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Vanadate oligomers: In vivo effects in hepatic vanadium accumulation and stress markers

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Abstract

The formation of vanadate oligomeric species is often disregarded in studies on vanadate effects in biological systems, particularly in vivo, even though they may interact with high affinity with many proteins. We report the effects in fish hepatic tissue of an acute intravenous exposure (12, 24 h and 7 days) to two vanadium(V) solutions, metavanadate and decavanadate, containing different vanadate oligomers administered at sub-lethal concentration (5 mM; 1 mg/kg). Decavanadate solution promotes a 5-fold increase (0.135 ± 0.053 μg V^{-1} dry tissues) in the vanadium content of the mitochondrial fraction 7 days after exposition, whereas no effects were observed after metavanadate solution administration. Reduced glutathione (GSH) levels did not change and the overall reactive oxygen species (ROS) production was decreased by 30% 24 h after decavanadate administration, while for metavanadate, GSH levels increased 35%, the overall ROS production was depressed by 40% and mitochondrial superoxide anion production decreased 45%. Decavanadate intoxication did not induce changes in the rate of lipid peroxidation till 12 h, but later increased 80%, which is similar to the increase observed for metavanadate after 24 h. Decameric vanadate administration clearly induces different effects than the other vanadate oligomeric species, pointing out the importance of taking into account the different vanadate oligomers in the evaluation of vanadium(V) effects in biological systems.

Keywords: Vanadate oligomers; Oxidative stress

1. Introduction

Recent biological studies about vanadium reveals its complex chemistry, being most of vanadium(V) biological importance associated with the monomeric vanadate (HVO_4^{2-}), due to its structural similarity with phosphate (HPO_4^{2-}) [1–3]. Besides monomeric vanadate species, other vanadate oligomers can occur in vanadate solutions, and such oligomers are known to promote different effects in enzymes activities, not only in vitro [4,5] but also in vivo [6,7]. Among vanadate oligomers, decameric vanadate, which may occur upon medium acidification, was considered by Stankiewicz et al. [4] as the vanadate oligomer with more biochemical relevance. Although unstable at physiologic pH, the slow rate of decameric vanadate decomposition allows studying its effects in biochemical systems [6–8].

Vanadium can induce the formation of reactive oxygen species (ROS) in biological systems through: (i) Fenton-like reactions [9]; (ii) vanadate bioreduction mediated by reduced glutathione (GSH), flavoenzymes or NAD(P)H oxidases with ROS as a by-product [10–15]; (iii) and recent evidences point out to an indirect promotion of ROS production, probably by interacting

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with mitochondria [16]. Moreover, vanadium toxicological effects in several cell types have been related with ROS production [16–19]. Nevertheless, vanadium was recently reviewed as "an element of atypical biological significance" [20], due to insulin mimetic properties of vanadium [16,21,22] and to the preventive effects of vanadium against animal carcinogens [23–26].

The role of vanadate oligomers in biological systems is still to be clarified and to our knowledge, in vivo toxicological studies of these species are almost inexistent. In this sense, in the present study we have analyzed the effects of an intravenous administration of two different vanadate solutions, namely metavanadate and decavanadate, on vanadium accumulation in liver and on overall oxidative stress markers such as: (i) reduced GSH content; (ii) overall rate of ROS production; (iii) mitochondrial superoxide anion radical ($O_2^{\bullet -}$) production and (iv) lipid peroxidation propagation.

2. Experimental

Specimens of *Halobactrachus didactylus* (toadfish), from both sexes, were caught in Ria Formosa lagoon (in the south coast of Portugal), with body weight 339.8 ± 117.7 and 130.3 ± 28.6 g, and body length 26.9 ± 3.2 and 20.2 ± 1.3 cm, for vanadium accumulation assays and for the overall oxidative stress studies, respectively. They were acclimatized for no less than 2 weeks, and fed with squid ad libitum. The animals were starved for 2 days before sacrifice. For vanadate accumulation assays: 10 individuals were divided in two groups (META and DECA groups) and treated with an intravenous injection of 1 mg/kg of vanadium (5 mM), as decavanadate and metavanadate, respectively; sub-groups of five individuals were sacrificed 1 day and 7 days after injection, respectively. Simultaneously, at the beginning of the experiment, a control group ($n = 5$) was sacrificed to determine basal values (Table 1). For overall oxidative stress studies: six individuals were divided in two groups (META and DECA groups) and treated with an intravenous injection of 1 mg/kg of vanadium (5 mM), as decavanadate and metavanadate, respectively; sub-groups of three individuals were sacrificed 12 and 24 h after injection, respectively. Simultaneously, at the beginning of the experiment, a control group ($n = 3$) was sacrificed to determine basal values (Table 2).

Metavanadate stock solution (50 mM, pH 6.7) was prepared from ammonium metavanadate purchased from Riedel-de-Haen. Decavanadate stock solution was obtained by adjusting the pH of the former solution to 4.0 [27]. Both metavanadate and decavanadate stock solutions were always adjusted to pH 7.0 immediately before using, diluted to the final concentration (5 mM) in physiological serum (NaCl 0.9%) and kept in ice before use. The concentrations of each vanadate species were obtained by integrating the respective areas of the NMR spectra and calculated by using an algorithm described elsewhere [27]. NMR spectroscopy measurements of vanadium were performed on a Bruker AM-400 MHz spectrometer at 105.2 MHz equipped with a 5-mm multinuclear inverse probe. ^{51}V NMR spectra of 5 mM metavanadate solution show that in addition to about 692 μM monomeric (V1), also contains 210 μM dimeric (V2), 830 μM tetrameric (V4) and lower amounts of pentameric (V5) species, whereas 5 mM decavanadate (equivalent to 500 M decameric species), contain mainly the characteristic peaks of decameric vanadate species (460 μM) and also monomeric (400 μM) species, i.e., 1:1 decameric:monomeric species. Whereas the metavanadate solution is stable, an estimate half-life of 12 h was estimated for decameric species that partially disintegrates in the injection medium (0.9% NaCl), in agreement to described elsewhere for 1 mM total vanadium [7].

To obtain mitochondrial and cytosolic fractions, tissues were removed, weighted and samples of 1 g were homogenized with ultra-turrax at maximum speed

Table 2
Reduced glutathione (GSH), reactive oxygen species (ROS), superoxide anion radical ($O_2^{\bullet -}$) and lipid peroxides basal/control values in hepatic tissue of *Halobactrachus didactylus*

	Basal/control values
Reduced GSH content	7.8 ± 3.0 nmol mg^{-1} protein
Overall ROS production	15.4 ± 6.1 a.u. min^{-1} mg^{-1} protein
Mitochondrial $O_2^{\bullet -}$ production	6.4 nmol $O_2^{\bullet -}$ min^{-1} mg^{-1} protein
Lipid peroxidation	46 ± 21 a.u. min^{-1} mg^{-1} protein

Values are presented as means ± SD ($n = 3$).

Table 1
Vanadium amounts in total, mitochondrial and cytosolic fractions of hepatic tissue from *Halobatrachus didactylus*

	CTRL	META		DECA	
Subcellular fractions	0 day	1 day	7 days	1 day	7 days
Total	0.434 ± 0.029	0.313 ± 0.180	0.269 ± 0.240	0.646 ± 0.112	1.168 ± 0.523[a]
Mitochondrial	0.023 ± 0.004	0.040 ± 0.008[a]	0.021 ± 0.011	0.051 ± 0.027	0.135 ± 0.053[a]
Cytosolic	0.022 ± 0.002	0.036 ± 0.014	0.023 ± 0.020	0.057 ± 0.011	0.066 ± 0.042[a]

CTRL, META and DECA refers to control, metavanadate (5 mM, 1 mg/kg) and decavanadate (5 mM, 1 mg/kg) group, respectively. 1 and 7 refer to time after administration in days. Values are presented as means ± SD ($n = 5$) and given in μg V g^{-1} dry tissue.

[a] Significantly different from control, $p < 0.05$.

(20,500 rpm) in a homogenization solution containing 10 ml of 20 mM tris(hydroxymethyl)aminometane (Tris) (pH 7.6), 0.5 M sucrose, 0.15 M KCl, 1 mM ethylenediaminetetraacetic acid dipotassium (EDTA), 1 mM dithiothreitol (DTT), and centrifuged at 460*g* for 10 min at 4 °C. The supernatants were then centrifuged at 10,000*g* for 30 min at 4 °C. The 10,000*g* supernatants were centrifuged at 50,000*g* for 120 min at 4 °C, and the resulting supernatants (named as the cytosolic fraction) were stored for vanadium accumulation assays. The pellets were resuspended in 5 ml of the homogenization buffer and again centrifuged at 10,000*g* for 30 min at 4 °C. The pellets containing the mitochondrial fraction were resuspended in 3 ml of homogenization solution and stored at −80 °C for determination of $O_2^{\bullet -}$ production.

For vanadium concentration measurements, aliquots of total, cytosolic and mitochondrial fractions were dried at 80 °C until constant weight and digested with 65% nitric acid (HNO_3) at the ratio of 20 ml per 1 g of dry weight tissue. After the digestion, the residue was resuspended with 1 ml 10% chloridric acid (HCl). Vanadium concentration were measured using a Varian AA20 atomic absorption spectrometer, equipped with a GTA-96 graphite furnace, with auto sampler working at a furnace program of 63 s with an argon gas flow of 3.0 l/min. The vanadium lamp was operated at 318.5 nm, with slit width of 0.2 nm and the instrument was calibrated with solutions containing 10, 20 and 40 ppb of vanadium. Calibration of the standards was obtained by successive dilutions of a Merck standard solution of vanadium 1000 mg/l. TORT-1 was used as standard reference material (1.4 ± 0.3 μg/g of vanadium).

Reduced GSH content was determined in fresh hepatic tissue homogenates, from the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) by sulfhydryl groups ($\varepsilon^{412} = 13.2\ mM^{-1}\ cm^{-1}$). Briefly, samples are supplemented with 20% of perchloric acid to precipitate proteins. The mix was then centrifuged at 16,000*g* for 10 min at 4 °C and the supernatant was neutralized with KOH. These two steps were repeated twice. The assay was performed in a medium containing 50 mM KH_2PO_4, pH 7.0, 100 mM KCl and 0.5 mM DTNB. The absorption value was registered after the addition of sample with a Shimadzu UV mini1240.

The overall rate of ROS production in hepatic tissue homogenates was calculated from the kinetics of increase of fluorescence ($\lambda_{exc} = 495$ nm, $\lambda_{em} = 520$ nm) using dichlorodihydrofluorescein diacetate (H_2DCFDA) as probe [14,28]. Fluorescence measurements were carried out under continuous stirring at 25 °C with a Perkin–Elmer 650-40 Fluorescence Spectrophotometer (Perkin–Elmer, Foster City, CA, USA). The assay started after sample addition and was performed in a medium containing 50 mM KH_2PO_4 (pH 7.0), 100 mM KCl and 2 ng/ml H_2DCFDA.

Mitochondrial superoxide anion radical production was measured from the rate of nitroblue tetrazolium (NBT) reduction, as indicated elsewhere [29]. The assay medium contains 50 mM KH_2PO_4 (pH 7.0), 100 mM KCl, 0.1 mM EDTA, 0.1 mM NBT and 120 μg of protein of mitochondrial fraction of *H. didactylus* hepatic tissue. Measurements were done at 560 nm with a Shimadzu UV mini1240. $O_2^{\bullet -}$ production was calculated from the decrease of the rate of NBT reduction after addition of 350 U SOD.

Evaluation of lipid peroxidation propagation on hepatic tissue homogenate was performed using *cis*-parinaric acid (AcPn) [30]. Fluorescence measurements ($\lambda_{exc} = 332$ nm, $\lambda_{em} = 416$ nm) were carried out under continuous stirring at 25 °C with a Perkin–Elmer 650-40 Fluorescence Spectrophotometer (Perkin–Elmer, Foster City, CA, USA). The assay medium contained 50 mM KH_2PO_4 (pH 7.0), 100 mM KCl, 12.5–25 μl of sample volume and 4 μM AcPn. Fluorescence readings started immediately after the addition of AcPn.

3. Results and discussion

Vanadium toxicity studies often disregard the formation of different vanadate oligomers known to have different biochemical effects and to interact with high affinity with many proteins such as, for example, myosin [5]. Few animal studies involving vanadium consider the contribution of different oligomeric vanadate species to vanadium toxicity. It has been recently shown that an acute exposure to decavanadate but not to other vanadate oligomers induced oxidative stress, lipid peroxidation and a different fate in vanadium intracellular accumulation [6,7]. These findings were further explored and the results described in the present article.

Our results demonstrated that there is a different pattern of vanadium accumulation when we compare metavanadate and decavanadate solutions administration, in total, mitochondrial and cytosolic fractions from *H. didactylus* hepatic tissue (Table 1). The control value for total vanadium accumulation in hepatic tissue, 0.43 (±0.03) μg/g dry tissue (Table 1), is within the range of values reported for fish [31]. The basal vanadium content in hepatic tissue is ~10 times higher than the value reported for cardiac tissue [6]. The subcellular distribution of vanadium in liver appears to be different than in heart, since mitochondrial and cytosolic fractions of hepatic tissue accumulate 10% of total vanadium, while in cardiac tissue the same fractions accumulate 20% [6]. However, hepatic vanadium accumulation, with intravenous administration, is different than the accumulation obtained in cardiac [6] and hepatic (data not shown) tissue when an intraperitoneal administration was performed. This finding is in agreement with the

observations that vanadium accumulation is dependent on the way of administration [32].

It was observed that metavanadate solution administration promoted weak increases in subcellular fractions after 24 h but not in total vanadium content, and after 7 days no significant differences between controls and metavanadate intoxicated individuals were found (Table 1). On the contrary, there were increases in vanadium concentration in all studied liver fractions after decavanadate solution administration. After 24 h the total vanadium content increased 50% ($p < 0.005$), while mitochondrial fraction and cytosolic fraction increased 122% and 155% ($p < 0.05$), respectively. The increase was much higher 7 days after exposition, particularly in cytosolic and mitochondrial fraction which had 2- and 5-fold increases ($p < 0.05$), respectively (Table 1). Several reports have pointed out that mitochondria is one of the target organelles for vanadium [33,34], and our results of vanadium subcellular distribution showed that the mitochondrial fraction tends to accumulate more vanadium after decavanadate solution administration. This result suggests that decavanadate is not behaving in the same way as other vanadate oligomers, and indicate that decameric vanadate species will not completely decompose into monomeric vanadate shortly after administration.

Previous studies with erythrocytes have shown that vanadate is transported inside through an unidentified anionic channel [32,35] or that it is incorporated in transferrin and albumin being rapidly transported to tissues [36]. However, data were not found regarding to decameric species. At pH 7, decameric vanadate might not be present in solution depending on several parameters such as vanadium concentration, ionic strength or temperature [8]. However, once formed at pH 4 and the pH adjusted to physiological pH, it decomposes very slowly with a half-time that can reach up to 15 h for the first order decomposition kinetics in serum [6,7]. This is an important feature, since decavanadate maintains its structure in serum for a significant amount of time. As decavanadate is eventually less permeant through the anionic channel than the other vanadate oligomers and likely to be bound with high affinity to proteins, therefore preventing its disintegration to vanadate oligomers and reduction to vanadyl, we suggest that the accumulation of vanadium in liver after decavanadate administration is a consequence of a higher level of exposure of liver to total vanadium. It is known that decavanadate binds with high affinity with proteins [4,5,8]. Moreover, it was demonstrated that, in certain experimental conditions (but at physiological pH), the half-life of 100 μM decameric vanadate species, as ascertained by UV/vis, increases from 5 to 15 h, in the presence of the calcium pump from sarcoplasmic reticulum (Aureliano and Gândara, in press). At these decavanadate concentrations and experimental conditions, UV/vis, is more sensitivite than NMR to follow the fate of decavanadate in solution.

In hepatic tissue the GSH status is differently affected upon administration of the two different vanadate solutions. Whereas basal GSH content (7.8 ± 3.0 nmol mg^{-1} protein) did not show significant changes after decavanadate solution administration (Table 2, Fig. 1), metavanadate solution administration increased GSH status up to 20% and 35%, after 12 and 24 h, respectively (Fig. 1). Since GSH was found to be involved in vanadate bioreduction [10–15], the levels of GSH should be affected when vanadate is administered. Indeed, it has been reported that the GSH pool is enhanced in the presence of vanadate [23,39].

On the other hand, GSH is known to be one of the most important intracellular anti-oxidants, being considered the largest component of an endogenous cellular "redox buffer" [37,38]. Therefore, differential effects of metavanadate and decavanadate on ROS production should be expected on the light of the previous data. Metavanadate-administered individuals showed a decrease of up to 40% in the overall rate of ROS production (Fig. 2), which is consistent with the observed increase in GSH content (Fig. 1). The decrease in ROS was observed between 12 and 24 h (Fig. 2). On the contrary, decavanadate-administered groups have a weak enhancement of ROS production 12 h after exposition (approx. 15% increase), but after 24 h ROS production decreased nearly 30%, mimicking the behavior observed with metavanadate solution (Fig. 2). The simplest explanation for this is that decomposition of decavanadate will produce a delayed exposure of the liver to metavanadate, although it is puzzling that no similar

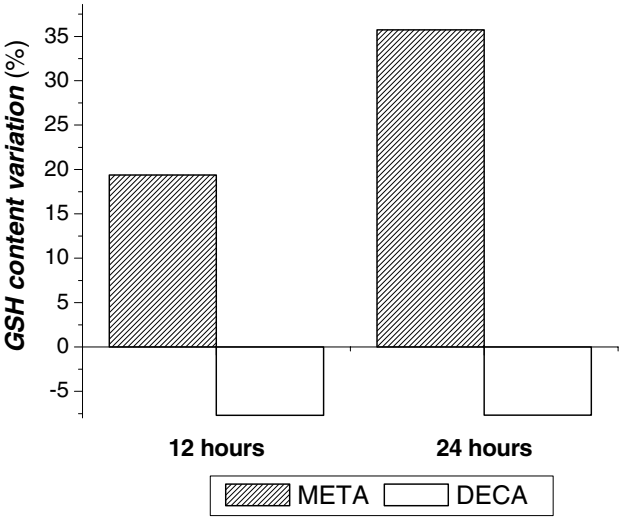

Fig. 1. Reduced glutathione content variation in the hepatic tissue of *Halobatrachus didactylus*. META and DECA refers to metavanadate (5 mM, 1 mg/kg) and decavanadate (5 mM, 1 mg/kg) groups, respectively. Variation is calculated based on basal values (Table 1). 12 and 24 refers to time after administration in hours.

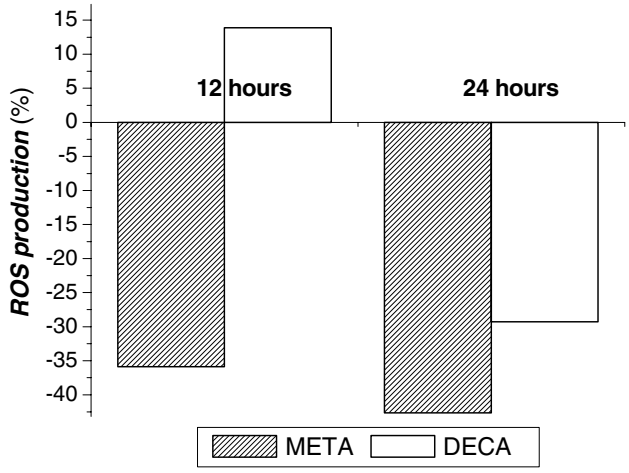

Fig. 2. Overall rate of ROS production variation in hepatic tissue of *Halobatrachus didactylus*. META and DECA refers to metavanadate (5 mM, 1 mg/kg) and decavanadate (5 mM, 1 mg/kg) groups, respectively. Variation is calculated based on basal values (Table 1). 12 and 24 refers to time after administration in hours.

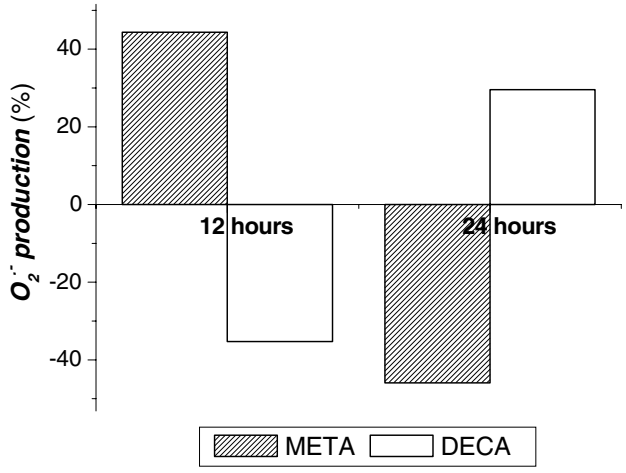

Fig. 3. Superoxide anion radical ($O_2^{\cdot-}$) production variation in the mitochondrial fraction from *Halobatrachus didactylus* hepatic tissue. META and DECA refers to metavanadate (5 mM, 1 mg/kg) and decavanadate (5 mM, 1 mg/kg) groups, respectively. Variation is calculated based on basal values (Table 1). 12 and 24 refers to time after administration in hours.

observations can be made regarding the GSH content described above (Fig. 1). Even though vanadium may participate in Fenton-like reactions [9], together with the proposed mechanisms of vanadate action that involves its bioreduction and ROS production [15,18,19,40], our results point out to a depression in the overall rate of ROS production. This observation is in good agreement with previous reports showing that vanadate supplementation diminished oxidative stress in certain experimental conditions, such as in rat-induced hepatocarcinogenesis [23] and in rat diabetic tissues [41].

Regarding superoxide anion radical production in mitochondrial fraction, once again it was observed that metavanadate and decavanadate promoted opposite effects. Twelve hours after exposition, metavanadate-administered individuals increased up to 45% in $O_2^{\cdot-}$ production rate, while decavanadate administration decreased 35% (Fig. 3). On other hand, after 24 h the results are reverted, decavanadate promoted a 30% increase, and metavanadate decreased $O_2^{\cdot-}$ production in 40% (Fig. 3). Thus, decameric vanadate species induced changes in $O_2^{\cdot-}$ mitochondrial production opposite to those produced by the oligomeric vanadate species that are present in metavanadate solution, from which we can suggest again different pathways of biological action for the different oligomers. Several proposed vanadate action pathways within cells involves $O_2^{\cdot-}$ production mediated by NADPH oxido-reductases from the respiratory chain [15,18,19,40]. If we consider the action and detoxification mechanism proposed for vanadate, where vanadate is reduced to vanadyl with production of $O_2^{\cdot-}$, it is possible that decavanadate participates in such reactions in a different manner. Note also that after 24 h, the increase in $O_2^{\cdot-}$ in decavanadate-administered individuals correlated with the increase of vanadium concentration in the mitochondrial fraction (Table 2, Fig. 3).

Lipid peroxidation is commonly observed following enhanced ROS production in the cellular medium. In metavanadate treated groups, it is observed an increase of 55% and 80% in lipid peroxidation propagation after 12 and 24 h, respectively (Fig. 4). Decameric species do not promote any significant change 12 h after administration, however, after 24 h the lipid peroxidation value is similar to metavanadate-administered individuals (Fig. 4). Without specifying which vanadate species were

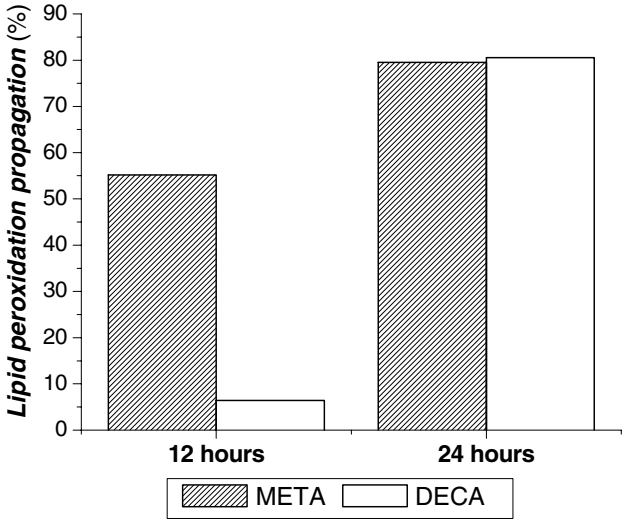

Fig. 4. Lipid peroxidation propagation variation in *Halobatrachus didactylus* hepatic tissue. META and DECA refers to metavanadate (5 mM, 1 mg/kg) and decavanadate (5 mM, 1 mg/kg) groups, respectively. Variation is calculated based on basal values (Table 1). 12 and 24 refers to time after exposition in hours.

present, it has been shown that vanadate can attenuate hepatic lipid peroxidation in induced hepatocarcinogenesis [23] and in 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis [42] and that vanadate does not change lipid peroxidation of control subjects [42]. We did not obtain any depression in lipid peroxidation in hepatic tissue after vanadate administration, even though there is maintenance of lipid peroxidation propagation until 12 h in decavanadate-administered individuals. Twenty-four hours of exposure to vanadate resulted in an increased rate of lipid peroxidation propagation, confirming the results obtain by our group with cardiac tissue [6]. Therefore, decavanadate seems to have a delayed effect in lipid peroxidation, probably due to its stability at physiological pH. Earlier cellular responses induced by decameric vanadate are currently being carried out looking for shorten times of exposition such as 1, 2 and 6 h.

4. Conclusions

We have shown that intravenous administration of two vanadate solutions, one of them containing decameric vanadate, did result in different vanadium accumulation in *H. didactylus* hepatic tissue. The results support the hypothesis of mitochondria as a target of decameric species, since vanadium content in mitochondrial fraction suffers a 5-fold increase and mitochondrial $O_2^{\bullet -}$ production was increased after decavanadate solution administration. Additionally it is shown that vanadate administration do not increase overall oxidative stress in liver. Lipid peroxidation propagation rate was increased by both vanadate solutions, suggesting that membrane lipids should be also viewed as vanadate targets.

Several experimental observations on vanadate oligomerization state in relation with ROS production in hepatic tissue were made in this work, however, more work is needed to clarify whether vanadate speciation in vivo could have different roles and fates. Nevertheless, the results highlight that different vanadate oligomers seem to follow, not only in vitro but also in vivo, different pathways, with different targets and effects. Although decameric vanadate is not taken into consideration to account for the effect of vanadium in most biological studies, it is to be noted that due to its unusual long stability at physiological pH, it may not completely decompose into monomeric vanadate before inducing changes in several cellular stress markers.

It is demonstrated that the administration of decameric vanadate species, although partially disintegrates in solution, induces different effects in liver oxidative stress when compared with the administration of metavanadate. This observation point out the importance of taking into account decameric vanadate in the evaluation of vanadium toxicity. It is believed that the present study about the contribution of decavanadate to vanadium toxicity may be useful to gain a deeper knowledge in vanadium biochemical effects.

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