Histol Histopathol (2016) 31: 433-439

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Myocardial Connexin-43 and N-Cadherin decrease during vanadium inhalation

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Summary. Particulate matter air pollution has considerably increased during the last decades; vanadium is a transition element adhered to this particulate matter, and the combustion of fossil fuels is the main source in the atmosphere.

It has been reported that air pollution and specifically vanadium exposure increases the probability of suffering arrhythmias; however the biological mechanism of such a relationship remains unknown. It has been established that a diminished presence of N-Cadherin alters the Connexin-43 arrangement, and the consequent altered presence of these proteins predisposes to ventricular heart rate problems.

We analyzed myocardial histology and the expression of N-Cadherin and Connexin-43 by immunohistochemistry in mouse that inhaled vanadium. Our results showed a significant and progressive reduction in both N-Cadherin and Connexin-43, as well as the presence of meganucleus; myofibrils disruption, and clumping in the exposed groups were also observed. Our findings add more information about a possible explanation for the arrythmogenic effect observed in dwellers of cities with high particulate matter atmospheric pollution.

Key words: Air pollution, Arrhythmias, Connexin-43, N-Cadherin, Vanadium inhalation, Myofibrils, Clumping

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DOI: 10.14670/HH-11-688

Introduction

Increased epidemiologic evidence links exposure to particulate matter (PM) with an increment in the incidence of myocardial infarction and arrhythmias (Brook et al., 2004; Farraj et al., 2011). Arrhythmias are a cause of increased mortality during acute pollution events (Gold et al., 2000; Fang et al., 2010; Gold and Mittleman, 2013). Several components of PM have been considered as the origin of these events because oxidative gases, organic compounds and transition metals adsorbed to the particles' carbon core are carried into the airways and transported to the pulmonary and systemic circulations.

ROFA (Residual oil fly Ash) is a waste product of fossil fuel combustion from boilers; it is rich in transition elements such as vanadium and a source of ambient fine particulate air pollutants (Ghio et al., 2002). About 25% of the inhaled vanadium is absorbed (Rodriguez-Mercado and Altamirano-Lozano, 2006) and according to Rehder (Rehder, 2015) when vanadium is in the bloodstream it is distributed into different organs such as myocardium, liver and kidneys. In addition, it has been reported that the effects of vanadium administration may persist several weeks after the element has been withdrawn (Barceloux, 1999).

Vanadium is a transition metal adhered to PM, which has been reported to predispose to the emergence of ventricular arrhythmias in occupational exposed population (Prakash, 1991). Other studies report heart rate variability in occupational exposed workers suggesting an effect on autonomic function (Dekker et

al., 1997). The pathophysiological mechanism proposed is a disturbance in the control of the autonomous system, advocating the inhibition of the parasympathetic branches (Shrey et al., 2011); however, there is no direct evidence for the possible mechanisms to explain the association between PM pollution and myocardial pathology.

Myocardiocytes are highly specialized cells with unique structures known as intercalated discs, composed by intercellular-junctions that facilitate the coordinated conduction of the electric stimuli generated in the heart's conducting system (Barrett et al., 2012). These intercellular-junctions are an ensemble of proteins such as Connexin-43 (Cx43) that electrically couple myocardiocytes for its coordinated depolarization, and its uncoupling has been associated with heart diseases (Li et al., 2005; Kalcheva et al., 2007); in addition, N-cadherin mediates the anchorage of myofibrils to the cellular membrane, maintaining the stability of the cell-to-cell communication (Luo and Radice, 2003).

It is known (Lerner et al., 2000; Meckert et al., 2005) that a specific loss of N-Cadherin leads to a decrease in myocardial Cx43 presence with consequent ventricular arrhythmias. Furthermore, it has been reported that in primary cell cultures of Syrian hamster embryos, vanadium exposure directly inhibits Cx-43 by tyrosine phosphorylation (Mikalsen and Kaalhus, 1998).

In this report we demonstrate that vanadium inhalation exerts a time-related decrease in Cx-43 and N-Cadherin, which may explain the association of increased incidence of myocardial pathology in cities with high PM atmospheric pollution.

Material and methods

CD-1 male mice weighing 33±2 g were housed in hanging plastic cages under controlled light conditions (12 h light / dark regime), and fed with Purina rat chow and water *ad libitum*. The mice were managed according to the Mexican official norm NOM-062-ZOO-1999 for the production, care and use of laboratory animals. Inhalation was performed as described by (Fortoul et al., 2009). Eighteen mice were randomly placed in an acrylic box, inhaling 0.02 M V_2O_5 (Sigma, St Louis USA) for one hour twice a week over twelve weeks. This exposure protocol was selected knowing that the half-life of vanadium is about 48 h. Controls (12 mice) inhaled only the vehicle –saline (sodium chloride [NaCl] 0.9%) - for the same period of time. Three exposed mice, and two controls were sacrificed 24 hours after the first inhalation and successively at 2, 4, 8, and 12 weeks. Animals were anesthetized with sodium pentobarbital and perfused via aorta with saline, followed by 4% paraformaldehyde (pH 7.4) in phosphate buffer (Colin-Barenque et al., 2015). Hearts were processed for light microscopy. Paraffin-embedded tissue sections were stained with Phosphotungstic acid-hematoxylin to evaluate myocardial striations.

Vanadium concentrations in the chamber were quantified as follows: a filter was positioned at the outlet of the ultranebulizer during the whole inhalation time at flow rate of 10 L/min. After each exposure, the filters were removed and weighed; the element was quantified following the same protocol as with tissue samples. Six filters for each exposure were evaluated as in (Fortoul et al., 2009). Samples were analyzed using a graphite furnace Atomic Absorption Spectrometer (Perkin Elmer Mod. 2380). The light source came from a hollow cathode lamp. Formaldehyde and the blanks were also analyzed to identify metal contamination from this source. Accuracy was assured by three random determinations of seven different standard solutions, prepared with the same chemical reactive used during the metal analysis. For vanadium, the wavelength was 318.4 nm, the detection limit was 0.37 ppm and the slit 0.7 nm, and each sample was analyzed in triplicate.

Tissue sections (5 μm) were placed in poly-L-lysine (SIGMA, St Louis, MO) coated slides. Antigen retrieval was achieved by incubation in citrate buffer (pH 7.4) at 120 lb for 3 min after which the slides were washed in phosphate-buffered saline (PBS). Endogenous peroxidase was blocked with 3% H_2O_2 (J.T. Baker, Phillipsburg NJ, USA) for 10 min. The sections were rinsed several times with PBS-Albumin, washed for 10 min in PBS, (MP Biomedical, Germany) and incubated for 1 h at 37°C in rabbit anti-N-Cadherin and separately in goat anti-Connexin-43 (abcam, Cambridge, MA, USA) diluted 1:100 in PBST- (PBS with 0.1% Tween 20 and Albumin). The sections were washed in PBS, and incubated for 30 min at 37°C with the biotynilated goat-anti-rabbit secondary antibody (Santacruz Biotechnology, California, USA), rinsed several times in PBS and incubated for 30 min at 37°C in HRP-streptavidin complex (Santacruz Biotechnology California, USA). Immunoreactivity was visualized by incubation in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Zymed Laboratories Inc, San Francisco, California, USA). Samples skipping primary antibody were also included as negative controls.

Evaluation of the immunoreactivity in myocardiocytes to N-Cadherin and Cx-43 was assessed on tissue sections, by color densitometry with an image analyzer, using the software Image-Pro-Plus version 6.0. (Media Cybernetics, Inc. Silver Spring, MD, USA) coupled to a digital camera (Evolution MP Color, Media Cybernetics) on a light microscope (Olympus BX51). From each animal, five fields (0.059 μm^2 each) randomly selected at x 400 were evaluated for color density, considering positive the presence of ochre color, when the developer for the reaction was diaminobenzidine (15 fields from each exposure time were evaluated, and 10 from controls). Color intensity was reported in pixel units (Fortoul et al., 2010).

Statistical analysis was conducted (ANOVA) to analyze the differences in the effect of vanadium on the immune-positive cells in the fields selected to N-Cadherin and Connexin-43 (Graph-Pad Prism v.4.0c, La Jolla California, USA). Dunnett's posttest was applied to

determine if the differences between controls and exposed groups were statistically significant ($p<0.05$). Also, a multiple linear regression analysis was performed to explain the changes in Cx43 and N-Cadherin with the time of exposure (Graph-Pad Prism v.4.0c, La Jolla California, USA). Myocardiocytes' nucleus morphometric measurements were evaluated with Macnification software V.1.6.1 (2008-2009 Orbicule). From each exposure time ten nuclei were measured for length and width and differences were statically analyzed (ANOVA, Dunnett's Posttest) to identify differences in nuclei's size in controls vs exposed groups.

Results

As was previously mentioned (Fortoul et al., 2009; Cano-Gutierrez et al., 2012; Ustarroz-Cano et al., 2012) the average concentration of vanadium in the chamber was 1436 $\mu g/m^3$ during the whole experiment. No macroscopic changes were observed when the hearts were extracted, neither in controls nor in exposed mice.

Myocardiocytes were identified in controls as large cells stuffed with ordered myofibrils in the cytoplasm. The periodicity of A and I bands was easily identified (Fig. 1A). In a cross section of the myocardiocytes the myofibrils are distributed in the cytoplasm (Fig. 1C). During the first week of exposure and through the length of the experiment exposed myocardiocytes were swollen and bands appeared disorganized (Fig. 1B). Also, large nuclei were observed in these cells (asterisk). In a cross section in exposed mice clumping of the myofibrils in some cells was observed (Fig. 1D). In the insert this abnormality is clearly distinguished (Fig. 1D). In Fig. 2, nuclei measurement demonstrated a significant difference in exposed myocardiocytes' nuclei width and length when compared with controls (Dunnett's $p<0.05$). The increase was more noticeable at week-8 through the length of the exposure, although the changes remained until the end of the experiment. In addition, in some

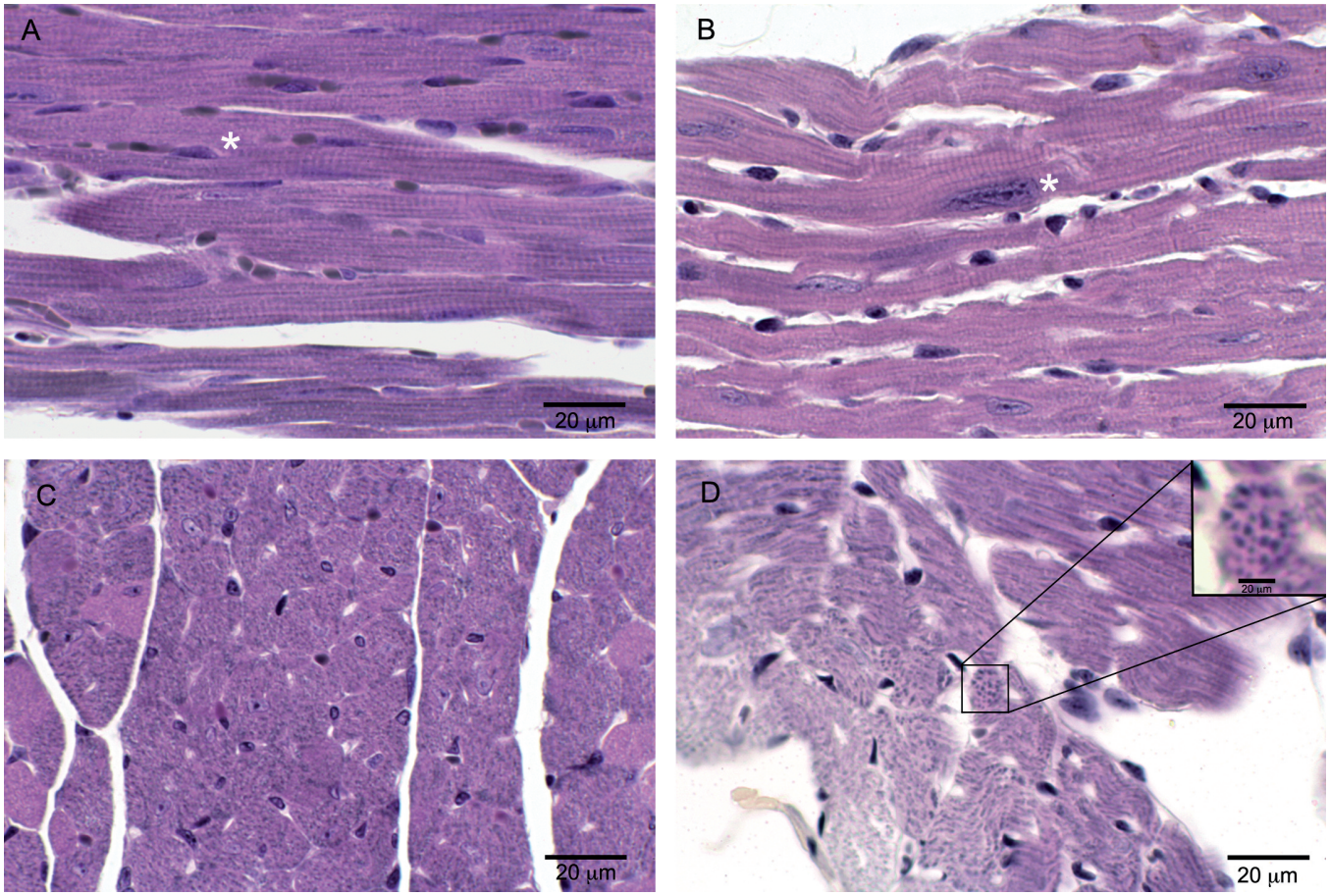

Fig. 1. A. Control myocardiocytes are characterized by a cytoplasm stuffed with ordered myofibrils, and a central nucleus. Bands A and I are easily identified. Dense chromatin nucleus is observed (asterisk). **C.** In cross-section in control myocardiocytes, myofibrils are regularly distributed in the cytoplasm. **B.** In exposed mice the myofibrils were disorganized; the presence of meganucleus was observed (asterisk). **D.** myocardiocytes cross-section in which myofibrillar clumping is notorious as it is shown in the insert (Phosphotungstic acid-hematoxilin).

myocardiocytes at 12-week exposure, myofibril clumps were observed in transverse sectioned myocardiocytes observed with more detail in the insertions (Fig. 3).

In control mice myocardiocyte N-Cadherine was noticed at the edges of the cell (Fig. 3A) and after a 12-week exposure time the ochre stain faded out (Fig. 3C). Cx-43 in controls was neatly located at the extremes of the cells in the intercalated disk (Fig. 3B), while in experimental mice the stain vanished (Fig. 3D). The observations were similar for both proteins.

Fig. 4 depicts the time-pattern for both proteins through the 12-week exposure time. An abrupt decrease in N-Cadherin preceded Connexin-43 decrement; this decrease was more evident at week four and it maintains this trend until the end of the experiment. Cx-43 begins its reduction at week eight and maintains this trend through the 12-week exposure. Multiple linear regression analysis indicated that Cx43 decreased as the time of exposure increased (r^2= 0.87, p<0.006); however for N-Cadherin the correlation was not statistically

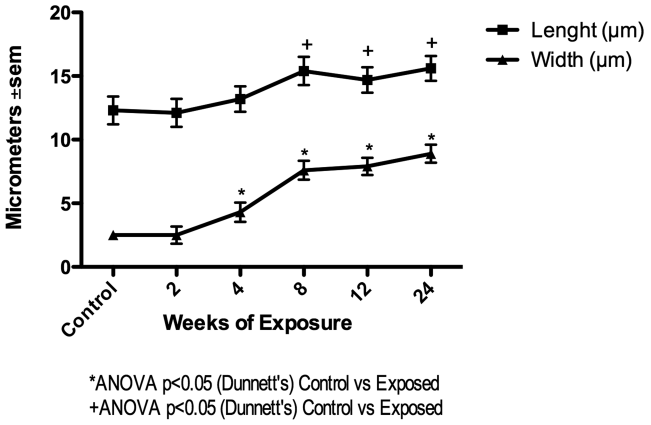

Fig. 2. Larger and wider nuclei are observed when controls are compared with exposed mice. Changes are observed in both parameters since the 8-week exposure. ANOVA p<0.05 (Dunnett's)* for width differences and + for length differences.

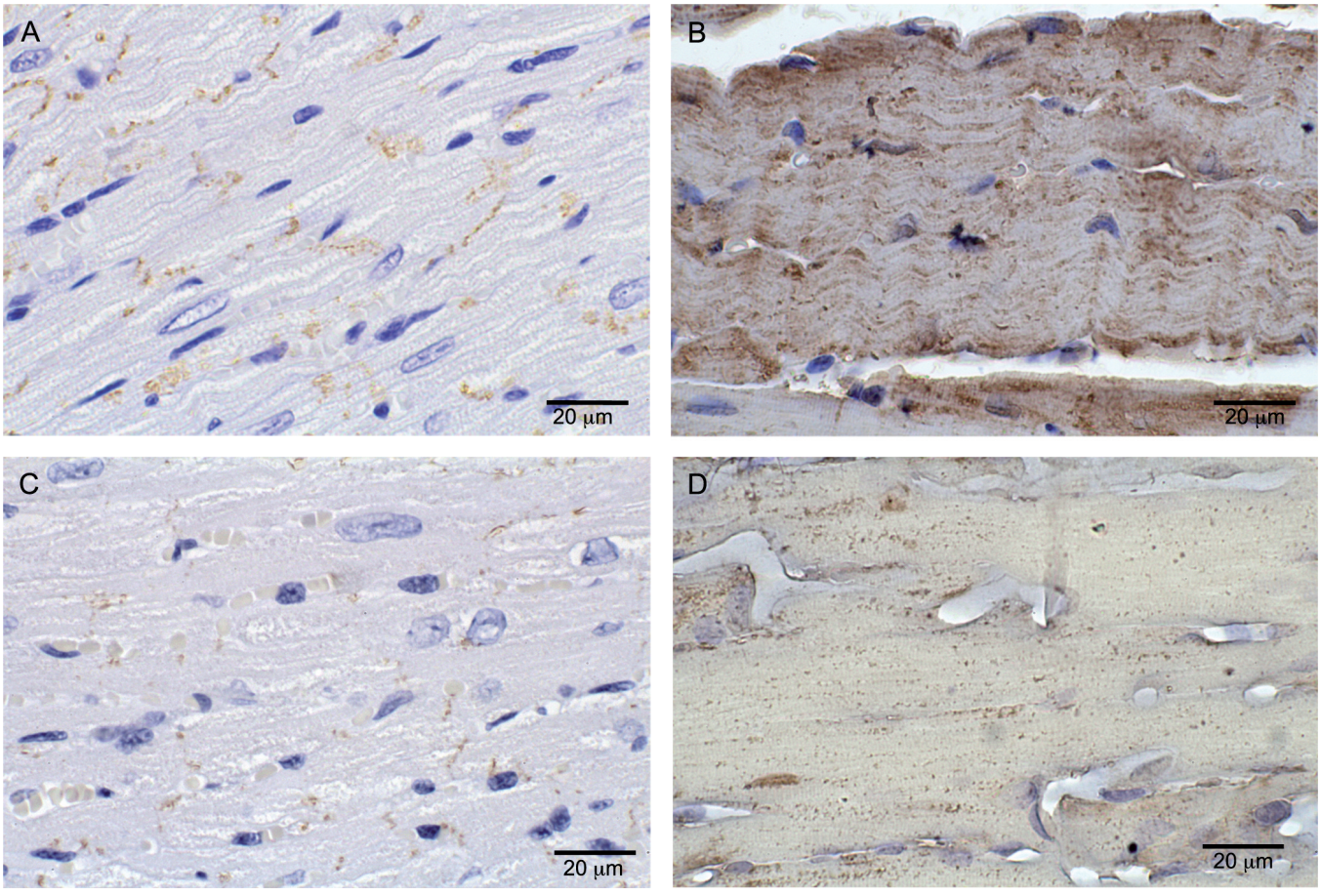

Fig. 3. A. Controls for Connexin 43, show the stain located in the intercalated disks in the cellular junctions, while in exposed myocardiocytes the stain reduces its intensity **(C). B.** Immunohistochemistry for N-Cadherin in control myocardiocytes showed a homogeneous distribution in the cytoplasm with a discrete predominance in the periphery of the cells. After a 12-week exposure time the stain almost disappeared **(D)**.

significant (r^2= 0.43, p NS).

Discussion

Our results indicate that inhaled vanadium causes a decrease in the presence of N-Cadherin and Cx-43 in a time-exposure mode. Also, histological changes such as edema, myofibrils' disorganization and clumping, as well as an increase in nuclear size, were evidenced in exposed mice compared with controls.

Cx-43 presence and function depends on many different factors that can be affected by vanadium. *In vitro* studies (Li et al., 2005) report that the reduction in the presence of Connexin-43 is enough to explain the conduction malfunction observed in myocardiocytes cultures. Farraj (Farraj et al., 2011) exposed rats to particulate matter measuring myocardium's miRNAs, and reported a decrease in miR-1 and miR-133. MiR-1 increases the possibilities of arrhythmias because it decreases kir2.1, which is the channel that controls the primary K^+ flux to maintain resting membrane potential and connexin-43. They do not report modifications in Cx-43 in their model, but their findings help to explain ours, and also suggest its association to vanadium exposure. It is possible that the differences found in their results and ours could be because of the use of mice in this experiment and the use of rats in theirs.

Besides vanadium effect on Cx-43, other reports indicate that vanadium exposure alters directly microtubule function, as well as other motor protein systems (Giepmans et al., 2001; Thier et al., 2003; Rodriguez-Lara et al., 2015). The direct relationship between N-Cadherin and Connexin-43 with microtubules is also a possible explanation for our findings, and it is supported by other authors' reports (Kirazov and Weiss, 1986; Thier et al., 2003; Shaw et al., 2007). In support of our histological observations, the absence of N-Cadherin decreases myocardiocytes contact and favors disruption of the myofibril organization (Shaw et al., 2007).

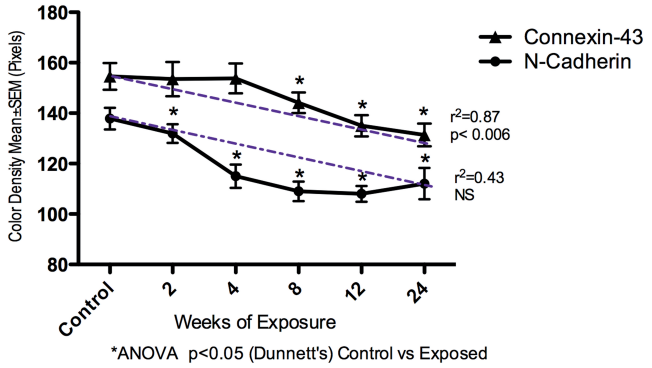

Fig. 4. Color density modification in Connexin-43 and N-Cadherin. A decrease in both proteins is observed. Connexin-43 shows a delayed decrease during the exposure reaching a plateau at 12-week exposure. N-Cadherin displays a sharply decrease from the beginning to the end of the experiment (ANOVA p<0.05 Dunnett's posttest. Controls vs exposed). A time-exposure correlation was observed (r^2=0.87 p<0.0006) for Connexin-43 but not for N-Cadherin (r^2=0.43 p NS).

Because Connexins are phosphoproteins and vanadium has a high affinity for phosphates sites, this could be another vanadium mechanism for disrupting connexin structure and function (Vinken et al., 2010) (Mikalsen and Kaalhus, 1998), and also an explanation for the higher correlation that we found with Cx-43 and vanadium time-exposure compared with N-Cadherin (Saffitz et al., 2000; Berthoud et al., 2004). To explain the differences in the decrease pattern observed for both proteins, we can suggest that N-cadherins were affected before Cx-43, as was reported by Matsushita (Matsushita et al., 1999) in ischemic myocardium.

These findings offer another possible explanation for the increased heart rate variability observed and attributable to particulate matter air pollution and vanadium exposure (Prakash, 1991; Gold et al., 2000). It is important to remark that because of the predominantly ventricular presence of Connexin-43, depleted hearts present arrhythmias by re-entry mechanisms, which would be manifested as extrasystoles and ventricular tachycardia. Other vanadium effects that might explain its implications in arrythmogenesis could be its capacity to inhibit the $2Na^+/3K^+$ pump (Valko et al., 2006).

On the other hand, in mice exposed to inhaled vanadium, thrombocytosis has been reported; a factor that might increase cardiac infarction risk and that has been independently associated with air pollution events and increased mortality rates (Gonzalez-Villalva et al., 2006). It is important to remember that arrhythmias also increase the risk of cardiac infarction by producing turbulent flow and embolus formation (Brook et al., 2004).

The concentration reported in our model (1436 $\mu g/m^3$) is in the range from 0.01 to 60 mg/m^3 mentioned by World Health Organization (WHO, 2000) in workplaces. High vanadium concentrations (>600 mg/kg) have also been detected in soils in the neighborhood of petrochemical plants in Mexico as a consequence of air-borne particulate emissions resulting from high levels of oil burning (Hernandez and Rodriguez, 2012). The concentration used in our experiment reflects the vanadium exposures that regularly occur among people living in many urban centers, as well as in occupational surroundings.

Finally, the morphologic changes observed in the myocardiocytes also reflect the interaction of vanadium with the cytoskeleton that supports the myofibrillar structure (Poelzing and Rosenbaum, 2004). The nuclear morphological changes correlate with those observed by Cano-Gutierrez et al. (2012) in hepatocytes after vanadium exposure, and are also supported by the endomitosis and polyploidization capability reported in myocardiocytes located in the periphery of myocardial infarcts reported in humans, which may increase nuclear size as it is observed in megakaryocytes (Meckert et al.,

2005). The loss of some nuclear proteins associated with nuclear morphology could be modified directly by vanadium (Rodriguez-Lara et al., 2013) or indirectly as a consequence of the oxidative stress induced by this element (Capo-chichi et al., 2009).

As Knuckles (Knuckles and Dreher, 2007) and us have shown, vanadium may exert its cardiotoxicity by a wide spectrum of mechanisms and makes us suggest that the sum of all the alterations previously mentioned could compromise cardiovascular function integrity.

It is important to mention that the study of inhaled vanadium myocardial effects is part of the evaluation of the systemic effects of this element. Previous studies from our group, in the same mouse experimental model, have demonstrated that vanadium inhalation affects different organs with similar underlying mechanisms, such as inflammation, oxidative stress, cytoskeleton changes, and genotoxicity; in addition immunotoxicity, reprotoxicity, teratogenicity and endothelial damage have also been reported (Montiel-Davalos et al., 2012; Fortoul et al., 2014). Therefore, we cannot assume that the changes we observed in myocardial tissue are specific; some are consequence of direct interaction with the structures that identify myocardiocytes –intercalated discs-, but others are the consequence of the inhalation of vanadium and its systemic implications.

Further studies are needed in order to gain a more profound understanding of the risks that particulate matter air pollution pose to cardiovascular function in dwellers of cities with this type of atmospheric pollution.

Acknowledgements. Authors thank Raquel Guerrero-Alquicira, from Departamento de Biología Celular y Tisular, School of Medicine, UNAM, for tissue-samples histological process; to Francisco Pasos-Nájera and Armando Zepeda-Rodriguez for the photographic image process. Thanks to Alejandra Núñez-Fortoul for reviewing and editing the manuscript.

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Accepted November 13, 2015