Original Article

DOI: 10.5582/bst.2019.01181

Anti-oxidant, anti-apoptotic, anti-hypoxic and anti-inflammatory conditions induced by PTY-2 against STZ-induced stress in islets

Shivani Srivastava[1], Harsh Pandey[1], Surya Kumar Singh[2], Yamini Bhusan Tripathi[1,*]

[1] *Department of Medicinal Chemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India;*
[2] *Department of Endocrinology and Metabolism, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.*

Summary **The earlier assessment of *Pueraria tuberosa* (PT) has shown anti-diabetic effects through enhancing incretin action and DPP-IV (Dipeptidyl peptidase-IV) inhibition. The aim of this work was to further explore the protective role of aqueous extract of *Pueraria tuberosa* tuber (PTY-2) against streptozotocin (STZ) induced islet stress in rats. Diabetes was induced by STZ (65 mg/kg body weight) in charles foster male rats. After 60 days of STZ administration, animals with blood glucose levels > 200 g/dL were considered as diabetic. All the rats were later divided into three groups: Group-1 (STZ untreated normal rats), Group-2 (Diabetic control), and Group-3 (PTY-2 [50 mg/100 g bw treatment for next 10 days to diabetic rats). The rats were then sacrificed after the 10th day of treatment accordingly. STZ treatment led to an increase in expression of Matrix metalloproteinases-9 (MMP-9), Tumour necrosis factor-α (Tnf-α), Hypoxia inducible factor-α (HIF-1α), Vascular endothelial growth factor (VEGF), Interleukin-6 (IL-6), Protein kinase C-ε (PKC-ε), Nuclear factor kappa-light-chain-enhancer of activated B-cells (NFkB), and Caspase-3. Reverse Transcriptase-PCR (RT-PCR), Immunohistochemistry and Western-Blot analysis showed an increase in the expressions of Superoxide Dismutase (SOD) and Nephrin, and a decrease in the expressions of NFkB, PKC-ε, TNF-α, MMP-9, HIF-1α, VEGF, Caspase-3 and IL-6 after 10 days of PTY-2 treatment. The results showed that PTY-2 favorably changed all the expressions *via* anti-oxidant, anti-apoptotic, anti-hypoxic and anti-inflammatory pathways, making itself as a protective agent against STZ induced islet stress. Further evaluation of PTY-2 might be helpful in establishing its role in the management of diabetes mellitus.**

Keywords: STZ, PTY 2, stress, expressions, diabetes, islets

1. Introduction

Mortality and morbidity due to diabetes mellitus (DM) are rising rapidly worldwide (*1,2*). Type 2 DM (T2DM) increases the risk of acute pancreatitis by 1.5-3 folds, and the use of anti-diabetic drugs decreases this excess risk (*2*). On the other hand, pancreatitis is one of the known risk factors for the onset of DM (*3-5*). Additionally, the onset of DM can be a symptom of pancreatic cancer as the latter is more common among newly diagnosed cases of T2DM. Furthermore, long-standing DM can increase the risk of occurrence of pancreatic cancer (*6-9*). Apart from the known etiological factors, pathological changes due to environmental factors or other unknown reasons can alter the gene expression and lead to diseases like DM, pancreatic cancer, acute or chronic pancreatitis (*10*). Both *in-vivo* and *in-vitro* models are being developed to understand the mechanisms underlying the profile change in gene expression. Many synthetic drugs and herbal formulations have been developed for the prevention and treatment of DM.

PTY-2, is being evaluated for its protective role in STZ induced islet stress. In our earlier studies, we had evaluated the role of PT in animal models of streptozotocin (STZ)-induced DM and in normoglycemic rats (*11-13*). PT has also been studied for its anti-

Released online in J-STAGE as advance publication October 8, 2019.

*Address correspondence to:
Prof. Yamini Bhusan Tripathi, Department of Medicinal Chemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India.
E-mail: yamini@bhu.ac.in, or yaminibiochemist6@gmail.com

inflammatory, anti-oxidant, nephro-protective, intestine-protective, hepato-protective, anti-hypertensive and anti-diabetic properties (*11-20,44*). The analysis of the actions makes hypothesis that there must be interconnected signaling pathways between anti-inflammatory, anti-hypoxic, anti-apoptotic, antioxidants and anti-diabetic genes for the effect of PT. Taking our research forwards, we have attempted to study the signaling pathways to understand the protective role of PTY-2 in islet damage.

Various markers like Nephrin, SOD, HIF-1α, TNF-α, MMP-9, Caspase-3, NF-kB, VEGF, PKC-ε, Caspase-3 and IL-6 have been used to study the effect of drugs or herbal products on DM. Earlier studies have demonstrated increased levels of VEGF, TNF-α, MMP-9, IL-6, NF-kB, PKC, and HIF-1α in inflammatory conditions, vascular lesions and DM (*21-25*). Excess generation of reactive oxygen species (ROS) and oxidative stress is one of the common etiological pathways in the development and progression of DM (*26,27*). Nephrin, a member of immunoglobulin super family, is a surface receptor that is specifically expressed in kidney, brain and pancreas (*28*). Nephrin plays an important role in beta cell survival signaling through the association with PI3-kinase, reported in mouse islet β-cells and mouse pancreatic beta-cell line (βTC-6 cells) (*29*). VEGF is a vital regulator of vascularization of islet cells, and the islet vascular system is critical for a normal secretion of insulin (*30,31*). Genetic studies have shown that normal VEGF and vascularization are important for adult islet cell function and β cell mass (*25*). The β cell-specific overexpression of VEGF causes rapid hypervascularization and hyperinnervation of the islet, leading to increased production of extracellular matrix components (ECM) (*32*). Hence, we can say that increased amount of VEGF is responsible for defective angiogenesis. MMPs are a large family of endopeptidases, and these are produced by stromal and inflammatory cells. Pancreatic MMPs (especially MMP-9) induce inflammation, and serum MMP-9 levels are an assessment marker of severity of pancreatitis (*33*). MMP-9 is usually involved in degradation and remodeling of ECM components (*22,34,35*). NF-kB, a nuclear transcription factor, regulates the transcription of various genes involved in inflammation mediation (*36*). The activation of NF-kB is an early pathological event in the development of insulin resistance (*37*). TNF-α, an inflammatory marker, is rapidly produced intracellularly with the activation of NF-kB and is known to have effects on diabetes and obesity (*21,38*). The PKC-ε belongs to the superfamily of isoforms of protein kinases. PKC-ε is involved in the development of insulin resistance, and its inhibition is associated with the improvement in glucose homeostasis in animal models (*39*). PKC-ε has a strong presence in islet cells, acinar cells, and ductal epithelium (*40*). Similarly, both IL-6 and HIF-1α are also known to play a pro-inflammatory role in the mediation of acute pancreatitis and pancreatic cancer (*23,24*). Hypoxia is an important cause of beta-cell loss and is measured by an increase in HIF-1α expression (41). Various gene knockout experiments have shown that caspase-3 is involved in beta cell apoptosis and that $Casp^{-/-}$ are protected from the development of DM (*42*).

As PT has multiple medicinal properties with several beneficial compositions, we have studied the protective effect of its total water extract rather than on its individual components. Because PT contains many steroids, triterpenoid, glycosides, carbohydrates, alkaloids, flavanoid, tannin, protein and amino acids, *e.g.*, daidzin, puerarin, puerarone, genistein, puetuberosanol, tuberostan, tuberosin, and puerarin 4',6'-diacetate as the main constituents (*12,14,43*). We planned to study the multi-targeted protective effect of PTY-2 on the islet damage among rats with STZ-induced stress.

2. Materials and Methods

2.1. *Materials*

The antibody of rabbit IL-6 (23 Kda) (08310): SAB1408591, mouse monoclonal VEGF (21 Kda) (JH-121): sc-57496, NF-κB p65 (D14E12) XP® Rabbit mAb #8242, rabbit polyclonal PKC-ε (SAB1300094), mouse monoclonal β-actin (A2228), mouse monoclonal Hif-1α (H6536-100 UG), rabbit monoclonal Caspase-3 (CASP 3 [D175] invitrogen), Mouse monoclonal MMP-9 and monoclonal anti-rabbit IgG (γ-chain specific)-peroxidise (A1949), pre-stained protein ladder (from Hi-Media Pvt. Ltd, Kolkata, India) along with PVDF membranes (from Millipore, catalog no. IPVH20200) were used for proteins expression analysis. STZ-S0130 was bought from Sigma-Aldrich, St Louis, USA. For RT-PCR, Trizol (Himedia, Pvt. Ltd, Kolkata, India), cDNA Kit (Fermentas), and Taq-polymerase (Genaxy Scientific Pvt.Ltd) were used.

2.2. *Sample preparation*

PT was purchased from Ayurvedic Pharmacy, Banaras Hindu University. Its authenticity has already been ascertained in our previous research (*44*). We extracted 30 g powder with eight volumes of distilled water. When the volume was reduced to ¼th, it was filtered with cloth. The total yield of PTY-2 obtained by this process was 30%.

2.3. *Study Design*

The protocol was approved by the Institute Ethical Committee (Dean/2015/CAEC/1266), Institute of Medical Sciences, Banaras Hindu University. After overnight fasting, Charles foster male rats of the same age group with body weight in the range of 120-130 grams were injected STZ (65 mg/kg body weight). STZ was prepared in chilled and fresh citrate buffer of pH

4.5. The blood glucose levels were checked using strips (Dr. Morepen) on the 5th day. Rats with blood glucose levels > 200 mg/dL were placed under diabetic group. In order to induce severe diabetes, we further left the rats (three rats per cage) for 55 days. On the 61st day, we divided the rats into three groups (n = 6): Group-1 (STZ untreated rats, *i.e*., age-matched normal rats), Group-2 (diabetic control), and Group-3 (PTY-2 at 50 mg/100 g bw treatment for next 10 days to diabetic rats). The rats were then sacrificed after 10 days of treatment. The pancreas was isolated and rinsed with PBS. Then, these were cut into two parts; one for histology (preserved in 10% formaldehyde) and the other was first crushed in liquid nitrogen and then stored in -80°C freezer for molecular study.

2.4. *RT-PCR*

RNA was extracted using trizol reagent from about 50 mg of pancreatic tissue with a homogenizer. Then 5 µg of total RNA was reverse-transcribed with superscript II RNase H-reverse transcriptase (RT) using random hexamers according to the instructions provided by the manufacturers (Fermentas Pvt. Ltd.). For SOD, 2 µL c-DNA, 0.2 mmol/L deoxynucleotide triphosphates (dNTPs), 1.5 mmol/L $MgCl_2$, 0.5 µmol/L of each primer, 2.5 µL 10X PCR buffer and 1 U Taq DNA polymerase were used. For Nephrin, 1 µL c-DNA, 0.2 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 1.2 µmol/L of each primer, 2.5 µL 10X PCR buffer and 1U Taq DNA polymerase were used. For matrix metallopeptidase 9 (MMP-9), 2 µl c-DNA, 200 umol/L dNTPs, 1.5 mmol/L $MgCl_2$, 0.4 µmol/L of each primer, 2 µL 10X PCR buffer and 2.5 U Taq DNA polymerase were used. For Tnf-α, 1 µL c-DNA, 200 umol/L dNTPs, 1.2 mmol/L $MgCl_2$, 0.6 µmol/L of each primer, 2 µL 10X PCR buffer and 2 U Taq DNA polymerase were used. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 0.1 µmol/L of each primer was used. The optical density of each expression was determined *via* alpha imager 2200 and presented as the ratio against GAPDH. All RT-PCR experiments were performed in triplicates (Table 1).

2.5 *Western blot analysis*

Pancreatic tissue was homogenized with chilled lysis buffer (50 mMTris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton, 0.1% SDS, 1 mM sodium orthovanadate, protease inhibitor cocktail and 1 mM PMSF). The homogenate was then centrifuged at 12,000 rpm at 4°C for 30 min. The protein estimation was done by Bradford method. 40 µg proteins with loading dye were separated in the polyacrylamide gel. The gel was then electro-transferred to PVDF membranes in transfer buffer (10X Tris-glycine-methanol and SDS-PAGE buffer) to stay overnight at 4°C at 45 V. The next day, PVDF membrane was blocked with 5% non-

Table 1. Details of PCR primer sequences, product size and thermal steps for expressions of TNF α, SOD, Nephrin, MMP 9 and GAPDH

Primers	Sequence	Product Size (bp)	RT PCR Thermal steps					
			Initial denaturation	Denature	Anneal	Extention	Final Extention
TNF α FORW	5'- CACCACGCTCTTCTGTCTACTGAAC -3'	546	No. of Cycle		30		1
TNF α REV	5'- CCGGACTCCGTGATGTCTAAGTACT -3'		Temp.(°C)	95	63	72	72
			Time	1 min	1 min	2 min	5 min
SOD FORW	5'-TCTAAGAAACATGGCGGTCC-3'	387	No. of Cycle		35		1
SOD REV	5'-CAGTTAGCAGGCCAGCAGAT-3'		Temp.(°C)	94	55	72	72
			Time	45 sec	30 sec	1.3 min	10 min
Nephrin FORW	5'-GTT CAG CTG GGAGAGACT GG-3'	340	No. of Cycle		43		1
Nephrin REV	5'-TTG GAC ATC CAG AGG GAC C-3'		Temp.(°C)	94	56	72	72
			Time	45 sec	45 sec	1 min	10 min
MMP 9 FORW	5'-TGTACCGTATGGTTACAC-3'	371	No. of Cycle		35		1
MMP 9 REV	5'CCGCGACACCAAACTGGAT3'		Temp.(°C)	94	58	72	72
			Time	1 min	90 sec	90 sec	7 min
GAPDH FORW	5'-CACGGCAAGTTCAATGGCACA-3'	244	No. of Cycle		35		1
GAPDH REV	5'-GAATTGTGAGGGAGATGCTC-3'		Temp.(°C)	94	58	72	75
			Time	30 sec	30 sec	45 sec	5 min

fat milk powder. The membrane was then incubated overnight with primary antibody diluted in TBST [IL-6 (1:1,000), PKC ε (1:500), VEGF (1:1,000), NF-kB (1:1,000), HIF 1α (1:1,000) & housekeeping gene β-actin (1:500)]. Then, on the next day, the blots were incubated with secondary antibody in TBST for one hour. Protein expression was detected through enhanced chemiluminescence (ECL) in LAS 500 Image Quant system (Wipro GE Healthcare, Hong Kong). The quantification was done by alpha imager 2200. The experiments were done in triplicates.

2.6. *Immunostaining*

The paraffin sections of pancreas were treated with xylene for 10 minutes to remove paraffin. The sections were rehydrated through 90%, 70% alcohol, and water by putting them for 5 minutes in each. Antigen retrieval was done by putting the citrate buffer dipped slides in EZ Retrieval System V.3 (Bio Genex). Sections were washed twice in citrate buffer and two times in 1X PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.4) for 10 minutes each, following which the sections were incubated in blocking solution [0.1% Triton X-100, 0.1% BSA, 10% FCS, 0.1% sodium deoxycholate and 0.02% Thiomersal (an anti-fungal agent), in 1X Phosphate Buffered Saline (PBS)] for 2 hours at room temperature and then transferred in primary antibodies, for overnight at 4°C. Tissues were washed in PBST (0.1% triton X in 1X PBS) with three changes of 10 minutes each. After the washing, the sections were incubated with anti-rabbit AF 546 (Red) and anti-mouse AF 488 (Green) (Invitrogen, USA) secondary antibody for 2 hours at room temperature. Sections were washed in PBS with Tween 20 (PBST) with three changes for 10 minutes each, counterstained with DAPI (1 µg/mL DAPI in 1X PBS), mounted in DABCO and examined under Zeiss LSM510 Meta confocal microscope. Image analysis was done by using Zen Black (2012) software.

2.7. *Statistical analysis*

One-way ANOVA test followed by post hoc analysis with Dunnett's test was done for each experiment. All results were expressed as means ± SD. Statistical significance was taken at $p \leq 0.05$.

3. Results

3.1. *PTY-2 response to islet stress*

3.1.1. *mRNA Expressions*

As compared to normal rats, the STZ-treated diabetic group showed a significant increase in TNF-α in pancreatic tissue, whereas the PTY-2 treatment significantly decreased the TNF-α expression as compared to diabetic control and increase as compared to normal. The MMP-9 expression also increased significantly in diabetic control as compared to normal rats, and there was a significant decrease after 10 days of PTY-2 treatment. On the contrary, both SOD and Nephrin expression decreased significantly in diabetic control rats as compared to normal. However, the PTY-2 treated group showed a significant increase in SOD expression as compared to diabetic control and a significant decrease as compared to normal. The Nephrin expression in PTY 2 treated rats increased significantly as compared to both normal and diabetic control (Figure 1 (a) and (b))

These results clearly indicate that in chronic diabetes, there is a significant increase in free radicals/ stress accompanied by an increase in pancreatic inflammation. Treatment with PTY-2 significantly reversed all these changes. Thus, any severe complications of severe diabetes like pancreatitis could be prevented by using PTY-2 as medicinal supplement.

3.2. *Protein Expressions*

3.2.1. *Western blot*

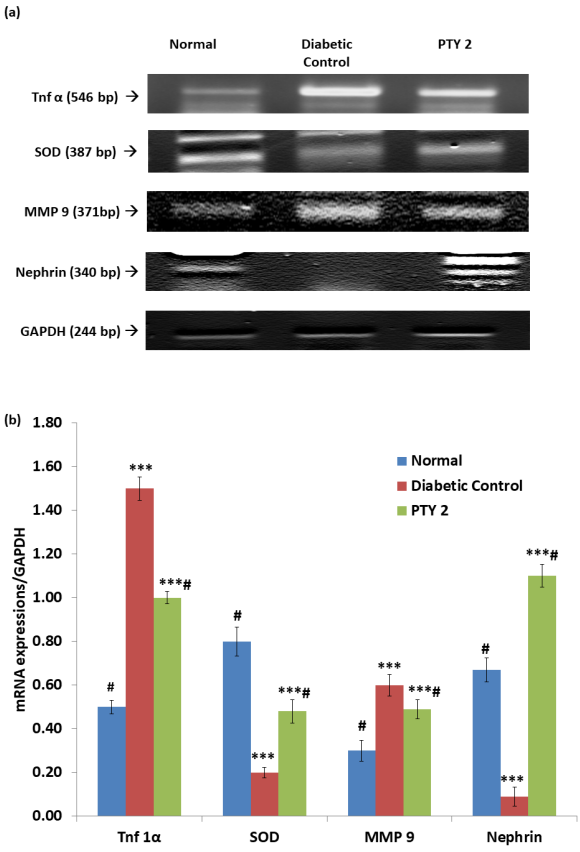

Figure 1. (a) mRNA expressions to investigate the effect of PTY-2 on STZ induced islet stress; (b) Densitometric analysis of RT-PCR product. Each value represents mean ± SD ($n = 6$); $^{***}p < 0.05$ compared with Normal, $^{\#}p < 0.05$ as compared with Diabetic Control.

For further validation, the protein expressions responsible for the induction of oxidative stress, hypoxia, apoptosis and inflammation of pancreatic tissues were estimated. The expressions of NF-kB, PKC ε, HIF-1α, VEGF and IL-6 were significantly increased in diabetic control as compared to normal rats. However, PTY-2 treatment significantly decreased all these expressions (Figure 2 (a) and (b)).

3.2.2. *Immunohistochemistry*

The expressions of MMP-9, HIF-1α, VEGF, IL-6, PKC-ε, NF-kB and Caspase-3 were significantly enhanced in diabetic control islets. The hazardous effects of STZ were down-regulated by 10 days of PTY-2 treatment. The Caspase-3, HIF-1α, MMP-9, IL-6, VEGF and PKC-ε expressions decreased significantly in PTY-2 treated group as compared to diabetic control and increased significantly as compared to the normal. The expression of NF-kB in PTY 2 treated group decreased significantly as compared to diabetic control, but non-

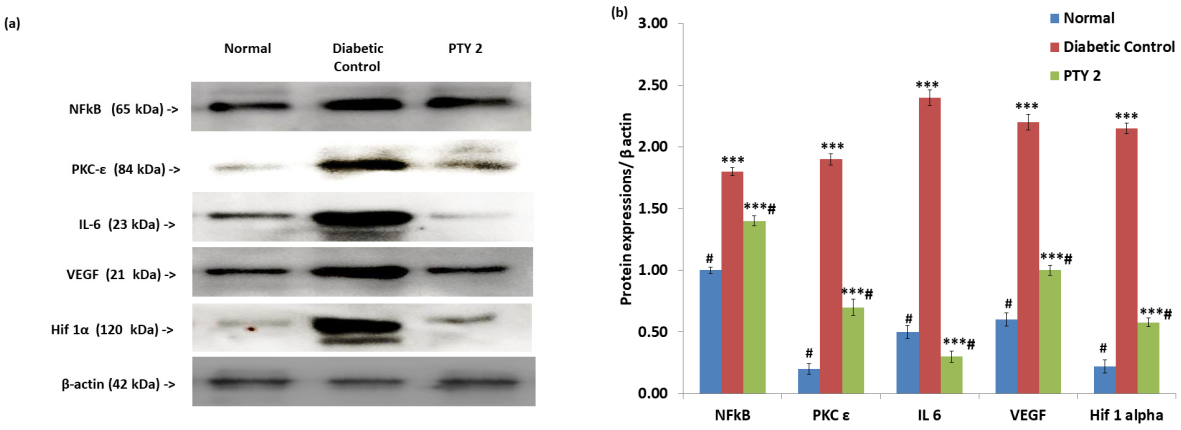

Figure 2. (a) Protein expressions to investigate the effect of PTY-2 on STZ induced islet stress; (b) Densitometric analysis of western blot product. Each value represent the mean ± SD (n = 6); $^{***}p < 0.05$ compared with Normal, $^{\#}p < 0.05$ compared with Diabetic Control.

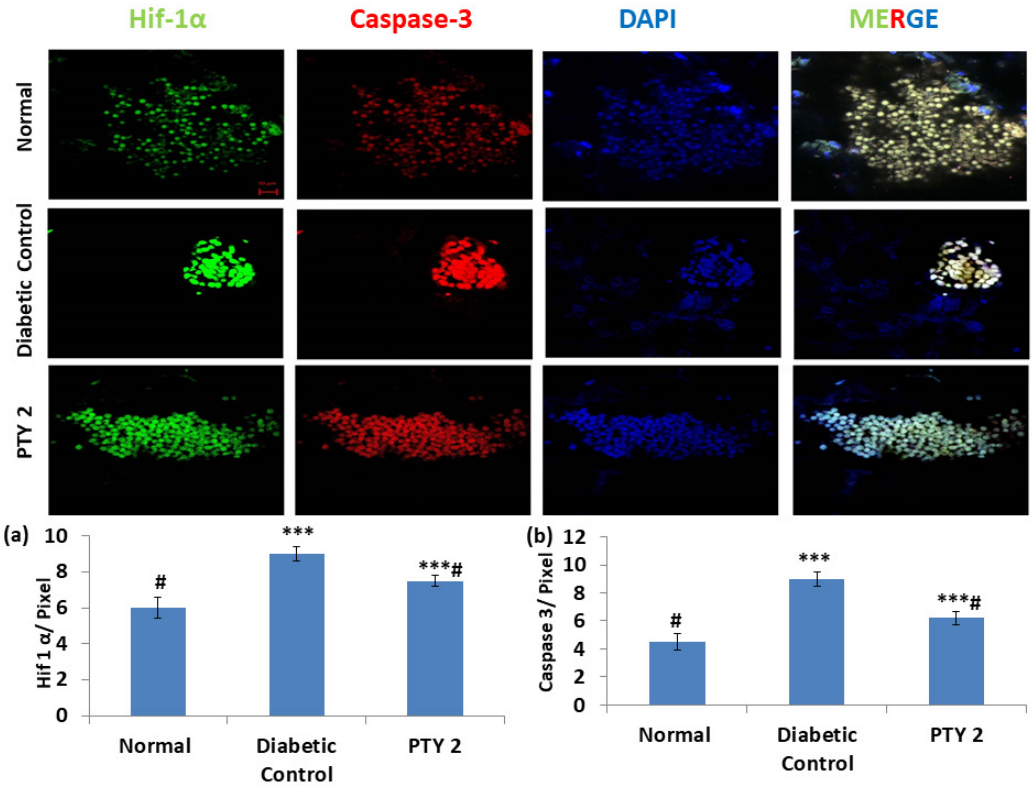

Figure 3. Immunohistochemistry analysis showed the effect of PTY-2 on the expression of (a) HIF-1α (green) and (b) Caspase 3 (red) in the islets of normal, diabetic control, and PTY-2-treated rats' pancreatic tissues. Both the expressions were merged with DAPI (blue). In comparison to diabetic control, PTY-2 down regulated the expression of both HIF-1α and Caspase 3. The images were taken at 63X magnification. Scale bar was 10 µm. The intensity was measured in pixel values. Each value represent the mean ± SD (n = 6); $^{***}p < 0.05$, compared with Normal, $^{\#}p < 0.05$, compared with Diabetic Control.

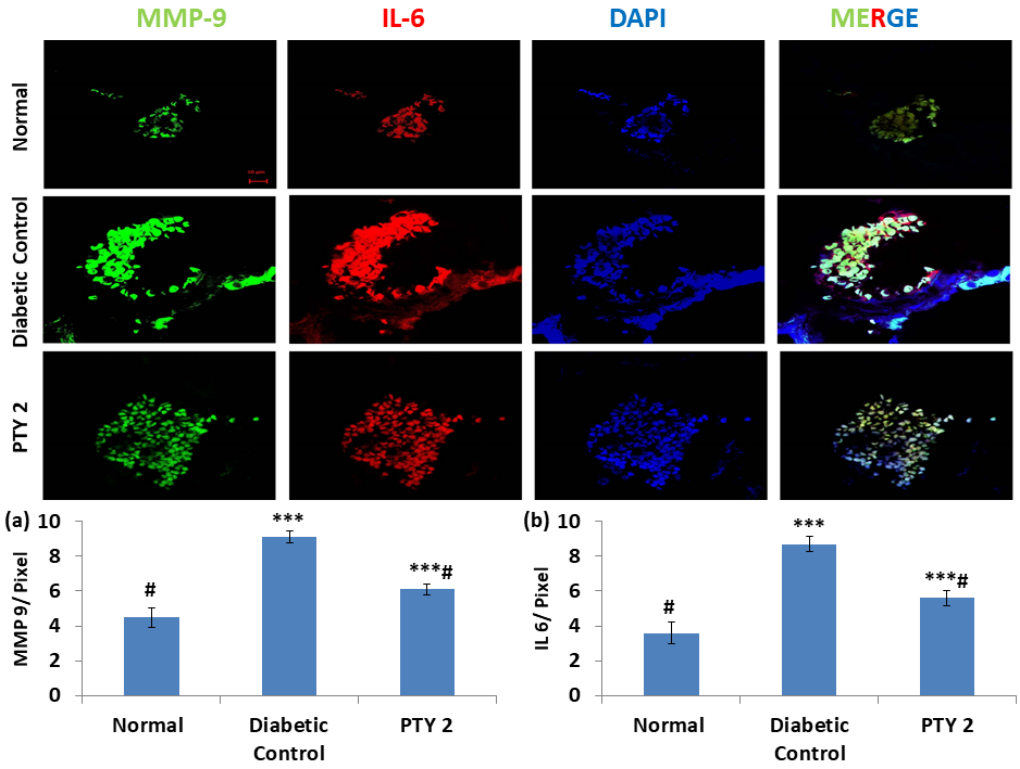

Figure 4. Immunohistochemistry analysis showed the effect of PTY-2 on the expression of (a) MMP-9 (green) and (b) IL-6 (red) in the islets of normal, diabetic control, and PTY-2-treated rats' pancreatic tissues. Both the expressions were merged with DAPI (blue). In comparison to diabetic control, PTY-2 down regulated the expression of both MMP-9 and IL-6. The images were taken at 63X magnification and scale bar was 10 μm. The intensity was measured in pixel values. Each value represent the mean ± SD ($n = 6$); $^{***}p < 0.05$, compared with Normal, $^{\#}p < 0.05$, compared with Diabetic Control.

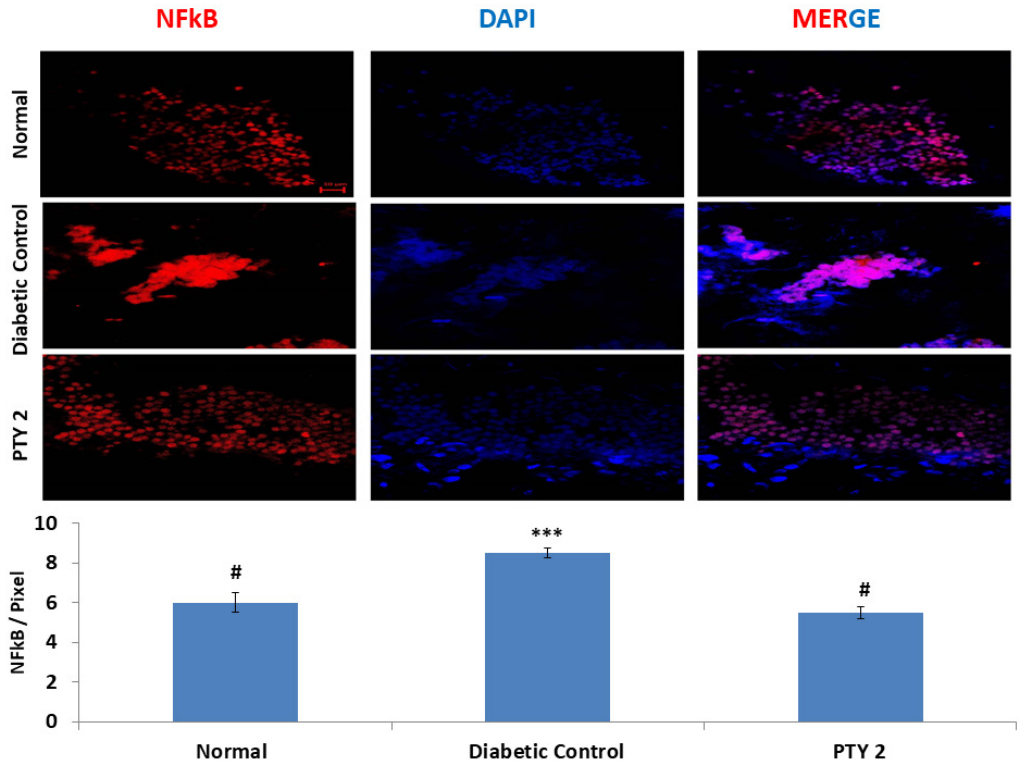

Figure 5. Immunohistochemistry analysis showed the effect of PTY-2 on the expression of NFkB (red) in the islets of normal, diabetic control, and PTY-2-treated rats' pancreatic tissues. The expression was merged with DAPI (blue) In comparison to diabetic control, PTY-2 down regulated the expression of NF-kB. The images were taken at 63X magnification and scale bar was 10 μm. The intensity was measured in pixel values. Each value represent the mean ± SD ($n = 6$); $^{***}p < 0.05$, compared with Normal, $^{\#}p < 0.05$, compared with Diabetic Control.

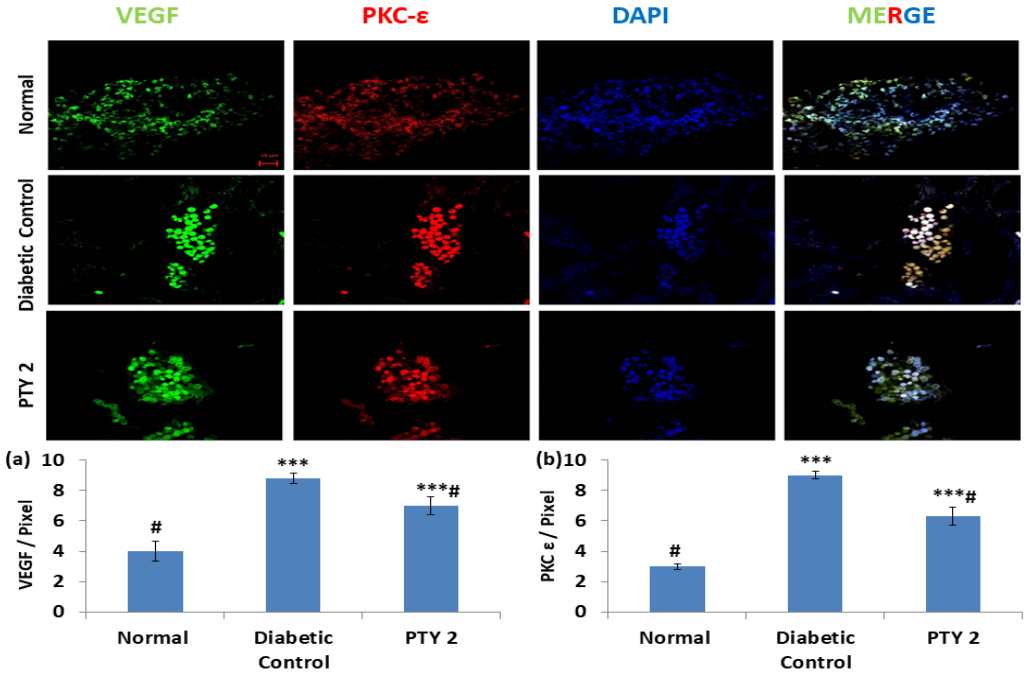

Figure 6. Immunohistochemistry analysis showed the effect of PTY-2 on the expression of (a) VEGF (green) and (b) PKC-ε (red) in the islets of normal, diabetic control, and PTY-2-treated rats' pancreatic tissues. Both the expressions were merged with DAPI (blue). In comparison to diabetic control, PTY-2 down regulated the expression of both VEGF and PKC ε. The images were taken at 63X magnification and scale bar was 10 μm. The intensity was measured in pixel values. Each value represent the mean ± SD ($n = 6$); $^{***}p < 0.05$, compared with Normal, $^{\#}p < 0.05$, compared with Diabetic Control.

significant to normal rats (Figures 3, 4, 5 and 6).

4. Discussion

The results of our study showed that, compared to the diabetic control group, the PTY-2 group had a favorable change in the expressions of biomarkers as assessed by RT-PCR, Western blot and Immunohistochemistry techniques. Analysis showed that STZ increased the expressions of MMP-9, TNF- α, HIF-1α, VEGF, IL-6, PKC-ε, NFkB and Caspase-3, which leads to the development of diabetic pathogenesis. But these expressions were significantly decreased by the treatment with PTY-2 for 10 days. Expression of SOD and Nephrin were significantly decreased among diabetic control rats, and these increased significantly after the administration of PTY-2. Thus, PTY-2 favorably changed the expressions of the biomarkers against islet stress.

Our earlier research had focused on the antidiabetic role of PT in STZ-induced diabetic model. We found that PT has an effect on inflammation (*15*) and hyperglycemia (*11-13*). Initially, the results showed that PTY-2 had hypoglycemic action because of inhibition of DPP-IV activity. In further research, we evaluated the effect on incretin receptors GLP-1R (Glucagon-like peptide 1 receptor), GIP-R (Glucose-dependent insulinotropic peptide receptor), and insulin. The results showed a significantly higher increase in plasma GLP-1 and GIP levels and a significant decrease in blood glucose concentrations after PTY-2 treatment (50 mg/100 g body weight) for 10 days. In the second study of chronic diabetes induced with STZ among rats, there was also a significant decrease in intestinal DPP-IV activity and an enhanced basal plasma insulin concentration in PTY-2 (earlier mentioned as PTWE) treated diabetic rats. Additionally, there was an increase in the number of islet cells and a significant increase in protein expression of insulin and B-cell lymphoma-2 (Bcl-2) in islet. According to *in silico* studies in our lab, Puerarone and Robinin were the two most effective phytochemicals for DPP-IV inhibition, and Tuberostan & Puererone of PTY-2 identified as the active component for GLP-1 and GIP receptors. Moreover, an *in-vivo* experiment showed that anti-inflammatory property of *Pueraria tuberosa* might be because of the scavenging of free radicals by increase in activity of SOD, and decrease in C-reactive protein levels (*15*).

Moving in the same direction of evaluation of the role of PTY-2 in chronic diabetes, we studied the effect of PTY-2 on the markers of oxidative stress, hypoxia, apoptosis and inflammation, which are known to play a significant role in the development and progression of pancreatitis and DM.

To understand and assimilate the results, the pathological changes in chronic diabetes along with the hypothetical mechanism of action of PTY-2 according to our study and previous reports have been diagrammatically shown in Graphical abstract (Figure 7). DM is a manifestation of abnormal metabolism and transport of glucose, and is associated with a decrease in insulin secretion and presence of insulin resistance. Further on, this leads to hyperglycemia and an increase

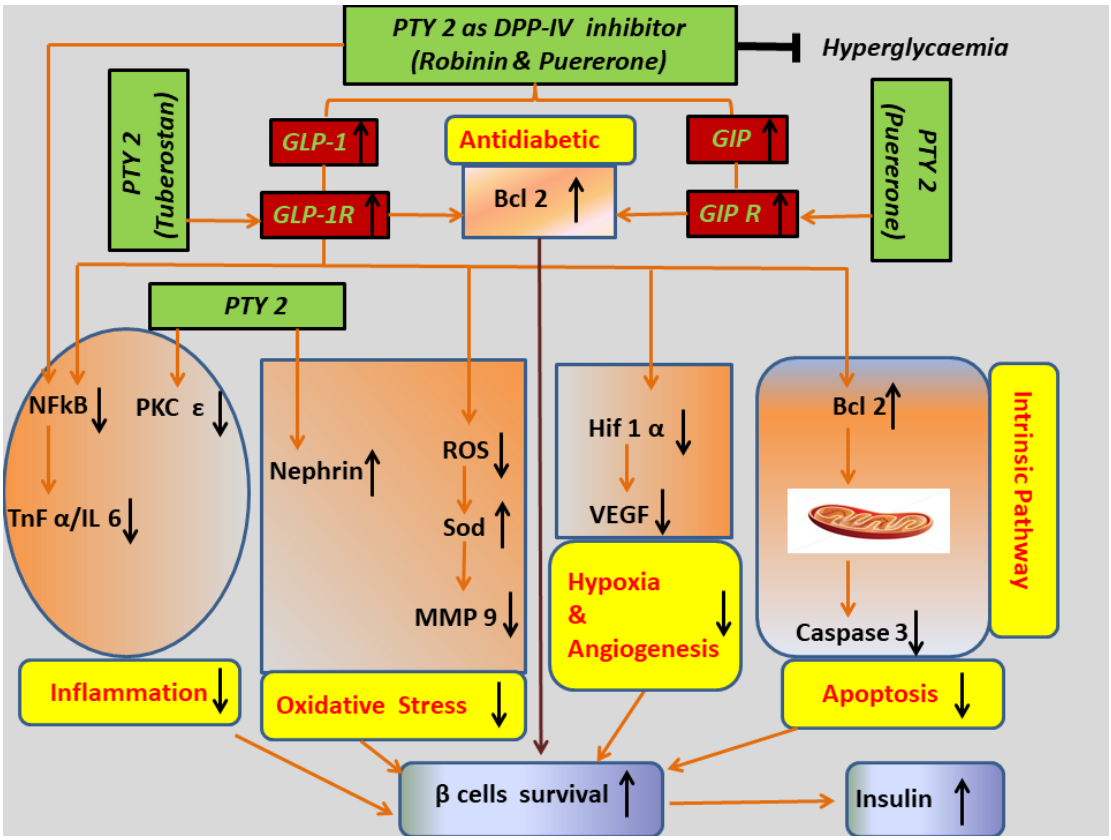

Figure 7. Mechanism of action of PTY-2 against STZ induced islet stress.

in the release of free fatty acids (FFAs) (*45*). All these inter-related steps decrease β-cell function and number, increase oxidative stress, induce apoptosis and endoplasmic reticulum stress, and increase the release of inflammatory cytokines (*45*). FFAs are known to induce the release of various interleukins, including IL-6, which further increase the release of free radicals and activate caspases. As shown in graphical abstract, NFκB, PKC-ε, TNF-α, and IL-6 are mediators of inflammation, and the administration of PTY-2 in our study was associated with a decrease in the expression of all these mediators. With the progression of diabetes, the balance between the pro-inflammatory, anti-inflammatory or protective mediators is disturbed (*45*). The increase in oxidative stress, measured by a decrease in SOD activity and an increase in ROS, was also decreased by PTY-2 administration. Other mediators of oxidative stress, *i.e.*, MMP-9 and VEGF, were also decreased. Hypoxia, which is measured with HIF 1α, is also an inducible factor of DM and was decreased by PTY-2 administration. Apoptosis, one of the critical pathological changes, is mediated by an increase in activity of caspases (*42*). PTY-2 led to a decrease in caspase-3 expression. Taking into consideration of our earlier evidence, it can be proposed that PTY-2 acts as a DPP-IV inhibitor, potentiates GLP-1 and GIP (*13*) mediated responses, and decreases inflammation, oxidative stress, apoptosis and hypoxia. GLP-1 agonists showed an inhibition of pro-inflammatory mediators in DM and other inflammatory conditions as well, in addition to their glucose-lowering potential (*46,47*).

MMP-9, one of the markers estimated in our study, deteriorates the inflammatory condition as it causes vascular injury, increases migration and cellular invasion by inflammatory cells (*22,33*). Both animal and human studies have shown an increase in MMP-9 expression in pancreatitis (*48,49*). MMP-9 acts as a diabetogenic factor by increasing proteolytic cleavage of insulin (*48,49*). Similar to our results, earlier studies have also shown an increase of MMP-9 activity in STZ induced models of DM (*22,48*). It is assumed that hyperglycemia induced oxidative stress induces the expression of MMP-9 in pancreas, and this can be counterbalanced with GLP-1 agonists (*22,50*). However, MMP-9 along with other paracrine factors is required for normal islet matrix turnover (*51*). IL-6, another biomarker, is also known to perform both inflammatory and protective roles (*45*). In type 1 DM, IL-6 participates in the regulation of balance between peripheral blood's regulatory T cells and Th17. In addition to this, IL-6 may contribute to both enhanced tissue insulin sensitivity and insulin resistance. Also, the increase of glucose concentration were found to co-exits with enhanced blood IL-6 concentration in patients with T2DM (*45*). However, PKC-ε inhibition/deletion is associated with an improvement in glucose homeostasis

(*39*). In a previous study, when the Psammomys (sand rats) were fed with high energy diet, they developed insulin resistance mimicking T2DM. Treatment with PKC-ε abrogated peptides prevented insulin resistance, hyperinsulinemia and pancreatic beta cell loss. It shows that the enhanced expression of PKC-ε in T2DM is associated with beta cell loss (*52*). In another study with culture of lipid-treated islets isolated from PKC-ε knockout (PKC-ε KO) mice, there occurred amplification of GSIS (glucose-stimulated-insulin-secretion), reinforcing the benefit of inhibition of PKC-ε (*39*). Our results also showed a significant increase of PKC-ε in the diabetic group, followed by a significant decrease in the PTY-2 group.

Pro-inflammatory and pro-apoptotic cytokines like IL-1β and TNF-α are involved in the development and progression of diabetes. NFκB is a transcription factor for mediating the cellular responses of inflammatory cytokines like IL-1β and TNF-α (*45*). NFkB pathways control cellular proliferation, inflammation, and immune responses through signal transduction (*21,53*). The activity of NFkB is increased in acute pancreatitis, and the longer duration of increased activity is associated with chronic diseases (*54-57*). Sitagliptin, a DPP-IV inhibitor used among T2DM patients, has shown anti-inflammatory action through the inhibition of NFκB, inflammatory cytokines and cell apoptosis (*58*). Mice deficient in NFκB have shown to be resistant to STZ induced diabetes (*59*).

TNF-α is a part of diabetes pathologenesis (*60*). The effects of TNF-α are mediated through the activation of NFkB pathway. An increase in the expression of TNF-α gene can enhance the risk of onset and progression of DM (*21*). Although the administration of TNF-α to animals is associated with insulin resistance and the regulation of TNF-α levels can improve insulin sensitivity, the status of TNF-α as a drug target for DM is still being evaluated. This might be possible with more understanding of the inter-relationships of the mediators in the pathogenesis of DM (*61*). A novel transcriptional inhibitor of TNF-α, MDL 201.449, has been found to reduce TNF-α mRNA levels dose dependently and prevent the development of hyperglycemia among mice following STZ injections (*73*).

Hyperglycemia also leads to the destabilization of HIF-1α, which is responsible for the regulation of the cellular responses to hypoxia. (*62,6*3). Hypoxia is an important cause of apoptosis and beta cell loss, and HIF-1α is an important indicator of beta cell loss (*64*). It is known that GLP-1R agonists (Extendin-4) improve islets survival through the activation of transcription factor, cAMP response element binding protein (CREB). A combination of CREB and Extendin-4 exerted enhanced anti-apoptotic action in cultured islets against hypoxia and cytokines. In an early phase, HIF-1α comes as a metabolic adaptation, but its prolonged activation enhances the expression of proapoptotic genes (64). Increased levels of caspases, along with the hypoxic state, are involved in beta cell apoptosis (*65*). Caspase-3, an important effector of the apoptotic pathways of DM, was also evaluated in our study *(42*). A study among Caspase-3 knockout ($Casp^{-/-}$) mice has shown that these mice were protected from the development of DM with a multiple low-dose administration of STZ, which, otherwise, causes selective β cell destruction and further triggers the immune reactions against islets (*42*). Studies with GLP-1 analogs among the animal models, *in-vitro* cell lines and human islet cells have shown a reduction in apoptosis, which was associated with a significant down-regulation of caspase-3 and up-regulation of bcl-2, and an increase in intracellular insulin content (*47,64,66,67*). In an earlier study, Puerarin, one of the components of PTY-2, decreased the expression of caspase-3 in osteoblasts of diabetic rats and improved the pathological changes (*68*).

HIF-1α is also a transcriptional activator of VEGF (*69*). VEGF, a pro-angiogenic growth factor, helps in the vascularization of the pancreatic islets (*31,70*). But overexpression of VEGF is fatal, as stated earlier. Oxidative stress, measured by the presence of ROS, is a promoter of angiogenesis (*70*). Currently, anti-VEGF therapy is approved for use in diabetic retinopathy (*71*). Additionally, the effect of DPP-IV inhibition and GLP-1 are being evaluated in diabetic ulcers and for cardiovascular protective role (*69,72*).

Hyperglycemia also impairs nephrin signaling by increasing its internalization and upregulates PKC-α expression. Thus, these expressions playing an interesting role against pancreatic β-cell loss in T2DM (*29*).

Currently, there is a need of antidiabetic agents with a wider spectrum of actions. As the roles of inflammation, oxidative stress, hypoxia, and apoptosis have become clearer over years, the currently available drugs should be re-evaluated for their effects on newer targets. Additionally, there is a need of newer agents which have action beyond the glucose-lowering potential. Various compounds have been studied for their role in the treatment of DM.

PTY-2 is a herbal medicine under evaluation for its role in DM. Along with these results, PTY-2 has also shown anti-diabetic action by inhibition of DPP-IV enzyme, by acting as incretins receptor agonist, and by decreasing β cell apoptosis. Further pre-clinical and clinical research can help in the utilization of PTY-2 as a treatment option in DM. PTY-2 can be a less costly treatment option as compared to the already available anti-diabetic synthetic drugs in market. As PTY-2 extract is composed of many phytochemicals, it can be effective for multiple diseases. On the other hand, the limitations of work shows that this study did not evaluate the role of individual phytochemicals of PTY-2 in DM. Overall, further post-translational studies are required to completely understand the protective effect of PTY-2 on islet.

5. Conclusion

Administration of PTY-2 for 10 days decreased the expressions of various biomarkers of oxidative stress, hypoxia, apoptosis and inflammation such as MMP-9, SOD, NFκB, VEGF, TNF-α, Caspase-3, IL-6, and HIF-1α as well as increased the expression of SOD and Nephrin among STZ-induced diabetic rats. Thus, PTY-2 protects diabetic islet through multi-targeted pathways. Further clinical research is needed to establish the role of PTY-2 in the treatment of DM.

Acknowledgements

We are thankful to the Head of Department, Dr. Amrita G. Kar, and all laboratory technicians of the Department of Pathology, IMS, BHU for their help in tissue slide preparations. Heartfelt thanks to ISLS, BHU for providing us the facility to perform Confocal Microscopy. Last but not least, we are grateful to Mrs. Durgavati Yadav, Mr. Vivek Pandey, Mrs. Rashmi Shukla, Mrs. Prerna Aditi and my escort Satish for helping us in sample processing.

References

1. World Health Organization. India: First to adapt the global monitoring framework on noncommunicable diseases (NCDs), 2015.
2. Yadav D, Lowenfels AB. The epidemiology of pancreatitis and pancreatic cancer. Gastroenterology. 2013; 144:1252-1261.
3. DiMagno MJ, DiMagno EP. Chronic pancreatitis. Curr Opin Gastroenterol. 2012; 28:523-531.
4. Alsamarrai A, Das SLM, Windsor JA, Petrov MS. Factors that affect risk for pancreatic disease in the general population: A systematic review and meta-analysis of prospective cohort studies. Clin Gastroenterol Hepatol. 2014; 12:1635-1644.e5.
5. Das SLM, Singh PP, Phillips ARJ, Murphy R, Windsor JA, Petrov MS. Newly diagnosed diabetes mellitus after acute pancreatitis: a systematic review and meta-analysis. Gut. 2014; 63:818-831.
6. Gupta S, Vittinghoff E, Bertenthal D, Corley D, Shen H, Walter LC, McQuaid K. New-onset diabetes and pancreatic cancer. Clin Gastroenterol Hepatol. 2006; 4:1366-1372.
7. Ben Q, Xu M, Ning X, Liu J, Hong S, Huang W, Zhang H, Li Z. Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. Eur J Cancer. 2011; 47:1928-1937.
8. Perrin MC, Terry MB, Kleinhaus K, Deutsch L, Yanetz R, Tiram E, Calderon R, Friedlander Y, Paltiel O, Harlap S. Gestational diabetes as a risk factor for pancreatic cancer: A prospective cohort study. BMC Med. 2007; 5:25.
9. Girman CJ, Kou TD, Cai B, Alexander CM, O'Neill EA, Williams-Herman DE, Katz L. Patients with type 2 diabetes mellitus have higher risk for acute pancreatitis compared with those without diabetes. Diabetes, Obes Metab. 2010; 12:766-771.
10. Rosendahl J, Bödeker H, Mössner J, Teich N. Hereditary chronic pancreatitis. Orphanet J Rare Dis. 2007; 2:1.
11. Srivastava S, Shree P, Pandey H, Tripathi YB. Incretin hormones receptor signaling plays the key role in antidiabetic potential of PTY-2 against STZ-induced pancreatitis. Biomed Pharmacother. 2018; 97:330-338.
12. Srivastava S, Koley TK, Singh SK, Tripathi YB. The tuber extract of *Pueraria tuberosa* Linn. competitively inhibits DPP-IV activity in normoglycemic rats. Int J Pharm Pharm. Sci. 2015; 7:7-11.
13. Srivastava S, Shree P, Tripathi YB. Active phytochemicals of *Pueraria tuberosa* for DPP-IV inhibition: *in silico* and experimental approach. J Diabetes Metab Disord. 2017; 16:46.
14. Tripathi AK, Kohli S. Anti-diabetic activity and phytochemical screening of crude extracts of *Pueraria tuberosa* DC. (FABACEAE) grown in India on STZ -induced diabetic rats. Asian J Med Pharm Res. 2013; 3:66-73.
15. Pandey N, Yadav D, Pandey V, Tripathi YB. Anti-inflammatory effect of *Pueraria tuberosa* extracts through improvement in activity of red blood cell anti-oxidant enzymes. Ayu. 2013; 34:297-301.
16. Srivastava S, Yadav D, Tripathi YB. DPP-IV Inhibitory potential of methanolic extract of *Pueraria tuberosa* in liver of alloxan induced diabetic model. Biosci Biotechnol Res Asia. 2018; 15:1-4.
17. Shukla R, Pandey N, Banerjee S, Tripathi YB. Effect of extract of *Pueraria tuberosa* on expression of hypoxia inducible factor-1α and vascular endothelial growth factor in kidney of diabetic rats. Biomed Pharmacother. 2017; 93:276-285.
18. Shukla R, Banerjee S, Tripathi YB. *Pueraria tuberosa* extract inhibits iNOS and IL-6 through suppression of PKC-α and NF-kB pathway in diabetes-induced nephropathy. J Pharm Pharmacol. 2018; 70:1102-1112.
19. Verma SK, Jain V, Singh DP. Effect of *Pueraria tuberosa* DC. (Indian Kudzu) on blood pressure, fibrinolysis and oxidative stress in patients with stage 1 hypertension. Pakistan J Biol Sci. 2012; 15:742-747.
20. Srivastava S, Pandey H, Singh SK, Tripathi YB. PTY 2 as DPP-IV inhibitor prevents intestinal cell's apoptosis. bioRxiv. 2019; doi:10.1101/670430.
21. Burke SJ, Lu D, Sparer TE, Karlstad MD, Collier JJ. Transcription of the gene encoding TNF-α is increased by IL-1β in rat and human islets and β-cell lines. Mol Immunol. 2014; 62:54-62.
22. Uemura S, Matsushita H, Li W, Glassford AJ, Asagami T, Lee KH, Harrison DG, Tsao PS. Diabetes mellitus enhances vascular matrix metalloproteinase activity: role of oxidative stress. Circ Res. 2001; 88:1291-1298.
23. Shibaji T, Nagao M, Ikeda N, Kanehiro H, Hisanaga M, Ko S, Fukumoto A, Nakajima Y. Prognostic significance of HIF-1 alpha overexpression in human pancreatic cancer. Anticancer Res. 2003; 23:4721-4727.
24. Pini M, Rhodes DH, Castellanos KJ, Hall AR, Cabay RJ, Chennuri R, Grady EF, Fantuzzi G. Role of IL-6 in the resolution of pancreatitis in obese mice. J Leukoc Biol. 2012; 91:957-966.
25. Reinert RB, Brissova M, Shostak A, Pan FC, Poffenberger G, Cai Q, Hundemer GL, Kantz J, Thompson CS, Dai C, McGuinness OP, Powers AC. Vascular endothelial growth factor-A and islet vascularization are necessary in developing, but not adult, pancreatic islets. Diabetes. 2013; 62:4154-4164.
26. Singh N, Bhardwaj P, Pandey RM, Saraya A. Oxidative

stress and antioxidant capacity in patients with chronic pancreatitis with and without diabetes mellitus. Indian J Gastroenterol. 2012; 31:226-231.
27. Dabhi B, Mistry KN. Oxidative stress and its association with TNF-α-308 G/C and IL-1α-889 C/T gene polymorphisms in patients with diabetes and diabetic nephropathy. Gene. 2015; 562:197-202.
28. Putaala H, Soininen R, Kilpeläinen P, Wartiovaara J, Tryggvason K. The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. Hum Mol Genet. 2001; 10:1-8.
29. Kapodistria K, Tsilibary EP, Politis P, Moustardas P, Charonis A, Kitsiou P. Nephrin, a transmembrane protein, is involved in pancreatic beta-cell survival signaling. Mol Cell Endocrinol. 2015; 400:112-128.
30. Watada H. Role of VEGF-A in pancreatic beta cells. Endocr J. 2010; 57:185-191.
31. Brissova M, Shostak A, Shiota M, Wiebe PO, Poffenberger G, Kantz J, Chen Z, Carr C, Jerome WG, Chen J, Baldwin HS, Nicholson W, Bader DM, Jetton T, Gannon M, Powers AC. Pancreatic islet production of vascular endothelial growth factor-A is essential for islet vascularization, revascularization, and function. Diabetes. 2006; 55:2974-2985.
32. Reinert RB, Cai Q, Hong JY, Plank JL, Aamodt K, Prasad N, Aramandla R, Dai C, Levy SE, Pozzi A, Labosky PA, Wright CVE, Brissova M, Powers AC. Vascular endothelial growth factor coordinates islet innervation *via* vascular scaffolding. Development. 2014; 141:1480-1491.
33. Zhen GD, Zhao LB, Wu SS, Chen MY, Li ZH, Zhou SZ, Li ZF. Associations of MMP-2 and MMP-9 gene polymorphism with ulinastatin efficacy in patients with severe acute pancreatitis. Biosci Rep. 2017; 37:BSR20160612.
34. De Palma AM, Verbeken E, Van Aelst I, Van den Steen PE, Opdenakker G, Neyts J. Increased gelatinase B/matrix metalloproteinase 9 (MMP-9) activity in a murine model of acute coxsackievirus B4-induced pancreatitis, Virology. 2008; 382:20-27.
35. Opdenakker G, Van den Steen PE, Van Damme J. Gelatinase B: a tuner and amplifier of immune functions. Trends Immunol. 2001; 22:571-579.
36. Rakonczay Z, Hegyi P, Takacs T, McCarroll J, Saluja AK. The role of NF- B activation in the pathogenesis of acute pancreatitis. Gut. 2008; 57: 259-267.
37. Patel S, Santani D. Role of NF-kappa B in the pathogenesis of diabetes and its associated complications. Pharmacol Rep. 2009; 61:595-603.
38. Swaroop JJ, Rajarajeswari D, Naidu JN. Association of TNF-α with insulin resistance in type 2 diabetes mellitus. Indian J Med Res. 2012; 135:127-130.
39. Cantley J, Burchfield JG, Pearson GL, Schmitz-Peiffer C, Leitges M, Biden TJ. Deletion of PKC epsilon selectively enhances the amplifying pathways of glucose-stimulated insulin secretion *via* increased lipolysis in mouse beta-Cells. Diabetes. 2009; 58:1826-1834.
40. Kim MJ, Lee YS, Lee KH, Min DS, Yoon SH, Hahn SJ, Kim MS, Jo YH. Site-specific localization of protein kinase C isoforms in rat pancreas. Pancreatology. 2001; 1:36-42.
41. Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, Lehmann R, Candinas D, Gassmann M, Weber M. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1α expression. FASEB J. 2002; 16:745-747.
42. Liadis N, Murakami K, Eweida M, Elford AR, Sheu L, Gaisano HY, Hakem R, Ohashi PS, Woo M. Caspase-3-dependent beta-cell apoptosis in the initiation of autoimmune diabetes mellitus. Mol Cell Biol. 2005; 25:3620-3629.
43. Asthana S, Agarwal T, Singothu S, Samal A, Banerjee I, Pal K, Pramanik K, Ray SS. Molecular docking and interactions of *Pueraria tuberosa* with vascular endothelial growth factor receptors. Indian J Pharm Sci. 2015; 77:439-445.
44. Pandey N, Tripathi YB. Antioxidant activity of tuberosin isolated from P*ueraria tuberose* Linn. J Inflamm (Lond). 2010; 7:47.
45. Cieślak M, Wojtczak A, Cieślak M. Role of pro-inflammatory cytokines of pancreatic islets and prospects of elaboration of new methods for the diabetes treatment. Acta Biochim Pol. 2015; 62:15-21.
46. Lee YS, Jun HS. Anti-inflammatory effects of GLP-1-based therapies beyond glucose control. Mediators Inflamm. 2016; 2016:1-11.
47. Farilla L, Hui H, Bertolotto C, Kang E, Bulotta A, Di Mario U, Perfetti R. Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker Diabetic rats. Endocrinology. 2002; 143: 4397-4408.
48. Descamps FJ, Martens E, Ballaux F, Geboes K, Opdenakker G. *In vivo* activation of gelatinase B/MMP-9 by trypsin in acute pancreatitis is a permissive factor in streptozotocin-induced diabetes. J Pathol. 2004; 204:555-561.
49. Descamps FJ, Van den Steen PE, Martens E, Ballaux F, Geboes K, Opdenakker G. Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin. FASEB J. 2003; 17: 887-889.
50. Ceriello A, La Sala L, De Nigris V, Pujadas G, Rondinelli M, Genovese S. GLP-1 reduces metalloproteinase-9 induced by both hyperglycemia and hypoglycemia in type 1 diabetes. The possible role of oxidative stress. Ther Clin Risk Manag. 2015; 11:901-903.
51. Christoffersson G, Waldén T, Sandberg M, Opdenakker G, Carlsson PO, Phillipson M. Matrix Metalloproteinase-9 is essential for physiological Beta cell function and islet vascularization in adult mice. Am J Pathol. 2015; 185:1094-1103.
52. Mack E, Ziv E, Reuveni H, Kalman R, Niv MY, Jörns A, Lenzen S, Shafrir E. Prevention of insulin resistance and beta-cell loss by abrogating PKCε-induced serine phosphorylation of muscle IRS-1 in Psammomys obesus. Diabetes Metab Res Rev. 2008; 24:577-584.
53. Croft M, Benedict CA, Ware CF. Clinical targeting of the TNF and TNFR superfamilies. Nat Rev Drug Discov. 2013; 12:147-168.
54. Chen W, Zheng Z, Duan J, Wang X, Wu S, Wang W, Xu L, Han S, Qiao Z. Quantitation of nuclear factor kappa B activation in pancreatic acinar cells during rat acute pancreatitis by flow cytometry. Int J Clin Exp Med. 2015; 8:10143-10151.
55. Steinle AU, Weidenbach H, Wagner M, Adler G, Schmid RM. NF-κB/Rel activation in cerulein pancreatitis. Gastroenterology. 1999; 116:420-430.
56. Algül H, Treiber M, Lesina M, Nakhai H, Saur D, Geisler F, Pfeifer A, Paxian S, Schmid RM. Pancreas-specific RelA/p65 truncation increases susceptibility of acini to inflammation-associated cell death following cerulein pancreatitis. J Clin Invest. 2007; 117:1490-1501.

57. Fantini L, Tomassetti P, Pezzilli R. Management of acute pancreatitis: Current knowledge and future perspectives. World J Emerg Surg. 2006; 1:16.
58. Hu X, Liu S, Liu X, Zhang J, Liang Y, Li Y. DPP-4 (CD26) inhibitor sitagliptin exerts anti-inflammatory effects on rat insulinoma (RINm) cells *via* suppressing NF-κB activation. Endocrine. 2017; 55:754-763.
59. Liuwantara D, Elliot M, Smith MW, Yam AO, Walters SN, Marino E, McShea A, Grey ST. Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. Diabetes. 2006; 55:2491-2501.
60. Mandrup-Poulsen T. Apoptotic signal transduction pathways in diabetes. Biochem Pharmacol. 2003; 66:1433-1440.
61. DE M. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. Trends Endocrinol Metab. 2000; 11: 21-27.
62. Catrina SB, Okamoto K, Pereira T, Brismar K, Poellinger L. Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. Diabetes. 2004; 53:3226-3232.
63. Bento CF, Pereira P. Regulation of hypoxia-inducible factor 1 and the loss of the cellular response to hypoxia in diabetes. Diabetologia. 2011; 54:1946-1956.
64. Velmurugan K, Balamurugan AN, Loganathan G, Ahmad A, Hering BJ, Pugazhenthi S. Antiapoptotic actions of exendin-4 against hypoxia and cytokines are augmented by CREB. Endocrinology. 2012; 153:1116-1128.
65. Greijer AE, van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J Clin Pathol. 2004; 57:1009-1014.
66. Farilla L, Bulotta A, Hirshberg B, Li Calzi S, Khoury N, Noushmehr H, Bertolotto C, Di Mario U, Harlan DM, Perfetti R. Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. Endocrinology. 2003; 144:5149-5158.
67. Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like Peptide-1 receptor signaling modulates β cell apoptosis. J Biol Chem. 2003; 278:471-478.
68. Liang J, Chen H, Pan W, Xu C. Puerarin inhibits caspase-3 expression in osteoblasts of diabetic rats. Mol Med Rep. 2012; 5:1419-1422.
69. Marfella R, Sasso FC, Rizzo MR, Paolisso P, Barbieri M, Padovano V, Carbonara O, Gualdiero P, Petronella P, Ferraraccio F, Petrella A, Canonico R, Campitiello F, Della Corte A, Paolisso G, Canonico S. Dipeptidyl peptidase 4 inhibition may facilitate healing of chronic foot ulcers in patients with type 2 diabetes. Exp Diabetes Res. 2012; 2012:892706.
70. El-Refaei MF, Abduljawad SH, Alghamdi AH. Alternative medicine in diabetes – role of angiogenesis, oxidative stress, and chronic inflammation. Rev Diabet Stud. 2014; 11:231-244.
71. Osaadon P, Fagan XJ, Lifshitz T, Levy J. A review of anti-VEGF agents for proliferative diabetic retinopathy. Eye. 2014; 28:510-520.
72. Xiao-Yun X, Zhao-Hui M, Ke C, Hong-Hui H, Yan-Hong X. Glucagon-like peptide-1 improves proliferation and differentiation of endothelial progenitor cells *via* upregulating VEGF generation. Med Sci Monit. 2011; 17:BR35-41.
73. Holstad M, Sandler S, A transcriptional inhibitor of TNF-α prevents diabetes induced by multiple low-dose streptozotocin injections in mice, J Autoimmun. 2001; 16:441-447.

(Received July 16, 2019; Revised September 24, 2019; Accepted October 4, 2019)