Toosendan Fructus ameliorates the pancreatic damage through the anti-inflammatory activity in non-obese diabetic mice

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ABSTRACT

Objectives : The present study was conducted to examine whether Toosendan Fructus has an ameliorative effect on diabetes-induced alterations such as oxidative stress and inflammation in the pancreas of non-obese diabetic (NOD) mice, a model of human type I diabetes.

Methods : Extracts of Toosendan Fructus (ETF) were administered to NOD mice at three doses (50 mg/kg, 100 mg/kg and 200 mg/kg). Mice at 18 weeks of age were measured glucose tolerance using intraperitoneal glucose tolerance test. After 28 weeks of ETF treatment, glucose, total cholesterol (TC), triglyceride (TG), and proinflammatory cytokines in serum, western blot analyses and a histopathological examination in pancreas tissue, and on the onset of diabetes were investigated.

Results : The results showed that levels of glucose, glucose tolerance, TC, TG , interferon-γ, interleukin (IL)-1 β, IL-6, and IL-12 in serum were down-regulated, while IL-4, IL-10, SOD, and catalase significantly increased. In addition, ETF improved protein expression of proinflammatory mediaters (such as cyclooxygenase-2, and inducible nitric oxide synthase) and a proapoptotic protein (caspase-3) in the pancreatic tissue. Also, in the groups treated with ETF (100 mg/kg or 200 mg/kg), insulitis and infiltration of granulocytes were alleviated.

Conclusions : Based on these results, the anti-diabetic effect of ETF may be due to its anti-inflammatory and antioxidant effect. Our findings support the therapeutic evidence for Toosendan Fructus ameliorating the development of diabetic pancreatic damage via regulating inflammation and apoptosis. Our future studies will be focused on the search for active compounds in these extracts.

Key words : Toosendanin fructose, Diabetes, inflammatory cytokine, pancreatic beta cell

Introduction

Non-obese diabetic (NOD) mice, which develop type 1 diabetes (T1D) spontaneously, is very similar to the human disorder, insulin-dependent diabetes mellitus (IDDM). Accordingly, diabetes in NOD mice is the most extensively studied model of autoimmune disease[1,2]. T1D is caused by the destruction of the insulin-producing β-cells through insulitis, which involves the infiltration of leukocytes into pancreatic islets[3,4]. In addition, the development of insulitis lead to the excess accumulation of inflammatory cells, a gradual decrease in β-cells mass, and the development of chronic hyperglycemia[5]. Recently, various research on herbal medicine has been studied as an alternative medicine for prevention and treating diabetes[6].

Toosendan Fructus is a fruit of *Melia toosendan* Sieb. et Zucc. Many compounds are contained in TF including β-sitosterol, toosendanin, kulinone ($C_{30}H_{48}O_2$), methylkulonate ($C_{31}H_{48}O_4$), melianol ($C_{30}H_{48}O_4$), melianodiol, melialactone, andazadiarachtin ($C_{35}H_{44}O_{16}$). Toosendanin in particular

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is a widely known major component. Toosendanin has displayed apoptosis inducing activity[8] and suppressed proliferation of cancer cells[9], anti−botulinum acivity[10], anti−microbial activity[11], inhibitory effect of releasing an acetylcholine[12], activation of drug metabolic enzymes and bile juice release in the liver[13], and anti−hyperlipidemia activity[14]. Recently two limonoids demonstrating anti− inflammation and analgesic properties were reported[15]. In addition, Toosendan Fructus (TF), a typical tranditional medicine, also reported antioxidative and anti−diabetic effect on blood glucose, lipid metabolism, and carbohydrate metabolism−related enzyme activities in the liver of streptozotocin−induced diabetic rats[7]. However, TF has not reported anti−diabetic activity through the anti− inflammatory activity in the pancreatic tissue of NOD mice.

Materials and Methods

1. Plant material

Toosendan Fructus was purchased from Omniherb Co. (Youngcheon, South Korea). Dry plant material was extracted with distilled water. TF (500 g) was powdered with a grinder, sieved through a mesh (pore size 0.5 μm) and boiled gently in 4 times their volume of distilled water for 3 h. The extract of TF (ETF) was then filtered, evaporated on a rotary evaporator (Buchi, Flawil, Switzerland), and lyophilized in a freeze dryer (Eyela FDU−540; Tokyo, Japan). The powdered extract was stored at −20 ℃. The percentage yield was about 16% (w/w). The ETF powder was freshly dissolved in distilled water before use.

2. Animals

A total of 60 mice (Orient, sungnam, Korea) used in the study, each weighing about 18g, were housed (one mouse per cage) under pathogen−free conditions in a temperature−controlled environment with a 12 h light/ dark cycle. The commercial diet were supplied by Diet Research (Bethlehem, PA, USA). Mice were divided into 5 groups by stratified random sampling to equalize the mean body weight of the groups. The groups ($n = 12$ in each group) were designated as follows: group A, normal (non−obese non diabetic) mice treated with distilled water (DW); group B, NOD mice treated with DW; group C, NOD mice treated with ETF at 50 mg/kg body weight/day; group D, NOD mice treated with ETF at 100 mg/kg body weight/day; group E, NOD

mice treated with ETF at 200 mg/kg body weight/day for 28 consecutive weeks. The approval and guide line for animal study was DHUARB2013−010.

3. Intraperitoneal glucose tolerance test (IPGTT)

Mice at 19 weeks of age (n = 8) were fasted for 12 h and then injected intraperitoneally with a single bolus of glucose (2 g/kg body weight)[16]. Glucose levels in blood collected from the tail vein were measured 0, 20, 40, 60, 90 and 120 min after glucose administration. Blood was immediately centrifuged (1,800 x g), the serum was separated and stored at −20 ℃ until assayed.

4. Determination of blood glucose, Total cholesterol (TC), and Triglyceride (TG)

For analysis of blood glucose, mice were bled from the orbital sinus following a 4−h fast approximately every 4 weeks from 5 weeks of age. Whole blood was immediately centrifuged to obtain serum. The concentration of glucose in the serum was measured using an enzymatic colorimetric assay (Glucose LiquiColor, Stanbio Laboratory, Boerne, TX, USA) followed by spectrophotometric analysis. Animals with fasting glucose levels greater than 300 mg/dl serum were diagnosed as diabetic. The study was terminated when the mice reached 33 weeks of age. For analysis of TC and TG, serum was collected once at 18 weeks from the tail vein, and analyzed with a commercial ELISA kit (Sigma, MO, USA).

5. Determination of body weight gain (g), and water and food consumption

Weight of each mouse was examined every week from 5 weeks to 33 weeks of age. Water consumption (ml/day [d]) and food intake (g/d) were determined every week. Water consumption was calculated by measuring total consumption (ml) during 1 week in a cage.

6. Assays for serum Cytokines

At 32 weeks of age, overnight−fasted mice were anesthetized to collecte blood samples. Serum insulin concentration was measured by ELISA (Mercodia, Inc., Winston−Salem, NC, USA). Cytokines in the serum were determined using a mouse cytokine array kit (Quansys Biosciences, West Logan, UT, USA), which included IL−1β, IL−4, IL−6, IL−10, IL−12.

7. Protein preparation and Western blot analyses

Pancreatic tissue in all mice was homogenized with ice-cold lysis buffer (pH 7.5) containing 137 mM NaCl, 20 mM Tris-HCl, 1% (v/v) Tween 20, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture were added. After centrifugation (10,500 x g at 4 ℃), protein concentration in each sample was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). SOD, catalase, cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS), caspase −3, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat anti-rabbit and goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Nitrocellulose membrane were purchased from Bio-Rad Laboratories (Tokyo, Japan). ECL Western Blotting Detection Reagents were purchased from GE Healthcare (Piscataway, NJ, USA). For the determination of SOD, catalase, COX2, iNOS and β-actin, protein sample 10 μg was electrophoresed through 8 − 12% sodium dodecylsulfate polyacrylamide gels (SDS-PAGE). Separated proteins were electrophoretically transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk for 1 h, and then incubated with primary antibodies to SOD, catalase, COX2, iNOS and β-actin overnight at 4 ℃. The blots were washed and incubated with secondary antibodies for 2 h at room temperature. Antigen-antibody complexes were visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (Fujifilm, Tokyo, Japan). Band densities were determined by Scion Image software (Scion Corporation, Frederick, MD, USA) and quantified as the ratio to β-actin. The protein levels of groups are expressed relative to those of normal mice.

8. Histopathological procedures and insulitis evaluation

Mice pancreases were fixed in 10% neutral buffered formalin, and then embedded in paraffin. Tissue sections (500 μm apart from each other) were deparaffinized, hydrolyzed and stained with hematoxylin and eosin. Islets in each section were assessed as described previously[17,18]. For histological scoring of insulitis, the sections were examined microscopically[19]. Insulitis of individual islets was blindly rated according to the scale of 0-4 as follows : 0, noinsulitis; 1, peri-insulitis; 2, moderate insulitis with less than one-half of the islet infiltrated; 3, more than one-half of the islet infiltrated; and 4, complete islet destruction. A mean of 10-20 islets per pancreatic sample was scored. Histological scoring of lymphocyte infiltration in submandibular glands was performed according to the scale of 0-3 as follows: 0, no infiltration; 1, light infiltration; 2, moderate infiltration; 3, severe infiltration.

9. Statistical analysis

All data were expressed as means ± standard error (S.E.M.) of three replicates. Statistical calculations were carried out with SPSS version 10.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was applied to determine differences between samples. Duncan's test was used to compare the data. Differences were considered significant at $p < 0.05$.

Results

1. Onset of type 1 diabetes and survival rate

The onset of diabetes mellitus (DM) was diagnosed when a mouse had glucose content of >300 mg/dl after fasting 4 h. Mouse deaths in the middle of the experiment were attributed to the incidence of DM because of the decreased weight induced by DM.

The DM onset ratios in the NOD control group were 58.3% at 17 weeks and 100% at 25 weeks. However, the onset ratios in the group treated with ETF at 50 mg/kg were 66.7% at 21 weeks, and 100% at 29 weeks; those in the group treated with ETF at 100 mg/kg were 50% at 17 weeks, and 100% at 33 weeks; those in the group treated ETF at 200 mg/kg were 41.7% at 17 weeks, and 100% at 33 weeks.

The survival rate of the normal group was 100% until the end of the experiment, whereas that of the NOD control group was 91.7% at 17 weeks, 83.3% at 21 weeks, 58.3% at 25 weeks, 33.3% at 29 weeks and 16.7% at 33 weeks. While the survival rate at 33 weeks was 25% in the treated with ETF at 50 mg/kg, it was 41.7% treated with ETF at 100 mg/kg. It increased by more than 8.3% and 25% in comparison with that of the NOD group. The survival rate of the group treated with ETF at 200 mg/kg was 50% at 33 weeks, i.e., an increase of 33.3% in comparison with the NOD control group, and an increase of 8.3% in comparison with the 100 mg/kg ETF group(Fig. 1).
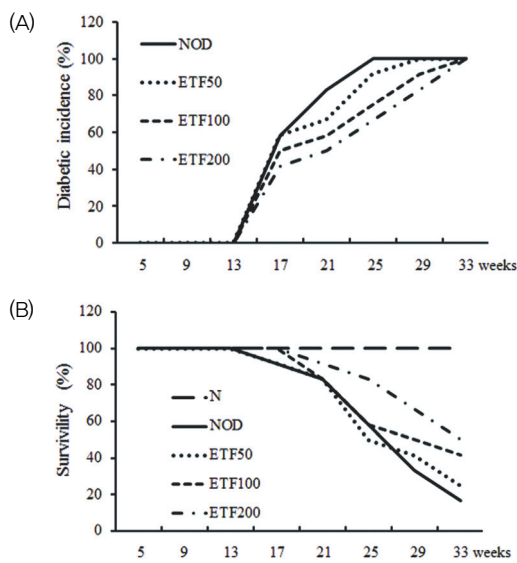
Fig. 1. Incidence of diabetes (A) and survival ratio (B) of NOD mice.

2. Changes in blood glucose

Between 17 and 33 weeks, blood glucose levels were significantly increased in NOD mice in comparison with those in normal mice; those in the group treated ETF at 50 mg/kg tended to be lower than in the NOD control group, but the differences were not significant.

As shown in Table 1, the group treated ETF at 100 mg/kg showed a significant decline in glucose level in comparison with NOD mice from 21 weeks, and the group treated ETF at 200 mg/kg showed significant decreases in glucose levels from 17 weeks(Table 1).

Table 1. Blood glucose levels (mg/dl) in normal and NOD mice treated or not with ETF

Group	Weeks							
	5	9	13	17	21	25	29	33
Normal	80.2±3	105.4±3	110.2±5	107.4±10	109.4±12	111.3±8	115.4±9	120.3±8
NOD control	82.5±2	103.6±3	135.4±9	250.4±8	367.4±18	420.1±15	435.7±25	451.2±34
NOD +ETF 50	80.6±5	105.4±8	126.4±9	224.9±9	335.4±20	375.4±17	395.4±30	398.1±28
NOD +ETF 100	81.2±2	106.4±4	120.4±8	204.1±8	300.2±17*	320.8±16*	344.6±34*	354.1±21*
NOD +ETF 200	79.3±3	102.3±3	121.5±5	190.4±8*	280.6±15*	298.8±21**	305.7±25*	321.5±22**

Normal, non-obese non diabetic mice treated with distilled water (DW); NOD control, NOD mice treated with DW; NOD + ETF 50, NOD mice treated with ETF at 50 mg/kg body weight/day; NOD + ETF 100, NOD mice treated with ETF at 100 mg/kg body weight/day; NOD + ETF 200, NOD mice treated with ETF at 200 mg/kg body weight/day. Data are the mean ± S.E. Significance: *$p < 0.05$, **$p < 0.01$ versus NOD control.

3. Changes in body weight, and food and water consumption

Body weight in the normal group was 17.7 ± 0.1 g at 5 weeks, and gradually increased to 36.5 ± 0.2 g at 33 weeks, whereas the NOD group showed significant body weight loss (26.0 ± 0.2 g at 21 weeks, and 22.4 ± 0.1 g at 33 weeks). Body weight in the group treated ETF respectively increased in comparison with the NOD control, especially in groups treated ETF at 100 mg/kg (25.6 ± 0.2 g) and 200 mg/kg (28.1 ± 0.2 g).

The water intake level of NOD mice was 0.173 ± 0.014 ml/g at 17 weeks, and was significantly higher than that of the normal group up to 33 weeks. In the groups treated ETF at 100 mg/kg and 200 mg/kg, water consumption was significantly lower than the NOD control. No significant changes in food intake were observed among NOD control and groups treated ETF(Table 2).

Table 2 Changes in water and food consumption and body weight

	Group	Weeks							
		5	9	13	17	21	25	29	33
Water (ml/g)	Normal	0.15±0.01	0.15±0.012	0.153±0.011	0.153±0.013	0.157±0.010	0.16±0.013	0.16±0.014	0.17±0.016
	NOD control	0.15±0.011	0.15±0.013	0.15±0.012	0.173±0.014	0.180±0.011	0.189±0.014	0.194±0.015	0.201±0.017
	NOD +ETF 50	0.15±0.011	0.15±0.013	0.15±0.012	0.17±0.014	0.174±0.011	0.185±0.014	0.186±0.015*	0.194±0.017*
	NOD +ETF 100	0.15±0.02	0.15±0.012	0.15±0.015	0.169±0.014	0.171±0.018*	0.179±0.016*	0.183±0.014**	0.191±0.016*
	NOD +ETF 200	0.15±0.013	0.15±0.014	0.151±0.016	0.177±0.014	0.17±0.018*	0.172±0.020*	0.177±0.016**	0.188±0.014**
Food (g)	Normal	2.41±0.24	2.43±0.16	2.46±0.18	2.56±0.34	2.6±0.24	2.68±0.24	2.71±0.16	2.81±0.21
	NOD control	2.38±0.15	2.47±0.16	2.53±0.21	2.48±0.3	2.43±0.24	2.5±0.21	2.57±0.19	2.61±0.13
	NOD +ETF 50	2.37±0.21	2.43±0.17	2.49±0.25	2.54±0.16	2.55±0.14	2.64±0.2	2.43±0.19	2.6±0.15
	NOD +ETF 100	2.39±0.17	2.46±0.16	2.51±0.2	2.46±0.21	2.5±0.22	2.43±0.16	2.61±0.18	2.68±0.27
	NOD +ETF 200	2.4±0.16	2.45±0.14	2.54±0.16	2.61±0.18	2.71±0.21	2.64±0.22	2.55±0.17	2.79±0.29
Body Weight (g)	Normal	17.7±0.1	20.4±0.1	24.5±0.3	26.1±0.2	30.4±0.2	32.5±0.2	34.8±0.1	36.5±0.2
	NOD control	17.9±0.1	21.5±0.2	24.1±0.1	25.4±0.2	26.0±0.2	25.4±0.3	24.7±0.2	22.4±0.1
	NOD +ETF 50	17.8±0.1	22.7±0.2	23.4±0.1	25.3±0.2	26.9±0.2	26.7±0.4	26.8±0.2*	23.7±0.1
	NOD +ETF 100	17.6±0.1	23.0±0.2	24.0±0.2	24.9±0.2	27.1±0.3	27.3±0.3*	27.0±0.2*	25.6±0.2*
	NOD +ETF 200	17.8±0.1	22.8±0.2	23.7±0.1	25.0±0.2	27.5±0.2*	28.4±0.3*	28.3±0.2*	28.1±0.2**

Normal, non-obese non diabetic mice treated with distilled water (DW); NOD control, NOD mice treated with DW; NOD + ETF 50, NOD mice treated with ETF at 50 mg/kg body weight/day; NOD + ETF 100, NOD mice treated with ETF at 100 mg/kg body weight/day; NOD + ETF 200, NOD mice treated with ETF at 200 mg/kg body weight/day. Data are the mean ± S.E.M. Significance: *$p < 0.05$, **$p < 0.01$ versus NOD control.

4. Changes in Total cholesterol (TC) and Triglyceride (TG) in serum

TC level was 88.5±7.8 mg/dl in normal group, 212.8 ± 17.6 mg/dl in NOD control, 209.0 ± 14.9 mg/dl in the group treated ETF at 50 mg/kg ETF, 187.6 ± 9.4 mg/dl in the group treated ETF at 100 mg/kg, and 173.1 ± 7.4 mg/dl in the group treated ETF at 200 mg/kg ETF. The values for the latter two groups were significantly lower than that in NOD control.

TG level in normal group was 2.6 ± 0.1 mg/dl, and was significantly increased (up to 5.8 ± 0.3 mg/dl) in NOD control. TG content in the groups treated with ETF at 100 mg/kg, 5.5 ± 0. mg/dl; 200 mg/kg, 5.1 ±

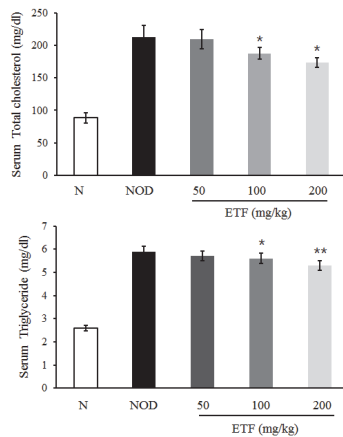0.2 mg/dl), were significantly lower than in NOD control(Fig. 2).

Fig. 2. ETF ameliorated the levels of serum TG and TC in NOD mice At 19 weeks, serum samples were collected and the levels of TG and TC were analyzed. Values are means ± S.E.M. *p〈0.05; **p 〈0.01 versus NOD control.

5. Intraperitoneal glucose tolerance test

The levels of blood glucose in NOD and ETF treated group were significantly increased compared to that in the normal group at all time points. Glucose levels significantly decreased from 60 min to 120 min in group treated ETF at 100 mg/kg and 200 mg/kg compared to NOD control(Table 3, Fig. 3).

Table 3. Glucose levels in Intraperitoneal glucose tolerance test

		Time (min)					
	Group	0	20	40	60	90	120
Glucose (mg/dl)	N	100±2.5	157±4.5	198±15.3	198±13.1	153±10.2	123±8.7
	NOD	112±4.1	234±15.4^C	304±20.6^C	345±28.1^C	334±21.4^C	324±16.8^C
	ETF50	105±3.5	223±17.9^C	285±18.2^C	320±24.1^C	305±25.1^C	295±23.9^C
	ETF100	111±2.1	209±20.1^C	274±23.1^C	295±19.5^{C,a}	278±17.6^{C,a}	250±17.4^{C,a}
	ETF200	109±3.0	195±13.5^{C,a}	260±20.4^{C,a}	274±23.4^{C,b}	257±14.8^{C,c}	234±18.8^{C,c}

Data are the means ± S.E.M. (n=10). Different letters indicate statistically significant differences: Ap〈0.05, Bp〈0.01, Cp〈0.001 versuss normal group; ap〈0.05, bp〈0.01, cp〈0.001 versus NOD control group.
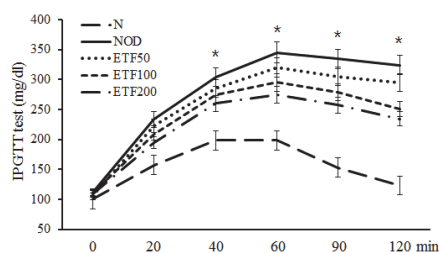

Fig. 3. Glucose tolerance in NOD mice treated with ETF. Values are means ± S.E.M. *p〈0.05 versus NOD control.

6. Cytokine levels in serum

The inflammatory cytokines such as IFN-γ, IL-1β,

IL-6 and IL-12 in NOD control were significantly increased in comparison with those of normal group. Administration of ETF significantly reduced the inflammation index, but the levels of anti-inflammatory cytokines (IL-4 and IL-10) were higher in the group treated ETF than in NOD control, but this differences were not significant(Table 4).

Table 4. Cytokine levels in serum on the NOD mice.

	Cytokines in serum					
Group	IFN-γ (pg/ml)	IL-1β (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (Units/ml)	IL-12 (pg/ml)
Normal	32.4±2.5	8.4±1.1	35.4±2.4	254.1±16.8	0.4±0.1	34.1±1.8
NOD control	124.4±6.1^C	35.1±2.8^C	190.3±11.2^C	2273.4±150.4^C	3.4±0.2^C	310.4±15.4^C
NOD+ETF 50	110.4±5.8^C	30.4±4.7^C	198.4±10.8^C	2078.4±217.5C,a	3.6±0.3^C	281.4±19.8^C
NOD+ETF 100	98.4±5.4^{C,b}	27.5±1.5^{C,b}	205.8±14.5^C	1889.7±130.4^{C,b}	3.8±0.6^C	250.4±23.1^{C,b}
NOD+ETF 200	79.1±8.3^{C,c}	25.8±2.7^{C,b}	221.4±18.1^C	1684.1±120.4^{C,c}	3.6±0.4^C	227.8±20.1^{C,c}

Data are the means ± S.E.M. (n=10). Different letters indicate statistically significant differences. Ap〈0.05, Bp〈0.01, Cp〈0.001 versus normal mice; ap〈0.05, bp〈0.01, cp〈0.001 versus NOD control group.

7. Western blot analysis of proteins in pancreatic tissue

The levels of iNOS and COX2 (proteins related to inflammation) in two ETF-treated groups (100 mg/kg and 200 mg/kg) were significantly lower than those in the NOD control group. ETF administration significantly increased the levels of SOD and catalase in the group treated ETF at 100 mg/kg and 200 mg/kg in comparison with that in NOD control(Fig. 4).
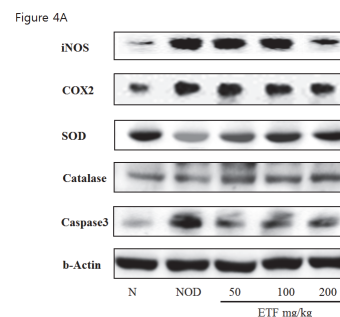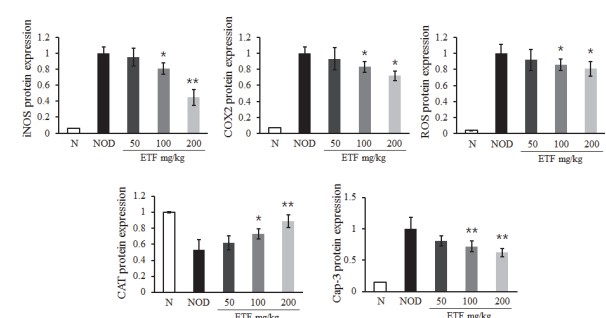

Fig. 4. iNOS, COX2, SOD, CAT and Cap-3 in pancreatic tissue. Values are means ± S.E.M. *p〈0.05; **p〈0.01 versus NOD control.

8. Insulitis evaluation

Photomicrographs of the pancreases showed a marked decrease in the number of islets of Langerhans, with atrophy of the islets, in the NOD control group. Histological examination showed that NOD mice at 33 weeks of age developed insulitis at various stages associated with lymphocyte infiltration. The average score of insulitis severity was similar between NOD controls and low-ETF-treated mice, whereas it was significantly lower in the medium- and high-ETF-treated groups than in the control group. These datas indicate that ETF treatment on NOD diabetes mouse inhibits a lymphocyte infiltration into pancreatic islets.

In all mice in the control and low-ETF-treated groups, various stages of lymphocyte infiltration were observed in submandibular glands, which was reduced in the medium- and high-ETF-treated groups. The severity of lymphocyte infiltration was lower in the high-ETF-treated group than in the control group(Fig. 5). Lymphocyte infiltration was rarely observed in parotid or sublingual glands in all groups(data not shown).
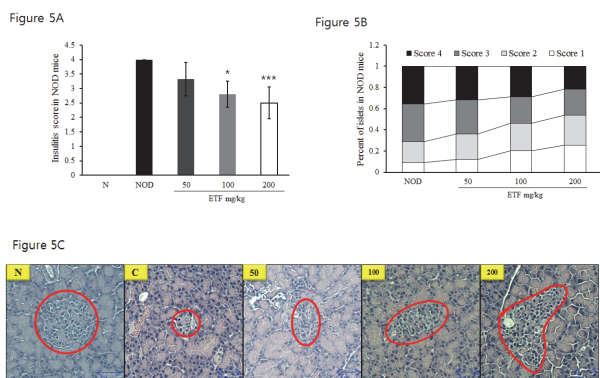

Fig. 5. The inhibitory effect of pancreatic insulitis (A), leukocyte infiltration score (B) and morphological data (C).
Values are means ± S.E.M. $^{*}p < 0.05$; $^{***}p < 0.001$ versus NOD control. (N), normal mice; (C), NOD control mice; (50), NOD mice treated ETF at 50 mg/kg ; (100), NOD mice treated ETF at 100 mg/kg; (200), NOD mice treated ETF at 200 mg/kg. (H-E staining, 200x magnification). Scale bar: 50 μm

Discussion

Type 1 and type 2 diabetes are characterized by progressive β-cells failure. Apoptosis is probably the main form of beta-cell death in both forms of the disease. It has been suggested that the mechanisms leading to cytokine-induced β-cell death in type 2 and type 1 diabetes, respectively, share the activation of a final common pathway involving interleukin (IL)-1β, nuclear factor (NF)-κB, and Fas. In the insulitis lesions in type 1 diabetes, invading immune cells produce cytokines, such as IL-1β, TNF-α, and IFN-γ. IL-1β and/or TNF-α plus IFN-γ induce β-cell apoptosis via the activation of β-cell gene networks under the control of the transcription factors NF-κB and STAT-1[20]. IFN-γ is a mediator of islet β-cell destruction *in vitro* as well as β-cell-destructive insulitis and IDDM[21]. Our study showed that a release of IFN-γ dose-dependently were downregulated in NOD mice treated with ETF.

Correlation studies between cytokines expressed in the islets and autoimmune diabetes development in NOD mice and BB rats have demonstrated that beta-cell destructive insulitis is associated with increased expression of proinflammatory cytokines (IL-1, TNF-α, and IFN-γ) and type 1 cytokines (IFN-γ, TNF-α, and IL-12), whereas non-destructive (benign) insulitis is associated with increased expression of type 2 cytokines (IL-4 and IL-10) and a type 3 cytokine (TGF-β). Cytokines (IL-1, TNF-α, TNF-β and IFN-γ) may be directly cytotoxic to β-cells by inducing nitric oxide and oxygen free radicals in the β-cells. NOD mice with targeted deletions of IL-12 and IFN-γ genes still develop IDDM, albeit less often, and the onset of the disease is delayed. In contrast, post-natal deletion of IL-12 and IFN-γ, and also IL-1, TNF-α, IL-2, and IL-6 (by systemic administration of neutralizing antibodies, soluble receptors or receptor antagonists, or receptor-targeted cytotoxic drugs) significantly decreases IDDM incidence in NOD mice[22].

Insulin-dependent diabetes mellitus (IDDM) is a disease that results from autoimmune destruction of insulin-producing β-cells in the pancreatic islets of Langerhans. The autoimmune response against islet β-cells is believed to result from a disorder of immunoregulation. According to this concept, a T helper 1 (Th1) subset of T cells and their cytokine products, i.e. Type 1 cytokines (IFN-γ and TNF-α), dominate over an immunoregulatory (suppressor) Th2 subset of T cells and their cytokine products, i.e. Type 2 cytokines (IL-4 and IL-10). This allows Type 1 cytokines to initiate a cascade of immune/inflammatory processes in the islets (insulitis), culminating in β-cell destruction. Type 1 cytokines activate (1) cytotoxic T cells that interact specifically with β-cells and destroy them, and (2) macrophages that produce proinflammatory cytokines (IL-1 and TNF-α), as well as oxygen and nitrogen free radicals that are highly toxic to β-cells. Furthermore, IL-1, TNF-α, and IFN-γ are cytotoxic to β-cells, largely by inducing the formation of oxygen free radicals, nitric oxide, and peroxynitrite in these

cells. Therefore, it would appear that prevention of β-cell destruction and IDDM should be aimed at stimulating the production and/or action of Type 2 cytokines, inhibiting the production and/or action of Type 1 cytokines, and inhibiting the production and/or action of oxygen and nitrogen free radicals in the pancreatic islets[23].

The nonspecific immune/inflammatory response involves β-cell destruction by T cell- and macrophage-derived proinflammatory cytokines (IL-1, TNF-α, TNF-β, IFN-γ) and free radicals/oxidants, e.g. O$_2$, H$_2$O$_2$, and NO. These inflammatory mediators are produced in T cells (both CD41 and CD81) and macrophages activated by cytokines (such as particularly IFN-γ, and also IL-2, TNF-α, and TNF-β) in T cells.

In addition, mRNA levels of iNOS correlate with mRNA levels of IL-1α and IFN-γ in islets of prediabetic NOD mice, and iNOS protein is expressed in islet-infiltrating macrophages and β-cells[24]. These findings suggest that IL-1 (from macrophages) and IFN-γ (from T cells) induce iNOS and consequent NO production by macrophages and by β-cells. In this way, macrophage-derived NO would be destructive to adjacent β-cells, and β-cell-derived NO would be self-destructive. Indeed, NO has been identified as an important mediator of β-cell damage by activated macrophages[25]. Also, release of IL-1 in intraislet by passenger macrophage activation *in vitro* leads to iNOS expression in b-cells and consequent impaires insulin secretion[26]. The levels of iNOS in two ETF-treated groups (100 mg/kg and 200 mg/kg) were significantly lower than those in the NOD control group. Thus, avariety of antioxidants (deferoxamine, nicotinamide, SOD, α-tocopherol, probucol, and lazaroid) have been reported to provide some protection against IDDM development in autoimmune diabetes-prone BB rats and/or NOD mice[27-33]. ETF administration increased the levels of SOD and catalase compared to NOD control.

In the present study, blood glucose, hyperlipidemia, glucose resistance and water consumption were significantly reduced in the group treated ETF in comparison with NOD control group. This indicates that ETF reduces clinical symptoms of diabetes by protecting pancreatic β-cells from injury. We analyzed both proinflammatory and anti-inflammatory cytokines. The levels of proinflammatory cytokines in serum (IFN-γ, IL-1β, IL-6 and IL-12) were significantly lower than in NOD control, whereas the levels of anti-inflammatory cytokines (IL-4 and IL-10) were increased by ETF in a dose-dependent manner, although this effect did not reach statistical significance. To investigate the protective effect of ETF on β-cells, proinflammatory proteins (iNOS and COX2), antioxidant biomarkers (SOD and catalase)

were analyzed by Western blotting. Cytokines such as IFN-γ, IL-1β, IL-6 and IL-12 induced oxidant stress to β-cell. ETF significantly decreased inflammatory proteins, and significantly increased SOD and catalase protein level in pancreas. In addition, proapoptic caspase-3 protein expression significantly suppressed in ETF 200 mg/kg. These results correspond to analysis of cytokines in serum and the histological insulitis score.

Conclusion

The present study was conducted to examine whether extracts of Toosendan Fructus (ETF) has an ameliorative effect on diabetes-induced alterations such as oxidative stress and inflammation in the pancreas of nonobese diabetic (NOD) mice, a model of human type 1 diabetes.

1. The administration of ETF delays the onset of type 1 diabetes and increase the survival rate dose-dependent manner in non-obese diabetic mice.

2. The administration of ETF showed significantly improved blood glucose levels and glucose tolerance.

3. The administration of ETF decreased proinflammatory cytokines, TG, and TC in serum.

4. The administration of ETF were significantly lower the levels of iNOS and COX2 (proteins related to inflammation) and increased the levels of SOD and catalase (proteins related to antioxidant).

5. The administration of ETF enhanced pancreatic insulitis through inhibiting lymphocyte infiltration into pancreatic islets.

We conclude that ETF could be a possible remedy for diabetes through and anti-oxidant effect and suppression of inflammation induced by oxidative stress in pancreatic β-cells. However, further studies are needed to elucidate the mechanism for this ETF action, which will provide valuable information for clinical trial to further evaluate its anti-diabetic potential in humans with type 1 diabetes

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