

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF PANCREATIC ISLET BETA CELLS OF DIABETIC RATS TREATED WITH ORAL VANADYL SULPHATE

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ABSTRACT

Vanadium salts have been suggested as a possible therapeutic agent for the treatment of diabetes. The aim of the present study was to clarify histological and immunohistochemical changes that occur in the pancreatic beta (β) cells of vanadyl sulphate (VS)-treated streptozotocin (STZ) induced diabetic rats.

Male Wistar rats were made diabetic by injecting a single intravenous dose of STZ (40 mg/kg) and were divided into two groups seven days after STZ injection. In the first group VS was administered via drinking water at a concentration of 1 mg/mL and treatment was maintained until normoglycemia appeared (DT). A second group of diabetic animals received distilled water for the same period and were considered as control diabetic (DC). One group of animals (NC) was injected intravenously with the same amount of vehicle as the diabetic rats and was considered as non-diabetic control. VS treatment was accompanied by amelioration of the signs of diabetes in DT rats while DC animals remained diabetic during this period.

Hematoxylin - Eosin stained pancreatic sections of DC rats showed a decrease in the number and size of islets and a disruption in their architecture. In DT rats the histological appearance of the islets was normal, their shape and size being within normal limits.

In horseradish peroxidase procedure (using guinea pig antiserum to insulin as primary antibody) performed on pancreatic islet paraffin sections of rats, insulin immunoreactivity was found in the majority of the islets in DT rats while in the islets of DC rats immunoreactivity was rare.

The results of this study indicated that amelioration of diabetes in vanadyl sulphate treated diabetic rats was accompanied with well preservation of islet structure and insulin immunoreactivity.

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INTRODUCTION

Diabetes mellitus is a chronic disease characterized

by a deficiency of insulin, the key hormone maintaining normoglycemia. It results from an absolute or relative deficiency in insulin secretion and a resistance of target

tissues to the action of insulin, in proportions that vary with the type of the disease. Human type 1 diabetes (IDDM) results from the selective destruction of insulin-producing pancreatic beta cells during islet inflammation. Cytokines and reactive radicals released during this process contribute to beta-cell death.¹ In contrast to other tissues, which rapidly regenerate, the adult insulin-producing pancreatic β cells are characterized by a limited proliferative capacity.²

Streptozotocin (STZ) is a glucose analogue that is selectively toxic to β cells in the pancreatic islets and specifically damages pancreatic β cells.³ This compound has been used extensively to produce an experimental model for diabetes mellitus in animals.^{4,5,6,7,8}

Vanadium salts, which diminish hyperglycemia and reverse the diabetic state in STZ induced diabetic rats, have been suggested as a possible therapeutic agent for the treatment of diabetes.⁴⁻⁸ These compounds can mimic many of the metabolic actions of insulin both *in vitro* and *in vivo* and improve glycemic control in human subjects with diabetes mellitus.^{9,10} Recent short-term clinical trials with vanadium salts also seem promising in patients suffering from type II (non-insulin-dependent) diabetes mellitus (NIDDM) in whom liver and peripheral insulin resistance was attenuated.^{9,10}

Previous studies have shown that chronic oral administration of vanadyl sulfate in diabetic rats has resulted in a sustained euglycemia following withdrawal of the drug and returning the tissue vanadium concentrations to values close to pre-treatment level.¹¹ We designed this study to clarify more clues about the cause of permanent normoglycemia seen in STZ diabetic rats after vanadyl treatment and withdrawal. We have hypothesized that it must be related to the effect of this compound on the intrinsic plasticity of the endocrine pancreas, β cell protective or proliferative activity. We assessed the effects of VS on islet β cell mass in DC rats by immunohistochemistry used in light microscopy technique.

MATERIAL AND METHODS

Glucose kit was purchased from Ziest Chem. Co. (Tehran, Iran). Primary antibody (guinea pig antiserum to insulin) and its negative control reagents (nonimmune guinea pig serum) were obtained from DAKO Corporation (Carpinteria, CA). Vanadyl sulphate ($\text{VOSO}_4 \cdot \text{H}_2\text{O}$) was from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, England) and Streptozotocin was from Pharmacia & Upjohn Company (Kalamazoo, Michigan, USA).

Treatment and maintenance of rats

Male Wistar rats (180-220 g) were obtained from Razi Institution (Karaj, Iran). Rats were maintained at $22 \pm 2^\circ\text{C}$ under conditions of controlled lighting (12h on 12h off)

and were maintained on standard laboratory chow and housed in individual wire-bottom cages. Diabetes was induced by a single intravenous injection of freshly prepared solution of streptozotocin (40 mg/kg body wt, i.v.) in 0.1 M citrate buffer (pH 4.5) through the lateral tail vein. Diabetic rats were divided into diabetic control ([DC], n=14) and diabetic treated ([DT], N=14) groups. One group of normal rats was injected with the same amount of vehicle as diabetic rats through the lateral tail vein and was considered as normal control ([NC], n=14) for the above groups.

Blood samples for analyses were taken from the nicked tail vein in microcentrifuge tubes. Plasma was separated by centrifugation and plasma factors measured immediately after plasma separation with a spectrophotometer (UV-3100/3100s, Shimadzu, Japan). To manifest equivalent degree in the severity of the disease, STZ-treated diabetic rats with plasma glucose levels in the range of 480-550 mg/dL, measured 7 days after STZ injection, were used further. Vanadyl sulphate was used in this study to induce normoglycemia in diabetic rats. Vanadyl sulphate was provided via drinking water to the DT group for 3 months beginning 7 days after STZ injection in concentrations of 0.5 mg/mL in the first week of the treatment and 1 mg/mL for the remaining period of the treatment. All of the animals had free access to food during the period of study. Plasma glucose and fluid intake were monitored frequently during and after withdrawal of the treatment. Normoglycemia in DT rats was defined as plasma glucose levels in the range of 90-170 mg/dL. Under these conditions, more than 90% of diabetic rats receiving vanadyl sulphate became normoglycemic during the three months of treatment. We continued treatment until permanent normoglycemia appeared. With the vanadyl sulphate treated rats, ~10% were excluded from the experiment because they remained hyperglycemic during the period of treatment. Diabetic treated animals were killed after normoglycemia appeared and had remained for two months after vanadyl withdrawal.

Histochemical study of islets

All of the animals were killed with an overdose of ether and the whole pancreas carefully dissected out and fixed in 10% neutral buffered formalin (pH 7.4) for 48 h at room temperature. Paraffin blocks were sectioned (five microns thick) and mounted on gelatinized slides coated by poly-L-lysine. Linked avidin biotin horseradish peroxidase method (LABS-HRP) using guinea pig antiserum to insulin as primary antibody was used for detecting insulin containing beta cells. For this purpose sections were deparaffinized and were initially blocked with blocking agent and subsequently incubated with the primary antibody (Clone N/A, Catalogue no 1542, DAKO

Corporation, Carpentry, USA) and its negative reagent (nonimmune guinea pig serum) at room temperature for 10 minutes, rinsed with three changes of PBS and then incubated with biotinylated secondary antibody. In the later stage sections were incubated with streptavidin conjugated horseradish peroxidase. The peroxidase reaction was developed with 25mg/dL deaminobenzidine in phosphate-buffered saline with 0.025% hydrogen peroxide for ten minutes at room temperature. Sections were examined by light microscopy.

Statistical analysis

Data was analyzed using SPSS & INSTAT software and presented as means \pm SE. For testing the homogeneity of variances Bartlett's test was used. In case of multiple comparisons, statistical significance of differences was calculated with one-way analysis of variance (ANOVA) followed by Tukey as a post hoc test.

RESULTS

Glycemia and fluid intake

A): VS treated diabetic rats

Changes in the plasma concentration of glucose and fluid intake in different groups of animals are shown in Table I. Plasma glucose and fluid intake that increased approximately five folds one week after STZ injection dropped to normal levels (125 ± 16 mg/dL, 25 ± 4 mL/day) after three months VS treatment. Thus STZ injection largely increased plasma glucose and fluid intake levels and VS treatment returned both of these diabetic signs (hyperglycemia and polydipsia) to normal levels. Diabetic animals responded to the VS treatment with two sensitivities; while the large majority of the diabetic animals displayed stable normoglycemic values, others showed fluctuating values and then found stable normoglycemia. One animal remained hyperglycemic even after long-term treatment.

B) Control diabetic rats

Plasma glucose and fluid intake levels in the control diabetic group of animals are shown in Table I. Plasma glucose and fluid intake in normal rats that was 110 ± 8 mg/dL and 27 ± 3 mL/day respectively, increased to 530 ± 28 mg/dL and 142 ± 12 mL/day one week after STZ injection. Control diabetic rats demonstrated an increase in plasma glucose levels that was approximately four folds that of control rats by three months post-STZ. DC animals remained diabetic during 3 to 5 months follow up. Mortality rate was high in this group of animals. We lost some of the DC animals during the period of study and some of them showed signs of severe diabetic state such as cataract and weight loss. All of the untreated

rats remained hyperglycemic during the period of study.

C) Normal control animals

Data in Table I shows that in untreated normal rats glycemia and fluid intake did not change significantly during the period of study. Animals in this group had normal plasma glucose and fluid intake levels by day 90.

Immunohistochemistry and light microscopy

Microscopic studies were performed when the treated rats were normoglycemic for two months. In light microscopy in DC animals (Fig. 2) the sizes of islets were smaller than normal and islets showed necrotic cells with pyknotic nuclei and dense eosinophilic cytoplasm. In DT animals (Fig. 3) pancreatic islet cells did not show any obvious changes as compared to normal rats (Fig. 1). Immunostaining of pancreatic islets for insulin showed a broad proportion of insulin - positive cells in DT rats (Fig 6.), which was comparable to that in normal animals (Fig. 4). Comparison of islet sections in the pancreas of DT rats with NC rats showed that VS treatment restored islet structure and preserved islet beta cells. Cellular degeneration of islet β -cells in DT animals was less marked than DC animals. Immunostaining was found in a scant number of the islet cells of DC animals (Fig. 5).

DISCUSSION

In previous studies we showed that in STZ induced diabetic rats VS treatment decreases the amount of insulin, which is needed to induce normoglycemia.⁷ We also showed that when diabetes is not severe VS treatment led to induction of normoglycemia that remained far be-

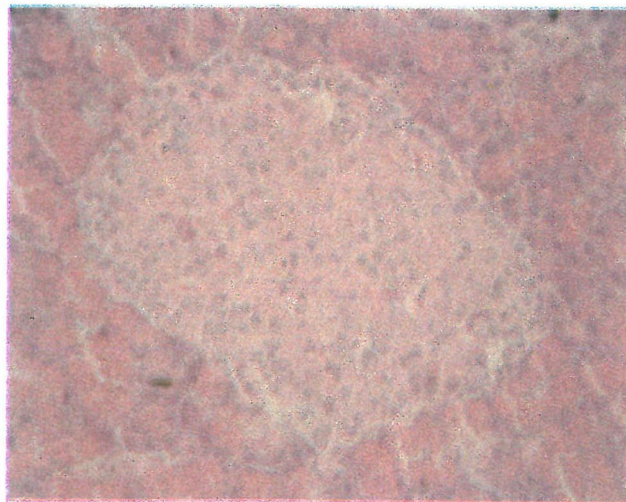


Fig. 1. Hematoxylin-Eosin stained pancreatic section of normal rats.

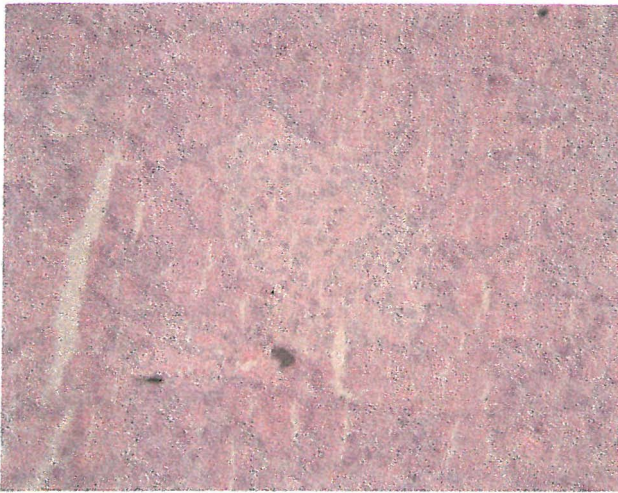


Fig. 2. Hematoxylin-Eosin stained pancreatic section of control diabetic rats.

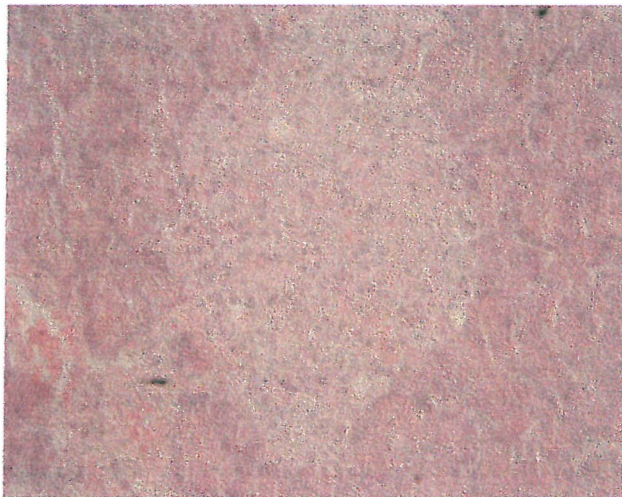


Fig. 3. Haematoxylin- Eosin stained pancreatic section of treated diabetic rats.

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yond VS withdrawal.¹³

Insulin mimetic action is not the cause of diabetic amelioration seen long after vanadium withdrawal. It was reported that in vanadyl treated and withdrawal diabetic rats normoglycemia existed when the concentration of vanadium in the various organs returned to pretreatment level.⁹

The well organized islet structure and high insulin immunoreactive positive cells in the islet of the DT animals and scant number of insulin immunoreactive positive cells in the islets of DC rats in this study indicates that VS treatment has a highly cell protective or replicate function in islet cells.

New pancreatic cells can be formed by neogenesis, or by replication of the preexisting differentiated cells.^{2,10,11} It is generally accepted that until late gestation, most β

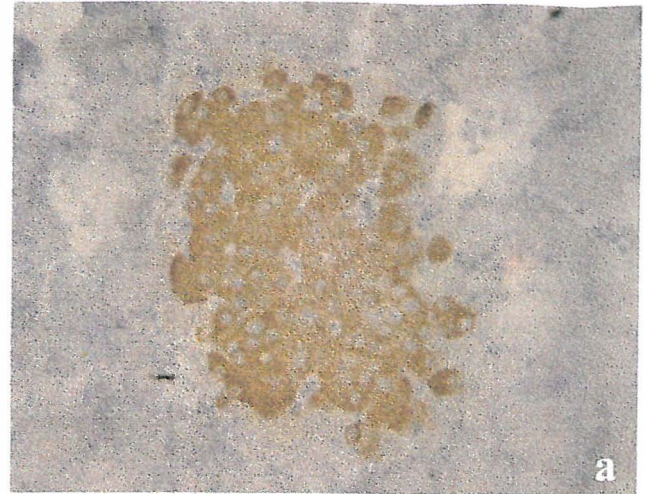


Fig 4. Light micrographs of normal pancreas islets.
a) Immunostained for insulin b) Negative control

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cells are the result of neogenesis and that after birth, most β cells are formed by replication. Beta cell regeneration is not a noteworthy feature in either human or animal models for type I diabetes, or in type II diabetes.¹⁰ If β cells could be induced to replicate at a higher rate, this might prove beneficial in maintaining normoglycemia, since the β cell mass is a major determinant of the total amount of insulin that can be secreted by the pancreas.

It seems that partial preservation of pancreatic β cells is both critical and sufficient for a long-term reversal of the diabetic state.¹² Vanadium pretreatment can not prevent STZ- induced β cell cytotoxicity.¹² The vanadium-induced amelioration of the diabetes may be the result of preserving a functional portion of pancreatic β cells that initially survived STZ toxicity, induction of islet neogenesis from undifferentiated precursor cells or transdifferentiation from other kinds of unknown cells, the process that occurs under special circumstances.^{10,11}

There is a potentially important reservoir of endocrine



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Fig. 5. Light micrographs of pancreas islets of diabetic control rats. a) Immunostained for insulin, b) Negative control.



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Fig. 6. Light micrographs of pancreas islets of diabetic treated rats. a) Immunostained for insulin, b) Negative control

Table I. Plasma glucose and fluid intake in different groups of animals during the period of study.

Group		Day	Plasma glucose (mg/dL)	Fluid intake (mL/day)
Diabetic	DC n=14	7	530±28 a n= 28	142±12a n= 28
		90	482±23 ab n= 14	174±18 ab n= 14
	DT n=14	7	530±28 a n= 28	142±12 a n= 28
		90	125±16 n= 14	25±4 n= 14
NC n= 14		7	110±8 n= 42	27±3 n= 42
		90	118±10 n= 14	28±4 n= 14

Data are mean ± SE from separate rats

^a $p < 0.01$ vs control group

^b $p < 0.01$ vs respective treated group

precursor cells that can be recruited to differentiate into β cells upon appropriate stimulation.¹⁰ One of the possible mechanisms by which VS could be linked to the permanent amelioration of diabetes seen in treatment with vanadyl derivatives is by preventing the full destruction of injured β cells or affecting the potent undifferentiated β cells such as duct cells.¹¹

Administration of β cell specific toxins such as streptozotocin results in an IDDM-like state with vast destruction of β cells and subsequent hyperglycemia. It has been shown that regeneration occurs in response to the damaging effects of β cell toxins¹³ or partial pancreatectomy.¹⁰ After subtotal pancreatectomy or when vast destruction of β cells occurs as a result of beta cell toxins, compensatory β cell regeneration is not of sufficient magnitude to reverse hyperglycemia.¹⁰ Hence it appears that extreme hyperglycemia or diabetes somehow represses compensatory β cell regeneration.

Vanadium compounds may promote the β cell regeneration that occurs in response to the damaging effects of beta cell toxins of magnitude that reverses hyperglycemia or may act as the appropriate stimulus for induction of islet neogenesis from potent islet precursor cells under special conditions. The ability of VS to preserve islet mass and to attenuate the diabetogenic effects of streptozotocin indicates that further *in vitro* and *in vivo* studies about the effect of VS on islet development and function are necessary, and may lead to therapeutic strategies in diabetes mellitus.

Further studies will be needed to elucidate the mechanisms through which VS causes streptozotocin resistance, in order to develop strategies based on VS treatment, which might employ VS to the advantage of treatment of diabetes. The possibility of manipulating β cell proliferation by trace elements such as vanadium and the significance of β -cell mitogenesis in relation to treatments of diabetes mellitus can be considered as a strategy for treatment of diabetes.

In summary regarding the results of this and our previous experiments we conclude that insulinomimetic properties of vanadium and their *in vivo* protective effect on pancreatic β cells gives the ability to this trace element to be considered as a beneficial substance in preventing or treatment of type 1 diabetes.

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