Article published online: 2022-06-14

Original Article

# Doxycycline: An Antibiotic with Brain Protective Function in Vanadium-Intoxicated Rats

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## Abstract

**Background:** Exposure to vanadium exhibits deleterious neurotoxicity. Doxycycline is a potential antioxidant that prevents the progression of disease through inhibition of lipid peroxidation. **Objectives:** This research investigates the neuroprotective effects of doxycycline, in different rat brain areas in an animal model intoxicated with vanadium. **Materials and Methods:** Male Sprague-Dawley rats were equally divided into the following four groups: control group, doxycycline-treated group, vanadium-treated group, and concomitant doses of doxycycline plus vanadium-treated group, all given orally for 10 consecutive days. The animals were watched daily for any signs of neurological defects. They were sacrificed by decapitation 24 h after the last dose. Brain was removed rapidly and dissected into cerebral cortex, cerebellum, and brain stem. Biochemical studies including the concentrations of phospholipids, cholesterol, cerebrosides, glutathione (GSH), acetylcholinesterase (AChE) activity, gangliosides, ascorbic acid, calcium, and lipid peroxidation levels were determined. **Results:** The results revealed that vanadium produced significant reduction in body and absolute brain weight, with neurological function deficits. Vanadium significantly decreased the concentrations of phospholipids, cholesterol, cerebrosides, and GSH and inhibited AChE activity together with significant increase in gangliosides, ascorbic acid, calcium, and lipid peroxidation levels compared to saline controls. Animals which were given the combined treatment of vanadium and doxycycline regained weight and became normal. Moreover, doxycycline reversed the effect of vanadium on the metabolic variables and inhibited lipid peroxidation nearing to normal levels to that of saline controls. **Conclusion:** These findings demonstrated the antioxidant or chelating action of doxycycline against vanadium neurotoxicity and its therapeutic potential to avert neurodegenerative changes in different rat brain areas.

**Keywords:** Doxycycline, lipid peroxidation, neuronal deficits, neurotoxicity, reactive oxygen species, vanadium

## Introduction

Vanadium, a transition metal having atomic number 23, is ubiquitous in the environment. It has been estimated that as much as 66,000 tons of vanadium are released into the atmosphere each year. The general population is increasingly exposed to this metal as a result of extensive use in heavy industries in the manufacture of pesticides and sulfuric acid, hardening of steel, and as a catalyst in the production of many materials.[1]

Furthermore, vanadium is a contaminant in fossil fuels; environmental exposure to high levels of vanadium occurs from crude oil spills, gas flaring, and automobile exhaust. Vanadium is extensively used in industries, therefore acute and/chronic occupational exposure occurs at production sites during the processing and refining of vanadium ores and sludges, manufacturing of vanadium-containing products, combustion of vanadium-rich fuels, and handling of catalysts in the chemical industry. The essentiality and toxicity of vanadium has been widely explored.[2]

In humans, vanadium toxicity induces central nervous system (CNS) perturbations, tremor, and impaired conditioned

Access this article online

Quick Response Code:

Website: journal.limu.edu.ly

DOI: 10.4103/LIUJ.LIUJ_29_20

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**How to cite this article:** Mohamed NA, Gassar ES, Abdulla SA, Elfakhri MM, Patel A, Haider SS. Doxycycline: An antibiotic with brain protective function in vanadium-intoxicated rats. Libyan Int Med Univ J 2020;5:37-47.

**Received:** 05-07-2020 **Revised:** 16-07-2020
**Accepted:** 24-07-2020 **Published:** 06-10-2020

reflexes, as well as congestion of the brain and spinal cord.[2] Vanadium exposure, regardless of the route and duration, has been demonstrated to have deleterious effects on neurons, glia, and myelin. A study[3] following chronic intraperitoneal vanadium exposure at 3 mg/kg in mice reported a generalized distribution of vanadium throughout the brain with predilection for the olfactory bulb, brain stem, and cerebellum. The authors have also demonstrated the presence of vanadium in the brain using laser ablation-inductively coupled plasma spectroscopy (LA-ICP-MS), which further evidences that vanadium does cross the blood–brain barrier. This study reported disruption of cortex with nuclear pyknosis; loss of pyramidal neurons and reduced apical dendrites in the hippocampus; and loss of cerebellar Purkinje cells. The morphological alterations were accompanied by astrogliosis and microgliosis.[3]

It is known that mammalian brain contains large amount of substances that are susceptible to free radical attack, such as unsaturated lipids and catecholamines, and relatively low levels of free radical-eliminating enzymes.[4] The occurrence of lipid peroxidation in the brain following vanadium administration is a proof to the role of oxidative stress in the neurotoxicity of the metal.[5] We have reported remarkably high levels of lipid peroxidation with preferential loss of oleic acid, linoleic acid, linolenic acid, arachidonic acid, phospholipids, and proteins in the discrete regions of brain of vanadium-exposed rats.[5]

The enormous progress in basic neuroscience research is focused on the development of novel candidates causing interaction in brain disease pathophysiology. Interestingly, antibiotics exert strong brain protective effect in various *in vivo* and *in vitro* models of neurodegenerative disease, such as stroke, Parkinson's, and Alzheimer's.[6] Doxycycline, a derivative of tetracycline and a chelator, is a broad-spectrum antibiotics with high lipophilic properties and has been used for its neuroprotective effect in a previous study. One potential mechanism by which doxycycline inhibits the progression of these diseases is by reducing oxidative stress through inhibition of lipid peroxidation and inflammatory responses.[7]

Therefore, this presentation evaluated the therapeutically potential effects of doxycycline, if any, on the occurrence of lipid peroxidation, status of brain lipids, levels of GSH, concentration of Vitamin C, levels of calcium, and activity of membrane bound enzyme acetylcholinesterase (AChE) in the various regions of rat brain following vanadium administration.

## Materials and Methods

### Chemicals

Acetylthiocholine, N-acetylneuraminic acid, 2-thiobarbituric acid, 5,5' dithiobis-2-nitrobenzoic acid, and 1,1,33-tetraethoxypropane were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Doxycycline hydrochloride was obtained from Sarvi Pharm., Guelma, Vilaya De Guelma, 24000, Algeria. Cholesterol and galactose were obtained from Merk, Darmstadt, FRG. Sodium metavanadate and all the other chemicals were purchased from BDH chemicals and were of analytical grade.

### Animals

Sixty-two male Sprague-Dawley rats (250–300 g) were obtained from the Central Animal House of the Faculty of Medicine, University of Benghazi, Benghazi. The animals were housed in stainless steel cages and adopted to laboratory conditions (25 ± 2°C, relative humidity 40%–60%, and 12-h light-dark cycle). The animals were allowed free access to laboratory pellet diet (National Company of Animal Feeds, Benghazi, Libya) and fresh water *ad libitum*. The study protocol was approved by the Animal Care and Use Research Ethics Committee, University of Benghazi, Libya.

### Mode of administration

Drugs were given to the animals by oral intubation by means of a small feeding tube through the mouth into the stomach. Distilled water was used as a vehicle for both vanadium and doxycycline. Drugs were given in volumes ranging between 0.25 and 0.30 ml/oral route.

### Experimental groups

The rats were randomly distributed into four experimental groups of 15 rats each. The details were as follows.

- Group 1: Saline-control group, received 0.2 ml of physiological saline orally for 10 consecutive days
- Group 2: Doxycycline-treated controls, received doxycycline (4 mg/kg body weight on day 1 followed by 2 mg/kg body weight hereafter) orally for 10 consecutive days. The sub-antibiotic dose (low-dose doxycycline) has been widely used in children and in periodontal treatment for enzymic inhibition and related anti-inflammatory properties[8]
- Group 3: Vanadium-treated group, received elemental vanadium (1.5 mg/body weight) as sodium metavanadate for 10 consecutive days. This dose of vanadium was dictated by previous results, which indicated that the administration of similar amounts of sodium metavanadate resulted in perturbations of brain lipids and occurrence of lipid peroxidation in discrete rate brain areas[9]
- Group 4: Doxycycline + vanadium-treated group, received concomitant dose of vanadium (1.5 mg vanadium/kg + doxycycline [4 mg/kg body weight on day 1 and 2 mg/kg body weight thereafter]) for consecutive 10 days.

Twenty-four hours after the last oral dose, the animals were sacrificed using cervical decapitation. The brain was carefully taken out, and the adhering blood clots were removed from the surface. Cerebral cortex, cerebellum, and brain stem were removed on an ice-cold surface and stored in deep freeze at −70°C. The relative brain weight was immediately calculated by the following formula.[10]

Absolute brain weight (g) = relative brain weight/body weight of rats on sacrifice day (g).

## Biochemical studies

### *Extraction of lipids from brain*

The discrete brain areas were weighed and homogenized in chloroform-methanol (2:1 v/v). The homogenizing medium was dictated by a previous method of Folch *et al*.[11] with a modification. Homogenates were shaken periodically for 1 h and filtered. The final volume of each extract was made up to 10 ml with fresh chloroform-methanol (2:1 v/v) mixture. The filtrates were washed with normal saline (2.5 ml) followed by vigorous shaking on a vortex mixer. The samples were stored at −20°C in a deep freeze for an overnight for extraction of lipids. The volume of both layers (aqueous and organic) was measured. The aqueous layer was used to estimate gangliosides, and the organic layer extract was used for the estimation of phospholipids, cerebrosides, and cholesterol.

### *Phospholipid determination*

The lipids in rat brain areas were extracted in chloroform: methanol (2 ml: 1 ml v/v). Aliquots (100 µl) from these samples were taken in the test tubes and added with 1 ml of 62% perchloric acid in a test tube and were digested at 70°C on a digester for 2 h. After cooling, the digested samples were added 1.5 ml of ammonium molybdate and 200 µl of aminonaphthol (prepared in sulfuric acid). The contents in the test tube were diluted with 7 ml of distilled water. The test tubes containing this assay mixture were mixed on a vortex mixer and incubated in a hot water bath (100°C). The absorbance was then measured spectrophotometrically at 660 nm.[12] The amount of phospholipids was calculated from standard calibration curve using phosphorous and was then expressed as mg of phosphate/g fresh brain tissue.

### *Cholesterol determination*

The lipids in rat brain areas were extracted in chloroform: methanol (2: 1 v/v). Aliquots (500 µl) from these samples were taken in the test tubes. These aliquots were added 4.5 ml of chloroform and 5 ml coloring agent (acetic anhydride: concentrated sulfuric acid, 50 ml: 5 ml v/v). This assay mixture was mixed on a vortex mixer and incubated in dark at 25°C for 30 min. The absorbance of green color was then measured spectrophotometrically at 660 nm.[13] The concentration of cholesterol was then calculated from standard calibration curve using cholesterol as standard and expressed as mg of cholesterol/g fresh brain weight.

### *Cerebroside estimation*

The lipids in rat brain areas were extracted in chloroform: methanol (2:1 v/v). Aliquots (100 µl) from these samples were taken in the test tubes. These aliquots were added 1 ml of aqueous phenol (2%) and 4 ml of concentrated sulfuric acid. The assay mixture was mixed on a vortex mixer and incubated at room temperature for 15 min. The absorbance of orange color was then measured spectrophotometric ally at 480 nm.[14] The concentration of cerebrosides was then calculated from standard calibration curve using cerebroside as standard and then expressed as mg of cerebrosides/g fresh brain weight.

### *Ganglioside estimation*

The lipids in rat brain areas were extracted in chloroform: methanol (2 ml: 1 ml v/v). This extract was washed with sodium saline to obtain the gangliosides. Sodium saline-phase layer (2 ml) was taken into a test tube. To this, 2 ml of resorcinol reagent was added (3% resorcinol [10 ml] + concentrated HCl [80 ml] + 0.1 M copper sulfate [250 µl] in a final volume of 100 ml). The tubes were incubated in a boiling water bath (100°C) for 30 min and cooled for 15 min. The gangliosides were extracted by adding 5 ml of extraction mixture (butyl acetate: n-butanol: 85 ml: 15 ml v/v) in the test tubes. They were mixed on a vortex mixture, and allowed to stand 10 min at room temperature for extraction. Light violet organic-phase layer was then measured in spectrophotometer at 580 nm.[15] The concentration of gangliosides was then calculated from standard calibration curve using ganglioside standard and then expressed as ganglioside mg/g of fresh brain tissue.

## Preparation of brain samples

Brain samples were prepared for the estimation of lipid peroxidation, GSH, ascorbic acid, calcium, and AChE enzyme.

The discrete brain areas from thirty animals were weighed and divided into equal parts. One part was homogenized in 0.15 M KCl (10% w/v) and was used for the estimation of lipid peroxidation, calcium, GSH, and ascorbic acid levels. Other part of the brain was homogenized in 0.2 M EDTA (10% w/v) buffer (pH 7.4) and centrifuged at 5000 rpm for 10 min. The supernatant obtained was used for the estimation of acetylcholine esterase activity.

### *Measurement of malondialdehyde level*

The content of malondialdehyde (MDA) formation (a product of lipid peroxidation) was estimated in the form of thiobarbituric acid-reactive substances (TBARS). The aliquots (200 µl) of brain homogenate were taken into test tubes. They were added 200 µl of sodium dodecyl sulfate (8.1%), 1.5 ml acetic acid (20%), and 1.5 ml of TBA (1%). The contents were mixed on a vortex mixer. The final volume of the reaction mixture was adjusted to 4 ml with distilled water. The tubes were mixed on a vortex mixer. The samples were heated in a hot water bath (100°C) for 60 min. After cooling under tap water, the samples were added distilled water (1 ml), extracted with 5 ml n-butanol: pyridine (15 ml: 1 ml v/v), mixed vigorously on a vortex mixer, and centrifuged (4000 rpm, 15 min). The MDA content in the n-butanol: pyridine layer was then spectrophotometrically determined at 535 nm.[16] MDA levels were calculated from the standard calibration curve using 1,1,3,3-tetra ethoxy propane and expressed as nanomoles of MDA/g fresh tissue.

### *Glutathione level determination*

The discrete brain area homogenates were obtained in 0.15 M KCl (10% w/v). The aliquots (500 µl) were mixed with 4% trichloroacetic acid (500 µl). Then, they were centrifuged for 15 min at 4000 rpm, and the supernatant was mixed with 0.4 M Tris buffer, pH (8.9) (1 ml), and dithio-nitro-benzoic

acid (DTNB) (100 µl). The tubes containing the assay mixture were mixed on a vortex mixer. The GSH levels were then spectrophotometrically determined at 412 nm.[17] GSH levels were calculated from standard calibration curve using GSH and expressed as mmol/fresh tissue.

### *Ascorbic acid determination*

The discrete brain areas were weighed and homogenized in 0.15 M KCl (10% w/v). The tissue homogenates (1 ml) were firstly mixed with phosphotungstic acid reagent (1 ml), mixed on a vortex mixer, incubated at room temperature for 10 min, and centrifuged (3000 rpm, 15 min). The supernatant (1 ml) was collected. The ascorbic acid was then spectrophotometrically determined at 535 nm.[18] Ascorbic acid levels were calculated from standard calibration curve using L-ascorbic acid and expressed as µg/g fresh tissue.

### *Determination of calcium concentration*

The discrete brain area homogenates in 0.15 M KCl (10% w/v) were centrifuged for 10 min at 4000 rpm at 4°C. The supernatants (250 µl) were mixed with enthanolamine buffer (1 mol/l, pH 10.6) and O-cresolpthalein complex one (0.32 mmol/l), followed by addition of distilled water (250 µl). The test tubes containing the assay mixture were mixed on a vortex mixer. The calcium concentration was then sperctrophotometrically determined at 546 nm.[19] Calcium was assayed by utilizing Boehringer Mannheim GmbH Diagnosta, Germany (Cat. No. MPR21553593). The total calcium levels were calculated from the standard calibration curve using calcium and expressed as mmol/g fresh tissue.

### *Measurement of acetylcholinesterase activity*

The activity of AChE enzyme was assayed in aliquots of homogenates tissues from rat brain area by utilizing kits supplied by Boehringer Mannheim (Cat no. MPR 21424117) GmbH Diagnosta Germany. The reaction mixture consisted of 20-µl supernatant, 100 µl of 0.25 mmol/l [5,5 dithiobis-(2-nitrobenzoic with 156 mmol/acetylcholine iodide acid], and 2.6 ml of 0.01 M sodium phosphate buffer (pH 7.2), and its absorbance was taken on ultraviolet-visible spectrophotometer at 412 nm.[20] The change in absorbance was calculated, and results were expressed as enzyme unit/g fresh brain tissue.

### Statistical analysis

The data were presented as means ± standard error of mean ($n$ = 15). Data were analyzed by one-way analysis of variance. When the analysis indicated a statistically significant difference ($P < 0.05$), the treated groups were compared to their respective controls. Statistical analyses were performed by $F$-test[21] for homogeneity of variance followed by $t$-test.

## Results

### General observations

The rats administered with physiological saline (controls) or doxycycline alone did not show any sign of illness. However, the rats administered with vanadium displayed signs of neurotoxicity such as akinesia, motoric disturbances, ataxia, convulsion, muscular fasciculation, asphyxia, lethargy, and diarrhea. On the other hand, doxycycline + vanadium-treated group of rats gained weight and appeared completely normal. These neurological deficits in vanadium neurotoxicity had been previously reported.[22]

### Body weight of the rats and their mortality

Rats in all the groups were weighed daily throughout the experiment [Table 1]. The vanadium group exhibited statistically significantly reduced body weight (−60 g; $P < 0/001$) on the day of sacrifice when compared with control group and −65 g when compared with doxycycline group. Doxycycline group alone exhibited (+1.70 g) gain in the body weights on the day of sacrifice when compared with the control group. On the other hand, vanadium + doxycycline-treated group exhibited gain in weight (+25 g) on the day of sacrifice when compared with the vanadium-treated group. The mortality of rats from vanadium administration was only two.

### Absolute and relative brain weight of the rats

Table 2 shows that there was no statistically significant difference ($P > 0.05$) in the absolute or relative brain weight of the animal across saline control and doxycycline groups. Whereas, the vanadium-administered group presented statistically significant decreases in absolute and relative brain weight compared to saline control and doxycycline-administered groups ($P < 0.05$). Furthermore, doxycycline + vanadium-administered group exhibited statistically significant differences ($P < 0.05$) in absolute and relative brain weights across saline control and doxycycline groups.

### Effects on phospholipid levels in the rat brain areas

Table 3 depicts that vanadium administration significantly depleted phospholipids in brain areas compared to saline controls in the following sequence: cerebral cortex (−35%; $P < 0.001$) > brain stem (21%; $P < 0.001$) > cerebellum (−19%; $P < 0.01$). The mean percentage depletion of phospholipids in brain areas was by − 25%. Doxycycline alone as compared to saline controls presented no effect *per se,* whereas concomitant (doxycycline + vanadium) treatment significantly prevented the effect of vanadium in brain areas in the following sequence: cerebral cortex (+15%; $P < 0.001$), followed by cerebellum (+9%; $P < 0.05$), brain stem (+6%; $P < 0.05$). The mean percentage protection in rat brain areas compared to vanadium-treated group was by 10%.

### Effects of doxycycline on cholesterol levels in the rat brain areas

Table 3 shows that vanadium administration significantly depleted the cholesterol levels in brain areas compared to saline controls in the following order: cerebral cortex (−35%; $P < 0.001$), brain stem (−21%; $P < 0.001$), and cerebellum (−19%; $P < 0.001$). The mean percentage depletion in brain areas between saline control and vanadium was by −25%. Doxycycline alone as compared to saline controls presented no effect *per se.* On the other hand, the concomitant (doxycycline + vanadium) administration exhibited protective effect in the following sequence: cerebral

**Table 1: Effect of doxycycline on body weight of rats and their morality following vanadium administration**

| Group | Treatment | Body weight on day 1 of treatment (g) | Body weight on sacrifice day (g) | Gain or loss in body weight (g) | Mortality |
|---|---|---|---|---|---|
| 1 | Normal saline, *n*=15 | 249±1.42 | 250±1.93 | ±0.4 | 0/15 |
| 2 | Doxycycline (2 mg/kg, oral), *n*=15 | 250±1.98 | 255±1.75 | ±2 | 0/15 |
| 3 | Vanadium (1.5 mg/kg, oral), *n*=17 | 250±1.65 | 190±1.16[a, b] | −24 | 2/17 |
| 4 | Doxycycline (2 mg/kg, oral) + vanadium (1.5 mg/kg, oral), *n*=15 | 247±1.86 | 215±0.87[a, b, c] | −13 | 0/15 |

*n*: Number of animals in each group. Values are expressed as mean±SEM. [a]*P*>0.2, Nonsignificantly different from control, [b]*P*<0.05, significantly different from doxycycline, [c]*P*<0.05, significantly different from vanadium. SEM: Standard error of mean

**Table 2: Effect of doxycycline on absolute and relative rat brain weight following vanadium administration**

| Group | Treatment | Absolute brain weight (g) | Relative brain weight ($\times 10^{-2}$) |
|---|---|---|---|
| 1 | Normal saline, *n*=15 | 1.71±0.05 | 6.84±0.17 |
| 2 | Doxycycline (2 mg/kg, oral), *n*=15 | 1.67±0.02 | 6.54±0.25 |
| 3 | Vanadium (1.5 mg/kg, oral), *n*=15 | 1.10±0.02[a, b] | 5.78±0.22[a, b] |
| 4 | Doxycycline (2 mg/kg, oral) + vanadium (1.5 mg/kg, oral), *n*=15 | 1.28±0.02[a, b] | 5.95±0.14[a, b] |

*n*: Number of animals in each group. Values are expressed as mean±SD. [a]*P*<0.05, significantly different from control, [b]*P*<0.05, significantly different from doxycycline, [c]*P*<0.05, significantly different from vanadium. SD: Standard deviation. (For calculation of relative brain weight, the reader is referred to the article by Adebiyi *et al.*2016[10] )

**Table 3: Effect of doxycycline on the levels of phospholipids, cholesterol, cerebrosides, and gangliosides in the rat brain areas following vanadium administration**

| Parameter | Groups | | | |
|---|---|---|---|---|
| | Control | Doxycycline | Vanadium | Doxycycline + vanadium |
| Cerebral cortex | | | | |
| Phospholipids (mg/g fresh tissue weight) | 50.59±1.36 | 50.32±0.78 | 32.86±3.02[a,b] | 37.67±0.77[a,b,c] |
| Cholesterol (mg/g fresh tissue weight) | 22.10±0.96 | 21.14±0.84 | 12.98±1.40[a,b] | 15.56±0.98[a,b,c] |
| Cerebrosides (mg/g fresh tissue weight) | 24.56±2.15 | 23.75±0.56 | 17.24±1.19[a,b] | 18.24±1.18[a,b] |
| Gangliosides (µg/g fresh tissue weight) | 275.38±8.65 | 270.25±15.06 | 320.17±40.65[a,b] | 312.77±19.31[a,b] |
| Cerebellum | | | | |
| Phospholipids (mg/g fresh tissue weight) | 52.65±2.49 | 51.20±5.60 | 42.80±5.49[a,b] | 46.65±0.74[a,b,c] |
| Cholesterol (mg/g fresh tissue weight) | 23.70±0.85 | 23.65±1.21 | 18.75±1.75[a,b] | 22.13±0.36[a,b,c] |
| Cerebrosides (mg/g fresh tissue weight) | 29.12±2.47 | 28.86±0.42 | 23.90±2.26[a,b] | 24.24±0.84[a,b] |
| Gangliosides (µg/g fresh tissue weight) | 246.45±17.45 | 242.81±14.20 | 354.55±12.15[a,b] | 352.08±3.55[a,b] |
| Brain stem | | | | |
| Phospholipids (mg/g fresh tissue weight) | 63.38±4.53 | 62.25±1.05 | 49.84±3.35[a,b] | 57.74±3.81[a,b,c] |
| Cholesterol (mg/g fresh tissue weight) | 33.46±1.66 | 32.70±1.22 | 26.15±3.71[a,b] | 29.52±0.39[a,b,c] |
| Cerebrosides (mg/g fresh tissue weight) | 37.10±2.72 | 37.16±0.75 | 30.12±1.96[a,b] | 39.12±1.38[a,b] |
| Gangliosides (µg/g fresh tissue weight) | 135.56±12.12 | 135.41±11.06 | 197.11±14.15[a,b] | 194.62±2.74[a,b] |

Vanadium (1.5 mg/kg/day), doxycycline (2 mg/kg/day), doxycycline (2 mg/kg/day) + vanadium (1.5 mg/kg/day), and normal saline. All doses were orally administered for 10 consecutive days. Values are expressed as±SEM for 15 animals. [a]*P*<0.05, significantly different from control group, [b]*P*<0.05, significantly different from doxycycline group, [c]*P*<0.05, significantly different from vanadium group. SEM: Standard error of mean

cortex (+20%; $P < 0.001$), brain stem (+13%; $P < 0.05$), and cerebellum (+8%; $P < 0.05$). The mean percentage protection in brain areas between vanadium and doxycycline + vanadium was by +14%.

### Effects on cerebroside levels in rat brain areas

Table 3 shows that vanadium administration significantly decreased the cerebroside level in brain areas compared to saline control in the following order: cerebral cortex (−30%; $P < 0.001$), brain stem (−19%; $P < 0.001$), and cerebellum (−18%; $P < 0.001$). The mean percentage depletion in brain areas between saline controls and vanadium was by −22%. Doxycycline alone presented no effect *per se* compared to saline controls. On the other hand, concomitant (doxycycline + vanadium) administration presented nonsignificant protection in the following sequence: cerebral cortex (+6%), brain stem (+6%), and cerebellum (+2%), compared to vanadium group. The mean percentage protection in brain regions by doxycycline + vanadium group as compared to vanadium group was by +5% only.

### Effects on ganglioside levels in rat brain areas

Table 3 shows that vanadium administration demonstrated statistically significant elevation in brain areas compared

to saline controls in the following order: brain stem (+46% $P < 0.001$), cerebellum (+44%; $P < 0.001$), and cerebral cortex (+16%; $P < 0.02$). The mean percentage change in brain regions between vanadium and saline controls was by +35%. Whereas, doxycycline alone presented no effect *per se* compared to saline controls. The combined administration (doxycycline + vanadium) to rats also presented no reversible effect *per se* in brain regions compared to the vanadium group.

### Effect on the occurrence of lipid peroxidation in rat brain areas

Table 4 shows that vanadium administration doubled the TBARS concentration in all brain areas as compared to controls injected with normal saline. The increase was in the following sequence: brain stem (+140%; $P < 0.001$), cerebellum (+115%; $P < 0.001$), and cerebral cortex (+112%; $P < 0.001$). The mean percentage increase in brain areas between controls and vanadium was by + 122%. Doxycycline administration alone decreased nonsignificantly the TBARS concentration, as compared to normal saline controls in the brain areas in the following sequence: brain stem (−11%), cerebellum (−8%), and cerebral cortex (−6%). On the other hand, a stringent blockade of the increase in TBARS contents was observed in the doxycycline + vanadium group, as compared to vanadium group in rat brain areas in the following order: cerebellum (−39%; $P < 0.001$), brain stem (−38%; $P < 0.001$), and cerebral cortex (−34%; $P < 0.001$). The mean percentage of TBARS concentration decrease in brain areas between the control and vanadium group was by +70%.

### Effects on glutathione levels in discrete rat brain areas

Table 4 shows that vanadium group statistically significantly decreased the GSH levels in all the brain areas as compared to normal saline controls in the following order: cerebellum (−36%; $P < 0.001$), cerebral cortex (−33%; $P < 0.001$), and brain stem (−29%: $P < 0.001$). The mean percentage decrease in GSH levels in brain areas between saline control and vanadium group was by − 33%. However, no significant difference was detected in GSH levels in brain areas between doxycycline and normal saline controls. Furthermore, in doxycycline + vanadium group, insignificant increases in GSH levels were discernible in the following sequence: cerebellum (+8), cerebral cortex (+7), and brain stem (+6%). The mean percentage increase between brain areas was by +7%.

### Effect on Vitamin C levels in discrete rat brain areas

Table 4 shows that vanadium administration resulted in highly significant increases in the levels of Vitamin C in all brain areas as compared to normal saline controls. This increase *per se* was in the following sequence: brain stem (+74%; $P < 0.001$), cerebral cortex (+66%; $P < 0.001$), and cerebellum (+50%; $P < 0.001$). The mean percentage increase in brain areas between controls and vanadium was by + 63%. No significant differences in ascorbic acid were discernible between doxycycline and saline controls, whereas statistically significant decreases in ascorbic levels were noticed in brain areas in the doxycycline + vanadium group, and the order was as follows: cerebral cortex (−15%; $P < 0.05$), cerebellum (−8%; $P < 0.05$), and brain stem (−6%;

**Table 4: Effect of doxycycline on the levels of lipid peroxidation, reduced glutathione, Vitamin C, calcium, and acetylcholinesterase activity in the rat brain areas following vanadium administration**

| Parameter | Groups | | | |
|---|---|---|---|---|
| | Control | Doxycycline | Vanadium | Doxycycline + vanadium |
| Cerebral cortex | | | | |
| Lipid peroxidation (nano mole MDA formed/g fresh tissue weight) | 228±26.44 | 214±22.67 | 483±24.89[a,b] | 321±37.24[a,b,c] |
| Reduced GSH (µmole/g/fresh tissue weight) | 0.522±0.031 | 0.510±0.011 | 0.349±0.022[a,b] | 0.374±0.015[a,b] |
| Vitamin C (µg/g fresh tissue weight) | 236.2±16.41 | 234.7±3.46 | 394.4±43.89[a,b] | 333.67±41.68[a,b,c] |
| AChE (unit/g fresh tissue weight) | 1071.91±48.91 | 1062.83±40.62 | 567.75±53.1[a,b] | 743.45±52.71[a,b,c] |
| Calcium (µmole/g fresh tissue weight) | 0.428±0.11 | 0.296±0.09 | 0.833±0.21[a,b] | 0.614±0.021[a,b,c] |
| Cerebellum | | | | |
| Lipid peroxidation (nano mole MDA formed/g fresh tissue weight) | 235±27.97 | 215±20.31 | 505±55.07[a,b] | 310±30.7[a,b,c] |
| Reduced GSH (µmole/g/fresh tissue weight) | 0.530±0.037 | 0.493±0.10 | 0.341±0.28[a,b] | 0.368±0.03[a,b] |
| Vitamin C (µg/g fresh tissue weight) | 220±10.21 | 217.2±6.45 | 330±29.58[a,b] | 280.55±44.4[a,b,c] |
| AChE (unit/g fresh tissue weight) | 1271.76±48.6 | 1263.05±70.74 | 687.87±58.95[a,b] | 984.11±60.7[a,b,c] |
| Calcium (µmole/g fresh tissue weight) | 0.571±0.21 | 0.317±0.071 | 0.793±0.02[a,b] | 0.664±0.012[a,b,c] |
| Brain stem | | | | |
| Lipid peroxidation (nmol MDA formed/g fresh tissue weight) | 216±24.77 | 196±27.31 | 517±51.18[a,b] | 318±38.78[a,b,c] |
| Reduced GSH (µmole/g/fresh tissue weight) | 0.485±0.030 | 0.470±0.011 | 0.340±0.035[a,b] | 0.359±0.02[a,b] |
| Vitamin C (µg/g fresh tissue weight) | 195±28.91 | 184±24.98 | 321±29.59[a,b] | 273.78±13.27[a,b,c] |
| AChE (unit/g fresh tissue weight) | 953.62±51.56 | 946.25±82.71 | 681.3±62.73[a,b] | 857.73±47.27[a,b,c] |
| Calcium µmole/g fresh tissue weight) | 0.523±0.08 | 0.397±0.04 | 1.03±0.89[a,b] | 0.754±0.11[a,b,c] |

Vanadium (1.5 mg/kg/day), doxycycline (2 mg/kg/day), doxycycline (2 mg/kg/day) + vanadium (1.5 mg/kg/day), and normal saline. All doses were orally administered for 12 consecutive days. Values are expressed as±SEM for 15 animals in each group. [a]$P<0.05$, significantly different from control group, [b]$P<0.05$, significantly different from doxycycline group, [c]$P<0.05$, significantly different from vanadium group. SEM: Standard error of mean, MDA: Malondialdehyde, AChE: Acetylcholinesterase, GSH: Glutathione

$P < 0.05$). The mean percentage decrease in Vitamin C levels in brain areas between doxyxycline + vanadium group and only vanadium-treated group was by 10%.

### Effect of doxycycline on acetylcholinesterase activity in discrete brain areas

Table 4 shows that vanadium administration significantly inhibited the activity of AchE in brain areas compared to saline controls. In cerebral cortex, AchE activity was strongly inhibited (−47%; $P < 0.001$) followed by cerebellum (−46%; $P < 0.001$) and brain stem (−29%; $P < 0.05$). The mean percentage decrease of AchE activity in brain areas between vanadium and normal saline controls was by − 41%. whereas, doxycycline alone presented no effect *per se* However, the combined administration doxycycline + vanadium exhibited highly significant increase in AchE activity in brain areas when compared to vanadium group in the following sequence: cerebellum (+43%; $P < 0.001$), cerebral cortex (+31%; $P < 0.001$), and brain stem (+26%; $P < 0.05$). The mean percentage increase in AchE in brain areas between doxycycline + vanadium group and vanadium group was by +33%.

### Effect on calcium levels in rat brain areas

Table 4 shows that vanadium administration presented with remarkably increased calcium levels in all brain areas, compared to the saline controls. The increase was in the following order: brain stem (+97%; $P < 0.001$), cerebral cortex (+95%; $P < 0.001$), and cerebellum (+39%; $P < 0.001$). The mean percentage increase was by + 77%. Whereas, administration of doxycycline alone exhibited substantial decrease in the calcium levels compared to saline controls in the following sequence: cerebellum (−45%; $P < 0.02$), cerebral cortex (−31%; $P < 0.05$), and brain stem (−24%; $P < 0.05$). The mean percentage decrease in the brain regions was by −33%. Moreover, combined administration of rats with doxycycline + vanadium had blocked the calcium increase in brain areas, compared to vanadium administered group. This blockage was in the following sequence: brain stem (−27%; $P < 0.01$), cerebral cortex (−26%; $P < 0.001$), and cerebellum (−23%; $P < 0.02$). The mean percentage blockage of calcium influx in brain areas in doxycycline + vanadium group as to compared to vanadium treated group was by −25%.

## Discussion

Vanadium, a metallic element of the first transition series, is abundant in the environment. Occupational exposure is common with workers at workplaces and among those living near vanadium-related industries such as metallurgical plants. Vanadium enters the brain through inhalation and different parental routes. Vanadium causes astrogliosis, depletion of oligodendrocites, neuroinflammation necrosis of ependymal layer and disarray of blood–brain barrier, demyelination, and various locomotor deficits. Vanadium compounds provoke apoptosis and DNA damage.[23] We have demonstrated that the majority of the toxic and molecular effects of vanadium in the brain are related to oxidative stress caused by the generation of reactive oxygen species.[5] Furthermore,[24] we have investigated the parallel effects of vanadium on morphology and biochemistry in rat brain. We observed chromatolysis, neural edema, and segmental demyelination in hypothalamus and other areas with an increased formation of MDA (lipid peroxidation) and decrease in other indices of antioxidant system. Other researchers have also testified our findings about the interplay of oxidative stress in the neurotoxicity of this metal.[25]

Many researchers have attempted to counteract the neurotoxicity of vanadium with a view to chelate the metal or obviating its oxidant action by the use of chelators such as tiron and desferoxamine.[26] Whereas, antioxidants such as Vitamin E, Vitamin C, erythropoietin, and selenium have also been used with varying levels of success in experimental animals.[9,22-24,27] In this context, the use of Vitamin E was of particular interest due to its fat solubility and membrane-protective properties.[9,25] In the present study, rats were administered a low dose of vanadium (1.5 mg/kg body weight). This dose of vanadium was exact equivalent to that administered by as[9] in earlier study over a period of 10 days. A desirable co-therapy with a chelator would prove to be a promising venture in the exploration of interplay of vanadium complexes, therefore, in this animal model, a therapeutic and nontoxic dose of doxycycline (2 mg/kg body weight)[8] was administered to target the rat brain areas in vanadium-induced oxidative damage and perturbations of other neurochemical variables.

The neurotoxic effects of vanadium in this study were presented with lameness, locomotor deficits, convulsions, and muscular weakness. These neurological disorders may be implicated to excessive production of reactive oxygen species. The anorexia and dehydration were through diarrhea, leading to the reduction in daily body weight. The reduction in brain weight might be due to neuronal losses. These observations are in consonance with our previous findings.[5,9] However, co-adminstration of doxycycline + vanadium presented with no signs of neurological defects. Rats recovered from anorexia and felt comfortable with food and began to eat more and reached a healthy weight when compared to those in the vanadium-administered group. It is thus likely that doxycycline protected rats against vanadium-induced neurological defects by improved muscle function and motoric coordination.

### Effect of doxycycline on lipids and occurrence of lipid peroxidation in rat brain areas following vanadium administration

Previously,[5] we have comprehensively studied the status of brain lipids following vanadium administration. Significant decreases in phospholipids (sphingomyelin, phosphotidyl choline and phosphotidyl ethanolamine, cholesterol, and cerebrosides) were discernible together

with preferential losses in unsaturated fatty acids such as oleic acid, linoleic acid, linolenic acid, and arachidonic acid in the different brain regions of vanadium-exposed rats. The present study is compatible with an earlier study.[5] Vanadium was capable of activating the calcium-dependent phospholipase $A_2$,[28] therefore, in our study lipids were depleted in rat brain areas following vanadium exposure. A noteworthy finding in our study was a significant increase in the concentration of gangliosides. The accumulation of these lipids in the brain was attributed to human genetic disease (the Gaucher disease). Does vanadium exposure inhibited the enzyme β-galactosidase? There is still a dearth of literature about this finding and remains to be of high current interest. This study is in congruence with our earlier report.[5]

Doxycycline co-treatment with vanadium protected the integrity of membrane lipids in the different regions of rat brain. The recovery of lipids was by 9% compared to vanadium-intoxicated group. Doxycycline is a chelator and lipid-loving drug, therefore, it is likely that it might have chelated vanadium metal and attenuated its neurotoxicity. On the contrary, it presented no protective effect *per se* against vanadium-induced elevation in brain ganglioside levels. Does vanadium neurotoxicity induced "Sphingolipidosis" via inhibition of lysosomal enzymes? It is a perplexing question and has open new horizons for future research.

The brain is rich in lipids and contains a high amount of polyunsaturated fatty acids in phospholipids of biological membranes, therefore it is quite susceptible to lipid peroxidation by reactive oxygen species. The aerobic metabolism in brain is fast but has relatively low levels of antioxidant enzymes.[29] Our previous studies have revealed that vanadium initiated remarkably high rate of oxidative damage following administration of sodium metavanadate ($NaVO_3$) in the discrete regions of rat brain.[5,22] These studies justified the notion that the vanadium neurotoxicity triggered the peroxidation of brain lipids with subsequent losses of polyunsaturated fatty acids in the neuronal membrane. The present investigation further verified our earlier studies that vanadium induced lipid peroxidation at a significantly high rate when compared to controls. In this study, doxycycline administration alone and concomitantly with vanadium demonstrated significant inhibition of lipid peroxidation in different regions of rat brain compared to that of vanadium-intoxicated group. Doxycycline is known to have neuroprotective effects.[30] In the combined group (doxycycline + vanadium), lipid peroxidation was significantly inhibited and levels of brain lipids were reversed (elevated) when compared with that of vanadium-administered group. It is thus likely that doxycycline by its antioxidant action has protected the neuronal membrane firstly by anatogizing the increased formation of free radicals and lipid peroxidation and secondly, by partial reversibility of lost membrane lipids in rat brain areas observed in vanadium-treated group.

### Effect of doxycycline on glutathione, ascorbic acid, calcium concentration, and acetylcholinesterase activity in rat brain areas following vanadium administration

The ability of a cell to maintain functional homeostasis on the induction of protective antioxidant enzymes and intracellular GSH plays a central role in defending cells against oxidative stress.[31] The GSH levels had significantly decreased in rat brain areas in vanadium-treated groups. The results are in congruence with our previous investigation[32] and indicated imbalance of the GSH redox couple. Reduced GSH molecule neutralizes free radicals by bonding through its extra electron to the reactive oxygen species molecules, and this phenomena captures free radicals.[31] On the other hand, co-treatment of rats demonstrated insignificant increases in GSH levels in various brain regions when compared with vanadium-treated groups. This is because of the high rate of induction of lipid peroxidation and inhibition of GSH reductase, therefore, substantial molecules of GSH are involved in antioxidant properties.

Ascorbic acid (Vitamin C) is a water-soluble vital antioxidant molecule in the brain. Ascorbic acid plays very important role in lipid-rich environment of the brain in sparing or recycling α-tocopherol by reducing the α-tocopherol radical back to α-tocopherol.[33] In the present investigation, vanadium-intoxicated group of rats exhibited remarkable elevations in the ascorbic acid concentration in the various regions of the brain. The results are well in congruence with the earlier report by us.[24] This event confirmed that in order to ameliorate vanadium neurotoxicity, ascorbic acid might have set an adaptive response to the increased lipid peroxidation, henceforth, its uptake was increased through cerebrospinal fluid in a recycling (oxidative-reductive) mechanism.[34] The noteworthy findings in co-administered (doxycycline + vanadium) group of rats were the reversal (decrease) of the ascorbic acid concentration in rat brain compared to vanadium-administered group. These findings suggested that depletion of ascorbic acid in the co-administered group could be because of its greater utilization in inhibiting lipid peroxidation, and henceforth, doxycycline conferred protection of brain against vanadium-induced free radical injury. Previously,[24] we have studied the effect of co-treatment of rats with L-ascorbic acid + vanadium, where L-ascorbic acid maintained the similar homeostatic mechanism in protecting rat brain against vanadium-induced free radical injury, therefore, our results are strongly supported by this evidence.[24]

Acetylcholine is an important neurotransmitter involved in many central functions including memory. Ach is degraded by AChE. This enzyme is influenced by changes in membrane microenvironment.[35] In previous study,[24] we have demonstrated inhibition of AChE in rat brain areas following sodium metavanadate intoxication. Furthermore, a WHO technical report[2] on vanadium has documented that AchE activity in the rat brain was diminished by intraperitoneal administration of graded doses of vanadyl sulfate. The

activity of AChE was also significantly inhibited in this study in rat brain areas following vanadium intoxication. There were curious parallels between our results and earlier report.[24] It is speculative that the neuronal membrane fluidity is altered and AchE concentration is build up in the brain as a consequence of lipid covalent binding and lipid peroxidative damage. The structural and functional integrity of neural membranes in crucial, therefore, alteration, if any, would eventually lead to nervous system pertubations. On the other hand, concomitant (doxycycline + vanadium) treatment of rats significantly reversed (elevated) the activity of AchE in various regions of the brain. It might be a consequence of neural membrane repair mechanism via inhibition of lipid peroxidation in this group. There is a dearth of information, and further research is warranted in future.

Thus far, the *in vivo* data pertaining to interaction between lipid peroxidation and calcium mediating damage to CNS is ill defined. The results of our investigation have revealed that in the vanadium-intoxicated rats, the calcium concentration in brain areas was significantly very high compared to that of saline controls. Our finding can be explained in light of a previous report, where in isolated rat brain synaptosomes, brain mitochondria, or cultured fetal spinal cord neurons, there were curious parallels between increase in $Ca^{2+}$ and markedly enhanced lipid peroxidation, and this phenomenon resulted in the disruption of neuronal function.[36] In this investigation, it is thus likely that vanadium might have triggered $Ca^{2+}$-dependent increase in brain membrane lipid peroxidation. However, in doxycycline-administered controls, the calcium concentration had significantly decreased in rat brain areas. This is possibly because of the decreased uptake of calcium by neurons in rat brain areas following the antioxidant action of doxycycline. $Ca^{2+}$ and lipid peroxidation are always considered combined entities in the pathophysiology of CNS trauma.[36] The co-administered (doxycycline + vanadium) group of rats also exhibited a significant decrease in the calcium concentration. This was curiously parallel with the inhibition of lipid peroxidation in brain areas when compared to vanadium-intoxicated rats. It is thus likely that the synergistic effect of high $Ca^{2+}$ and enhanced lipid peroxidation is blocked by the antioxidant action of doxycycline that was damaging the neuronal membranes. Another possibility is that doxycycline might have directly scavenged reactive oxygen species to inhibit lipid peroxidation in neural membrane, therefore $Ca^{2+}$ uptake into brain cells is reduced.

The brain is a marvel of complexity in structure and function. In the present investigation, the administration of vanadium dose demonstrated critical variations in heterogeneity in brain areas and their unique roles in the neuronal basis of cognitive functions. For example, cerebral cortex demonstrated substantial perturbations in brain lipids, ascorbic acid, and calcium concentrations, but concomitant treatment (doxycycline + vanadium) had a reverse effect on these to that of vanadium-intoxicated group. Whereas, brain stem demonstrated high induction of lipid peroxidation in vanadium neurotoxicity, but concomitant treatment (doxycycline + vanadium) had inhibited lipid peroxidation with a decreased calcium concentration. Furthermore, in vanadium-treated group, the cerebellum exhibited substantial inhibition in the activity of AchE enzyme, but concomitant treatment (doxycycline + vanadium) had reversed effects to that of vanadium. These neurochemical alterations consequently increase the susceptibility of the nervous system to lipid peroxidative damage, which might have caused deficits in voluntary movement, balance control, convulsions, muscle tone, and motor activity in vanadium-intoxicated rats, but vanadium-induced CNS deficits were attenuated by doxycycline as muscle function and coordination had improved in concomitantly treated group.

## Conclusion

The studies described herein report the potential neuroprotective role of doxycycline in vanadium neurotoxicity in rat brain areas. This antibiotic had reversed the effects of vanadium neurotoxicity such as decrease in phospholipids, cholesterol, cerebrosides, and GSH and inhibition of AchE together with elevation in gangliosides, ascorbic acid, and calcium levels. Therefore, doxycycline offers a therapeutic option to ameliorate the findings of secondary brain damage and edema. The enormous release of vanadium into environment, through fossil fuels burning, is increasing and assessment of its effects on human health should be taken into consideration. Further studies are in progress to lend a better insight into the therapeutic role of doxycycline in ameliorating deleterious vanadium neurotoxicity in rat brain.

### The limitation of this study

This study should have included the effect of vanadium alone and its combination with doxycycline on brain antioxidant enzymes. This would be proposed as a further extension of this study.

### Acknowledgments

This research was supported by the Department of Biochemistry, University of Benghazi, Libya.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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## ملخص المقال باللغة العربية

### دوكسيسيكلين: مضاد حيوي له وظيفة حماية الدماغ في فئران متسممة بالفاناديوم

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**الخلفية**: التعرض للفاناديوم يسبب سمية عصبية ضارة. الدوكسيسيكلين مضاد حيوي وهو أحد مضادات الأكسدة المحتملة التي تمنع تطور السمية العصبية من خلال تثبيط فوق الأكسدة الدهنية.

**الأهداف**: يدرس هذا البحث التأثيرات الوقائية العصبية للدوكسيسيكلين في مناطق مختلفة من دماغ الفئران في نموذج فئران متسممة بالفاناديوم.

**المواد والطرق**: تم تقسيم ذكور فئران سبراغ-داولي بالتساوي إلى المجموعات الأربع التالية: المجموعة الضابطة، المجموعة المعالجة بالدوكسيسيكلين، المجموعة المعالجة بالفاناديوم، والمجموعة المعالجة بالدوكسيسيكلين بالإضافة إلى الفاناديوم، وكلها أعطيت عن طريق الفم لمدة 10 أيام متتالية. تمت مراقبة الحيوانات يوميًا بحثًا عن أي علامات على وجود عيوب عصبية. تم قتل الفئران بقطع الرأس بعد 24 ساعة من آخر جرعة. تمت إزالة الدماغ بسرعة وتشريحه إلى قشرة دماغية ومخيخ وجذع دماغ. تم إجراء الدراسات البيوكيميائية بما في ذلك تركيزات الفسفوليبيد، والكوليسترول، والـ سيريبْرُوزيد، والجلوتاثيون، ونشاط أسيتيل كولينستراز، والجانغليوسيدات، وحمض الأسكوربيك، والكالسيوم، ومستويات بيروكسيد الدهون.

**النتائج**: أظهرت النتائج أن الفاناديوم أدي إلى انخفاض كبير في وزن الجسم والدماغ، مع قصور في الوظائف العصبية. قلل الفاناديوم بشكل كبير من تركيزات الفسفوليبيد والكوليسترول والـ سيريبْرُوزيد والجلوتاثيون، وتثبيط نشاط أسيتيل كولينستراز مع زيادة كبيرة في الجانغليوسيدات وحمض الأسكوربيك، ومستويات الكالسيوم، ومستويات بيروكسيد الدهون مقارنة بمجموعة الضوابط. استعادت الحيوانات التي أعطيت العلاج المشترك للفاناديوم والدوكسيسيكلين وزنها وأصبحت طبيعية. علاوة على ذلك، عكس الدوكسيسيكلين تأثير الفاناديوم على المتغيرات الأيضية وتثبيط بيروكسيد الدهون الذي أقترب من المستويات الطبيعية لمجموعة الضوابط.

**الخلاصة**: أظهرت هذه النتائج العمل المضاد للأكسدة أو التأثر المخلبي للدوكسيسيكلين ضد السمية العصبية للفاناديوم وقدرته العلاجية على تجنب التغيرات العصبية التنكسية في مناطق دماغ الفئران المختلفة.

**الكلمات المفتاحية**: الدوكسيسيكلين، بيروكسيد الدهن، عجز الخلايا العصبية، السمية العصبية، أنواع الأكسجين التفاعلية، الفاناديوم.