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Anti-hyperglycemic, Immunomodulatory and Anti-oxidant Efficacy of Vasoactive Intestinal Peptide in Streptozotocin-Induced Diabetic Mice

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Abstract: The purpose of this study was to assess the effects of vasoactive intestinal peptide (VIP) administration on the glycemic state, insulin secretion, various pro-inflammatory and anti-inflammatory cytokines, liver pathology and oxidative stress as well as antioxidant defense system in streptozotocin-induced diabetic mice. VIP was intraperitoneally administered to normal and streptozotocin-induced diabetic CD1 mice at a dose level of 41.6 ng kg^{-1} b.wt., 3 times/week for 8 consecutive weeks to form a total dose of 1 μg kg^{-1} b.wt. at the end of the experiment. The VIP was found to have anti-hyperglycemic and insulinotropic properties and improving effects on the islets of Langerhans and liver pathology of diabetic mice. These VIP ameliorating-changes may be mediated via its augmenting effect on anti-inflammatory cytokines (IL-10 and IL-12) and decreasing effect on pro-inflammatory cytokine, TNF-α, as well as its potency in suppressing the oxidative stress and enhancing the anti-oxidant defense system. However, further clinical studies are required to assess the efficacy and safety of VIP in diabetic human beings.

Key words: Diabetes mellitus, vasoactive intestinal peptide, oxidative stress, cytokines

INTRODUCTION

Insulin-Dependent Diabetes Mellitus (IDDM), type 1 diabetes mellitus, is a chronic inflammatory disorder characterized by autoimmune destruction of beta-cells and absolute insulin deficiency resulting in a series of complex metabolic disturbances (Eisenbarth, 2004; Poirot *et al*., 2004; Fidan *et al*., 2005). It can be induced in laboratory animals by injection of chemicals, like streptozotocin (Ahmed *et al*., 2007; Haidara *et al*., 2009), which selectively destroys the β-cells in the pancreas. Animal models of IDDM are characterized by several disorders of both humoral and cellular immunological parameters (Marliss *et al*., 1982; Kolb *et al*., 1986; Mordes *et al*., 1987). IDDM is associated with cellular infiltration and an inflammatory response in the islets of Langerhans (Nielsen *et al*., 1999). Cellular components of this infiltration include monocytes, macrophages and $CD4^+$ and $CD8^+$ T-cells (Mandrup-Poulsen, 1996). Several cytokines are expressed from these infiltrated cells in humans or animal models with insulin dependent diabetes mellitus (Mandrup-Poulsen, 1996; Rabinovitch, 1998; Rabinovitch and Suarez-Pinzon, 1998). The pattern of network in which these cytokines act is very complex. A specific cytokine might either amplify or counteract the effects of other cytokines (Holstad, 2001). In general, the balance between T-helper (Th)-1 type and T-helper (Th)-2 is crucial in the pathogenesis of IDDM (Rabinovitch, 1998; Amirshahrokhi *et al*., 2008). A shift toward the proinflammatory Th-1 cytokines promotes insulitis and IDDM (Rabinovitch *et al*., 1995; Amirshahrokhi *et al*., 2008). Previous experimental data suggested that pro-inflammatory and inflammatory cytokines, interleukin 1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-12 and nitric oxide (NO) could play a fundamental role in a progressive autoimmune destruction of β-cells in the islets of Langerhans in IDDM (Nerup *et al*., 1994; Mandup-

Poulsen, 1996; Eizirik *et al*., 1996; Kubisch *et al*., 1997; Nicoletti *et al*., 1999; Wen *et al*., 2006). Th2 anti-inflammatory cytokines, IL-4, IL-5 and IL-10, have been found to suppress insulitis, beta-cell damage and IDDM in multiple-low-dose streptozotocin (MLDS)-induced diabetic mice and in non-obese diabetic (NOD) mice (Pennline *et al*., 1994; Cameron *et al*., 1997; Amirshahrokhi *et al*., 2008). Thus, the preponderance of Th2-anti-inflammatory cytokines may prevent the incidence, or delay the onset, of IDDM.

There is evidence that oxidative stress might be implicated in promoting a state of systemic inflammation and an increase of inflammatory cytokines in diabetes mellitus (Gumieniczek *et al*., 2006). Diabetes induced in Sprague-Dawley rats by streptozotocin caused a significant increase in blood lipid peroxidation associated with profound elevation of pro-inflammatory cytokines, TNF-α and IL-6 levels, similar to those in diabetic patients (Jain *et al*., 2007; Vinson, 2007); the treatment of these animals with antioxidants decreased these elevations. Nielsen *et al*. (1999) suggested that cytokine-free radicals in β-cells may be involved in β-cells-specific destruction in IDDM. Sumoski *et al*. (1989) and Tabatabaie *et al*. (2003) postulated that the free-radicals evoked by pro-inflammatory cytokines may be strongly involved in the destruction of β-cells in the course of type 1 diabetes development. Also, the reactive oxygen species are considered as a strong stimulus for the release of cytokines (Vassilakopoulos *et al*., 2003).

Vasoactive intestinal peptide (VIP), a neuropeptide synthesized by immune cells, can modulate several immune aspects including the function of cells which are involved in the inflammatory response (Metwali *et al*., 2002). It has been shown that VIP promotes Th2 differentiation, inhibits Th1 responses, inhibits *in vitro* and *in vivo* production of the pro-inflammatory cytokines TNF-α, IL-2, IL-6, IL-12, IFN-γ and of Nitric oxide (NO) and stimulates the production of the anti-inflammatory cytokines IL-10 and IL-1Ra (Sun and Ganea, 1993; Hernandez *et al*., 1997; Dewit *et al*., 1998; Martinez *et al*., 1998; Xin and Sriram, 1998; Delgado *et al*., 1999a-d, 2002). In addition to the immunoregulatory role, VIP was found to evoke profound increases in insulin secretion from the pancreas of normal and diabetic rats (Adeghate *et al*., 2001; Winzell and Ahren, 2007). In addition, there are many reports that revealed a deficiency of VIP content and VIP receptors in different body tissues of diabetic animals (Noda *et al*., 1990; Adeghate *et al*., 2001).

The study aims to assess the effect of VIP on the glycemic state, insulin secretion, immunoregulation and oxidative stress as well as on liver and pancreas histological changes of diabetic mice.

MATERIALS AND METHODS

Date and Location of the Research

The research was conducted in the Zoology Department, Faculty of Science, Beni-Sueif University, Egypt in the period between 1 April/2007 and 1 October/2008.

Animals

Male 8-week old CD1 albino mice were supplied from the Schistosome Biological Supply Center (SBSC), Theodore Bilharz Research Institute (TBRI, Imbaba, Giza, Egypt). Mice were maintained under suitable living conditions in the animal house (Zoology Department, Faculty of Science, Beni-Suef University, Egypt) at controlled temperature (20-25 °C and natural daily 12 h light-dark cycles throughout the experiment. They were supplied with food pellets, which contain at least 25.4% protein and tap water *ad libitum*. They were also kept under observation for 1 week before the onset of the experiment to exclude any intercurrent infection and for proper acclimatization. All animal procedures follow the animal research bioethics of SBSC, TBRI and are in accordance with the recommendations for the proper care and use of laboratory animals (CCAC, 1993).

Vasoactive Active Intestinal Peptide (VIP)

Synthetic porcine VIP was obtained from Sigma Chemical Co., St. Louis, MO, USA. It was reconstituted in sterile 0.9% saline and 0.1% gelatin containing 0.5% albumin as described by Karlsson and Ahren (1990) and Allam (2007). The peptide was divided into aliquots and immediately stored at -70°C until use.

Induction of Insulin-Dependent Diabetes Mellitus

Experimental diabetes mellitus was induced in overnight fasted CD1 albino mice by intraperitoneal injection of streptozotocin (STZ) (Sigma Chemical company, USA) at dose level of 150 mg kg^{-1} body weight (b.wt.) dissolved in citrate buffer (pH 4.5) (Miyamoto *et al*., 2008). Ten days after STZ injection, blood samples were obtained from lateral tail vein of mice that had been deprived of food for 10-12 and 2 h after oral glucose loading (3 g kg^{-1} b.wt.). Mice having 2 h serum glucose concentration that ranged from 180 to 300 mg dL^{-1} were considered mild diabetics and included in the experiment.

Experimental Design

Experimental animals used in this study were allocated into 4 groups. Both the normal treated and diabetic treated groups were intraperitoneally administered VIP at a dose level of 41.6 ng kg^{-1} b.wt. 3 times/week for 8 consecutive weeks. These consecutive doses form a total dose of 1 μg kg^{-1} b.wt. at the end of the experiment (Allam, 2007). The other two groups consisted of normal and diabetic control mice and were given the equivalent amount of the vehicle in which VIP is dissolved at the same time as the treated groups.

Blood and Tissue Sampling

Blood samples were obtained from the lateral tail vein of normal, diabetic, normal treated and diabetic treated mice deprived of food overnight at fasting state and after 2 h of oral glucose loading (3 g kg^{-1} b.wt.) at the day before sacrifice. Serum glucose concentration was measured. The mice were sacrificed and blood samples were collected from the lateral jugular vein. Sera were separated from the clotted blood samples after centrifugation at 3000 rpm for 15 min, then were divided into aliquots and stored at -70°C until use. The liver and pancreas from each mouse were immediately excised and rinsed in ice-cold saline solution. Portion of each liver and pancreas were fixed in 10% neutral buffered formalin for preparation of hematoxylin and eosin-stained sections. Another portion of liver was homogenized by Teflon homogenizer (Glas-Col, USA) in 0.9% saline (0.5 g tissue/5 mL saline), centrifuged at 3000 rpm for 15 min and the supernatant was decanted and stored at -70°C until use for different biochemical estimations.

Histopathological Investigations

The fixed organs were transferred to the National Cancer Institute, Cairo University, Egypt for blocking, sectioning at 5 μm thickness and staining with hematoxylin and eosin. The stained sections were examined to detect histological changes.

Cytokine Measurement

Cytokines level was measured by using sandwich Enzyme-Linked Immunosorbent Assay (ELISA) in the Virology Research Laboratory (Faculty of Veterinary, Beni-Suef University, Egypt). Cytokine concentrations for mouse IL-10, IL-12 +p40 and TNF-α were determined with commercially available reagents and ELISA kits purchased from BioSource International (Camarillo, California, USA). Cytokine concentrations were determined using a standard curve obtained from the known concentration of cytokine standards included in each assay plate according to the manufacture's instructions.

Biochemical Analysis

All biochemical determinations were performed in the Zoology Department Research Laboratory (Faculty of Science, Beni-Suef University, Egypt) except for those mentioned elsewhere. Humalyzer 2000 Chemistry Analyzer (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) was used for spectrophotometric and colorimetric determinations.

Serum glucose concentration was determined according to the enzymatic method described by Trinder (1969) using reagent kits obtained from BioMerieux Chemical Company (France). Serum insulin and C-peptide concentrations were measured according to the methods of Marschner *et al*. (1974) and Bonser and Garcia-Webb (1984), respectively, in the Radioactive Isotopes Unit, National Research Center (Dokki, Cairo, Egypt) using radioimmunoassay kits supplied by Diagnostic Products Corporation (Los Angeles, USA) through Gamma Trade Company (Mohandeseen, Cairo, Egypt).

Hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the homogenate supernatant were estimated according to the kinetic method of Bergmyeyer *et al*. (1978), using reagent kits purchased from Spinreact Company (Spain). Hepatic γ-glutamyl transferase (γ-GT) was determined according to the kinetic method of Young (1990) and Tietz (1991) using reagent kits obtained from BioSystems (Spain). Liver LDH activity was determined according to the method described by Tietz (1986), using reagent kits purchased from Dialab (Wien, Austria).

The amount of collagen in the liver samples was determined from its hydroxyproline content by the method of Neuman and Logan (1950a , b). Briefly, the defatted and dried samples were hydrolyzed by autoclaving in 2 mL of 6N HCl at 50 pounds pressure. The protein hydrolysates were used for the determination of hydroxyproline and the percentage of collagen of a tissue sample was calculated by using the 7.46 factor of Neuman and Logan (1950b) and Baykal-Erkilic *et al*. (1995). Meanwhile, a standard curve was constructed in order to determine the reproducibility of the assay.

The levels of hepatic reduced glutathione (GSH), total thiol and lipid peroxidation products were determined by the methods of Bentler *et al*. (1963), Koster *et al*. (1986) and Preuss *et al*. (1998), respectively. Liver catalase was determined according to the technique of Cohen *et al*. (1970). The results of catalase activity were expressed in terms of the first-order reaction rate constant (k) as indicated by Cohen *et al*. (1970). Heme peroxidases (myeloperoxidase and eosinophil peroxidase) activity in the liver of normal and diabetic control mice and diabetic mice treated with VIP was assayed according to the methods of Chance and Maehley (1955) and Kar and Mishra (1976) with some modifications, using pyrogallol as a substrate (Status, 1958; Lee *et al*., 1988; Pruitt *et al*., 1990; Conyers and Kidwell, 1991; Joseph *et al*., 1992) in the presence of H_2O_2. To 25 µL of supernatant, 2 mL phosphate buffer solution (pH 6.8), 100 µL 1.4% pyrogallol and 100 µL 1.1 mmole H_2O_2 were added. After exactly 5 min, the density of color of formed purpurogallin was measured against the blank by taking absorbance at 420 nm. The enzyme activity for each sample was obtained from the standard curve performed by using different dilutions of heme peroxidase (EC.1.11.1.7) (Sigma-Aldrich Company, USA).

Serum Carbohydrate Antigen (CA) 19.9 concentration was estimated by an immunoradiometric assay kit (Coat-A-Count GI-MA IRMA, DPC, USA) according to the method of Frebourg *et al*. (1988). α-Fetoprotein (AFP) in liver homogenate supernatant was determined by a radioimmunoassay (RIA) kit (Double antibody kit, DPC, USA) according to the methods of Waldmann and Mclntire (1974) and Krikpatrick *et al*. (1977). Both CA19.9 and AFP concentrations were measured in the Radioactive Isotopes Unit, National Research Center (Dokki, Cairo, Egypt)

Statistical Analysis

The data were analyzed using one way Analysis of Variance (ANOVA) followed by LSD analysis to compare various groups with each other using PC-STAT program, University of Georgia, USA

(Rao *et al*., 1985). The results were expressed as Mean±SE and values of $p>0.05$ were considered statistically insignificant, while those of $p<0.05$ and $p<0.01$ were considered statistically significant and highly significant, respectively. F-probability for each variable expressed the general effect between groups.

RESULTS AND DISCUSSION

Biochemical Results

The diabetic animals exhibited a significant elevation ($p<0.01$) in serum glucose levels at fasting state and 2 h after oral glucose loading concomitant with the depletion ($p<0.01$) of insulin and C-peptide levels at fasting state. The treatment of diabetic mice with VIP for 8 weeks induced a potential amelioration ($p<0.01$) of these variables. With regards to one way-ANOVA of serum glucose, insulin and C-peptide levels, it was found that the effect between groups was very highly significant ($p<0.001$) throughout the experiment (Table 1).

Hepatic ALT, AST and LDH activities exhibited significant increases in the diabetic mice while γ-GT activity was potentially decreased ($p<0.05$). The administration of VIP to diabetic mice induced profound depletion ($p<0.01$) of the elevated ALT activity to reach value that was significantly below ($p<0.05$) the normal level. AST, LDH and γ-GT activities, altered in diabetic mice, were potentially ameliorated as a result of treatment with VIP. However the effect of VIP on ALT, AST and LDH activities of diabetic mice was significant and the effect on γ-GT activity was non-significant. With regards one-way ANOVA, the general effect between groups was found to be very highly significant ($p<0.001$) on ALT and AST activities and highly significant ($p<0.01$) on LDH and γ-GT activities throughout the experiment (Table 2).

Table 1: Effect of VIP administration on serum glucose, insulin and C-peptide levels of normal and diabetic male mice

	Parameters			
	Glucose (mg dL^{-1})			
Groups	Fasting	2 h	Insulin (μ IU mL^{-1})	C-peptide (pmole L^{-1})
Normal	66.73±2.14^{e}	103.97±2.27^{d}	16.25±0.56^{a}	278.33±2.79^{ab}
Normal treated with VIP	64.53±1.79^{e}	98.28±2.19^{d}	16.00±0.32^{a}	270.00±1.67^{bc}
Diabetic	140.15±7.27^{c}	271.58±21.79^{a}	8.50±0.22^{c}	176.25±2.64^{d}
Diabetic treated with VIP	79.70±5.45^{de}	197.79±3.04^{b}	12.67±0.21^{b}	282.50±1.71^{a}
F-probability	p<0.001		p<0.001	p<0.001
LSD at the 5% level	24.694		1.05	6.96
LSD at the 1% level	33.256		1.43	9.49

Data are expressed as Mean±SE. Number of animals in each group is 10. Mean values with the same superscript letter(s) are not significantly different

Table 2: Effect of VIP administration on liver enzyme activities of normal and diabetic male mice

	Parameters			
Groups	ALT (mU/100 mg)	AST (mU/100 mg)	LDH (mU/100 mg)	γ-GT (mU g^{-1})
Normal	379.77±28.59^{b}	237.20±4.24^{c}	47.43±2.10^{b}	53.08±6.89^{a}
Normal treated with VIP	416.28±44.39^{ab}	252.00±3.10^{c}	47.40±1.57^{b}	44.26±1.61^{a}
Diabetic	494.15±38.44^{a}	477.45±45.68^{a}	59.75±5.68^{a}	33.87±0.90^{b}
Diabetic treated with VIP	245.75±23.32^{c}	348.13±0.73^{b}	37.77±3.28^{b}	44.07±0.73^{ab}
F-probability	p<0.001	p<0.001	p<0.01	p<0.01
LSD at the 5% level	102.30	68.102	10.420	10.314
LSD at the 1% level	139.52	92.880	14.220	14.066

Data are expressed as Mean±SE. Number of animals in each group is 10. Mean values with the same superscript letter(s) are not significantly different

Table 3: Effect of VIP administration on liver collagen, serum CA19.9 and AFP levels of normal and diabetic male mice

	Parameters		
Groups	Liver collagen (µg/100 mg tissue)	Serum CA19.9 (U mL^{-1})	Liver AFP (U g^{-1})
Normal	4.27±0.10^{a}	6.38±0.13^{a}	7.83±0.04^{a}
Normal treated with VIP	4.60±0.38^{a}	4.95±0.25^{b}	5.02±0.71^{b}
Diabetic	5.05±0.58^{a}	6.65±0.11^{a}	6.33±0.82^{ab}
Diabetic treated with VIP	4.70±0.46^{a}	6.23±0.09^{a}	5.40±0.18^{b}
F-probability	p>0.05	p<0.001	p<0.01
LSD at the 5% level	--	0.467	1.623
LSD at the 1% level	--	0.637	2.213

Data are expressed as Mean±SE. Number of animals in each group is 10. Mean values with the same superscript letter(s) are not significantly different

Table 4: Effect of VIP administration on various liver oxidative stress markers of normal and diabetic male mice

	Parameters				
Groups	Total thiol (nmol/100 mg)	GSH (nmol/100 mg)	LPO (nmol MDA/100 mg)	Peroxidase (U g^{-1})	Catalase ($k.10^{2}$)
Normal	190.77±22.35^{ab}	41.36±1.28^{b}	54.75±8.46^{b}	29.16±3.71^{b}	54.69±3.41^{a}
Normal treated with VIP	175.05±26.14^{a}	53.84±1.75^{a}	64.65±0.35^{b}	31.29±2.35^{b}	53.51±3.94^{a}
Diabetic	108.79±15.31^{c}	37.65±0.07^{c}	94.45±3.78^{a}	42.35±1.31^{a}	24.16±1.77^{b}
Diabetic treated with VIP	148.81±7.54^{b}	44.57±1.34^{b}	67.60±1.52^{b}	46.07±3.27^{a}	31.54±0.59^{b}
F-probability	p<0.01	p<0.001	p<0.001	p<0.001	p<0.001
LSD at the 5% level	45.537	3.313	13.870	8.237	8.169
LSD at the 1% level	62.106	4.518	18.910	11.235	11.141

Data are expressed as Mean±SE. Number of animals in each group is ten. Mean values with the same superscript letter(s) are not significantly different

As indicated in Table 3, the diabetic mice exhibited a non-significant change in liver collagen, serum Carbohydrate Antigen (CA)19.9 and liver AFP levels in comparison with normal animals. The administration of VIP to diabetic mice induced a non-significant effect on liver collagen, serum CA19.9 and liver AFP levels while the administration of VIP to normal mice produced significant decreases of serum CA19.9 and liver AFP levels. One-way ANOVA indicated that the effect between groups on liver collagen, liver AFP and serum CA19.9 antigen was non-significant ($p>0.05$), highly significant ($p<0.01$) and very highly significant ($p<0.001$), respectively.

Concerning oxidative stress and antioxidant defense system (Table 4), the liver lipid peroxidation and peroxidase activity were remarkably elevated ($p<0.01$) while the total thiol, glutathione content as well as catalase activity were significantly decreased in diabetic mice. The treatment of the diabetic animals with VIP induced a significant amelioration of total thiol ($p<0.05$), glutathione ($p<0.01$) contents and lipid peroxidation ($p<0.01$). Also, the administration of VIP to normal mice significantly increased the hepatic glutathione content. One-way ANOVA indicated that the effect between groups on total thiol was highly significant ($p<0.01$), but the effect between groups on the other tested oxidative stress variables was very highly significant ($p<0.001$) throughout the experiment.

Changes in Cytokine Levels

The diabetic mice exhibited a marked increase in serum IL-10, IL-12 and TNF-α levels. However, while the administration of VIP to diabetic mice induced more increase in IL-10 level, it produced a significant decrease in the elevated IL-12 and TNF-α concentrations. Also, the treatment of normal animals with VIP produced an enormous elevation ($p<0.01$) of IL-10 concentration and remarkable decreases in IL-12 ($p<0.01$) and TNF-α ($p<0.05$) levels. One-way ANOVA depicted that while the effect between groups on TNF-α was highly significant ($p<0.01$), it was very highly significant ($p<0.001$) on serum IL-10 and IL-12 levels (Table 5).

Table 5: Effect of VIP administration on serum IL-10, IL-12 and TNF-α levels of normal and diabetic male mice

	Parameters		
	IL-10	IL-12	TNF-α
Groups	(pg mL^{-1})		
Normal	18.40±1.79^{d}	100.90±3.19^{b}	86.26±3.10^{ab}
Normal treated with VIP	147.40±2.68^{b}	68.93±5.48^{c}	77.35±5.97^{b}
Diabetic	94.40±2.68^{c}	122.18±4.47^{a}	99.57±9.13^{a}
Diabetic treated with VIP	178.00±6.24^{a}	108.61±2.05^{b}	29.53±2.53^{c}
F-probability	p<0.001	p<0.001	p<0.010
LSD at the 5% level	10.872	11.835	17.135
LSD at the 1% level	14.828	16.142	23.370

Data are expressed as Mean±SE. Number of animals in each group is 10. Mean values which share the same superscript letter(s), are not significantly different

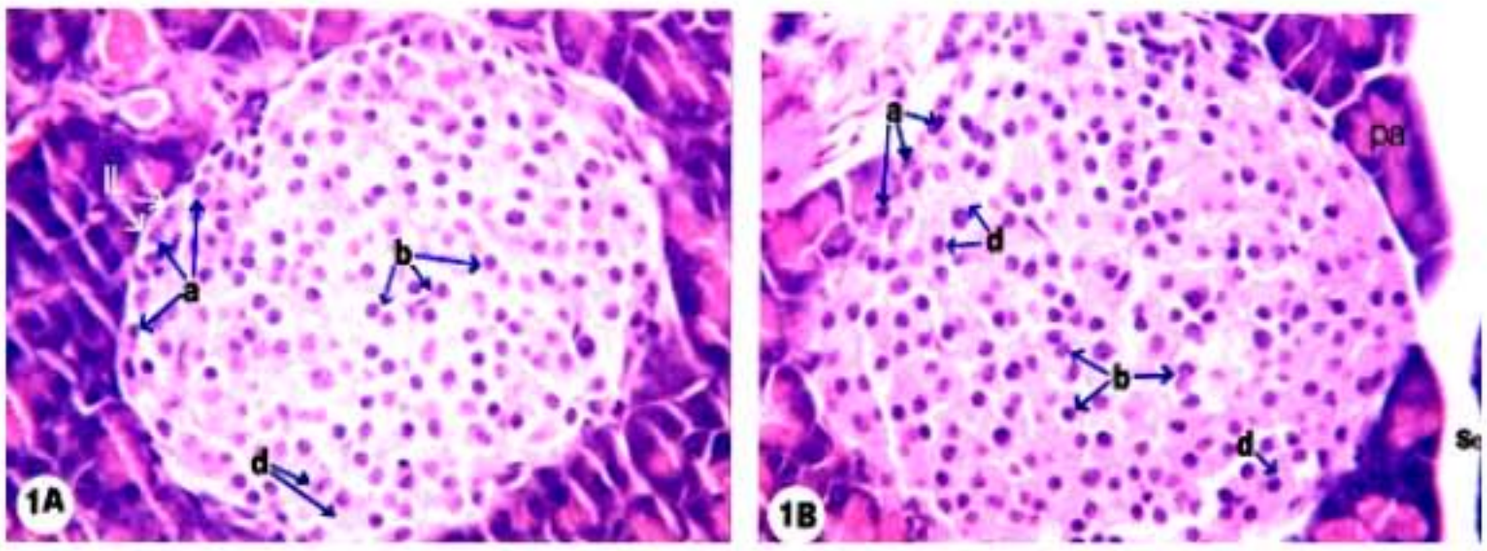

Fig. 1 (A, B): Sections of pancreas of normal mice showing normal architecture of islets of Langerhans (IL). The islets contain alpha cells (a) at the periphery, beta-cells (b) in the core of the islets and delta-cells (d) found adjacent to alpha cells and of relatively larger size (Fig. 1A and 1B, X400)

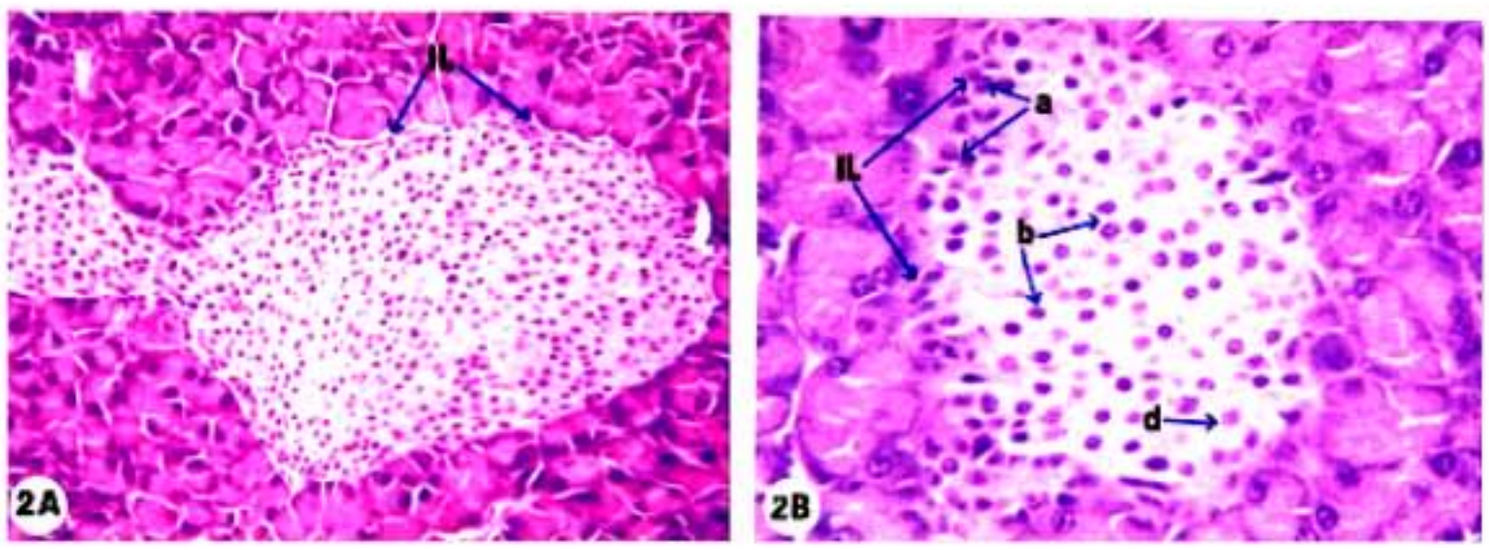

Fig. 2 (A, B): Section of pancreas of normal mice treated with VIP showing islets of larger size with highly proliferated cells (hyperplasia) in some animals (Fig. 2A, X100) and normal islets in others (Fig. 2B, X400). Alpha (a), beta (b) and delta (d) cells are clearly noticed

Histopathological Changes

The islets of Langerhans (IL) of normal mice showed normal architecture (Fig. 1A, B). The treatment of normal mice with VIP induced rapid proliferation of the islets cells and a marked increase in the islets size (Fig. 2A) in some animals. However, others showed normal islets (Fig. 2B).

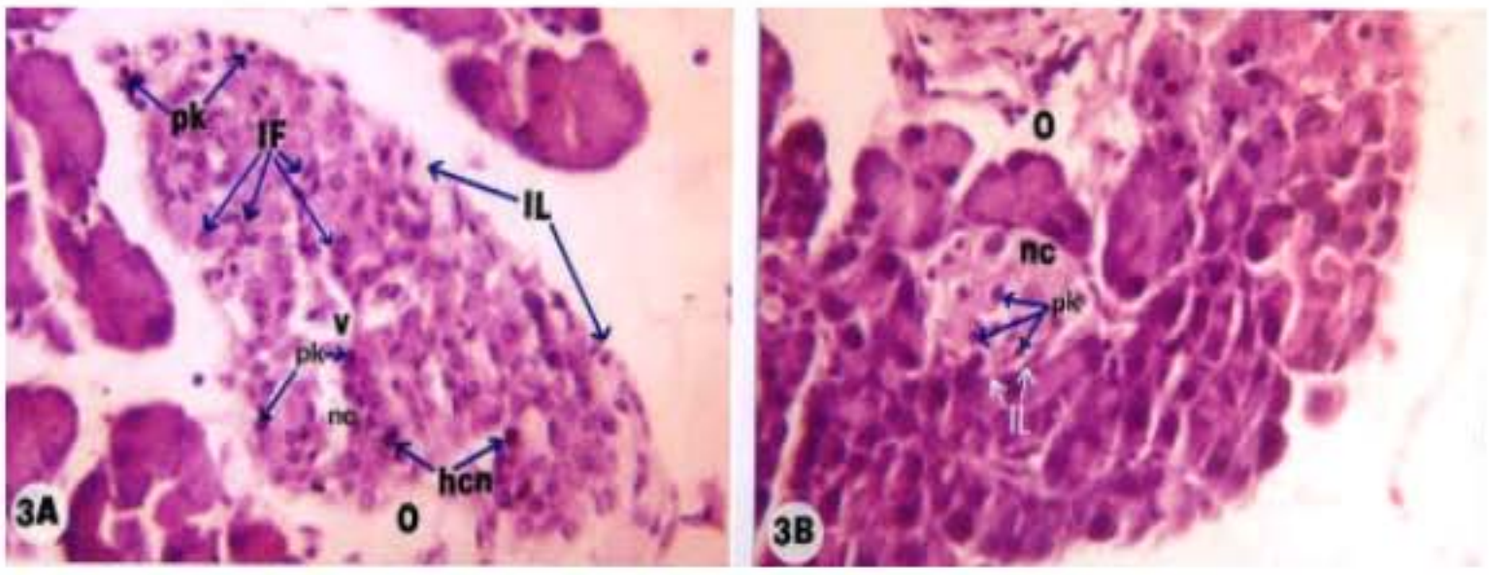

Fig. 3 (A, B): Sections of pancreas of streptozotocin diabetic mice showing disrupted islets (IL) architecture, necrosis (nc), pyknotic nuclei (pk), vacuolations (V), hyperchromatic nuclei (hcn), oedema (O) and inflammatory cells (IF) infiltration in the islets of Langerhans (Fig. 3A, B, X400). The islets appeared with an enormous reduced size in some mice (Fig. 3B, X400)

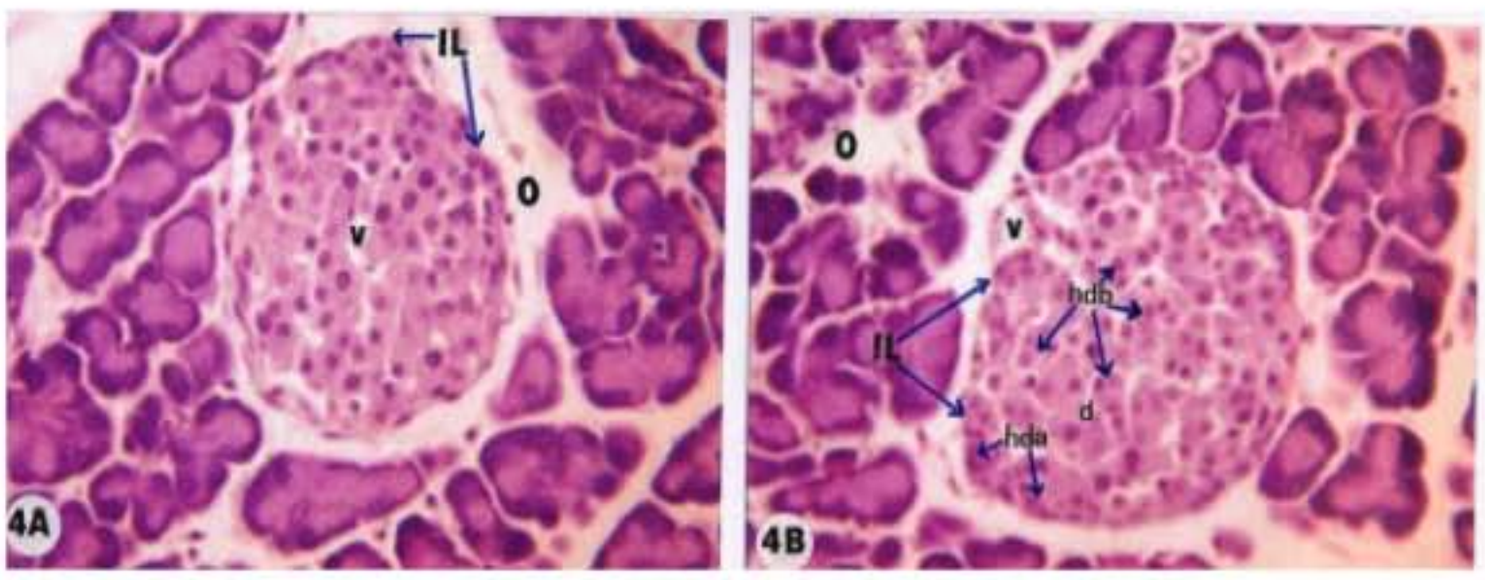

Fig. 4 (A, B): Sections of pancreas of streptozotocin-induced diabetic mice treated with VIP showing marked amelioration of islets (IL) architecture associated with increased number of islet cells (Fig. 4A, B, X400). The islets in some mice showed highly divided alpha- cells (hda) and highly divided beta-cells (hdb) and delta cells (d) (Fig. 4B, X400). Oedema was also noticed

In the diabetic mice, the islets exhibited disrupted architecture, marked reduction in size (Fig. 3B), necrotic (nc) foci, vacuolations (V), many pyknotic (pk) and hyperchromatic (hcn) nuclei, inflammatory cells (IF) infiltration and oedema (O) (Fig. 3A, B). The treatment of these diabetic animals with VIP produced a marked amelioration of the mentioned lesions (Fig. 4A, B). The islets attained a more organized architecture with less vacuolations (V). Oedema was also observed. The islets in some diabetic mice treated wit VIP showed highly divided alpha cells (hda) and highly proliferated beta cells (hdb) (Fig. 4B).

The liver of normal mice showed normal architecture (Fig. 5). The liver of normal mice treated with VIP showed well organized histological architecture with no deleterious changes (Fig. 6).

The histological architecture of diabetic mice liver showed tremendous disrupted changes (Fig. 7A, B). Many hepatocytes are megalic (mh) with hydropic (hc), vacuolated (v) or ballooned (b) cytoplasm. They also exhibited karyorrhexia (kx) in some animals and have atrophied (an), degenerated

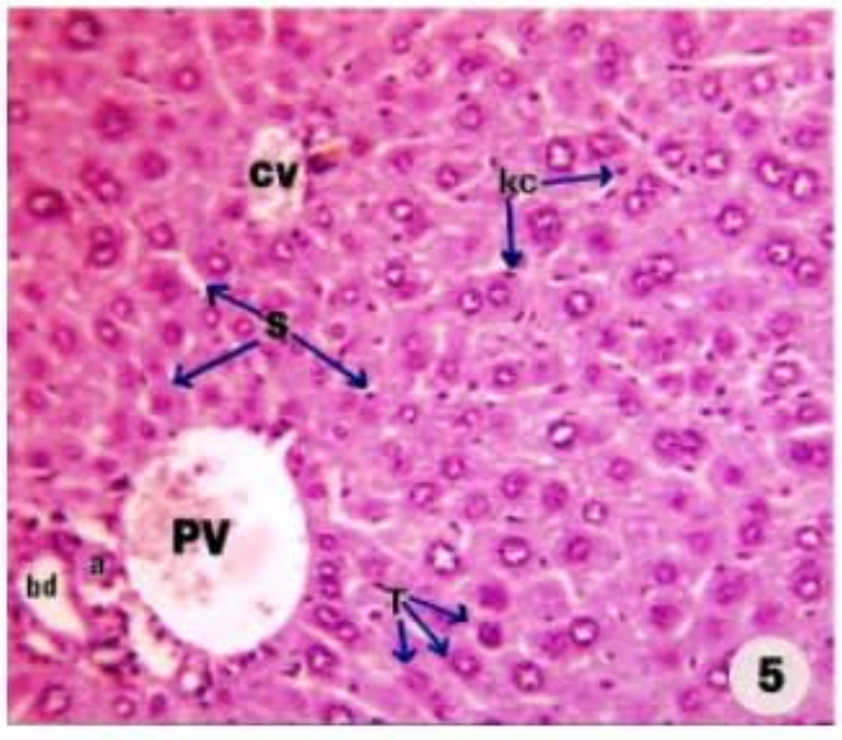

Fig. 5: Section of liver of normal mice showing normal histological structure. Each hepatic lobule consists of plates of hepatic cells or trabeculae (T) radiating from a Central Vein (CV). Hepatic sinusoids (s) and kupffer cells (kc) are located between hepatic trabeculae. Between various hepatic lobules in the portal triads or area, hepatic Portal Vein (PV), hepatic artery (a) and bile ductile (bd) were observed. X100

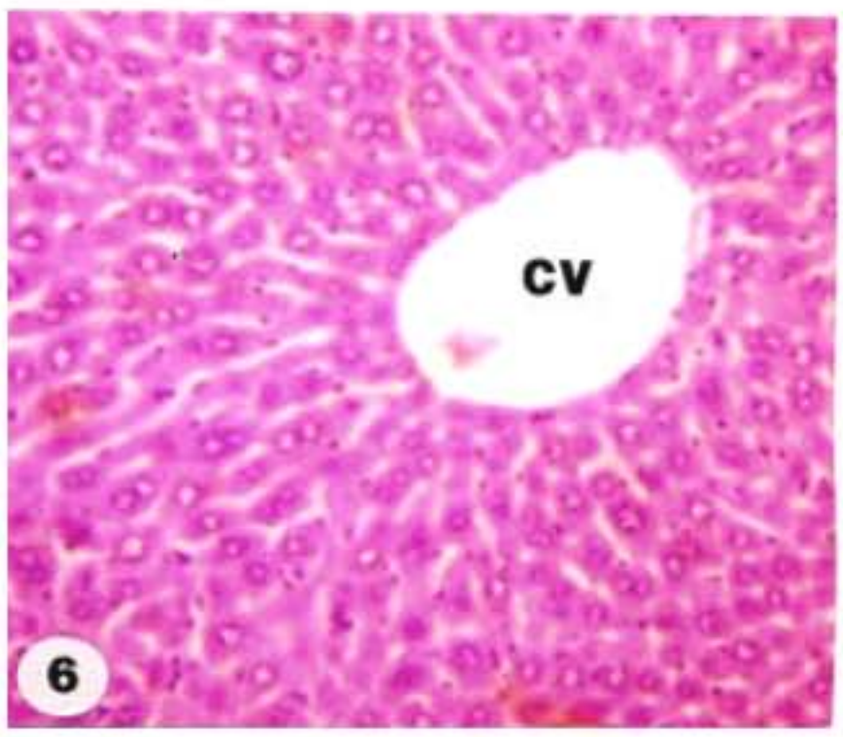

Fig. 6: Section of liver of normal mice treated with VIP normal histological architecture of the tissue. CV: Central vein. X100

(dn) and hypertrophied (hn) nuclei in others. The liver tissue further exhibited hyperemic central veins (hcv), peri-vasculzar albuminous material (al) and inflammatory cells (IF) infiltration in the portal triads as well as central vein and portal vein dilatation. The administration of VIP to diabetic mice induced a potential improvement in the liver histology (Fig. 8A, B). However, mild inflammation (IF) and necrosis (nc) were still noticed in addition to the presence of few hyperchromatic (hcn) and pyknotic nuclei in the hepatocytes.

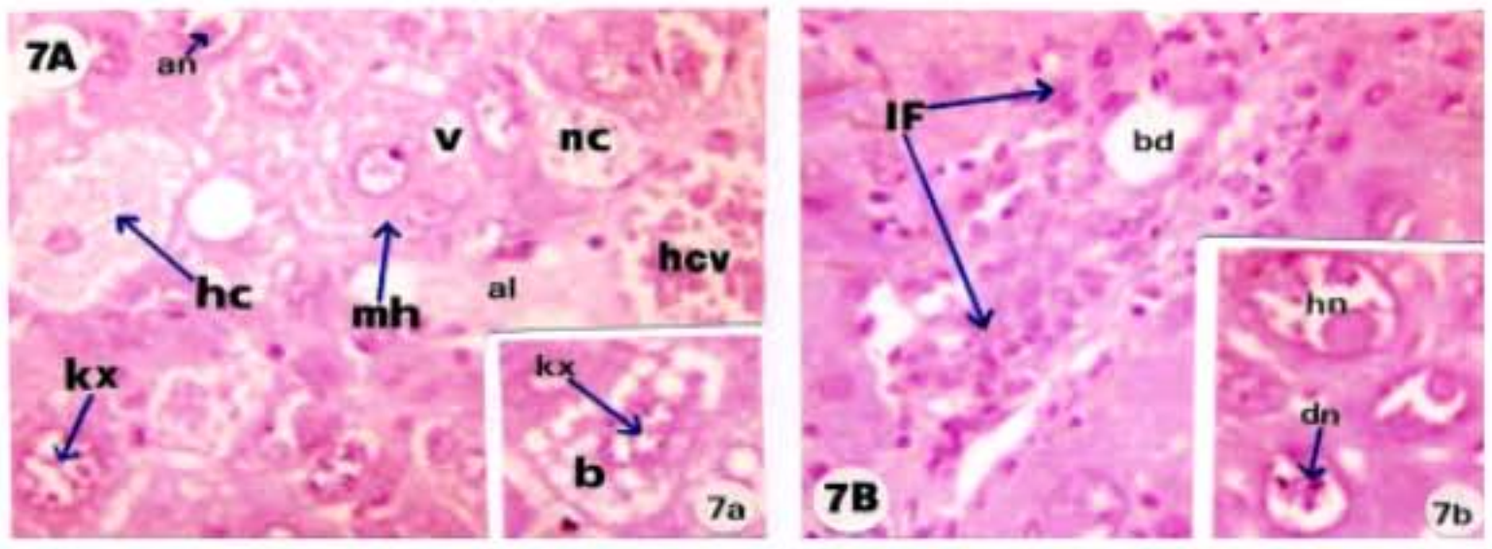

Fig. 7 (A, B): Sections of liver of diabetic mice showing many histological perturbations including hyperemic central vein (hcv), peri-vascular albuminous material (al), necrosis (nc), hepatocyte hydropic cytoplasm (hc), karyorrhexia (kx), atrophied nuclei (an), megalic hepatocytes (mh), vacuolated cytoplasm (V) and ballooning (b) in some mice (Fig. 7A and a, X400) as well as inflammatory cells infiltration (IF) in the portal areas, hypertrophied nuclei (hn) and degenerated nuclei (dn) in others (Fig. 7B and 7b, X400)

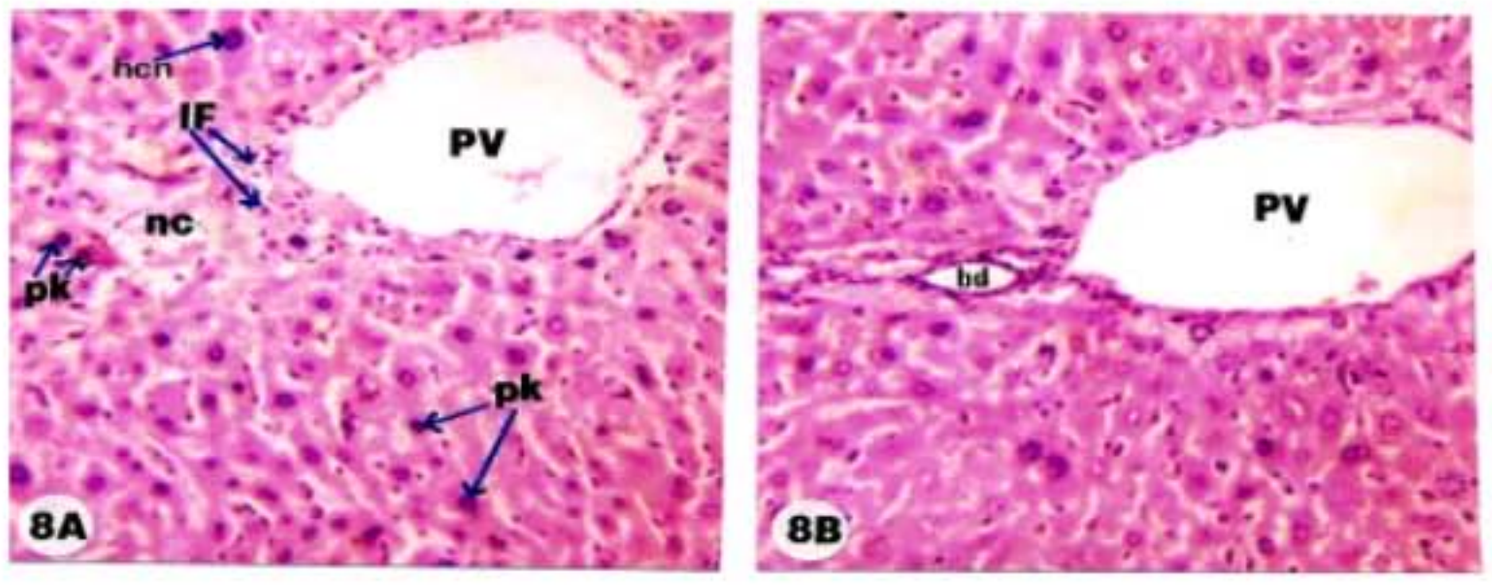

Fig. 8 (A, B): Sections of liver of diabetic mice treated with VIP showing potential amelioration of histological architecture. Most of histological lesions noticed in the diabetic mice were alleviated. Only mild inflammatory cells (IF) infiltration around Portal Vein (PV), small necrotic areas (nc) and few hyperchromatic (hcn) and pyknotic (pk) nuclei were noticed (Fig. 8A, B, X100)

DISCUSSION

There is good recent evidence that the immune system may be involved in the pathogenesis of IDDM in humans as well as in animal models (Fidan *et al*., 2005; Ugochukwa and Figgers, 2007) including an inflammatory response and an autoimmune destruction of β-cells in the pancreatic islet of Langerhans (Bergholdt *et al*., 2002; Fidan *et al*., 2005). Recent studies in humans and animal models suggested that autoimmune diabetes is associated with an imbalance between pro-inflammatory Th1-cytokines and anti-inflammatory Th2 cytokines in the immune system (Lo *et al*., 2004). It was suggested that the more production of pro-inflammatory cytokines (IL-12, IL-1β, IL- 2, TNF-α and

IFN-γ) may induce β-cells destruction whereas the more production of anti-inflammatory Th2-cytokines (IL-4, IL-10 and IL-13) in pancreatic islets may protect against β-cells destruction (Cnop *et al*., 2005; Amirshahrokhi *et al*., 2008). Thus, targeting these cytokines seems an attractive option/strategy for therapy of IDDM. This could possibly aid in delaying the onset of the disease and in preventing β-cell damage. Streptozotocin-induced diabetes in animals, a well characterized model of IDDM, may be a useful tool for studying the disease and its treatment. VIP is assessed as a therapy in this study as it has immunoregulatory effect and may influence Th1/Th2-cytokines (Ahmed and Allam, 2008). In addition, it has been reported that VIP was remarkably reduced in different tissues of STZ-induced diabetic rats and mice (Noda *et al*., 1990; Nowak *et al*., 1995; Adeghate *et al*., 2001).

In the current study, serum glucose concentration at a fasting state and 2 h post-oral glucose loading was profoundly elevated in the diabetic mice as compared with normal animals. This change was associated with a significant deficiency of circulating insulin and C-peptide level, which was due to the deleterious damage of β-cells in the islets of Langerhans in the pancreas after streptozotocin (STZ) injection. The histological results of the islets of Langerhans of streptozotocin-induced diabetic mice of this study showed an enormous reduced islet size and the presence of necrosis, vacuolations, invading inflammatory cells, oedema, nuclear pyknosis and hyperchromatic nuclei. Several mechanisms were reported by various authors to explain the action of streptozotocin to induce β-cells damage and in turn to decrease blood insulin and C-peptide levels. These include, (1) an increase in the oxidative stress and production of reactive oxygen and nitrogen species, (2) an induction of DNA strand breaks, (3) a depletion of NAD, (4) an inhibition of ATP synthesis and/or (5) a perturbed immune regulation (Okamoto, 1985; Pusztai *et al*., 1996; Amirshahrokhi *et al*., 2008). Like and Rossini (1976) reported that STZ initiated a cell-mediated immune reaction. The present results support the suggestions which indicate the roles of the immune system and oxidative stress in the destruction of the islet cells because there are inflammatory cells infiltration in the islets and an increase in lipid peroxidation as well as suppression of antioxidant defense system in STZ-induced diabetic mice. Many authors revealed the roles of the inflammatory and pro-inflammatory cytokines released from the islet infiltrating leukocytes in the destruction of β-cells. Ugochukwu and Figgers (2007), Jain *et al*. (2007) and Guo *et al*. (2007) found that inflammatory and pro-inflammatory cytokines were upregulated in STZ-diabetes. Pehuet-Figoni *et al*. (1992) suggested that TNF-α, which was remarkably increased in STZ diabetic mice in the current study, could play a fundamental role in the progressive autoimmune destruction of β-cells in IDDM. Kaneto *et al*. (1995) also stated that in the presence of increased TNF-α level, large amounts of NO were produced and DNA cleavage occurred more noticeably in the islets of Langerhans. The NO-induced DNA damage is considered as an important step in the destruction and dysfunction of pancreatic β-cells caused by inflammatory stimulation or treatment with STZ (Kaneto *et al*., 1995). Furthermore, Lukic *et al*. (1998) and Amirshahrokhi *et al*. (2008) reported that multiple-low doses of STZ diabetes, a model of IDDM, is induced by Th1 lymphocytes that secrete soluble effector molecules that activates macrophages and promote destruction of β-cells possibly by both nitric oxide and non-nitric oxide - mediated mechanisms. It was also found by Wen *et al*. (2006) that the high glucose levels led to a potential increase in the mRNA expression of inflammatory and pro-inflammatory mediators (TNF-α, IFN-γ, IL-1β, IL- 6, IL-12 and IL-18), from mouse peritoneal macrophage, with IL-12 showing the highest activation (5.4-fold) in a time- and dose-dependent manner. In addition to the diabetogenic action of STZ, it was postulated that chronic and persistent elevated levels of glucose induce the rate of beta-cells damage and apoptosis (Butler *et al*., 2003; Mellado-Gil and Aguilar-Diosdado, 2004; Cnop *et al*., 2005). IL-12, which was significantly increased in STZ-diabetic mice in the present study, was assumed by Rothe and Kolb (1998) to upregulate IFN-γ from T-cells and Natural Killer (NK) cells and polarizes Th0-cells into a Th1-secretory

phenotype. Thus, it is an important pathogenetic movens in eliciting the type 1 cytokine-mediated anti β-cells response leading to islets inflammation and insulitis. IL-10 in STZ-injected mice has been reported by Mueller *et al*. (1996) to have a general immunostimulatory effect, but did not lead to the activation of β-cell-specific T cells. Thus, those authors suggested that IL-10 may have a critical role in the development of diabetes *in vivo* despite its established immunosuppressive activities *in vitro*. Although, several publications reported the anti-inflammatory role of this cytokine (Pennline *et al*., 1994; Cameron *et al*., 1997; Nicoletti *et al*., 1997; Amirshahrokhi *et al*., 2008), the present study may support the pre-described suggestion since serum IL-10 level was 5-fold increased in the present diabetic mice in such condition. This controversy may be due to the evidence that IL-10 is a multifunctional cytokine with adverse effects on different immune cells (Moore *et al*., 2001). In accordance with the results of oxidative stress, Azuma *et al*. (2007) reported that lipid peroxidation products, TBARS were increased in STZ-induced diabetes. This increase in lipid peroxidation together with the attenuated antioxidant defense system, marked in the present study by a decrease in total thiol and glutathione contents as well as catalase activity, have been reported to be involved, at least partially, in the destruction of hepatocytes and islet cells in the course of type 1 diabetes development (Tabatabaie *et al*., 2003). Also, the suppressed antioxidant activity makes β-cells and hepatocytes more sensitive to destruction by oxygen and nitrogen reactive species their formation is evoked by inflammatory and pro-inflammatory cytokines (Xu *et al*., 1999). In contrast to catalase, peroxidase activity was increased in the diabetic mice of this study. This marked increase in hepatic peroxidase activity may be the result of the release of eosinophil peroxidase from eosinophil granulocytes and/or myeloperoxidase from mononuclear phagocytes in the regions of inflammation in addition to stimulation of its production from activated Kupffer cells (Weiss *et al*., 1983; Anderson *et al*., 1997).

The treatment of streptozotocin-induced diabetic mice with VIP for 8 consecutive weeks, in the present study, induced a potential amelioration of the elevated serum glucose at fasting state and 2 hours post-oral glucose loading secondary to the improvement of the serum insulin levels. The parallel increase of serum level of C-peptide, a beta-cells function marker, as a result of treatment of diabetic mice, indicates that VIP may be an insulinotropic agent. These results are in accordance with those of Adeghate *et al*. (2001), who found that VIP evoked an increase of insulin secretion in STZ-induced diabetic mice. It is worth mentioning that the effect of VIP in the pancreatic islets to increase insulin secretion seems to occur *via* adenylate cyclase signaling pathway (Winzell and Ahren, 2007). The activation of adenylate cyclase leads to an elevation of cyclic adenosine monophosphate (cAMP) which in turn activates protein kinase A (PKA) to promote insulin secretion *via* several different pathways (Winzell and Ahren, 2007). The improvement of insulin and C-peptide levels in the diabetic mice treated with VIP is associated with improved pancreatic islets histological architecture and increased volume of islets and number of β-cells as compared to the diabetic control. The recovery of β-cells is believed to be a consequence of either multiplication of β-cells that remained survived after streptozotocin poisoning or a new formation of β-cells from the duct epithelium of the exocrine portion of pancreas (Rerup, 1970; Ahmed, 2003). The present study also revealed that the inflammatory response, indicated by invading inflammatory cells, disappeared in the diabetic animals treated with VIP. Thus, it can be suggested that the alleviation of the islets histology in diabetic mice treated with VIP may be attributed to (1) the decrease in inflammatory and pro-inflammatory cytokines (IL-1β, IL-12, IFN-γ and TNF-α), (2) the increase in anti-inflammatory cytokines (IL-4, IL-10 and IL-13), (3) suppression of the oxidative stress and (4) and enhancement in the antioxidant defense system. This opinion is strongly supported by the results of the present study because the treatment of diabetic mice with VIP produced significant decreases in serum TNF-α and IL-12, potentially increased liver total thiol, glutathione and catalase levels and suppressed lipid peroxidation. In this regard, it was stated by several authors that the decrease in reactive oxygen species and the improvement of antioxidant defense system might be therapeutic tools to interfere with the

development of autoimmune diabetes and destruction of β-cells at multiple levels, including lymphocyte proliferation and adhesion as well as the production of pro-inflammatory and cytotoxic mediators (Lenzen *et al*., 1996; Lukic *et al*., 2003; Stosic-Grujicic *et al*., 2004; Jain *et al*., 2007; Vinson, 2007). It was also reported by Wen *et al.* (2006) that the alleviation in the oxidative stress markers and the inflammatory response in diabetic mice may be secondary to the improvement in the hyperglycemic and metabolic states and the reverse may also be true.

It is interesting to find, in the present study, that VIP induced a remarkable proliferation of both alpha- and beta-cells and a detectable enlargement of islets size in some tested normal and diabetic mice as compared with their corresponding controls. However, further investigations at molecular levels are required to elucidate the mechanism(s) by which VIP acts to induce this process.

With regards to the liver histological architecture in the diabetic mice, many lesions are detected including, necrotic foci: vacuolations, inflammatory cells infiltration in the portal area, hyperemic blood vessels, megalic hepatocytes, hydropic cytoplasm, ballooning, leakage of albuminous material to peri-vascular area, karyorrhexia, karyopyknosis and some degenerated, hypertrophied and atrophied nuclei. These liver histological alterations are in accordance with those obtained by other authors (Eskander *et al*., 1995; Ahmed, 2001). Also, these histological perturbations are consistent with a profound decrease of hepatic γ-GT activity due to its increased leakage from necrotic tissue to blood (Ahmed and Mostafa, 2003). However these histological deteriorations in diabetic mice, liver ALT, AST and LDH activities were profoundly increased. Ahmed (2001) and Abdel Reheim *et al*. (2007) attributed the increase in the activities of these liver enzymes in diabetic animals to their greater need for gluconeogenic substrate. Balistreri and Rej (1996) and Ahmed (2001) explained that drugs as streptozotocin may serve as haptens and may combine with liver cell membrane antigens, thus providing a locus for immunologically directed hepatocyte injury; in this situation, a marked infiltration of inflammatory cells occurred. Also, the histological lesions in liver of diabetic mice were consistent with the increase in serum pro-inflammatory cytokines IL-12 and TNF-α as well as an increase in lipid peroxidation and attenuation of the antioxidant defense system which in turn lead to damage and necrosis of hepatocytes.

In the present study, the liver fibrotic index, collagen content as well as tumor markers, serum CA19.9 and liver AFP levels were not significantly affected in diabetic mice as compared to normal. Also, the treatment of diabetic animals with VIP also induced non-significant changes.

The treatment of streptozotocin-induced diabetic mice with VIP for 8 weeks induced a marked amelioration of liver histological architecture where only small necrotic foci, mild inflammatory response and few pyknotic and hyperchromatic nuclei were still found. This improvement in liver histological lesions in the diabetic mice treated with VIP was associated with (1) a decrease in serum pro-inflammatory cytokines, IL-12 and TNF-α, (2) an increase in the anti-inflammatory cytokines, IL-10 (3) decreased production of lipid peroxides and (4) enhancement of anti-oxidant defense system. These data are in accordance with other publications, which stated that VIP inhibits the transcription (Delgado and Ganea, 1999) and production of pro-inflammatory cytokine, IL-12, from macrophages (Xin and Sriram, 1998; Gomariz *et al*., 2000) and from Th1-cells (Wang *et al*., 1999). Also, Delgado *et al*. (1998, 1999d, e) reported that VIP inhibits TNF-α production *in vitro* and *in vivo* from LPS-stimulated macrophages through the binding to VIP/pituitary adenylate cyclase-activating peptide (VPAC) receptor 1 that leads to activation of the adenylate cyclase system and reduction of the nuclear factor-κB (NF-κB) binding. It has also reported that VIP stimulates *in vitro* and *in vivo* production of the anti-inflammatory cytokine IL-10 (Delgado *et al*., 1999b, Gomariz *et al*., 2000; Allam, 2007; Larocca *et al*., 2007). In addition to increasing IL-10 production from Th2 cells, VIP treatment can increase production of regulatory T cells, which produce high levels of IL-10 (Voice *et al*., 2002; Chorny *et al*., 2005; Delgado *et al*., 2005; Reinke and Fabry, 2006). The stimulation of IL-10 production by VIP administration attenuates the production of pro-inflammatory cytokines including

TNF-α and NO (Nicoletti *et al*., 1997). Consequently, VIP strongly reduces inflammatory response and exerts its anti-inflammatory function in several ways: (1) direct inhibition of pro-inflammatory cytokine production (TNF-α, IL-6 and IL-12); (2) up-regulation of IL-10 production (a potent anti-inflammatory cytokine), (3) inhibition of expression and release of pro-inflammatory chemokines from activated macrophages and microglia, (4) inhibition of B7.1/B7.2 expression in activated macrophages and dendritic cells and subsequent inhibition of their stimulatory activity for antigen-specific T cells; and (5) inhibition of Th1 responses (reduction in both the amounts of Th1 cytokines and the number of cytokine-producing Th1 cells) (Delgado *et al*., 2004). Similarly to its action to increase insulin secretion, it was reported that VIP-immunomodulatory effects may operate through a cAMP-dependent mechanism (Weinstock *et al*., 1991).

In conclusion, it can be suggested that VIP may have anti-hyperglycemic activity, insulinotropic action and ameliorating effects on islets and liver histological architecture in addition to its immunomodulatory action in streptozotocin-induced diabetic mice.

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