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Original article

Synthesis, characterization and antidiabetic properties of N^1-2,4-dihydroxybenzylidene-N^4-2-hydroxybenzylidene-*S*-methyl-thiosemicarbazidato-oxovanadium(IV)

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Abstract

A new oxovanadium(IV) chelate [VOL] (L: N^1-2,4-dihydroxybenzylidene-N^4-2-hydroxybenzylidene-*S*-methyl-thiosemicarbazidato) was synthesized and characterized by elemental analysis, conductivity and magnetic measurements, UV–vis, IR, EPR spectroscopy and mass spectrometry. The biochemical and immunohistochemical effects of the administration of the vanadium complex (VOL) into the pancreas of normal and streptozotocin-induced diabetic rats were profoundly investigated. The animals were randomly divided into four groups. Group I: control (intact) animals. Group II: control animals administered with VOL. Group III: STZ-induced diabetic animals. Group IV: STZ-induced diabetic animals administered with VOL. VOL was given to some of the experimental animals by gavage at a dose of 0.2 mM/kg every day for 12 days. Blood samples were collected from animals, on 0 and 1, 6 and 12 days after STZ injection. On day 12, the pancreatic tissues were taken from the animals. The tissue sections were labelled with streptavidin biotin peroxidase technique for insulin. In the diabetic group, the blood glucose levels, aspartate and alanine transaminases, alkaline phosphatase activities were increased. But, in the diabetic + VOL groups, the blood glucose levels, aspartate and alanine transaminases, alkaline phosphatase activities were reduced. In the diabetic group, a decrease in the pancreatic glutathione levels, glutathione peroxidase and superoxide dismutase activities and an increase in the pancreatic lipid peroxidation level and catalase activities were observed. The administration of VOL to the diabetic rats reversed this diabetic effect due to its insulinomimetic effects. According to the immunohistochemical and biochemical results obtained, it was concluded that VOL can regenerate B cells of the pancreas in experimental diabetes and has an antidiabetic and protective effects on the pancreas.

Keywords: *S*-Methylthiosemicarbazone-oxovanadium; Structural characterization; In vivo studies; Antidiabetic effects on pancreas; Biochemical and immunohistochemical studies

1. Introduction

Thiosemicarbazone derivatives have been of great importance because of their biological activities. Their biological potential has been thought to improve selectively certain biological systems due to their ability to chelate with trace metals. For antitumor, antibacterial and antiHIV activities thiosemicarbazones are of primary influence [1,2], antibacterial [3,4] and anti-HIV activities [5] are considered to be of primar influence. Also vanadium element has a biological significance, and therefore a large number of vanadium compounds have raised considerable interest in pharmacology [6–8]. Some biological activities of the vanadium containing complexes are antiamoebic [9], antitumor [10], antiviral [11,12] and exclusively antidiabetic effects [13–16].

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In recent years, glucose-lowering effect of vanadium compounds has been the subject of medicinal research and a wide variety of vanadium(IV) complexes have been tested as candidates for antidiabetic treatments. Thus, many VO^{2+} chelates have been synthesized using multifarious ligands which have *OOOO*, *SSSS*, *SNSN*, *SOSO* or *ONNO* donor sets [17]. Most preferred ligands are 3-hydroxy-4-pyran (maltol) derivatives [18–21]. The others, kojic acid [22], picolinic acid [23,24], biguanide [25], acetylaceton [26,27] and imidazole derivatives [28], are known to show the effects of insulinomimetic vanadium complexes.

A good glycemic control with available therapies continues to be an elusive goal. Thus, there is a need to find effective, orally active drugs that mimic or enhance the properties of insulin [25]. Therefore, many researchers in this field have been trying to find orally active therapeutic compounds. Previous studies have revealed that chromium, manganese, selenium, zinc and vanadium ions show both in vitro insulinomimetic action and in vivo antidiabetic activity in animal experiments [29–32]. The potential of vanadium complexes in controlling numerous clinical symptoms involved in diabetes both in humans and in experimental animals and the possibility of administering such oral route are of great importance [33,34]. It is known that these compounds show some toxic effects [35]. For this reason, many investigators tested new vanadium complexes in order to identify an agent that could be characterized by its lowest toxic effect and highest efficacy as an antidiabetic drug [36–39].

For the first time, we explained the insulinomimetic potential of a new VO^{2+} chelate in *ONNO* type obtained from a thiosemicarbazone ligand (L). The chelate, N^1-2,4-dihydroxybenzylidene-N^4-2-hydroxybenzylidene-*S*-methyl-thiosemicarbazidato-oxovanadium(IV) [VOL], was synthesized and characterized by elemental analysis, molar conductivity and magnetic susceptibility measurements, electronic, infrared and mass spectroscopies (Fig. 1). The present study was to investigate the biochemical and immunohistochemical effects of the administration of VOL to the pancreas of normal and streptozotocin-induced diabetic rats.

2. Results

2.1. Some physical properties of the oxovanadium complex

The interaction of 2,4-dihydroxybenzaldehyde *S*-methyl-thiosemicarbazone and 2-hydroxybenzaldehyde in the presence of VO^{2+} ion in 1:1:1 molar ratio in ethanol yielded a stable solid complex of the formula VOL. The brown complex can be isolated in the form of fine powder crystal, and sometimes as a mixture of amorphous and very fine crystals. The product is very poorly soluble in water and alcohol, but is very much soluble in donor solvents, such as DMF and DMSO.

The relative low molar conductivity of the oxovanadium chelate indicates a structure with non-ionic character.

2.2. Spectral data

The electronic spectra of the oxovanadium complex recorded in DMSO show the charge transfer and d–d bands. The bands in the 242–267 and 314–350 nm regions can be assigned to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions associated with the thiosemicarbazone moiety and two aromatic rings. The d–d bands observed at 418, 802 and 958 nm may be attributed to the $b_2(d_{xy}, d_{xz}) \rightarrow a_1^*(d_{xy})$, $b_2(d_{x^2-y^2}) \rightarrow e_\pi^*(d_{xz}, d_{yz})$ and $a_1^*(d_{xy}) \rightarrow e_\pi^*(d_{xz}, d_{yz})$ transitions, respectively. The obtained electronic spectrum indicates a structure with five-coordinates, probably square pyramidal geometry [40].

The 1.64 BM value of magnetic susceptibility at room temperature confirms that the vanadium ion is in the V(IV) state. At room temperature, the EPR spectra of VOL were recorded for powder and solution forms. The first-derivative X-band EPR signal registered for powder form of the VO^{2+} complex is shown in Fig. 2. An intensive single curve is observed without asymmetry. The signal does not have any hyperfine peaks. The EPR signal has a resonance field of $H_r = 3483$ G and an effective *g*-value of 2.01084.

The first-derivative EPR signal taken for the VO^{2+} complex in chloroform solution is shown in Fig. 3. The signal contains 8 well-resolved hyperfine peaks as expected due to the interaction between the electronic spin of magnetic electron and nuclear spin of V^{4+} ($I = 7/2$) ion. Hyperfine lines do not show symmetrical character, that is, the width and amplitude

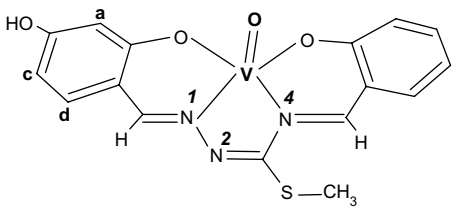

Fig. 1. The oxovanadium(IV) complex [VOL].

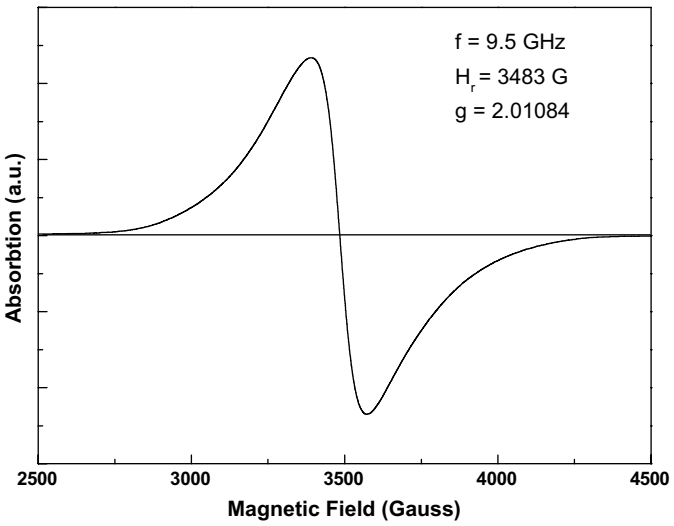

Fig. 2. X-band ESR spectrum of VOL in powder.

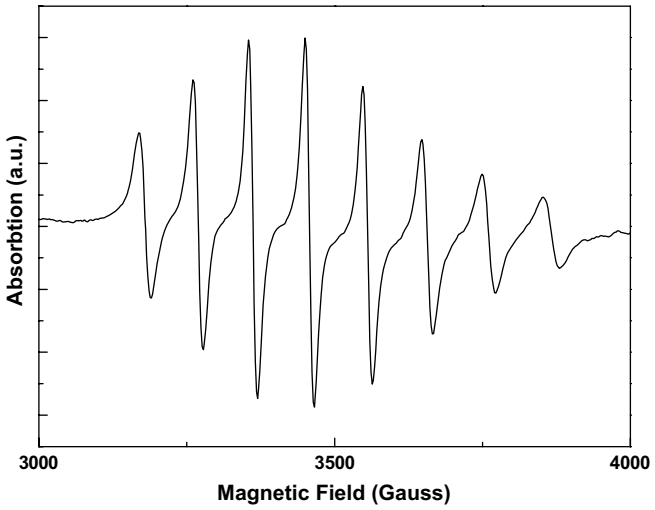

Fig. 3. X-band ESR spectrum of VOL in chloroform.

of every peak change from low field region to high field region as in literature [41,42]. Also a varying shift from a constant hyperfine splitting (A_0) value between each transition line that is the isotropic Breit–Rabi case is observed.

Significant line-narrowing effects for the hyperfine peaks were observed. The reason for line-narrowing effects must be the decrease in the magnitude of dipolar interaction between the magnetic moments of atom and electron. Since the chloroform solution increases the average separation between the magnetic ions, the dipolar effects get weaker and line-narrowing effect is expected in this case.

In the infrared spectra of 2,4-dihydroxybenzaldehyde *S*-methylthiosemicarbazone (L_1) the ν_a(NH), ν_s(NH) and $\delta(NH_2)$ bands were observed at 3445, 3337 and 1624 cm^{-1}, respectively. The bands are absent after chelation. The infrared spectrum of the oxovanadium complex shows that the strong ν(C=N) bands are at 1605, 1593 and 1578 cm^{-1}. In the spectrum of complex, the occurrence of a new azomethine group (N^4=C) by the condensation of the second aldehyde and the thioamide nitrogen can be monitored (Fig. 1). The bands seen at 1605 and 1593 cm^{-1} can be attributed to $\nu(C{=}N^1)$ and $\nu(N^4{=}C)$, respectively. Although it is difficult to distinguish the different azomethine bands of complex molecule, it can be proposed that the first azomethine bands ($C{=}N^1$) shifts towards lower energies ca. 20 cm^{-1} on chelation [43]. The medium (C–O) bands are between 1123 and 1146 cm^{-1} because these vibrations in the spectrum of metal complex shift to 25–30 cm^{-1} lower frequencies by coordination of the phenolic oxygen to the VO^{2+}. In addition, the characteristic ν(V=O) and ν(V–O) bands can be monitored at 985 and 477–434 cm^{-1}, respectively.

In the mass spectrum, the M (394) and M + 1 (395) peaks were clearly determined and it was possible to distinguish some of the fragments which are present in complex structure such as $-CH_3$, $-CH{=}N-N{=}C{=}S$ and $-C_6H_4(OH)_2$. The analytical and spectral data indicate the chelate structure of the VOL in Fig. 1.

2.3. Biochemical results

The mean body weight levels of four groups are given in Table 1. Before inducing diabetes, there was no significant difference in body weight levels between four groups on day 0 ($P_{ANOVA} = 0.433$). In the control (intact) and control animals administered with VOL body weight did not change remarkably at days 0, 1, 6 and 12 ($P_{t\text{-test}} = 0.801$, $P_{t\text{-test}} = 0.253$). Body weight in the diabetic group showed a notable decrease on days 1, 6 and 12 compared to day 0 ($P_{t\text{-test}} = 0.034$). The administration of VOL to diabetic rats resulted in a decrease in the level of body weight ($P_{t\text{-test}} = 0.085$).

Table 2 gives the change in blood glucose levels of control and experimental rats at regular intervals during the experimental period. Before induction of diabetes, the blood glucose levels of all groups were similar ($P_{ANOVA} = 0.893$). After STZ injection, a significant increase was observed in the blood glucose levels of STZ treated rats on days 1, 6 and 12 as compared to day 0 ($P_{t\text{-test}} = 0.0001$). VOL treatment did not produce any change in the blood glucose levels of the control rats ($P_{t\text{-test}} = 0.322$). The administration of VOL to diabetic rats resulted in a significant decrease in the level of blood glucose ($P_{t\text{-test}} = 0.0001$).

The mean serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) activities are given in Table 3. According to Table 3, there were no significant differences in the AST, ALT and ALP activities of the four groups on day 0 ($P_{ANOVA} = 0.348$, $P_{ANOVA} = 0.111$, $P_{ANOVA} = 0.605$), respectively. In STZ-diabetic rats, a significant increase in the AST, ALT and ALP activities were observed on day 12 compared to day 0 ($P_{t\text{-test}} = 0.001$, $P_{t\text{-test}} = 0.0001$, $P_{t\text{-test}} = 0.0001$), respectively.

Table 1
Mean levels of body weight for all groups (*g*)[a]

Groups	*n*	Day 0	Day 1	Day 6	Day 12	$P_{t\text{-test}}$
Control	5	247.81 ± 31.15	256.37 ± 32.22	259.10 ± 28.77	267.87 ± 33.81	0.801
Control + VOL	5	255.47 ± 17.29	262.71 ± 16.36	268.63 ± 14.82	279.08 ± 17.44	0.253
Diabetic	6	238.70 ± 26.37	215.16 ± 22.40	202.84 ± 26.88	194.01 ± 25.66	0.034
Diabetic + VOL	5	223.80 ± 5.41	203.42 ± 7.48	191.12 ± 23.25	187.37 ± 25.53	0.085
P_{ANOVA}		0.433	0.001	0.0001	0.0001	

n = Number of animals.
[a] Mean ± SD.

Table 2
Mean levels of blood glucose for all groups (mg/dl)[a]

Groups	n	Day 0	Day 1	Day 6	Day 12	$P_{t\text{-test}}$
Control	5	87.79 ± 2.90	90.58 ± 9.78	92.75 ± 7.82	91.80 ± 10.11	0.850
Control + VOL	5	83.64 ± 7.58	92.92 ± 8.62	90.64 ± 10.41	92.84 ± 8.03	0.322
Diabetic	6	84.60 ± 11.05	286.50 ± 25.78	307.99 ± 56.75	266.98 ± 78.83	0.0001
Diabetic + VOL	5	86.34 ± 9.50	251.89 ± 58.72	104.13 ± 21.61	99.17 ± 13.73	0.0001
P_{ANOVA}		0.893	0.0001	0.0001	0.0001	

n = Number of animals.
[a] Mean ± SD.

VOL administration caused remarkable decreases in serum AST, ALT and ALP activities in diabetic groups.

The change in the activities of pancreatic superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GP_x) in all the experimental and control rats is summarized in Table 4. A significant decrease was observed in the activities of GP_x and SOD in pancreas of diabetic rats ($P_{t\text{-test}} = 0.0001$, $P_{t\text{-test}} = 0.006$). The GP_x and SOD activities significantly increased in diabetic group administered with VOL when compared with diabetic group without VOL ($P_{t\text{-test}} = 0.0001$, $P_{t\text{-test}} = 0.003$). An increase in the activity of CAT was observed in the pancreas of diabetic rats when compared with the pancreas of control rats. The administration of VOL to diabetic rats resulted in a significant decrease in the activity of CAT ($P_{t\text{-test}} = 0.0001$).

Table 5 gives the levels of lipid peroxidation (LPO) and glutathione (GSH) in the pancreas of normal and treated groups of rats. In the diabetic groups, LPO levels were higher than those in the other groups ($P_{ANOVA} = 0.222$). There was a non-significant increase in the levels of LPO in pancreas of diabetic rats when compared to normal rats. The administration of VOL was found to reduce pancreas LPO levels in diabetic rats ($P_{t\text{-test}} = 0.045$). A significant difference in the pancreas GSH levels of all groups was observed ($P_{ANOVA} = 0.001$). In diabetic rats, a significant decrease in pancreas GSH levels was observed when compared with control groups ($P_{t\text{-test}} = 0.002$). The administration of VOL was found to increase pancreas GSH levels in diabetic rats ($P_{t\text{-test}} = 0.378$).

2.4. Immunohistochemical results

The control group administered with VOL was not different from the intact control group considering the insulin immunoreactivity in B cells ($P = 0.700$) (Fig. 4A, B). In pancreatic islets of the diabetic group, a decrease in the number of immunoreactive B cells was observed in comparison to the control group and control group with VOL ($P = 0.036$) (Table 6). Hypertrophic cells were observed in pancreatic islets of the diabetic animals. On the other hand, pancreatic islets of the diabetic group administered with VOL showed an increase in the number of immunoreactive B cells in comparison to the diabetic group, statistically ($P = 0.032$) (Fig. 4C, D). In this study, a number of insulin-immunoreactive cells were distinguished in some islets of the STZ-diabetic rats administered with vanadium. We also observed numerous single insulin-immunoreactive cells close in the ducts in STZ-diabetic rats administered with VOL.

3. Discussion

STZ-induced diabetes is characterized by severe loss in body weight. In this study, a decrease in the body weight of the diabetic rats was observed. These results were in accordance with the previously reported study after treatment of STZ-diabetic rats [44]. The decrease in body weight observed in uncontrolled diabetics might be the result of protein wasting due to the unavailability of carbohydrate for utilization as an energy source [45]. Our previous studies have shown that vanadyl sulfate treatment causes body weight gain [44]. In the diabetic group administered with VOL of our present study, a gradual reduction in body weight over days 1–12 was observed, but the reduction in the body weight of the diabetic rats was not changed with VOL.

The blood glucose level in the control group was maintained within the normal range. When control rats received a daily oral administration of VOL at a dose of 0.2 mM/kg for 12 days, the blood glucose level did not decrease as

Table 3
Serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) activities in control and treated groups of rats

Groups	AST (U/l)[a]		$P_{t\text{-test}}$	ALT (U/l)[a]		$P_{t\text{-test}}$	ALP (U/l)[a]		$P_{t\text{-test}}$
	Day 0	Day 12		Day 0	Day 12		Day 0	Day 12	
Control	109.95 ± 14.07	113.23 ± 19.31	0.812	46.35 ± 8.64	55.84 ± 7.77	0.095	73.52 ± 29.77	81.29 ± 32.10	0.702
Control + VOL	105.35 ± 0.18	110.05 ± 9.37	0.503	40.80 ± 4.99	53.36 ± 6.35	0.008	65.45 ± 6.06	92.05 ± 12.00	0.002
Diabetic	102.14 ± 7.40	183.33 ± 20.00*	0.001	49.05 ± 3.81	174.70 ± 20.01**	0.0001	74.68 ± 11.87	293.74 ± 45.16***	0.0001
Diabetic + VOL	93.90 ± 4.75	142.12 ± 10.44	0.0001	40.31 ± 7.29	109.40 ± 37.59	0.005	79.62 ± 3.25	107.42 ± 6.02	0.001
P_{ANOVA}	0.348	0.0001		0.111	0.0001		0.605	0.0001	

*·**·***$P_{t\text{-test}} = 0.0001$ versus control group.
[a] Mean ± SD.

Table 4
Activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) in the pancreas of control and treated groups of rats

Groups	n	GPx (U/mg protein)[a]	$P_{t\text{-test}}$	SOD (U/mg protein)[a]	$P_{t\text{-test}}$	CAT (U/mg protein)[a]	$P_{t\text{-test}}$
Control	5	116.46 ± 8.64	0.0001	46.50 ± 7.06	0.003	636 ± 0.49	0.035
Control + VOL	5	74.90 ± 3.83		28.63 ± 1.70		545 ± 0.57	
Diabetic	6	68.38 ± 12.29*	0.0001	34.30 ± 0.97**	0.003	706 ± 0.72***	0.0001
Diabetic + VOL	5	175.92 ± 17.61		37.50 ± 1.41		356 ± 0.88	
P_{ANOVA}		0.0001		0.0001		0.0001	

n = Number of animals; *$P_{t\text{-test}} = 0.0001$ versus control groups; **$P_{t\text{-test}} = 0.006$ versus control groups; ***$P_{t\text{-test}} = 0.111$ versus control groups.
[a] Mean ± SD.

compared with the control (intact) rats, suggesting that VOL did not affect the blood glucose level of control rats. It is well known that blood glucose levels are elevated in diabetic rats [44]. In this study, an increase in blood glucose levels of diabetic rats was observed. Twelve days after administration of VOL, the blood glucose level significantly decreased in comparison with the diabetic group. The experimental results clearly indicate that VOL has hypoglycemic effect on STZ-diabetic rats. Vanadium acts on the stimulation of glucose into cells and thus a decrease in the blood glucose level was found [8]. VOL administration attenuates hyperglycemia and maintains persistent normoglycemia in diabetic rats. This phenomenon showed that VOL could be associated with an increased number of B cells in each islet. Several studies on the B cell regeneration have reported two different pathways. The proliferation of intra-islet B cell and the differentiation from extra-islet precursor cell [46]. It is possible that VOL treatment had caused the regeneration of endocrine and exocrine pancreatic B cells. According to the immunohistochemical results obtained, VOL can regenerate B cells of insulin dependent diabetic rats, because we observed numerous single insulin-immunoreactive cells close in ducts of exocrine pancreas of STZ-diabetic rats administered with VOL. These data also support that duct cells might be a source for regenerating islet cells.

The liver is the most important organ in the metabolism of drugs and other substances. Common biochemical markers of liver damage are known to increase the activity of some enzymes like AST, ALT and ALP in the blood. The increase in the activities of serum AST, ALT and ALP indicated that diabetes may be induced due to liver dysfunction [47]. The increase in the activities of AST, ALT and ALP in serum may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream, which gives an indication on the hepatotoxic effect of STZ [48]. In our study, these values decreased by administration of VOL. The decrease in those increasing enzyme activity shows that VOL prevented damage in the liver.

Hyperglycemia can lead to both a rise in reactive oxygen species (ROS) production and attenuation of free radical scavenging compounds [49]. Antioxidant enzymes appear to be important for cell defense against oxidative damage [49]. Catalase is a heme protein, which catalyzes the reduction of hydrogen peroxides and protects tissues from highly reactive hydroxyl radicals [50]. The altered balance of the antioxidant enzymes caused an increase in catalase, which may be responsible for the inadequacy of the antioxidant defenses in combating ROS-mediated damage. The increased activities of catalase may be a response to the increased production of H_2O_2 and $O_2^{\bullet -}$ by the autooxidation of glucose and non-enzymatic glycation. The increase in CAT activity in pancreas of this study may reflect a condition of higher oxidative stress and a consequent increase in endogenous hydrogen peroxide. We found that oral administration of VOL to diabetic rats could reverse the elevated CAT activity in pancreas. The decreased activity of CAT, due to the oral administration of VOL probably indicates decreased endogenous hydrogen peroxide production. Excess amounts of peroxide are assumed to be present since CAT protects cells against high hydrogen peroxide levels, whereas GP_x is sensitive to low hydrogen peroxide concentrations [51]. CAT is responsible for scavenging or detoxification of H_2O_2, whereas GP_x scavenges H_2O_2 and lipid peroxides [52]. The opposing responses of catalase and GP_x, both of which breakdown H_2O_2, are in agreement with those reported in kidneys of diabetic rats [53].

Table 5
Lipid peroxidation (LPO) and glutathione (GSH) levels in the pancreas of control and treated groups of rats

Groups	n	LPO (nmol MDA/mg protein)[a]	$P_{t\text{-test}}$	GSH (nmol GSH/mg protein)[a]	$P_{t\text{-test}}$
Control	5	0.19 ± 0.07	0.766	27.04 ± 0.93	0.001
Control + VOL	5	0.17 ± 0.05		21.50 ± 0.81	
Diabetic	6	0.25 ± 0.03*	0.045	22.06 ± 1.51**	0.378
Diabetic + VOL	5	0.16 ± 0.06		23.07 ± 1.88	
P_{ANOVA}		0.222		0.001	

n = Number of animals; *$P_{t\text{-test}} = 0.222$ versus control groups; **$P_{t\text{-test}} = 0.002$ versus control groups.
[a] Mean ± SD.

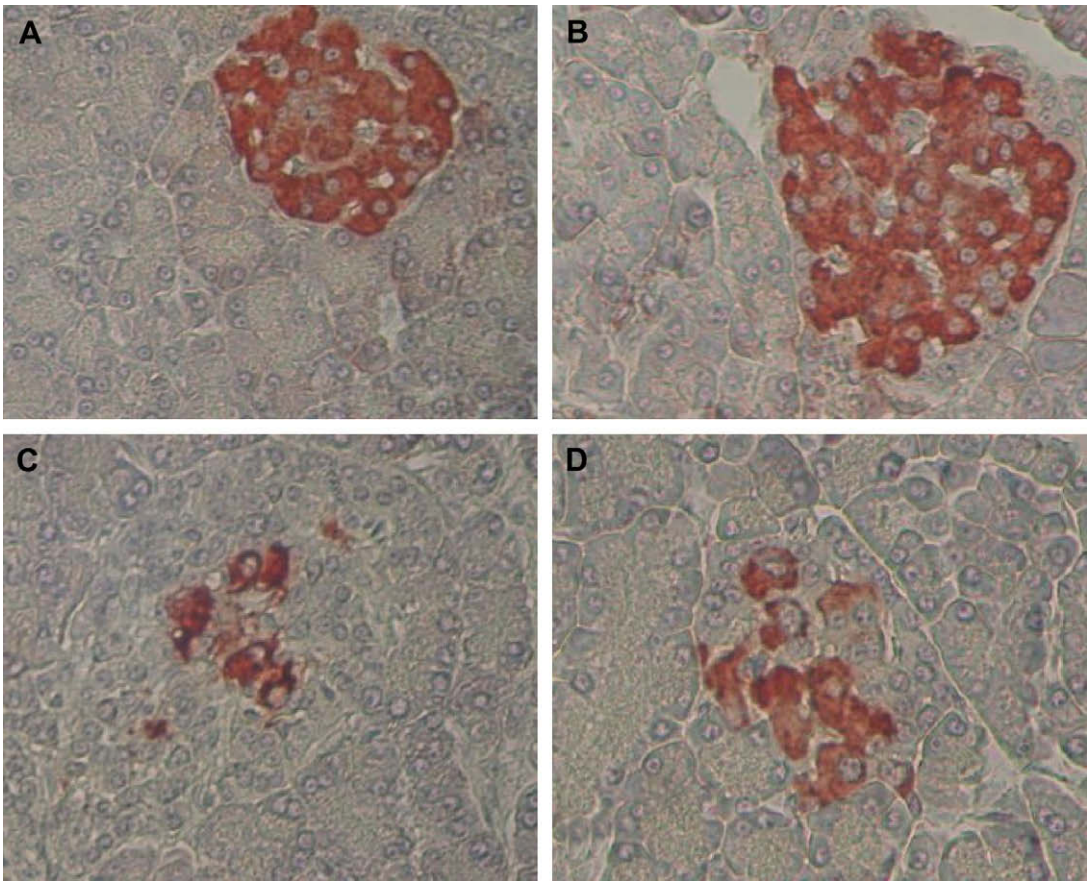

Fig. 4. Immunoreactive beta cells in Langerhans islet localized immunohistochemically with streptavidin biotin peroxidase technique. A control (intact) rat (A), a control rat administered with VOL (B), STZ-diabetic rat (C), and diabetic rat administered with VOL (D). Magnifications: ×400.

Glutathione peroxidase, an enzyme with selenium, has a key role in enzymatic defense systems and acts on peroxides (H_2O_2, lipid or organic peroxides) to remove them. It catalyzes the reduction of hydrogen peroxide and hydroperoxides to nontoxic metabolites. The activity of GP_x was observed to decrease significantly in diabetic rats [54]. The depletion in the activity of GP_x may result in the involvement of deleterious oxidative changes due to the accumulation of toxic products. Other studies also reported a decrease in the activity of GP_x enzymes in tissues of diabetic rats [54]. The depression of glutathione peroxidase activity observed in diabetic pancreas has been shown to be an important adaptive response to increased peroxidative stress [55]. From the present study, it was observed that VOL therapy could effectively normalize the activity of GP_x. This suggests that increased oxidative stress during diabetes (involving increased O_2 and H_2O_2 production) was controlled.

Table 6
Mean of insulin immunohistochemistry for all groups

Groups	*n*	Mean ± SD
Control	3	24.17 ± 11.91
Control + VOL	3	38.23 ± 18.52
Diabetic	5	3.90 ± 1.19
Diabetic + VOL	5	7.67 ± 3.24

Values are mean ± SD; *n* = number of animals.

Superoxide dismutase, one of the main enzymes of the enzymatic antioxidant defense system, responsible for the protection against the increase in free radical production has been demonstrated to be reduced in diabetic groups. SOD scavenged the superoxide radical by converting it into H_2O_2 and molecular oxygen. It is known that the SOD activity is low in diabetes mellitus [56]. Our study has results supporting this literature data. The reduced activity of SOD could be due to its depletion or inhibition as a result of the increased production of free radicals [57]. The administration of VOL increased the activity of SOD and may help to control free radicals in diabetic rats.

Lipid peroxidation is another characteristic parameter of chronic diabetes. The increased free radicals may react with polyunsaturated fatty acids in cell membranes leading to LPO [55]. LPO is supposed to cause the destruction and damage of cell membranes leading to changes in membrane permeability and fluidity, enhancing the protein degradation rates [58]. In our study, the LPO was elevated in diabetic rats as reported earlier [55]. An increase in the level of LPO in the pancreas of diabetic rats suggested increased levels of oxygen radicals that could be due to their increased production or decreased destruction [56]. Elevated free radical levels in

diabetes could be due to an increase in blood glucose levels. It has been reported that the vanadyl ion can act as a scavenger of oxyradicals and thus could prevent liver [59] and heart dysfunctions [60]. In our study, it was presented that the VOL treatment decreased lipid peroxidation in the diabetic group. According to the data, VOL has a protective effect by decreasing lipid peroxides.

One of the most important intracellular antioxidant systems is the glutathione redox cycle. Reduced glutathione normally presented in millimolar concentrations in all cells is known to protect the cellular system against the toxic effects of lipid peroxidation. GSH functions as a direct free radical scavenger, as a cosubstrate for GPx activity and as a cofactor for many enzyme-form conjugates in endo- and exo-xenobiotic reactions [61]. Chronic hyperglycemia induced toxicity may also decrease the level of GSH in tissues and plasma [56,59]. We have observed a decrease in the level of GSH in the pancreas during diabetes. Other studies and another study we had conducted have also reported decreased levels of tissue GSH in STZ-diabetic rats [56,59]. The activity of GP_x in this study was also similarly lowered in diabetic rats. This may be attributed to the unavailability of reduced GSH. The administration of VOL increased the content of reduced GSH in the pancreas of diabetic rats. The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies reactive oxygen species generated from exposure to STZ.

The restoration of pancreatic antioxidant enzymes and normalization of lipid peroxidation and glutathione levels underline the amelioration of hyperglycemia induced oxidative stress by the oxovanadium(IV) chelate (VOL). The immunohistochemical examinations of the pancreas highlight the efficacy of the vanadium chelate in the attenuation of oxidative stress in the pancreas. The alterations in the antioxidant status of the pancreas of STZ-induced diabetic rats were reversed by VOL, which has an insulinomimetic effect and attributed to its restoration of glucose metabolism.

In conclusion, it can be said that the insulinomimetic activity of VOL appears as the cause of its protective effect on pancreatic tissue defense system. The present study demonstrates firstly that a oxovanadium(IV) chelate derived from thiosemicarbazone could be useful as a potential antidiabetic agent. It can also be suggested that this vanadium chelate is a significant hope in the search for an alternative medicine to insulin.

In conclusion, the insulinomimetic effect of VOL is the major claims for the protective effect of VOL chelate on pancreatic tissue defense system.

4. Experimental protocols

4.1. Materials, methods and apparatus

All chemicals and solvents were of reagent grade. The elemental analyses were determined on a Thermo Finnigan Flash EA 1112 Series Elementary Analyser. Molar conductivity was measured using a digital CMD 750 conductivity meter. Magnetic measurement was carried out at room temperature by the Gouy technique with an MK I model device obtained from Sherwood Scientific.

IR spectra were recorded (KBr disks) on a Mattson 1000 FT-IR spectrometer. ^{1}H NMR spectra were obtained on a Varian INOVA 500 MHz spectrometer. UV–vis spectra were recorded on ATI-Unicam UV2 spectrophotometer. EPR spectra have been registered by a conventional X-band ($\nu = 9.5$–9.7 GHz) Bruker EMX model spectrometer employing an ac magnetic modulation technique. LC–APCI-MS analyses were carried out in positive and negative ion modes using a Thermo Finnigan LCQ Advantage MAX LC/MS/MS. The mobile phase consisted of a gradient mixture of 60% MeOH and 40% H_2O. Hypersil Betabasic-8 (5 μ, 100 mm × 4.6 mm) column was used at a flow rate of 0.2 ml/min at 25 °C. APCI-MS inlet conditions in the +/− ion mode: heated capillary temperature, 200/200 °C; vaporizer temperature, 250/250 °C; sheath gas flow rate, 50/50 units; capillary voltage, 3/−8 V and tube lens offset, 45/−35 V.

4.2. Synthesis

4.2.1. 2,4-Dihydroxybenzaldehyde S-methylthiosemicarbazone

2,4-Dihydroxybenzaldehyde *S*-methylthiosemicarbazone was prepared according to the literature [62] and characterized by melting points and spectral data. The colour, yield, m.p., R_f, ^{1}H NMR and IR data of L_I were given as follows: pinkish-cream, 94%, 179–180 °C, 0.492 (CH_2Cl_2), ^{1}H NMR (DMSO-d_6, 25 °C, ppm): 11.67, 11.02 (*cis*/*trans* ratio: 5/2, s, 1H, OH), 9.75 (s, 1H, OH), 8.32, 8.20 (*syn*/*anti* ratio: 2/3, s, 1H, CH=N^1), 6.71, 6.65 (*syn*/*anti* ratio: 1/1, s, 2H, NH_2), 7.28, 7.15 (*syn*/*anti* ratio: 1/1, d, *J*: 8.25, 1H, d), 6.33, 6.29 (dd, *J*: 1.80, *J*: 7.84, 1H, c), 6.294 (splitted d, *J*: 2.44, *J*: 6.84, 1H, a), 2.42, 2.38 (*cis*/*trans* ratio: 3/2, s, 3H, S–CH_3), FT-IR (KBr, cm^{-1}): ν_a(NH) 3445, ν_s(NH) 3337, ν(OH) 3495, δ(NH) 1624, ν(C=N^1), ν(N^2=C) 1608, 1585, ν(C–O) 1177–1150.

4.2.2. N^1-2,4-Dihydroxybenzylidene-N^4-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV) [VOL]

2,4-Dihydroxybenzaldehyde-*S*-methylisothiosemicarbazone (1.12 g, 5 mmol) and 2-hydroxybenzaldehyde (0.5 ml, approx. 5.5 mmol) were dissolved in 25 ml of ethanol. The solution was then added stirring to a solution of 1.26 g (5 mmol) $VOSO_4 \cdot 5H_2O$ and orthoformic ester (6 ml) in ethanol (25 ml). After 1 h at room temperature, 8.5 ml of triethylamine was added into the reaction mixture. After 48 h, the brown precipitate was filtered off, washed with ethanol–ether (1:1, 10 ml) and dried in vacuo over P_2O_5.

The colour, yield, m.p., R_f, magnetic moment, molar conductance (in DMSO at 25 ± 1 °C), Elemental analysis, UV–vis, IR and APCI-mass data were given as follows: brown, 60% (1.31 g), m.p. > 380 °C, R_f: 0.515 (Et_2O), μ_{eff}: 1.64, 17.5 Ω^{-1} cm^2 mol^{-1}, $C_{16}H_{13}N_3O_4SV$ found (calc.): C, 48.82 (48.74); H, 3.24 (3.30); N, 10.59 (10.66); S, 8.23 (8.12), UV–vis (λ nm/ε in DMSO): 245/25 083, 314/28 826, 350/22 412, 418/20 246, 802/160 and 958/42, IR (KBr, cm^{-1}):

ν(OH) 3410, ν(C=N) 1605, 1593, 1578, ν(C–O) 1146–1123, ν(V=O) 985, ν(V–O) 477–434, mass: 515, 496. *m/z* (+c APCI) 394 (M^+, 18.65), 395 (MH^+, 100.00), 379 ($M^+ - CH_3$, 15.96), 309 ($M^+ - CH{=}N{-}N{=}C{=}S$, 5.87). *m/z* (−c APCI) 394 (M^+, 32.85), 395 (MH^+, 10.43), 393 ($M^+ - H$, 100.00), 379 ($M^+ - CH_3$, 5.18), 378 ($MH^+ - CH_3$, 4.52), 363 ($MH^+ - S-$, 5.06), 311 ($M^+ - VO_2$, 1.23), 284 ($M^+ - C_6H_4(OH)_2$, 1.05).

4.3. Animals and experimental design

In the experiments, male Swiss albino rats were used. The rats which are 3–3.5 months old were randomly divided into four groups. Group I: control (intact) animals ($n = 5$). Group II: control animals administered with VOL ($n = 5$). Group III: STZ-induced diabetic animals ($n = 6$). Group IV: STZ-induced diabetic rats treated with VOL. VOL of 0.2 mM/kg daily dose was administered by gavage technique to rats for 12 days, after the experimental animals were made diabetic. On day 12, the pancreatic tissues were taken under ether anesthesia from animals that were fasted overnight.

For the preparation of diabetic rats, diabetes was induced by a single intraperitoneal injection of STZ (65 mg/kg). STZ was dissolved in a freshly prepared 0.01 M citrate buffer (pH 4.5).

The experiments were reviewed and approved by the Animal Care and Use Institute's Committees of Istanbul University. They were maintained under standard environmental conditions and fed with laboratory pellet chow and given water ad libitum.

4.4. Biochemical assays

The biochemical investigations were made in blood and pancreatic tissue. The blood samples of rats were collected from the tail vein on days 0, 1, 6 and 12. Fasting blood glucose levels (after 18 h period of fasting) were determined by *o*-toluidine methods [63]. Serum AST and ALT activities were assessed by the Reitman–Frankel method [64]. Serum ALP activities were determined by two-point method [65]. The body weight of all rats was measured at days 0, 1, 6 and 12. At the end of the experimental period, the animals were fasted overnight and then sacrificed. For biochemical analysis, pancreatic samples were dissected out and immediately washed in ice-cold with 0.9% saline and homogenized in 0.9% saline with a glass homogenizer to make up to 10% homogenate (w/v). The homogenates were centrifuged and the clear supernatants were used for protein, glutathione (GSH), lipid peroxidation (LPO) and enzymes analyses. Pancreatic LPO and GSH levels were determined by the methods of Ledwozyw [66] and Beutler using Ellman's reagent [67], respectively. The catalase activity (CAT) was measured in the tissue according to the method of Aebi [68]. The glutathione peroxidase (GP_x) activity was determined by the method described by Paglia and Valentine [69] and modified by Wendel [70]. The superoxide dismutase (SOD) activity was assayed by the method described by Mylroie et al. [71]. The levels of pancreas total protein were measured by the method of Lowry using bovine serum albumin as standard [72].

4.5. Immunohistochemistry studies

The pancreatic tissue samples were fixed in Bouin, dehydrated in a graded series of ethanol, and embedded in paraffin wax before sectioning. Sections were dewaxed and rehydrated. After a washing step in phosphate-buffered saline (PBS), sections were immersed in a solution of 3% H_2O_2 for 10 min. The sections were then pre-incubated with non-immune serum for 20 min. They were labelled with streptavidin biotin following incubation with primary monoclonal anti-insulin clone antibody (dilution 1/1500 $\mu g\,ml^{-1}$). The localization of the antigen was indicated by a red colour obtained with 3-amino-9-ethyl-carbazole (AEC) as a chromogenic substrate for peroxidase activity. Primary anti-insulin antibody (Sigma I-2018) and Histostain-Plus kit (Zymed Code 85-9943) were used for immunohistochemistry, and the slides were counterstained with hematoxylin. The specificity of the immunohistochemical staining was checked by the omission of the primary antibody or by using an inappropriate antibody (anti-gastrin). All these controls gave negative results. The control pancreas sections with (+) signals were used as a positive control. Insulin-immunoreactive cells were counted in all Langerhans islets seen in two sections for each animal. The mean count of insulin-immunoreactive cells was determined with the total insulin-immunoreactive cells, by dividing count of total Langerhans islets. Two different blinded observers evaluated the immunoreactivity.

4.6. Statistical analysis

The biochemical results were evaluated using an unpaired *t*-test and ANOVA using the NCSS statistical computer package. The significance of changes in insulin-immunoreactive cells was evaluated statistically using the Kruskal–Wallis and Mann–Whitney test. Results were expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

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