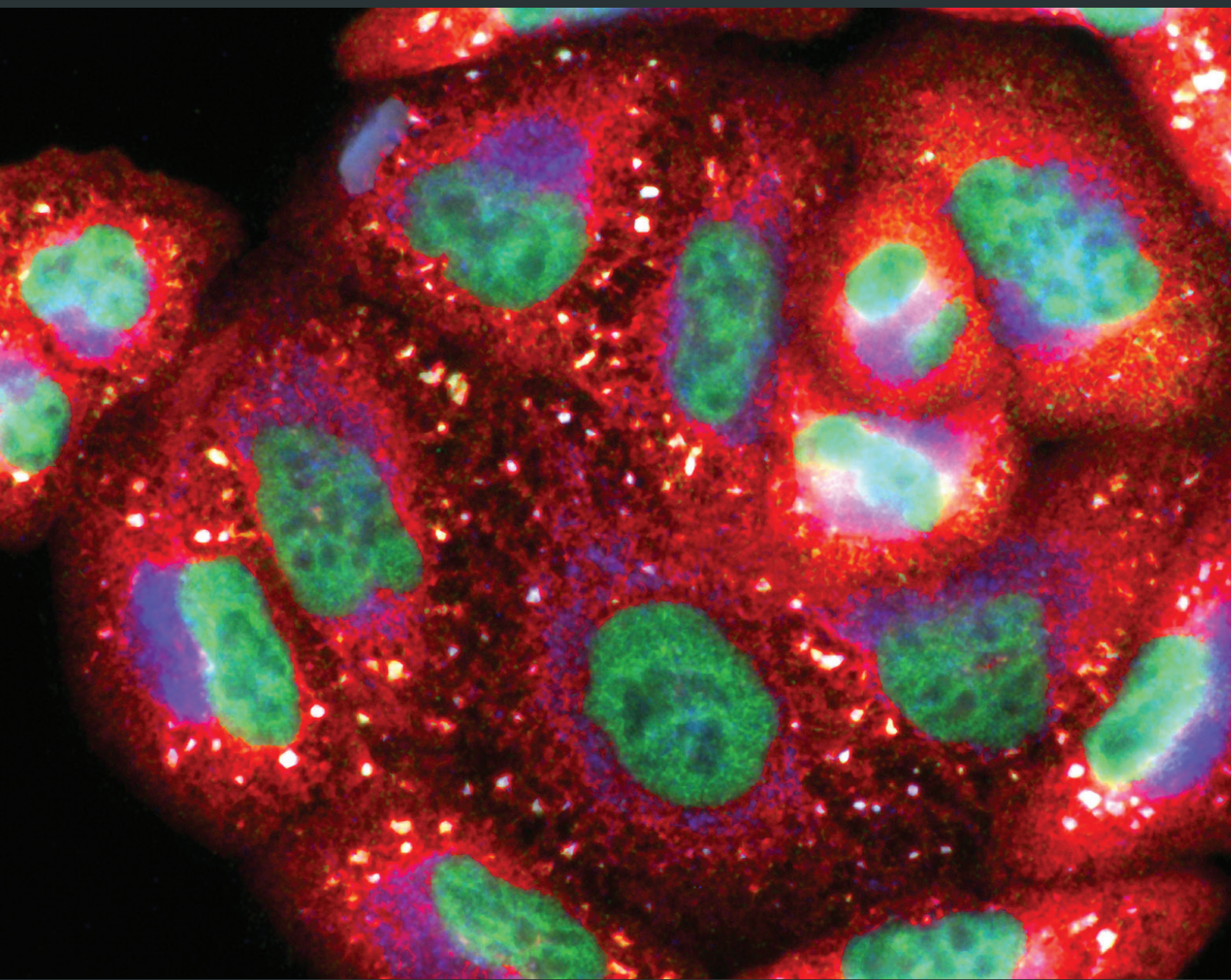
Oxidative Medicine and Cellular Longevity

Vanadium Toxicological Potential versus Its Pharmacological Activity: New Developments and Research

Guest Editors: Agnieszka Ścibior, Juan Llopis, Alvin A. Holder, and Mario Altamirano-Lozano



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Editorial Board

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Editorial

Vanadium Toxicological Potential versus Its Pharmacological Activity: New Developments and Research

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Vanadium, which arouses interest of many research centers worldwide, is a biologically essential redox-active metal with an ability to evoke diametrically opposite effects. Due to its dual character and multidirectional action, vanadium receives a great deal of attention of pharmacologists and researchers from different scientific disciplines.

Despite the fact that extensive knowledge about vanadium has been gathered, many aspects of its action still require to be clearly defined. At present, there is a lack of sufficient data about the mechanisms of absorption, transport, and excretion of this metal. Relatively little is also known about the consequences and mechanisms of mutual interactions of vanadium with other elements, particularly those that have antioxidant potential. The question of interactions between elements, which may take place at different levels and can be used to elucidate the cellular mechanisms of response to combinations of metals, is always up to date and still an important issue in toxicology. The essentiality of vanadium to humans and the mechanisms of its toxic action also need to be elucidated. The above-mentioned aspects and likewise the pharmacological activity of vanadium, which raises hopes for use thereof in the treatment of certain diseases in humans, including diabetes, osteoporosis, and certain types of cancer as well as parasitic and infectious diseases, need to be further studied in order to fully understand and elucidate the toxicology as well as the biological activity and pharmacological potential of this element. More details in this research field may help to estimate the balance between adverse effects of vanadium (which has a relatively narrow margin of safety) and its potential therapeutic properties and to determine better its toxicity and therapeutic intervals which, in turn, allows us to devise and develop possible procedures of treatment of certain illnesses.

In this special issue, an attempt has been made to include reports that update our knowledge about the role of vanadium in toxicological processes and pharmacological applications and identify gaps in this research field, which would ensure better understanding of the specific behavior of this element. The special issue on vanadium compiles six (6) excellent manuscripts including reviews and research articles, which provide current and comprehensive knowledge about some aspects of the action of this metal.

The review article by M. Aureliano covers recent advances in the understanding of decavanadate toxicology and pharmacological applications. The Author (a) discusses in depth the modes of action of decavanadate (V_{10}) through oxidative stress, effects on mitochondria, sarcoplasmic reticulum, and cytoskeleton, (b) provides information about some aspects of action of decavanadate (V_{10}) and vanadate (V_1), and (c) highlights the significance of understanding the V_{10} toxicology and pharmacological activities as important targets to elucidate the biological activities of several polyoxometalates in order to make them available and safe for clinical use. In turn, the review article by Tsave et al. focuses on the role

of vanadium in cellular and molecular immunology. The Authors nicely and concisely illustrate (a) the effects of selected vanadium species in the immune system processes, (b) forms of vanadium exhibiting immunogenic activity, and (c) the key role(s) of vanadium in promoting innate and adaptive immunity as well as (d) current obstacles to be overcome by specifically designed vanadium metallodrugs in cancer immunotherapeutics. The research data collected in this review highlight the synthetic and structural bioinorganic profile of vanadium along with its biological activity attributes, collectively formulating the significant potential of unique structure-based and immune process-specific vanadodrugs for the detection, prevention, and treatment of immune system aberrations.

As far as the research articles are concerned, the report of Treviño et al. presents metforminium decavanadate (MetfDeca) as a potential metallopharmaceutical drug for the treatment of diabetes mellitus. The Authors showed efficiency of MetfDeca in improving serum profiles of carbohydrates and lipids and revealed a protective effect of MetfDeca on pancreatic beta cells of rats with model type 1 diabetes mellitus. In turn, the research article of García-Rodrígez et al. illustrates the *in vivo* effects of vanadium (as vanadium pentoxide) and certain antioxidants (ascorbic acid and alpha-tocopherol) on apoptotic, cytotoxic, and genotoxic activity in mice. The Authors demonstrated that both these antioxidants were able to protect cells against vanadium pentoxide-induced genetic damage. The research article of Christensen et al., in turn, presents the effectiveness of bisphosphonate and vanadium-bisphosphonate compounds against axenic *Leishmania tarentolae*. Specifically, the Authors showed that two polyoxometalates (POMs) with nitrogen containing bisphosphonate ligands: vanadium/alendronate $[V_5(Ale)_2]$ and vanadium/zoledronate $[V_3(Zol)_3]$ complexes were effective in inhibiting the growth of *L. tarentolae* and suggested that $V_3(Zol)_3$ may be effective in a skin cream formulation as a weekly or daily treatment for cutaneous leishmaniasis. Finally, the report of Folarin et al. describes the effects of administration of vanadium on memory in mice. The Authors indicated that mice exposed to vanadium exhibited no difference in learning abilities but had significant loss in memory acumen after 3 months of exposure. They also revealed that the memory deficit induced by chronic administration of vanadium in mice is reversible, but only after a long period of vanadium withdrawal.

We believe that the information provided in this special issue will be of interest to readers who are interested in vanadium and to those of Oxidative Medicine and Cellular Longevity in general.

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Agnieszka Ścibior
Juan Llopis
Alvin A. Holder
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Research Article

In Vivo Effects of Vanadium Pentoxide and Antioxidants (Ascorbic Acid and Alpha-Tocopherol) on Apoptotic, Cytotoxic, and Genotoxic Damage in Peripheral Blood of Mice

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This study was conducted to investigate the effects of vanadium pentoxide (V_2O_5), ascorbic acid (AA), and alpha-tocopherol (α-TOH) on apoptotic, cytotoxic, and genotoxic activity. Groups of five Hsd:ICR mice were treated with the following: (a) vehicle, distilled water; (b) vehicle, corn oil; (c) AA, 100 mg/kg intraperitoneally (ip); (d) α-TOH, 20 mg/kg by gavage; (e) V_2O_5, 40 mg/kg by ip injection; (f) AA + V_2O_5; and (g) α-TOH + V_2O_5. Genotoxic damage was evaluated by examining micronucleated polychromatic erythrocytes (MN-PCE) obtained from the caudal vein at 0, 24, 48, and 72 h after treatments. Induction of apoptosis and cell viability were assessed at 48 h after treatment in nucleated cells of peripheral blood. Treatment with AA alone reduced basal MN-PCE, while V_2O_5 treatment marginally increased MN-PCE at all times after injection. Antioxidants treatments prior to V_2O_5 administration decreased MN-PCE compared to the V_2O_5 group, with the most significant effect in the AA + V_2O_5 group. The apoptotic cells increased with all treatments, suggesting that this process may contribute to the elimination of the cells with V_2O_5-induced DNA damage (MN-PCE). The necrotic cells only increased in the V_2O_5 group. Therefore, antioxidants such as AA and α-TOH can be used effectively to protect or reduce the genotoxic effects induced by vanadium compounds like V_2O_5.

1. Introduction

For several decades, vanadium was considered a low-toxicity essential trace element with antidiabetic and anticarcinogenic properties [1, 2]. However, in 2006, the International Association for Research on Cancer (IARC) classified vanadium pentoxide (V_2O_5) as a Group 2B substance (possibly carcinogenic to humans) based on results in experimental animals [3]. Three years later, in 2009, the American Council of Government and Industrial Hygienists (ACGIH) placed V_2O_5 in category A3 (confirmed animal carcinogen with unknown relevance to humans) [4]. Today, there is disagreement regarding the carcinogenic responses to V_2O_5 and evidence supporting that a genotoxic mode of action is still insufficient [5]. The general consensus is that while both positive and negative results have been reported, the weight of evidence suggests that V_2O_5 has the potential to induce aneuploidy, micronucleus (MN), and chromosomal aberrations in some cells *in vitro* and *in vivo* (somatic cells) [3, 6, 7].

Among the handful of proposed mechanisms of vanadium(V) toxicity, which include interference with protein phosphatase and kinase activity and inhibition of DNA repair, the induction of oxidative stress is of particular importance for biological systems [7–9]. The genotoxicity associated with oxidative stress is based on the oxidative mechanism of reduction of vanadium(V), generating reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$) [10]. Since antioxidants are able to inactivate highly reactive molecules such as ROS that are generated during various biochemical processes in the cells [11], substances with antioxidant properties emerge as putative preventatives and coadjuvants in the treatment of chronic degenerative diseases related to oxidative stress and DNA damage [12].

A large number of antioxidants have been shown to reduce the clastogenicity of drugs and pesticides in experimental animals [13–15]; these include ascorbic acid (AA) and alpha-tocopherol (α-TOH). The beneficial properties of AA were highlighted by Cameron and Pauling in the 1970s, who suggested that high doses of AA (>10 g/day) cure and prevent cancer by promoting collagen synthesis [16]. However, researchers now suggest that AA actually prevents cancer by neutralizing ROS before they can damage DNA and initiate tumor growth; AA may also act as a prooxidant, helping the body's own ROS destroy early-stage tumors [17–19]. Similarly, it has been shown that α-TOH is effective in reducing the effects of various genotoxic compounds [20, 21]. In terms of bioavailability and bioactivity, α-TOH is biologically and functionally the most important and most active antioxidant of all the vitamin E isoforms in humans because it effectively minimizes oxidative stress and regulates lipid peroxidation and toxic effects of ROS in biological systems [22–24]. Since the 1960s and similarly to AA, it has been observed that dietary α-TOH supplementation is somewhat effective in suppressing carcinogen-induced cancers in rodents [25].

Although the antioxidants AA and α-TOH have shown great potential in reducing some cancers and genotoxic effects induced by different chemicals, there is no information on their effect against V_2O_5-induced genotoxicity, cytotoxicity, and apoptosis *in vivo*. Therefore, in this study we evaluated AA (water-soluble) and α-TOH (lipid-soluble) in order to identify and understand their possible beneficial effects against V_2O_5-induced genotoxicity and cytotoxicity using the MN technique and the analyses of apoptosis, necrosis, and cell viability in peripheral blood of Hsd:ICR mice.

2. Materials and Methods

2.1. Chemicals. The following chemical and reagent tests were obtained from Sigma Chemicals Co. (St. Louis, MO, USA): V_2O_5 (CAS number 1314-62-1), acridine orange (AO) (CAS number 10127-02-3), ethidium bromide (EB) (CAS number 1239-45-8), α-tocopherol (α-TOH) (CAS number 10127-02-3), and ascorbic acid (AA) (CAS number 50-81-7). The corn oil (delivery vehicle for fat-soluble compounds) also was obtained from Sigma Chemicals Co. (CAS number 8001-30-7).

2.2. Animals. Two- to three-month-old Hsd:ICR male mice (28–35 g) were used in the experiments. The animals were kept under controlled temperature (22°C) with a 12-12 h light-dark period (light 07:00–19:00 h). The mice had free access to food (Purina®-Mexico chow for small rodents) and water. All of the mice were obtained from Harlan at "Facultad de Química, Universidad Nacional Autónoma de México" (UNAM) and were acclimated for a two-week period. The Bioethics Committee of the "Facultad de Estudios Superiores-Zaragoza," UNAM, approved the experimental protocols used in this study.

2.3. Experimental Design. The doses of AA and α-TOH were based on results obtained in a previous study, in which doses of 100 and 20 mg/kg of AA and α-TOH, respectively, did not increase MN in polychromatic erythrocytes (PCE) [26]. The V_2O_5 dosage was selected according to previous studies in which a 40 mg/kg dose administered intraperitoneally (ip) induced MN-PCE in the peripheral blood of mice [27].

The AA and V_2O_5 were prepared in solution by dissolving the dry compounds in sterile distilled water, and the α-TOH was dissolved in corn oil (vehicle for lipid-soluble compounds). The solutions (0.25 mL) were administered immediately after preparation of the compounds. The control groups were treated identically, using vehicle only (sterile distilled water or corn oil). The evaluation criteria and work conditions were set up according to OECD guidelines (test number 474), Food and Drug Administration (FDA) guidelines, Environmental Protection Agency (EPA) guidelines, and guidelines for the testing of chemicals specified by the Collaborative Study Group for the Micronucleus Test (CSGMT) and the Mammalian Mutagenesis Study Group of the Environmental Society of Japan (JEMS.MMS) for the short-term mouse peripheral blood micronucleus test [28, 29].

After establishing treatment doses, the effects of AA and α-TOH on genotoxic damage in V_2O_5-treated mice were evaluated. These assessments were performed by MN-PCE kinetic, apoptosis, and cell viability analyses. Mice were assigned randomly to one of the following groups ($N = 5$ mice per each group) according to the protocol described in Figure 1.

2.4. Micronucleus Assay. Slides were covered with AO and prepared in accordance with the technique described by Hayashi et al. [30]. Briefly, AO was dissolved in distilled water at a concentration of 1 mg/mL, and 10 μL of this solution was placed on a preheated (approximately 70°C) clean glass slide. The AO was evenly distributed on the slide by moving a glass rod back and forth over the slide, which was then air-dried. The AO-coated glass slides were stored in a dark, dry location at room temperature prior to experimental use.

To evaluate MN after treatment, 5 μL of peripheral blood samples was collected by piercing a tail blood vessel of the mice once every 24 h over a four-day period (0 to 72 h). The samples were placed directly on AO-treated slides [30]. Afterwards, a coverslip (24 × 50 mm) was placed over the sample and its edges were sealed with rubber cement. All of the prepared slides were kept in plastic boxes in the dark at 4°C. While these slide preparations cannot be stored permanently, they can be stored for several days in refrigeration if the coverslip has been sealed. Two slides were prepared for each mouse, and analyses of the slides were conducted after 12 h. The MN-PCE analysis was based on 2,000 PCE per mouse, and the presence of MN-PCE was considered genotoxic damage [29]. In parallel, the relative proportion of PCE to normochromatic erythrocytes (NCE) was analyzed in 1,000 erythrocytes.

The evaluations were made by identifying PCE, NCE, and MN-PCE under a fluorescent microscope (Nikon OPTIPHOT-2) with blue (480 nm) excitation and a barrier filter emission (515–530 nm). The differential AO staining distinguished PCE from NCE because PCE were stained,

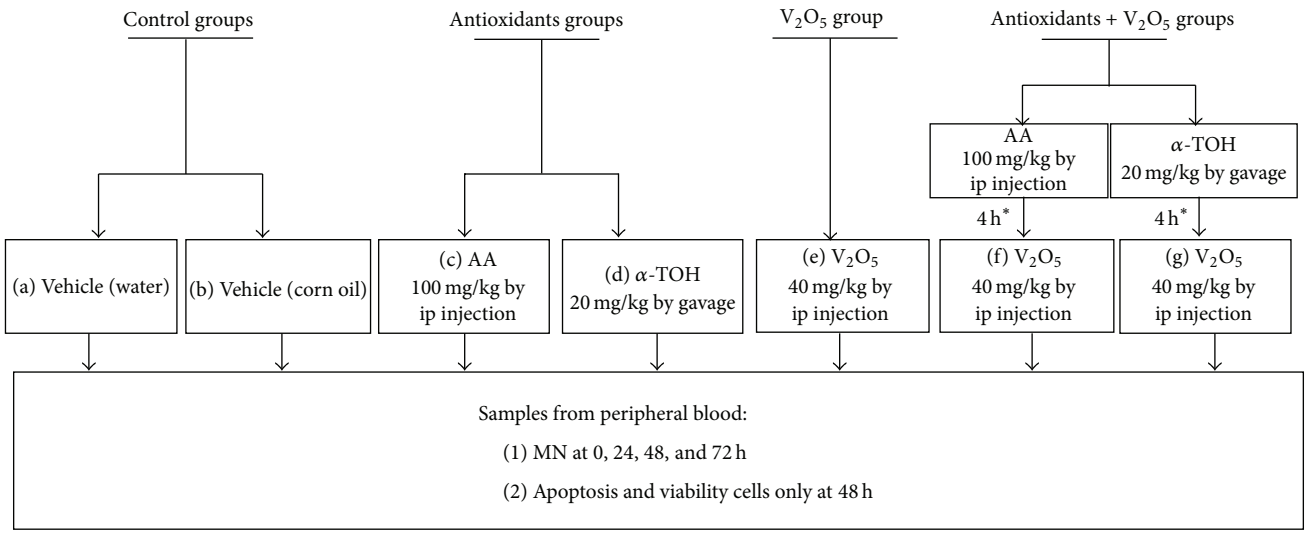

Figure 1: Experimental protocol. Mice were assigned at random to one of the following groups ($N = 5$ mice per group). For evaluations of MN, all animals were sampled before administering treatments (0 h) and at 24, 48, and 72 h after treatments. As for apoptosis and cell viability only at 48 h samples after treatments were taken. *The antioxidants were administrated 4 h before the injection of V_2O_5.

showing orange fluorescence due to the presence of ribosomal RNA, while NCE did not stain at all. The AO also allowed the identification of MN-PCE, which showed yellow fluorescence due to their DNA content. To compare the kinetics of MN-PCE induction, the data were analyzed using the "net induction frequency" (NIF) [31].

2.5. Apoptosis and Cell Viability Analyses. To evaluate apoptosis and cell viability, we used differential acridine orange/ethidium bromide (AO/EB) staining following the technique previously adapted for peripheral blood [12]. Blood samples (100 μL) were collected by piercing a tail blood vessel of the mice prior to treatment and 48 h after treatment. Heparin (10 μL) was added to the blood samples, and 20 μL of AO/EB dye mix (100 μL/mL AO and 100 μL/mL EB, both prepared in phosphate buffered saline (PBS)) was then added. The suspension was concentrated via centrifugation (4,500 g) and the cell pellet was resuspended in 10 μL of PBS and plated on a clean slide; a coverslip (24 × 24 mm) was immediately placed on the slide. Two slides for each mouse were prepared, and the analysis was conducted immediately. The apoptotic and cell viability assessments were based on 200 nucleated cells per mouse [32].

Necrotic, apoptotic (early and late), and viable cells were identified using a fluorescent microscope (Nikon OPTIPHOT-2) with blue (480 nm) excitation and a barrier filter emission (515–530 nm) at 40x magnification. With the differential AO/EB staining, it is possible to distinguish between viable and nonviable cells based on membrane integrity. These dyes were used to identify cells that have undergone apoptosis and to distinguish between cells in the early and late stages of apoptosis, also based on membrane integrity (Figure 2). AO intercalates into the DNA, giving it a green appearance. This dye also binds to RNA, but because it cannot intercalate, the RNA stains red-orange.

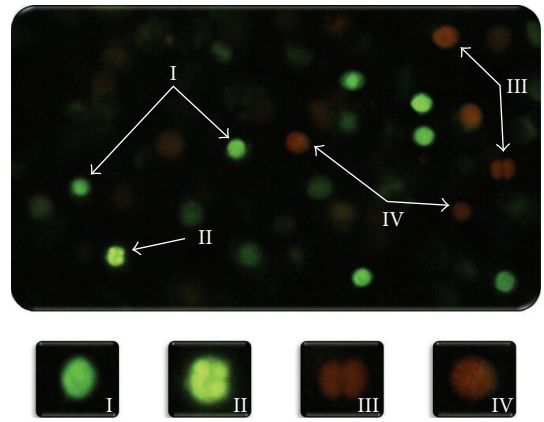

Figure 2: Morphology of viable cells (early apoptotic and nonapoptotic cells) and nonviable cells (late apoptotic and necrotic cells) assessed with AO/EB staining (40x). Viable cells stain uniformly showing green (I). Early apoptotic cells with intact plasma membranes appear green, with "dots" of condensed chromatin that are highly visible within (II). Late apoptotic cells are stained showing bright green-orange because membrane blebbing starts to occur. EB can enter the cells (III). Necrotic cells are stained emitting bright orange due to the entry of EB into these cells (IV).

Thus, viable cells appear bright green. EB is only taken up by nonviable cells and also intercalates into DNA, making it appear orange. However, EB only binds weakly to RNA, which may result in a slightly red appearance. Thus, nonviable cells have bright orange nuclei because the EB overwhelms AO staining and their cytoplasm appears dark red (if any content remains). Both normal and early apoptotic nuclei in viable cells exhibit bright green fluorescence (Figure 2, I-II). In contrast, necrotic or late apoptotic nuclei in nonviable cells emit bright orange fluorescence (Figure 2, III-IV).

Table 1: Averages of the MN-PCE induction in peripheral blood of mice.

Treatment	Dose (mg/kg)	N	Time analysis (hours)	MN-PCE/1,000 cells^ (mean ± SD)	ANOVA
Control (water)	0	5	0	0.5 ± 0.3	
			24	0.8 ± 0.2	
			48	0.9 ± 0.4	
			72	1.2 ± 0.2	
Control (corn oil)	0	5	0	0.2 ± 0.2	
			24	0.3 ± 0.3	
			48	0.4 ± 0.4	
			72	0.3 ± 0.3	
AA*	100	5	0	0.5 ± 0.3	
			24	0.2 ± 0.2	
			48	0.3 ± 0.2	
			72	0.3 ± 0.4	
α-TOH*	20	5	0	0.3 ± 0.4	
			24	0.5 ± 0.3	
			48	0.3 ± 0.2	
			72	0.3 ± 0.2	
V_2O_5	40	5	0	1.4 ± 0.4	
			24	2.2 ± 0.2	a, c, d
			48	3.0 ± 0.6	a, b, c, d
			72	3.9 ± 0.8	a, b, c, d
AA + V_2O_5	100 + 40	5	0	1.1 ± 0.2	
			24	0.9 ± 0.4	
			48	0.7 ± 0.2	
			72	0.8 ± 0.4	
α-TOH + V_2O_5	20 + 40	5	0	0.7 ± 0.4	
			24	1.0 ± 0.3	
			48	1.2 ± 0.2	
			72	0.8 ± 0.2	

[a] $p < 0.05$ versus controls; [b] $p < 0.05$ versus V_2O_5 0 h; [c] $p < 0.05$ versus AA + V_2O_5; [d] $p < 0.05$ versus α-TOH + V_2O_5.
*AA: vehicle water, distilled; α-TOH: vehicle corn oil.
^2,000 PCE were evaluated in each mouse (5 mice per group).

The nuclei of viable cells with intact membranes were uniformly stained green (Figure 2, I). Early apoptotic cells with intact membranes, but in which the DNA has begun to fragment, still exhibit green nuclei because the EB cannot enter the cell, but chromatin condensation is visible as bright green patches in the nuclei (Figure 2, II). As the cell progresses through the apoptotic pathway and membrane blebbing begins to occur, EB permeates the cell, producing a green-orange stained cell (Figure 2, IV). Late apoptotic cells show bright orange patches of condensed chromatin in the nuclei; this distinguishes them from necrotic cells, which stain uniformly orange (Figure 2, III) [12, 32].

2.6. Statistical Analysis. The results of MN-PCE induction, the PCE/NCE ratio, the NIF of MN-PCE, the cell viability (viable/nonviable cells), and the necrotic and apoptotic cells (early/late) are expressed as the mean ± standard deviation (SD). The results from the various treatments were compared by an ANOVA/ANCOVA followed by Tukey's test. SPSS/PC V18TM software was used for the statistical analyses. For all of the analyses, $p < 0.05$ was considered to be significant.

3. Results

The MN-PCE averages are shown in Table 1. Although an increase of MN-PCE is observed in the control group treated with distilled water, there were no significant effects in either control group (water and corn oil vehicles). The antioxidants did not markedly affect the average MN-PCE in treated mice (Table 1). Treatment of V_2O_5 significantly increased the averages of MN-PCE at all times after injection, with the greatest increase at 72 h (about 4 MN-PCE). When the treatment included antioxidants (AA or α-TOH) and V_2O_5, the number of MN-PCE observed at 24, 48, and 72 h after treatment was lower than when treated with V_2O_5 alone.

As shown in Table 1, the baseline MN-PCE varied between groups (time 0), which obscured actual MN-PCE increases. Therefore, calculation of the NIF was performed to improve the ability to determine net MN-PCE induction. This calculation subtracts the frequencies of MN-PCE prior to treatment from the frequencies following treatment, thereby eliminating baseline MN-PCE variability among treatment groups at time 0 (Table 1). Data represent "the absolute value"

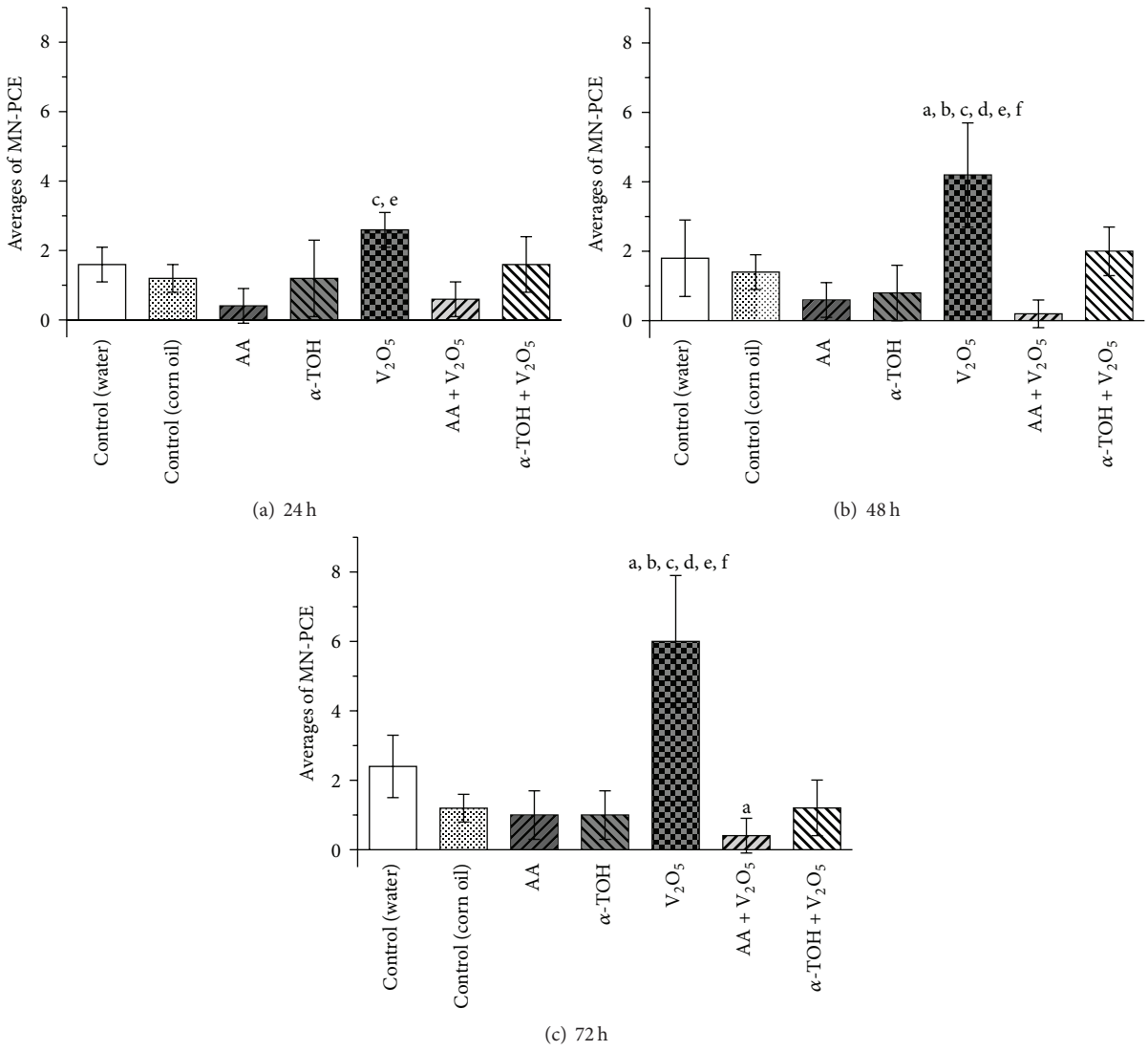

Figure 3: Effect of antioxidants (AA and α-TOH) on the MN-PCE NIF in peripheral blood of mice at different times: (a) 24 h, (b) 48 h, and (c) 72 h after treatment with V_2O_5. Data represent "the absolute value" of the averages of MN-PCE frequencies obtained at 24, 48, and 72 h per group minus the averages of MN-PCE frequencies at 0 h per group. ANCOVA: [a]$p < 0.05$ versus control (water); [b]$p < 0.05$ versus control (corn oil); [c]$p < 0.05$ versus AA; [d]$p < 0.05$ versus α-TOH; [e]$p < 0.05$ versus AA + V_2O_5; [f]$p < 0.05$ versus α-TOH + V_2O_5. 2,000 PCE were evaluated in each mouse (5 mice per group).

of the averages of MN-PCE frequencies and were analyzed as follows:

$$\begin{aligned}\text{NIF} &= \big|\text{average of MN-PCE frequencies measured at time } x_i \\ &\quad - \text{average of MN-PCE frequencies measured at time } 0\big|,\end{aligned} \tag{1}$$

where x_i is evaluation at 24, 48, or 72 h per group and time 0 is evaluation at 0 h (before treatment) per group.

Data represent the average MN-PCE frequencies at 24, 48, and 72 h per group, minus the average MN-PCE frequencies at 0 h per group. Figure 3 shows the NIF of MN-PCE values for all groups at 24, 48, and 72 h after treatment. The frequencies of MN-PCE in samples from the group treated with AA were lower than the control at 24, 48, and 72 h (75, 67, and 58% reduction, resp.) after treatment. In the groups that combined treatments with antioxidants and V_2O_5, a significant reduction in the frequencies of MN-PCE was detected: the AA reduced by 77% at 24 h and a complete reduction was observed at 48 and 72 h, while α-TOH reduced by 38, 52, and 80% at 24, 48, and 72 h, respectively.

PCE/NCE ratio is shown in Table 2. Treatments with antioxidants and antioxidants + V_2O_5 decreased the frequencies of PCE compared to controls. This decrease was more significant when antioxidants were administered alone. Treatment of V_2O_5 did not affect the average MN-PCE in the mice (Table 2). The cytotoxic effects were simultaneously assessed by apoptosis, necrosis, and cell viability directly in nucleated cell of peripheral blood of mice at 48 h after treatment.

Table 2: Averages of the PCE/NCE ratio in peripheral blood of mice.

Treatment	Dose (mg/kg)	N	Time analysis (hours)	PCE/NCE 1,000 cells^ (mean ± SD)	ANOVA
Control (water)	0	5	0	127.4 ± 5.9	
			24	123.2 ± 11.2	
			48	133.6 ± 10.1	
			72	128.8 ± 4.9	
Control (corn oil)	0	5	0	115.0 ± 3.9	
			24	115.5 ± 4.0	
			48	111.7 ± 3.4	
			72	114.0 ± 2.2	
AA*	100	5	0	100.6 ± 2.0	
			24	68.8 ± 1.9	a, b
			48	67.2 ± 3.0	a, b
			72	68.2 ± 0.8	a, b
α-TOH*	20	5	0	103.4 ± 3.6	
			24	75.4 ± 3.4	a, c
			48	66.2 ± 7.0	a, c
			72	67.8 ± 2.4	a, c
V_2O_5	40	5	0	123.8 ± 6.6	
			24	125.4 ± 4.2	d, f
			48	124.8 ± 4.3	d, f
			72	136.6 ± 3.0	d, f
AA + V_2O_5	100 + 40	5	0	101.2 ± 4.7	
			24	95.8 ± 5.3	a
			48	88.4 ± 1.3	a
			72	87.6 ± 1.9	a
α-TOH + V_2O_5	20 + 40	5	0	116.0 ± 4.4	
			24	87.0 ± 3.4	a, e
			48	84.6 ± 3.4	a, e
			72	87.4 ± 2.2	a, e

[a] $p < 0.05$ versus control; [b] $p < 0.05$ versus AA 0 h; [c] $p < 0.05$ versus α-TOH 0 h; [d] $p < 0.05$ versus AA + V_2O_5; [e] $p < 0.05$ versus AA + V_2O_5 0 h; [f] $p < 0.05$ versus α-TOH + V_2O_5.

*AA: vehicle water, distilled; α-TOH: vehicle corn oil.

^1,000 erythrocytes were evaluated in each mouse (5 mice per group).

Unlike the results obtained in the PCE/NCE ratio, cell viability also decreased in treatment with V_2O_5 alone, which was more significant than in the other treatments (excluding treatment with α-TOH + V_2O_5) (Figure 4). All treatments significantly increased apoptotic cell frequency, with the highest increases in the V_2O_5 and combined groups. Late apoptotic cells were mainly identified in antioxidant and antioxidants + V_2O_5 treatments. Lower average early apoptotic cells were found in the treatments with antioxidants compared to those in the control group, and this reduction was significant in the treatment with AA. Although in the AA-treated group the decrease in early apoptotic cells was statistically significant, the increase in late apoptotic cells was significant (Table 3). In the V_2O_5 and combined treatments, an increase in both early and late apoptotic cells was observed, being greater in the late apoptotic cells. The necrotic cells increased significantly only in the treatment with V_2O_5 alone (Table 3).

No mice exposed to V_2O_5 died, and no clinical signs of toxicity were observed.

4. Discussion

Although V_2O_5 is considered a possible carcinogen in humans based on evidence of lung carcinogenesis in mice [33], the information regarding the genotoxic potential of V_2O_5 in models *in vivo* is limited and inconclusive [7]. In this study we observed that the administration of 40 mg/kg of V_2O_5 via ip injection increases the frequencies of MN-PCE in peripheral blood. This is consistent with several studies that also evaluated MN-PCE in experimental animals treated with soluble vanadium compounds (Na_3VO_4, SVO_5, and NH_4VO_3) administered orally [34, 35] and particularly by inhalation of V_2O_5 in males [36]. However, the maximum increases we observed were around 4/1,000 MN-PCE, which are lower than induction by other metals clearly identified as genotoxic agents such as Cr(VI) [31, 37, 38].

The rodent micronucleus assay is used in regulatory test batteries to predict the carcinogenicity of chemical agents through their ability to produce genotoxicity *in vivo*. If a compound increases MN frequencies it is often regarded

Table 3: Evaluations at 48 h in peripheral blood of mice. Averages of early and late apoptotic and necrotic cells per group.

Group	Dose (mg/kg)	N	$\overline{x} \pm$ SD		
			Early apoptotic	Late apoptotic	Necrotic
Control (water)	0	5	12.6 ± 1.7	1.2 ± 0.4	0.8 ± 0.4
Control (corn oil)	0	5	10.6 ± 2.1	1.0 ± 0.0	0.6 ± 0.5
AA	100	5	1.0 ± 0.7^{a}	33.4 ± 2.5^{a}	0.2 ± 0.4
α-TOH	20	5	6.8 ± 3.2	37.8 ± 4.1^{b}	0.2 ± 0.4
V_2O_5	40	5	$21.2 \pm 5.6^{a,b}$	$40.8 \pm 5.4^{a,b}$	$2.4 \pm 1.3^{a,b,c,d}$
AA + V_2O_5	100 + 40	5	23.8 ± 2.4^{a}	34.2 ± 1.9^{a}	0.8 ± 0.5
α-TOH + V_2O_5	20 + 40	5	25.0 ± 3.5^{b}	41.6 ± 4.6^{b}	0.6 ± 0.5

[a] $p < 0.05$ versus control (water); [b] $p < 0.05$ versus control (corn oil); [c] $p < 0.05$ versus AA + V_2O_5; [d] $p < 0.05$ versus α-TOH + V_2O_5. Nonviable cells include apoptotic and necrotic cells. 200 nucleated cells were evaluated in each mouse (5 mice per group).

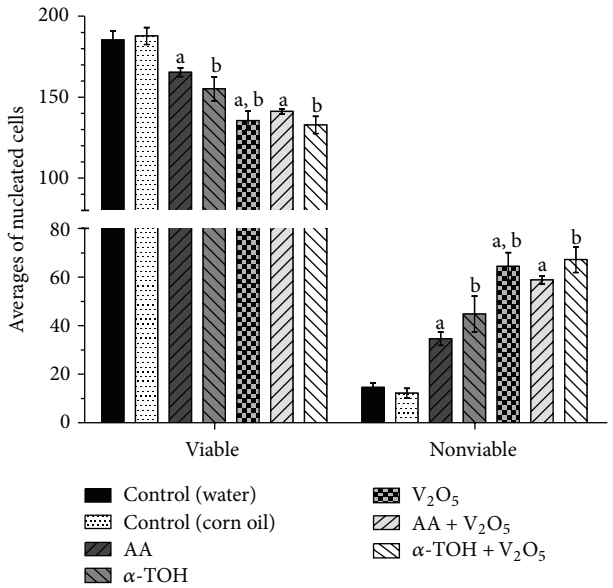

Figure 4: Evaluations at 48 h in peripheral blood of mice. Averages of viable and nonviable nucleated cells. [a] $p < 0.05$ versus control (water); [b] $p < 0.05$ versus control (corn oil); [c] $p < 0.05$ versus AA + V_2O_5; [d] $p < 0.05$ versus α-TOH + V_2O_5. Nonviable cells include apoptotic and necrotic cells. 200 nucleated cells were evaluated in each mouse (5 mice per group).

as definitive evidence of *in vivo* genotoxicity, making it a probable carcinogen [39, 40]. However, it is important that marginal results in the induction of MN be taken with reservation, since there is evidence that compound-related disturbances in rodent physiology, such as body temperature and erythroblast toxicity, can also modify MN frequencies and increase erythropoiesis by stimulating cell division in bone marrow and peripheral blood [39]. These increases in MN may therefore not be a result of direct, intrinsic genotoxic properties of the agent. For this reason, the EPA Gene-Tox Program and the Collaborative Study Group for the Micronucleus Test have proposed a threshold of 4/1,000 MN-PCE increase to define a compound as a genotoxic agent and a threshold of 7.5/1,000 MN-PCE increase to designate it as a positive control agent [28, 29, 40]. Similarly, a range between 0 and 3 MN-PCE has been proposed for the control group in order to consider individual variation among test subjects. Thus, while we did find higher induction of MN-PCE in the water control group compared to the corn oil control group at all evaluation times, the increase was within this proposed control range (spontaneous micronucleus in PCE from untreated animals) [39, 40].

When assessing the PCE/NCE ratio in the group treated with V_2O_5 alone, no changes were observed compared to the control group. The PCE/NCE ratio is suggested by MN assay because it is an indicator of cytotoxicity [40]. However, while finding reduced PCE frequency is indicative of cytotoxic effects, negative results must be interpreted with caution because when toxicity occurs during erythropoiesis, the mechanisms of cell division can be activated and mask the effect [39, 40]. Moreover, it has been observed that vanadium compounds can produce lipid peroxidation in the erythrocyte membrane, leading to hemolysis, which could interfere with the erythroid differentiation process [41, 42]. The effects on erythropoiesis could therefore be related to the marginal increase in MN-PCE observed in mice treated with V_2O_5. For this reason, we also assessed cell viability in nucleated peripheral blood cells using the differential AO/EB staining technique. The dual fluorochrome assay is an indicator of cell metabolism and death caused by cell membrane injury [12, 32]. With this analysis, a decrease in the viable cells at 48 h in mice treated with V_2O_5 was observed, suggesting a cytotoxic effect. However, cytotoxic effects of V_2O_5 have not been found in *in vitro* assays in lymphocytes and human mucosal cells [43] or *in vivo* in bone marrow [33]. Although Rojas-Lemus et al. [36] observed a decrease in cell viability in mice 24 h after inhalation of V_2O_5 during acute phase, this did not persist for more than a week in peripheral blood leukocytes.

On the other hand, we observed that the administration of 40 mg/kg of V_2O_5 via ip injection increased the frequency of apoptotic cells. The apoptotic activity was indicated by the increased frequencies of early and especially late apoptotic cells 48 h after treatment with V_2O_5. Anticancer properties have been attributed to vanadium(V) compounds, and apoptosis has been identified as one of the ways to eliminate tumor cells [7]. Vanadium compounds activate different signaling pathways in normal and cancer cells, acting mainly through inactivation of PTPs and/or activation of PTKs. Activation of cellular signaling pathways converges

downstream to cooperatively modulate the transcriptional activity of NF-κB or by the suppression of genes involved in cell cycle regulation, DNA repair, and apoptosis [1, 44, 45]. Although it has been suggested that, in p53-defective rodent cells, such as L5178Y, MN induction may be independent of apoptosis [46], apoptosis may contribute to the elimination of micronucleated cells and hence lead to a marginal induction of MN-PCE when administering V_2O_5. However, in the mice treated with V_2O_5, counts of necrotic cells increased significantly, leading to inflammatory processes. It has been suggested that it is the combination of oxidative stress, inflammation, and genotoxicity that makes this element a possible carcinogen [47].

Recent studies have shown that vanadium(V) in mice induces genotoxic damage and apoptosis through oxidative stress [7, 8, 48]. The *in vivo* administration of AA or α-TOH prior to V_2O_5 injection decreased MN-PCE formation compared to V_2O_5 alone (Figure 2). The ways in which the antioxidants protect cells against V_2O_5-induced genetic damage may be related to its oxide-reductive properties. AA is a potent antioxidant (reducing agent) that is capable of scavenging free radicals of reactive oxygen and nitrogen species that have the potential to damage nucleic acids and promote carcinogenesis [49, 50]. Thus, the combination of antioxidant agents such as AA or α-TOH with V_2O_5 could protect cells from genetic damage. The genotoxicity of vanadium(V) is due to its reduction by NADH to vanadium(IV), generating $^{\bullet}$OH [10]. Vanadate reacts with thiols to produce V(IV) and thiyl radicals (vanadyl). During catalysis in the reaction of 2-deoxyguanosine with molecular oxygen, 8-hydroxydeoxyguanosine is formed, causing DNA strand breaks [51]. Thus, ascorbate could react with ROS, quenching and converting them into poorly reactive semidehydroascorbate radicals, which cause no DNA damage [52–54], which is reflected in the reduction of MN-PCE (Figure 2). Vitamin E belongs to the family of lipid-soluble vitamins, of which α-TOH is the most active form, and like AA, it is a powerful biological antioxidant that may effectively minimize oxidative stress, lipid peroxidation, and toxic effects of ROS in biological systems [24]. Our data demonstrate that AA and α-TOH protected cells against V_2O_5-induced genetic damage. The reduction of MN-PCE observed with AA and α-TOH was more effective than with the administration of high-antioxidant beverages such as green tea [55], red wine [56], and particularly their antioxidant components such as polyphenols [12, 57]. The particular finding regarding the effects of AA was that it reduced the basal MN-PCE, and that presented the strongest protection against genotoxic damage induced by V_2O_5.

Both antioxidants tended to reduce the basal early apoptotic cells, and this effect was significant for the AA group. However, the antioxidants increased late apoptotic cells significantly (Table 3), which could be related to the decrease of MN-PCE observed when antioxidants were administered alone as compared with the control group and its own time 0 of evaluation (when no treatments had yet been administered). Apoptosis is a normal and essential aspect of organ development and remodeling that is initiated at birth and continues throughout life [58]. Thus, apoptosis may play an essential role as a protective mechanism against genotoxic agents by removing genetically damaged cells.

Although numerous reports are available in the literature on the cytotoxic and anticarcinogenic effects of antioxidants in different tumor model systems, the molecular mechanisms underlying the anticarcinogenic potential of antioxidants are not completely understood. Specific forms of vitamin E display apoptotic activity against a wide range of cancer cell types while having little or no effect on normal cell function or viability [59]. Similarly, Naidu [19] demonstrated that ascorbyl stearate inhibited cell proliferation by interfering with the cell cycle, reversing the phenotype and inducing apoptosis in human brain tumor glioblastoma (T98G) cells. Therefore, it has been postulated that the mechanism to explain the chemopreventive potential of antioxidants is their chemical ability to target specific cellular signaling pathways that regulate cellular proliferation and apoptosis [60]. This is consistent with our results, in which the administration of AA or α-TOH alone elevated the frequencies of apoptotic cells significantly, and their administration prior to treatment of V_2O_5 increased apoptosis even further (Figure 4 and Table 3). The main increases were observed in the late apoptotic cells. The interactions between antioxidants and V_2O_5 suggest that their influence is neither additive nor antagonistic (Figure 4 and Table 3). In other studies it was observed that the apoptosis-inducing activity of antioxidants might be synergistically enhanced by a combined treatment with chemopreventive [61] or genotoxic agents [62]. The enhanced induction of apoptosis following a combined treatment suggests that this process may contribute to the elimination of the cells with V_2O_5-induced DNA damage (MN-PCE).

Some compounds, including vanadium(V) oxide, have emerged as therapeutic drugs for cancer, since intracellular cascade mechanisms may be involved in causing apoptotic cell death. Low levels of ROS can induce activation of transcription factors, promoting mRNA formation and encoding proteins known to be regulated by vanadium; however, high levels of ROS are cytotoxic to the cells and trigger apoptotic mechanisms. It has therefore been proposed that vanadium compounds be used against malignancies, since their cytotoxic effects against cancer cell lines by generating ROS and Reactive Nitrogen Species have already been shown [63, 64]. The ability to overcome the adverse effects of vanadium compounds during therapeutic action is thus a crucial issue for its future use in medicine [64]. In addition, the low costs of vanadium-based drugs make the use of vanadium compounds very promising. Our findings strongly suggest that both AA and α-TOH can be used effectively in therapy either alone (antioxidants) or in combination with other agents like V_2O_5 to reduce its genotoxicity. Additional studies are required to determine the specific intracellular sites of action that these antioxidants target in order to fully understand the specific mechanisms of action mediating their antigenotoxic and apoptotic effects, as well as to further clarify their potential value as chemotherapeutic agents in the prevention and treatment of diseases related with genotoxic damage, including some cancers.

Abbreviations

α-TOH:	Alpha-tocopherol
AO:	Acridine orange
AA:	Ascorbic acid
EB:	Ethidium bromide
FDA:	Food and Drug Administration
ip:	Intraperitoneal
MN:	Micronucleus
MN-PCE:	Micronucleated polychromatic erythrocytes
NCE:	Normochromatic erythrocytes
NIF:	Net induction frequency
$^{\bullet}$OH:	Hydroxyl radical
PCE:	Polychromatic erythrocytes
ROS:	Reactive oxygen species
V_2O_5:	Vanadium pentoxide.

Competing Interests

The authors of this paper declare that they have no direct financial relationship with the commercial identities mentioned in this paper that might lead to a conflict of interests.

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References

[1] A. M. Evangelou, "Vanadium in cancer treatment," *Critical Reviews in Oncology/Hematology*, vol. 42, no. 3, pp. 249–265, 2002.

[2] B. Mukherjee, B. Patra, S. Mahapatra, P. Banerjee, A. Tiwari, and M. Chatterjee, "Vanadium—an element of atypical biological significance," *Toxicology Letters*, vol. 150, no. 2, pp. 135–143, 2004.

[3] International Agency for Research on Cancer (IARC), *Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide and Vanadium Pentoxide*, vol. 86 of *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, International Agency for Research on Cancer (IARC), Lyon, France, 2006.

[4] American Conference of Governmental Industrial Hygienists (ACGIH), "Vanadium pentoxide: chemical substances 7th edition documentation, 9," in *Appendix B: Threshold Limit Values (TLVs®) and Biological Exposure Indices (BEIs®)*, 2012, http://www.nsc.org/facultyportal/Documents/fih-6e-appendix-b.pdf.

[5] T. B. Starr, J. A. MacGregor, K. D. Ehman, and A. I. Nikiforov, "Vanadium pentoxide: use of relevant historical control data shows no evidence for a carcinogenic response in F344/N rats," *Regulatory Toxicology and Pharmacology*, vol. 64, no. 1, pp. 155–160, 2012.

[6] F. L. Assem and L. S. Levy, "A review of current toxicological concerns on vanadium pentoxide and other vanadium compounds: gaps in knowledge and directions for future research," *Journal of Toxicology and Environmental Health, Part B: Critical Reviews*, vol. 12, no. 4, pp. 289–306, 2009.

[7] F. L. Assem and A. Oskarsson, "Vanadium," in *Handbook on the Toxicology of Metals*, pp. 1347–1367, 4th edition, 2015.

[8] L. A. Soriano-Agueda, C. Ortega-Moo, J. Garza, J. A. Guevara-García, and R. Vargas, "Formation of reactive oxygen species by vanadium complexes," *Computational and Theoretical Chemistry*, vol. 1077, pp. 99–105, 2016.

[9] J. B. Mailhes, C. Hilliard, J. W. Fuseler, and S. N. London, "Vanadate, an inhibitor of tyrosine phosphatases, induced premature anaphase in oocytes and aneuploidy and polyploidy in mouse bone marrow cells," *Mutation Research*, vol. 538, no. 1-2, pp. 101–107, 2003.

[10] X. Shi and N. S. Dalal, "Hydroxyl radical generation in the NADH/microsomal reduction of vanadate," *Free Radical Research*, vol. 17, no. 6, pp. 369–376, 1992.

[11] W. F. Costa and J. C. Nepomuceno, "Protective effects of a mixture of antioxidant vitamins and minerals on the genotoxicity of doxorubicin in somatic cells of *Drosophila melanogaster*," *Environmental and Molecular Mutagenesis*, vol. 47, no. 1, pp. 18–24, 2006.

[12] M. D. C. García-Rodríguez, M. M. Carvente-Juárez, and M. A. Altamirano-Lozano, "Antigenotoxic and apoptotic activity of green tea polyphenol extracts on hexavalent chromium-induced DNA damage in peripheral blood of CD-1 mice: analysis with differential acridine orange/ethidium bromide staining," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 486419, 9 pages, 2013.

[13] L. M. Antunes and C. S. Takahashi, "Effects of high doses of vitamins C and E against doxorubicin-induced chromosomal damage in Wistar rat bone marrow cells," *Mutation Research*, vol. 419, no. 1–3, pp. 137–143, 1998.

[14] Y. H. Siddique, T. Beg, and M. Afzal, "Antigenotoxic effects of ascorbic acid against megestrol acetate-induced genotoxicity in mice," *Human and Experimental Toxicology*, vol. 24, no. 3, pp. 121–127, 2005.

[15] M. Singh, P. Kaur, R. Sandhir, and R. Kiran, "Protective effects of vitamin E against atrazine-induced genotoxicity in rats," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 654, no. 2, pp. 145–149, 2008.

[16] E. Cameron and L. Pauling, *Cancer and Vitamin C*, W.W. Norton & Company, Inc, New York, NY, USA, 1979.

[17] G. Block, "Vitamin C and cancer prevention: the epidemiologic evidence," *The American Journal of Clinical Nutrition*, vol. 53, no. 1, pp. 270S–282S, 1991.

[18] B. Frei, "Reactive oxygen species and antioxidant vitamins: mechanisms of action," *The American Journal of Medicine*, vol. 97, no. 3, pp. 5S–13S, 1994.

[19] K. A. Naidu, "Vitamin C in human health and disease is still a mystery? An overview," *Nutrition Journal*, vol. 2, article 7, 2003.

[20] M. Sugiyama, K. Tsuzuki, K. Matsumoto, and R. Ogura, "Effect of vitamin E on cytotoxicity, DNA single strand breaks, chromosomal aberrations, and mutation in Chinese hamster V-79 cells exposed to ultraviolet-B light," *Photochemistry and Photobiology*, vol. 56, no. 1, pp. 31–34, 1992.

[21] J. Lunec, E. Halligan, N. Mistry, and K. Karakoula, "Effect of vitamin E on gene expression changes in diet-related carcinogenesis," *Annals of the New York Academy of Sciences*, vol. 1031, pp. 169–183, 2004.

[22] L. Packer, "Protective role of vitamin E in biological systems," *American Journal of Clinical Nutrition*, vol. 53, no. 4, pp. 1050S–1055S, 1991.

[23] H. J. Kayden and M. G. Traber, "Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans," *Journal of Lipid Research*, vol. 34, no. 3, pp. 343–358, 1993.

[24] A. Ogutcu, M. Uzunhisarcikli, S. Kalender, D. Durak, F. Bayrakdar, and Y. Kalender, "The effects of organophosphate insecticide diazinon on malondialdehyde levels and myocardial cells in rat heart tissue and protective role of vitamin E," *Pesticide Biochemistry and Physiology*, vol. 86, no. 2, pp. 93–98, 2006.

[25] S. L. Haber and R. W. Wissler, "Effect of vitamin E on carcinogenicity of methylcholanthrene.," *Experimental Biology and Medicine*, vol. 111, no. 3, pp. 774–775, 1962.

[26] M. García-Rodríguez, G. Serrano-Reyes, and M. Altamirano-Lozano, "Comparative study in vivo of the genotoxic damage induced by CrO_3 and the effects of the antioxidants: ascorbic acid, alfa-tocopherol and beta-carotene," *Free Radical Biology and Medicine*, vol. 53, no. 1, article S216, 2012.

[27] M. A. Altamirano-Lozano, A. R. Montaño-Rodríguez, G. P. García-Cárdenas, P. Peralta-García, and M. C. García-Rodríguez, "Estudio de las frecuencias de micronúcleos en sangre periférica de ratón CD-1 tratados con trióxido de cromo, sulfato de talio y pentóxido de vanadio in vivo," *Revista Internacional de Contaminación Ambiental*, vol. 29, no. 4, p. 154, 2013.

[28] J. A. Heddle, M. Hite, B. Kirkhart et al., "The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program," *Mutation Research*, vol. 123, no. 1, pp. 61–118, 1983.

[29] CSGMT (The Collaborative Study Group for the Micronucleus Test), "Protocol recommended by the CSGMT/JEMS.MMS for the short-term mouse peripheral blood micronucleus test. The Collaborative Study Group for the Micronucleus Test (CSGMT) (CSGMT/JEMS.MMS, The Mammalian Mutagenesis Study Group of the Environmental Mutagen Society of Japan)," *Mutagenesis*, vol. 10, no. 3, pp. 153–159, 1995.

[30] M. Hayashi, T. Morita, Y. Kodama, T. Sofuni, and M. Ishidate Jr., "The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides," *Mutation Research Letters*, vol. 245, no. 4, pp. 245–249, 1990.

[31] M. C. García-Rodríguez, V. López-Santiago, and M. Altamirano-Lozano, "Effect of chlorophyllin on chromium trioxide-induced micronuclei in polychromatic erythrocytes in mouse peripheral blood," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 496, no. 1-2, pp. 145–151, 2001.

[32] A. J. McGahon, S. J. Martin, R. P. Bissonnette et al., "The end of the (cell) line: methods for the study of apoptosis *in vitro*," in *Methods in Cell Biology*, vol. 46, chapter 9, pp. 153–185, 1995.

[33] NTP, "Toxicology and carcinogenesis studies of vanadium pentoxide," NTP TR 507, National Toxicology Program, Research Triangle Park, NC, USA, 2002.

[34] R. Ciranni, M. Antonetti, and L. Migliore, "Vanadium salts induce cytogenetic effects in in vivo treated mice," *Mutation Research*, vol. 343, no. 1, pp. 53–60, 1995.

[35] P. Leopardi, P. Villani, E. Cordelli, E. Siniscalchi, E. Veschetti, and R. Crebelli, "Assessment of the in vivo genotoxicity of vanadate: analysis of micronuclei and DNA damage induced in mice by oral exposure," *Toxicology Letters*, vol. 158, no. 1, pp. 39–49, 2005.

[36] M. Rojas-Lemus, M. Altamirano-Lozano, and T. I. Fortoul, "Sex differences in blood genotoxic and cytotoxic effects as a consequence of vanadium inhalation: micronucleus assay evaluation," *Journal of Applied Toxicology*, vol. 34, no. 3, pp. 258–264, 2014.

[37] M. C. García-Rodríguez, G. P. García-Cárdenas, A. R. Montaño-Rodríguez, and M. A. Altamirano-Lozano, "Cytotoxic and genotoxic effects of exposure to heavy metals (chromium [VI] and thallium [I]) of mice CD-1 strain: micronucleus, apoptosis and cell viability," *Acta Universitaria*, vol. 24, no. 2, pp. 91–96, 2014.

[38] EPA. Environmental Protection Agency, "Toxicological review of hexavalent chromium," in *Support of Summary Information on the Integrated Risk information System (IRIS)*, Office of Research and Development, Washington, DC, USA, 2010.

[39] D. J. Tweats, D. Blakey, R. H. Heflich et al., "Report of the IWGT working group on strategies and interpretation of regulatory in vivo tests. I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 627, no. 1, pp. 78–91, 2007.

[40] G. Krishna and M. Hayashi, "In vivo rodent micronucleus assay: protocol, conduct and data interpretation," *Mutation Research*, vol. 455, no. 1-2, pp. 155–166, 2000.

[41] G. R. Hogan, "Peripheral erythrocyte levels, hemolysis and three vanadium compounds," *Experientia*, vol. 46, no. 5, pp. 444–446, 1990.

[42] M. V. Aguirre, J. A. Juaristi, M. A. Alvarez, and N. C. Brandan, "Characteristics of in vivo murine erythropoietic response to sodium orthovanadate," *Chemico-Biological Interactions*, vol. 156, no. 1, pp. 55–68, 2005.

[43] N. H. Kleinsasser, P. Dirschedl, R. Staudenmaier, U. A. Harréus, and B. C. Wallner, "Genotoxic effects of vanadium pentoxide on human peripheral lymphocytes and mucosal cells of the upper aerodigestive tract," *International Journal of Environmental Health Research*, vol. 13, no. 4, pp. 373–379, 2003.

[44] F. C. R. Manning and S. R. Patierno, "Apoptosis: inhibitor or instigator of carcinogenesis?" *Cancer Investigation*, vol. 14, no. 5, pp. 455–465, 1996.

[45] D. Assimakopoulos, E. Kolettas, N. Zagorianakou, A. Evangelou, A. Skevas, and N. J. Agnantis, "Prognostic significance of p53 in the cancer of the larynx," *Anticancer Research*, vol. 20, no. 5, pp. 3555–3564, 2000.

[46] J. Whitwell, R. Smith, K. Jenner et al., "Relationships between p53 status, apoptosis and induction of micronuclei in different human and mouse cell lines in vitro: implications for improving existing assays," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 789-790, pp. 7–27, 2015.

[47] T. I. Fortoul, V. Rodriguez-Lara, A. González-Villalva et al., "Inhalation of vanadium pentoxide and its toxic effects in a mouse model," *Inorganica Chimica Acta*, vol. 420, pp. 8–15, 2014.

[48] C. Huang, Z. Zhang, M. Ding et al., "Vanadate induces p53 transactivation through hydrogen peroxide and causes apoptosis," *The Journal of Biological Chemistry*, vol. 275, no. 42, pp. 32516–32522, 2000.

[49] J. W. Crott and M. Fenech, "Effect of vitamin C supplementation on chromosome damage, apoptosis and necrosis *ex vivo*," *Carcinogenesis*, vol. 20, no. 6, pp. 1035–1041, 1999.

[50] A. C. Mamede, S. D. Tavares, A. M. Abrantes, J. Trindade, J. M. Maia, and M. F. Botelho, "The role of vitamins in cancer: a review," *Nutrition and Cancer*, vol. 63, no. 4, pp. 479–494, 2011.

[51] X. Shi, H. Jiang, Y. Mao, J. Ye, and U. Saffiotti, "Vanadium(IV)-mediated free radical generation and related 2′-deoxyguanosine hydroxylation and DNA damage," *Toxicology*, vol. 106, no. 1–3, pp. 27–38, 1996.

[52] M. C. Putchala, P. Ramani, H. J. Sherlin, P. Premkumar, and A. Natesan, "Ascorbic acid and its pro-oxidant activity as a therapy for tumours of oral cavity—a systematic review," *Archives of Oral Biology*, vol. 58, no. 6, pp. 563–574, 2013.

[53] D. C. Crans, B. Baruah, E. Gaidamauskas, B. G. Lemons, B. B. Lorenz, and M. D. Johnson, "Impairment of ascorbic acid's anti-oxidant properties in confined media: inter and intramolecular reactions with air and vanadate at acidic pH," *Journal of Inorganic Biochemistry*, vol. 102, no. 5-6, pp. 1334–1347, 2008.

[54] D. C. Horton, D. VanDerveer, J. Krzystek et al., "Spectroscopic characterization of *L*-ascorbic acid-induced reduction of vanadium(V) dipicolinates: formation of vanadium(III) and vanadium(IV) complexes from vanadium(V) dipicolinate derivatives," *Inorganica Chimica Acta*, vol. 420, pp. 112–119, 2014.

[55] M. C. García-Rodríguez, R. E. Vilches-Larrea, T. Nicolás-Mendez, and M. A. Altamirano-Lozano, "Green tea and its role on chemoprevention in vivo of genotoxic damage induced by carcinogenic metals (chromium [VI])," *Nutricion Hospitalaria*, vol. 27, no. 4, pp. 1204–1212, 2012.

[56] M. D. C. García-Rodríguez, R. A. Mateos-Nava, and M. Altamirano-Lozano, "*In vivo* effect of red wine undiluted, diluted (75%) and alcohol-free on the genotoxic damage induced by potential carcinogenic metals: chromium [VI]," *Nutrición Hospitalaria*, vol. 32, no. 4, pp. 1645–1652, 2015.

[57] M. D. C. García-Rodríguez, T. Nicolás-Méndez, A. R. Montaño-Rodríguez, and M. A. Altamirano-Lozano, "Antigenotoxic effects of (−)-epigallocatechin-3-gallate (EGCG), quercetin, and rutin on chromium trioxide-induced micronuclei in the polychromatic erythrocytes of mouse peripheral blood," *Journal of Toxicology and Environmental Health Part A*, vol. 77, no. 6, pp. 324–336, 2014.

[58] M. Raff, "Cell suicide for beginners," *Nature*, vol. 396, no. 6707, pp. 119–122, 1998.

[59] P. W. Sylvester, "Vitamin E and apoptosis," *Vitamins and Hormones*, vol. 76, pp. 329–356, 2007.

[60] J. F. Kerr, A. H. Wyllie, and A. R. Currie, "Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics," *British Journal of Cancer*, vol. 26, no. 4, pp. 239–257, 1972.

[61] Y. Gao, W. Li, L. Jia, B. Li, Y. C. Chen, and Y. Tu, "Enhancement of (−)-epigallocatechin-3-gallate and theaflavin-3-3′-digallate induced apoptosis by ascorbic acid in human lung adenocarcinoma SPC-A-1 cells and esophageal carcinoma Eca-109 cells via MAPK pathways," *Biochemical and Biophysical Research Communications*, vol. 438, no. 2, pp. 370–374, 2013.

[62] M. C. García-Rodríguez, A. R. Montaño-Rodríguez, and M. A. Altamirano-Lozano, "Modulation of hexavalent chromium-induced genotoxic damage in peripheral blood of mice by epigallocatechin-3-gallate (EGCG) and its relationship to the apoptotic activity," *Journal of Toxicology and Environmental Health, Part A: Current Issues*, vol. 79, no. 1, pp. 28–38, 2016.

[63] E. Kioseoglou, S. Petanidis, C. Gabriel, and A. Salifoglou, "The chemistry and biology of vanadium compounds in cancer therapeutics," *Coordination Chemistry Reviews*, vol. 301-302, pp. 87–105, 2015.

[64] J. C. Pessoa, S. Etcheverry, and D. Gambino, "Vanadium compounds in medicine," *Coordination Chemistry Reviews*, vol. 301-302, pp. 24–48, 2015.

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Review Article

Role of Vanadium in Cellular and Molecular Immunology: Association with Immune-Related Inflammation and Pharmacotoxicology Mechanisms

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Over the last decade, a diverse spectrum of vanadium compounds has arisen as anti-inflammatory therapeutic metallodrugs targeting various diseases. Recent studies have demonstrated that select well-defined vanadium species are involved in many immune-driven molecular mechanisms that regulate and influence immune responses. In addition, advances in cell immunotherapy have relied on the use of metallodrugs to create a "safe," highly regulated, environment for optimal control of immune response. Emerging findings include optimal regulation of B/T cell signaling and expression of immune suppressive or anti-inflammatory cytokines, critical for immune cell effector functions. Furthermore, in-depth perusals have explored NF-κB and Toll-like receptor signaling mechanisms in order to enhance adaptive immune responses and promote recruitment or conversion of inflammatory cells to immunodeficient tissues. Consequently, well-defined vanadium metallodrugs, poised to access and resensitize the immune microenvironment, interact with various biomolecular targets, such as B cells, T cells, interleukin markers, and transcription factors, thereby influencing and affecting immune signaling. A synthetically formulated and structure-based (bio)chemical reactivity account of vanadoforms emerges as a plausible strategy for designing drugs characterized by selectivity and specificity, with respect to the cellular molecular targets intimately linked to immune responses, thereby giving rise to a challenging field linked to the development of immune system vanadodrugs.

1. Introduction

In the past decades, several metallodrugs have been developed to target human pathophysiologies, with platinum, copper, vanadium, gold, ruthenium, and yttrium, among select metal ions, serving as the basis of such pharmaceuticals [1, 2]. Representative examples of therapeutic metallodrugs include Y-90 (Zevalin) used in the treatment of non-Hodgkin's lymphoma, sodium aurothiomalate (Myochrysine, Myocrisin, and Tauredon) or aurothioglucose (Aureotan, Solganal, Solganol, and Auromyose) used in rheumatoid arthritis patients, and bismuth subsalicylate (Pepto-Bismol), a widely used drug for the treatment of gastrointestinal disorders [1]. Beyond those, the successful platinum-based metallodrugs (cisplatin, carboplatin, and oxaliplatin) as antitumor agents were burdened by undesirable toxic side effects and appearance of chemoresistance. Both of them emerged as dire problems forcing the development of alternative metallodrugs with

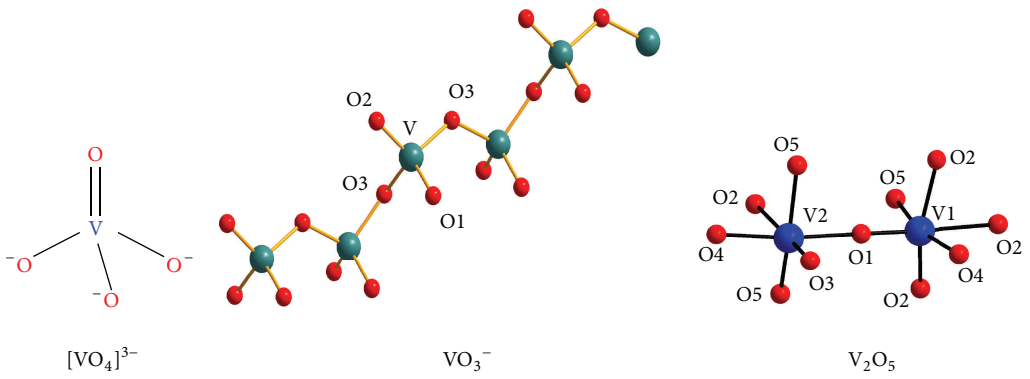
distinct modes of action and fewer or no side effects [3]. Vanadium is a known metal of high physiological, environmental, and industrial importance. It is an early first-row transition metal (Group 5 with $Z = 23$), with an electronic configuration of $[Ar]3d^3 4s^2$, having two natural isotopes, ^{51}V and ^{50}V. Its presence in biological systems in the marine and terrestrial environment has been well-established over the years [4]. It is encountered, among others, in vanadium-dependent haloperoxidases and alternative nitrogenases [5]. Moreover, various vanadium species have been found to exhibit significant effects as external cofactors, inhibiting the function of a wide range of enzymes (glyceraldehyde-3-phosphate dehydrogenase, lipoprotein lipase, tyrosine phosphorylase, glucose-6-phosphate dehydrogenase, glycogen synthase, adenylate cyclase, and cytochrome oxidase) and stimulating the function of others (Na^+-K^+-ATPase, H^+/K^+-ATPase, myosin ATPase, dynein, adenylate kinase, phosphofructokinase, and choline esterase) [6, 7]. From the biological point of view, the oxidation states V(IV) and V(V) appear to be of strong interest, with cationic and anionic complexes thereof forming in the physiological pH range (pH 2–8). *In vivo*, a key redox interplay emerges between the physiologically relevant V(V) and V(IV) oxidation states, with medium equilibria defining their distribution intra- and extracellularly. V(III), on the other hand, is present in ascidians and fan worms, but it is not present in higher organisms [8, 9]. Its emergence in biological media under reduced conditions, however, leaves a lot to be scrutinized with respect to potential roles in bioprocesses [10, 11] currently elusive or unknown. Nevertheless, the majority of mammalian tissues contain approximately 20 nM vanadium. Consequently, involvement of a biogenic metal ion, such as vanadium, in immune-regulating mechanisms, including immune suppression and inflammation downregulation, formulates a well-defined platform for research into future effective and efficient immunotherapy [12, 13]. In this respect, the herein elaborated account presents new facets of the merit that vanadium holds as a metallodrug in immunotherapy, based on currently held views and knowledge emerging from ongoing research in the fields of (bio)chemical and medical interest (Figure 1). The various forms of vanadium thus far employed in immune-related pathologies (a) necessitate an orderly account of its (bio)chemical activity at the cellular and molecular level, (b) signify a structure-based elaboration of its involvement in immune system interactions and responses, and (c) point out significant factors entering future design of new vanadodrugs capable of atoxically, selectively, and specifically targeting cellular molecular loci, intimately influencing immunophysiology and contributing to immunopharmaceuticals in a host of relevant diseases (Figure 2).

2. The Role of Vanadium in B Cell Signaling

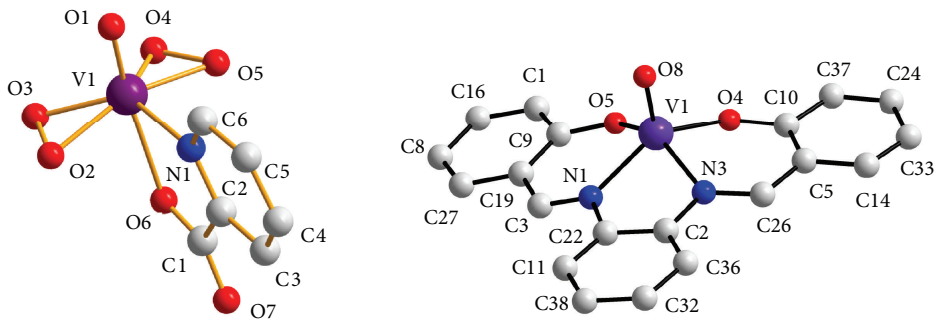
A number of recent studies have noted the role of vanadium in B cell signaling. This association triggers activation of multiple signaling cascades involving kinases, GTPases, and transcription factors [14]. This, in turn, results in changes in cell metabolism, gene expression, and cytoskeletal organization that regulate cellular mechanisms such as survival, tolerance, apoptosis, proliferation, and differentiation into antibody-producing cells or memory B cells. In this regard, a recent study has shown that vanadium treatment significantly proliferated splenocytes and expansion of B cells accounted for increased immune response and high number of splenocytes [15]. Vanadium treatment showed potency in amplifying the production of IFN-γ and total IgG in irradiated splenocytes, which correlated with the expansion of B cells. In agreement with previous reports, the number of $CD3^+$, $CD4^+$, and $CD8^+$ cells of splenocytes was not affected. The number of $CD11b^+$ and Gr-1^+ cells in splenocytes also showed no difference upon vanadium treatment. However, the $CD45R/B220^+$ B cell population expanded to significant levels in irradiated mice treated with sodium metavanadate ($NaVO_3$) (Figure 1) (Table 1). Consistent with the results from irradiated mice, 0.245 μM $NaVO_3$ treatment markedly enlarged the population of $CD45R/B220^+$ B cells of both non-irradiated and irradiated splenocytes and enhanced activation of immune B cell signaling. The effect of sodium orthovanadate (Na_3VO_4) (Figure 1) (Table 1) on the enhancement of DNA synthesis by T and B cell mitogenic agents was also studied using murine thymocytes and splenocytes [16]. Addition of orthovanadate to thymocyte cultures inhibited the mitogenic response in a concentration-dependent fashion. On the other hand, DNA synthesis, induced in thymocytes by pokeweed lectin and periodate treatment, was essentially uninhibited at the lower vanadate concentrations that were markedly effective for concanavalin A-induced synthesis. In addition, no significant inhibition of mitogenesis of splenic B cells in response to lipopolysaccharide and dextran was detectable at lower orthovanadate concentrations. In the absence of added mitogens, orthovanadate was found to be mitogenic for a subpopulation of thymus cells but not for splenocytes or T cell-enriched splenocyte populations. Evidently, the results suggest that (a) vanadate affects mitogenic responses in lymphocytes and (b) the interaction of vanadate with T and B cells is distinctly different, thus modulating B cell immune response. Histological studies indicating the presence of morphologically normal B cells in islets from vanadium-treated diabetic animals suggest, however, that vanadium treatment might not only mimic the effects of insulin, but also, at least partially, prevent and/or treat B cell lesions [17]. Induced vanadyl sulphate accumulation in bone, kidney, and liver prevented some alterations classically associated with diabetes, without causing further notable changes in various blood parameters or the histology of various tissues. In summary, the findings indicate that vanadium could be useful as a potential immunostimulating agent.

3. Regulation of T Cell Signaling

Vanadium-induced immune activation also involves T lymphocytes that (a) play a central role in cell-mediated immunity and (b) are characterized by the presence of a T cell receptor (TCR) on their cell surface [18]. T cells are essential for human immunity and almost every aspect of the adaptive immune response is controlled by them. Vanadium can influence T cell signaling by changing the number of mature



$[VO_4]^{3-}$ VO_3^- V_2O_5



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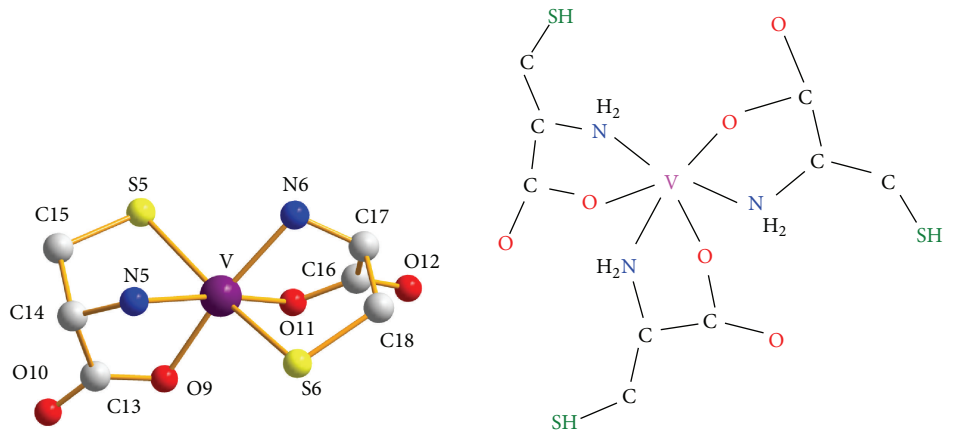

CV(III)-I CV(III)-II

Two forms of Ti-Al-V alloy implant surfaces

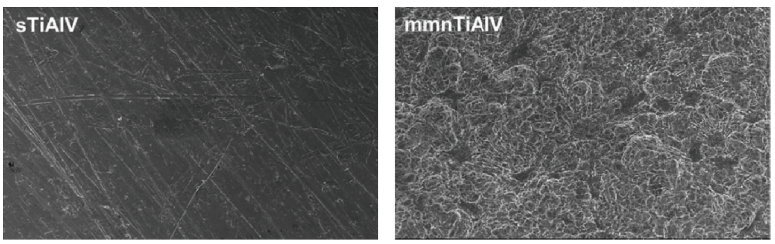

Figure 1: Vanadium forms exhibiting immunogenic activity.

Table 1: Vanadium oxidation state (V(V, IV, III)) and form-specific effects in immune system processes ([A] *in vitro*, [B] *in vivo*).

Vanadium species, compound	Immune system process, pathology, disease	Vanadium effect	Vanadium dose
Sodium metavanadate (V(V))	Immune system activation against γ-irradiation in mice	(1) Amplification of production of IFN-c and total IgG in irradiated splenocytes (2) Expansion of B cells accounting for increased number of splenocytes	0–3.99 μM[15,B]
	NF-κB/JNK signaling	Activation of both NF-κB and c-Jun N-terminal kinase (JNK)	0–80 μM[30,A]
Ammonium metavanadate (V(V))	T cell signaling	(1) Concentration-dependent inhibition-proliferation of splenic T cells	5–60 ppm[19,B]
		(2) Immune system function of local intestinal mucosa in broilers could be affected	5–60 mg/kg[20,B]
	Cellular immune function	Reduction of percentage of peripheral blood T-cell subsets and proliferation function and serum interleukin-2 content	5–60 ppm[26,B]
Vanadium pentoxide (V(V)) Ammonium metavanadate (V(V))	T lymphocyte activation Immunocompetence	(1) Inhibition of secretion of proinflammatory cytokines (IL-1, TNF-α, etc.) (2) Effect of production-release of major immunoregulatory cytokines and disruption of cell-mediated immunity	1 fM–100 μM[27,A]
Vanadium pentoxide (V(V))	Autoimmunity	(1) Thymic dysfunction (2) T cell negative selection in mice	0.02 M[21,B]
	Impairment of function of immunoregulatory NK cells	IL-2-mediated dysregulation of signaling pathways in NK cells	25–400 μM[25,A]
Sodium orthovanadate (V(V))	B cell signaling	Enhancement of DNA synthesis by T and B cell mitogenic agents	0–1000 μM[16,B]
	T cell signaling	(1) Enhancement of inducible forms of CREB in both resting and antigen-stimulated T cells (2) Enhancement of AP-1 activity in primary T lymphocytes	10–100 μM[22,B]
"Pervanadate" (V(V)-peroxido species)	NF-κB signaling	NF-κB activation through tyrosine phosphorylation	50–250 μM[31,A]
Bis(peroxido)vanadium species (Bpv) (V(V))	NF-κB signaling	(1) NF-κB activation	10 μM[29,A]
		(2) Neuroprotection	0.2 mg/kg/day[41,B]
Vanadyl sulphate (V(IV))	B cell morphology	(1) B cell morphology maintenance (2) Prevention and/or treatment of B cell lesions induced by streptozotocin treatment	0.25, 0.50, 0.75, 1.00 mg/mL[17,B]
Vanadium(IV) oxido complex N,N′-Bis(salicylidene)-O-phenylenediamine vanadium(IV) oxide (V(IV))	NF-κB signaling	Modulation of both ERK and NF-κB pathways	7–25 μM[28,A]
Vanadium(III)-(L-cysteine) (V(III))	Antioxidant defense, inflammation	(1) Prevention of cisplatin generation of ROS (2) Restoration of renal antioxidant enzymes (3) Chemoprotectant in cisplatin therapy	1–10 μM[40,A] 1 mg/kg[40,B]
Ti-Al-V alloy surfaces	TLR signaling	(1) Reduction of TLR4 mRNA (2) Proinflammatory interleukins, cell death, and apoptosis	N/A[35,A]

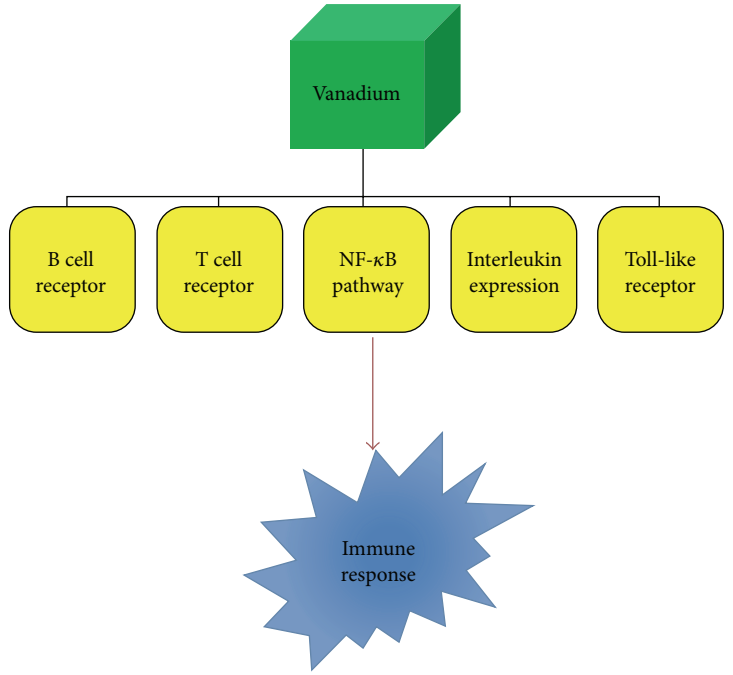

Figure 2: Vanadium influences several immune-related pathways, thereby sculpturing immune response.

T cells migrating from the thymus to the spleen. Because of that, secretion of IL-2 and IL-6 is affected (Figure 3). Furthermore, ammonium metavanadate (NH_4VO_3) (Table 1) inhibits the proliferation activity of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ splenic T cells and depresses their activity in broilers [19]. According to this study, vanadium can affect the percentage of splenic T cell subsets, the proliferation of splenic T cells, and serum IL-2 and IL-6 content. Vanadium in excess of 30 ppm reduces T cell population, serum IL-2 and IL-6 content, and proliferation of splenic T cells, which means that cellular immune function is finally impaired in broilers. Contrary to that, vanadium concentration < 30 ppm increases the T cell population and serum IL-2 and IL-6 contents, thereby improving cellular immune function. It is speculated that vanadium influences T cell subsets by modulating the thymic selection function, as there are lesions observed in the thymus where T cells are activated and differentiated [20]. Findings reveal that vanadium can affect expression of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells in both ileac lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes (IELs), implying that the immune function of local intestinal mucosa in broilers could be affected by vanadium treatment. In addition, vanadium can also modify immune CD11c and MHC-II expression in thymic dendritic cells by decreasing the presence of CD11c surface marker on mouse thymic dendritic cells as a result of vanadium pentoxide (V_2O_5) exposure. It is surmised that this decrease might induce dysfunction, including possible negative selection of T cells, which could increase the presence of autoreactive clones in the exposed host [21]. On an equal footing, vanadium has been reported to alter $CD4^+$ T helper (T_h) cell expression, serving as an important initiator and regulator of cellular and humoral immune responses against infectious microorganisms and other antigens. Sodium orthovanadate exposure (a) enhanced inducible forms of CREB (cAMP response element-binding protein) in both resting and antigen-stimulated T cells, followed by activation of the p50/p65 heterodimeric form of NF-κB, and (b) inhibited activation of NFAT (nuclear factor of activated T cells) and affected levels of its constitutive DNA-binding activity in resting lymph node T cells, whereas it enhanced AP-1 activity in transgenic mouse $CD4^+$ T lymphocytes [22].

4. Shaping Cytokine-Interleukin Response

Cytokine interleukins belong to a family of immunomodulatory proteins that elicit a wide variety of immune responses in various tissues and organs [23, 24]. Over the past years, vanadium has been shown to interact with several IL members. A notable example is IL-2, which plays a key role in regulating immune system tolerance and immunity, primarily via its direct effects on T cells. Using an (IL-2)-independent human NK-92MI cell line that is phenotypically considered an NK bright cell line, studies have shown that vanadium pentoxide (V_2O_5) (Figure 1) inhibited secretion of select proinflammatory cytokines and cell proliferation, induced apoptosis, and modified the IL-2 receptor signaling pathway [25] (Table 1). Vanadium also inhibited IL-10 and IFN-γ secretion, but mostly only after a 24 h exposure and primarily at higher doses tested. In a similar manner, it was found that dietary vanadium in excess of 30 mg/kg (a) reduces the population and proliferation of T cells and interleukin-2 (IL-2) content in the spleen and serum and (b) causes lesions in the spleen and bursa of Fabricius in broilers [26]. Likewise,

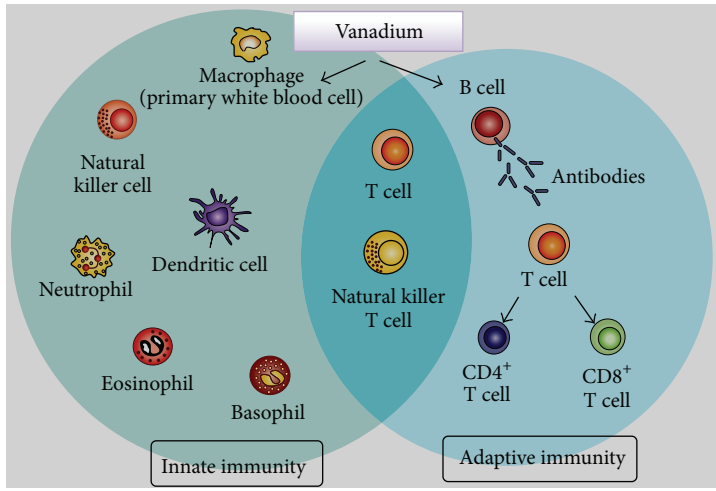

Figure 3: Key role(s) of vanadium in promoting innate and adaptive immunity.

vanadium was shown to downregulate specific interleukin expression, mainly IL-6, IL-10, TNF-α, and IFN-γ, in the cecal tonsil. IL-6, as a proinflammatory cytokine, acts as a mediator of fever, acute phase response, and is responsible for stimulating acute phase protein synthesis as well as the production of neutrophils in the bone marrow. It supports B cell growth of and is antagonistic to regulatory T cells. On the other hand, IL-10 is a cytokine with numerous, pleiotropic, effects responsible for immunoregulation and inflammation. It reduces Th1 cytokine expression, MHC class II antigens, and macrophage-induced costimulatory molecules by blocking NF-κB activity [23, 24]. It also promotes B cell survival, proliferation, and antibody production. Ammonium metavanadate and vanadium pentoxide (Figure 1) were shown to affect production-release of similar immunoregulatory cytokines and disrupt cell-mediated immunity. Specifically, release of the (IL-1)/(TNF-α)-regulating prostanoid PGE_2 was significantly increased at the highest vanadate concentration, although LPS-stimulated PGE_2 production was unaffected. These results indicate that, *in vitro*, pentavalent vanadium (V(V)) can interfere with immunoregulatory mediators critical for maintaining host immunocompetence [27].

5. Targeting the NF-κB Signaling Pathway

In recent years, several studies have demonstrated that NF-κB might be a very important target for vanadium with regard to the influence of cell signaling mechanisms and gene expression. Vanadium has the ability to interact with several transcription factors and influence the activity of the cell cycle, oncogenes, or tumor suppressor genes. V(IV) complex species (Figure 1) seem to promote differentiation and mineralization of the mesenchymal stem cells via activation of the NF-κB/ERK signaling pathway and subsequent enhancement of the NF-κB mediated action. Moreover, it has been demonstrated that ERK is implicated in the rise of the transcriptional activity of NF-κB. Thus, it is possible that V(IV) modulates both ERK and NF-κB pathways, and each pathway would act in concert to stimulate osteoblasts [28]. Likewise, bis(peroxido)vanadium species (Bpv) (Figure 1) (Table 1), a phosphotyrosine phosphatase inhibitor, induces myogenic cells to acquire a gene expression profile and differentiation potential consistent with the phenotype of circulating precursors, while maintaining their myogenic potential. These effects are mediated by NF-κB activation through the Tyr42-IκB-alpha phosphorylation, as shown by the expression of the dominant negative mutant form of the p50 NF-κB subunit [29]. Moreover, treatment of macrophages with sodium metavanadate results in the activation of both NF-κB and c-Jun N-terminal kinase (JNK) [30]. The activity of IκB kinase-beta (IKKbeta) was significantly elevated concurrently with the increased degradation of IκB-α and enhanced NF-κB activity in cells exposed to metavanadate. Thus, both IKK and SAPK/ERK kinase 1 (SEK1), an intermediate kinase within the MEKK1 to c-Jun N-terminal kinase (JNK) cascade, are involved in vanadate-induced NF-κB activation. Finally, "pervanadate" (V(V)-peroxido) was also shown to activate the DNA-binding activity of NF-κB, through (a) tyrosine phosphorylation and (b) expression of the T cell tyrosine kinase $p56^{lck}$, but not degradation of IκB-α [31] (Table 1). Evidently, suitably configured vanadium species of both oxidation states (V(IV) and V(V)) are in a position to support distinct influence patterns of reactivity in key NF-κB signaling pathways.

6. Subverting Toll-Like Receptor Signaling

Toll-like receptors (TLRs) constitute a distinct type of pattern recognition receptors (PRR) playing a crucial role in innate immune response [32]. Triggering TLRs to generate an immune response is therefore a primary goal in immunotherapy. To this end, certain metallodrugs are able to elicit an immune response in various immune cell types via Toll-like receptors (TLRs) and, correspondingly, their receptor agonists [33, 34]. Recently, texture-specific vanadium-containing

alloy materials (mmnTi-Al-V), reflecting implant materials, were shown to diminish TLR expression, exhibiting an 8-fold reduction in mRNAs for Toll-like receptor-4. Treated cells had reduced levels of proinflammatory interleukins and higher mRNAs for factors strongly associated with cell apoptosis [35] (Figure 1) (Table 1). Under normal conditions, TLR ligation and dimerization activate signaling cascades and subsequent production of proinflammatory cytokines, interferons, ROS, and proteases. Signaling involves recruitment of adaptor proteins MyD88, MAL, TRIF, or TRAM. The MyD88-dependent pathway is required for all TLRs except for TLR3, and MyD88 signaling involves a serine kinase (IL-1R)-associated kinase (IRAK), TNFR-associated factor 6 (TRAF6), and (TGF-β)-activated kinase 1 (TAK-1) sequence followed by activation of nuclear factor NF-κB and activator protein 1 (AP-1) transcription factors via the IKK and MAPK pathways, respectively [36]. TLR-targeting therapies, employing metallodrugs currently under development and clinical trials, and better understanding of the mechanisms of TLR-targeting therapies are thus expected to allow more specific treatments to be developed, thereby improving treatment options for immunoinflammatory disorders.

7. Role in Inflammation-Related Immunopathology

Activation of the inflammatory cascade involves immune cell mediators, transcription factors, and chemokines [37]. Inflammation is characterized by upregulation in the systemic concentrations of inflammation-related cytokines such as IL-6, IL-8, IL-18, TNF-α, and C-reactive protein (CRP) [38, 39]. Accumulating evidence reveals that vanadium can downregulate inflammatory reactions both *in vitro* and *in vivo*. To this end, recent findings have shown that vanadium administration reduced serum creatinine and blood urea nitrogen levels, suggesting amelioration of renal dysfunction [40]. Moreover, vanadium(III)-(L-cysteine) (VC-III) (Figure 1) (Table 1) treatment significantly prevented CDDP (*cis*-diamminedichloroplatinum(II))-induced generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and onset of lipid peroxidation in kidney tissues of experimental mice. In addition, vanadium also substantially restored CDDP-induced depleted activities of the renal antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and glutathione (reduced) levels. Histopathological analysis also confirmed reduced expression of proinflammatory mediators such as NF-κB, COX-2, and IL-6. VC-III administration also stimulated the Nrf2-mediated antioxidant defense system through promotion of downstream antioxidant enzymes, such as HO-1. Moreover, vanadium treatment significantly enhanced CDDP-mediated cytotoxicity in MCF-7 and NCI-H520 human cancer cell lines. Thus, VC-III can serve as a suitable chemoprotectant and increase the therapeutic window of CDDP in cancer patients. Furthermore, bis(peroxido)vanadium is able to prevent neuronic inflammation on cerebral ischemia. Data reveal that bis(peroxido)vanadium (Bpv), a specific inhibitor of PTEN's phosphatase activity, exhibits powerful neuroprotective properties [41]. Treatment with Bpv significantly increased IL-10 levels and decreased TNF-α concentration in the ischemic boundary zone of the cerebral cortex. Likewise, vanadium(III)-(L-cysteine) treatment significantly reduced PTEN mRNA and protein levels and increased PI3K, Akt, and p-GSK-3β protein expression in the ischemic boundary zone of the cerebral cortex. These results (a) demonstrate the neuroprotective effects of bis(peroxido)vanadium on cerebral ischemia and reperfusion injury of ischemic stroke rats and (b) show that vanadium is associated with reduction of inflammatory mediator production and upregulation of PTEN downstream proteins PI3K, Akt, and p-GSK-3β.

8. Pharmacotoxicology Mechanisms

Increasing evidence shows that complex vanadium species possess structural characteristics that justify their chemical reactivity at the biological level, thereby rendering them viable candidates for immune system disease metallodrugs [42, 43]. In order for vanadium compounds to be effective, atoxic well-defined forms of that metal ion encompassing selected physicochemical characteristics should be examined carefully in terms of their availability, selectivity, and specificity, followed by long-term epidemiological studies and controlled clinical trials. For such well-defined forms to emerge as immunomodulatory agents, key factors should be taken into consideration in the design and subsequent synthetic efforts. Such factors include (a) the nature of vanadium itself (inorganic forms at various oxidation states, metal-organic complex species, organometallic forms, etc.), (b) the nature of ligands-substrates bound to vanadium (e.g., peroxido, oxido, and nonperoxido organic chelators of variable O,N-containing tethers), (c) the oxidation state of vanadium (with V(IV) and V(V) representing the well-established physiological forms in human biological fluids, and V(III) awaiting further perusal), (d) the hydrophilicity-hydrophobicity of the ligands-substrates as well as the arising vanadium complex inorganic-organic species, thereby allowing access to specified molecular loci of action, and (e) the binary and ternary complex metal-organic nature of vanadium bestowing appropriate chemical reactivity where and when such is needed to counteract carcinogenic activity. The aforementioned collective properties formulate the chemical profile of vanadium that will configure its biological reactivity and consequently adhere to the selectivity and specificity needs of the immune system target site(s) of anti-inflammatory action. The need for such approaches to new atoxic vanadium compounds exemplifies the motivation for commensurate research efforts currently underway (Figure 4). In line with the emergence of select vanadium species, capable of delivering immunogenic activity, studies on the identification of immune system specific sites of interaction of vanadium with biomolecular targets in the cell should be conducted, shedding light onto the chemistry associated with the biological activity of vanadium in its various selected atoxic forms (Table 1). Current research data presented in this review highlight vanadium's synthetic and structural bioinorganic profile along with its biological activity attributes,

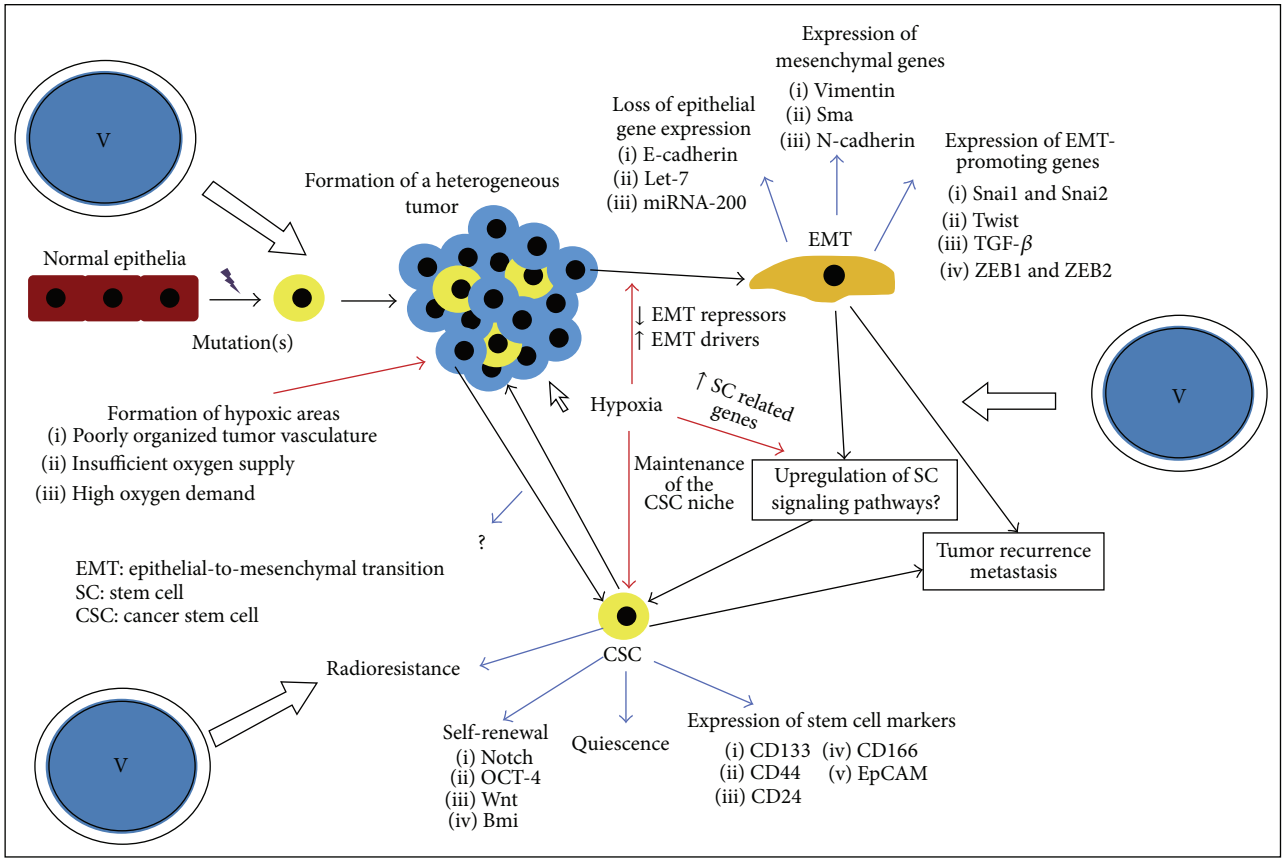

Figure 4: Current obstacles to overcome by specifically designed vanadium metallodrugs in cancer immunotherapeutics.

collectively formulating the significant potential of unique structure-based and immune process-specific vanadodrugs for the detection, prevention, and treatment of immune system aberrations.

9. Conclusions

Overall, specified vanadium complex species are involved in key mechanisms of immune regulation and can be used as promising metallodrugs toward future immunotherapy. Therefore, significant merit emerges toward further studies attempting to (a) design new vanadodrugs and (b) decipher the potential role that vanadium species have in interactions with immune system modulators as well as other transcription factors influencing immune signaling. Concurrently, vanadium regulation of B and T cell signaling emerges as a useful tool in probing modulatory mechanisms of inflammation suppression and their (in)direct implication in immunotherapeutic approaches. In addition, activation of certain interleukins, including IL-2, IL-4, IL-6, and IL-10 by vanadium denotes their specific contribution to immunometabolic processes, thereby warranting further perusal into the development of diagnostic and immunotherapeutic tools in immunopathological disorders. Numerous advances have contributed to the understanding of the cellular and molecular pathways involved in immune-related inflammation and stand as groundwork toward further investigations linking interleukin involvement to inflammation-driven immune response. Sculpting the immune response using metallodrugs may thus be a challenging goal toward future immunotherapies. The collective data mentioned in the current review reflect apt examples of vanadium-based approaches in cancer immunotherapy and related diseases. To this end, better understanding of the molecular signaling pathways used by vanadium interjection in immune surveillance, immune-driven inflammation, and immune cells stands as a well-defined platform for targeted research into future effective and efficient vanadium-based immunotherapy. Defined into such a well-formulated framework, vanadium-linked approaches in immunotherapy have merit, deserve due attention, and warrant further investigation.

Abbreviations

ATPase: Adenosine triphosphatase
GTPase: Guanosine triphosphatase
AP-1: Activator protein 1
LPLs: Lamina propria lymphocytes
IELs: Intraepithelial lymphocytes
IFN-γ: Interferon-gamma
TCR: T cell receptor

TNF-α:	Tumor necrosis factor alpha
MHC:	Major histocompatibility complex
PGE_2:	Prostaglandin E_2
ERK:	Extracellular signal-regulated kinases
IKKB:	Inhibitor of nuclear factor kappa-B kinase subunit beta
JNK:	c-Jun N-terminal kinase
MEKK1:	Mitogen-activated protein kinase kinase kinase 1
SAPK:	Stress-activated protein kinase
NF-κB:	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NFAT:	Nuclear factor of activated T cells
CDDP:	*cis*-Diamminedichloroplatinum(II)
MYD-88:	Myeloid differentiation primary response gene 88
COX-2:	Cyclooxygenase-2
Nrf-2:	Nuclear factor (erythroid-derived 2)-like 2
CRP:	C-reactive protein
Bpv:	Bis(peroxido)vanadium
PTEN:	Phosphatase and tensin homolog
PI3K:	Phosphoinositide 3-kinase
Akt:	Serine-threonine kinase-protein kinase B
p-GSK-3β:	Phosphorylated glycogen synthase kinase 3 beta
mmnTi-Al-V:	Macro-/micro-/nanotextured rough Ti-Al-V.

Competing Interests

The authors have no conflict of interests to declare.

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References

[1] K. D. Mjos and C. Orvig, "Metallodrugs in medicinal inorganic chemistry," *Chemical Reviews*, vol. 114, no. 8, pp. 4540–4563, 2014.

[2] J. J. Soldevila-Barreda and P. J. Sadler, "Approaches to the design of catalytic metallodrugs," *Current Opinion in Chemical Biology*, vol. 25, pp. 172–183, 2015.

[3] S. Spreckelmeyer, C. Orvig, and A. Casini, "Cellular transport mechanisms of cytotoxic metallodrugs: an overview beyond cisplatin," *Molecules*, vol. 19, no. 10, pp. 15584–15610, 2014.

[4] D. Rehder, "Vanadium. Its role for humans," *Metal Ions in Life Sciences*, vol. 13, pp. 139–169, 2013.

[5] E. V. Fedorova, A. V. Buryakina, N. M. Vorobieva, and N. I. Baranova, "The vanadium compounds: chemistry, synthesis, insulinomimetic properties," *Biomeditsinskaya Khimiya*, vol. 60, no. 4, pp. 416–429, 2014.

[6] J. Korbecki, I. Baranowska-Bosiacka, I. Gutowska, and D. Chlubek, "Biochemical and medical importance of vanadium compounds," *Acta Biochimica Polonica*, vol. 59, no. 2, pp. 195–200, 2012.

[7] E. Kioseoglou, S. Petanidis, C. Gabriel, and A. Salifoglou, "The chemistry and biology of vanadium compounds in cancer therapeutics," *Coordination Chemistry Reviews*, vol. 301-302, pp. 87–105, 2015.

[8] M. Imtiaz, M. S. Rizwan, S. Xiong et al., "Vanadium, recent advancements and research prospects: a review," *Environment International*, vol. 80, pp. 79–88, 2015.

[9] B. Mukherjee, B. Patra, S. Mahapatra, P. Banerjee, A. Tiwari, and M. Chatterjee, "Vanadium—an element of atypical biological significance," *Toxicology Letters*, vol. 150, no. 2, pp. 135–143, 2004.

[10] D. Rehder, J. Costa Pessoa, C. F. G. C. Geraldes et al., "In vitro study of the insulin-mimetic behaviour of vanadium(IV, V) coordination compounds," *Journal of Biological Inorganic Chemistry*, vol. 7, no. 4-5, pp. 384–396, 2002.

[11] C. Gabriel, J. Venetis, M. Kaliva et al., "Probing for missing links in the binary and ternary V(V)-citrate-(H_2O_2) systems: synthetic efforts and in vitro insulin mimetic activity studies," *Journal of Inorganic Biochemistry*, vol. 103, no. 4, pp. 503–516, 2009.

[12] D. C. Crans, J. J. Smee, E. Gaidamauskas, and L. Yang, "The chemistry and biochemistry of vanadium and the biological activities exerted by vanadium compounds," *Chemical Reviews*, vol. 104, no. 2, pp. 849–902, 2004.

[13] I. Zwolak, "Vanadium carcinogenic, immunotoxic and neurotoxic effects: a review of in vitro studies," *Toxicology Mechanisms and Methods*, vol. 24, no. 1, pp. 1–12, 2014.

[14] M. Valko, H. Morris, and M. T. D. Cronin, "Metals, toxicity and oxidative stress," *Current Medicinal Chemistry*, vol. 12, no. 10, pp. 1161–1208, 2005.

[15] D. Ha, H. Joo, G. Ahn et al., "Jeju ground water containing vanadium induced immune activation on splenocytes of low dose γ-rays-irradiated mice," *Food and Chemical Toxicology*, vol. 50, no. 6, pp. 2097–2105, 2012.

[16] M. Ramanadham and M. Kern, "Differential effect of vanadate on DNA synthesis induced by mitogens in T and B lymphocytes," *Molecular and Cellular Biochemistry*, vol. 51, no. 1, pp. 67–71, 1983.

[17] J. J. Mongold, G. H. Cros, L. Vian et al., "Toxicological aspects of vanadyl sulphate on diabetic rats: effects on vanadium levels and pancreatic B-cell morphology," *Pharmacology and Toxicology*, vol. 67, no. 3, pp. 192–198, 1990.

[18] B. Malissen, C. Grégoire, M. Malissen, and R. Roncagalli, "Integrative biology of T cell activation," *Nature Immunology*, vol. 15, no. 9, pp. 790–797, 2014.

[19] W. Cui, H. Cui, X. Peng et al., "Excess dietary vanadium induces the changes of subsets and proliferation of splenic T cells in broilers," *Biological Trace Element Research*, vol. 143, no. 2, pp. 932–938, 2011.

[20] K. Wang, H. Cui, Y. Deng et al., "Effect of dietary vanadium on the ileac T cells and contents of cytokines in broilers," *Biological Trace Element Research*, vol. 147, no. 1–3, pp. 113–119, 2012.

[21] M. Ustarroz-Cano, I. García-Peláez, G. Piñón-Zárate, M. Herrera-Enríquez, G. Soldevila, and T. I. Fortoul, "CD11c decrease in mouse thymic dendritic cells after vanadium inhalation," *Journal of Immunotoxicology*, vol. 9, no. 4, pp. 374–380, 2012.

[22] K. Lee, X. Shen, and R. König, "Effects of cadmium and vanadium ions on antigen-induced signaling in CD4+ T cells," *Toxicology*, vol. 169, no. 1, pp. 53–65, 2001.

[23] D. Anestakis, S. Petanidis, S. Kalyvas et al., "Mechanisms and applications of interleukins in cancer immunotherapy," *International Journal of Molecular Sciences*, vol. 16, no. 1, pp. 1691–1710, 2015.

[24] J. L. Stow and R. Z. Murray, "Intracellular trafficking and secretion of inflammatory cytokines," *Cytokine and Growth Factor Reviews*, vol. 24, no. 3, pp. 227–239, 2013.

[25] F. Gallardo-Vera, D. Diaz, M. Tapia-Rodriguez et al., "Vanadium pentoxide prevents NK-92MI cell proliferation and IFN-γ secretion through sustained JAK3 phosphorylation," *Journal of Immunotoxicology*, vol. 13, no. 1, pp. 27–37, 2016.

[26] W. Cui, H.-M. Cui, X. Peng, Z. Zuo, X. Liu, and B. Wu, "Effect of vanadium on the subset and proliferation of peripheral blood T cells, and serum interleukin-2 content in broilers," *Biological Trace Element Research*, vol. 141, no. 1–3, pp. 192–199, 2011.

[27] M. D. Cohen, E. Parsons, R. B. Schlesinger, and J. T. Zelikoff, "Immunotoxicity of in vitro vanadium exposures: effects on interleukin-1, tumor necrosis factor-α, and prostaglandin E2 production by WEHI-3 macrophages," *International Journal of Immunopharmacology*, vol. 15, no. 3, pp. 437–446, 1993.

[28] S. Srivastava, N. Kumar, and P. Roy, "Role of ERK/NF-κB in vanadium (IV) oxide mediated osteoblast differentiation in C3H10t1/2 cells," *Biochimie*, vol. 101, no. 1, pp. 132–144, 2014.

[29] L. Castaldi, C. Serra, F. Moretti et al., "Bisperoxovanadium, a phospho-tyrosine phosphatase inhibitor, reprograms myogenic cells to acquire a pluripotent, circulating phenotype," *The FASEB Journal*, vol. 21, no. 13, pp. 3573–3583, 2007.

[30] F. Chen, L. M. Demers, V. Vallyathan et al., "Vanadate induction of NF-κB involves IκB kinase β and SAPK/ERK kinase 1 in macrophages," *The Journal of Biological Chemistry*, vol. 274, no. 29, pp. 20307–20312, 1999.

[31] V. Imbert, R. A. Rupec, A. Livolsi et al., "Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α," *Cell*, vol. 86, no. 5, pp. 787–798, 1996.

[32] M. J. Jiménez-Dalmaroni, M. E. Gerswhin, and I. E. Adamopoulos, "The critical role of toll-like receptors—from microbial recognition to autoimmunity: a comprehensive review," *Autoimmunity Reviews*, vol. 15, no. 1, pp. 1–8, 2016.

[33] S. Deng, S. Zhu, Y. Qiao et al., "Recent advances in the role of toll-like receptors and TLR agonists in immunotherapy for human glioma," *Protein and Cell*, vol. 5, no. 12, pp. 899–911, 2014.

[34] S. H. Aalaei-Andabili, M. Fabbri, and N. Rezaei, "Reciprocal effects of Toll-like receptors and miRNAs on biological processes in human health and disease: a systematic review," *Immunotherapy*, vol. 5, no. 10, pp. 1127–1142, 2013.

[35] R. Olivares-Navarrete, S. L. Hyzy, P. J. Slosar, J. M. Schneider, Z. Schwartz, and B. D. Boyan, "Implant materials generate different peri-implant inflammatory factors: PEEK promotes fibrosis and Micro-textured titanium promotes osteogenic factors," *Spine*, vol. 40, no. 6, pp. 399–404, 2015.

[36] E. Kay, R. S. Scotland, and J. R. Whiteford, "Toll-like receptors: role in inflammation and therapeutic potential," *BioFactors*, vol. 40, no. 3, pp. 284–294, 2014.

[37] S. Muralidharan and P. Mandrekar, "Cellular stress response and innate immune signaling: integrating pathways in host defense and inflammation," *Journal of Leukocyte Biology*, vol. 94, no. 6, pp. 1167–1184, 2013.

[38] R. Divekar and H. Kita, "Recent advances in epithelium-derived cytokines (IL-33, IL-25, and thymic stromal lymphopoietin) and allergic inflammation," *Current Opinion in Allergy and Clinical Immunology*, vol. 15, no. 1, pp. 98–103, 2015.

[39] P. Fietta, E. Costa, and G. Delsante, "Interleukins (ILs), a fascinating family of cytokines. Part I: ILs from IL-1 to IL-19," *Theoretical biology forum*, vol. 107, no. 1-2, pp. 13–45, 2014.

[40] A. Basu, S. Singha Roy, A. Bhattacharjee et al., "Vanadium(III)-L-cysteine protects cisplatin-induced nephropathy through activation of Nrf2/HO-1 pathway," *Free Radical Research*, vol. 50, no. 1, pp. 39–55, 2015.

[41] L.-L. Mao, D.-L. Hao, X.-W. Mao et al., "Neuroprotective effects of bisperoxovanadium on cerebral ischemia by inflammation inhibition," *Neuroscience Letters*, vol. 602, pp. 120–125, 2015.

[42] J. C. Pessoa, S. Etcheverry, and D. Gambino, "Vanadium compounds in medicine," *Coordination Chemistry Reviews*, vol. 301-302, pp. 24–48, 2015.

[43] J. C. Pessoa, E. Garribba, M. F. A. Santos, and T. Santos-Silva, "Vanadium and proteins: uptake, transport, structure, activity and function," *Coordination Chemistry Reviews*, vol. 301-302, pp. 49–86, 2015.

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Research Article

Metforminium Decavanadate as a Potential Metallopharmaceutical Drug for the Treatment of Diabetes Mellitus

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New potential drugs based on vanadium are being developed as possible treatments for diabetes mellitus (DM) and its complications. In this regard, our working group developed metforminium decavanadate (MetfDeca), a compound with hypoglycemic and hypolipidemic properties. MetfDeca was evaluated in models of type 1 and type 2 diabetes mellitus, on male Wistar rats. Alloxan-induction was employed to produce DM1 model, while a hypercaloric-diet was employed to generate DM2 model. Two-month treatments with 3.7 μg (2.5 μM)/300 g/twice a week for DM2 and 7.18 μg (4.8 μM)/300 g/twice a week for DM1 of MetfDeca, respectively, were administered. The resulting pharmacological data showed nontoxicological effects on liver and kidney. At the same time, MetfDeca showed an improvement of carbohydrates and lipids in tissues and serum. MetfDeca treatment was better than the monotherapies with metformin for DM2 and insulin for DM1. Additionally, MetfDeca showed a protective effect on pancreatic beta cells of DM1 rats, suggesting a possible regeneration of these cells, since they recovered their insulin levels. Therefore, MetfDeca could be considered not only as an insulin-mimetic agent, but also as an insulin-enhancing agent. Efforts to elucidate the mechanism of action of this compound are now in progress.

1. Introduction

The use of metal-based drugs as therapeutic agents dates back to ancient cultures who lived in Mesopotamia, India, Egypt, and China, as early as 2500 B.C. [1]. Making a great leap in the history of metals in medicine up to Renaissance era, it is important to mention the work of Paracelsus and his concept of doses-response, coming from the introduction of mercury, arsenic, tin, lead, and antimony (among others) into the *Materia medica*: "All substances are poisons: there is none which is not a poison. The right dose differentiates a poison and a remedy" [2]. For this reason, the deep understanding about chemistry of metals within biological systems is very important.

Currently, the metal-based drugs are being used for the treatment of a variety of pathologies such as diabetes, cancer,

rheumatoid arthritis, and inflammatory and cardiovascular diseases [3–5]. The health status of a person and physiological disorders and diseases are usually related to the presence or absence of metal ions and/or their corresponding complexes formed with biomolecules in the body. Thus, the metals called trace elements are necessary for many biological processes. In this sense, vanadium is a biometal capable of improving the metabolism of lipids and carbohydrates, as it has been reported for some of their compounds.

Despite the fact that the physiological pathways are unclear, it is now known that vanadate has different pharmacokinetics compared to the decavanadate and therefore different pharmacological activities, although, in relation with the carbohydrate and lipid metabolism, these act very similar. Although the vanadium intracellular concentration (+5, vanadate) is very low for the decavanadate species to be formed, it has been described that V10 was formed in acidic compartments in *Saccharomyces cerevisiae* that were grown in media containing vanadate. Thus it has been proposed that once it is formed the rate of decavanadate decomposition is slow (half-life time of hours) enough to allow observing its effects not only in vitro, but also in vivo [6–10].

Under these considerations the metallopharmaceuticals based on vanadium with potential hypoglycemic features could be considered as an admissible alternative for the treatment of diseases involving glycemic control, both in patients being insulin-requiring and insulin-independent diabetes mellitus types 1 (DM1) and 2 (DM2), respectively, and in patients with other diseases that have in common carbohydrate and lipid disorders [11].

Actually, diabetes mellitus represents a global public health problem. It has been estimated that 347 millions of people have diabetes mellitus until 2014. This pathology is defined as a metabolic disorder characterized by chronic hyperglycemia with disturbances in the metabolism of carbohydrates, lipids, and proteins resulting from defects in insulin secretion, its action, or both. Additionally, it is worth mentioning that DM represents the end stage of a chronic and progressive heterogeneous syndrome characterized by a series of metabolic disorders (dysglycemia and dyslipidemia), promoted by environmental factors, genetic susceptibility, insulin resistance, and a decrease in hormone secretion by pancreatic β cells [12, 13]. This pathology is a major cause of morbidity and mortality around the world, including Mexico [14].

Most patients diagnosed with DM2 should begin their therapy with lifestyle changes (lifestyle counseling, weight-loss education, exercise, etc.). When these lifestyles are not enough to achieve or maintain the glycemic levels, metformin monotherapy should be added soon after diagnosis, unless there are contraindications or intolerance. Metformin possesses a long list of evidences detailing its efficacy and safety; moreover it is very inexpensive and may reduce risk of cardiovascular events [15]. It has also been reported that metformin consumption has some disadvantages, such as gastrointestinal side effects (diarrhea, abdominal cramping), risk of lactic acidosis (rare), vitamin B12 deficiency, acid reflux, chronic kidney disease, hypoxia, and dehydration [16–18]. Additionally, we have reported that chronic administration of metformin showed multiorgan complications in the development of steatosis. By contrast, metforminium decavanadate (MetfDeca) presented in Figure 1 showed an improvement in the intracellular biochemical behavior and also a recovering of the adequate levels of lipids and carbohydrates [11].

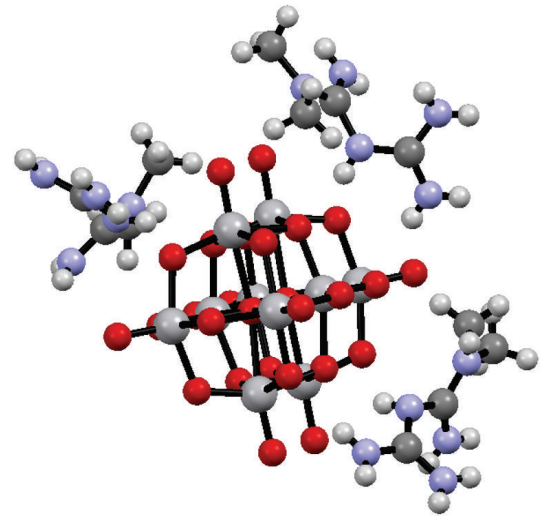

Figure 1: Ball and stick representation of metforminium decavanadate $(H_2Metf)_3[V_{10}O_{28}]\cdot 8H_2O$. Water molecules are omitted for clarity [11].

Therefore, in the research of the vanadium-based metallopharmaceuticals both their beneficial physiological function and their potential toxicity should be considered [10, 19]. For these reasons, in this paper we focused on the role of MetfDeca as a potential new drug for the treatment of DM (and related disorders) and its toxicity effects in rat models of hyperglycemia, both requesting insulin and insulin-independent, since they are altered in the metabolism of carbohydrates and lipids.

2. Material and Methods

Male Wistar rats (70–100 g) were provided by the Bioterium "Claude Bernard," of the Benemérita Universidad Autónoma de Puebla. The rats were housed in a climate-controlled and light-regulated facility with 12/12 h day/night cycles with free access to food and water "ad libitum." All procedures described in this study are in accordance with the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care NOM-062-ZOO-1999. Every effort was made to minimize the number of animals used and to ensure the minimal animal pain and/or discomfort. The animals were conditioned with normal calorie diet by 15 days. The diet used was 5001 of the LabDiet (Laboratory Rodent Diet); its composition can be consulted on the manufacturer's website. Upon reaching the ideal weight to each study, animals were randomly separated into different groups.

2.1. Dose-Response Curve and Toxicological Effects of MetfDeca. 60 male Wistar rats of 300 to 320 g in weight underwent an intraperitoneally application of alloxan (150 mg/kg)

and were monitored starting from the third day after administration of glucose and insulin (as indicated by the commercial kits). When hyperglycemia (HG) above 200 mg/dL was presented, the animals were appointed to the various working groups ($n = 10$) which were administered with doses of MetfDeca of 0.0, 0.7, 1.4, 2.8, 5, and 10 μM. The oral doses were administered twice a week for a month; the dilutions were realized with sterile water. The glucose was measured once a week to establish hypoglycemic action of MetfDeca.

Once they completed the treatment, animals were sacrificed and serum was collected in BD Vacutainer® Venous Blood Collection system, centrifuged to 2500 r.p.m. during 5 min; glucose quantification was used for determination of a dose-response curve. Meanwhile, insulin, triglycerides, cholesterol, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γGT), alkaline phosphatase (ALP), sodium, potassium, urea, and creatinine assays were done as control of hypoglycemic, hypolipemic, and toxicological effects. For the toxicological effects, 10 intact rats of the same weight and age were used as negative control group (intact).

2.2. Effect of MetfDeca on Insulin-Independent Model (DM2). 80 male Wistar rats between 100 and 120 g were randomly divided into two groups: normal-calorie (NC) $n = 20$ and hypercaloric (HC) $n = 60$. The NC group was fed a balanced diet (Rodent Lab diet 5001) and the HC group was fed a diet high in calories (Patent: MX/E/2013/047377), which was designed with 71.4% carbohydrates, demonstrated by bromatological analysis. After 3 months of feeding with high-calorie diet, metabolically altered animals were validated. The validation was realized by body weight, abdominal perimeter and length from tip of nose to base of the tail, BMI (body mass index), body fat percentage, and serum parameters. Body weight of NC rats was 300 g, meanwhile HC rats reached 350–400 g [11].

The metabolically deregulated animals with HC diet were subdivided in 3 subgroups of $n = 20$, when these presented a minimal value of fasting glucose of 150 mg/dL and/or above 200 mg/dL in oral glucose tolerance test (OGTT, 1.75 g of glucose anhydrous/kg) and dyslipidemia characterized by hypertriglyceridemia and decline of high density lipoprotein cholesterol (HDL-C). In rats fed with high-calorie diet for 3 months, an oral glucose tolerance test (OGTT) was carried out after 4–6 h of fasting; an anhydrous glucose load of 1.75 g/kg was orally administered. Then glycemia was measured at 0, 30, 60, and 90 min by cardiac puncture previous anesthesia with ketamine + xylazine. OGTT was realized for the control group (diet NC, without treatment), group I-I (diet HC, without treatment), group I-I + metformin (diet HC, daily oral metformin 200 mg/kg), and group I-I + MetfDeca (diet HC, two times a week of MetfDeca 3.7 μg/300 g (2.5 μM) orally). Two months later, treatments ended and serum analysis was performed for HbA1c, OGTT, triglycerides, free fatty acids, cholesterol, and its fractions HDL-C, LDL-C, and VLDL-C. For which, animals were anesthetized (at a dose of ketamine + xylazine 0.2 mL/100 g intraperitoneally). 700 μL of blood (approximately) was collected by intracardiac puncture with a BD Vacutainer Venous Blood Collection system and serum was separated for analysis. Immediately, rats were sacrificed and perfused with cold isotonic saline, and biopsies from tissues (liver, heart, kidney, and muscle) were taken and stored at −70°C for subsequent determination of glycogen and triglycerides according to established techniques [11].

2.3. Effect of MetfDeca on Insulin-Requiring Model (DM1). 60 male Wistar rats of 300 to 320 g in weight underwent a dose of 150 mg/kg of alloxan intraperitoneally. The model was validated when rats presented glucose levels above 300 mg/dL and hyperglycemia with hypoinsulinemia <5 μU/mL. The groups were conformed ($n = 20$) in the next order: control group (without hyperglycemia), group I-R (hyperglycemia without treatment), group I-R + insulin (daily subcutaneous insulin, 2 IU per 100 mg/dL glucose), and group I-R + MetfDeca (7.18 μg/300 g (4.8 μM) of oral MetfDeca twice a week). Two months later, treatments ended. The insulin administered was a Humalog® Mix 75/25 (75% insulin lispro protamine suspension and 25% insulin lispro injection (rDNA origin)) which is a mixture of insulin lispro solution, a rapid-acting blood glucose-lowering agent, and insulin lispro protamine suspension, an intermediate-acting blood glucose-lowering agent. Two months later, treatments ended and serum analysis was performed. Similarly, to insulin-independent model (DM2), the extraction of serum and tissues was carried out. The serum and tissues extraction were carried out as previously described.

2.4. Biochemical Assays. The concentrations in serum of glucose, triglycerides, cholesterol, Low Density Lipoprotein-Cholesterol (LDL-C), Very Low Density Lipoprotein-Cholesterol (VLDL-C), High Density Lipoprotein-Cholesterol (HDL-C), total bilirubin, aspartate transaminase (AST), alanine aminotransferase (ALT), Gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), urea, and creatinine in serum were determined with a semiautomatic analyzer BTS-350 (BioSystems). HbA1c analysis was performed using an I-Chroma Analyzer, which used immunofluorescence methodology. Electrolytes were measured in a Plus Lyte II analyzer of ion selective electrodes. Free fatty acid (FFA) concentration was determined according to the method described by Brunk and Swanson, 1981 [20]. Plasma insulin concentration was determined by an ELISA immunoassay (Diagnostica International Company), with the resulting antibody antigen complex assessed at 415 nm in a Stat fax 2600 plate reader (WinerLab Group). Insulin concentrations were obtained from a standard curve with a range of 0–200 μUI/mL. The samples of oral glucose tolerance test (OGTT) were collected at the times 0, 30, 60, and 90 min after glucose load and stored in Eppendorf tubes. Samples were centrifuged at 8000 rpm for 10 min, and the serum was separated and frozen at −70°C until analysis. At the time of analysis, sera were thawed at room temperature.

2.5. Tissue Determinations. The biopsies from tissues (liver, heart, kidney, and adipose) were homogenized 100 mg in 800 μL of isotonic saline solution (ISS) and proceeded to

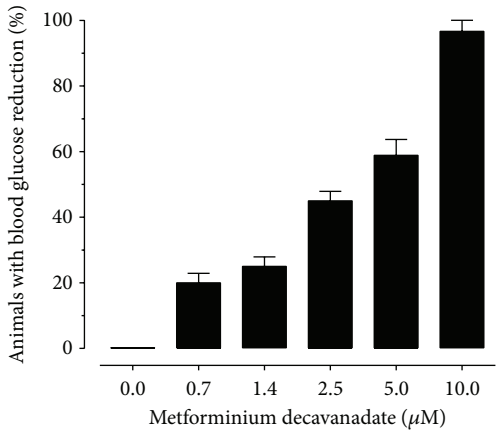

Figure 2: Administration of MetfDeca showing a lowering in blood glucose levels of hyperglycemic rats. The animals ($n = 10$/group) were administered with this compound at doses (0.7, 1.4, 2.5, 5, and 10 μM) for 4 weeks in rats with alloxan-induced hyperglycemia (150 mg/kg). The graph shows the percentage of animals with reduced levels of glucose.

perform the protocol for the determination of glycogen by the technique described by Bennett et al. in 2007 [21]. Additionally, a second dilution was made for triglycerides content, in which the homogenate was diluted 1 : 2 with ISS and the protocol described by the manufacturer for the triglyceride kit was followed.

2.6. Statistical Analysis. Results were expressed as mean ± standard error of the mean (SEM). The results of toxicological effect model were evaluated and interpreted by statistical Student's "t" distribution at a significance level of $p \leq 0.05$. Meanwhile, the biochemistry characterization and tissue measurements were evaluated by one-way ANOVA test considering $p \leq 0.05$ significant, with Bonferroni post hoc test.

3. Results

The administration of MetfDeca in hyperglycemic rats showed a decrease in blood glucose levels in dependence of the concentrations administered of compound. Quantitative analysis indicated that a concentration of 0.7 and 1.4 μM of MetfDeca caused a reduction of glucose levels in the 20 and 25% of the animals, respectively. Likewise, the concentrations of 2.5, 5, and 10 μM generated a reduction in serum glucose which corresponded to 45, 52, and 97%, respectively, as shown in Figure 2.

According to the results, the concentration of 5 μM reduces glucose levels in 50% of subjects administered with MetfDeca. To perform the mathematical model which assigned the exact concentration of the compound that corresponded to effective dose 50 (ED_{50}), we found that 4.8 μM or 7.18 μg/300 g is the adequate ED_{50}. In this sense, the dose of 5 μM for being the closest to ED_{50} was selected to assess the consequences in the metabolism of lipids and carbohydrates, as well as toxicological features.

Nonclinical studies for drug development according to European Medicine Agency include basic goals as identifying the pharmacological properties, in this particular case, glucose control and antilipemic features, and additionally understanding the toxicological profile as marked for the directive 2001/83/EC. Therefore, we carry out the basic toxicological profile of kidney and liver function, as well as triglycerides, cholesterol, and glucose-insulin.

The evaluation of features on metabolic control that MetfDeca possesses was assessed by the quantification of glucose that increases in rats with alloxan administration (150%); meanwhile, animals with alloxan and 5 μM of compound recovered basal levels of the metabolite. With respect to insulin levels, rats with alloxan induction diminish in 86%. Rats with the compound did not show differences versus intact control. Triglycerides decrease in alloxan group and recovered to the normal levels in rats with administration of the compound. However, cholesterol did not show changes between groups (Table 1).

On the other hand, in Table 1, liver toxicological profile which is characterized by increases in enzymatic activity of AST, ALT, ALP, and γGT was found in animals with only administration of alloxan, which similarly was the behavior of the levels of total bilirubin; all results were statistically significant $p \leq 0.05$. When alloxan + 5.0 μM of compound was analyzed, rats showed improvements on bilirubin levels, even below the control group, same case in AST and ALP activity with $p < 0.05$, while ALT remained significantly high ($p \leq 0.05$).

Likewise, renal profile framed by urea, creatinine, sodium, and potassium concentrations were assessed (Table 1). Rat with alloxan increases significantly its levels of urea and creatinine in 25% and 86% more than control group; sodium and potassium decreased significantly in 9% and 25% as signs of altered renal function. Meanwhile, rats with additional treatment of the compound show recovery in the electrolyte balance (not entirely for sodium), but not in the nonprotein nitrogen levels which remain higher.

The preclinical development of a drug often includes pharmacology studies, in which the efficacy is assessed in appropriate animal models. In this study insulin-independent rats were used, as well as insulin-requiring rats to prove therapeutic efficiency of the MetfDeca. In order to justify the dosage used in clinical trials with models altered on lipids and carbohydrates.

Figure 3 shows results obtained in both models. In Figure 3(a) glucose tolerance in insulin-independent model with respect to the control group was demonstrated. The hypercaloric diet caused an increases in area under curve (AUC, dotted line) and a displacement to the right, characteristic of insulin resistance and metabolic syndrome. Both, the metformin and MetfDeca treatments diminished 23% and 27% the AUC, being significant to $p \leq 0.05$ (AUC of NC = 4337; I-I + Metf = 3339; I-I + MetfDeca = 2182). Insulin (Figure 3(b)) in I-I group also showed an increase in AUC, which corresponded to 57% (AUC of I-I = 6815). The treatment with metformin returns to a normal secretion of the hormone, while treatment with MetfDeca reduces it until 50% of the AUC of the insulin secretion versus control

Table 1: Metabolite control, renal and hepatic toxicological profile with dose of 5.0 μM MetfDeca.

Metabolite	Control	Alloxan + 0.0 μM (MetfDeca)	Alloxan + 5.0 μM (MetfDeca)
Insulin (μU/mL)	25.1 ± 5.2	3.4 ± 0.3▼	23.6 ± 3.6
Glucose (mg/dL)	122.4 ± 2.7	300 ± 13.7▲	129 ± 4.6
Triglycerides (mg/dL)	96.3 ± 5.1	55 ± 3.4▼	138 ± 9.6▲
Cholesterol (mg/dL)	62.1 ± 2.4	58 ± 1.8	65 ± 3.6
Total bilirubin (mg/dL)	0.7 ± 0.1	0.95 ± 0.1▲	0.51 ± 0.25
AST (U/L)	230 ± 16	316.5 ± 2.3▲	117.9 ± 3.1▼
ALT (U/L)	60 ± 1.65	86.4 ± 2.5▲	91.1 ± 1.2▲
γGT (U/L)	7.3 ± 0.75	14.6 ± 0.29▲	8.7 ± 0.21
ALP (U/L)	316 ± 23	620.9 ± 4.9▲	267.3 ± 8.1▼
Sodium (mmol/L)	144 ± 0.1	131.2 ± 0.5▼	137.5 ± 1.5▼
Potassium (mmol/L)	6.6 ± 0.1	4.91 ± 0.7▼	6.8 ± 0.4
Urea	36 ± 0.41	45 ± 3.6▲	67 ± 2.3▲
Creatinine	0.53 ± 0.01	0.96 ± 0.1▲	0.8 ± 0.09▲

The serum parameters are the average of 10 separate experimental animals ± SEM. ▲ indicates significant difference with values above the control group, while ▼ indicates significant difference with values below the control group with $p \leq 0.05$ by Student's t-test. AST: aspartate transaminase, ALT: alanine aminotransferase, γGT: Gamma-glutamyl transpeptidase, and ALP: alkaline phosphatase.

group. Additionally, A1c fraction of the glycohemoglobin (Figure 3(c)) in the I-I group showed increase in 35% and a reduction without differences with respect to the control group in the animals with metformin and MetfDeca treatments.

Likewise, in insulin-requiring model evaluation of the handling of carbohydrate showed that I-R group increased in 300% of the AUC. The therapeutic efficiency of insulin and MetfDeca was compared with I-R group, while NC group only was used as reference of management therapeutic goal. Lispro insulin of rapid-acting and intermediate-acting was insufficient to control, because the AUC remained 250% above NC group but improved 18% with respect to I-R group (AUC of NC = 4337; I-R = 10842). The administration of MetfDeca observed similar behavior compared to insulin treatment (Figure 3(d)) (AUC of I-R + Metf = 8829; I-R + MetfDeca = 9075). Insulin concentration is showed in Figure 3(e), and I-R group presented very low levels of the hormone <2 μUI/mL. Meanwhile, as it was expected insulin levels go down in the presence of glucose load until similar values to the I-R group. Insulin in MetfDeca group surprisingly presented normal levels of the hormone in fasting (19 μUI/mL); however, the response does not achieve diminished plasmatic glucose; evidence suggests a recovery function of the β cells. This fact was corroborated by the HbA1c quantification; MetfDeca group showed better regulation with respect to the group with insulin treatment, and I-R group (Figure 3(f); 6.9%, 8.9%, and 13.9%, resp.).

The management of carbohydrates was confirmed by glycogen measurement in different tissues, in which recovery is indicative of effectiveness of the treatment. In this sense, insulin-independent model was evaluated; the animals fed with hypercaloric diet showed an increase of the glycogen stored in liver (259%), muscle (257%), heart (264%), renal cortex (70%), and renal medulla (200%). Metformin administration on other hand contrary to expectations does not reduce these levels, despite clear improvement of glucose levels in serum; glycogen levels in liver, muscle, heart, renal cortex, and medulla (107%, 400%, 580%, 88%, and 103%, resp.) were higher than NC group (significant, $p < 0.05$) Finally, treatment with MetfDeca showed better regulation on glycogen and management of carbohydrates, since in liver and muscle they remain 25% and 33% more in relation to NC, respectively. In renal cortex, glycogen content diminishes in 38%. Meanwhile, heart and renal medulla keep their glycogen levels significantly high in 98% and 68%, respectively; $p < 0.05$ (Figures 4(a)–4(d)).

In the DM1 model (I-R) a decrease of glycogen was observed, contrary to the one observed in DM2 model. In this case I-R group showed an important reduction of glycogen content, due to administration of alloxan which caused a depletion of serum insulin; glycogen formation is dependent on hormone signaling. Liver presented a significant reduction which correspond to 91%; muscle went down in 70%, and heart went down in 85%; and in kidney, the glycogen of the cortex diminishes 83%, while in medulla the decrement corresponded to 71%, all with statistic significant difference of $p < 0.05$. On the other hand, insulin treatment (daily subcutaneous insulin, 2 IU per 100 mg/dL glucose) which improved serum glucose management, however, in tissues did not show an important improvement; in liver and heart of I-R + insulin group with respect to I-R group, the glycogen stored increased 100% and 42%, respectively; however, muscle glycogen was not recovered and even remained 20% lower than I-R group. Kidney did not show differences versus I-R group. The group administered with MetfDeca improved with respect to I-R group in liver (178%), heart (213%), and renal cortex (147%). In renal medulla there are no changes regarding I-R group; meanwhile the glycogen in muscle was reduced the most with 50% below in the I-R group (Figures 4(e)–4(h)). Remarkably, in all treatments cases shown, a recovery regarding to NC group was not found.

The profile of serum lipids in which treatments were contrasted against intact animals (NC group) and impairment

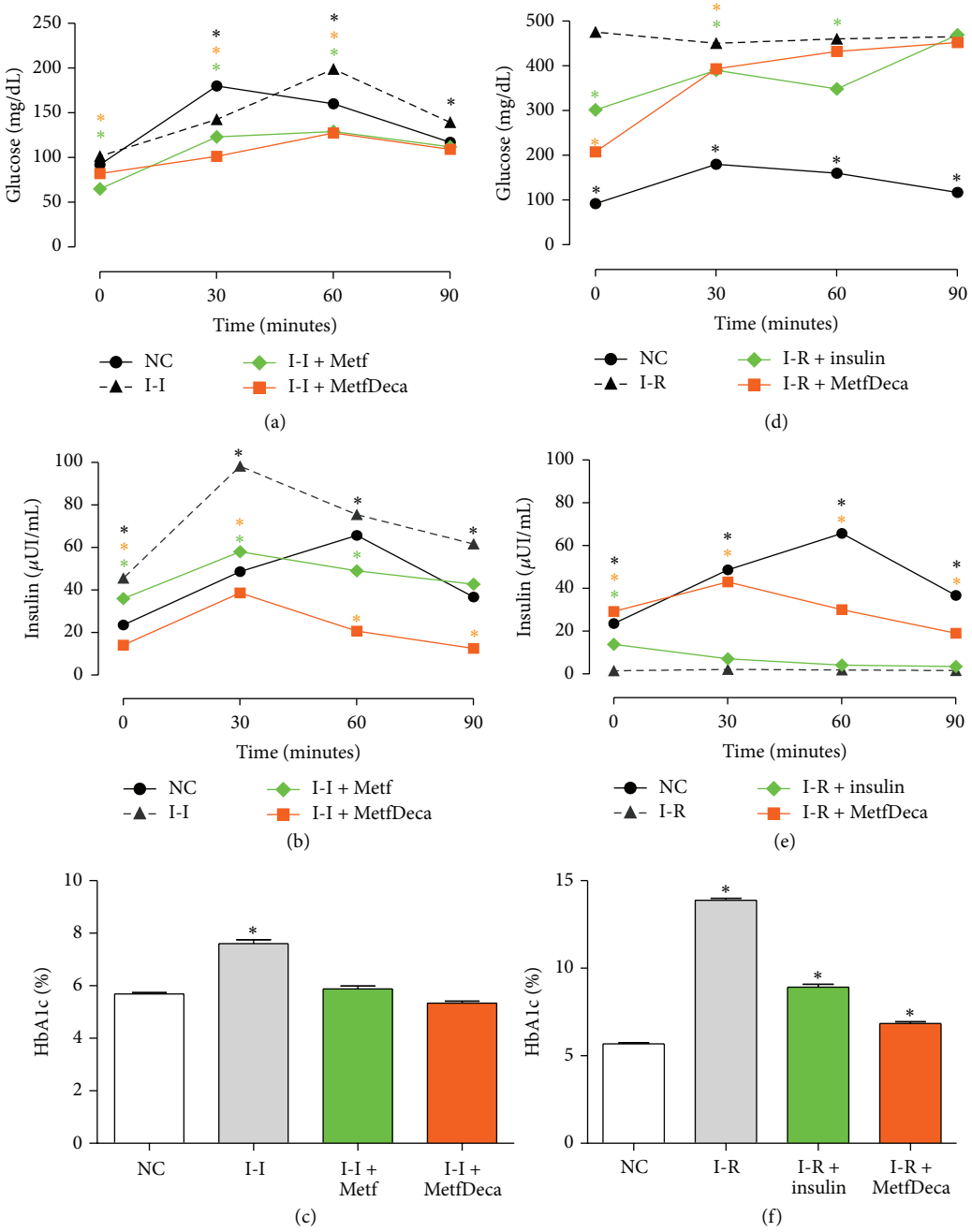

Figure 3: Oral glucose tolerance, insulin response, and fraction A1c of the glycosylated hemoglobin in different groups to two months of treatment. (a–c) Independent insulin (I-I) model. (d–f) Insulin-requiring (I-R) model. The results shown are the average of 5 different experiments ± SEM. ∗ indicates significant difference from the control group $p \leq 0.05$ group by ANOVA test with a Bonferroni post hoc test.

groups of DM2 (I-I) and DM1 (I-R) were measured with the goal to get to know the effect of MetfDeca. In the first section of Table 2 results of I-I model are shown that increased lipid profile in all parameters (TL 67%, TG 113%, Chol 37%, VLDL-C 28%, LDL-C 183%, and FFA 110%), except to HDL-C, which diminish 26.6%. When animals were treated with metformin the levels of total lipids, triglycerides, VLDL-C, LDL-C, and FFA were regulated; even HDL-C showed an increase of 26%; however, levels of cholesterol remained elevated slightly (23%); obviously, lipid profile improved considerably with regard to I-I group. Meanwhile, I-I + MetfDeca group normalized its concentration of total lipids, cholesterol, LDL-C, HDL-C, and FFA, but not in triglycerides concentration which remained increased in 70%, same case for VLDL-C with 39%; in relation to I-I group TL, cholesterol, LDL-C, and FFA showed a significantly reduction $p \leq 0.05$.

In the DM1 model (I-R) we observed a complete lipid decompensation, classical of the diabetes. I-R group showed

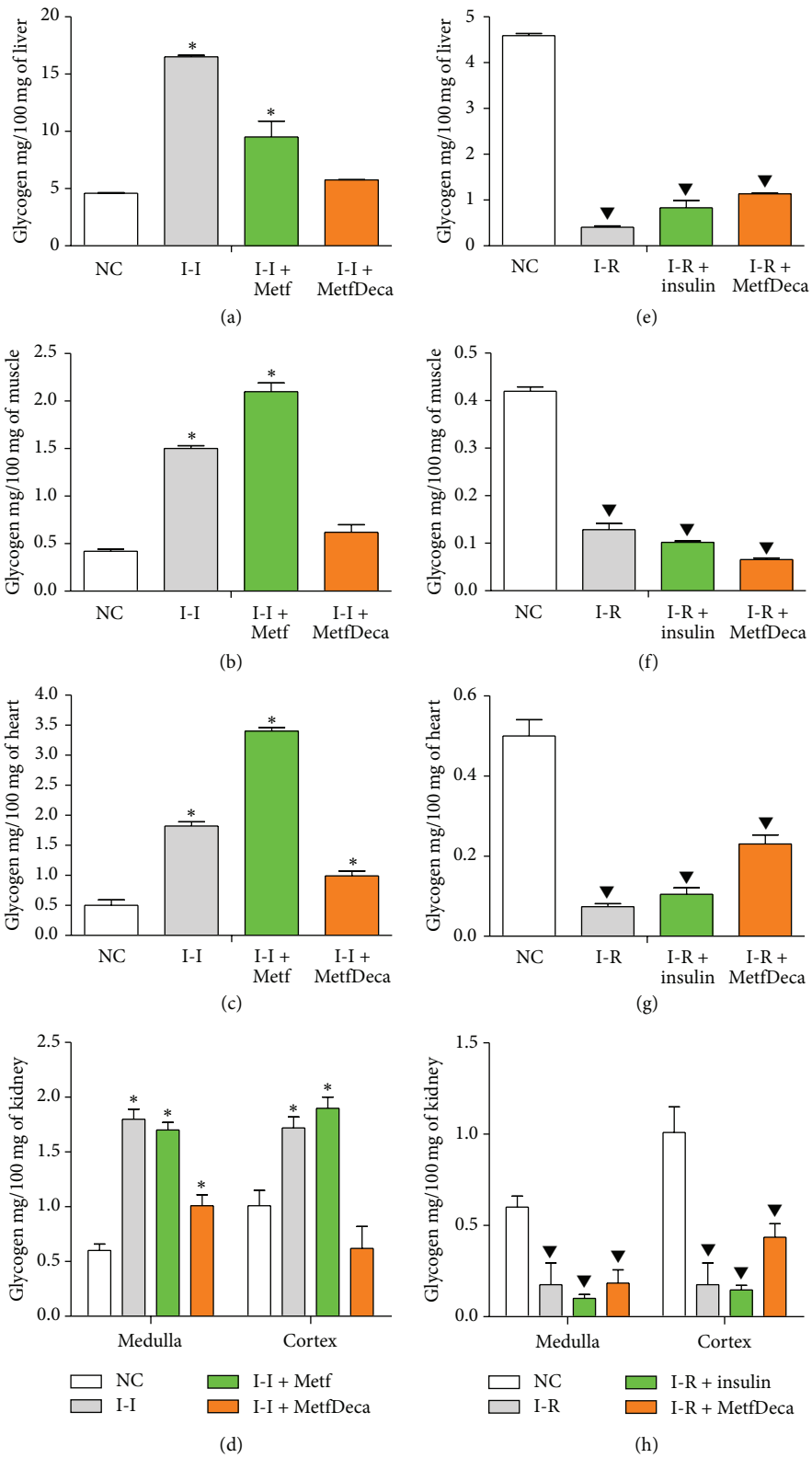

FIGURE 4: Glycogen content in different tissues from groups to two months with treatments. (a–d) Independent insulin (I-I) model. (e–h) Insulin-requiring (I-R) model. The results shown are the average of 20 different experiments ± SEM. ∗ indicates significant difference above from the control group (NC); meanwhile ▼ indicates significant difference below from the control group (NC), both with $p \leq 0.05$ group by ANOVA test with a Bonferroni post hoc test.

Table 2: Serum lipids in independent insulin model and insulin-requiring model with pharmacological treatments and MetfDeca.

Metabolite	NC ($n = 20$)	I-I ($n = 20$)	I-I + Metf ($n = 20$)	I-I + MetfDeca ($n = 20$)	I-R ($n = 20$)	I-R + insulin ($n = 20$)	I-R + MetfDeca ($n = 20$)
Total lipids (mg/dL)	176 ± 11	294 ± 20▲	190 ± 12↓	203 ± 20↓	546 ± 28▲	502 ± 21▲	223 ± 16▲↓
Triglycerides (mg/dL)	63.5 ± 5.0	135 ± 13▲	68 ± 6.2↓	108 ± 5▲	360 ± 10▲	340 ± 13▲	75 ± 3↓
Cholesterol (mg/dL)	89.5 ± 2.4	123 ± 14▲	110 ± 6.9▲	87 ± 3↓	173 ± 8▲	145 ± 18▲	135 ± 11▲↓
VLDL-C (mg/dL)	16.5 ± 1.8	21.2 ± 2.0▲	20 ± 1.5	23 ± 2▲	58.4 ± 2▲	29.1 ± 1.3▲↓	17 ± 2↓
LDL-C (mg/dL)	23 ± 2.6	65.1 ± 2.8▲	26.9 ± 2.5↓	24 ± 3↓	21 ± 1.8	31.4 ± 3▲	73 ± 5▲
HDL-C (mg/dL)	50 ± 6.5	36.7 ± 1.1▼	63.1 ± 3.2▲	40 ± 5	93.6 ± 11▲	84.5 ± 6▲	45 ± 2.4↓
FFA (mg/dL)	2.5 ± 0.6	5.5 ± 1.2▲	2.8 ± 0.4↓	2.3 ± 0.2↓	11.5 ± 1.2▲	3.2 ± 0.3↓	4.8 ± 0.8▲↓

The results shown are the average of 20 separate experimental animals ± SEM. ▲ indicates significant difference with values above the control group with normal calorie diet, ▼ indicates significant difference with values below the control group with normal calorie diet, while ↓ indicates significant difference with respect to groups I-I and I-R, $p \leq 0.05$ one-way ANOVA test with Bonferroni post hoc test.

increases in TL (210%), triglycerides (467%), cholesterol (93%), VLDL-C (254%), HDL-C (87%), and FFA (360%); LDL-C did not show difference. Insulin treatment only reduced FFA levels, while the dyslipidemia remains present. Finally, MetfDeca treatment improved significantly lipid profile with respect to I-R group; however, TL maintained levels significantly above of NC group only in 27%, cholesterol in 51%, LDL-C in 271%, and FFA in 92%, as shown in Table 2.

Pharmaceutical treatments most of the time are only limited to investigate serum effects; however, it is very important to consider the different tissues in relation to the metabolite analyzed. Therefore, triglycerides content in liver, muscle, heart, and kidney of experimental animals was also quantified as it is shown in Figure 5. In this sense, I-I group increases its triglycerides content in liver in 55.6%, muscle in 187%, heart in 130%, renal cortex in 130%, and renal medulla in 152%; these levels demonstrate multitissues steatosis. Metformin treatment only improves triglyceride levels in kidney which showed similar values to NC group. In liver, muscle, and heart metformin promoted a major increase of triglycerides of the 175%, 357%, and 162%. Meanwhile, with the MetfDeca treatment the distribution and levels of triglycerides in liver, heart, and kidney were regulated, although, in muscle which showed improvement this remained with a 100% more TG in relation to NC group. MetfDeca manages a better cellular lipid homeostasis than metformin treatment, which apparently produces a triglyceride redistribution toward other tissues.

In the animals administered with alloxan to get the insulin-requiring model, triglycerides levels were not affected in liver and kidney; however, in muscle and heart content showed a decrease of the 47% and 43%, respectively. Insulin treatment produced an overstoring in heart, renal cortex, and renal medulla from 132%, 29%, and 91%, respectively. While liver level remained unaffected and even in muscle went down more (63.5%) in relation to NC group. Meanwhile, MetfDeca treatment showed the lowest level of triglycerides in liver (32%), although in muscle the triglycerides only remained low in 22%. Contrary in heart, renal cortex, and renal medulla, content increased in 58.5%, 12%, and 17% above from NC group.

4. Discussion

In the guidelines to develop new medicinal products for treatment of diabetes mellitus specific strategies and steps should be considered, as pharmacodynamic data, in which the contribution to therapeutic and/or toxic effects should be discussed [22]. In this sense, the MetfDeca showed an important decrease of glycemia levels after 4 weeks of administration in the hyperglycemic alloxan-induced model, since decavanadate complexes have been described as insulin mimetics [6, 9–11, 23]. The most usual substances to induce diabetes in the rat are alloxan and streptozotocin (STZ) [24]. Despite the fact that STZ model is extensively recognized, this not only causes beta cell necrosis but also produces DNA alkylation in different tissues; cytotoxic effect is related to transport capacity of glucose through the facilitated glucose transporters GLUT2 and GLUT1 in pancreas, liver, kidney, and brain, mainly, producing cell death, structural and metabolic changes in these tissues, complicating the correct evaluation of therapeutic strategies [24–27]. Although the cytotoxic action of both STZ and alloxan is mediated by reactive oxygen species, the source of their generation is different. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide and molecular oxygen. Thereafter highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β cells and could alter cells of different tissues; however the antioxidant defense prevents the severe injuries, so it is more suitable for studying therapeutic strategies on insulin-requiring diabetes mellitus [24].

As it was expected damage in exocrine pancreas was observed in rats with alloxan administration, because hyperglycemia with hypoinsulinemia was induced. It is important to emphasize that insulin signaling is associated with hepatic lipogenesis and mobility of triglycerides into VLDL; therefore, low levels of the hormone in DM1 early models produce lower levels of serum triglycerides, although these are accumulated in liver, as shown Table 1 [28–30]. Biochemical changes provoked by alloxan in blood have been

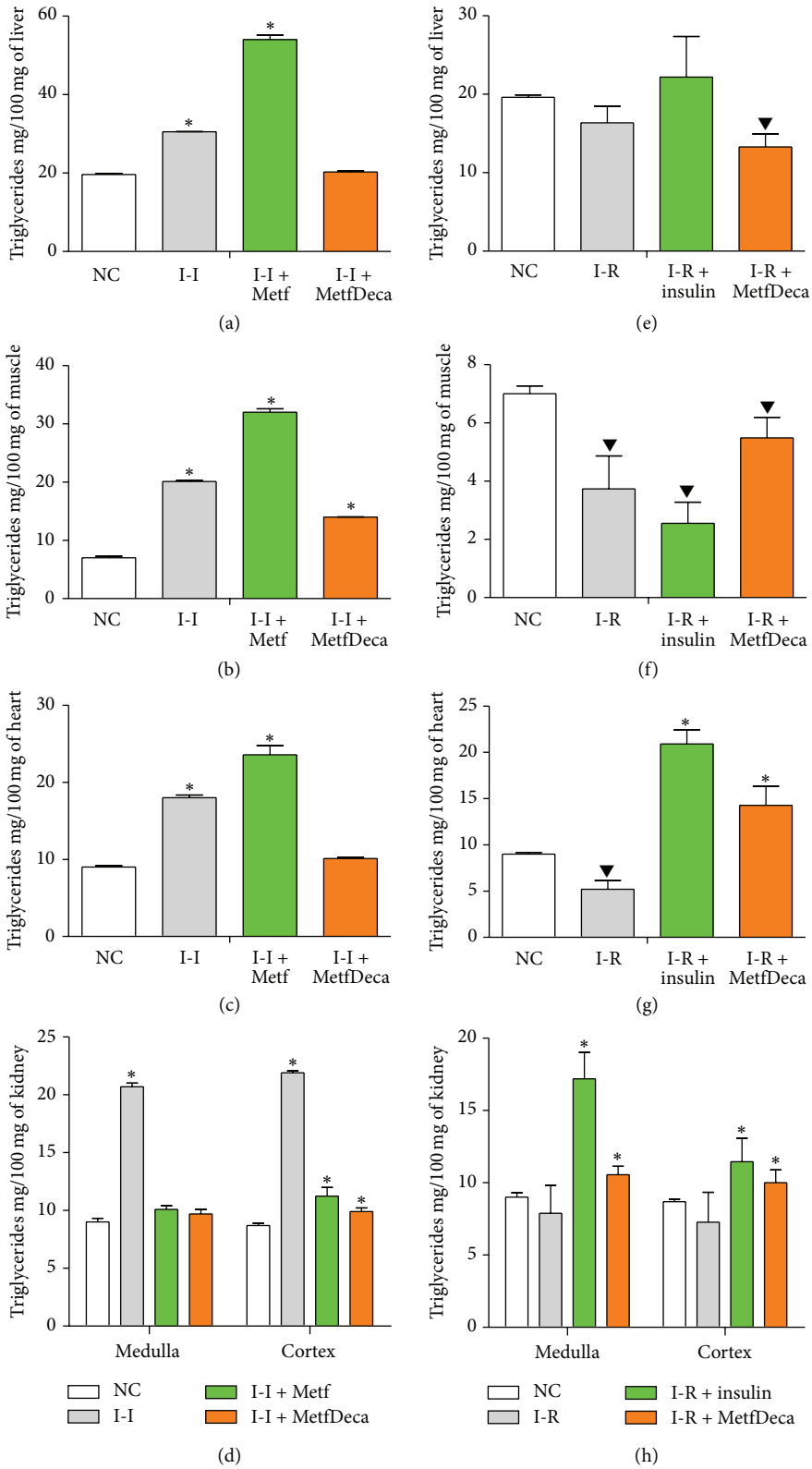

Figure 5: Triglyceride content in different tissues from groups to two months with treatments. (a–d) Independent insulin (I-I) model. (e–h) Insulin-requiring (I-R) model. The results shown are the average of 20 different experiments ± SEM. ∗ indicates significant difference above from the control group (NC); meanwhile ▼ indicates significant difference below from the control group (NC), both with $p \leq 0.05$ group by ANOVA test with a Bonferroni post hoc test.

associated with morphological and ultrastructural lesions in the liver that largely resembled chronic liver disease in humans linked to diabetes mellitus. Liver changes ranged from the fatty degeneration of liver cells to steatohepatitis and periportal fibrosis. Therefore, an increase in the hepatic enzymatic activity as has been reported in other studies exists, in which alloxan-induced diabetic animals increase blood levels of AST and ALT in the first 2 weeks after treatment, but only ALT remained significantly elevated until 26 weeks after diabetes induction [31, 32]. In this regard, we observed increased hepatic transaminases, γGT, and ALP, as well as bilirubin, which by its levels could be interpreted as hepatic inflammation, but not as a toxic effect that derived in necrosis caused by alloxan administration.

On the other hand, kidney is the other organ involved in detoxification process and hidroelectrolitic homeostasis. Alloxan-induced diabetic rats showed increases of serum urea and creatinine classical in diabetic kidney, as well as loss of sodium and potassium, indicative of kidney function impairment [31, 33, 34]. Evidence suggests that hyperglycemia is associated with kidney damage since early stages, combined with an increase of reactive oxygen species and the resulting oxidative stress is thought to play a key role in the pathogenesis of this disorder [35]. Likewise, it has been demonstrated that alloxan causes both tubular and glomerular changes in structure, which imply a lost in the functions [33, 35]. In addition, a marked increase in the activity of Na^+/K^+-ATPase has been observed in the diabetic kidney probably because of an adaptation of nephrons to maintain electrolyte homeostasis in diabetes in face of the increased glomerular filtration rate (GFR) and osmotic diuresis [36].

After the conclusion that the dose with hypoglycemic feature of MetfDeca was 5 μM/300 g/2 times a week, the metabolic response was evaluated, and surprisingly the fasting insulinemia level was normalized in the diabetic group. The insulinemia level of diabetic rats was normalized. Indeed, the decrease of blood glucose levels registered in diabetic rats exposed to vanadium compound may be related to the concomitant increase of plasma insulin concentrations. These results are in accordance with previous studies, in which insulin levels were recovered [37], fact attributable to the effectiveness of vanadium compounds [38, 39]. Protection on β cell could be due to antioxidant feature that has been described, in which antioxidant stress markers as superoxide dismutase, catalase, and glutathione peroxidase are dependent on the nature of the polyoxovanadates present, besides the concentration administered, because higher levels of decavanadate can produce an increase of superoxide anion and cellular damage associated with Fenton-like reactions as it is observed in transitional elements [6, 23]. Taking advantage of the minimal concentration of decavanadate administered, cells with vanadium uptake can improve oxidation of energetic molecules such as glucose and lipids and through insulinemia restarted the conversion of these energetic molecules in energetic stored triglycerides, which are mobilized into the bloodstream. Liver is the main organ involved in this mechanism, our results showed that, in rats treated with MetfDeca for one month, liver recovered almost totally its enzymatic activity, probably associated with improvement in metabolic conditions and reduction of oxidative stress, with exception of ALT that remain increased. ALT levels are in concordance with urea behavior because this transaminase is involved in the transferring of NH_3^+ groups to α-ketoglutarate to form glutamate which take part in gluconeogenesis, generating urea as final product. Uremia is considered a kidney damage marker; however, in conditions of sodium loss, Henle's loop used urea as countercurrent osmotic gradient to achieve sodium recovery [40, 41]; for this reason rats administered with MetfDeca improve sodium and potassium levels, but also creatinine serum almost achieved normal values. Pharmacodynamical data obtained have showed that contribution to therapeutic effect of MetfDeca could be appropriated, because toxic effects are to some extent negligible.

Insulin-mimetic attributes of decavanadate were challenged in two models with different metabolic disturbances, but with hyperglycemia in common. Usually, only the serum biochemical behavior of glucose and lipids is considered but does not take into account the intracellular behavior of these molecules, which are controlled by insulin signaling; thus, when we talk about insulin-mimetic activity, these actions should be considered to clarify the limitations of vanadium compounds.

First, an insulin-independent model was developed, which possesses clinical phenotype of glycemic response impaired, A1c glycohemoglobin elevated, and dyslipidemia with hypertriglyceridemia and cholesterolemia (VLDL and LDL fractions elevated with HDL decrement), despite hyperinsulinemia present as it happens in type 2 diabetes mellitus. Additionally, classical complications by insulin signaling also were presented: increases in glycogen levels were observed in liver, muscle, heart, and kidney in cortex and medulla zones, and steatosis in the evaluated tissues (triglycerides stored) was also observed.

American Diabetes Association as well as European Association for the Study of Diabetes recommended metformin monotherapy as initial pharmacological treatment in type 2 diabetes mellitus, due to the fact that it has a long-standing evidence base for efficacy and safety, is inexpensive, and may reduce risk of cardiovascular events; position statement evaluated the data and developed recommendations, including advantages and disadvantages, for antihyperglycemic agents for type 2 diabetic patients [13]. However, its study is based mainly on serum evidences and not on tissues behavior. Previously, we reported risk of developing lactic acidosis and alterations in multiple tissues in triglycerides and glycogen storages [11]. In this work, we reproduced independent insulin model in Wistar rats that were treated with metformin monotherapy following pharmacological recommendations of ADA. The subjects studied observed regulation on glucose tolerance, insulin secretion, A1c glycohemoglobin fraction, and dyslipidemia. However, intracellular glycogen in liver, muscle, heart, and kidney were not improved, same case to triglycerides stored in these tissues; this last phenomenon is recognized as steatosis and is common in excess of metabolic needs. Steatosis can generate lipotoxicity by lipotoxic intermediates such as ceramide and acylcarnitine. Collectively these events favor oxidative stress

and apoptosis, and mitochondria become damaged further compromising ATP production; lipotoxicity is related also to endoplasmic reticulum stress through mechanisms related to oxidative stress and mitochondrial dysfunction and by inducing an inflammatory response [42, 43].

Moreover, other groups with same independent insulin conditions were treated with pharmacological monotherapy of MetfDeca 3.7 μg/300 g (2.5 μM) two times a week, a dosage of 48,000 times less than metformin. In these studies the subjects also observed improvement on glucose tolerance and regulation in insulin secretion with A1c fraction of glycohemoglobin normalized, despite the fact that they remain fed with HC diet. Glycogen stored is showed to be regulated in almost all tissues that were evaluated; minor increases remain in heart and kidney medulla. Likewise, dyslipidemia profile was improvement, although triglycerides did not reach values as normocaloric group. However, steatosis was removed in all tissues evaluated, and minimal levels remain in muscle and kidney cortex, but these do not have biological impact, because they mean a recovery of insulin resistance previously generated by HC consumption [44]. Our results showed that decavanadate in combination with metformin has a biological activity, although the exact mechanism is unknown completely; evidence has demonstrated an improvement in insulin signaling, as in the case of protein kinase B (PKB) that favors the regulation of signals with subsequent inhibition of lipolysis; this argument is supported in inhibition of protein tyrosine phosphatase 1B (PTP 1B); this enzyme provides a negative feedback by catalyzing the dephosphorylation of the insulin receptors. The dephosphorylation of the insulin receptor slows the intake of the glucose from the blood by not allowing the other proteins in the insulin transduction pathway to be activated and consequently not to do their job of transferring the signal to the other proteins in the pathway, so it favored the correct insulin signaling [45–47]. Pharmacological action of decavanadate and metformin in low dosage which can act simultaneously in different tissues and organs favoring the oxidation and burning of carbohydrates and lipids in a more regulated way thus has an advantage over the counter ion alone as monotherapy [11]. However, this model has the presence of insulin, so it could not know if MetfDeca is an insulin-mimetic agent or only improves the cellular environment and then insulin present does its work.

In order to investigate insulin-mimetics effects of decavanadate we develop a model insulin-requiring by alloxan injection in which observed insulin depletion with hyperglycemia that produces an increase of glycohemoglobin occurs in type 1 diabetes mellitus. Normally, when insulin binds to its receptor, it activates the glycogen synthesis by inhibiting the enzymes that slow down the PI3K pathway such as PKA enzyme. At the same time, it will promote the function of the enzymes that provide a positive feedback for the pathway like the AKT. The inactivating enzymes that stop the reaction and activating enzymes that provide a positive feedback will increase glycogen, lipid, and protein synthesis, therefore promoting the normal glucose intake. Therefore, if insulin signaling is missing, glycogen synthesis is diminished as shown in Figures 4(e)–4(h). Additionally, DM1 usually has a tendency for the late development of dyslipidemia associated with cardiovascular risk factors and microvascular complications [48–51]. Both dyslipidemia and impairment of stored triglycerides were observed in this study; mostly effect was presented by muscle and heart (Table 2 and Figures 5(e)–5(h)). Thus, DM1 model was validated as suggested by the guidelines to pharmaceutical development.

Recommended therapy for type 1 diabetes by international associations consists of initiation and management of insulin therapy with insulin analogs to achieve desired glycemic goals [13]. Following the guide, the initiation and management of insulin therapy in rats of the DM1 model were administered daily subcutaneous insulin, 2 IU per each 100 mg/dL glycemia. Insulin administered was a Humalog Mix 75/25 (rDNA origin) that is a mixture of insulin of rapid-acting (25%) and insulin lispro protamine suspension that acts as an intermediate agent. Glycemia with insulin treatment was recovered gradually; however, therapy apparently was good but insufficient because fasting glucose did not return to appropriate levels; furthermore, basal insulin levels also remain diminished, and in consequence, DM1 model did not recover its glucose tolerance, not even complete regulation in A1c glycohemoglobin levels; same case occurs for lipids profile in which there are improved parameters, but not to required levels. When these situations occur the guide recommended three injections of rapid-acting insulin analog administered just before eating or regular human insulin premixed formulations (70/30) that are rapid-acting insulin analogs, but their pharmacodynamic profiles are suboptimal for the coverage of postprandial glucose and its monitoring must be monitoring completely [13]; for this reason in an animal model it is difficult to be implemented; however, adjustments were made in insulin administered dosage based on the prevailing blood glucose levels in each animal, until understanding of the pharmacodynamic profile of formulation Humalog Mix 75/25. After glycogen and triglycerides in tissues were quantified, data showed minimal increase of glycogen levels that correspond to a cellular oxidative dynamic appropriate in which reserves of carbohydrates and triglycerides are employed to obtain energy [52]. In this regard, muscle presented the features aforementioned; however, heart and kidney presented an overstored triglycerides, precisely in relation to low capacity of energetic spend promoted by insulin administration, in contrast with liver which had no difference between groups. This apparent discordance between ideas is in relation to insulin which stimulates intracellular triglyceride synthesis while inhibiting lipolysis in tissues with low energy obtained by lipids [53–55].

Pharmacological dosage of MetfDeca administered in DM1 model showed an improvement in glucose tolerance and insulin secretion, in fact, rats previously insulin-depleted showed an important secretion of the hormone after MetfDeca administration, in accordance with other study, suggesting a recovery of insulin secretion ability by Langerhans islets, as well as an insulinotropic property of the vanadium compound studied here [37]. Previously, processes of beta cells regeneration from extraislet precursor cells in mouse model induced by selective perfusion of alloxan

have been reported [54, 55]. Indeed, in our DM1 model, MetfDeca treatment might stimulate beta cell proliferation from intraislet endocrine cells, as well as differentiation from extraislet precursor cells. Therefore, MetfDeca promoted a best reduction in A1c fraction of glycohemoglobin that corroborated beneficial actions of MetfDeca. Vanadium compounds have shown that they can influence glucose and lipid metabolism by insulin-dependent or insulin-independent biochemical pathways [56, 57]. Insulin-independent mechanism of vanadium compounds is mediated through activation of PKB/AKT kinase leading to the glucose uptake by the GLUT4 transporter [58, 59]. Also, activation of PKB/AKT stimulates the phosphorylation of GSK3, resulting in the stimulation of glycogen synthesis [57, 60]. However, we observed a partial glycogen restauration, but better than insulin treatment.

In relation to lipids profile MetfDeca improved all parameters measured, even better than insulin treatment, although it was not enough to resemble the intact control group. Lipids behavior suggests an improvement in tissues in relation to energy obtaining mode, because rates of hepatic triglyceride synthesis from fatty acid esterification are dependent on substrate flux and independent of circulating plasma insulin concentrations; thus, when serum FFA diminish liver lost flux of prime matter to build triglycerides, results suggest strongly that MetfDeca induced lipidic burning, as in DM2 model [61, 62]. This last idea was supported by the triglyceride content in tissues; these showed a subtle regulation in each tissue evaluated. Apparently, decavanadate could induce the formation of ATPase dimers, eventually relevant to ATPase activity, and at same time can interact with mitochondrial complex III, limiting electron chain which diminishes ATP formation so that energy obtaining would be by lipid burning (β-oxidation); the induction of the production of ROS would be expected [6, 7, 9, 46].

5. Conclusion

A potential metallopharmaceutical MetfDeca has been developed and in this study it has been shown to be a good alternative that could be considered as adjuvant in the treatment of diseases that presented hyperglycemia and dyslipidemia, because low concentrations do not generate toxicity conditions and it is very efficient as hypoglycemic and hypolipidemic agent due to properly redistributing the excess of these molecules to different organs in a similar way to insulin. However, although MetfDeca has been considered as an insulin-mimetic agent, actually it acts as an insulin-enhancer agent in many aspects, ranging from protection to the possible generation of beta cells, but highlighted its intracellular mechanisms in the management of lipids. Therefore, pharmaceutical goals as pharmacodynamic data and therapeutic contributions as well as toxic effects have been analyzed. Next step should be the precise description of the action mechanism. Work is in progress in this regard.

Competing Interests

The authors declare that they have no competing interests.

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References

[1] H. Zhao and Y. Ning, "China's ancient gold drugs," *Gold Bulletin*, vol. 34, no. 1, pp. 24–29, 2001.

[2] Paracelsus, *Four Treatises of Theophrastus von Hohenheim Called Paracelsus*, Translated by: H. Sigerist et al., Edited by: H. Sigerist, Johns Hopkins University Press, Baltimore, Md, USA, 1996.

[3] P. J. Sadler and Z. Guo, "Metal complexes in medicine: design and mechanism of action," *Pure and Applied Chemistry*, vol. 70, no. 4, pp. 863–871, 1998.

[4] C. Orvig and M. J. Abrams, "Medicinal inorganic chemistry: introduction," *Chemical Reviews*, vol. 99, no. 9, pp. 2202–2204, 1999.

[5] S. K. Bharti and S. K. Singh, "Recent developments in the field of anticancer metallopharmaceuticals," *International Journal of PharmTech Research*, vol. 1, no. 4, pp. 1406–1420, 2009.

[6] M. Aureliano and R. M. C. Gândara, "Decavanadate effects in biological systems," *Journal of Inorganic Biochemistry*, vol. 99, no. 5, pp. 979–985, 2005.

[7] M. Aureliano and D. C. Crans, "Decavanadate $V_{10}O_{28}^{-6}$ and oxovanadates: oxometalates with many biological activities," *Journal of Inorganic Biochemistry*, vol. 103, no. 4, pp. 536–546, 2009.

[8] M. Aureliano, "Recent perspectives into biochemistry of decavanadate," *World Journal of Biological Chemistry*, vol. 2, no. 10, pp. 215–225, 2011.

[9] G. R. Willsky and D. A. White, "Metabolism of added orthovanadate to vanadyl and high-molecular-weight vanadates by *Saccharomyces cerevisiae*," *The Journal of Biological Chemistry*, vol. 259, no. 21, pp. 13273–13281, 1984.

[10] M. Aureliano, "Decavanadate toxicology and pharmacological activities: V_{10} or V_1, both or none?" *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 6103457, 8 pages, 2016.

[11] S. Treviño, E. Sánchez-Lara, V. E. Sarmiento-Ortega et al., "Hypoglycemic, lipid-lowering and metabolic regulation activities of metforminium decavanadate $(H_2Metf)_3$ $[V_{10}O_{28}]\cdot 8H_2O$ hypercaloric using carbohydrate and lipid-induced deregulation in Wistar rats as biological model," *Journal of Inorganic Biochemistry*, vol. 147, pp. 85–92, 2015.

[12] World Health Organization (WHO), "Screening for type 2 diabetes. Report of a World Health Organization and International Diabetes Federation meeting," WHO/NMH/MNC/03.1, 2009.

[13] American Diabetes Association, "Standards of medical care in diabetes 2015," *Diabetes care*, vol. 37, pp. 14–15, 2015.

[14] Federación Mexicana de Diabetes A.C., http://fmdiabetes.org/wp-content/uploads/2014/11/diabetes2013INEGI.pdf.

[15] M. E. Almiron, S. C. Gamarra, and M. S. González, "Diabetes," *Journal of Postgraduate Medicine Chair*, vol. 1, no. 152, pp. 23–27, 2005.

[16] L. Xu, Z. Huang, X. He, X. Wan, D. Fang, and Y. Li, "Adverse effect of metformin therapy on serum vitamin B12 and folate: short-term treatment causes disadvantages?" *Medical Hypotheses*, vol. 81, no. 2, pp. 149–151, 2013.

[17] A. Strózik, A. Stęposz, M. Basiak, M. Drozdz, and B. Okopień, "Multifactorial effects of vildagliptin added to ongoing metformin therapy in patients with type 2 diabetes mellitus," *Pharmacological Reports*, vol. 67, no. 1, pp. 24–31, 2015.

[18] J. de Jager, A. Kooy, P. Lehert et al., "Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial," *British Medical Journal*, vol. 340, Article ID c2181, 2010.

[19] D. Rehder, "The future of/for vanadium," *Dalton Transactions*, vol. 42, no. 33, pp. 11749–11761, 2013.

[20] S. D. Brunk and J. R. Swanson, "Colorimetric method for free fatty acids in serum validated by comparison with gas chromatography," *Clinical Chemistry*, vol. 27, no. 6, pp. 924–926, 1981.

[21] L. W. Bennett, R. W. Keirs, E. D. Peebles, and P. D. Gerard, "Methodologies of tissue preservation and analysis of the glycogen content of the broiler chick liver," *Poultry Science*, vol. 86, no. 12, pp. 2653–2665, 2007.

[22] Committee for Medicinal Products for Human Use (CHMP), *Guideline on Clinical Investigation of Medicinal Products in the Treatment or Prevention of Diabetes Mellitus*, vol. 1, European Medicines Agency, London, UK, 2012, CPMP/EWP/1080/00 Rev.

[23] S. S. Soares, H. Martins, R. O. Duarte et al., "Vanadium distribution, lipid peroxidation and oxidative stress markers upon decavanadate in vivo administration," *Journal of Inorganic Biochemistry*, vol. 101, no. 1, pp. 80–88, 2007.

[24] T. Szkudelski, "The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas," *Physiological Research*, vol. 50, no. 6, pp. 537–546, 2001.

[25] W. J. Schnedl, S. Ferber, J. H. Johnson, and C. B. Newgard, "STZ transport and cytotoxicity, specific enhancement in GLUT2-expressing cells," *Diabetes*, vol. 43, no. 11, pp. 1326–1333, 1994.

[26] C. A. Delaney, A. Dunger, M. D. Matteo, J. M. Cunningham, M. H. L. Green, and I. C. Green, "Comparison of inhibition of glucose-stimulated insulin secretion in rat islets of Langerhans by Streptozotocin and methyl and ethyl nitrosoureas and methanesulphonates. Lack of correlation with nitric oxide-releasing or O^6-alkylating ability," *Biochemical Pharmacology*, vol. 50, no. 12, pp. 2015–2020, 1995.

[27] M. Elsner, B. Guldbakke, M. Tiedge, R. Munday, and S. Lenzen, "Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin," *Diabetologia*, vol. 43, no. 12, pp. 1528–1533, 2000.

[28] J.-M. Schwarz, P. Linfoot, D. Dare, and K. Aghajanian, "Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets," *American Journal of Clinical Nutrition*, vol. 77, no. 1, pp. 43–50, 2003.

[29] K. L. Donnelly, C. I. Smith, S. J. Schwarzenberg, J. Jessurun, M. D. Boldt, and E. J. Parks, "Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease," *The Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1343–1351, 2005.

[30] J. E. Lambert, M. A. Ramos-Roman, J. D. Browning, and E. J. Parks, "Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease," *Gastroenterology*, vol. 146, no. 3, pp. 726–735, 2014.

[31] A. Adesokan, O. Oyewole, and B. M. S. Turay, "Kidney and liver function parameters in alloxan-induced diabetic rats treated with aloe barbadensis juice extract," *Sierra Leone Journal of Biomedical Research*, vol. 1, no. 1, pp. 33–37, 2010.

[32] A. N. Lucchesi, L. L. Cassettari, and C. T. Spadella, "Alloxan-induced diabetes causes morphological and ultrastructural changes in rat liver that resemble the natural history of chronic fatty liver disease in humans," *Journal of Diabetes Research*, vol. 2015, Article ID 494578, 11 pages, 2015.

[33] M. Pourghasem, E. Nasiri, and H. Shafi, "Early renal histological changes in alloxan-induced diabetic rats," *International Journal of Molecular and Cellular Medicine*, vol. 3, no. 1, pp. 11–15, 2014.

[34] J. Das and P. C. Sil, "Taurine ameliorates alloxan-induced diabetic renal injury, oxidative stress-related signaling pathways and apoptosis in rats," *Amino Acids*, vol. 43, no. 4, pp. 1509–1523, 2012.

[35] K. Winiarska, K. Szymanski, P. Gorniak, M. Dudziak, and J. Bryla, "Hypoglycaemic, antioxidative and nephroprotective effects of taurine in alloxan diabetic rabbits," *Biochimie*, vol. 91, no. 2, pp. 261–270, 2009.

[36] M. R. Siddiqui, K. Moorthy, A. Taha, M. E. Hussain, and N. Z. Baquer, "Low doses of vanadate and *Trigonella* synergistically regulate Na^+/K^+-ATPase activity and GLUT4 translocation in alloxan-diabetic rats," *Molecular and Cellular Biochemistry*, vol. 285, no. 1-2, pp. 17–27, 2006.

[37] S. Missaoui, K. B. Rhouma, M.-T. Yacoubi, M. Sakly, and O. Tebourbi, "Vanadyl sulfate treatment stimulates proliferation and regeneration of beta cells in pancreatic islets," *Journal of Diabetes Research*, vol. 2014, Article ID 540242, 7 pages, 2014.

[38] M. Soveid, G. A. Dehghani, and G. R. Omrani, "Long- term efficacy and safety of vanadium in the treatment of type 1 diabetes," *Archives of Iranian Medicine*, vol. 16, no. 7, pp. 408–411, 2013.

[39] J. Meyerovitch, Z. Farfel, J. Sack, and Y. Shechter, "Oral administration of vanadate normalizes blood glucose levels in streptozotocin-treated rats. Characterization and mode of action," *Journal of Biological Chemistry*, vol. 262, no. 14, pp. 6658–6662, 1987.

[40] T. L. Pallone, M. R. Turner, A. Edwards, and R. L. Jamison, "Countercurrent exchange in the renal medulla," *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, vol. 284, no. 5, pp. R1153–R1175, 2003.

[41] H. Tsukaguchi, C. Shayakul, U. V. Berger, and M. A. Hediger, "Urea transporters in kidney: molecular analysis and contribution to the urinary concentrating process," *The American Journal of Physiology—Renal Physiology*, vol. 275, no. 3, pp. F319–F324, 1998.

[42] Y. Zhang and J. Ren, "Role of cardiac steatosis and lipotoxicity in obesity cardiomyopathy," *Hypertension*, vol. 57, no. 2, pp. 148–150, 2011.

[43] D. H. van Raalte, N. J. van der Zijl, and M. Diamant, "Pancreatic steatosis in humans: cause or marker of lipotoxicity?" *Current Opinion in Clinical Nutrition & Metabolic Care*, vol. 13, no. 4, pp. 478–485, 2010.

[44] P. Xie, A. K. G. Kadegowda, Y. Ma et al., "Muscle-specific deletion of comparative gene identification-58 (CGI-58) causes muscle steatosis but improves insulin sensitivity in male mice," *Endocrinology*, vol. 156, no. 5, pp. 1648–1658, 2015.

[45] B. F. Harland and B. A. Harden-Williams, "Is vanadium of human nutritional importance yet?" *Journal of the American Dietetic Association*, vol. 94, no. 8, pp. 891–894, 1994.

[46] J. Nilsson, A. A. Shteinman, E. Degerman et al., "Salicylamide and salicylglycine oxidovanadium complexes with insulin-mimetic properties," *Journal of Inorganic Biochemistry*, vol. 105, no. 12, pp. 1795–1800, 2011.

[47] D. C. Crans, R. L. Bunch, and L. A. Theisen, "Interaction of trace levels of vanadium(IV) and vanadium(V) in biological systems," *Journal of the American Chemical Society*, vol. 111, no. 19, pp. 7597–7607, 1989.

[48] J. J. Chillarón, J. A. Flores-Le-Roux, A. Goday et al., "Metabolic syndrome and type-1 diabetes mellitus: prevalence and associated factors," *Revista Española de Cardiología*, vol. 63, no. 4, pp. 423–429, 2010.

[49] R. Nishimura, R. E. LaPorte, J. S. Dorman, N. Tajima, D. Becker, and T. J. Orchard, "Mortality trends in type 1 diabetes: the allegheny county (Pennsylvania) registry 1965–1999," *Diabetes Care*, vol. 24, no. 5, pp. 823–827, 2001.

[50] P. Hovind, L. Tarnow, K. Rossing et al., "Decreasing incidence of severe diabetic microangiopathy in type 1 diabetes," *Diabetes Care*, vol. 26, no. 4, pp. 1258–1264, 2003.

[51] G. Pambianco, T. Costacou, D. Ellis, D. J. Becker, R. Klein, and T. J. Orchard, "The 30-year natural history of type 1 diabetes complications: the Pittsburgh epidemiology of diabetes complications study experience," *Diabetes*, vol. 55, no. 5, pp. 1463–1469, 2006.

[52] B. Essén-Gustavsson and P. A. Tesh, "Glycogen and triglyceride utilization in relation to muscle metabolic characteristics in men performing heavy-resistance exercise," *European Journal of Applied Physiology*, vol. 61, no. 1-2, pp. 5–10, 1990.

[53] S. Ohfuji, "Generalized muscular steatosis (pseudohypertrophy) in a heifer," *Comparative Clinical Pathology*, vol. 22, no. 4, pp. 785–788, 2013.

[54] I. Lingvay, E. D. Roe, J. Duong, D. Leonard, and L. S. Szczepaniak, "Effect of insulin versus triple oral therapy on the progression of hepatic steatosis in type 2 diabetes," *Journal of Investigative Medicine*, vol. 60, no. 7, pp. 1059–1063, 2012.

[55] M. Waguri, K. Yamamoto, J.-I. Miyagawa et al., "Demonstration of two different processes of β-cell regeneration in a new diabetic mouse model induced by selective perfusion of alloxan," *Diabetes*, vol. 46, no. 8, pp. 1281–1290, 1997.

[56] A. B. Goldfine, M.-E. Patti, L. Zuberi et al., "Metabolic effects of vanadyl sulfate in humans with non-insulin-dependent diabetes mellitus: in vivo and in vitro studies," *Metabolism*, vol. 49, no. 3, pp. 400–410, 2000.

[57] M. C. Cam, R. W. Brownsey, and J. H. McNeill, "Mechanisms of vanadium action: insulin-mimetic or insulin-enhancing agent?" *Canadian Journal of Physiology and Pharmacology*, vol. 78, no. 10, pp. 829–847, 2000.

[58] A. Mohammad, V. Sharma, and J. H. McNeill, "Vanadium increases GLUT4 in diabetic rat skeletal muscle," *Molecular and Cellular Biochemistry*, vol. 233, no. 1-2, pp. 139–143, 2002.

[59] G. Vardatsikos, M. Z. Mehdi, and A. K. Srivastava, "Bis(maltolato)-oxovanadium (IV)-induced phosphorylation of PKB, GSK-3 and FOXO1 contributes toits glucoregulatory responses," *International Journal of Molecular Medicine*, vol. 24, no. 3, pp. 303–309, 2009.

[60] S. Semiz and J. H. McNeill, "Oral treatment with vanadium of Zucker fatty rats activates muscle glycogen synthesis and insulin-stimulated protein phosphatase-1 activity," *Molecular and Cellular Biochemistry*, vol. 236, no. 1-2, pp. 123–131, 2002.

[61] D. F. Vatner, S. K. Majumdar, N. Kumashiro et al., "Insulin-independent regulation of hepatic triglyceride synthesis by fatty acids," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 4, pp. 1143–1148, 2015.

[62] D. L. Topping and P. A. Mayes, "Insulin and non-esterified fatty acids. Acute regulators of lipogenesis in perfused rat liver," *Biochemical Journal*, vol. 204, no. 2, pp. 433–439, 1982.

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Research Article

Studies of the Effectiveness of Bisphosphonate and Vanadium-Bisphosphonate Compounds *In Vitro* against Axenic *Leishmania tarentolae*

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Leishmaniasis is a disease that is a significant problem for people, especially in tropical regions of the world. Current drug therapies to treat the disease are expensive, not very effective, and/or of significant side effects. A series of alkyl bisphosphonate compounds and one amino bisphosphonate compound, as well as alendronate and zoledronate, were tested as potential agents against *Leishmania tarentolae*. Also, two polyoxometalates (POMs) with nitrogen-containing bisphosphonate ligands, vanadium/alendronate ($V_5(Ale)_2$) and vanadium/zoledronate ($V_3(Zol)_3$), were tested against *L. tarentolae* and compared to the results of the alendronate and zoledronate ligands alone. Of the compounds evaluated in this study, the $V_5(Ale)_2$ and $V_3(Zol)_3$ complexes were most effective in inhibiting the growth of *L. tarentolae*. The $V_5(Ale)_2$ complex had a larger impact on cell growth than either alendronate or orthovanadate alone, whereas zoledronate itself has a significant effect on cell growth, which may contribute to the activity of the $V_3(Zol)_3$ complex.

1. Introduction

Leishmaniasis is a disease caused by the *Leishmania* protozoan parasite that occurs in the tropical regions of Africa and Asia as well as Central and South America [1]. This disease is a significant problem for people in some 80–90 countries; it is estimated that 1.3 million new cases and 20,000–30,000 deaths from the diseases can be expected each year [1]. *Leishmania* are parasitic trypanosomatids along with *Trypanosoma brucei* and *Trypanosoma cruzi* [2]. The three genera are a specific group of disease-causing kinetoplastid protozoa with a single flagellum. Kinetoplast protozoa are characterized by a single mitochondrion near the flagellum which contains DNA in a small compartment called a kinetoplast [2]. More than 20 different species of *Leishmania* can infect humans, and there are approximately 30 species of sand flies, the alternate host, that can spread the disease to humans. Other species of *Leishmania* can infect dogs, cats, goats, reptiles, and other animals [3]. Since the publication of the genomes of several species of *Leishmania* [4–7], there has been even more widespread study of the organisms and possible therapeutic avenues for leishmaniasis.

Drug therapies, including pentavalent antimonials, pentamidine (Nebupent™), amphotericin B (Fungizone™), and miltefosine (Miltex™), are currently employed to treat leishmaniasis, each with different mechanisms of action [8]. The pentavalent antimonial drugs are believed to inhibit parasitic glycolysis, fatty acid beta-oxidation, and ADP phosphorylation and their exact mechanism of activity is not known [8] although trypanothione *S*-transferase has been shown to play a key role [9, 10]. Amphotericin B is an antifungal agent causing parasitic cell lysis. Pentamidine interferes with the replication and transcription of genetic material in the parasite's mitochondria, and miltefosine is believed to disrupt parasitic

cell surface receptors and change inositol, phospholipase, and protein kinase C metabolism [8]. These therapies are mostly given systemically and not topically. However, none of these drugs have been found to be satisfactory in meeting the needs of drug therapy, which are to be effective, economical, and have minimal side effects. Amphotericin B and miltefosine are expensive drugs and cause complications, and disease resistance to pentavalent antimonials is now widespread in India [11–14]. Development of better drugs for the treatment of leishmaniasis is needed because it would improve the quality of life for millions of people; thus new therapy targets should prove useful. One possible option is bisphosphonate compounds.

Bisphosphonate compounds are reported to have antibacterial, herbicidal, anticancer, and antiparasitic properties, and they are also reported to be involved in the activation of T cells [15–17]. Bisphosphonates derived from fatty acids have been reported to adversely affect *T. cruzi* and other trypanosomatids [15, 16]. Therefore, evaluation of the effects of bisphosphonate compounds on *Leishmania* parasites is warranted. Docampo and Moreno [17] report that some bisphosphonate compounds inhibit the growth of trypanosomatid parasites including *Leishmania donovani* both *in vitro* and *in vivo*. Studies point to the ability of bisphosphonates to inhibit the farnesyl pyrophosphate synthase (FPPS) enzyme in the parasite's cytosol as their mechanism of activity [17]. *Leishmania major* promastigotes, genetically modified to overexpress the FPPS enzyme, were less affected by the bisphosphonate risedronate, and the effect of the bisphosphonate on the parasite decreased as the enzyme's activity increased [17]. Several nitrogen-containing bisphosphonate compounds, namely, alendronate, zoledronate, ibandronate, and risedronate (Figure 1, **8**–**11**), often in their acid form, are currently used to treat conditions including osteoporosis, Paget's disease, hypercalcemia, bone tumors, and other bone diseases [18]. These four nitrogen-containing bisphosphonate compounds are geminal bisphosphonates with a P-C-P backbone [19]. Bisphosphonates, then, are worth examining as antileishmanial therapeutic agents.

Metal complexes have also been examined as antileishmanial agents, including complexes containing vanadium [20–24]. Vanadium is a well-known phosphatase inhibitor [25–29], and we have previously examined phosphatase inhibition by vanadium complexes as an avenue for anti-*Leishmania* impacts by vanadium complexes [21, 22]. Given the effective metal-complexing behavior of bisphosphonates, some synergistic effects of bisphosphonates and metal complexes may be expected. For example, polyoxometalates (POMs; reviewed by [30]), anionic metal and oxygen clusters, are known to adversely affect tumors and viruses as well [15] and POMs of Mo^{V} or Mo^{VI} ion complexes with bisphosphonates have been studied against three different human tumor cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (lung large cell), and SF-268 (central nervous system glioblastoma) [19]. Compain et al. [19] found that a Mo^{VI}-alendronate complex was most effective against all three lines and that the Mo-POM and bisphosphonate ligand have a synergistic effect. El Moll et al. [15, 31] found similar metal-bisphosphonate efficacy against the same cell lines in an extended study, with the most effective being V-zoledronate complexes.

Demoro et al. [32] reported that copper, cobalt, manganese, and nickel metal complexes with the bisphosphonate ligand alendronate or pamidronate were effective against *Trypanosoma cruzi* amastigotes, the trypanosomatid that causes Chagas disease [32]. Fernández et al. [33] found that V^{IV} complexes adversely affected *T. cruzi*, but V^{V} complexes were ineffective against the parasite. Their data also indicated that the effectiveness of the compounds was related to the stability of the V^{IV} complexes [33].

Docampo and Moreno [17] note the existence of a proton translocating pyrophosphatase (V-H^+-PPase) enzyme localized in the acidocalcisome in some parasitic protozoa including *L. donovani* and *L. amazonensis* as another potential drug target for bisphosphonate compounds. The acidocalcisome is an organelle with a high concentration of calcium and phosphate ions found in pathogenic microorganisms, green algae and slime molds. Bisphosphonate compounds are analogs of pyrophosphates and have been shown to inhibit V-H^+-PPase in mung bean plants [17]. Docampo and Moreno speculate that bisphosphonates targeting this enzyme activity could be a new direction in the treatment of leishmaniasis with bisphosphonates [17].

Here we report the effects of a series of four alkyl bisphosphonate compounds, one amino alkyl bisphosphonate compound, as well as alendronate (Ale) and zoledronate (Zol), on axenic *Leishmania tarentolae*. Two polyoxometalates (POMs), vanadium/alendronate ($(NH_4)_2Rb_2[V_5O_9(OH)_2(H_2O)(O_3PC(C_3H_9N)OPO_3)_2]\cdot 8H_2O$, **6**, $V_5(Ale)_2$) and vanadium/zoledronate ($Na_3[V_3O_4(O_3PC(C_4H_6N_2)OPO_3)_2]\cdot 8H_2O$, **7**, $V_3(Zol)_3$), were also tested with *Leishmania tarentolae* and results were compared to those with the alendronate and zoledronate ligands alone. We expected to determine whether (or not) these bisphosphonate compounds would have a negative effect on the *L. tarentolae* parasite *in vitro*.

2. Materials and Methods

2.1. Chemicals and Materials. The following bisphosphonate compounds (Figure 1) were tested with axenic *Leishmania tarentolae* in culture: 1,3-propyl bisphosphonate (**1**), 1,4-butyl bisphosphonate (**2**), 1,5-pentyl bisphosphonate (**3**), 1,6-hexyl bisphosphonate (**4**) (**1**–**4** obtained from acids provided by A. Herlinger, Loyola University, Chicago, IL), and 1-aminodecane-1,1-bisphosphonate (**5**, commercially available from Sigma-Aldrich Chemical Co., St. Louis, MO). Sodium alendronate ($Na\mathbf{8}\cdot 3H_2O$, Alfa Chemical, Berkshire, England) and zoledronic acid hydrate (**9**, Santa Cruz Biotechnology, CA) were obtained from commercial sources. Vanadium POM bisphosphonate analogues $V_5(Ale)_2$ ($(NH_4)_2Rb_2[V_5O_9(OH)_2(H_2O)(O_3PC(C_3H_9N)OPO_3)_2]\cdot 8H_2O$, **6**) and $V_3(Zol)_3$ ($Na_3[V_3O_4(O_3PC(C_4H_6N_2)OPO_3)_2]\cdot 8H_2O$, **7**) formed from alendronate or zoledronate, respectively, were synthesized as previously described [15].

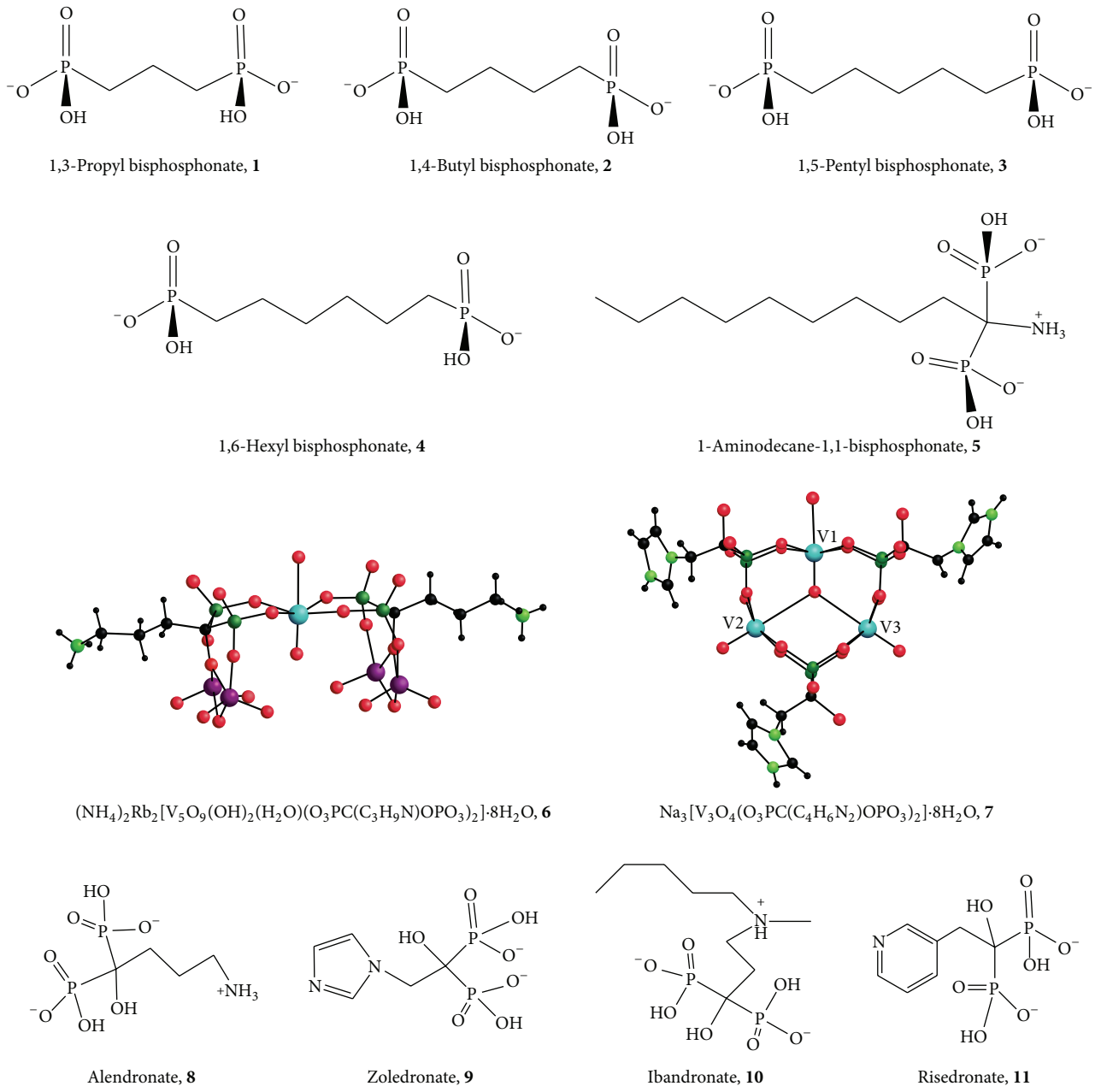

Figure 1: Bisphosphonate compounds. Compounds **1**–**5** are expected to be doubly deprotonated at the experimental pH (7.5) resulting in a net negative charge.

2.2. Leishmania tarentolae. *Leishmania tarentolae* is a species of *Leishmania* parasitic protozoa that infects reptiles and has been shown to be a good model system for testing promising compounds that have anti-*Leishmania* activity [34], although there are differences in the genomes [7] and different species of trypanosomes are known to react differently to the same treatment [16]. *Leishmania tarentolae* (ATCC 30143) have a predictable growth pattern which lends itself to visual observation of cell health. The parasites were grown at room temperature in 25 cm^2 canted flasks (Corning, Inc.; Product number 430372) in sterile brain heart infusion (BHI) medium (BHI; Becton, Dickinson and Co., Sparks, MD; Product number 211059). BHI powder (18.5 g) was mixed with 500 mL of nanopure water and then autoclaved for 21 min. at 250°F and 20 pounds per square inch of pressure using the gravity setting. After autoclaving and cooling, 2 mL of 2.5 mM sterile hemin and 5 mL penicillin/streptomycin (10,000 units/mL and 10 mg/mL, resp.; Sigma-Aldrich Chemical Co., St. Louis, MO) were added to the growth medium using the standard methods of Morgenthaler et al. [35].

The parasites were observed microscopically to monitor the effect of each compound on the parasite. A Jenco International, Inc. (Portland, OR) inverted compound microscope Model CP-2A1 was used for microscopic evaluation of the parasite. The microscope could be adjusted to focus on cells at the bottom, middle, or upper parts of the culture flask

which allowed observations of the parasite throughout the culture medium. Images of cells (at 400x magnification) were taken with a Kodak EasyShare C743 digital camera using close up or video mode. The spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Product number M5655-1G, Sigma-Aldrich Chemical Co., St. Louis, MO) cell viability assay [36] was also used as a quantitative measure of cell mitochondrial activity and therefore indirectly measured cell growth. Sample absorbance at 595 nm was determined with an iMark microplate reader (BioRad Laboratories, Hercules, CA). The BHI growth medium alone was considered as a blank value subtracted from the sample absorbance (BHI and cells). Results are reported as corrected absorbance mean ± standard deviation (SD) for $n = 4$ replicates. Microscopic analysis of the cells for motility, shape, and clumping was also employed.

2.3. Sample Preparation of Test Compounds. The seven test compounds (Figure 1) were evaluated for their effect on axenic *Leishmania tarentolae* cells in culture using a uniform cell population. To help adjust for variations in cell number and viability, large cultures were grown in 500 mL of BHI growth medium. The cells were allowed to grow in a shaker incubator for three days at room temperature before distribution as uniform 10 mL aliquots to the Corning flasks. The test compounds were dissolved in 67 mM Tris-Cl buffer, pH 7.5, or dimethyl sulfoxide (DMSO) as indicated; typically a stock solution was prepared by dissolving material in appropriate solvent at 100x final desired concentration. Control cells from the same large culture were grown without added compound or with 1% (v/v) added DMSO to control for that solvent when required. Tested concentrations were based on preliminary studies with sphingomyelinase (not shown) for **1**–**5**. Complex **5** was ineffective at lower concentration [37] so the concentration was increased to correlate with the work previously reported by Compain et al. with Ale [19]. Concentrations for POMs **6** and **7** were chosen based on averages of previously reported values [15].

Cells were incubated with each of the four alkyl bisphosphonates at a final concentration of 10 mM. Cells were also incubated with 1-aminodecane-1,1-bisphosphonate (**5** at 130 μM final concentration), or cells were incubated with 1.0 mM final concentrations of the vanadium POM bisphosphonate compounds (**6**, **7**). However, $V_3(Zol)_3$ (**7**) was not fully soluble in buffer or medium even following sonication despite its solubility in water, warming to 37°C, and vortexing; therefore, the actual solution concentration in the experimental flasks was *lower* than 1.0 mM, but we report the maximum possible value to show the effectiveness of the complex. Seven blank samples, one for each compound, were also prepared by adding the same experimental concentration of each compound to 10 mL of sterile BHI with no cells. For the 1-aminodecane-1,1-bisphosphonate compound (**5**), DMSO alone was added to the blank. The blank for the control cells was BHI growth medium.

The pKa values for two of the alkyl bisphosphonate compounds, 1,3-propyl bisphosphonate and 1,4-butyl bisphosphonate [38], indicate that the molecules are predominantly doubly deprotonated at the experimental pH level of 7.5, and it is expected that 1,5-pentyl bisphosphonate and 1,6-hexyl bisphosphonate are also doubly deprotonated under our experimental conditions.

3. Results

3.1. Importance of Standard Sampling Conditions via Examining Effect on L. tarentolae of 1-Aminodecane-1,1-bisphosphonate. The importance of conducting all tests on uniform batches of cells is illustrated in the growth curves of *Leishmania tarentolae* incubated with and without 1-aminodecane-1,1-bisphosphonate (**5**), a known inhibitor of a phosphodiesterase enzyme and reported to inhibit enzymes involved in membrane phospholipid turnover [39, 40]. Data for two separate experiments are shown in Figures 2(a) and 2(b). For each experiment, the compound was dissolved in 100% DMSO and was added to one flask to give a final concentration of 0.01 mM compound and 1% DMSO. In another flask, cells were grown without the compound with an equal amount of DMSO. The growth curve data suggest that with the added compound (on day 2 of culture) the culture does not result in the same number of viable cells at stationary phase because the maximum absorbance value is lower and the apparent time to stationary phase differs. However, the log phase growth in both cultures appears comparable because the slopes of the growth curve during log phase are similar within and between experiments. Also, the culture with the added compound appears to undergo senescence sooner, suggesting that cells are not dividing or are dying more readily. This experiment was repeated several times, and all time growth curves of cell cultures with and without the compound were plotted. The four replicate experiments resulted in results which were not significantly different (using Student's t-test). The mean slope of the log phase of growth with the compound was 1.37 ± 0.81 relative to 1.07 ± 0.54 without the compound whereas the mean maximum absorbance value at 595 nm (A_{595}) with the compound was 1.38 ± 0.41 relative to 1.52 ± 0.64 without the compound. Thus we conclude that this compound had little effect on the *Leishmania* culture, while also determining that different cultures produce difference cells in all phases of growth. To reduce cell culture variability, subsequent work was all completed with single batches of cell cultures and day 3 aged cells were used for subsequent experiments.

*3.2. L. tarentolae and Test Compounds **1**–**3** Hours after Compound Addition.* A 500 mL batch of cells was prepared and on day 3 of culture the batch was separated into equal volumes in flasks, and different test compounds (**1**–**7**) were added to different flasks. Concentrations of intervention compounds were chosen based on preliminary results, as described in Section 2.3. The cells were examined microscopically within one hour after compound addition. In all of the experimental flasks the cells appeared to have much lower motility compared to the control cells; this reduction in motility leads to more cells found on the bottom of the flask. The cells in the control flask were active and undulating; thus fewer cells end up on the flask bottom. Figure 3 shows control cells

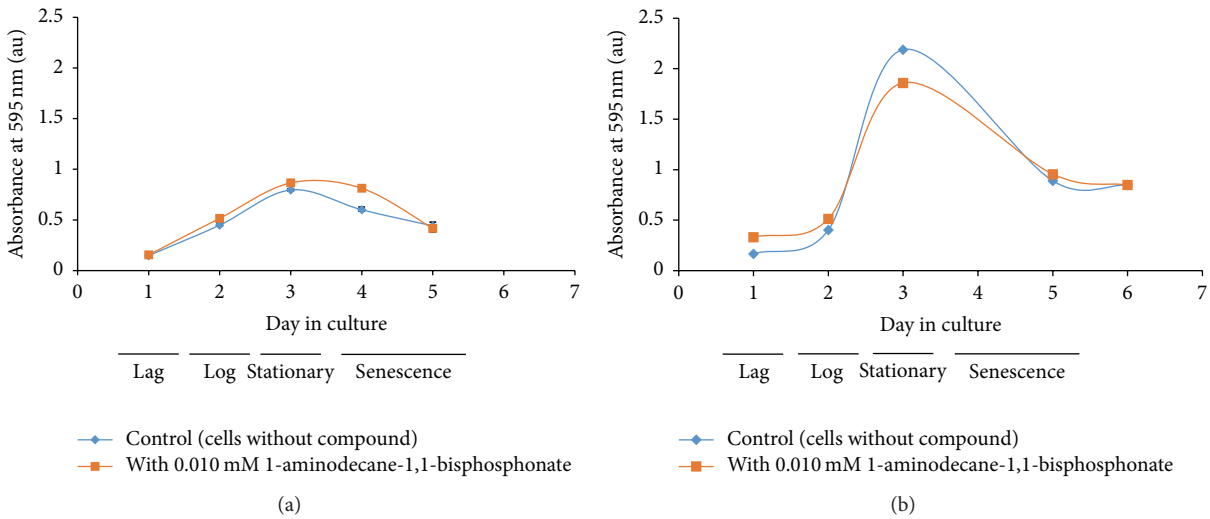

Figure 2: Cell growth curve (absorbance using MTT reagent versus day in culture) of *Leishmania tarentolae* with and without 1-aminodecane-1,1,-bisphosphonate (**5**). (a) and (b) are data from two representative experiments. Compound is added on day 2.

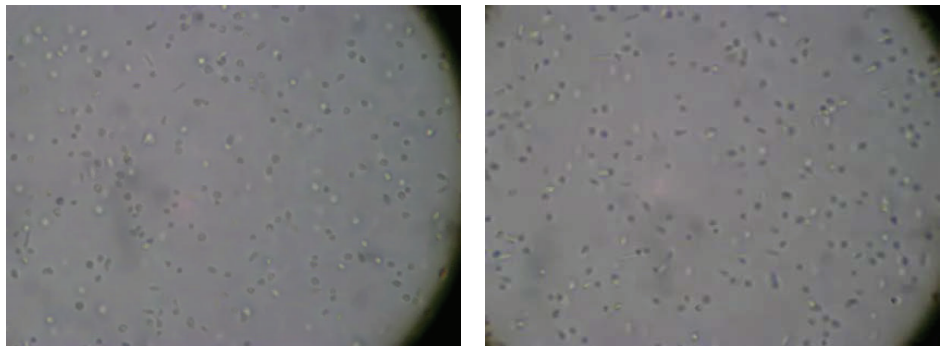

Figure 3: *Leishmania* cells one hour after 10.0 mM 1,4-butyl bisphosphonate addition (**2**) (400x).

and cells incubated with 10.0 mM 1,4-butyl bisphosphonate (**2**). A noticeable motility difference is not obvious in these still photographs; however, these pictures are part of a video file in which the difference in motility was very obvious (see Supplementary Videos in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9025627; control cells or cells 1 hour after addition of 1,4-butyl bisphosphonate).

Three hours after compound addition, 1 mL samples from each flask and its companion blank flask were withdrawn for the MTT cell viability test. Figure 4 shows the resulting corrected MTT absorbance values; additional results, including those as percent of control, are displayed in Figure S1. Samples from flasks with the alkyl bisphosphonates (**1**–**4**) had about 35% less absorbance than the control cells. A sample of cells with the 1-aminodecane-1,1-bisphosphonate (**5**) showed a 28% decrease in absorbance. A sample from cells incubated with $V_5(Ale)_2$ (**6**) had 34% lower absorbance than the control, and a sample from cells with $V_3(Zol)_3$ (**7**) had a 60% decrease in absorbance. Because the MTT assay indirectly measures active mitochondrial reductases to process the MTT reagent, these data suggest a rapid detrimental effect of the test compounds on the test cells. Student's t-test ($p \leq 0.05$) performed with the data statistically distinguished three separate groups based on mean ± SD values. The control is group 1 (∗), six of the seven experimental compounds are group 2 (∗∗), and the last compound, $V_3(Zol)_3$ (**7**, Figure 1), is group 3 (∗∗∗). Data indicate that at three hours after compound addition there was a statistically significant decrease in corrected absorbance value relative to control cells with the $V_3(Zol)_3$ (**7**,Figure 1) treated cells being the most reduced (by some 60% relative to control).

3.3. L. tarentolae and Test Compounds 24–27 Hours after Compound Addition. On the second day of the experiment, the cell cultures (day 4 cells) were examined microscopically, and the cells in flasks with the alkyl bisphosphonates (**1**–**4**) were in poor condition (low motility and more circular shape) with many apparently lifeless cells on the bottom of the flask. The other samples appeared in varying degrees of distress. Figure 5 shows the condition of the cells incubated with 1,4-butyl bisphosphonate (**2**), $V_5(Ale)_2$ (**6**), and $V_3(Zol)_3$ (**7**) (Figures 5(b), 5(c), and 5(d)).

The control sample shows clumping of cells, which is typical during the stationary and senescence phase of *Leishmania*

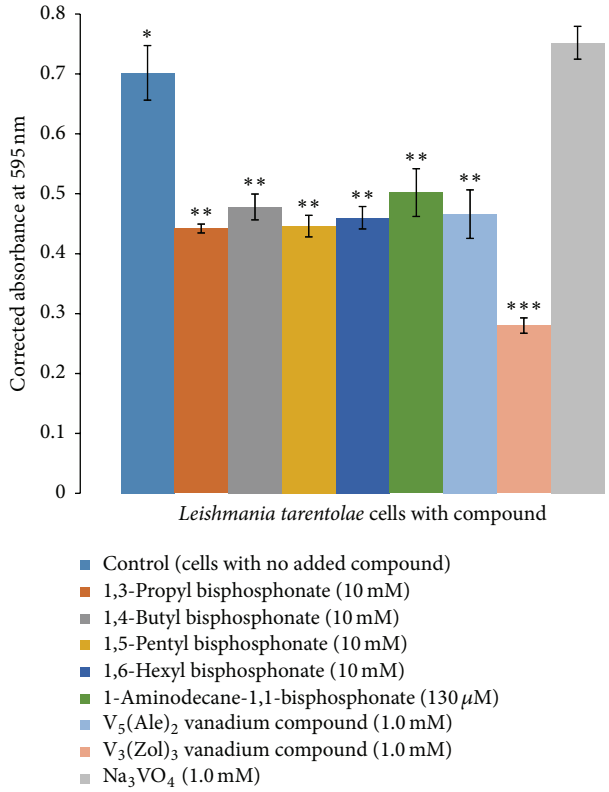

FIGURE 4: MTT cell viability assay of day 3 cells incubated 3 hours with test compounds (mean ± SD, $n = 4$).

growth [22]. Clumping occurs as the cell population increases causing depletion of nutrients and build-up of waste products. The cells incubated with these test compounds showed much less clumping, suggesting that cells are not responding normally. The cells incubated with compounds are more round than the control cells, which also suggests less cell vitality.

One mL samples from each flask sample were withdrawn for viability analysis. At 27 hours after compound addition, MTT results showed that samples from flasks with the alkyl bisphosphonates (**1**–**4**) had approximately 45% less absorbance than the control cells. The samples from flasks with the 1-aminodecane-1,1-bisphosphonate (**5**) or the $V_5(Ale)_2$ (**6**) addition exhibited a 27% decrease in absorbance relative to control cells. The sample from cells treated with $V_3(Zol)_3$ (**7**) had a 37% decrease in absorbance compared to the control cells (Figures 6 and S2).

Student's t-test ($p \leq 0.05$) statistically distinguished four separate groups: the control is group 1 (∗), the alkyl bisphosphonates (**1**–**4**) are group 2 (∗∗), the 1-aminodecane-1,1-bisphosphonate (**5**) and $V_5(Ale)_2$ (**6**) compounds comprise group 3 (∗ ∗ ∗), and the $V_3(Zol)_3$ (**7**) is group 4 (∗ ∗ ∗∗). Thus at 27 hours after adding the various compounds there was a statistically significant decrease in corrected absorbance value for all tested compounds relative to the same age control cells. Cells with the $V_3(Zol)_3$ (**7**) compound appear to be able to recover from the effects of a single addition of this compound (from a 60% decrease in absorbance to a 37% decrease) in about 24 hours. However, this compound was not very soluble in either the buffer in which it was dissolved for the stock solution nor in the BHI cell medium. The solid compound was observed in the flask during the experiment suggesting that its effective concentration in the culture decreased as the experiment progressed. Therefore, this experiment does not accurately measure the concentration dependent effect of $V_3(Zol)_3$ particularly on *L. tarentolae*. Lower concentrations (100–200 μM) were ineffective inhibitors of comparison enzymes [15], so they were not pursued further in this study. It appears that the other test compounds also adversely affect the parasite at the stationary and senescence stages because there is a significant decline in MTT response on experimental day two, which is day 5 of culture, typically shown as late in the culture growth phase.

3.4. Incubation of L. tarentolae with Alendronate, Zoledronate, and Orthovanadate. *L. tarentolae* were incubated with each of the ligands associated with the experimental vanadium compounds as well as the standard sodium orthovanadate, Na_3VO_4. Cells incubated with 0.02–2.0 mM alendronate (**8**) for 27 hours or zoledronate (**9**) at 0.03–0.3 mM for 27 hours exhibited no loss of cell viability or microscopic changes (data not shown). There was no significant difference between the cells grown without alendronate or cells grown with alendronate at concentrations of 0.02 mM, 0.2 mM, or 2.0 mM. The cells treated with 0.03 and 0.3 mM concentrations of zoledronate were not different from control cells after 27 hours of incubation. However, a decrease of approximately 50% in cell viability was observed with cells incubated with 3.0 mM zoledronate (**9**) for 27 hours as compared to the control cells. These data suggest that the adverse effects we observed on *L. tarentolae* after 27 hours of incubation with either vanadium bisphosphonate compound at 1.0 mM are due to the intact complexes **6** and **7** themselves and not due to dissociated ligands **8** or **9**, respectively.

3.5. Effects of Varying Dosages of 1,4-Butyl Bisphosphonate and $V_5(Ale)_2$ on Leishmania. As a representative sample two of the test compounds, 1,4-butyl bisphosphonate (**2**) and $V_5(Ale)_2$ (**6**), were selected to test in dose-response experiments with *L. tarentolae* using the cell batch method. Ten flasks were prepared for each compound: five with 10 mL of day 3 cells in BHI and five with 10 mL BHI for use as the appropriate blank. Compounds were prepared as earlier described, and concentrations of compounds (0.1–10.0 mM for 1,4-butyl bisphosphonate (**2**) and 0.01 to 1.0 mM for $V_5(Ale)_2$ (**6**)) were added to flasks with cells in BHI and the BHI only flasks. After the compounds were added, the cells were left undisturbed overnight. Samples from each flask were evaluated microscopically (Figure 7 and Supporting Video) and tested for MTT cell viability (Figures 8 and 9). Microscopically, the control cells appeared healthy with actively moving parasites; however, the cells with the highest concentration of 1,4-butyl bisphosphonate (**2**, Figure 1) on day 2 after addition (Figure 7(b) and Supporting Video) appeared round and stationary on the flask bottom which suggests cell stress and even death. The cells with the highest concentration of $V_5(Ale)_2$ (**6**) on day 2 after addition appeared dense and

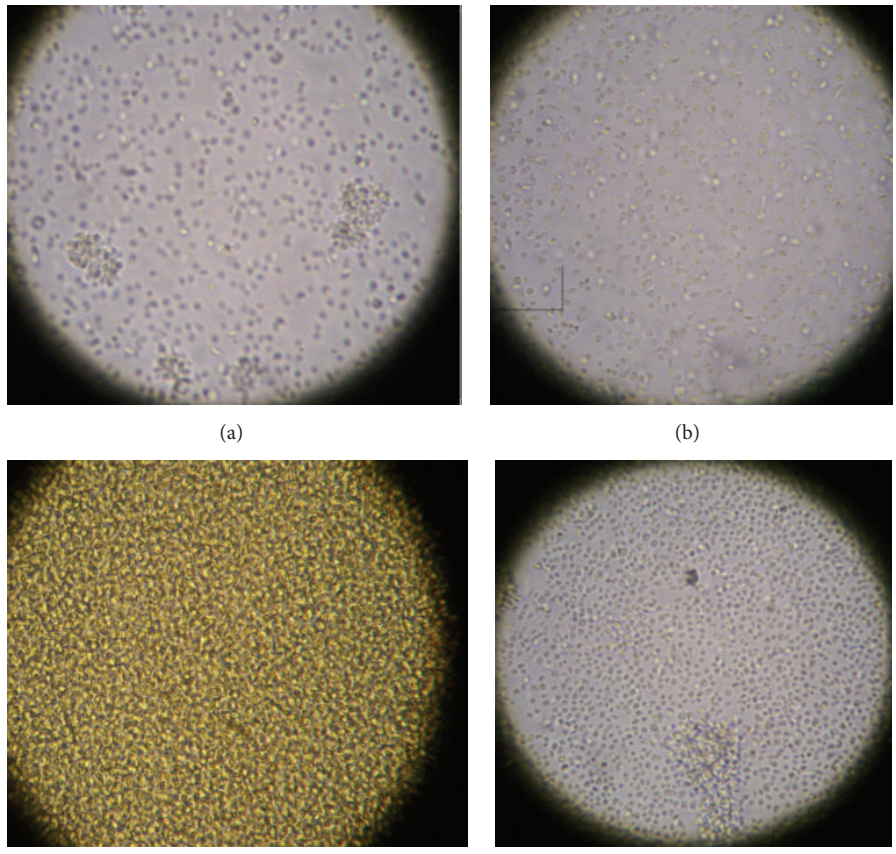

Figure 5: Microscopic observation of day 2 after addition of *Leishmania tarentolae* (a) control cells and cells incubated with representative test compounds (b) 1,4-butyl bisphosphonate (**2**, 10.0 mM), (c) $V_5(Ale)_2$ (**6**, 1.0 mM), and (d) $V_3(Zol)_3$ (**7**, 1.0 mM) for 24 hours (400x). See Figure 1 for structures.

lifeless on the bottom of the flask relative to control cells (Figure 7(c)).

The MTT analysis of samples from flasks with 1,4-butyl bisphosphonate (**2**) on day one after addition (Figure 8) showed that those with the least amount of compound (0.1 to 1.0 mM) had MTT absorbance values statistically the same as the control cell absorbance. However, the MTT response from cells with the highest compound concentration of **2** (10.0 mM) had an absorbance value 75% less than the control. On day two (Figure 8) after addition, the samples with the least concentrated amounts of compound **2** again had absorbance values not different from the control sample, and cells in these flasks appeared to be growing and dividing like control cells. However, with the highest compound concentration of **2** (10.0 mM), an absorbance value 90% lower than the control was measured which indicates that the cells have a greatly reduced viability.

On day one, the MTT absorbance of the samples incubated with $V_5(Ale)_2$ (**6**) at any concentration (Figure 9) was not different from the cells in the control flask. However on day 2, there was a more profound effect. Overall, the absorbance compared to the control was 46% less in cells with 1.0 mM of the compound, but there was also a marked decrease (30%) in absorbance, for cells with 0.1 mM of compound, compared to control cells. This suggests that $V_5(Ale)_2$ (**6**) affects the cells at a much smaller concentration than does 1,4-butyl bisphosphonate (**2**) as shown in Figure 8.

4. Discussion

Using axenic *Leishmania tarentolae* 1-aminodecane-1,1-bisphosphonate (**5**, Figure 1) was confirmed to be effective at reducing viability in this study; previously Roth et al. [40] had reported that this compound and other bisphosphonates prevented bacterial infections in rats. The alkyl bisphosphonates (**1**–**4**) at 10.0 mM concentration and vanadium POM compounds at 1.0 mM concentration (**6** and **7**) were moderately effective at inhibiting both *Leishmania* motility and viability for at least 27 hours after the addition of a single dose to cultures of cells in log phase of growth. Both microscopic observations and viability tests indicate negative effects with a single dose on axenic cells. These compounds then may represent new therapeutic directions for *Leishmania* work. However, at this time, the mechanism of activity has not been established.

Compain et al. [19] tested POMs of Mo^{V} or Mo^{VI} ions and bisphosphonates on three different human tumor cell lines and found that the compound with the lowest IC_{50} (10 μM)

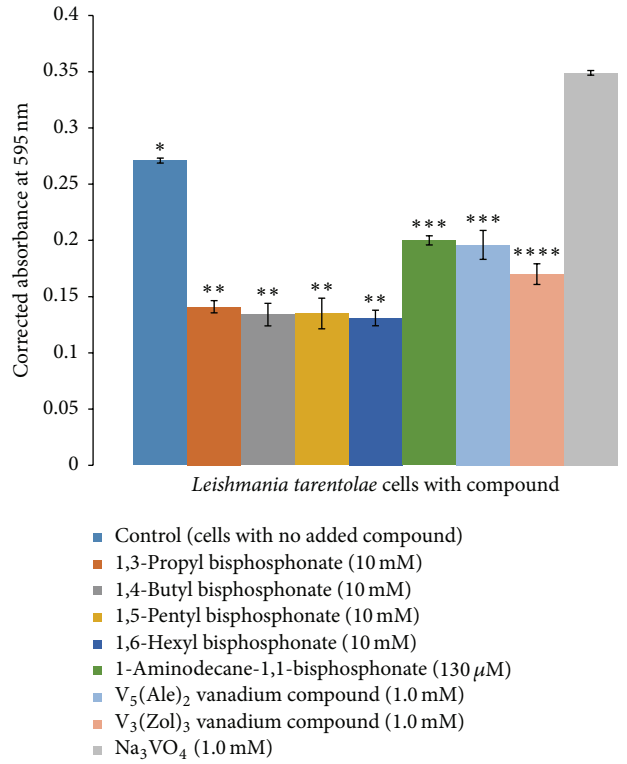

Figure 6: MTT cell viability assay of cells incubated with test compounds after 27 hours (mean ± SD, $n = 4$).

for all three types of tumor cells was a compound with a Mo^{VI} ion and alendronate ligands. Because the alendronate ligands alone had an average IC_{50} of 160 μM when tested on the tumor cells, this suggests that the POM and the alendronate ligands together have a synergistic effect on the tumor cells [19, 31].

The two vanadium POM compounds tested in this study, $V_5(Ale)_2$ (**6**) and $V_3(Zol)_3$ (**7**), have been tested on three tumor cell lines by El Moll et al. [15]. The cell lines are MCF-7 (breast adenocarcinoma), NCI-H460 (lung large cell), and SF-268 (central nervous system glioblastoma). The $V_5(Ale)_2$ (**6**) compound averaged an IC_{50} of 0.6 μM or a 1.2 μM IC_{50} value per one bisphosphonate ligand for the three cell lines. The $V_3(Zol)_3$ (**7**) compound IC_{50} for the three cells lines was 0.3 μM or a 0.9 μM IC_{50} value per one bisphosphonate ligand [15]. In comparison the alendronate (**8**) and zoledronate (**9**) ligands alone when tested by El Moll et al. averaged an IC_{50} value of 150 μM and 9.4 μM, respectively, per compound which is also the per bisphosphonate ligand value. These data as well as the high activity of a fully inorganic compound suggest synergy between vanadium and ligands are less obvious than for the Mo compounds [15, 31]. El Moll et al. also note that they tested vanadium, molybdenum, and tungsten POMs, but the vanadium POMs had the lowest IC_{50} values [15].

Speciation of metal complexes, especially with vanadium [41–43], has been shown to be critical in efficacy of the active species [28]. Interactions of the vanadium with buffers, metabolites, substrates, and enzymes in assay conditions are also well established [44–48]. As such, studies with the POM, VO_4^{3-}, and the ligands individually were performed in each case. Our data show that the alendronate (**8**) ligand alone did not affect the growth of *Leishmania tarentolae* at the experimental concentrations of 0.02, 0.20, or 2.0 mM. The vanadium/alendronate compound, $V_5(Ale)_2$ (**6**), however, adversely affected *Leishmania tarentolae* at a concentration of 1.0 mM after three hours incubation with the compound; there was a 34% decrease in absorbance compared to control cells (Figure S1). This effect was also seen after 27 hours with the compound; the cells incubated with $V_5(Ale)_2$ (**6**) had 27% less absorbance compared to control cells (Figure S2).

The zoledronate (**9**) ligand alone was ineffective at 0.03 to 0.30 mM concentrations, but it did adversely affect the *Leishmania* cells when incubated at 3.0 mM. The absorbance values of cells incubated with 3.0 mM zoledronate for 27 hours were 50% less than control cells. After three hours of incubation with the POM $V_3(Zol)_3$ (**7**), the cells had 60% less absorbance than control cells, and the experimental cells had 37% less absorbance than control cells after 27 hours of incubation with the compound. However, because of the lack of solubility of $V_3(Zol)_3$ (**7**) in cell growth media, the compound could have more adverse effect on the cells than the data indicate.

It is not surprising that zoledronate (**9**) alone at 3.0 mM concentration had an adverse effect on *L. tarentolae* because zoledronate has been reported to be a potent compound. As reported previously, amino bisphosphonates such as alendronate ($R = (CH_2)_3NH_3^+$) are 10–100 times more potent in clinical use than when the R group is CH_3. However, bisphosphonates with a nitrogen atom in a heterocyclic ring such as zoledronate ($R = CH_2(N_2C_3H_3)$) are up to 10,000 times more potent than when R is a methyl group [15].

The difference in potency reported here also is reflected in the dosage of two drugs used to treat osteoporosis. The Physician Desk Reference reports that, in humans, a daily dose of 10 mg alendronate sodium (Fosamax®) is used to treat osteoporosis [49]. In contrast, a 5 mg yearly dose of zoledronate (Reclast®) is used to treat osteoporosis in a human [50].

5. Conclusions

A series of alkyl bisphosphonate compounds and one amino bisphosphonate compound, as well as alendronate and zoledronate, were tested as potential agents against *Leishmania tarentolae*. Given the potency of both zoledronate and the $V_3(Zol)_3$ compounds as well as the difficulty in solubilizing the $V_3(Zol)_3$ in aqueous solutions, we speculate that, if safety and efficacy tests warrant, this POM compound may be effective in a skin cream formulation as a weekly or daily treatment for cutaneous leishmaniasis. This would allow more than a single dose to be easily applied. This is of importance since the majority of *Leishmania* infections are of the cutaneous type [51]. These compounds also have the added advantage of being quite stable at room temperature; thus easy storage and transportation to areas where infections by *Leishmania* are serious problems.

Future studies should examine the effect of the experimental compounds tested in this study on the proton

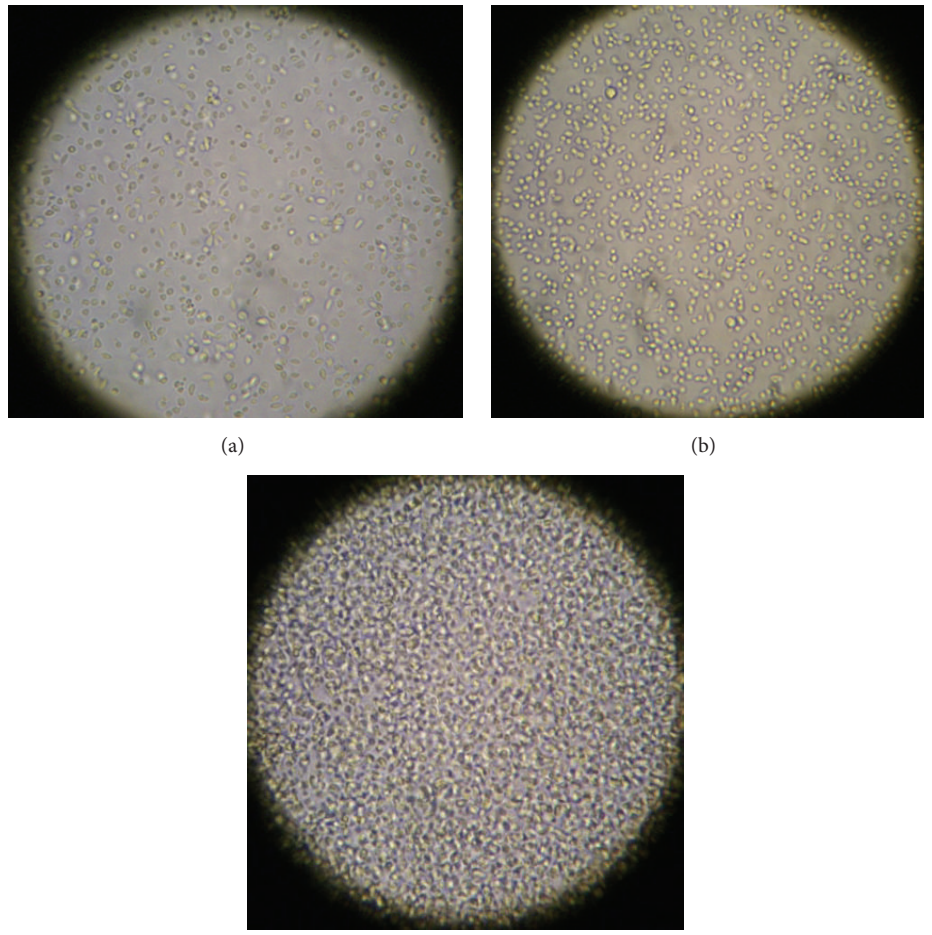

Figure 7: Microscopic evaluation of day 2 after addition of *Leishmania tarentolae* incubated with (b) 1,4-butyl bisphosphonate (**2**, 10 mM) and $V_5(Ale)_2$ (**6**, 1.0 mM) compared to (a) control cells (400x).

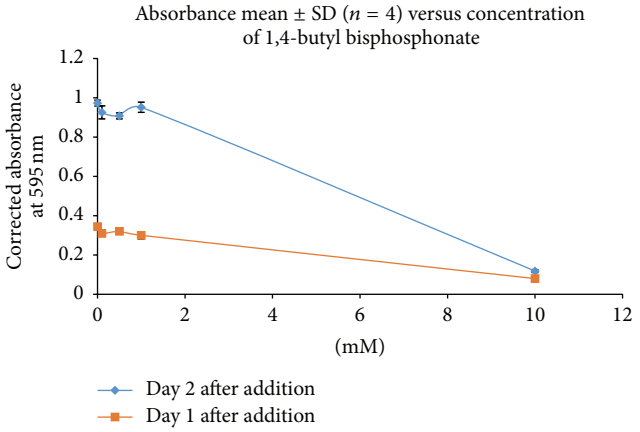

Figure 8: MTT cell viability of *Leishmania tarentolae* incubated with 1,4-butyl bisphosphonate (**2**, Figure 1) compared to control cells.

translocating pyrophosphatase (V-H^+-PPase) enzyme which is localized in the acidocalcisome of at least some of the species of *Leishmania* [17]. These bisphosphonate compounds, which are analogs of pyrophosphates, have been shown to inhibit V-H^+-PPase in other organisms [17] and may be involved in the inhibition of the growth of *Leishmania* in these current studies.

Abbreviations

Ale:	Alendronate, **8**
BHI:	Brain-heart infusion
DMSO:	Dimethylsulfoxide
FPPS:	Farnesyl pyrophosphate synthase
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
POM:	Polyoxometalate
*p*NPP:	*para*-nitrophenylphosphate
PTP:	Protein tyrosine phosphatase
SIM:	Schneider's Insect Medium
TES:	*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tris:	tris(hydroxymethyl)aminomethane hydrochloride
V-H^+-PPase:	Proton translocating pyrophosphatase

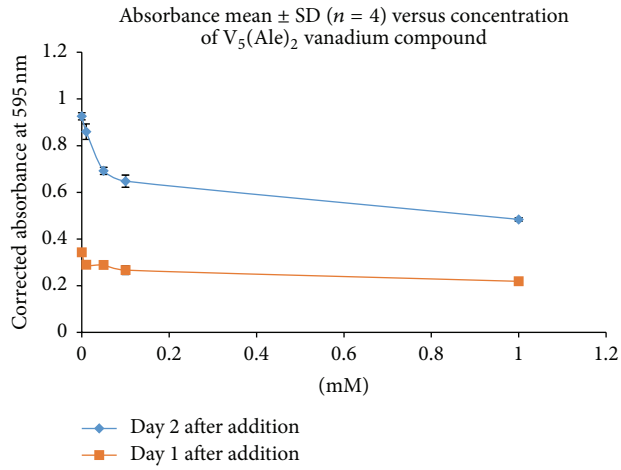

Figure 9: MTT data of *Leishmania tarentolae* incubated with $V_5(Ale)_2$ (**6**) compared to control cells.

$V_3(Zol)_3$: $Na_3[V_3O_4(O_3PC(C_4H_6N_2)OPO_3)_2]\cdot 8H_2O$, **7**
$V_5(Ale)_2$: $(NH_4)_2Rb_2[V_5O_9(OH)_2(H_2O)(O_3PC(C_3H_9N)OPO_3)_2]\cdot 8H_2O$, **6**
Zol: Zoledronate, **9**.

Disclosure

This work was presented in part at the 9th International Vanadium Symposium, Padova, Italy, June 30–July 2, 2014.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

The work presented here was initially designed primarily by Amy T. Christensen and Marjorie A. Jones. Follow-up studies were suggested by Craig C. McLauchlan and Anne Dolbecq. Anne Dolbecq and Pierre Mialane prepared and provided the POM vanadium complexes. The initial paper draft was prepared by Amy T. Christensen as part of her M.S. thesis and revised by Craig C. McLauchlan and Marjorie A. Jones. Anne Dolbecq provided a critical review of the paper. Figures were prepared by Amy T. Christensen and revised by Craig C. McLauchlan. The revised paper was revised primarily by Craig C. McLauchlan with the assistance of all authors. All the authors had final approval of the submitted version of the paper.

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References

[1] June 2011, http://www.who.int/leishmaniasis/en/.

[2] J. Lukeš, D. L. Guilbride, J. Votýpka, A. Zíková, R. Benne, and P. T. Englund, "Kinetoplast DNA network: evolution of an improbable structure," *Eukaryotic Cell*, vol. 1, no. 4, pp. 495–502, 2002.

[3] January 2011, http://www.cdc.gov/parasites/leishmaniasis/.

[4] A. C. Ivens, C. S. Peacock, E. A. Worthey et al., "The genome of the kinetoplastid parasite, *Leishmania major*," *Science*, vol. 309, no. 5733, pp. 436–442, 2005.

[5] C. S. Peacock, K. Seeger, D. Harris et al., "Comparative genomic analysis of three *Leishmania* species that cause diverse human disease," *Nature Genetics*, vol. 39, no. 7, pp. 839–847, 2007.

[6] T. Downing, H. Imamura, S. Decuypere et al., "Whole genome sequencing of multiple *Leishmania donovani* clinical isolates provides insights into population structure and mechanisms of drug resistance," *Genome Research*, vol. 21, no. 12, pp. 2143–2156, 2011.

[7] F. Raymond, S. Boisvert, G. Roy et al., "Genome sequencing of the lizard parasite *Leishmania tarentolae* reveals loss of genes associated to the intracellular stage of human pathogenic species," *Nucleic Acids Research*, vol. 40, no. 3, pp. 1131–1147, 2012.

[8] L. Monzote, "Current treatment of leishmaniasis: a review," *The Open Antimicrobial Agents Journal*, vol. 1, pp. 9–19, 2009.

[9] G. Mandal, S. Wyllie, N. Singh, S. Sundar, A. H. Fairlamb, and M. Chatterjee, "Increased levels of thiols protect antimony unresponsive Leishmania donovani field isolates against reactive oxygen species generated by trivalent antimony," *Parasitology*, vol. 134, no. 12, pp. 1679–1687, 2007.

[10] S. Wyllie, T. J. Vickers, and A. H. Fairlamb, "Roles of trypanothione S-transferase and tryparedoxin peroxidase in resistance to antimonials," *Antimicrobial Agents and Chemotherapy*, vol. 52, no. 4, pp. 1359–1365, 2008.

[11] R. Lira, S. Sundar, A. Makharia et al., "Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of *Leishmania donovani*," *Journal of Infectious Diseases*, vol. 180, no. 2, pp. 564–567, 1999.

[12] S. Sundar, D. K. More, M. K. Singh et al., "Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic," *Clinical Infectious Diseases*, vol. 31, no. 4, pp. 1104–1107, 2000.

[13] S. L. Croft, S. Sundar, and A. H. Fairlamb, "Drug resistance in leishmaniasis," *Clinical Microbiology Reviews*, vol. 19, no. 1, pp. 111–126, 2006.

[14] N. Singh, M. Kumar, and R. K. Singh, "Leishmaniasis: current status of available drugs and new potential drug targets," *Asian Pacific Journal of Tropical Medicine*, vol. 5, no. 6, pp. 485–497, 2012.

[15] H. El Moll, W. Zhu, E. Oldfield et al., "Polyoxometalates functionalized by bisphosphonate ligands: synthesis, structural, magnetic, and spectroscopic characterizations and activity on tumor cell lines," *Inorganic Chemistry*, vol. 51, no. 14, pp. 7921–7931, 2012.

[16] S. H. Szajnman, V. S. Rosso, L. Malayil et al., "1-(Fluoroalkylidene)-1,1-bisphosphonic acids are potent and selective inhibitors of the enzymatic activity of *Toxoplasma gondii* farnesyl pyrophosphate synthase," *Organic & Biomolecular Chemistry*, vol. 10, no. 7, pp. 1424–1433, 2012.

[17] R. Docampo and S. N. J. Moreno, "The acidocalcisome as a target for chemotherapeutic agents in protozoan parasites," *Current Pharmaceutical Design*, vol. 14, no. 9, pp. 882–888, 2008.

[18] P. Sieber, P. Lardelli, C. A. Kraenzlin, M. E. Kraenzlin, and C. Meier, "Intravenous bisphosphonates for postmenopausal osteoporosis: safety profiles of zoledronic acid and ibandronate in clinical practice," *Clinical Drug Investigation*, vol. 33, no. 2, pp. 117–122, 2013.

[19] J.-D. Compain, P. Mialane, J. Marrot et al., "Tetra-to dodecanuclear oxomolybdate complexes with functionalized bisphosphonate ligands: activity in killing tumor cells," *Chemistry—A European Journal*, vol. 16, no. 46, pp. 13741–13748, 2010.

[20] D. Gambino, "Potentiality of vanadium compounds as antiparasitic agents," *Coordination Chemistry Reviews*, vol. 255, no. 19-20, pp. 2193–2203, 2011.

[21] T. L. Turner, V. H. Nguyen, C. C. McLauchlan et al., "Inhibitory effects of decavanadate on several enzymes and *Leishmania tarentolae in vitro*," *Journal of Inorganic Biochemistry*, vol. 108, no. 3, pp. 96–104, 2012.

[22] R. S. Mendez, B. M. Dorsey, C. C. McLauchlan et al., "Vanadium complexes are *in vitro* inhibitors of *Leishmania* secreted acid phosphatases," *International Journal of Chemistry*, vol. 6, no. 1, pp. 35–49, 2014.

[23] I. O. Adriazola, A. E. D. Amaral, J. C. Amorim et al., "Macrophage activation and leishmanicidal activity by galactomannan and its oxovanadium (IV/V) complex in vitro," *Journal of Inorganic Biochemistry*, vol. 132, pp. 45–51, 2014.

[24] P. d. Machado, V. Z. Mota, A. C. Cavalli et al., "High selective antileishmanial activity of vanadium complex with stilbene derivative," *Acta Tropica*, vol. 148, pp. 120–127, 2015.

[25] V. Lopez, T. Stevens, and R. N. Lindquist, "Vanadium ion inhibition of alkaline phosphatase-catalyzed phosphate ester hydrolysis," *Archives of Biochemistry and Biophysics*, vol. 175, no. 1, pp. 31–38, 1976.

[26] L. E. Seargeant and R. A. Stinson, "Inhibition of human alkaline phosphatases by vanadate," *Biochemical Journal*, vol. 181, no. 1, pp. 247–250, 1979.

[27] D. W. Boyd, K. Kustin, and M. Niwa, "Do vanadate polyanions inhibit phosphotransferase enzymes?" *Biochimica et Biophysica Acta (BBA)—Protein Structure and Molecular Enzymology*, vol. 827, no. 3, pp. 472–475, 1985.

[28] C. C. McLauchlan, B. J. Peters, G. R. Willsky, and D. C. Crans, "Vanadium-phosphatase complexes: phosphatase inhibitors favor the trigonal bipyramidal transition state geometries," *Coordination Chemistry Reviews*, vol. 301-302, pp. 163–199, 2015.

[29] C. C. McLauchlan, J. D. Hooker, M. A. Jones et al., "Inhibition of acid, alkaline, and tyrosine (PTP1B) phosphatases by novel vanadium complexes," *Journal of Inorganic Biochemistry*, vol. 104, no. 3, pp. 274–281, 2010.

[30] M. T. Pope and A. Müller, "Polyoxometalate chemistry: an old field with new dimensions in several disciplines," *Angewandte Chemie—International Edition*, vol. 30, no. 1, pp. 34–48, 1991.

[31] A. Saad, W. Zhu, G. Rousseau et al., "Polyoxomolybdate bisphosphonate heterometallic complexes: synthesis, structure, and activity on a breast cancer cell line," *Chemistry*, vol. 21, no. 29, pp. 10537–10547, 2015.

[32] B. Demoro, F. Caruso, M. Rossi et al., "Bisphosphonate metal complexes as selective inhibitors of *Trypanosoma cruzi* farnesyl diphosphate synthase," *Dalton Transactions*, vol. 41, no. 21, pp. 6468–6476, 2012.

[33] M. Fernández, L. Becco, I. Correia et al., "Oxidovanadium(IV) and dioxidovanadium(V) complexes of tridentate salicylaldehyde semicarbazones: searching for prospective antitrypanosomal agents," *Journal of Inorganic Biochemistry*, vol. 127, pp. 150–160, 2013.

[34] V. M. Taylor, D. L. Muñoz, D. L. Cedeño, I. D. Vélez, M. A. Jones, and S. M. Robledo, "*Leishmania tarentolae*: utility as an *in vitro* model for screening of antileishmanial agents," *Experimental Parasitology*, vol. 126, no. 4, pp. 471–475, 2010.

[35] J. B. Morgenthaler, S. J. Peters, D. L. Cedeño et al., "Carbaporphyrin ketals as potential agents for a new photodynamic therapy treatment of leishmaniasis," *Bioorganic & Medicinal Chemistry*, vol. 16, no. 14, pp. 7033–7038, 2008.

[36] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[37] A. T. Christensen, Thesis, Illinois State University, Normal, Ill, USA, 2015.

[38] L. D. Freedman and G. O. Doak, "The preparation and properties of phosphonic acids," *Chemical Reviews*, vol. 57, no. 3, pp. 479–523, 1957.

[39] D. Vizitiu, A. G. Kriste, A. Stewart Campbell, and G. R. J. Thatcher, "Inhibition of phosphatidylinositol-specific phospholipase C: studies on synthetic substrates, inhibitors and a synthetic enzyme," *Journal of Molecular Recognition*, vol. 9, no. 2, pp. 197–209, 1996.

[40] A. G. Roth, D. Drescher, Y. Yang, S. Reamer, S. Uhlig, and C. Arenz, "Potent and selective inhibition of acid sphingomyelinase by bisphosphonates," *Angewandte Chemie—International Edition*, vol. 48, no. 41, pp. 7560–7563, 2009.

[41] L. Pettersson, I. Andersson, and B. Hedman, "Multicomponent polyanions. 37. A potentiometric and ^{51}V-NMR study of equilibria in the H^+-HVO_4^{2-} system in 3.0 M-Na(ClO_4) medium covering the range 1≤-1g[H^+]≤10," *Chemica Scripta*, vol. 25, pp. 309–317, 1985.

[42] L. Pettersson, B. Hedman, I. Andersson et al., "Multicomponent polyanions. 34. P potentiometric and ^{51}V NMR study of equilibria in the H^+-HVO_4^{2-} system in the 0.6 M Na(Cl) medium covering the range $1 \leq -1g[H^+] \leq 10$," *Chemica Scripta*, vol. 22, pp. 254–264, 1983.

[43] D. C. Crans, J. J. Smee, E. Gaidamauskas, and L. Yang, "The chemistry and biochemistry of vanadium and the biological activities exerted by vanadium compounds," *Chemical Reviews*, vol. 104, no. 2, pp. 849–902, 2004.

[44] N. D. Chasteen, "Vanadyl(IV) EPR spin probes inorganic and biochemical aspects," in *Biological Magnetic Resonance*, L. J. Berliner and J. Reuben, Eds., pp. 53–119, Springer, New York, NY, USA, 1981.

[45] N. D. Chasteen, "The biochemistry of vanadium," in *Copper, Molybdenum, and Vanadium in Biological Systems*, B. A. Averill, L. R. Briggs, N. D. Chasteen et al., Eds., vol. 53 of *Structure and Bonding*, pp. 105–138, Springer, Berlin, Germany, 1983.

[46] D. C. Crans, R. L. Bunch, and L. A. Theisen, "Interaction of trace levels of vanadium(IV) and vanadium(V) in biological systems," *Journal of the American Chemical Society*, vol. 111, no. 19, pp. 7597–7607, 1989.

[47] A. Selling, I. Andersson, L. Pettersson, C. M. Schramm, S. L. Downey, and J. H. Grate, "Multicomponent polyanions. 47. The aqueous vanadophosphate system," *Inorganic Chemistry*, vol. 33, no. 14, pp. 3141–3150, 1994.

[48] D. C. Crans, "Enzyme interactions with labile oxovanadates and other polyoxometalates," *Comments on Inorganic Chemistry*, vol. 16, no. 1-2, pp. 35–76, 1994.

[49] Fosamax (alendronate sodium), October 2014, http://www.pdr.net.

[50] Reclast (zoledronate), October 2014, http://www.pdr.net.

[51] J. Alvar, I. D. Vélez, C. Bern et al., "Leishmaniasis worldwide and global estimates of its incidence," *PLoS ONE*, vol. 7, no. 5, Article ID e35671, 2012.

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Review Article

Decavanadate Toxicology and Pharmacological Activities: V_{10} or V_1, Both or None?

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This review covers recent advances in the understanding of decavanadate toxicology and pharmacological applications. Toxicological *in vivo* studies point out that V_{10} induces several changes in several oxidative stress parameters, different from the ones observed for vanadate (V_1). In *in vitro* studies with mitochondria, a particularly potent V_{10} effect, in comparison with V_1, was observed in the mitochondrial depolarization (IC_{50} = 40 nM) and oxygen consumption (99 nM). It is suggested that mitochondrial membrane depolarization is a key event in decavanadate induction of necrotic cardiomyocytes death. Furthermore, only decavanadate species and not V_1 potently inhibited myosin ATPase activity stimulated by actin (IC_{50} = 0.75 μM) whereas exhibiting lower inhibition activities for Ca^{2+}-ATPase activity (15 μM) and actin polymerization (17 μM). Because both calcium pump and actin decavanadate interactions lead to its stabilization, it is likely that V_{10} interacts at specific locations with these proteins that protect against hydrolysis but, on the other hand, it may induce V_{10} reduction to oxidovanadium(IV). Putting it all together, it is suggested that the pharmacological applications of V_{10} species and compounds whose mechanism of action is still to be clarified might involve besides V_{10} and V_1 also vanadium(IV) species.

1. Introduction

The number of review papers and chapters reporting decavanadate biochemistry and biological activities has clearly increased since 2005 [1–5]. Besides the biological activities, decavanadate ($V_{10}O_{28}^{6-}$) as well as other polyoxometalates (POMs) has a wide range of environmental, chemical, and industrial uses and applications in catalysis, nanomaterials, prevention of corrosion, smart glasses, macromolecular crystallography, and food chemistry, among others [6–9].

Decavanadate (V_{10}) species are usually not taken into account in vanadium toxicological studies, although they are well known to affect the activity of several enzymes and to impact lipidic structures [1]. Besides, *in vivo* decavanadate toxicological studies remain seldom [1, 10]. One eventual reason is the consideration that almost 98% of vanadium in cells is present as oxidovanadium(IV), also known as vanadyl (+4 oxidation state), being the intracellular concentration of vanadium (+5, vanadate) very low to decavanadate species be formed. Previously, it was described that V_{10} was formed in acidic compartments in *Saccharomyces cerevisiae* that were grown in media containing vanadate [11]. It has been proposed that once formed the rate of decavanadate decomposition is slow (half-life time of hours) enough to allow observing its effects not only *in vitro* [12], but also *in vivo* [1, 10]. Furthermore, it was suggested that decameric vanadate can be stabilized upon interaction with cytoskeleton and membrane proteins and therefore its contribution to vanadium biochemistry and pharmacological activities can be enlarged [13]. For instance, it was described that rat adipocytes accumulate much more glucose upon decavanadate incubation than with known insulin mimetic agents such as bis(maltolato)oxovanadium(IV) (BMOV) [14]. Besides the insulin mimetic behavior, decavanadate and recent decavanadate compounds show several pharmacological activities such as anticancer, antibacterial, and antivirus [2, 15–17]. These recent findings, which are now briefly reviewed, are evaluated and several hypotheses and V_{10} modes of action

through oxidative stress, effects in mitochondria, sarcoplasmic reticulum, and cytoskeleton, among other biological and pharmacological activities are analyzed.

2. Decavanadate and Oxidative Stress

In the last years, our research group has performed novel *in vivo* studies with decavanadate in order to understand the contribution of decameric vanadate species to vanadium toxic effects [1, 10]. First, at the specific experimental conditions, it was confirmed, using spectroscopy methodologies, if decavanadate is, or not, completely disintegrated into vanadate before inducing changes in several stress markers [1]. Secondly, following decavanadate solutions *in vivo* administration it was evaluated and also compared with monomeric vanadate solutions, several oxidative stress parameters, namely, reduced GSH content, overall rate of ROS production, lipid peroxidation, and antioxidant enzyme activities [1, 10].

First of all, it was concluded that the effects induced by both vanadate and decavanadate depend not only on the concentration but also on other experimental parameters such as the exposure time, cellular fraction, type of tissue, mode of administration, and species of animal [1, 3, 10]. Secondly, in the majority of the studies decavanadate clearly induced more, different, and, in many times, opposite effects than the ones observed for vanadate [3]. Thirdly, oxidative stress induced by decavanadate may be also due to decavanadate decomposition into vanadate [1, 3]. For instance, it was observed that the increase in GSH content upon decavanadate exposure was observed in experimental conditions where V_{10} is almost totally decomposed. The same suggestion was made for the increase in ROS production, with vanadate causing a larger increase in the first hour (150%) whereas decavanadate only caused also an increase (80%) after 12 hours, probably after dissociation into monomeric species [3].

It is known that the cellular detoxification mechanism proposed for vanadate involves bioreduction of vanadate to vanadyl by glutathione (GSH) [18]. Therefore, GSH is an important cellular antioxidant defense system and directly or indirectly regulates the levels of ROS [19, 20]. However, it is proposed that the mechanism for decavanadate detoxification is not the same, as it was suggested for the mechanism of thiol compounds oxidation by similar POMs [21]. Eventually, vanadate reduction by GSH may be delayed if decavanadate species are present. Hence, putative differences in the reactivity towards GSH may explain, at least in part, the different effects that vanadate and decavanadate solutions have in GSH levels and in ROS production. In the Fenton-like reactions vanadate is reduced to vanadyl with production of $O_2^{\bullet -}$ [22]. It is possible that decavanadate participates in such reactions as well as in the GSH oxidation in a different manner and/or extension. On the other hand, lipid peroxidation is commonly described as a consequence of oxidative damage caused by ROS [19, 23]. It was described that lipid peroxidation propagation increased by 55% and 80% after 12 and 24 hours, respectively, in liver mitochondria on exposure to vanadate [1, 3] whereas no increase was evident after 12 hours in the case of decavanadate exposure. However, after 24 hours the effect induced by the latter was the same as that of vanadate [1, 3]. Similar oxidative stress behavior has been described in cardiac tissue [3], confirming that decavanadate seems to have a delayed effect on lipid peroxidation probably due to its decomposition into vanadate. Furthermore, for longer periods after exposure (seven days), decavanadate clearly differs from vanadate once it keeps the levels of lipid peroxidation high [3]. Regarding the antioxidants enzymes, it was suggested globally that decavanadate exposition induces a decrease in mitochondrial antioxidant enzymes activities such as SOD and catalase activities, whereas opposite or no effects were observed for vanadate [3]. Therefore, it is suggested that decavanadate species exposure follows different pathways than vanadate for the generation of reactive oxygen species and interferes differently with some of the enzymes involved in antioxidant defenses in cells. Besides, decavanadate slow decomposition would also induced delayed oxidative stress responses through vanadate species.

3. Mitochondria and Decavanadate Toxicity

Vanadium is a pollutant, and its toxic mechanisms are related to the production of oxidative stress [24]. Mitochondria provide the majority of the energy produced by aerobic organisms and are also often referred to as a major ROS production site. Therefore, mitochondria are a key issue for decavanadate toxicity and a tool to evaluate changes in several oxidative stress parameters, as described in the above section. Several studies pointed out mitochondria as a potential target for vanadium [25, 26] and variety of vanadium compounds, that is, vanadyl sulphate ($VOSO_4$), sodium metavanadate ($NaVO_3$), and vanadyl complexes with organic ligands [27]. Regarding decavanadate *in vivo* studies, it was suggested that the mitochondrial fraction tends to accumulate more vanadium upon decavanadate than upon vanadate administration, besides inducing different changes in mitochondrial antioxidant enzymes activities [1, 3]. This observation was further explored and *in vitro* studies were performed using cardiac mitochondria [28]. These studies showed that decavanadate inhibits mitochondrial respiration and induces mitochondrial membrane depolarization at nM range of decavanadate concentrations (IC_{50} values 40–100 nM) [28, 29]. Decavanadate effects on mitochondrial membrane depolarization and oxygen consumption are about hundredfold more strongly than monomeric vanadate [28]. The heart mitochondria from the fish (*Sparus aurata*) have been shown to be less sensitive to decavanadate than rat heart mitochondria, with IC_{50} values for decavanadate towards membrane depolarization and oxygen consumption that were about four times higher (196 and 400 nM, resp.) than the values found in the rat mitochondria studies (39 and 99 nM, resp.) [3, 28]. One the other side, μM range of vanadate concentration is needed to induce the same effects: IC_{50} of 25 μM and 50 μM, respectively, for instance, for fish heart mitochondria. The effects induced by decavanadate are not due to the uncoupling of the mitochondria or associated with the mitochondrial permeability transition pore (MPTP),

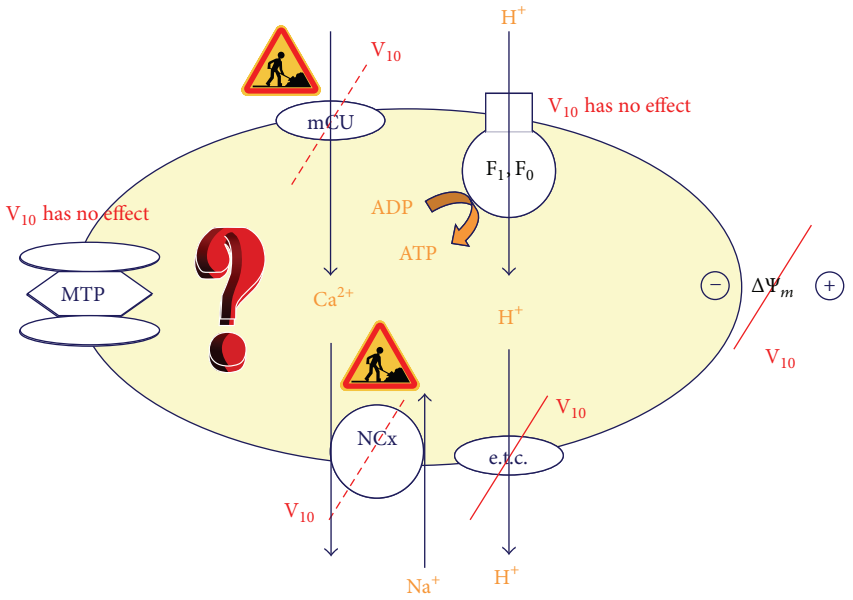

Figure 1: Mitochondria: a target for decavanadate (V_{10}). Oxygen consumption and membrane depolarization are strongly affected whereas no effects were found for MTP and ATP synthase. To our knowledge, V_{10} effects on Ca^{2+} uniporter and Na^+/Ca^{2+} exchanger were not yet described. F_1F_0, F_1F_0-ATP synthase; e.t.c., electron transport chain; mCU, Ca^{2+} uniporter; NCx, Na^+/Ca^{2+} exchanger; MTP, mitochondrial transition pore; Ψ_m, membrane potential.

once the respiratory rate control was not changed or the inhibitor cyclosporine did not prevent effects induced upon decavanadate incubation.

Once the hypothesis that V_{10} affects respiratory complexes I and II is excluded, we considered the possibility that decameric vanadate may interfere with complex III (ubiquinol : cytochrome *c* oxidoreductase). The observed changes in the absorbance at 500–550 nm (0.05 OD increases) upon incubation with V_{10} pointed out that the cytochrome *b* redox state is altered and suggested that complex III is shifted to a more oxidized steady-state. Thus, decavanadate (20 μM total vanadium concentration, i.e., decavanadate) affects the redox state of complex III cytochrome *b*, similarly as the well-known respiratory inhibitor antimycin-A [3, 28, 29]. With a similar total concentration of vanadate (20 μM) no effect was observed [28, 29]. Notice that the respiratory rate control was not changed for either rat or fish heart mitochondria in the presence of either vanadium solution (5.0 ± 0.1) nor did the ADP/O values for pyruvate or malate; the vanadate solutions do not induce uncoupling of the mitochondria [28]. Furthermore, 2 μM decavanadate did not affect NADH content, FoF1-ATPase, and cytochrome *c* oxidase, nor did it affect respiratory complexes I and II, pointing out to a specific V_{10} interaction with complex III (cytochrome *b*) from mitochondrial respiratory chain [3, 28]. The V_{10} effects can be summarized in the scheme present at Figure 1. By inducing mitochondria membrane depolarization and/or by inhibiting mitochondria respiratory chain, it is expected that V_{10} prevents the production of anion superoxide. In fact, both V_{10}-induced mitochondrial depolarization and a decrease of mitochondrial superoxide anion generation can themselves account for a V_{10} antioxidant effect. A potential role for decavanadate in accepting electrons instead of oxygen is suggested. Conversely, V_{10} interaction with complex III would induce a leakage of electrons to molecular oxygen; it would be expected to induce the production of ROS.

Recently, the formation of vanadyl species upon decavanadate incubation with mitochondria (unpublished results), as analyzed by EPR, was observed, whereas no signals were detected upon vanadate incubation. It has been described previously that decavanadate may interact with NADH oxidation by oxygen catalyzed by several enzymes present in membrane systems such as the plasma membrane of red blood cells or rat liver microsomes that leads to reduction of cytochrome *c* [30] and to vanadate reduction. In other studies, decavanadate has been described to be reduced by a specific isocitrate dehydrogenase pointing out to a redox role for decavanadate [31]. These studies suggest an increasing of NADH oxidation by decavanadate, consequent oxygen reduction to H_2O_2, and concomitant reduction to vanadyl species [30, 31].

4. Decavanadate Toxicity Induces Apoptosis or Necrosis?

Vanadium causes a variety of toxic effects such as hematological and biochemical changes [10, 32]. Several studies have shown vanadate effects varying from stimulation of cell growth to induction of cell death [3, 20, 24, 32, 33]. In most cases, the vanadate effect on cell proliferation was biphasic, being cytotoxic for cells over a concentration range of 50 to 100 μM [34]. By targeting mitochondria decavanadate might induce directly or indirectly processes of cell death. Besides, the inhibition of the mitochondrial respiratory chain

described above by decavanadate can lead to sustained mitochondrial depolarization which is lethal for cells demanding a high supply of metabolic energy. Mitochondria are well-known organelle responsible for many features and processes of cell death, such as apoptosis and necrosis, and calcium homeostasis. Cell death is of course a parameter of toxicity and therefore questions arise: did the decavanadate toxicity effects induce cell death? In which way? The answer can be found on the studies that described that, upon incubation for 24 h with either decavanadate or vanadate, the concentrations were found to produce 50% loss of cell viability (1 μM V_{10}, and 10 μM, resp.). Both vanadate species induce cardiomyocytes necrotic cell death, whereas no significant caspase-3 activation was observed [29]. It was also observed that the concentration needed for 50% mitochondrial depolarization was 0.65 μM for V_{10} and 6.5 μM for V_1, that is, only slightly lower than the value obtained for vanadate induced 50% loss of cell viability [29]. Furthermore, depolarization of mitochondria was clearly observed even from 6 hours after addition of decavanadate to cardiomyocytes, suggesting a leading role of mitochondrial depolarization in V_{10}-induced cardiomyocytes death and pointing out as an early event in decavanadate induced necrotic cell death of cardiomyocytes.

It is known that mitochondrial membrane depolarization leads to mitochondrial calcium release [35] and also IP_3-mediated endoplasmic reticulum release in cardiomyocytes [36]. In fact, it was observed that the incubation of both decavanadate and vanadate with cardiomyocytes induces a rise of the basal cytosolic Ca^{2+} from 60 ± 10 nM to 200–250 nM, upon 24 h incubation with 1 μM V_{10} or 10 μM V_1 [29]. These results are in agreement with earlier studies showing that vanadate increased intracellular Ca^{2+} in cultured aortic smooth muscle cells, thereby affecting the vascular tone [37]. In the heart, the release of Ca^{2+} from intracellular stores leads to an increase of heart rate and cardiac inotropism and to vasodilatation [36–38]. Thus, it is strongly suggested that mitochondrial membrane depolarization is a key event in decavanadate induced cardiomyocytes death. As referred to above, the effects described for decavanadate, after 24 hours of incubation, may be due to vanadate upon decavanadate slow decomposition.

5. Sarcoplasmic Reticulum and Decavanadate

Sarcoplasmic reticulum (SR) plays a crucial role in calcium homeostasis and in regulating the process of muscle contraction. SR Ca^{2+}-ATPase is known to be responsible for actively transporting calcium ion, at ATP expenses, into SR lumen, and it plays a major role in the muscle relaxation process. The high sensitivity of the sarco/endoplasmic Ca^{2+}-pumps to vanadate is well documented [39] providing also a simple explanation for the sustained rise of basal cytosolic Ca^{2+} concentration after incubation with vanadate and decavanadate solutions, as described above. However, it was demonstrated more than twenty years ago that decameric vanadate has specific features and interactions with SR Ca^{2+}-ATPase, for instance, by inducing protein crystallization [40]. Besides, it was described that only decavanadate is able to inhibit SR Ca^{2+}-ATPase calcium uptake, whereas no effects were observed for V_1 [41]. Using several different methodologies, it was suggested that decavanadate interaction with the Ca^{2+}-ATPase is noncompetitive versus ATP and that it inhibits strongly the ATPase activity (IC_{50} = 15 μM), in comparison with V_1 (IC_{50} = 80 μM) [12, 39]. In the absence or in the presence of the natural ligand ATP, the interaction of V_{10} with the pump induces vanadate reduction, as analyzed using EPR spectroscopy [42]. During these studies, protein cysteine oxidation was detected upon V_{10} incubation, suggesting the involvement of cysteines at the V_{10} binding site as well as the participating of vanadyl species on the process of enzymatic inhibition. It is well established that the V_{10} binding site, which is formed by three proteins domains [43], is located at the cell cytoplasm side. V_{10} can interact with proteins by electrostatic interaction or by hydrogen bonding but the specific residues involved in V_{10}-SR Ca^{2+}-ATPase interaction, perhaps a cysteine residue, are yet to be totally clarified.

Once decavanadate binding site is located at the cytoplasmic site, V_{10} species would need to cross the SR membrane in order to bind to the E1E2 ATPase. Whereas the interaction between V_1 and the E1E2 ATPase is only favored by the E2 conformation, V_{10} binds with all the two conformations E1 and E2, been or not phosphorylated, thus interacting also with E1P and E2P [12]. Therefore, it is suggested that decavanadate can affect all the steps of the mechanism of calcium translocation by the E1E2 ATPase. Perhaps due to this particularity, V_{10} interaction with the ion pumps might also occur through the extracellular side, as is the case with several other drugs that impact these proteins [3]. By targeting ion pumps without needing to cross the membrane, decavanadate can more rapidly induce changes in calcium homeostasis with implications in, for example, muscle contraction, calcium accumulation in mitochondria, and concomitantly ROS production and cell death.

Studies with the SR Ca^{2+}-ATPase were also performed upon decavanadate *in vivo* intravenous administration [1, 3]. Thus, measurements of the skeletal muscle SR Ca^{2+}-ATPase activity, performed 48 hours upon administration of decavanadate (70.4 ± 6.65 nmol Pi/min/mg), showed that the ATP hydrolysis is significantly increased (52%), whereas vanadate solution exposure decreased it by 15%. These results seem in opposition to previously decavanadate and vanadate inhibition studies performed *in vitro* with the SR Ca^{2+}-ATPase pump. It is difficult to explain why the sarcoplasmic reticulum vesicles prepared from animals previously exposed to V_{10} present higher ATPase activity and are opposite to the ones observed for vanadate. Vanadate is known for its ability to increase the contractile force of heart muscle through its inotropic effect [37, 38, 44]. Apparently, V_{10} affects differently the calcium homeostasis and the samples used contain more calcium or V_{10} interaction would induce the formation of ATPase dimers, eventually relevant for ATPase activity. These very interesting observations that need further studies point out that different responses obtained upon *in vivo* administration cannot be always associated with *in vitro* studies and prove that great care must be taken with

extrapolations from *in vitro* to *in vivo* conditions and vice versa.

6. Decavanadate and Muscle Contraction

Several recent review papers clearly point out that decavanadate presents many biological activities affecting several biological processes and biochemical mechanism including the mechanism of muscle contraction and its regulation [1, 3]. Skeletal muscle cells and vanadium are historically strongly connected to each other, since vanadium was identified as an impurity in commercial ATP prepared from equine muscle [45]. However, the essentiality of vanadium in muscle and globally in humans is yet to be clarified [46]. Myosin, the major ATPase of muscle, interacts with actin during the process of muscle contraction. Although some aspects are poorly understood, during the contractile cycle, the rate limiting step of the ATP hydrolysis is the release of Pi from myosin, which is accelerated by the rebinding of actin [47]. It has originally demonstrated that, in the absence of actin, vanadate inhibits myosin ATPase activity [48]. However, only decavanadate inhibits myosin ATPase activity stimulated by actin [49]. A simple mechanism for the experimentally observed noncompetitive inhibition pattern of V_{10} towards both ATP and actin, as it does not interfere with the nucleotide binding site or with actin binding surface, is by acting as a "back-door" blocking the actomyosin cycle, most likely, in the prehydrolysis state [1, 49, 50].

When we compare the effects of decavanadate on myosin ATPase, Ca^{2+}-ATPase, actin polymerization, and myosin ATPase activity stimulated by actin, the latter presented the higher decavanadate inhibitory capacity with an IC_{50} value of 0.75 μM, whereas higher inhibitory IC_{50} values were found for Ca^{2+}-ATPase activity (15 μM) and for actin polymerization (68 μM) [3, 12, 13, 49]. It was suggested that skeletal muscle myosin contains a high affinity decavanadate binding site, being a potential target for decavanadate [49].

Recent studies also described a specific decavanadate interaction with the actin monomer, G-actin, at the ATP binding site [3, 50]. Actin is one of the most abundant proteins in cells, being involved in many cellular and biological processes. It has been described that vanadium induces actin cytoskeleton damage associated with impaired fertility [51]. However, the studies about "vanadium and actin," and more specifically with "decavanadate and actin," remain scarce [1, 3, 50]. As it was above described for the V_{10}-SR Ca^{2+}-ATPase interaction, also the decavanadate interaction with G-actin leads to cysteine oxidation and vanadyl formation, whereas no reductions were observed upon vanadate incubation [1, 3]. In contrast to the calcium pump, ATP prevents the formation of vanadyl species, confirming that V_{10} binds to the ATP binding site. Both decavanadate and vanadyl inhibit actin polymerization. It was further observed that actin contains a high affinity binding site for vanadyl, as it happens with other proteins such as transferrin and albumin. Therefore, it is suggested that, in the absence of ATP, decavanadate interactions with actin lead to vanadyl protein binding, although the mechanism by which decavanadate inhibits actin polymerization, for instance, through vanadyl formation, is yet to be clarified.

7. Decavanadate Pharmacological Activities

The majority of the studies described above support the concerns over the potential risk of the use of vanadyl sulphate in athletes as a sport supplement [44]. In fact, once vanadium is slowly eliminated from mammalian tissues [52], chronic consumption of vanadium compounds, such as vanadyl sulphate, may eventually reach the toxic levels to cardiomyocytes. Furthermore, decavanadate, vanadate, or vanadyl interferes, although differently, with muscle proteins and with the process of muscle contraction and its regulation. The processes by which vanadyl compounds, commonly used by the bodybuilders, increase muscle mass and enhance muscle power are not understood. In fact, the biochemical processes involved whether being related with muscle bioenergetics, metabolism, and functionality of the contractile systems or through the increasing of the muscle fibers is almost completely unknown. The role of vanadium in muscle cells and its essentiality to humans still remains a mystery.

Regarding the use of vanadate species as anticancer agents and in the chemotherapy of multidrug-resistant tumors, also the studies described above might be in contradiction with this possibility due to its toxic effects, being manifested even at very low concentrations. Nevertheless, as described briefly below, the pharmacological activities of decavanadate, and decavanadate compounds as antidiabetic, antivirus, antibacterial, and antitumor agents, is actually a matter of increasing interest.

For V_{10} alone, it was reported that rat adipocytes incubated with decavanadate at 37°C, thus favoring V_{10} decomposition, accumulate much more glucose than with other known insulin mimetic agents such as BMOV or vanadate [13]. It is suggested that the agents (enzymes, receptors, pumps, or channels) involved in the early events of the process of glucose transport can be enhanced and/or potentiated by V_{10}. However, to our knowledge, the V_{10} mechanism or contribution as an insulin mimetic agent or enhancer is yet to be totally clarified. Eventually, as it was referred for vanadate, decavanadate insulin mimetic effects are probably induced through the inhibition of tyrosine phosphatase (PTP) [53]. Moreover, it was speculated that V_{10} could have a role in treating *Leishmania* diseases through PTP inhibition [53]. Another mechanism includes the use of decavanadate compounds as a prodrug of peroxovanadate insulin mimetics [54]. Crystallization of decavanadate in a spatially selective manner within the protein cages of virions is the most cited paper regarding V_{10} in biology [17]. As it was described in a fundamental review, the antiviral and antitumor activities are the dominant activities of POMs in pharmacology and medicine [55]. It seems that POMs such as decavanadate are able to inhibit the virus activities by preventing the virus-cell host binding [56]. POMs low toxicity toward human body and their high solubility in water are main factors that contributed to their development as drugs.

A chitosan-decavanadate complex with antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was recently described [57]. Chitosan is famous for its antimicrobial activity as it inhibits the mRNA synthesis after penetration into the nuclei of the microorganism. On the other hand, decavanadate is known for its inhibition of ion pumps causing a disturbance in the molecular transport across the membrane thus devastating the bacteria metabolism, presenting altogether an antibacterial inhibition of 12.5 μM. The antitumour activity of decavanadate is less understood and more recent than the antiviral one. New decavanadate complexes have been synthetized and tested their antitumor activity *in vitro* against human lung carcinoma cells (A549) and murine leukaemia cells (P388) [58]. Both compounds exhibited lower inhibition than cisplatin compounds, whereas the decavanadate compound with a higher lipophilic effect, thus enhancing its penetration through the lipid bilayer of the cell membrane, showed higher inhibitory activity [58]. The cytotoxicity of both V_{10} compounds was tested on human normal hepatocytes being more or equally toxic against normal cells compared to effective against cancer cells. Other decavanadate complexes were reported as antitumor agents, showing apoptotic mechanism of cell death and also lower activities than platin compounds [15, 16, 58].

Although the antitumor activity of V_{10} compounds against a large number of tumor cells has been reported, it looks as if their mechanisms of action are still difficult to understand. It was described that polyoxometalates are able to inhibit the tumor growth by inducing apoptosis. Some studies suggest that POMs entered into the mitochondrion leading to the inhibition of ATP synthesis [55]. Although it is speculated that V_{10} effects in mitochondria can be applied for other POMs, these studies are apparently in opposition with the studies described above regarding the process of cell death induced by decavanadate in cardiomyocytes.

Notice that, in the majority of the studies described above, the stability of decavanadate compounds, at the several experimental conditions, was not performed or takes in consideration its putative reduction or decomposition into vanadate species. As described above, although decavanadate toxicological and pharmacological applications differ from vanadate, we cannot exclude a participation of monomeric vanadate. Furthermore, decavanadate toxicity effects and pharmacological activities can be due, at least in part, to V_{10} reduction to vanadyl species. Therefore, although some decavanadate compounds have been shown to be stable, care must be taken before attributing them the toxicity effects or the pharmacological activities [1, 3].

It is known that abnormal levels of alkaline phosphatases (ALP) in the serum are detected in cancer patients since tumors are abnormal cellular growth proliferating faster than a normal cell. Inhibition of ALP will affect tumor cell metabolism and function and therefore POMs were assessed for their inhibitory effect on ALP activity and as putative antitumor agent [59]. V_{10} also demonstrated inhibition on several alkaline phosphatases, suggesting that decavanadate, similarly to other POMs, inhibits abnormal cellular growth proliferating. Despite promising results against virus, bacteria, and tumor cells, polyoxometalates and V_{10} are not yet tested in clinical trials. This may be due to the lack of understanding of its mechanism of action. Besides, to be approved as a drug the polyoxometalate or the V_{10} compounds must show higher activity against tumor cells and very low toxicity toward normal cells.

8. Conclusions

Oxidative stress induced by decavanadate would occur in organisms more often than expected. Decavanadate mechanisms to induce stress might involve the interaction with ion pumps, mitochondria, and specific biochemical processes. The mechanism of necrotic cell death induced by decavanadate is proposed to be mediated through mitochondrial membrane depolarization. The simultaneous effects in ion pumps and in mitochondria promoted by decavanadate lead to an intracellular calcium increase, changes in ROS producing, and inhibition on antioxidant enzymes activities, namely, SOD and catalase. Several major proteins in muscle contraction and its regulation are molecular targets for decavanadate. Particularly interesting is the proposed backdoor mechanism of V_{10} myosin ATPase inhibition stimulated by actin and also the inhibition of actin polymerization by decavanadate, although the latter process is still to be clarified. Some decavanadate compounds seem not suited for antitumour activity since their cytotoxicity was higher than its inhibitory rate of tumor cell growth. However, decavanadate was used with success in antibacterial activity and described to present many other pharmacological applications such as antidiabetic agent besides against virus activities. Putting it all together, it is proposed that the understanding of decavanadate toxicology and pharmacological activities may be useful, at least in part, to elucidate the biological activities of several polyoxometalates in order to make them available and safe for clinical use.

Abbreviations

ALP: Alkaline phosphatase
BMOV: Bis(maltolato)oxovanadium(IV)
GSH: Reduced glutathione
MPTP: Mitochondrial permeability transition pore
$NaVO_3$: Sodium metavanadate
POMs: Polyoxometalates
PTP: Protein tyrosine phosphatase
ROS: Reactive oxygen species
SOD: Superoxide dismutase
SR: Sarcoplasmic reticulum
$VOSO_4$: Vanadyl sulphate
V_1: Vanadate, monomeric vanadate containing 1 vanadate unit
V_{10}: Decavanadate, vanadate oligomer containing 10 vanadate units.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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References

[1] M. Aureliano, "Recent perspectives into biochemistry of decavanadate," *World Journal of Biological Chemistry*, vol. 2, no. 10, pp. 215–225, 2011.

[2] S. Toumi, N. Ratel-Ramond, and S. Akriche, "Decavanadate cage-like cluster templated by organic counter cation: synthesis, characterization and its antimicrobial effect against Gram positive *E. Feacium*," *Journal of Cluster Science*, vol. 26, no. 5, pp. 1821–1831, 2015.

[3] M. Aureliano and C. A. Ohlin, "Decavanadate in vitro and in vivo effects: facts and opinions," *Journal of Inorganic Biochemistry*, vol. 137, pp. 123–130, 2014.

[4] Y. Hayashi, "Hetero and lacunary polyoxovanadate chemistry: synthesis, reactivity and structural aspects," *Coordination Chemistry Reviews*, vol. 255, no. 19-20, pp. 2270–2280, 2011.

[5] X. Chen, S. Yan, H. Wang, Z. Hu, X. Wang, and M. Huo, "Aerobic oxidation of starch catalyzed by isopolyoxovanadate $Na_4Co(H_2O)_6V_{10}O_{28}$," *Carbohydrate Polymers*, vol. 117, pp. 673–680, 2015.

[6] L. Mohapatra and K. M. Parida, "Dramatic activities of vanadate intercalated bismuth doped LDH for solar light photocatalysis," *Physical Chemistry Chemical Physics*, vol. 16, no. 32, pp. 16985–16996, 2014.

[7] A. Bijelic and A. Rompel, "The use of polyoxometalates in protein crystallography—an attempt to widen a well-known bottleneck," *Coordination Chemistry Reviews*, vol. 299, pp. 22–38, 2015.

[8] B. N. Chen, R. Xing, F. Wang, A. P. Zheng, and L. Wang, "Inhibitory effects of α-Na8SiW11CoO40 on tyrosinase and its application in controlling browning of fresh-cut apples," *Food Chemistry*, vol. 188, pp. 177–183, 2015.

[9] S. Yerra, B. K. Tripuramallu, and S. K. Das, "Decavanadate-based discrete compound and coordination polymer: synthesis, crystal structures, spectroscopy and nano-materials," *Polyhedron*, vol. 81, pp. 147–153, 2014.

[10] G. Borges, P. Mendonça, N. Joaquim, J. M. Coucelo, and M. Aureliano, "Acute effects of vanadate oligomers on heart, kidney, and liver histology in the Lusitanian toadfish (*Halobatrachus didactylus*)," *Archives of Environmental Contamination and Toxicology*, vol. 45, no. 3, pp. 415–422, 2003.

[11] G. R. Willsky, D. A. White, and B. C. McCabe, "Metabolism of added orthovanadate to vanadyl and high-molecular-weight vanadates by *Saccharomyces cerevisiae*," *The Journal of Biological Chemistry*, vol. 259, no. 21, pp. 13273–13281, 1984.

[12] G. Fraqueza, C. A. Ohlin, W. H. Casey, and M. Aureliano, "Sarcoplasmic reticulum calcium ATPase interactions with decaniobate, decavanadate, vanadate, tungstate and molybdate," *Journal of Inorganic Biochemistry*, vol. 107, no. 1, pp. 82–89, 2012.

[13] S. Ramos, M. Manuel, T. Tiago et al., "Decavanadate interactions with actin: inhibition of G-actin polymerization and stabilization of decameric vanadate," *Journal of Inorganic Biochemistry*, vol. 100, no. 11, pp. 1734–1743, 2006.

[14] M. J. Pereira, E. Carvalho, J. W. Eriksson, D. C. Crans, and M. Aureliano, "Effects of decavanadate and insulin enhancing vanadium compounds on glucose uptake in isolated rat adipocytes," *Journal of Inorganic Biochemistry*, vol. 103, no. 12, pp. 1687–1692, 2009.

[15] A. Galani, V. Tsitsias, D. Stellas, V. Psycharis, C. P. Raptopoulou, and A. Karaliota, "Two novel compounds of vanadium and molybdenum with carnitine exhibiting potential pharmacological use," *Journal of Inorganic Biochemistry*, vol. 142, pp. 109–117, 2015.

[16] E. Kioseoglou, C. Gabriel, S. Petanidis et al., "Binary decavanadate-betaine composite materials of potential anticarcinogenic activity," *Zeitschrift fur Anorganische und Allgemeine Chemie*, vol. 639, no. 8-9, pp. 1407–1416, 2013.

[17] T. Douglas and M. Young, "Host–guest encapsulation of materials by assembled virus protein cages," *Nature*, vol. 393, no. 6681, pp. 152–155, 1998.

[18] W. Legrum, "The mode of reduction of vanadate(+V) to oxovanadium (+IV) by glutathione and cysteine," *Toxicology*, vol. 42, no. 2-3, pp. 281–289, 1986.

[19] L. S. Capella, M. R. Gefé, E. F. Silva et al., "Mechanisms of vanadate-induced cellular toxicity: role of cellular glutathione and NADPH," *Archives of Biochemistry and Biophysics*, vol. 406, no. 1, pp. 65–72, 2002.

[20] Z. Zhang, S. S. Leonard, C. Huang, V. Vallyathan, V. Castranova, and X. Shi, "Role of reactive oxygen species and MAPKs in vanadate-induced G(2)/M phase arrest," *Free Radical Biology and Medicine*, vol. 34, no. 10, pp. 1333–1342, 2003.

[21] S. Chakrabarty and R. Banerjee, "Kinetics and mechanism of oxidation of 2-mercaptoethanol by the heteropolyoxovanadate $[MnV_{13}O_{38}]^{7-}$," *International Journal of Chemical Kinetics*, vol. 47, no. 1, pp. 13–18, 2015.

[22] S. J. Stohs and D. Bagchi, "Oxidative mechanisms in the toxicity of metal ions," *Free Radical Biology and Medicine*, vol. 18, no. 2, pp. 321–336, 1995.

[23] J. Z. Byczkowski and A. P. Kulkarni, "Oxidative stress and prooxidant biological effects of vanadium," in *Vanadium in the Environment, Part 1: Chemistry and Biochemistry*, J. O. Nriagu, Ed., pp. 235–263, John Wiley & Sons, New York, NY, USA, 1998.

[24] L. Colin-Barenque, J. Pedraza-Chaverri, O. Medina-Campos et al., "Functional and morphological olfactory bulb modifications in mice after vanadium inhalation," *Toxicologic Pathology*, vol. 43, no. 2, pp. 282–291, 2015.

[25] J. Edel and E. Sabbioni, "Accumulation, distribution and form of vanadate in the tissues and organelles of the mussel *Mytilus edulis* and the goldfish *Carassius auratus*," *Science of the Total Environment*, vol. 133, no. 1-2, pp. 139–151, 1993.

[26] W. M. Bracken, R. P. Sharma, and Y. Y. Elsner, "Vanadium accumulation and subcellular distribution in relation to vanadate induced cytotoxicity in vitro," *Cell Biology and Toxicology*, vol. 1, no. 4, pp. 259–268, 1985.

[27] Y. Zhao, L. Ye, H. Liu et al., "Vanadium compounds induced mitochondria permeability transition pore (PTP) opening related to oxidative stress," *Journal of Inorganic Biochemistry*, vol. 104, no. 4, pp. 371–378, 2010.

[28] S. S. Soares, C. Gutiérrez-Merino, and M. Aureliano, "Mitochondria as a target for decavanadate toxicity in *Sparus aurata* heart," *Aquatic Toxicology*, vol. 83, no. 1, pp. 1–9, 2007.

[29] S. S. Soares, F. Henao, M. Aureliano, and C. Gutiérrez-Merino, "Vanadate induces necrotic death in neonatal rat

cardiomyocytes through mitochondrial membrane depolarization," *Chemical Research in Toxicology*, vol. 21, no. 3, pp. 607–618, 2008.

[30] T. Ramasarma and A. V. S. Rao, "Decavanadate interacts with microsomal NADH oxidation system and enhances cytochrome c reduction," *Molecular and Cellular Biochemistry*, vol. 281, no. 1-2, pp. 139–144, 2006.

[31] A. V. S. Rao and T. Ramasarma, "NADH-dependent decavanadate reductase, an alternative activity of NADP-specific isocitrate dehydrogenase protein," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1474, no. 3, pp. 321–330, 2000.

[32] S. K. Ghosh, R. Saha, and B. Saha, "Toxicity of inorganic vanadium compounds," *Research on Chemical Intermediates*, vol. 41, no. 7, pp. 4873–4897, 2015.

[33] L. S. Capella, J. S. M. Alcantara, V. Moura-Neto, A. G. Lopes, and M. A. M. Capella, "Vanadate is toxic to adherent-growing multidrug-resistant cells," *Tumor Biology*, vol. 21, no. 1, pp. 54–62, 2000.

[34] S. B. Etcheverry and A. N. Cortizo, "Bioactivity of vanadium compounds on cells in culture," in *Vanadium in the Environment, Part 1: Chemistry and Biochemistry*, J. O. Nriagu, Ed., pp. 359–395, John Wiley & Sons, New York, NY, USA, 1998.

[35] C. M. O'Reilly, K. E. Fogarty, R. M. Drummond, R. A. Tuft, and J. V. Walsh Jr., "Spontaneous mitochondrial depolarizations are independent of SR Ca^{2+} release," *American Journal of Physiology—Cell Physiology*, vol. 286, no. 5, pp. C1139–C1151, 2004.

[36] B. J. Poindexter, J. R. Smith, L. M. Buja, and R. J. Bick, "Calcium signalling mechanisms in dedifferentiated cardiac myocytes: comparison with neonatal and adult cardiomyocytes," *Cell Calcium*, vol. 30, pp. 373–382, 2001.

[37] L. Sandirasegarane and V. Gopalakrishnan, "Vanadate increases cytosolic free calcium in rat aortic smooth muscle cells," *Life Sciences*, vol. 56, no. 7, pp. PL169–PL174, 1995.

[38] E. Braunwald, "Vanadate increases cytosolic free calcium in rat aortic smooth muscle cells," *Cardioscience*, vol. 5, pp. 139–144, 1994.

[39] P. Caroni and E. Carafoli, "The Ca^{2+}-pumping ATPase of heart sarcolemma. Characterization, calmodulin dependence, and partial purification," *Journal of Biological Chemistry*, vol. 256, no. 7, pp. 3263–3270, 1981.

[40] L. Dux and A. Martonosi, "Two-dimensional arrays of protein in sarcoplasmic reticulum and purified Ca^{2+}-ATPase vesicles treated with vanadate," *The Journal of Biological Chemistry*, vol. 258, no. 4, pp. 2599–2603, 1983.

[41] M. Aureliano and V. M. C. Madeira, "Interactions of vanadate oligomers with sarcoplasmic reticulum Ca^{2+}-ATPase," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1221, no. 3, pp. 259–271, 1994.

[42] G. Fraqueza, L. A. E. Batista de Carvalho, M. P. M. Marques et al., "Decavanadate, decaniobate, tungstate and molybdate interactions with sarcoplasmic reticulum Ca^{2+}-ATPase: quercetin prevents cysteine oxidation by vanadate but does not reverse ATPase inhibition," *Dalton Transactions*, vol. 41, no. 41, pp. 12749–12758, 2012.

[43] S. Hua, G. Inesi, and C. Toyoshima, "Distinct topologies of mono- and decavanadate binding and photo-oxidative cleavage in the sarcoplasmic reticulum ATPase," *The Journal of Biological Chemistry*, vol. 275, no. 39, pp. 30546–30550, 2000.

[44] J. P. Fawcett, S. J. Farquhar, R. J. Walker, T. Thou, G. Lowe, and A. Goulding, "The effect of oral vanadyl sulphate on body composition and performance in weight-training athletes," *International Journal of Sport Nutrition*, vol. 6, pp. 382–390, 1996.

[45] L. Josephson and L. C. Cantley Jr., "Isolation of a potent (Na-K)ATPase inhibitor from striated muscle," *Biochemistry*, vol. 16, no. 21, pp. 4572–4578, 1977.

[46] K. Gruzewska, A. Michno, T. Pawelczyk, and H. Bielarczyk, "Essentiality and toxicity of vanadium supplements in health and pathology," *Journal of Physiology and Pharmacology*, vol. 65, no. 5, pp. 603–611, 2014.

[47] A. Månsson, D. Rassier, and G. Tsiavaliaris, "Poorly understood aspects of striated muscle contraction," *BioMed Research International*, vol. 2015, Article ID 245154, 28 pages, 2015.

[48] C. C. Goodno, "Inhibition of myosin ATPase by vanadate ion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 76, no. 6, pp. 2620–2624, 1979.

[49] T. Tiago, M. Aureliano, and C. Gutiérrez-Merino, "Decavanadate binding to a high affinity site near the myosin catalytic centre inhibits F-actin-stimulated myosin ATPase activity," *Biochemistry*, vol. 43, no. 18, pp. 5551–5561, 2004.

[50] S. Ramos, R. O. Duarte, J. J. G. Moura, and M. Aureliano, "Decavanadate interactions with actin: cysteine oxidation and vanadyl formation," *Dalton Transactions*, no. 38, pp. 7985–7994, 2009.

[51] V. Rodriguez-Lara, A. Morales-Rivero, A. M. Rivera-Cambas, and T. I. Fortoul, "Vanadium inhalation induces actin changes in mice testicular cells," *Toxicology and Industrial Health*, pp. 1–8, 2013.

[52] D. G. Barceloux, "Vanadium," *Journal of Toxicology: Clinical Toxicology*, vol. 37, no. 2, pp. 265–278, 1999.

[53] T. L. Turner, V. H. Nguyen, C. C. McLauchlan et al., "Inhibitory effects of decavanadate on several enzymes and *Leishmania tarentolae* in vitro," *Journal of Inorganic Biochemistry*, vol. 108, pp. 96–104, 2012.

[54] F. Yraola, S. Garcia-Vicente, L. Marti, F. Albericio, A. Zorzano, and M. Royo, "Understanding the mechanism of action of the novel SSAO substrate $(C_7NH_{10})_6(V_{10}O_{28})\cdot 2H_2O$, a prodrug of peroxovanadate insulin mimetics," *Chemical Biology and Drug Design*, vol. 69, no. 6, pp. 423–428, 2007.

[55] J. T. Rhule, C. L. Hill, D. A. Judd, and R. F. Schinazi, "Polyoxometalates in medicine," *Chemical Reviews*, vol. 98, no. 1, pp. 327–357, 1998.

[56] T. Yamase, "Anti-tumor, -viral, and -bacterial activities of polyoxometalates for realizing an inorganic drug," *Journal of Materials Chemistry*, vol. 15, no. 45, pp. 4773–4782, 2005.

[57] Y.-T. Li, C.-Y. Zhu, Z.-Y. Wu, M. Jiang, and C.-W. Yan, "Synthesis, crystal structures and anticancer activities of two decavanadate compounds," *Transition Metal Chemistry*, vol. 35, no. 5, pp. 597–603, 2010.

[58] F. Zhai, X. Wang, D. Li, H. Zhang, R. Li, and L. Song, "Synthesis and biological evaluation of decavanadate $Na_4Co(H_2O)_6V_{10}O_{28}\cdot 18H_2O$," *Biomedicine & Pharmacotherapy*, vol. 63, no. 1, pp. 51–55, 2009.

[59] R. Raza, A. Matin, S. Sarwar et al., "Polyoxometalates as potent and selective inhibitors of alkaline phosphatases with profound anticancer and amoebicidal activities," *Dalton Transactions*, vol. 41, no. 47, pp. 14329–14336, 2012.

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Research Article

Memory Deficit Recovery after Chronic Vanadium Exposure in Mice

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Vanadium is a transitional metal with an ability to generate reactive oxygen species in the biological system. This work was designed to assess memory deficits in mice chronically exposed to vanadium. A total of 132 male BALB/c mice (4 weeks old) were used for the experiment and were divided into three major groups of vanadium treated, matched controls, and animals exposed to vanadium for three months and thereafter vanadium was withdrawn. Animals were tested using Morris water maze and forelimb grip test at 3, 6, 9, and 12 months of age. The results showed that animals across the groups showed no difference in learning but had significant loss in memory abilities after 3 months of vanadium exposure and this trend continued in all vanadium-exposed groups relative to the controls. Animals exposed to vanadium for three months recovered significantly only 9 months after vanadium withdrawal. There was no significant difference in latency to fall in the forelimb grip test between vanadium-exposed groups and the controls in all age groups. In conclusion, we have shown that chronic administration of vanadium in mice leads to memory deficit which is reversible but only after a long period of vanadium withdrawal.

1. Introduction

There has been an increased awareness of accumulating levels of toxic metals in the atmosphere and their relationship with health [1–4]. More pertinent in this regard is the increasing wave of neurodegenerative diseases and the roles that metal exposure may be playing in the pathogenesis of these diseases [5]. Vanadium is a transitional metal of atomic number 23. The metal has outer orbitals that contain eleven electrons in shell 3 and two electrons in shell 4. This arrangement allows for numerous electronic exchange reactions and consequently the formation of a wide range of organic and inorganic complexes that contains vanadium in different oxidation states [6]. Vanadium and its agents are important in several industrial processes. Vanadium is used to improve the hardness, malleability, and fatigue resistance of steel [7]; and vanadium alloys are highly valued in the production of aerospace products [8]. In addition, vanadium agents are utilised in the production of glass, pigments, varnishes, reducing agents, and inks [8] and in fertilizers production [9] amongst others. Vanadium is widely distributed in the environment [10] with exposure to the metal occurring through vanadium mining sites and as contaminants during the mining of some heavy metals [8]. In addition, vanadium is released into the atmosphere through forest fires, volcanic emissions, and burning of fossil fuels in vanadium-contaminated crude as seen in Venezuela, the Arabian Gulf, the Gulf of Mexico, and the Nigerian Niger Delta [1, 6, 11]. Vanadium has been reported as the most abundant trace metal in petroleum samples [12] and accumulates in the soil, groundwater, and vegetation, being consumed by animals and humans [13]. The study of the neurotoxic profile of vanadium has been on the rise in recent years [14–17]. Behavioral changes associated with vanadium exposure include lethargy, tremor, anger hostility, depression-dejection, and various locomotor deficits [10, 14, 15]. In fact, Naylor [18] reported raised levels of vanadium in different body samples of manic and depressed patients while Avila-Costa et al. [19] reported

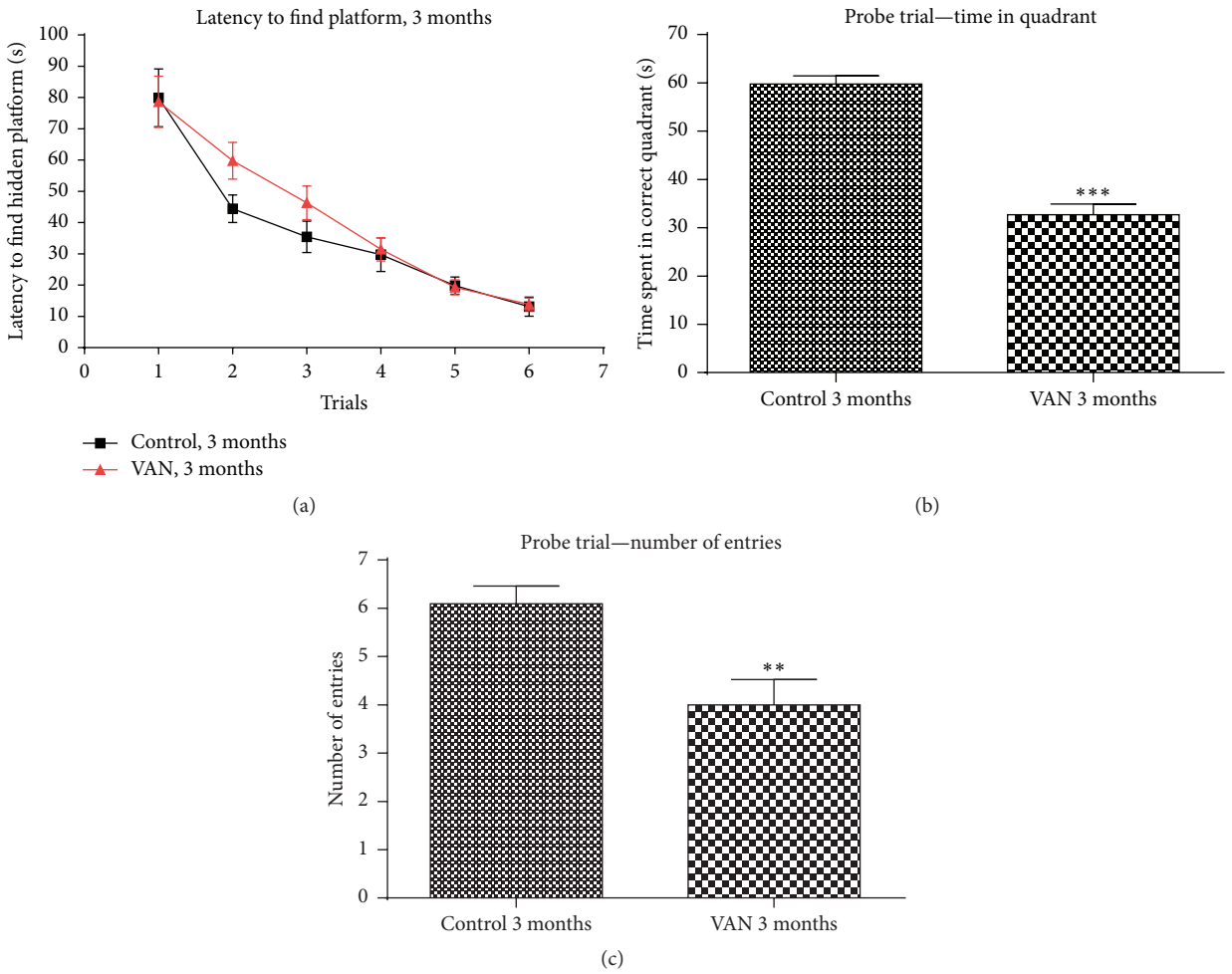

Figure 1: Effect of intermittent vanadium (VAN) treatment for three months on learning and memory in mice: 12 male mice were exposed thrice a week for three months to VAN (3 mg/kg b.w.) with equal number and age matched controls. The ability to learn the location of the hidden platform was unperturbed because they gradually spent shorter times to locate the platform with successive training trials. There was no statistically significant difference between the groups in their learning ability (a) however; the length of time in target quadrant ($^{***}P < 0.001$) and annulus crossings of the VAN group ($^{**}P < 0.01$) were significantly decreased when compared to the controls ((b) and (c)).

time dependent loss of dendritic spines, necrotic like cell death, and profound alterations in the hippocampal CA1 neuropile. Few studies have however reported progressive memory loss induced by vanadium exposure. Most animal experiments on behavioral deficits involving vanadium exposure have been based on acute exposure while in reality many patients occupationally [6] and environmentally [1] exposed to vanadium are so exposed for decades or even a life time. This work is designed to assess memory deficits in rats chronically exposed to vanadium.

2. Materials and Methods

2.1. Animal Design. A total of 132 male BALB/c mice (4 weeks old) were used for the experiment which covered a period of 12 months. The animals were assigned to one of the following animal groups: vanadium- (V-) treated, withdrawal, and control groups. Animal experiments were done in accordance with University of Ibadan Ethical Research Committee guidelines for use of research animals. V-treated group consisted of four subgroups of twelve separate animals each. The subgroups are designated as VAN3, VAN6, VAN9, and VAN12. The mice (from 4 weeks old) were injected with 3 mg/kg b.w./day of vanadium (sodium metavanadate), i.p. thrice a week for 3, 6, 9, and 12 months. After the treatment, the animals in each group ($n = 12$) were subjected to behavioral analysis which included test for cognitive and memory function by using Morris water maze and forelimb grip strength tests; these were carried out on every animal in each group. Withdrawal group consisted of a total of three groups of 12 animals each. The subgroups are designated as W3, W6, and W9. The mice (from 4 weeks old) were injected with 3 mg/kg b.w./day of vanadium (sodium metavanadate), i.p. thrice a week only for the first three months and then vanadium administration was stopped. After the treatment, the animals in each group were subjected to behavioral analysis which included tests for cognitive and memory function as mentioned earlier 3(W3), 6(W6), and 9(W9) months after

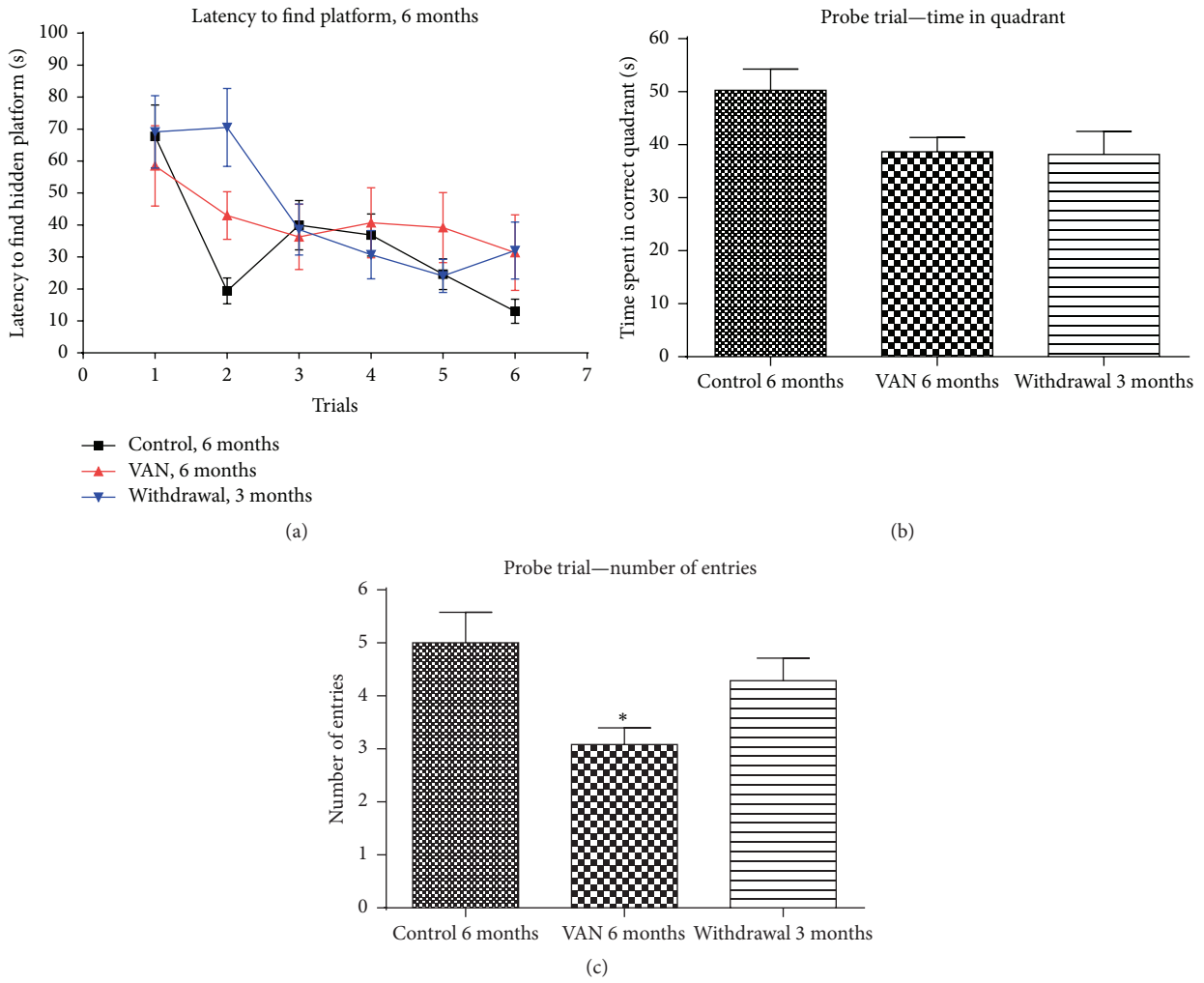

Figure 2: Effect of intermittent vanadium (VAN) treatment for six months on learning and memory in mice: 12 male mice were exposed thrice a week for six months to VAN (3 mg/kg b.w.) with equal number and age matched controls. The withdrawal group were exposed to VAN for three months but replaced with water thereafter. All the groups of the 6-month-old mice (C6, VAN6, and W3) were able to learn the location of the hidden platform and there was no statistically significant difference among the groups in their ability to learn (a) however; the memory retention of the V6 group was significantly decreased when compared to the control group ($^{*}P < 0.05$) ((b) and (c)).

the vanadium treatment had been withdrawn. Control group consisted of four subgroups of twelve different animals each. The subgroups are designated as C3, C6, C9, and C12. The mice (from 4 weeks old) were injected with sterile water, i.p. thrice a week for 3, 6, 9, and 12 months which was volume matched with the V-treated group. After the treatment, the animals were subjected to behavioral analysis which included test for cognitive and memory function as mentioned earlier in V-treated group.

2.2. Behavioral Tests

2.2.1. Forelimb Grip Strength Test. This test involves the forepaws of the mice being placed on a horizontally suspended wire (measuring 2 mm in diameter and 1 m in length), placed one meter above a soft bedding-filled landing area. The latency to fall (i.e., length of time each mice was able to stay suspended before falling off the wire) was recorded with a stopwatch. A maximum time of 2 minutes was given to each mouse after which it was removed and each mouse had two trials. This test reflects muscular strength and balance in the animals [20, 21].

2.2.2. Modified Morris Water Maze Test. The modified Morris water maze is a circular pool of water (110 cm in diameter, 30 cm in height) with a hidden circular escape platform (12 cm in diameter) which the mouse must learn its location using contextual and visual cues. This tests hippocampal-dependent spatial learning and memory in rodents [22, 23]. The task is based on the principle that rodents are highly motivated to escape from a water environment by the fastest, most direct route. The pool was marked North, South, East, and West and the hidden platform was placed in a particular spot. Each mouse was dropped into the pool and expected to

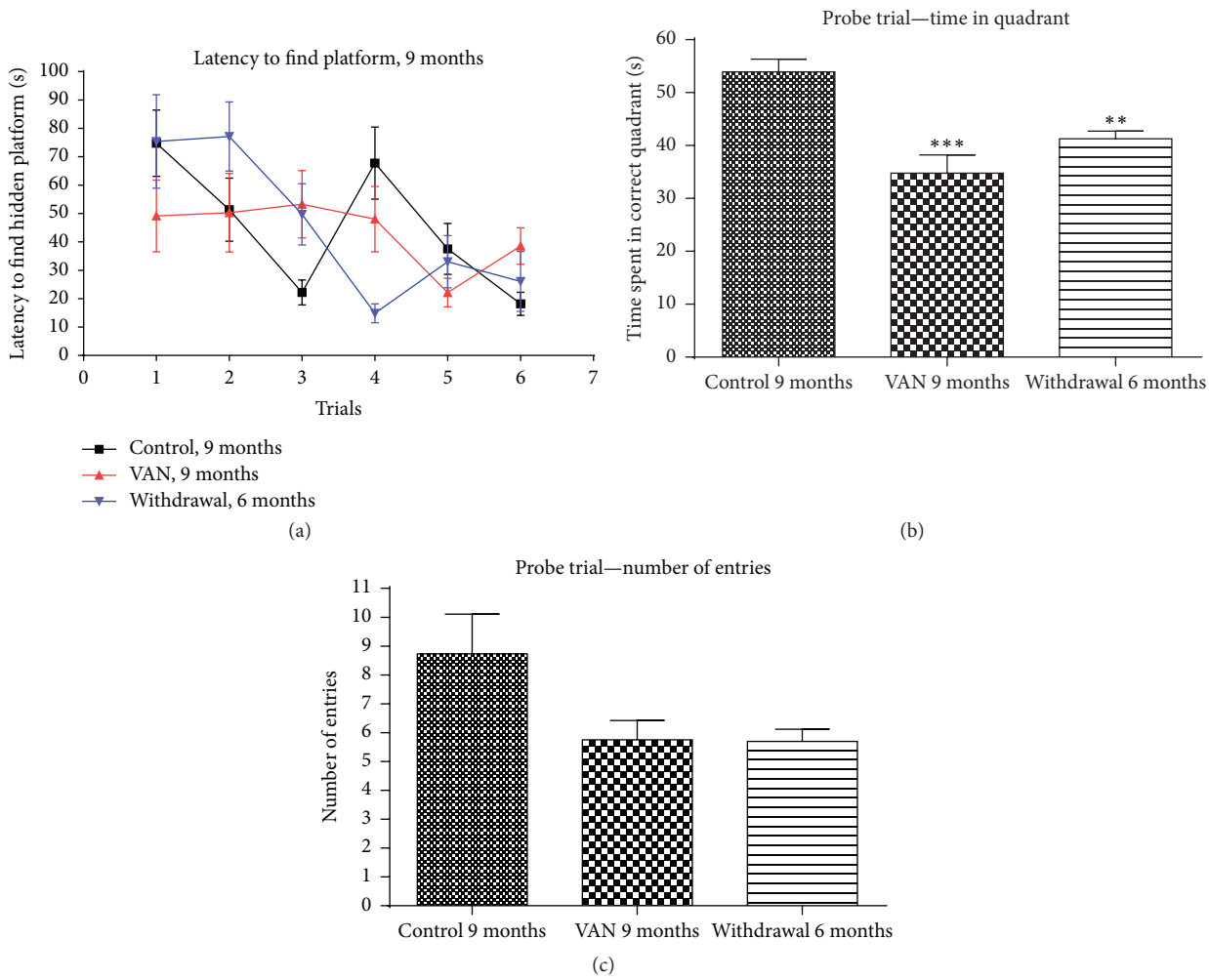

Figure 3: Effect of intermittent vanadium (VAN) treatment for nine months on learning and memory in mice: 12 male mice were exposed thrice a week for nine months to VAN (3 mg/kg b.w.) with equal number and age matched controls. The withdrawal group were exposed to VAN for three months but replaced with water thereafter. All the groups of the 9-month-old rats (C9, VAN9, and W6) were able to learn the location of the hidden platform with no statistically significant difference among them (a). However, there was a statistically significant decrease in memory retention in vanadium ($^{***}P < 0.001$) and withdrawal ($^{**}P < 0.01$) groups relative to control ((b) and (c)). The W6 group showed some recovery ((b) and (c)) in comparison to the VAN9 group but was still significantly less than the controls.

find the platform, and the length of time it takes to find the platform was recorded. If it did not find the platform after 60 seconds, the mouse was guided to the platform and allowed to stay there for 15 seconds. Each mouse went through three trials per day for two consecutive days. This test is a measure of the learning ability of the mouse. On the third day, a single probe trial was given to test the mouse's spatial memory in the water maze while the platform was removed. Its memory of the initial location of the escape platform was tested by measuring the time it spent in the target quadrant and the number of times it crossed the island zone where the platform was initially located. This latter record is a test of its memory ability. The test was recorded manually by two observers using a stopwatch. The Morris water maze was introduced as an instrument with particular sensitivity to the effects of hippocampal lesions in rodents [23, 24].

3. Results

The results obtained in this study are as described in Figures 1–5. Animals across the groups show no difference in learning abilities but significant loss in memory abilities after 3 months of vanadium exposure and this trend continued in all vanadium-exposed groups relative to control (Figures 1–4). Animals exposed to vanadium for three months after which treatment was withdrawn recovered significantly only 9 months after vanadium withdrawal. There was no significant difference in latency to fall in the forelimb grip test between vanadium-exposed groups and their respective controls in all age groups. There was however a significant reduction in latency to fall in vanadium withdrawal group W6 (nine months old) relative to their controls (Figure 5).

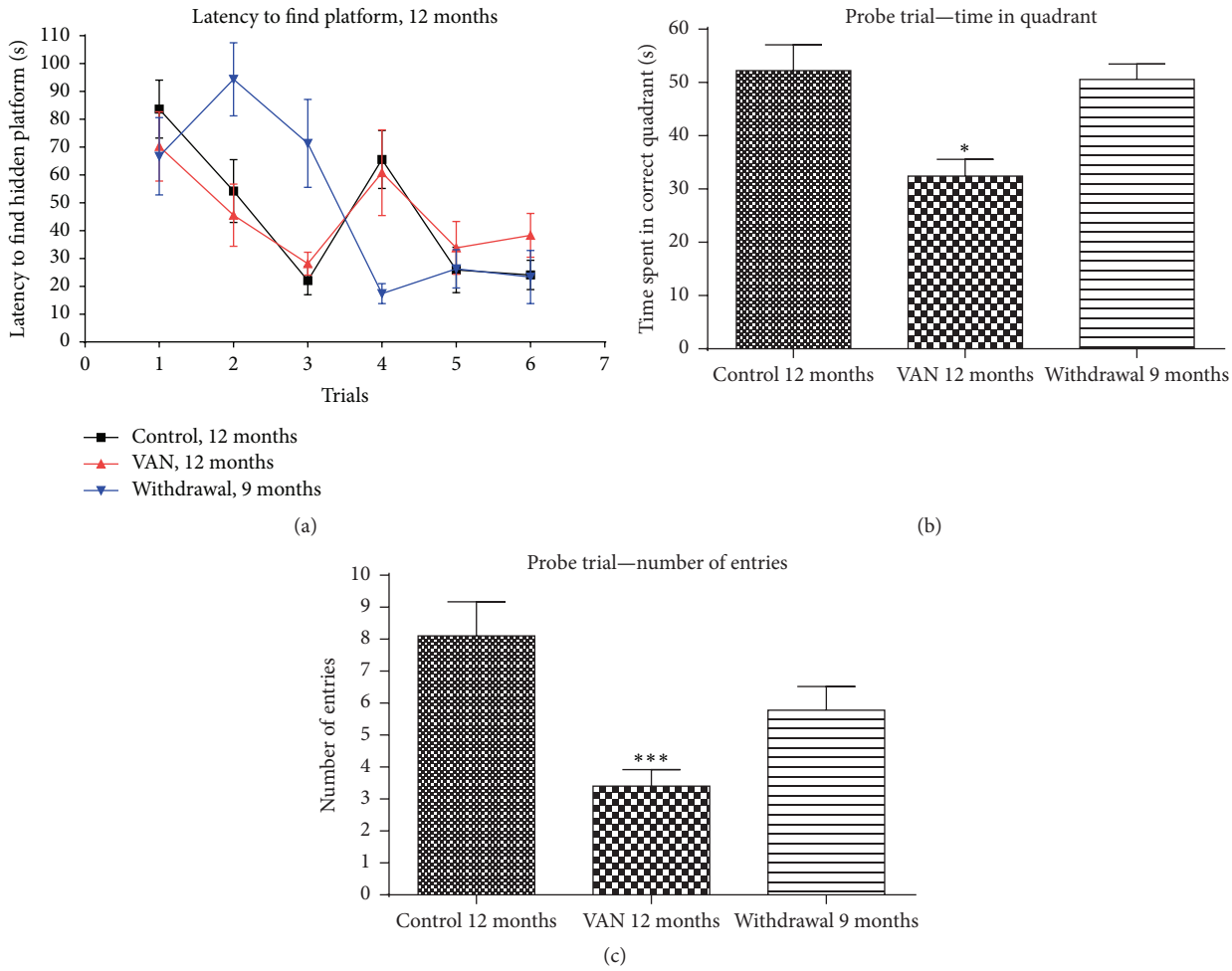

FIGURE 4: Effect of intermittent vanadium (VAN) treatment for twelve months on learning and memory in mice: 12 male mice were exposed thrice a week for twelve months to VAN (3 mg/kg b.w.) with equal number and age matched controls. The withdrawal group were exposed to VAN for three months but replaced with water thereafter. All the groups of the 12-month-old rats (C12, V12, and W9) were able to learn the location of the hidden platform with no statistically significant difference among them. However, there was statistically significant decrease in memory retention in V12 ($^{*}P < 0.05$ and $^{***}P < 0.001$) relative to controls ((b) and (c)). The W9 group showed a remarkable recovery in memory retention being significantly higher ($P < 0.01$) than the V12 (b) and were comparable to the controls.

4. Discussion

In this study, vanadium administration led to significant loss in memory abilities as early as 3 months after exposure and this was consistent till 12 months. Previous studies using the Morris water maze have reported reduced memory scores after metal exposures. Haider et al. [25] showed a dose dependent impairment in memory function in cadmium-treated rats relative to controls and Lu et al. [26] demonstrated significantly longer escape latency in manganese exposed rats when compared to controls. There are reports of behavioral deficits after vanadium exposure [16, 17, 27]; however, few have reported on memory deficits using the Morris water maze. In addition, our data showed that long term vanadium exposure (3–12 months) leads to significant memory deficits which persist long after exposure has stopped. This could have implications for people who are occupationally or environmentally exposed to vanadium over a long period of time. Of note is the huge population in the Arabian Gulf and Nigerian Niger Delta who have been exposed for decades to vanadium-contaminated crude burning in relatively confined ecosystem [1]. A striking observation in this study is the significant improvement in memory scores observed in mice exposed to vanadium for 3 months after a withdrawal period of 9 months, but not earlier. This implies the reversibility of vanadium mediated effects in the brain after vanadium withdrawal, despite being only after a long withdrawal period. A reversal of tissue damaging effects (not the brain) of vanadium after withdrawal has been reported by Olopade et al. [28]. Barker et al. [29] reported that previous benzodiazepine users will likely experience benefits of improved cognitive functioning after withdrawal but will not experience full restitution of functions; similarly, deficits resulting from vanadium exposure though reversible could take a long time before full restitution is obtained. Also, observed in this study was the fact that although vanadium exposure led to memory

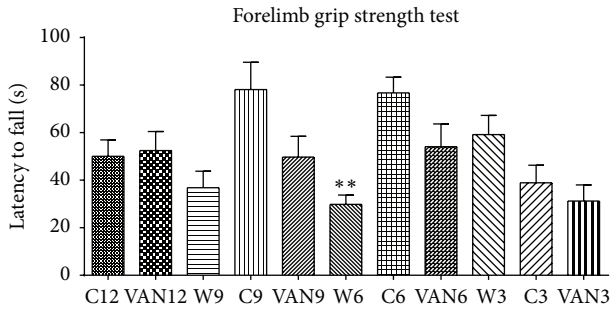

Figure 5: Effect of intermittent vanadium (VAN) treatment for twelve months on learning and memory in mice: 12 male mice each were exposed thrice a week for three, six, nine, and twelve months to VAN (3 mg/kg b.w.) with equal number and age matched controls. The withdrawal group were exposed to VAN for three months but replaced with water thereafter. All the groups of 3–12-month-old mice were subjected to forelimb grip test with 6–12 months reported here. There was no significant difference in latency to fall in the forelimb grip test between vanadium-exposed groups and their respective controls in all age groups. There was a significant reduction in latency to fall in vanadium withdrawal group W6 ($^{**}P < 0.01$) relative to their controls.

deficits, the ability of the mice to learn was largely unaffected. All the groups of mice were capable of learning the spatial location of the escape platform, shown by the progressive reduction observed in escape latency with subsequent trials. However, in contrast to controls, the vanadium-exposed mice were impaired in their ability to remember the escape position during the probe trial. Impaired spatial retention of vanadium-exposed mice during the probe trials was observed by comparison of annulus crossings over the trained position and time spent in trained quadrant. The Morris water maze (MWM) is a test of spatial learning which is assessed across repeated trials and reference memory which is determined by preference for the platform area when the platform is absent (probe trial). There is extensive evidence of its validity as a measure of hippocampal-dependent spatial navigation and reference memory [30] and its specificity as a measure of place learning. Consolidation of memory requires a fully functional hippocampus which converts the working/short term memory into long term memory [31]. This long term potentiation seems to be the focus of neurological deficit observed in vanadium-exposed mice as they showed a significantly reduced ability to consolidate the memory of the location of the escape platform. Consolidation of memory is dependent on phosphorylation of hippocampal mitogen activated protein kinase (MAPK)/ERK and subsequent synaptic plasticity [32]. Standing on the premises that hippocampal ERK phosphorylation has been shown to be required during recent spatial learning and memory, Leon et al. [33] reported that its inhibition leads to failure of consolidation of memory, though it did not affect spatial acquisition. We propose that the deficit in memory consolidation observed in vanadium-exposed mice is due to a disruption in this process. Vanadium exposure has been found to lead to the loss of dendritic spines and necrotic-like cell death in the hippocampus [19] and this could also be the possible cause. The recovery seen in withdrawal group after 9 months could be related to adult neurogenesis or a plastic reorganization of neuronal networks compensating for possible early neuronal losses [34]. The forelimb grip test did not show any significant changes in the vanadium-exposed animals relative to controls. Though forelimb grip test has shown reduced muscular strength after vanadium exposure, the difference was not significant. It seems that long term vanadium exposure may not affect muscular activity and coordination to a large degree. A human study by Charles et al. [35] did not provide evidence that occupational exposure to pesticides, solvents, and metals adversely affected hand-grip strength in the studied population. The histological analysis of the different brain regions of these mice is ongoing in our laboratory and this will hopefully shed more light on the pathogenesis of memory loss seen in this study. In conclusion, we have shown that chronic administration of vanadium in mice leads to memory deficits which are reversible only after a long period of vanadium withdrawal.

5. Conclusion

This work has shown that mice exposed to vanadium over a period of time exhibited no difference in learning abilities but had significant loss in memory acumen after 3 months of exposure.

Our work also revealed that the memory deficit induced by chronic administration of vanadium in mice is reversible but only after a long period of vanadium withdrawal.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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References

[1] J. O. Olopade and J. R. Connor, "Vanadium and neurotocixity: a review," *Current Topics in Toxicology*, vol. 7, pp. 33–39, 2010.

[2] J. J. Wirth and R. S. Mijal, "Adverse effects of low level heavy metal exposure on male reproductive function," *Systems Biology in Reproductive Medicine*, vol. 56, no. 2, pp. 147–167, 2010.

[3] M. Corradi and A. Mutti, "Metal ions affecting the pulmonary and cardiovascular systems," *Metal Ions in Life Sciences*, vol. 8, pp. 81–105, 2011.

[4] K. Jomova and M. Valko, "Advances in metal-induced oxidative stress and human disease," *Toxicology*, vol. 283, no. 2-3, pp. 65–87, 2011.

[5] S. Modgil, D. K. Lahiri, V. L. Sharma, and A. Anand, "Review: role of early life exposure and environment on neurodegeneration: implications on brain disorders," *Translational Neurodegeneration*, vol. 3, article 9, 2014.

[6] T. I. Fortoul, V. Rodriguez-Lara, A. González-Villalva et al., "Inhalation of vanadium pentoxide and its toxic effects in a mouse model," *Inorganica Chimica Acta*, vol. 420, pp. 8–15, 2014.

[7] D. G. Barceloux, "Vanadium," *Journal of Toxicology: Clinical Toxicology*, vol. 37, no. 2, pp. 265–278, 1999.

[8] R. R. Moskalyk and A. M. Alfantazi, "Processing of vanadium: a review," *Minerals Engineering*, vol. 16, no. 9, pp. 793–805, 2003.

[9] J. J. Rodríguez-Mercado and M. A. Altamirano-Lozano, "Vanadium: pollution, metabolism and genotoxicity," *Revista Internacional de Contaminacion Ambiental*, vol. 22, no. 4, pp. 173–189, 2006.

[10] P. N. Saxena, J. Arya, N. Saxena, and A. Shukla, "Vanadium intoxication in albino rat based on haematobiochemistry and behaviouristic changes," *International Journal of Environmental Engineering and Management*, vol. 4, no. 4, pp. 293–300, 2013.

[11] M. M. Sasi, S. S. Haider, M. El-Fakhri, and K. M. Gwarsha, "Microchromatographic analysis of lipid protein and occurrence of lipid peroxidation in various brains area of vanadium exposed rats. A possible mechanism of vanadium neurotoxicity," *Neurotoxicology*, vol. 15, no. 2, pp. 413–420, 1994.

[12] F. A. C. Amorim, B. Welz, A. C. S. Costa, F. G. Lepri, M. G. R. Vale, and S. L. C. Ferreira, "Determination of vanadium in petroleum and petroleum products using atomic spectrometric techniques," *Talanta*, vol. 72, no. 2, pp. 349–359, 2007.

[13] K. Pyrzyńska and T. Wierzbicki, "Determination of vanadium species in environmental samples," *Talanta*, vol. 64, no. 4, pp. 823–829, 2004.

[14] H. Li, D. Zhou, Q. Zhang et al., "Vanadium exposure-induced neurobehavioral alterations among Chinese workers," *NeuroToxicology*, vol. 36, no. 10, pp. 49–54, 2013.

[15] O. Mustapha, B. Oke, N. Offen, A.-L. Sirén, and J. Olopade, "Neurobehavioral and cytotoxic effects of vanadium during oligodendrocyte maturation: a protective role for erythropoietin," *Environmental Toxicology and Pharmacology*, vol. 38, no. 1, pp. 98–111, 2014.

[16] J. O. Olopade, I. O. Fatola, and F. E. Olopade, "Vertical administration induces behavioural and neuro morphological changes: protective role of vitamin E," *Nigeria Journal of Physiological Sciences*, vol. 26, pp. 55–60, 2011.

[17] M. Soazo and G. B. Garcia, "Vanadium exposure through lactation produces behavioral alterations and CNS myelin deficit in neonatal rats," *Neurotoxicology and Teratology*, vol. 29, no. 4, pp. 503–510, 2007.

[18] G. J. Naylor, "Reversal of vanadate-induced inhibition of Na-K ATPase: a possible explanation of the therapeutic effect of carbamazepine in affective illness," *Journal of Affective Disorders*, vol. 8, no. 1, pp. 91–93, 1985.

[19] M. R. Avila-Costa, T. I. Fortoul, G. Niño-Cabrera et al., "Hippocampal cell alterations induced by the inhalation of vanadium pentoxide (V_2O_5) promote memory deterioration," *Neuro Toxicology*, vol. 27, no. 6, pp. 1007–1012, 2006.

[20] K. L. K. Tamashiro, T. Wakayama, R. J. Blanchard, D. C. Blanchard, and R. Yanagimachi, "Postnatal growth and behavioral development of mice cloned from adult cumulus cells," *Biology of Reproduction*, vol. 63, no. 1, pp. 328–334, 2000.

[21] N. Van Wijk, E. Rijntjes, and B. J. M. Van De Heijning, "Perinatal and chronic hypothyroidism impair behavioural development in male and female rats," *Experimental Physiology*, vol. 93, no. 11, pp. 1199–1209, 2008.

[22] M. Shabani, M. Nazeri, S. Parsania et al., "Walnut consumption protects rats against cisplatin-induced neurotoxicity," *NeuroToxicology*, vol. 33, no. 5, pp. 1314–1321, 2012.

[23] R. D'Hooge and P. P. De Deyn, "Applications of the Morris water maze in the study of learning and memory," *Brain Research Reviews*, vol. 36, no. 1, pp. 60–90, 2001.

[24] L. Golchin, L. Golchin, A. A. Vahidi, and M. Shabani, "Hippocampus and cerebellum function following imipenem treatment in male and female rats: evaluation of sex differences during developmental stage," *Pakistan Journal of Biological Sciences*, vol. 16, no. 4, pp. 151–159, 2013.

[25] S. Haider, L. Anis, Z. Batool et al., "Short term cadmium administration dose dependently elicits immediate biochemical, neurochemical and neurobehavioral dysfunction in male rats," *Metabolic Brain Disease*, vol. 30, no. 1, pp. 83–92, 2015.

[26] C.-L. Lu, S. Tang, Z.-J. Meng et al., "Taurine improves the spatial learning and memory ability impaired by sub-chronic manganese exposure," *Journal of Biomedical Science*, vol. 21, no. 1, article 51, 2014.

[27] B. Todorich, J. O. Olopade, N. Surguladze, X. Zhang, E. Neely, and J. R. Connor, "The mechanism of vanadium-mediated developmental hypomyelination is related to destruction of oligodendrocyte progenitors through a relationship with ferritin and iron," *Neurotoxicity Research*, vol. 19, no. 3, pp. 361–373, 2011.

[28] J. Olopade, O. Owolabi, O. Aina, and S. Onwuka, "The effect of alpha tocopherol on body organs of neonatal rats exposed to vanadium," *Journal of Applied Sciences and Environmental Management*, vol. 15, no. 1, pp. 5–8, 2011.

[29] M. J. Barker, K. M. Greenwood, M. Jackson, and S. F. Crowe, "Persistence of cognitive effects after withdrawal from long-term benzodiazepine use: a meta-analysis," *Archives of Clinical Neuropsychology*, vol. 19, no. 3, pp. 437–454, 2004.

[30] R. G. M. Morris, "An attempt to dissociate 'spatial-mapping' and 'working-memory' theories of hippocampal function," in *Neurobiology of the Hippocampus*, W. Seifert, Ed., pp. 405–432, Academic Press, New York, NY, USA, 1993.

[31] A. D. Redish and D. S. Touretzky, "The role of the hippocampus in solving the Morris water maze," *Neural Computation*, vol. 10, no. 1, pp. 73–111, 1998.

[32] E. R. Kandel, "The molecular biology of memory storage: a dialogue between genes and synapses," *Science*, vol. 294, no. 5544, pp. 1030–1038, 2001.

[33] W. C. Leon, M. A. Bruno, S. Allard, K. Nader, and A. C. Cuello, "Engagement of the PFC in consolidation and recall of recent spatial memory," *Learning and Memory*, vol. 17, no. 6, pp. 297–305, 2010.

[34] D. F. Wozniak, R. E. Hartman, M. P. Boyle et al., "Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults," *Neurobiology of Disease*, vol. 17, no. 3, pp. 403–414, 2004.

[35] L. E. Charles, C. M. Burchfiel, D. Fekedulegn et al., "Occupational and other risk factors for hand-grip strength: the Honolulu-Asia Aging study," *Occupational and Environmental Medicine*, vol. 63, no. 12, pp. 820–827, 2006.