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The effects of N(1)-2,4-dihydroxybenzylidene-N-(4) hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV) on testicular damage in streptozotocin-induced diabetic rats

Sevim TUNALI[1] *, Tulay BAL-DEMIRCI[2], Bahri ÜLKÜSEVEN[2]*, Refiye YANARDAG[1]

[1] Department of Chemistry, Biochemistry Division, Faculty of Engineering, Istanbul University-Cerrahpasa, Istanbul, Turkey.
[2] Department of Chemistry, Inorganic Chemistry Division, Faculty of Engineering, Istanbul University-Cerrahpasa, Istanbul, Turkey.
* Corresponding Author. E-mail: stunali@iuc.edu.tr (S.T.); Tel. +90-212-473 70 70/17791

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ABSTRACT: Diabetes mellitus (DM) is a serious metabolic disorder that has negative effects on male sexual and reproductive functions in humans and animals. The purpose of current research is to demonstrate the effect of N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV) (VOL) on testicular damage in male rats with streptozotocin (STZ)-induced diabetes. Male Swiss albino rats were randomly grouped as follows: Control (intact) group animals; control group animals given VOL (0.2 mM/kg/day) for 12 days; STZ-induced diabetic animals; diabetic animals given VOL group, at same dose and time. Experimental diabetes was induced with a single dose of 65 mg/kg intraperitoneal STZ injection. On day 12, overnight fasted animals were sacrificed and testis tissues (right and left) were collected and homogenized in 0.9 % saline. After centrifugation, protein levels and non-enzymatic parameters such as glutathione, lipid peroxidation, protein carbonyl, as well as the activities of alkaline phosphatase, myeloperoxidase and enzymatic antioxidants were determined. Based on the results obtained, VOL was shown to be a potentially beneficial compound in the amelioration of damaged testicular tissue of male diabetic rats after 12 days of administration. Our results suggest that VOL may be a promising candidate for the development of new generation antidiabetic drugs, and its administration to diabetic rats may be a suitable candidate in reducing testicular damage.

KEYWORDS: Diabetes mellitus; oxovanadium complex; testicular damage; oxidative stress.

1. INTRODUCTION

Vanadium, an important transition element in glucose and lipid metabolism, is an insulin-mimetic, antilipidemic, and potent stress alleviating agent in Diabetes mellitus (DM) [1]. As knowledge about the biological importance and potentials of vanadium compounds becomes widespread, interest in the synthesis of new compounds and complexes of this element for therapeutic purposes is increasing [2-4]. Besides the medical and therapeutic activities of vanadium compounds, several coordination complexes including vanadyl (IV) ions have been studied in vitro and in vivo as insulin-mimetic agents [5].

Oxidative stress, caused by hyperglycemia, has been suggested to be an important factor in production of excessive reactive oxygen species (ROS). Disproportionately increased free radicals in diabetes by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins declined antioxidant defense mechanisms and lead to damage of cellular organelles and enzymes [6]. This condition is associated with various pathological situations such as micro and macrovascular complications [7], nephropathy [8], cataract formation [9] and cardiovascular diseases [10].

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The developed complication of DM by oxidative stress consequently affect male reproductive functions [11]. In addition, researches on birth rates in developed countries have clearly demonstrated that increased DM incidence is intimately connected to falling birth and infertility rates [12,13]. Hyperglycemia decreases sperm motility and semen volume, it influences sperm counts and morphology, and as well as disrupt seminiferous tubular morphology [14,15]. The occurrence of male infertility in diabetes (characterized by hyperglycemia), is closely related to both increased production of ROS and impairment of the activity of testicular antioxidant enzymes [16,17]. Hence, oxidative stress in testicular tissue induce apoptosis of endothelium of germ cells, lipid peroxidation, and the oxidative damage of DNA and proteins [18,19]. Besides that, damage to the seminiferous tubules and serious decrease in sperm quality is observed in the reproductive organs of streptozotocin (STZ)-induced diabetic animals [20,21]. STZ, a diabetogenic agent widely used to induce experimental diabetes in animals, causes characteristic changes in blood insulin and glucose concentrations by acting on pancreatic beta cells [22].

Vanadium and vanadium compounds have been reported to mimic the action of insulin [5,7]. The most accepted opinion about this behavior as insulin-like agent is considered to be based on the protein tyrosine phosphatase (PTPs) inhibition [23].

In view of the fact that oxidative stress is known to be the one of the factors in the DM, we aim to scrutinize the effect of VOL (a newly developed compound) on ROS level and antioxidant capacity of the testicular tissue of STZ-induced diabetic animal model.

2. RESULTS

STZ-induced DM causes weight loss and muscle wasting occurs due to the increase in catabolytic reactions [5, 24]. The data from this set of animals, concerning body weights and fasting blood glucose levels have been previously published with respect to the synthesis, characterization and antidiabetic properties of VOL [5]. The body weight data were significant between all groups for 1, 6 and 12 days (P_{ANOVA} = 0.001; P_{ANOVA} = 0.0001; P_{ANOVA} = 0.0001 respectively). The loss seen in body weight of the diabetic animals was prevented by VOL administration which could be attributed to its anti diabetic effect (Table 1). Accordingly, significance was also observed between groups at day 1, 6 and day 12 for blood glucose levels (P_{ANOVA} = 0.0001; P_{ANOVA} = 0.0001; P_{ANOVA} = 0.0001 respectively). Blood glucose levels which were increased by STZ administration were diminished by VOL treatment (Table 2) [5].

Table 1. Mean levels of body weight for all groups (g)* (Yanardag et al., 2009) [5].

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	

*Data were presented as mean ± SD.

Abbreviations: n, number of animals; SD., standard deviation; ANOVA, analysis of variance; VOL, N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV); g., gram.

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Table 2. Mean levels of blood glucose levels for all groups (mg/dl)* (Yanardag et al., 2009) [5].

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	

*Data were presented as mean ± SD.

Abbreviations: n, number of animals; SD., standard deviation; ANOVA, analysis of variance; VOL, N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV).

With respect to testicular oxidative stress indices, lower level of glutathione (GSH) in STZ-induced diabetic group was observed ($p < 0.01$), whereas the levels of malondialdehyde (MDA) and protein carbonyl (PC) were elevated in comparison to intact rats ($p < 0.005$; $p < 0.005$). VOL administration to the diabetic animals significantly increased GSH level ($p < 0.05$) and significantly lowered the raised MDA and PC contents ($p < 0.05$; $p < 0.05$) (Table 3).

Table 4 represents the activities of ALP and MPO of all experimental groups. There was a significant alteration in the activities of alkaline phosphatase (ALP) and myeloperoxidase (MPO) in diabetic rats when compared to control group ($p < 0.01$; $p < 0.0001$). VOL treatment of diabetic group significantly prevented the increased activities of ALP and MPO resulting from diabetes ($p < 0.0001$; $p < 0.005$) (Table 4). Meanwhile, the activity of MPO in control animals given VOL complex significantly increased in comparison to intact control animals ($p < 0.0001$).

Table 3. Testis GSH, LPO and PC levels in control and experimental groups.

Groups	GSH (nmol GSH/mg protein)*	LPO (nmol MDA/mg protein)*	PC (nmol Carbonyl/mg protein)*
Control	0.98 ± 0.27	1.11 ± 0.24	0.34 ± 0.03
Control + VOL	0.71± 0.13	1.04 ± 0.27	0.32 ± 0.02
Diabetic	0.46 ± 0.12[a]	1.82 ± 0.27[c]	0.43 ± 0.02[c]
Diabetic + VOL	0.86 ± 0.08[b]	1.21 ± 0.39[b]	0.36 ± 0.04[b]
P_{ANOVA}	0.004	0.004	0.003

*Data were presented as mean ± SD.

[a]$p < 0.01$ versus to control animals; [b]$p < 0.05$ versus to diabetic animals; [c]$p < 0.005$ versus to control animals.

Abbreviations: GSH, reduced glutathione; LPO, lipid peroxidation; PC, protein carbonyl; SD., standard deviation; ANOVA, analysis of variance; VOL,N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV).

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Table 4. Testis ALP and MPO activities in control and experimental groups.

Groups	ALP (U/mg protein)*	MPO (U/g tissue)*
Control	41.94 ± 3.31	0.08 ± 0.01
Control + VOL	45.14 ± 4.17	0.23 ± 0.01[c]
Diabetic	53.16 ± 4.52[a]	0.15 ± 0.01[c]
Diabetic + VOL	25.39 ± 2.40[b]	0.10 ± 0.06[d]
P_{ANOVA}	0.0001	0.0001

*Data were presented as mean ± SD.

[a]p < 0.01 versus to control animals; [b]p < 0.0001 versus to diabetic animals; [c]p < 0.0001 versus to control animals; [d]p < 0.005 versus to diabetic animals.

Abbreviations: ALP, alkaline phosphatase; MPO, myeloperoxidase; SD., standard deviation; ANOVA, analysis of variance; VOL, N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV).

A significant change in enzymatic oxidative stress bio-markers of diabetic group when compared to control animals was observed (Table 5). A significant decrease in the activities of all enzymatic antioxidant enzymes in the STZ-induced hyperglycemic group as compared to the control animals testicular tissues was noticed (p < 0.0001; p < 0.0001; p < 0.05; p < 0.0001). On the other hand, VOL treatment to the diabetic rats significantly elevated the activities of catalase (CAT), glutathione peroxidase (GPx), glutathone-S-transferase (GST), and superoxide dismutase (SOD) (p < 0.05; p < 0.0001; p < 0.0001; p < 0.05).

Table 5. Testis CAT, GPx, GST and SOD activities in control and experimental groups.

Groups	CAT (U/mg protein)*	GPx (U/g protein)*	GST (U/mg protein)*	SOD (U/mg protein)*
Control	24.54 ± 0.34	0.40 ± 0.05	1.800 ± 0.02	15.18 ± 4.42
Control + VOL	18.05 ± 1.58[a]	1.40 ± 0.11[a]	0.150 ± 0.01[e]	8.92 ± 1.24[a]
Diabetic	19.63 ± 1.38[b]	0.13 ± 0.03[b]	0.153 ± 0.01[f]	12.63 ± 3.34[b]
Diabetic + VOL	22.51 ± 1.77[c]	0.22 ± 0.02[d]	0.206 ± 0.01[d]	16.94 ± 1.15[c]
P_{ANOVA}	0.0001	0.0001	0.0001	0.0001

*Data were presented as mean ± S.D.

[a]p < 0.005 versus to control animals; [b]p < 0.0001 versus to control animals; [c]p < 0.05 versus to diabetic animals; [d]P < 0.0001 versus to diabetic animals; [e]P < 0.05 versus to control animals; [f]P < 0.05 versus to control animals.

Abbreviations: CAT; catalase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; SOD, superoxide dismutase; SD., standard deviation; ANOVA, analysis of variance; VOL, N(1)-2,4-dihydroxybenzylidene-N(4)-2-hydroxybenzylidene-S-methyl-thiosemicarbazidato-oxovanadium(IV).

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In addition, a significant change in the activities of CAT, GPx, GST and SOD was observed in VOL administered control animals. The activities of CAT, GST and SOD of VOL treated control animals significantly decreased when compared to intact animals ($p < 0.005$; $p < 0.05$; $p < 0.005$). In the contrary to that, GPx activity increased significantly in VOL treated animals when compared to intact rats ($p < 0.005$) (Table 5).

3. DISCUSSION

DM, characterized by hyperglycemia, is a multifactorial disease. Nowadays, there is great concern that the increasing incidence of diabetes may affect the reproductive function of many men in active reproductive age [25]. The seen loss of body weight and increased blood glucose level of the diabetic animals were prevented by VOL, and this findings were discussed in detail in our previous research [5]. Numerous studies, both in vitro and in vivo, have demonstrated the insulin-mimetic properties of vanadium and its inorganic and organic compounds [7,8,26].

Experimental studies on vanadium compounds with different coordination geometries have shown that vanadium complexes with five coordination numbers are stronger inhibitors than those with coordination numbers six or seven [27]. In our previous researches and the current study, the new oxovanadium(IV) complex-VOL has been found to have a healing effect on many biochemical parameters in diabetic testicular tissue, as well as normalising hyperglycaemia [5,28,29]. This effect may in part be due to the inhibition of PTPs by vanadium in various ways. The PTPs active sites has a cysteine residue, and their catalytically mechanism is formed via fosfocysteine as an intermediate [23,27]. On the other hand, in the form of compound, vanadium taken into the cell binds to the active site of PTP. This is resulting with the inhibition of the enzyme and signal transduction pathways used in glucose uptake are activated [23, 30]. Vanadium mimics insulin in this way and causing the reduction of blood sugar concentration in the body [30].

Reduced glutathione is an endogenous tripeptide that plays an important role in inactivating hydrogen peroxide and lipid peroxides in the biological system. Reduced glutathione level is the marker of the oxidative status. Moreover, glutathione and its constituent cysteine are effective compounds for decarbonylation [31]. Thiols cause reduction in the levels of protein carbonyl content, since they affect protein conformation, activity and function [32]. In the current study, vanadium complex caused a rise in the level of GSH, while PC content was significantly decreased. The opposite of these was observed in the hyperglycemic group, GSH concentration decreased in the diabetic group as compared to the intact group, while a significant increase in the PC level was observed. Similar to our findings, the decreased level of GSH in the diabetic testicular tissue was also reported by Bal et al., (2011) [33]. This may be as a result of the cleavage of GSH into cysteine, a decline in GSH synthesis, or a decrease in cellular antioxidant reserves as a result of reactions such as mixed disulfide formation [34].

Sperm cells contain high concentrations of specific lipid compounds such as plasmalogen, sphingomyelin and polyunsaturated fatty acids. Insufficiency of antioxidant mechanisms makes polyunsaturated fatty acids more vulnerable to oxidative damage by the excessively produced ROS species in DM and causes damage to testicular tissue [35,36]. Furthermore, the negative effects of LPO in membrane structure can influence the ability of spermatozoa to take part in membrane fusion events that are relevant for fertilization. Reports provides evidence that defective sperm function is commonly induced by oxidative stress. Thus, affecting sperm motility via lipid peroxidation and altered DNA integrity [37]. In the present investigation, there is show significantly high LPO level in diabetic testicular tissue. Related finding like ours has also been reported in the study with diabetic testicular damage ameliorated by diosgenin [38]. Oral administration of VOL to diabetic rats caused a significant reduction of LPO level. This beneficial effect of the vanadium complex may be due to its hypoglycemic effect.

Vanadium and its compounds have been found to have an inhibitory effect on PTPs as well as enzymes that especially catalyze phosphate reactions [39]. Various phosphatases such as acid phosphatase, alkaline phosphatase and tyrosine-protein phosphatases can be inhibited by vanadium compounds. Alkaline

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phosphatases are membrane bond glycoprotein enzymes which catalyse the hydrolysis of phosphate monoesters from molecules. They also plays a role in the transfer of phosphate groups to hydroxyl groups of organic molecules. The catalytic mechanism of such enzymes is the phosphorylation of a serine residue on the active side and subsequent transfer of the phosphate group to organic acceptor or water molecule [40]. The reason why vanadium and its complexes inhibit such enzymes is due to the physicochemical similarity of phosphate and vanadate, and their participation in similar reactions. Despite the structural similarity, it is important to emphasize that coordination geometries of vanadium complexes shows a greater flexibility. Besides that, as mentioned beforere, this strong inhibiton can be due to five numbered coordination geometry of vanadium complexes [27,40]. In the current study, the new oxovanadium complex-VOL demonstrates a five coordination number. The inhibition of ALP in the diabetic group given VOL may therefore suggest that VOL has a role in protecting testicular tissue against STZ-induced diabetic oxidative injury.

MPO is the enzyme that catalyzes the production of reactive oxygen species including hypochlorous acid (HOCl) in the presence of hydrogen peroxide and halides. The findings of Aratani (2018), obtained from real-time PCR techniques showed that the ram's testicles and bulbourethral glands expresses the MPO gene [41,42]. Further, a different research observed that MPO activity is significantly higher in the testicular tissue of the rats with non-insulin dependent diabetes mellitus (NIDDM), thereby revealing the association of the MPO gene with T2DM [43,44]. Khosravi et al., (2019) also demonstrated an increased activity of MPO (a biomarker and consistent indicator of neutrophil proliferation and occurrence of inflammation) in diabetic animal testis [38]. In agreement with these findings, we observed an increased MPO activity it the diabetic animals testicular tissues. VOL administration which is associated with lower inflammation was effective in the improvement of MPO activity in testicular tissues of diabetic rats.

The pathological state in DM is strictly associated with an imbalance of ROS production and their effect on enzymatic antioxidant defense system in tissues [45]. The seminal vesicles have been shown to secrete CAT, GPx, GR, SOD, and GSH into the seminal fluid, as an additional antioxidative support for the spermatozoa [46,47]. Consistent with our findings on enzymatic antioxidant activities in diabetic testicular rat tissue, there is reported the decreased activity of antioxidant enzymes CAT, GPx and SOD [38]. The mechanism responsible for the protective effect of vanadium on testicular function may involve the enhancement of intracellular GSH level and increased activities of CAT, GPx, GST and SOD [46,48]. Additionally, the probability that vanadium mimics the insulin is partially owing to changes in prooxidant/oxidant balance [49,50]. The competitive behavior of vanadate with respect to phosphate is likely an indication of its insulin-mimetic or insulin-enhancing effect of vanadium compounds. In this way, an enhancement of the antioxidant defense of the testis may counteract the effects of the excessive oxidative stress.

A remarkable finding in our study was the observation that VOL administration to control animals resulted in reduction of the activities of testicular CAT, GST and SOD. Whereas, the activities of MPO and GPx significantly increased. The changes noticed upon vanadium treatment may be as a result of increased formation of oxygen-free radicals, which in turn overwhelm the body's antioxidant defense system. Findings of the present study showing altered enzymatic activities suggests that administration of vanadium complex may cause toxic effects in normal testicular tissue metabolism. Similar observations have been shown in different studies of vanadium toxicity [51], however a more comprehensive study is need to be conducted to assess the safe and effective doses of VOL.

4. CONCLUSION

Modern pharmacological research suggests that testicular damage are caused by hyperglycemic-induced oxidative stress, apoptosis, and disorders associated with endocrine metabolism. Therefore, researchers have recently tried to control the development of hyperglycemic related reproductive damage by focusing on the synthesis of new chemical compounds that may mimic and replace insulin. The present study provided information on the effects of VOL, a novel vanadium complex, on diabetic testicular tissue, which can be presented as a possible therapeutic alternative in reducing diabetes-related male infertility.

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5. MATERIALS AND METHODS

5.1. Synthesis of VOL

The oxovanadium(IV) complex (VOL) (Figure 1) was freshly prepared by starting from S-methyl-isothiosemicarbazide as earlier described [5]. In first step, 2-hydroxybenzaldehyde S-methyl-isothiosemicarbazone was obtained from reaction of S-methyl-isothiosemicarbazide (0.460 g) and 2,4-dihydroxyl-benzaldehyde (0.604 g). Then, the thiosemicarbazone (1mmol) and 2-hydroxybenzaldehyde (1 mmol) were dissolved in ethanol (50 mL). Vanadyl sulphate ($VOSO_4.5H_2O$, 1 mmol) dissolved in 25 mL alcohol was added to the mixture. After stirring for 5 hours, the brownish-powder formed was filtered. The crude product was recrystallized from alcohol-ether (1:1) and checked its purity by TLC. The chemical structure of VOL was confirmed using elemental analysis and infrared spectrum. A yield of 64%, and m.p. > 380oC was obtained. For C16H13N3O4SV (calc.): C, 48.82 (48.74); H, 3.24 (3.32); N, 10.59 (10.64); S, 8.23 (8.14). IR (ATR, cm1): (OH) 3410, (C=N) 1606, 1594, 1574, (CO) 1146, (V=O) 984, (VO) 478-436.

5.2. Experimental animals and study design

Animals: The experiments were reviewed and approved by Animal Care and Use Institute's Committee of the Istanbul University. Twenty-one male Swiss albino strain rats (200 ± 50 g) were kept in normal temperature (22 ± 2°C), and fed with standard chow and water ad libitum.

Inducement of diabetes: Diabetes was induced by a single intraperitoneal dose of streptozotocin (STZ) (65 mg/kg body weight) dissolved in cold citrate buffer (0.1 M, pH 4.5) [52]. After 24 hour of STZ injection, DM was confirmed by measuring fasting blood glucose levels from the rats tail. Rats with a fasting blood glucose level above 250 mg/dL were considered as diabetic. The part of the study related to weight and blood glucose values was detailed in our previous research [5].

Experimental Protocol: The rats were randomly divided into four groups as follows: two control groups divided as (1) control group-intact (n=5) and (2) treated control-received VOL (n=5) (0.2 mM/kg/day); two diabetic groups separated as (3) diabetic control (n=6) (65 mg/kg body weight) and (4) VOL treated diabetic group (n=5) (receiving the same doses of STZ and VOL). On the last day of the experiment (day 12), the mice were sacrificed under anesthesia.

5.3. Biochemical assays

Both testicular tissues, right and left, were homogenized in a cold saline solution and centrifuged at 10,000×g for 10 minutes. The obtained clear upper phase was used for estimation of biochemical parameters.

Total protein determination: Testis total protein level was determined by the method of Lowry [53]. Briefly, alkaline proteins are reacted with copper ions and then reduced by Folin reactive. The absorbance of the product was evaluated at 500 nm by a spectrophotometer and calculated to express the results of the parameters per protein.

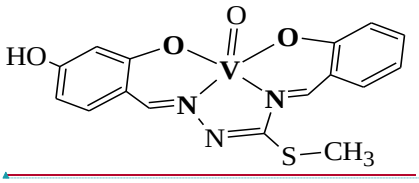

Figure 1. The VOL, a thiosemicarbazone-based oxovanadium(IV) complex.

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Glutathione determination: GSH level was quantified by measuring the content of −SH groups via spectrophotometric technique according to the Beutler (1971) at 412 nm [54]. Metaphosphoric acid was used for protein precipitation, and 5,5′-dithiobis-2-nitrobenzoic acid was used for color development. The results were expressed as nmol GSH/mg protein.

Lipid peroxidation determination: Testis malondialdehyde (MDA) level, an end product of lipid peroxidation (LPO), was determined as thiobarbituric acid reactive substances by the method of Ledwozyw (1986) by measuring the formation of malondialdehyde (MDA) [55]. LPO was expressed in terms of MDA equivalents as nmol MDA/mg protein.

PC determination: Testis tissue PC was determined spectrophotometrically by the method of Levine et al. (1990) [56]. Testis homogenate samples (0.5 ml) were incubated in dark at room temperature with 2 ml DNPH (10 mM) for 1 h and mixed every 15 min. About 2.5 ml 20% tricholoroacetic acetic (TCA) were added and samples were kept in ice for 5 min. After centrifugation, the supernatants were discarded and precipitates were washed three times with a mixture of 2 ml of ethanol and ethyl acetate (1:1). The final precipitates were treated with 1 ml of guanidine hydrochloride (6 M) and incubated at 37°C for 10 min. The absorbances were read at 370 nm, and the results expressed in nmol carbonyl/mg protein.

ALP determination: The specific activities of alkaline phosphatase (ALP) were determined by the method of Walter and Schutt (1974) [57]. Under optimum conditions for measurements of the ALP, the absorbance was monitored at 405 nm. Enzyme activities were expressed as U/mg protein.

MPO determination: Enzymatic activity of MPO was determined by the method of Wei and Frenkel (1991) [58]. This involves a solution of tissue homogenate, phenol, H_2O_2, and 4-aminoantipyrine as color-generating substance. One unit of enzyme activity was defined as the amount of the MPO present per gram of protein which caused a change in absorbance per minute at 460 nm and 37°C. The results were expressed as U/mg protein.

CAT determination: Testis catalase (CAT) activity was determined by the method of Aebi [59]. The method is based on converting hydrogen peroxide (H_2O_2) to water by the effect of CAT. The absorbance of the samples was measured in 240 nm by a spectrophotometer. The results were expressed as U/mg protein.

GPx determination: GPx activity of testis tissues was determined by the method described by Paglia and Valentine (1967) and modified by Wendel (1981) [60,61]. GPx catalyzes the conversion of hydrogen peroxide (H_2O_2) to water. The generated glutathione disulfide (GSSG) is reduced to GSH with consumption of nicotinamide adenine dinucleotide phosphate (NADPH) by glutathione reductase. During the oxidation of NADPH to NADP, the decrease in absorbance was measured spectrophotometrically at a wavelength of 366 nm. GPx activity was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}, and the results are expressed as U/g protein.

GST determination: GST is a metabolizing enzyme that plays a prominent role in the detoxification of oxidized metabolites and may serve as an antioxidant. GST enzyme activity was measured according to the method of Habig and Jacoby (1981) [62]. GST catalyzes the reaction between GSH and 1-chloro-2,4-dinitrobenzene. The resultant product formed was monitored spectrophotometrically at 340 nm and 25°C. The level of GST activities in testicular tissue was evaluated by an extinction coefficient of 0.625 mM^{-1} cm^{-1}, and expressed as U/mg protein.

SOD determination: SOD activity was assayed according to the method described by Mylroie, Collins, Umbles, and Kyle (1986) [63]. The reaction mixture contains potassium phosphate buffer (pH 7.8), o-dianisidine dihydrochloride, and riboflavin. The reaction was initiated by the addition of riboflavin to the reaction mixture and the absorbance of the mixture was monitored immediately at a wavelength of 460 nm. Enzyme activity was determined using the SOD standard and activities in testicular tissue were expressed as U/mg protein.

The analysis of each sample was made in duplicate.

5.4. Statistical analyses

Results were evaluated using an unpaired t test and analysis of variance (ANOVA) using the NCSS statistical computer package. The values were expressed as mean ± SD. $p < 0.05$ was considered as significant.

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