Food and Chemical Toxicology 73 (2014) 113–118

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Exposure to bis(maltolato)oxovanadium(IV) increases levels of hepcidin mRNA and impairs the homeostasis of iron but not that of manganese

Cristina Sánchez-González [a], Lorenzo Rivas-García [a], Carlos López-Chaves [a], Alba Rodríguez-Nogales [b], Francesca Algieri [b], Julio Gálvez [b], Jorge Gómez-Aracena [c], Laura Vera-Ramírez [d], Maria Montes-Bayon [e], Alfredo Sanz-Medel [e], Juan Llopis [a,*]

[a] Department of Physiology, Faculty of Pharmacy, University of Granada, 18071 Granada, Spain
[b] CIBERehd, Department of Pharmacology, ibs.GRANADA, University of Granada, 18071 Granada, Spain
[c] Department of Preventive Medicine and Public Health, Faculty of Medicine, University of Malaga, 29071 Malaga, Spain
[d] GENyO Center, Pfizer-University of Granada and Andalusian Government Centre for Genomics and Oncology, Granada, Spain
[e] Department of Analytical Chemistry, Faculty of Chemistry, University of Oviedo, 33007 Oviedo, Spain

ARTICLE INFO

Article history:
Received 5 June 2014
Accepted 17 August 2014
Available online 25 August 2014

Keywords:
Vanadium
Hepcidin
Iron
Manganese
Rat

ABSTRACT

The aim of this study was to examine whether alterations in iron homeostasis, caused by exposure to vanadium, are related to changes in the gene expression of hepatic hepcidin. Two groups of rats were examined: control and vanadium-exposed. Vanadium, as bis(maltolato)oxovanadium(IV) was supplied in the drinking water. The experiment had a duration of five weeks. Iron and manganese were measured in excreta, serum and tissues. Leptin, ferritin, IL-1β, IL-6, TNF-α, red blood cells, haemoglobin and haematocrit were determined. Protein carbonyl group levels and hepcidin gene expression were determined in the liver. In the vanadium-exposed rats, iron absorption, serum iron and leptin and all haematological parameters decreased. Levels of IL-6, TNF-α and ferritin in serum and of iron in the liver, spleen and heart increased. In the liver, levels of protein carbonyl groups and hepcidin mRNA were also higher in the vanadium-exposed group. Exposure to vanadium did not modify manganese homeostasis. The results obtained from this study provide the first evidence that bis(maltolato)oxovanadium(IV) produces an increase in the gene expression of the hepcidin, possibly caused by an inflammatory process. Both factors could be the cause of alterations in Fe homeostasis and the appearance of anaemia. However, Mn homeostasis was not affected.

1. Introduction

Vanadium (V) is a widely distributed element, which is essential for some living organisms, but its role as a micronutrient, its essentiality and its biological and pharmacological activity are as yet incompletely understood. Rising levels of V in the environment, due to its widespread use in diverse industrial processes, its frequent use as a supplement by athletes and body builders to improve performance (Barceloux, 1999), and growing interest in the pharmacological effects (glucose-lowering agent) of some V compounds, have led to the study of V metabolism becoming an important area of current investigation (Domingo et al., 1991, 1995; Oster et al., 1993; Nriagu, 1998; Thompson and Orvig, 2006; Michibata, 2012). Among the V compounds, it has been reported that bis(maltolato)oxovanadium(IV) (BMOV) has proved to be two to three times more effective than vanadyl sulphate as glucose-lowering agent and was better tolerated in animal models (Thompson and Orvig, 2006). However, it has been recommended that the use of V compounds should be avoided until more information is available on possible toxic effects arising from chronic treatment (Domingo, 2000).

At the present time, many metabolic aspects have yet to be established, such as the digestive and metabolic interactions with other trace elements involved in antioxidant defence. Moreover, V exposure has been related with oxidative stress and inflammation changes. The appearance of oxidative stress is associated with an inflammatory response. Vanadium exposure has been related with cytokine production (Dye et al., 1999), inflammatory changes in the upper and lower respiratory tracts (Pierce et al., 1996; Ghio et al., 2005; Cohen et al., 2010) and lung tumours (Assem and Levy, 2012). However, other authors have suggested that V may mediate

* Corresponding author. Address: Department of Physiology, Campus Cartuja, University of Granada, E-18071 Granada, Spain. Tel.: +34 958241000x20320, +34 626797356; fax: +34 958 248959.
E-mail address: jllopis@ugr.es (J. Llopis).

http://dx.doi.org/10.1016/j.fct.2014.08.011
0278-6915/

the inhibition of different forms of cancer (Das et al., 2012). Therefore, the use of V as a potential insulin-mimetic agent is subject to evaluation of the toxicity associated with its inflammatory effects.

Studies have also examined the effects of V on iron (Fe) homeostasis and haematological parameters. It is generally agreed that the increased intake of V leads to Fe overload, mainly in the spleen and liver (Thompson et al., 2002; Scibior et al., 2012; Sánchez-González et al., 2014), together with a deterioration in haematological parameters (Domingo et al., 1995; Scibior et al., 2006; Sánchez-González et al., 2012a).

In view of the absence of information about a possible relation between V and hepcidin, we studied whether exposure to V modifies the gene expression of hepcidin. Hepcidin is a peptide hormone that is synthesised mainly in the hepatocytes. It acts on the ferroportin transporter. This peptide inhibits ferroportin-dependent Fe efflux from the enterocytes, hepatocytes and macrophages into the plasma (Ganz and Nemeth, 2006, 2011). Hepcidin synthesis is regulated by hepatic and extracellular Fe, by the requirements of Fe for erythropoiesis and by inflammatory processes. The binding of hepcidin to ferroportin provokes the internalisation and degradation of the transporter (Nemeth et al., 2004a; De Domenico et al., 2007). Inadequate hepcidin synthesis may explain many forms of Fe overload disease, whereas high levels of hepcidin produced by prolonged inflammatory stimuli can account for hypoferraemia and anaemia of chronic disease (Weiss and Goodnough, 2005; De Domenico et al., 2007).

Bartnika recently suggested that manganese (Mn), a metal that is transported by DMT-1, may also modulate the expression of hepcidin, and that changes in the plasma levels of Mn could attenuate or increase those of hepcidin (Bartnikas, 2012). However, this author also observed that physiological plasma levels of Mn are much lower (about 100 times lower) than the concentrations shown to be capable of increasing the expression of hepcidin in vitro. It has also been shown that Mn is a substrate for ferroportin, that the transport seems to be unidirectional and that the Mn efflux is concentration dependent (Yin et al., 2010; Madejczyk and Ballatori, 2012). However, Mitchell et al. (2014), in oocytes expressing ferroportin, did not observe any stimulated efflux of ^{54}Mn when this metal was injected at either 5 μM or 500 μM.

In view of these considerations, the aim of this study is to examine whether alterations in Fe homeostasis and haematological changes, caused by exposure to V, supplied as (BMOV), are related to changes in the gene expression of hepcidin and whether these changes affect the homeostasis of Mn. The results obtained could help clarify the role of V as a micronutrient and the question of its toxicity.

2. Materials and methods

2.1. Animals and diets

In this experiment, carried out over a period of five weeks, male Wistar rats weighing 190–220 g (Charles River Laboratories, L'Abresde, France) were randomly divided into 2 groups. In the control group (C), 9 healthy rats were fed the AIN93 M semisynthetic diet, containing 44.5 mg Fe, 12.8 mg Mn and 60 mg V per kg food and were allowed free access to drinking water (Milli-Q quality) throughout the experimental period. The group exposed to vanadium (V) was constituted of 10 healthy rats fed the same AIN93M diet but given drinking water containing 0.75 mg BMOV/mL. Every day at 9 a.m., the surplus water from the previous day was removed and the amount consumed was calculated. Water and V intake is described in Table 1. This compound was selected for the study because it has been reported to be two to three times more effective than vanadyl sulphate as glucose-lowering agent and was better tolerated and nontoxic in animal models (Caravan et al., 1995; Thompson and Orvig, 2006). In all cases, the BMOV solution was prepared and administered daily in the drinking water for 35 days, a sufficient time for the effects of V to become apparent (Oster et al., 1993; Majithiya et al., 2005; Tas et al., 2007; Sánchez-González et al., 2012a). BMOV was synthesised according to published procedures (McNeill et al., 1992).

Table 1
Digestive utilisation of Fe and Mn on days 28–35 of study.

Groups	C	V
Body weight on day 35	296 ± 30	236 ± 24[a]
Food intake (g/day)	15.5 ± 2.5	13.2 ± 1.6[a]
Energy intake (kcal/day)	56 ± 9,0	47 ± 5,8[a]
Water intake (mL/day)	16.6 ± 4.3	8.3 ± 1.9[a]
V intake (μg/day)	1 ± 0.2	1038 ± 208[a]
Digestive utilisation of Fe		
Intake Fe (I) (μg/day)	668 ± 88	587 ± 73[a]
Faecal Fe (F) (μg/day)	497 ± 75	480 ± 54
Absorbed Fe (μg/day)	171 ± 78	107 ± 58[a]
Absorption Fe (%)	26 ± 8.1	18 ± 6.6[a]
Digestive utilisation of Mn		
Intake Mn (I) (μg/day)	192 ± 25	169 ± 21[a]
Faecal Mn (F) (μg/day)	124 ± 25	115 ± 22
Absorbed Mn (μg/day)	68 ± 17	54 ± 11[a]
Absorption Mn (%)	35 ± 9	32 ± 10

Values shown are means ± SD, C (control rats); V (rats exposed to 1 mg V/day). Biological indices calculated: absorbed as $[I - F]$ and absorption (%), as $[(I - F)/I] \times 100$.

[a] C vs. V, $P < 0.05$.

On day 35, after overnight fasting, the rats were anaesthetised by intraperitoneal injection of 5 mg sodium pentobarbital/100 g body weight (Sigma–Aldrich, St Louis, MO, USA) and exsanguinated by cannulating the posterior aorta. Blood was collected and centrifuged at 1200*g* for 15 min to separate the serum. The liver (right medial lobe), kidney, spleen and heart were removed, weighed, placed in polyethylene vials and stored at −80 °C. During the last seven days of the experimental period, the faeces were collected every 24 h and stored at −80 °C in polyethylene bottles for subsequent analysis. All animals were housed from day 0 of the experiment in individual metabolic cages designed for the separate collection of faeces and urine. The cages were located in a well-ventilated, temperature-controlled room (21 ± 2 °C) with relative humidity of 40–60%, and a 12-h light:dark period.

All experiments were carried out in accordance with Directional Guides Related to Animal Housing and Care (European Community Council, 1986) and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

2.2. Analytical methods

V, Fe and Mn in the diet, serum and tissues were determined by collision reaction cell ICP–MS (He/H_2 mode) (Agilent 7500, Tokyo, Japan) after digestion of the corresponding material using a microwave oven (Milestone, Sorisole, Italy). All the plastic containers used in the analysis were previously cleaned with super-pure nitric acid and ultra-pure water (18.2 Ω) obtained using a Milli Q system (Millipore, Bedford, MA, USA). Samples of 0.2–0.4 g of dry tissue or serum, were prepared by digestion with HNO_3 and H_2O_2 (super-pure quality; Merck, Darmstadt, Germany), in a microwave digester (Milestone, Sorisole, Italy). When the sample had been digested, the extract was collected and made up to a final volume of 50 ml for subsequent analysis. Calibration curves were prepared following the Ga addition technique as an internal standard, using stock solutions of 1000 mg/l of each element (Merck). The accuracy of the method was evaluated by the analysis of suitable certified reference materials, Seronorm (Billingstad, Norway) and NIST 8414 (Gaithersburg, MD, USA), and by recovery studies in samples of organs enriched with multi-element standards. The percentage of CV obtained was 5.6% for V, 1.3% for Fe and 2.9% for Mn. For each element, we used the mean of five separate determinations of this reference material.

2.3. Measurement of haematological parameters

Red blood cell (RBC), haemoglobin (Hb) and haematocrit (Hct) counts were determined using a Sysmex KX-21 automatic haematology analyser (Sysmex, Japan). Mean cell haemoglobin (MCH) and mean cell volume (MCV) were calculated from the RCB, Hb and Hct.

2.4. Determination of biochemical and proinflammatory parameters, and protein carbonyl groups

Ferritin, leptin, interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) were determined in serum. Ferritin (Spinreact Ref 1107040DS, Girona, Spain) was determined using a BS-200 Chemistry Analyser (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Hamburg, Germany). Leptin, interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) were

determined using the kit Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel, RECYTMAG-65K, with the Luminex xMAP detection system (EMD, Millipore Corporation, Billerica, MA, USA). The analysis was performed using a Luminex system (Millipore, Germany). Assays were performed according to the manufacturer's instructions.

The levels of protein carbonyl groups were assessed using Protein Carbonyl Kit (Cayman Chemical Company, MI, USA). Briefly, 100 µl of the homogenate from the right medial lobe of the liver were transferred to two tubes. One tube was the sample tube and the other was the control tube. After adding 400 µl of 2,4-dinitrophenylhydrazine (DNPH) to the sample tube and 400 µl of 2.5 M HCl to the control tube, both of them were incubated in the dark at room temperature for 1 h. Afterwards, 0.5 ml of 20% trichloroacetic acid was added to each tube and incubated in ice for 5 min. This mixture was centrifuged at 10,000*g* for 10 min at +4 °C, obtaining a pellet that was resuspended in 0.5 ml of 10% trichloroacetic acid and incubated in ice for 5 min and again centrifuged at 10,000*g* for 10 min at +4 °C. The pellet obtained was resuspended in 0.5 ml of (1:1) ethanol/ethyl acetate mixture and centrifuged at 10,000*g* for 10 min at +4 °C twice. Finally, the pellet obtained was resuspended in 250 µl of guanidine hydrochloride and centrifuged at 10,000*g* for 10 min at +4 °C, obtaining a supernatant of which 220 µl were transferred to a 96-well plate and absorbance read (Infinite® 200 PRO NanoQuant. Tecan Group Ltd., Switzerland) at 370 nm. The protein content of the supernatant solutions was determined by the Bradford method (1976), using bovine serum albumin as the standard.

2.5. Analysis of hepcidin gene expression in liver samples by RT-qPCR

Total RNA from liver samples from 9 control rats and 8 rats exposed to V was isolated using Trizol® following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer and 2 µg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). Real time quantitative PCR amplification and detection was performed on optical-grade 48well plates in an Eco™ Real-Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, the KAPA SYBR ® FAST qPCR Master Mix (Illumina, CA, USA). The primer sequences for HAMP, which encoded the hepcidin protein, were: forward 3′-GTTGTCTGCTCTGTCTGATGC-5′, reverse 3′-GAATCACGGTAGAACGGAAGA-5′. To normalise mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured, with the following primer sequences: forward 3′-CCATCACCATCTTCCAGGAG-5′, reverse 3′-CCTGCTTCACCACCTTCTTG-5′. The mRNA relative quantisation was calculated using the ΔΔCt method.

2.6. Statistical analysis

Descriptive statistical parameters (means and standard deviations) were obtained for each of the variables studied. Statistical comparisons among the four groups were performed by the analysis of the variance (ANOVA) test. All analyses were performed using the Statistical Package for Social Science 15.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at a probability level <5%.

3. Results

The reasons for analysing BMOV, the specific doses used and observations on toxicity problems observed in the animals have been described in a previous publication (Sánchez et al., 2011; Sánchez-González et al., 2012b). The stability study of the BMOV in water has been described in Caravan et al. (1995).

The mean ingestion of V (at 1 mg V/day) as BMOV led to the reductions the weight gain by ≈20% and food and water intake by 15% and 50%, respectively (Table 1). Table 1 also shows the changes caused in the digestive utilisation of Fe and Mn. The following biological indices were calculated: absorbed as $[I - F]$, and absorption (%), as $[(I - F)/I] \times 100$; where I = intake and F = faecal excretion The rats that consumed BMOV presented a decreased intake of Fe (14.8%) and Mn (14.6%), due to the reduced the food intake. V significantly reduced Fe absorbed (by 37%) and the percentage of Fe absorption (31%). Absorbed Mn also decreased significantly, although by only 21%, there were no significant changes in the percentage of absorption.

Table 2 shows the effect of BMOV on biochemical and haematological parameters. As expected, the intake of the complex provoked a significant increase in serum levels of V. It also produced a decrease in serum levels of Fe, but no significant changes were observed in the serum levels of Mn. The BMOV had an important effect on the serum levels of leptin, which fell by 64%. However, circulating levels of IL-6, TNF-α and ferritin increased, by 67%, 67% and 15%, respectively, while those of IL-1β remained unchanged. In the treated rats, red blood cells, haemoglobin (Hb), haematocrit (Hct), mean cell haemoglobin (MCH) and mean cell volume (MCV) all fell significantly, with Hb being the blood parameter that was most significantly affected by the treatment.

Table 2
Haematological and biochemical parameters in serum on day 35.

Groups	C	V
Biochemical parameters		
V (µg /L)	2.3 ± 0.5	456 ± 73[a]
Fe (mg/L)	1.8 ± 0.14	1.5 ± 0.22[a]
Mn (µg/L)	1.6 ± 0.4	1.4 ± 0.5
Leptin (ng/L)	16.3 ± 4.4	5.9 ± 2.3[a]
IL-1β (pg/mL)	79 ± 45	66 ± 29
IL-6 (pg/mL)	127 ± 69	212 ± 37[a]
TNF-α (pg/mL)	7.7 ± 2.0	12.9 ± 3.3[a]
Ferritin (ng/mL)	210 ± 30	242 ± 25[a]
Haematological parameters		
Red blood cells ($\times 10^6$/mm^3)	9 ± 1	7 ± 1[a]
Hb (g/dL)	16 ± 3	12 ± 1[a]
Hct (%)	47 ± 8	37 ± 6[a]
MCV (fL)	55 ± 0.6	52 ± 1[a]
MCH (pg)	19 ± 0.5	17 ± 0.4[a]

Values shown are means ± SD, C (control rats); V (rats exposed to 1 mg V/day).
[a] C vs. V. $P < 0.05$. Haemoglobin (Hb), haematocrit (Hct), mean cell haemoglobin (MCH), mean cell volume (MCV).

Table 3 shows the fresh weight and the V, Fe and Mn content of the tissues studied. The BMOV provoked a decrease in liver weight, whereas no significant changes were observed in the weight of the kidney, spleen or heart. The hepato-somatic ratio was calculated by the formula: liver weight × (body weight − liver weight)$^{-1}$ × 100; this ratio was found to be the same for both groups of animals (controls 2.8 ± 0.5 rats, rats exposed to V 2.8 ± 0.2). These results indicate that the lower weight of the liver in the exposed rats is a consequence of the lower weight gain following BMOV consumption.

BMOV intake produced a significant increase in V content in all the organs studied. The greatest accumulation occurred in the

Table 3
Vanadium, iron and manganese content in liver, spleen and heart (mg/kg dry tissue) and protein carbonyl groups level on day 35.

Groups	C	V
Liver		
Fresh weight (g)	8.3 ± 1.6	6.6 ± 1.0[a]
V (mg/kg dry tissue)	0.043 ± 0.01	8.8 ± 2.5[a]
Fe (mg/kg dry tissue)	404 ± 54	531 ± 91[a]
Mn (mg/kg dry tissue)	8.2 ± 1.2	8.3 ± 1.0
Protein carbonyl groups (pmol/mg protein)	134 ± 33	221 ± 73[a]
Kidney		
Fresh weight (g)	0.9 ± 0.1	0.9 ± 0.1
V (mg/kg dry tissue)	0.12 ± 0.02	28 ± 6[a]
Fe (mg/kg dry tissue)	255 ± 43	255 ± 23
Mn (mg/kg dry tissue)	3.5 ± 0.5	3.8 ± 0.2
Spleen		
Fresh weight (g)	0.56 ± 0.10	0.54 ± 0.12
V (mg/kg dry tissue)	0.048 ± 0.01	0.19 ± 0.18[a]
Fe (mg/kg dry tissue)	3043 ± 246	3648 ± 324[a]
Mn (mg/kg dry tissue)	0.63 ± 0.05	0.66 ± 0.07
Heart		
Fresh weight (g)	0.9 ± 0.2	0.8 ± 0.1
V (mg/kg dry tissue)	0.007 ± 0.002	1.25 ± 0.18[a]
Fe (mg/kg dry tissue)	340 ± 26	391 ± 31[a]
Mn (mg/kg dry tissue)	1.2 ± 0.14	1.4 ± 0.13

Values shown are means ± SD, C (control rats); V (rats exposed to 1 mg V/day).
[a] C vs. V, $P < 0.05$.

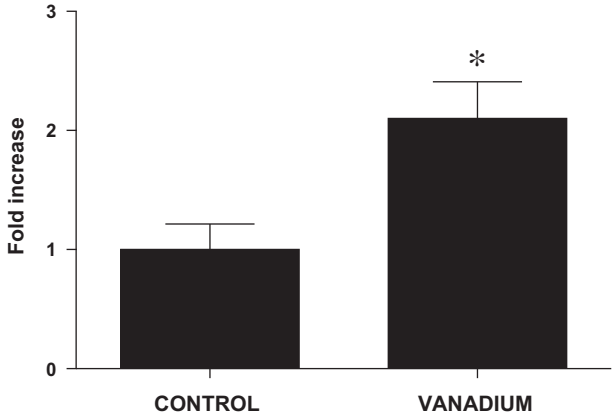

Fig. 1. Effect of V intake for five weeks on the hepatic expression of hepcidin, analysed by RT-PCR in rats, revealing a twofold increase in V-treated rats in comparison with the control group. Data (Control $n = 9$; exposed to BMOV $n = 8$) are expressed as means ± SEM; $^{*}P < 0.05$ vs. control group).

kidney, followed by the liver and the heart, and the lowest accumulation was in the spleen. In relation to changes in the tissues, the Fe content increased in the liver, spleen and heart, but there were no changes in the kidney. The spleen presented the highest content of Fe per kg dry tissue, but the largest percentage increase took place in the liver (31%), whereas in the spleen this increase was 20% and in the heart it was 15%. There were no significant changes in Mn content in any of the tissues studied (Table 3). Table 3 also shows that BMOV intake also led to a significant increase in the content of hepatic protein carbonyl groups (by ≈65%, from values in liver of 134 ± 33 pmol/mg protein). This study also addressed the effect of BMOV intake for five weeks on the hepatic expression of hepcidin, as revealed by RT-PCR in rats. We observed a twofold increase in the V-treated rats in comparison with the control group (Fig. 1).

4. Discussion

The aim of the present study was to determine whether alterations in Fe homeostasis and haematological changes caused by exposure to V are related to changes in the secretion of hepcidin, and whether these changes affect Mn homeostasis. Our results revealed, for the first time, that BMOV provokes an increase in the gene expression of hepcidin, which is associated with the presence of an inflammatory process, alterations in Fe homeostasis and the appearance of anaemia. However, Mn homeostasis was not affected by these changes.

In our study, V was found to decrease food, energy and water intake (Table 1), effects that are associated with lower weight gain as previously reported (Sánchez-González et al., 2012a; Oster et al., 1993; Scibior et al., 2006) and lower liver weight. Vanadium also decreased serum levels of leptin in comparison with the untreated rats (Table 2). Leptin is an adipokine that is synthesised mainly by the adipose tissue, in amounts directly related to the mass of adipose tissue. This hormone participates in the regulation of appetite (reducing food intake) and in that of energy expenditure (Jéquier, 2002; Almanza-Pérez et al., 2008). The lower weight gain by the V-treated group indicates that these rats have a lower adipose tissue mass, which would account for the lower serum leptin levels recorded. However, the decrease in leptin levels should have caused an increase in food intake and body weight. The opposite effect actually occurred, possibly due to the direct action of the ion on the central nervous system. Other authors have reported that V enhances the transduction of the leptin signal (Wilsey et al., 2006), which would explain why lower levels of leptin were accompanied by a reduction in food intake and body weight in our study.

In our experimental conditions, the exposure to V significantly decreased Fe absorption (Table 1), due to various factors. On the one hand, the reduction in food intake led to a corresponding reduction in absorbed Fe, although this decreased food intake (≈15%) is insufficient to fully explain the decrease in Fe absorption, as is evidenced by the significant decrease in absorption expressed as a percentage (Table 1). On the other hand, divalent metal transporter-1 (DMT-1) is known to be expressed in significant quantities in the duodenal enterocytes (Gunshin et al., 1997). This transporter is essential in the absorption of Fe^{2+}, and also carries V (as V^{3+} or VO^{2+}) (Mackenzie et al., 2007). However, there is little information about whether V may block or modify transporter expression. Ghio et al. (2005) reported an increased expression of the DMT1-IRE isoform in lung epithelial cells following the instillation of ferric ammonium citrate. However, its expression decreased after exposure to $VOSO_4$, which would decrease the ability to capture Fe, thus facilitating the emergence of ROS and lung diseases. It was recently observed that when rats were exposed to $NaVO_3$, supplied in their drinking water, DMT-1 levels in the liver and in the cerebral hemispheres were not significantly altered, while they decreased in the kidney (Scibior et al., 2014). To analyse the possibility that V intake may interfere with Fe intestinal absorption, blocking and/or modifying the expression of DMT-1, we studied the digestive absorption of Mn, a cation which is also transported by DMT-1. Mackenzie et al. (2007) studied the mechanism by which Mn is transported by DMT-1, and showed that Mn^{2+} presents evoked currents similar to those of Fe^{2+}. It has also been shown, in broilers, that DMT-1 plays a very important role in Mn absorption within the duodenum and jejunum (Bai et al., 2008). Alterations in the homeostasis of Mn would indicate an effect of V on the carrier protein DMT-1. However, our results showed no differences in Mn absorption expressed as a percentage in V treated rats. Therefore, the lower absolute quantities absorbed are a direct result of the lower intake (Table 1). These findings lead us to believe that V neither blocks nor modifies transporter expression in the intestine and that the decrease in Fe absorption is due to other causes.

To further examine the causes underlying the decrease in Fe absorption, we studied changes in the gene expression of hepcidin in the liver (Fig. 1), finding that V intake provoked a significant increase in hepcidin expression, thus aggravating the degradation of ferroportin and inhibiting the ferroportin-dependent Fe efflux from the enterocytes, hepatocytes and macrophages toward the plasma. This outcome would explain the decrease in intestinal Fe absorption, the hypoferraemia, the Fe sequestration in the tissues and the higher circulating levels of ferritin (Tables 2 and 3). The increase in circulating ferritin reflects the existence of higher levels of ferritin in the tissues. In turn, this facilitates Fe sequestration in the tissues and may be related to an attempt by the organism to control the oxidative stress associated with exposure to the metal (Ghio et al., 1998). Fe overload in the tissues in response to exposure to V has been observed by other authors in studies of non-diabetic rats (Thompson et al., 2002; Scibior et al., 2012, 2014) and of diabetic rats (Sánchez-González et al., 2014).

There are conflicting views regarding the haematological changes provoked by V in non-diabetic rats. Some authors have found these values to decrease, while others report them unchanged. It has been observed that V produces peroxidative changes in the erythrocyte membrane and liver (Scibior et al., 2006, 2013). However, other authors have found no such changes (Dai et al., 1995). Caloric restriction is known to reduce oxidative stress and damage (Qiu et al., 2010), to inhibit the inflammatory response and to enhance the cell-mediated immune function

(Wang et al., 2013). Therefore, the lower caloric intake resulting from the ingestion of V might contribute to decreasing the production of reactive oxygen species (ROS) and proinflammatory cytokines. However, in a previous study we reported that V decreased the activity of various antioxidant enzymes and provoked an increase in the lipid-peroxidation level (MDA) in the liver (Sánchez-González et al., 2012a). These circumstances could cause malfunctions in the liver by the overproduction of ROS. We also examined protein carbonyl group levels in the liver, which were found to increase after the exposure to V (Table 3). This would suggest that the treatment also provoked oxidative damage to the liver proteins. The appearance of oxidative stress is associated with an inflammatory response, and V exposure has been related with inflammatory changes in respiratory tracts (Pierce et al., 1996). The generation of oxidative stress is critical to the induction of V-induced effects on cytokine production (Dye et al., 1999). In our study, treatment with V significantly increased levels of IL-6 and TNF-α.

Various studies have examined alterations in Fe metabolism in the lung following exposure to environmental particles containing various metals, including V. These studies show that the metals present in such particles that affect Fe homeostasis and their redox behaviour and properties are crucial determinants of immunotoxic effects in the lung. Specifically, V^V has a significant local effect, causing significant increases in cytokines, which may help explain the toxic effects of these metals when they are inhaled (Ghio and Cohen, 2005; Prophete et al., 2006; Cohen et al., 2010). It has been reported that the exposure of human bronchial epithelial cells to residual oil fly ash containing V, Ni and Fe produces significant increases in IL-8, IL-6 and TNF-α (Carter et al., 1997) and that the phosphorylation of MAPK proteins may mediate the metal-induced expression of inflammatory proteins in these cells (Samet et al., 1998).

Studies have reported that the expression of hepcidin can be modulated in different conditions, including inflammation, infection, hypoxia or low cellular Fe. In fact, it has been shown that human hepcidin promoters contain a consensus HRE that makes this sensitive to alterations in the HIF system, a negative regulator of hepcidin expression and provides a mechanism whereby hypoxia may lead to decrease in hepcidin expression (Peyssonnaux et al., 2007). In support of our results here, in vitro studies have revealed that hepcidin transcription also involves interactions between functional metal response elements (MREs) in its promoter and the MRE-binding transcription factor-1. Analysis of hepcidin mRNA and protein levels in hepatoma cells suggests that its expression may be regulated by divalent metal ions, with zinc inducing maximal effects on hepcidin levels (Balesaria et al., 2010). Similar actions could be involved in the increased hepcidin expression in hepatic tissue after the prolonged intake of V by rats. Moreover, the HIF system is activated in Fe-deficient states (Prophete et al., 2006) and in our case the V produces an overload of hepatic Fe.

The synthesis of hepcidin in hepatocytes is known to be transcriptionally regulated by IL-6 (Nemeth et al., 2004b). The increased production of hepcidin mediated by inflammation is responsible for the Fe sequestration in the tissues and the hypoferraemia that develops during inflammatory disease, thus limiting the availability of Fe for erythropoiesis and contributing to anaemia by inflammation or chronic disease (Ganz and Nemeth, 2011). In our study, V caused the appearance of a moderate anaemic process, with decreases in haematological parameters such as the number of red cells, Hb, Hct, MCH and MCV in comparison with the control group (Table 2). These results agree with those reported in previous studies (Scibior et al., 2006, 2012). Although the stimulation of hepcidin production by IL-6 is well defined, other effects of oxidative stress and inflammation may contribute to the onset of anaemia, including the reduction in the half life of the erythrocytes due to haemolysis, the activation of macrophages and erythropoietic suppression due to the direct effect of cytokines on erythrocyte precursors and a partial inhibition of erythropoietin (Ganz and Nemeth, 2011).

It has been suggested that Mn might modulate the expression of hepcidin, and therefore changes in the plasma levels of Mn could attenuate or increase hepcidin content (Bartnikas, 2012). It has also been reported that Mn is a substrate for ferroportin, the transport of which seems to be unidirectional (Yin et al., 2010; Madejczyk and Ballatori, 2012). However, recently Mitchell et al. (2014) observed that oocytes expressing ferroportin did not stimulate the efflux of ^{54}Mn when this metal was injected. In the present study, no changes were observed in Mn absorption (Table 1), plasma levels (Table 2) or content in the different tissues studied (Table 3). Therefore, in our experimental conditions the Mn does not seem to be involved in the increased expression of hepcidin, and this increase did not produce significant changes in the homeostasis of Mn.

5. Conclusions

The results obtained from this study provide the first evidence that V given to healthy rats at a dose of 1 mg V per day in the form of bis(maltolato)oxovanadium(IV) (BMOV) provokes an increase in the gene expression of hepcidin, possibly caused by an inflammatory process. Both factors could be the cause of alterations in Fe homeostasis and the appearance of anaemia. However, Mn homeostasis was not affected by these changes. Nevertheless, further studies are needed to better determine the effects arising from these interactions in order to establish the role of V, both as a micronutrient and to reduce its toxic effects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

We are grateful for support received from the Consejeria de Innovacion, Ciencia y Empresa, Andalusian Regional Government (Project P06-CTS-01435), and from the Spanish Ministry of Economy and Competitivity (SAF2011-29648); the CIBERehd is funded by the Instituto de Salud Carlos III and GK Harding for translating the manuscript into English. This paper forms part of the Doctoral Programme in Nutrition and Food Science of the University of Granada.

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