetic-metformin group, diabetic rats treated with 500 mg/kg of metformin; Group E: diabetic-*Stevia* group, diabetic rats treated with 400 mg/kg *Stevia*; and Group F: pre-*Stevia*-diabetic group: a healthy group treated with 400 mg/kg of *Stevia* at first for 30 days and then inducing diabetes for 7 days to evaluate preventive effect of *Stevia*. All doses were administered by oral gavage once a day at 8:00 am. Sample blood was taken from the hearts of the slaughtered rats upon the completion of the treatment period to be centrifuged at 3000 rpm for 10 m. The samples were immediately isolated and kept at −80°C for analysis.

Fasting serum glucoseand malondialdehyde (MDA) measurement

Diagnostic colorimetric kits (BioSystem, Spain) were used to measure fasting serum Glucose by an enzymatic colorimetric assay with a DIRUI (CS-T240, China) auto-clinical chemistry-analyzer30 days of the completion of the treatment. Hagar *et al.*[10] method was used to measure the MDA level. The acetal form (TEP or 154 1,1’,3,3’-tetraethoxy propane) was hydrolyzed to form MDA at 95 °C. The TEP standard provided a 10mM stock solution in Tris-HCl and was diluted 1/500 (v/v) in water. A standard curve at 532 nm was used to detect MDA concentrations.

Preparation of tissues for stereological analysis

One month after the treatment, the pancreases were quickly removed, trimmed of adipose tissue, and weighed by sensitive scales. The primary volume, V (primary), of the pancreas, was measured using the Sherle’s method[14] (Fig. 1A). Sherle’s method was used to measure the primary volume. Then, the sections were prepared by the orientation method according to Noorafshan and colleague research[15] (Fig. 1B). The 5- and 20 μm sections (Fig. 1 C & D) of the circular pieces of the pancreas were embedded in a paraffin block. Gomori's aldehyde fuchsin was used to stain tissue sections to assess the shrinkage size of the pancreas with the following formula:

$$\text{Degree of shrinkage (Dsh)} = 1 - \left(\frac{\text{Area after}}{\text{Area before}}\right)^{1.5}$$

The point-counting method (Fig. 1) was used to assess the volume density of the Langerhans islets with Delesse’s formula[16]:

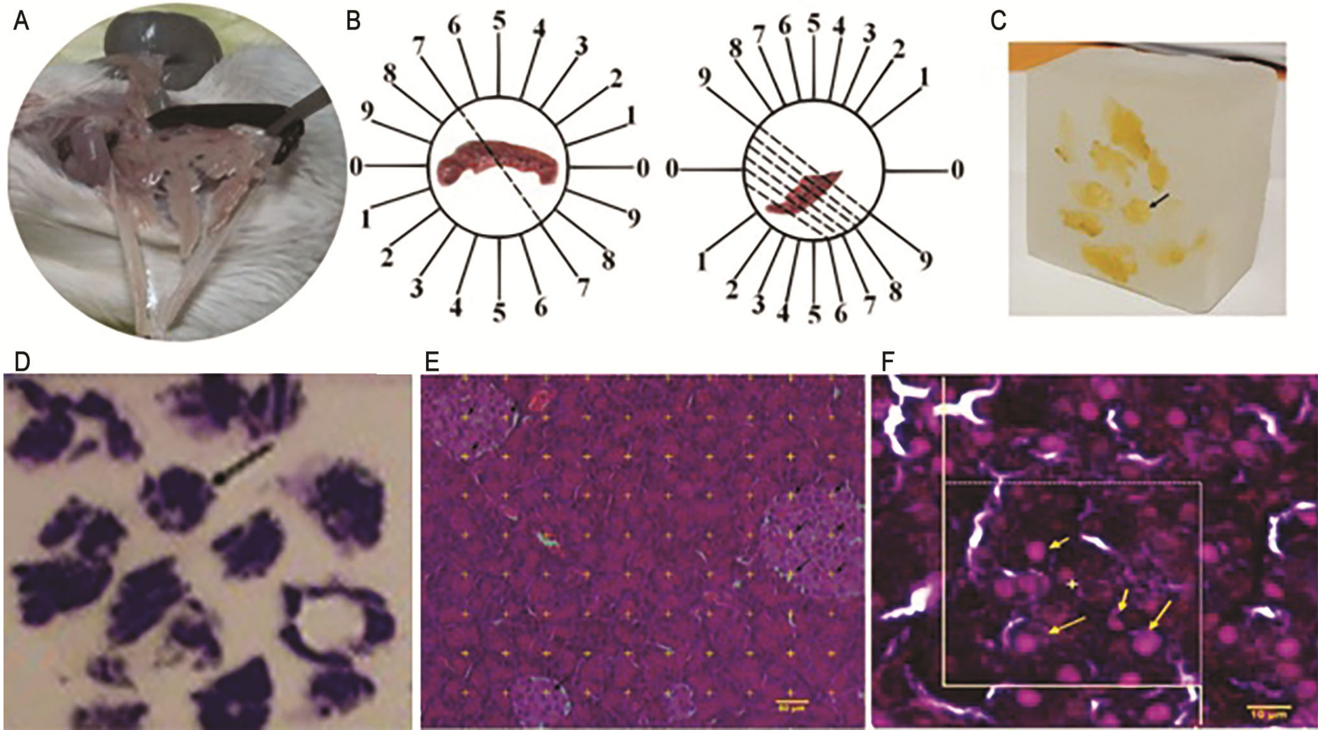

Fig. 1 — Preparation of rat pancreatic tissue for stereological analysis. (A) Isolation of the rat's pancreatic tissue (arrow); (B) Sherle's method; (C and D) Orientator method was utilized to obtain isotropic uniform random sections of the pancreas. (E) Point counting method (The accepted points which hits the right upper corner of each cross of the targeted islets were counted). (F) Optical disector method (In this method, the cells were counted which were placed inside or on the accepted line (Dotted lines) and not rejected lines (lower and left borders.

$$Vv(Langerhans\ islets) = \sum_{i=1}^{n} p\ (islets) / \sum_{i=1}^{n} (pancreas)$$

where "$\sum_{i=1}^{n} p\ (Islet)$" was the number of the test points falling on the Langerhans isles and "$\sum_{i=1}^{n} p$ (pancreas)" was the total points hitting the pancreas sections. The absolute volume of Langerhans islets was assessed with the following formula[17]:

$$V_{absolute}(Langerhans\ islets) = V(pancreas) \times Vv(Langerhans\ islets)$$

An optical dissector method was used to determine the number of β-cells on the thickness of 20 μm. With the following formula:

$$Nv = \frac{\sum_{i=1}^{n} Q}{\sum_{i=1}^{n} P \times h \times (\frac{a}{f})} \times \frac{t}{BA}$$

where "$\sum_{i=1}^{n} Q$" was the number of the follicles counted in all the disectors, "h" was the height of the optical Disector, "a/f" was the area of the counting frame, "$\sum_{i=1}^{n} P$" was the total number of the counted frames, "BA" or block advance was the setting of the microtome to cut the paraffin block, and "t" was the mean of the final section thickness[18]. To evaluate the total number of the β-cells, the following formula was applied[17]:

$N_{(Cells)} = N_{V(Cells/Islets)} \times V_{(Islets)} \times D_{(The\ degree\ of\ shrinkage)}$

Nucleator method was used to estimate the volume of beta cells using the following formula[19]:

$$V = \frac{4}{3}\pi \bar{L}_n^3$$

where $\bar{L}_n^3$ was :$\bar{L}_n^3 = \frac{L_1^3 + L_2^3 + L_3^3 + L_4^3}{4}$

Statistical analysis

Our findings were analyzed by SPSS (Version 23.0; SPSS Inc., Chicago, USA). Stereological parameters were compared by one-way ANOVA, and Tukey's test was used as a post-hoc test. Differences were considered significant when P-values were less than 0.05.

Result

Fasting serum glucose level

Intraperitoneal injection of STZ (60 mg/kg) increased serum glucose levels in diabetic rats

significantly (P <0.05). Likewise, the hypoglycemic effect of orally administered *Stevia* (400 mg/kg) on diabetic and healthy rats was significant. Unexpected significant changes in blood glucose levels were observed after the administration of *Stevia* extract in healthy rats followed by injection of the STZ, which seems to have a protective effect. Furthermore, FBS was controlled by metformin (500 mg/kg) better than by *Stevia* (Table 1).

Bodyweight

The weight of diabetic rats induced by STZ in all groups decreased irrespective of the treatment regime.

Table 1 — Evaluation of body wt. (g), serum FBS (mg/dL) and pancreatic malondialdehyde (nmol/mL) in different experimental groups

Groups	Body weight (g)	Serum FBS (mg/dL)	MDA (nmol/mL)
Healthy control	285.7±11.97[a]	77.5±3.80[a]	0.386±0.013[a]
Healthy *Stevia*	271.8±1.90[ac]	74.8±3.03[a]	0.381±0.018[a]
Diabetic control	208.5±8.30[b]	316±40.30[b]	1.065±0.057[b]
Diabetic-metformin	282.67±+5.8[ac]	89.1±10.50[a]	0.930±0.048 [b]
Diabetic-*Stevia*	248.5±24.22[ac]	233.3±61.4[c]	0.471±0.041[ac]
Pre-*Stevia*- diabetic	244±21.00[bc]	256.6±13.2[c]	0.616±0.086[c]

[[a,b,c]There was no significant difference between groups, which have at least one similar letter. Dissimilar letters indicate a significant difference between groups(P <0.05)]

As depicted in Table 1, there are no significant differences in body weight of our treated groups (*Stevia* or Metformin) and healthy one.

Serum lipid peroxidation

A higher increase in MDA was observed among diabetic rats than the normoglycemic state (Table 1). Yet, treatment with *Stevia* (400 mg/kg) decreased MDA in both healthy and diabetic rats compared to diabetic control. Metformin (500 mg/kg) was not as effective as *Stevia* in this regard. The protective effect of the peroxidation of lipid on rats pre-treated with *Stevia* was the same among the diabetic ones treated with *Stevia*, and so was the damaging effect of oxidative stress-induced with STZ. On the other hand, MDA in rats pre-treated with *Stevia* significantly is higher than the healthy group treated by *Stevia* (P <0.05).

Photomicrograph of the rat's pancreas histology

Qualitative histological evaluation demonstrated in Fig. 2. The number of islets significantly decreased, also beta cells were vacuolated and degenerated with severe atrophy in the diabetic group (C) induced by STZ. Improvement and regeneration of beta cells were determined in *Stevia* and Metformin groups

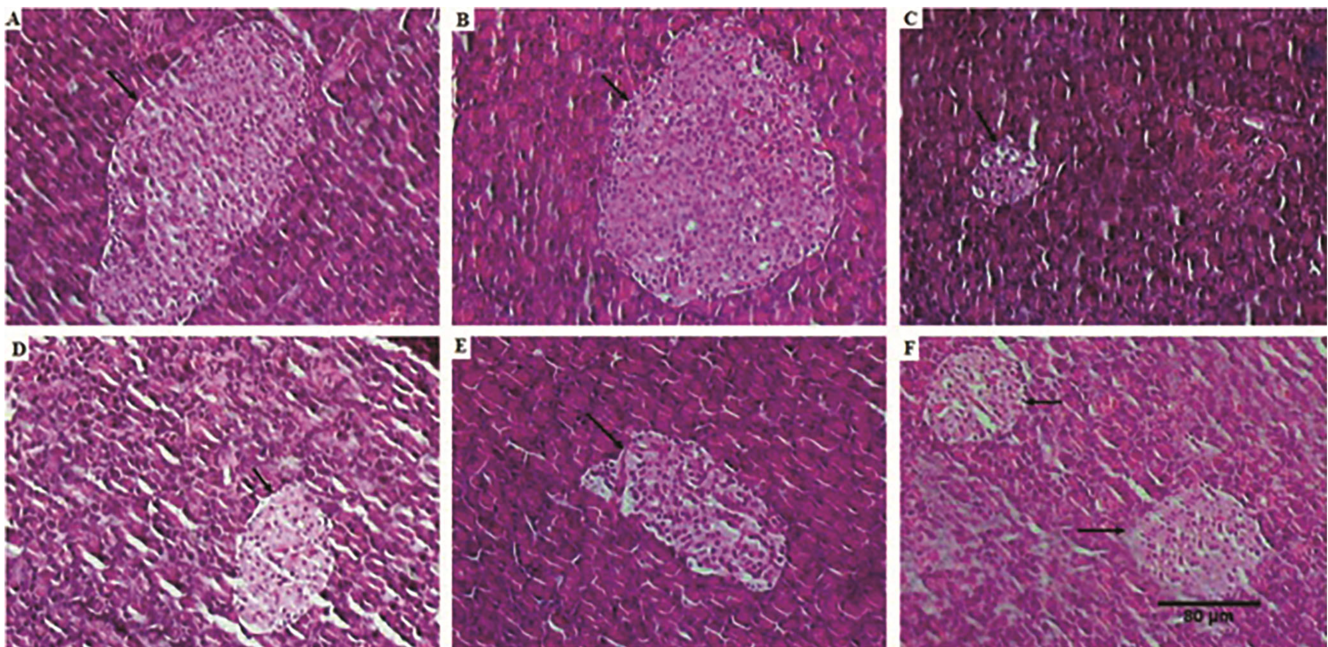

Fig. 2 — Photomicrograph of the rat's pancreas histology in different groups. (A) The healthy group: The pancreas showed a normal appearance; (B) The healthy group (treated with 400 mg/kg of *Stevia*) without any qualified changes in the pancreas compared to a healthy one; (C) The diabetic control group with severe distinct atrophy, also massive volume reduction in Langerhans islets; (D & E) diabetic groups treated with 500 mg/kg of metformin or *stevia* 400 mg/kg which protected islets from damaging side effects of STZ; and (F) pre-*stevia*-diabetic group: a healthy group (treated with 400 mg/kg of *Stevia*) at first for 30 days and then inducing diabetes for 7 days which did not show significant changes in stereological parameters except the volume of the pancreas.

(D, E). Based on our investigation the size and number of islets in rats pre-treated with *Stevia* (F)were improved compared with diabetic ones.

Effect of aquatic extract of *Stevia* on stereological parameters

Pancreas volume

Based on stereological evaluation the volume of the pancreas significantly decreased in the diabetic rats compared to other groups (Fig. 3). It is worth mentioning that no significant differences were observed between our treated groups and healthy rats.

Volume of pancreatic islets

Our results showed that the volume of pancreatic islets significantly decreased in diabetic rats except for the healthy and diabetic rats treated with *Stevia* (P <0.05). Interestingly, a non-significant increase was observed in pancreatic islets volume in rats treated with metformin, further suggesting that *Stevia* had protective effects (Fig. 3).

Count of β cells

The number of β cells significantly decreased in diabetic rats compared to other groups. An unexpected significant improvement in the number of β-cells in healthy and diabetic rats treated with *Stevia* (P <0.05). As shown in Fig. 3, a non-significant increase was observed in the count of β cells in the diabetic group pre-treated with *Stevia* and the one treated with metformin.

β-cells volume

The stereological results demonstrated that the volume of β cells significantly elevated in diabetic control rats compared with normoglycemic ones, healthy rats treated with *Stevia* (P <0.001), and diabetic rats treated with *Stevia* (P <0.05). But in the diabetic group pre-treated with *Stevia*, and the group treated with the metformin, a non-significant increase was observed in the volume of β cells (Fig. 3).

Discussion

With the increasing incidence of diabetes in recent years, especially the involvement of children, extensive research work on herbal medicines with at least side effects has received much attention[20]. The candyleaf, *Stevia rebaudiana* Bertoni, as a natural sweet herb with zero calories, is used in the treatment of several diseases including hypertension, diabetes, obesity, inflammation, oxidative stress, dental caries, microbial infections, and cancer[21]. In the present study, an aquatic extract of *Stevia* could regulate blood glucose and decrease serum MDA as an indicator factor in oxidative stress in diabetic conditions. We focused on the pancreas as the main organ involved in managing blood glucose to evaluate the protective also the preventive effect of *Stevia* by stereological analysis. Our data revealed that *Stevia* improves the destructive effects of STZ on the pancreas by increasing the volume of tissue and islets also the number and volume of β-cells. According to the results, the protective effect of *Stevia* is much more than preventive effects in diabetic pancreas induced by STZ-NA. Our results demonstrated a significant increase in blood glucose levels in rats was induced by the STZ-NA injection. STZ damages the β-cell by sudden depletion of ATP andnicotinamide adenine dinucleotide (NAD^+)following high activity of poly (ADP-ribose) polymerase (PARP-1) enzyme. NA injection elevated β -cell protection for STZ effects via PARP-1 inhibition and it also needed to NAD^+ formation, which altogether, led to a model of type II diabetes in pre-diabetic rats[22].

Based on the previous findings, *Stevia* derivatives (stevioside) reduce blood sugar levels[23]. Similarly, *Stevia* derivatives (stevioside and steviol) have anti-hyperglycemic effects via changes in cAMP levels as they affect plasma membrane K^+ATP-Sensitive

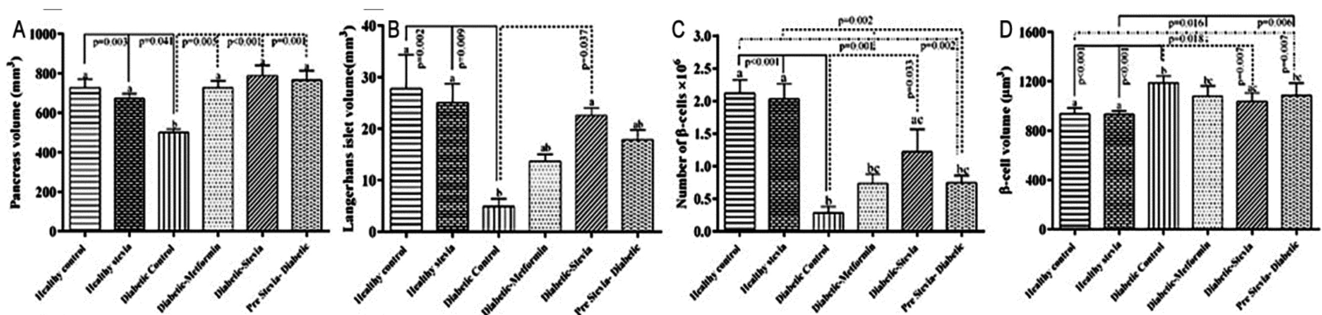

Fig. 3 — Estimation of stereological parameters in experimental groups. (A) The volume of pancrease, (B) The vulome of langerhanse islets, (C) The number of β-cells , and (D) The volume of β-cells. There was no significant difference between columns, which have at least one similar letter[a,b,c]. However, dissimilar letters indicate a significant difference (P <0.05).

channel natural process, which in turn, stimulates insulin secretion[24].

Some researchers have reported that *Stevia* (and its derivatives) can decrease the blood glucose levels by stimulating insulin secretion[25], reducing protein and gene expression levels of phosphoenolpyruvate carboxykinase (PEPCK)[26] and decreasing insulin resistance[27], decreasing the activity of gluconeogenic enzymes (glucose-6-phosphatase and fructose1,6-bis-phosphatase) and glycogenesis or increasing glycolysis enzyme (hexokinase and glucose-6-phosphate dehydrogenase) in STZ-NA induced diabetic rats[28].

Furthermore, our previous published data has shown that the aquatic extract of *Stevia* not only reduces liver enzymes ALT, AST, MDA levels and regulates catalase activity but also significantly increases insulin and PPARγ expression in diabetic rat's pancreas[10]. Thus, the *Stevia* extract can adjust the amount of glucose in diabetic rats (induced by STZ or STZ-NA) via various signaling pathways in any organs. Also, our results showed that an increase in the number of β-cells can be one of the mechanisms involved in the hypoglycemic effects of *Stevia*.

In agreement with our results, Pari & Satheesh reported that the plasma level of glucose was decreased in rats induced with STZ-NA treated with metformin[29]. In addition,Bayat *et al.* Mokaram *et al*. showed that FBS significantly decline in STZ-NA induced diabetic rats treated by metformin and *Stevia* for 30 days[12].

It is accepted that hyperglycemia causes high reactive oxygen species (ROS) production and causes an imbalance of oxidative stress/antioxidative properties, which in turn, leads to high malondialdehydelevels in serum[30]. According to our results, the quantity of serum MDA increased in the diabetic group (2.7-fold increase) compared with healthy rats and healthy rats treated with *Stevia* (Table 1). Malondialdehyde increased in diabetic rats while, *Stevia* administration slowed down the increase in MDA levels due to its proven anti-oxidant properties and high amount of phenol as well as flavonoid in aquatic extract of *Stevia*[31]. Furthermore, diabetic rats pre-treated with *Stevia* showed a significant decrease in serum MDA level compared with diabetic ones and less strong decease in healthy rats, suggesting that the extract of *Stevia* can have a protective effect, as it prevents deteriorating changes that oxidative stress-induced in diabetic rats due to injection of STZ. Longer consumption of *Stevia* extract, say up to 7 months, can effectively protect rats from the induction of diabetes by raising the body's antioxidant potential properties in serum also in the whole body.

It is generally accepted that metformin decreases the FBS level in non-insulin dependent Diabetes mellitusby increasing insulin sensitivity[32]. Metformin raises the potential of receptors for binding, insulin receptors phosphorylation, and tyrosine kinase activity[33]. Metformin exerts an anti-hyperglycemic effect through several mechanisms including hepatic glucose production suppression, increased insulin-mediated glucose consumption and a decrease in fatty acid oxidation[34]. We found that metformin significantly improved FBS and body weight in diabetic groups. However, we found no significant differences in pancreatic MDA levels in diabetic rats and metformin-treated ones. The results are consistent with the reports that metformin produced no significant effects on the antioxidant enzyme (Catalase, SOD) and TBARS (lipid peroxidation level), so it did not offer protection of pancreas against oxidative stress[35]. According to our results, metformin showed better anti-hyperglycemic effects compared with *Stevia*, while *Stevia* improved MDA level much better than metformin possibly because of its antioxidant, anti-inflammatory properties.

Earlier studies have demonstrated that due to its antioxidant, anti-hyperglycemic effects, *Stevia* decreases the complications of diabetes on such tissues as the pancreas[36], testis[37], liver[38], heart[39], and kidney[40], although its exact mechanism is not clear. To our best knowledge, there is little information about the effects of *Stevia* extract on the stereological parameters. Quantitative microscopic or stereological studies provide critical information about the effects of various drugs on different tissues and also preserve the microenvironment feature of the cells, particularly in the special tissues[40,41].

The above stereological investigation on the basic quantitative characteristics of the pancreas in rats and their changes in different groups revealed that pancreatic islet volume and pancreatic weight and volume were shrunk in the diabetic group. Thet STZ effects on pancreatic islets could be because of selective degradation of pancreatic islets β-cells (the maximum ingredient in the pancreatic islet).

Microvascular diseases are one of the complications of diabetes that affect islets bloodstream and subsequently, in pancreatic islets, cause the atrophic alteration[14,19].

Beta-cell growth a process whereby preexisting β cells are replicated from precursor cells and beta-cell death needs are in constant balance in healthy human beings and animals. Beta-cell hypertrophy is the result of the imbalance between the said processes normally induced by diabetes[42]. Here, we observed that *Stevia* extracts could improve atrophic islets and β-cells volume in diabetic rats resulting in hypertrophic changes in β-cells. The changes, though observed in all groups, were the most profound in the diabetic group.

In agreement with our data, renal stereological study showed that a high dose of the bitter fraction of *Stevia* can improve kidney structural changes (hypertrophy both volume and length of tubules) possibly due to its antioxidant and anti-hyperglycemic effects in diabetic mice[40]. Also, *Allium saralicum* can improve blood sugar levels and renal changes in STZ-induced diabetic mice[43]. On the other hand, Mahmoudzadeh *et al.*[44], have demonstrated that diabetic rats were treated with an aquatic extract of *Eucalyptus globulus* in a dose-dependent manner that could compensate for the diabetic damages and improved pancreatic mass, islets mass, beta-cell mass, and volume density of beta-cells/islets. Another study revealed that the administration of oral *Arnebia euchroma* extract, although it cannot protect and heal the pancreatic changes due to diabetes-induced by STZ, it improves volumes of pancreatic islets, and beta-cells population beside its antihyperglycemic effects[14]. Heidari Z *et al.*[45], showed that short-term pre-treated sodium tungstate can protect beta-cells in STZ-induced diabetic rats. In line with our results, in a stereological study in diabetic rats, Gholizadeh *et al.*[11] revealed that aquatic extract of *Stevia* improves productivity and reduces damages to the testis by elevating the weight and volume of the testis, total volumes of germinal epithelium, and sperm count and motility.

Results of this study suggest that metformin is not as effective as *Stevia* in significantly improving the number of β-cells and the volume of islets though it has a limited effect on peripheral tissues, which helped to control the level of blood glucose. We also found that rats pre-treated with *Stevia* did not benefit from the protective effects of changes in islets and β-cells volume and the number of β-cells. Similarly, *Stevia* could not reverse the destructive effects of STZ on pancreases nor could it protect beta cells from the damaging effects of diabetes. Yet, Salehi *et al.*[46] have demonstrated that treatment with quercetin (25 mg/kg/day 3 days before injected 75 mg/kg STZ) and administration of quercetin for one month significantly protected pancreatic β-cells integrity. This discrepancy might be due to the difference between the treatment duration in our study and theirs. Hence, it becomes necessary further to investigate whether prolonged treatment with *Stevia* extract would yield different results. Nonetheless, evidence has been provided for the effect of oral consumption of *Otostegia persica* extract on a volume of pancreas, islets, and β-cells in diabetic rats[47]. Likewise, Mahmoudzadeh *et al.*[48] reported that *Tamarindus indica* Linn. partially restored pancreatic beta-cells and repaired damages induced by STZ in rats while Moezi *et al.*[49] have shown that extract of *Amygdalus lycioides* (1000 mg/kg) increases potentially in the numerical density of beta cells and help to regenerate the pancreas.

Conclusion

In this stereological study, we evaluated the protective and prevention effects of *Stevia* oral extract on the pancreas of diabetic rats.30-day oral consumption of aquatic extract of *Stevia* in STZ-NA induced diabetic rats can control the blood sugar level in hyperglycemic conditions and attenuate oxidative stress by reduction of MDA due to its antioxidant capacity. On the other hand, based on our stereological evaluation *Stevia* improves the destructive effects of STZ on the pancreas by increasing the volume of tissue and islets also the number and volume of β-cells. It can be concluded that it helps the pancreatic islets and remaining beta cells to overcome and prevent some pathologic changes such as hypertrophy, degranulation, and loss of capacity induced by STZ in the pancreas. Therefore, *Stevia* can be a good therapeuticcandidate even recommended as a drug supplement as an antidiabetic drug to cure diabetic complications.

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Conflict of interest

Authors declare no competing interests.

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