Int. J. Morphol.,
40(1):10-17, 2022.

Copper Induces Damage, Oxidative Stress and Cell Death in Endothelium of Chronic Intoxicated Wistar Rats

El Cobre Induce Daño, Estrés Oxidativo y Muerte Celular en el Endotelio de Ratas Wistar Intoxicadas

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FERNÁNDEZ-RODARTE, B. A.; SOTO-DOMÍNGUEZ, A.; GONZÁLEZ-NAVARRO, A.; MELLADO-AYALA, A.; PIÑA-MENDOZA, E. I.; RODRÍGUEZ-ROCHA, H.; MONTES-DE-OCA-LUNA, R.; GUERRA-LEAL, J. D.; GUZMÁN-LÓPEZ, S. & ELIZONDO-OMAÑA, R. E. Copper induces damage, oxidative stress and cell death in endothelium of chronic intoxicated Wistar rats. *Int. J. Morphol., 40(1)*:10-17, 2022.

SUMMARY: Reactive Oxygen Species (ROS) are part of the functional balance of various systems, they can generate cellular damage by oxidative stress associated with disease processes such as atherosclerosis, cardiovascular disease, diabetes, and aging. Some studies report that copper induces damage to the endothelium, which could be associated with cardiovascular pathologies. This study was an experimental comparative, prospective, longitudinal, and controlled clinical trial in a murine animal model. Twenty-four male Wistar rats were included, the distribution of the groups was time-depending chronic exposition to copper, and a control group. Results show gradual alterations in the groups treated with copper: areas with loss of the endothelium, signs of disorganization of smooth muscle fibers in the tunica media, as well as areas with the fragmentation of the elastic sheets. A significant statistical difference was observed in the active- Caspase-3 analysis expression in the aortic endothelium and endothelium of the capillaries and arterioles of the lung between the control group vs 300 ppm of copper. Expression of eNOS was detected in the endothelium of the aorta and vessels of the lung. Our study shows histological changes in the walls of the great vessels of intoxicated rats with copper, and the increment of inflammatory cells in the alveoli of the study model, mainly at a high dose of copper exposition. These results will be useful to understand more about the mediators involved in the effect of copper over endothelium and cardiovascular diseases in chronic intoxication in humans.

KEY WORDS: Oxidative stress; Copper; Chronic intoxication; Damage; eNOS; Cell death.

INTRODUCTION

Reactive Oxygen Species (ROS) are part of the functional balance of various systems (Carvajal Carvajal, 2019), they can generate cellular damage, and are associated with disease processes such as atherosclerosis, cardiovascular disease, diabetes, and aging (Lushchak, 2014). In the same way, oxidative stress, together with the decrease in nitric oxide (NO), are associated with the development of atherosclerosis in vascular grafts used for revascularization with a vein graft (Guzik *et al*., 2005).

It has been shown that non-oxidative stress by free radicals is also a precursor of diseases such as thrombophilia induced by metals like iron or copper (Niemman *et al*., 2017).Some studies report that copper induces damage to the endothelium, which could be associated with cardiovascular pathologies (Gaetke *et al*., 2014). Regarding the mechanism of damage, it is known that copper ions generate ROS, which are partially responsible for cell damage. Furthermore, copper causes a decrease in glutathione levels and an alteration in thiol levels in endothelial cell cultures (Hultberg *et al*., 1997). Changes in the expression of the endothelial nitric oxide synthase (eNOS) gene have been observed in the presence of copper ions in endothelial tissue of the umbilical vein (Hultberg *et al*.).

The cupric ion (Cu^{2+}) is a powerful catalyst in the NO oxidation reaction. In hypertension, oxidative stress promotes endothelial dysfunction, vascular remodeling, and inflammation, leading to vascular damage (Furman *et al*., 1999). Increased ROS production or decreased defense capacity of antioxidant mechanisms contribute to vascular oxidative stress, an important factor in the pathogenesis of

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hypertension (Montezano *et al*., 2015). Copper, also contributes to the generation of ROS and these generate cell damage; NO when reacting with the superoxide ion (O_{2}^{-}) generates peroxynitrite (Cat *et al*., 2013). In turn, peroxynitrite acts as positive feedback, causing the release of ions copper, which generates even greater ROS release and greater cellular damage. The damage ends up generating pathologies such as inflammation, cancer, cardiovascular diseases, diabetes, atherosclerosis, and neurological diseases (Gray *et al*., 2013).

Furthermore, NO is one of the molecules synthesized by the endothelium that regulates a greater number of local homeostatic processes (Badimón & Martínez-González, 2006). NO could be classified as an atheroprotective molecule of endothelial origin: vasodilator, platelet anti-aggregate, an inhibitor of the proliferation of CML, antioxidant and inhibitor of expression of Cell Adhesion Molecules (CAM),and monocyte adhesion (Beckman *et al*., 1990). Therefore, through the alteration of endothelial NO production, vascular homeostasis is deeply disturbed and the development of atherosclerotic lesions is enhanced. The decrease in NO-dependent dilation is the most common manifestation, early endothelial dysfunction is observed in patients with various risk factors, such as hypercholesterolemia, diabetes, or homocystinuria (Furman *et al*.). The alteration of endothelium-dependent dilation due to hypercholesterolemia may also be due to a decrease in the bioavailability of NO (Montezano *et al*.). Plane *et al*. (1997) determined that copper acts as an inducer of the production of NO (Plane *et al*.). To date, few studies have analyzed the mediators involved in endothelial damage induced by chronic copper intoxication *in vivo*. Therefore, the objective of this study was to evaluate the effects of chronic exposition to copper on the generation of oxidative stress, cell damage, and apoptosis induction in endothelium in an in vivo murine model.

MATERIAL AND METHOD

Study type and design. This study was an experimental comparative, prospective, longitudinal, and controlled clinical trial in a murine animal model. This work was developed in the Departments of Human Anatomy, and Histology, both in the Faculty of Medicine, UANL. The research protocol was approved by the Ethics Committee and the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) in the Faculty of Medicine, UANL with approval number AH15-019. The handling of the experimental animals was performed according to NOM-062-ZOO-1999.

Characteristics of the population and sample size. Twenty-four male Wistar rats were included, young adults (age at the beginning of the study between 5-6 months, weight 400-500 g), skeletally mature, and clinically healthy. The sample size was determined according to Charan & Biswas (2013), employing a hypothesis test and difference of two means or with the mean of a reference value, with a za value of 1.96 with a significance level of 95 % for two tails, and a zβ value of 0.84 with a power of 80 %, a sample of 8 units per group was obtained.

Distribution of the animals. All the experimental animals (n = 24) were randomly distributed into six experimental groups (n = 4, each). Negative control groups receive just water for 180 or 300 days, experimental groups received copper sulfate ($CuSO_4$) in drinking water at concentrations of 100 ppm Cu^{2+} or 300 ppm Cu^{2+} during 180 or 300 days. The concentrations were according with Maynard *et al*. (2009). All the animals were kept in cages with constant 12-hour light and dark cycles, with access to water and food *ad libitum*. Temperature and humidity were kept at a constant level (22ºC and 60 %, respectively).

Sample collect. The rats of the experimental and control groups were euthanized by inhalation of CO_2 in a closed chamber according to recommendations of the National and International Regulations (NOM-062-ZOO-1999 and UFAW). Verification of death was performed in a triple manner: confirming the absence of respiration by observation, absence of pulse, and absence of movement for five minutes. Subsequently, a thoracotomy was performed accessing the heart, and it was perfused with 4 % formaldehyde to fix the tissues and organs. Next, aorta and lung samples were obtained and preserved in 4 % formaldehyde at room temperature (RT) for further processing, and morphological and immunohistochemical analysis.

Morphological analysis. The aorta was evaluated in the arch, and samples of lung were taken from the same lobe. After fixation, the conventional histological technique was applied and the pieces were included in paraffin blocks, then by using a microtome, histological sections of 5-μm thickness were obtained and stained with Hematoxylin and Eosin (H&E). The slides were analyzed with a bright field light microscope (Nikon Eclipse 50i) and a Digital Sight dDS-2Mu image analysis system. The morphological analysis included the presence of inflammatory cells, hyperplasia of any of the blood vessel tunics, general tissue alterations, organization of the elastic fibers, cellular or extracellular matrix alterations, alterations in the morphology of the vascular wall, analysis of the endothelial cells, and tissue damage.

Evaluation of oxidative stress. To evaluate the oxidative stress levels generated by chronic copper administration, the presence of eNOS was determined by immunohistochemistry (IHC) using a rabbit anti-eNOS antibody (ab66127, Abcam, Cambridge, MA) as the primary antibody. The Mouse and rabbit specific HRP/DAB (ABC) detection IHC kit (ab64264, Abcam) detection system was used. Positivity was identified with 3,3'diaminobenzidine (DAB), and the nuclei were contrasted with Gill's hematoxylin. The slides were analyzed with a bright field light microscope (Nikon Eclipse 50i) and a Digital Sight dDS-2Mu image analysis system. Subsequently, 32 fields/group were taken (8 fields per slide, 1 slide per animal, 4 animals/group), and densitometric analysis was performed using the ImageJ v1.51 program according tothe methodology already described (Soto-Domínguez *et al*., 2018).

Assessment of cell death. For the evaluation of cell death induced by chronic copper intoxication, the presence of active-Caspase-3 was identified by IHC using the active anti-Caspase-3 antibody (ab66110, Abcam, Cambridge, MA). The positivity detection, contrast, and quantification system were using the previously mentioned method.

Statistical Analysis. It was performed using the SPSS computer program (Version 19.00) for Windows XP (SPSS, Chicago, IL). The results were statistically analyzed using tests for non-parametric data (Chi-square test and Mann-Whitney test) considering a value of $p \leq 0.05$ as significant.

RESULTS

Treatment with copper induces alterations in the wall of the aorta. In this study, gradual alterations were observed in the groups treated with copper, it stands out that in the exposure to 100 ppm at 180 days, areas with loss of the endothelium, signs of disorganization of smooth muscle fibers in the tunica media, as well as areas with the fragmentation of the elastic sheets. These alterations increased in the group treated at 100 ppm and were even more pronounced time-dependent in the groups treated with 300 ppm during 300 days. In the control groups, normal histological characteristics were observed (Fig. 1).

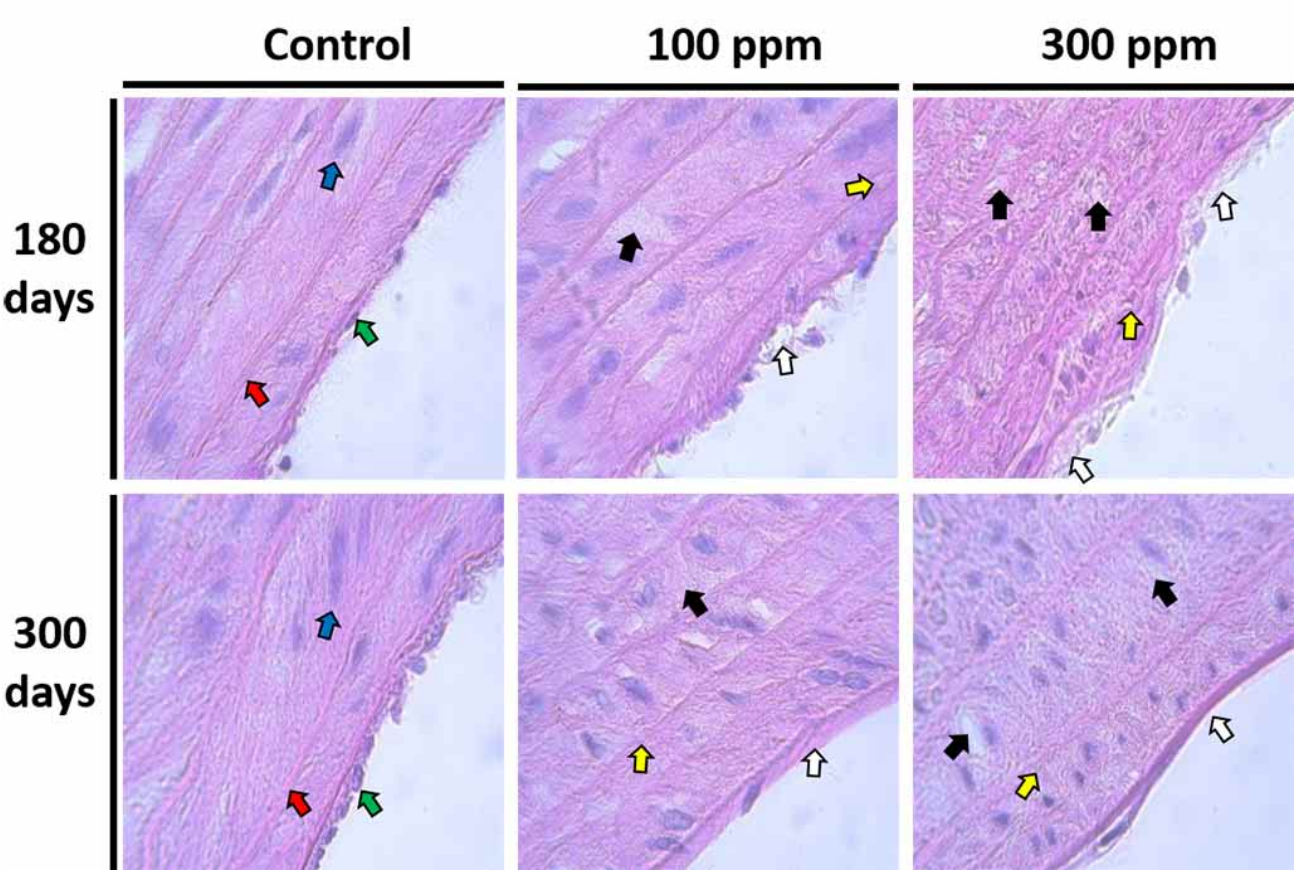

Fig. 1. Chronic exposure to copper causes histological alterations in the wall of the aorta. Areas with loss of continuity of the endothelium (white arrows), disorganization of muscle fibers (black arrows), disorganization of elastic fibers (yellow arrows). Endothelium (green arrows), elastic fibers (red arrows), muscle fibers (blue fibers). H&E, 100X magnification.

Exposure to copper induces increased expression of eNOS in the aorta. In this study, eNOS detection was also performed in aortic endothelium on exposure to copper. In the immunohistochemical analysis, an increase in the expression of this protein was observed in the groups treated at 100 ppm time-dependent compared with the control groups; however, this expression decreased in the groups treated at 300 ppm (Fig. 2). These results were confirmed by performing the morphometric analysis, and when comparing the treated groups with each other no statistically significant difference was observed (Fig. 2).

Chronic exposure to copper causes Caspase-3 expression to activate the aortic endothelium. In the active Caspase-3 analysis, expression was observed in the aortic endothelium, highlighting a greater positivity at 100 ppm to 180 that increased at this concentration at 300 days, which decreased in the 300 ppm groups (Fig. 2). When statistically comparing the treated groups, a significant statistical difference was observed between the control group vs 300 ppm (Fig. 2).

Chronic exposure to copper induces morphological changes in the lung. On the other hand, we also analyzed the morphological changes induced by chronic exposure to copper in the lung. The group exposed to 100 ppm showed interstitial cellular infiltrate and pneumonitis, dilatation of the interalveolar capillaries, and thickening of the septa with slight signs of fibrosis. These alterations were increased in the group exposed to 300 ppm time-dependent, it is

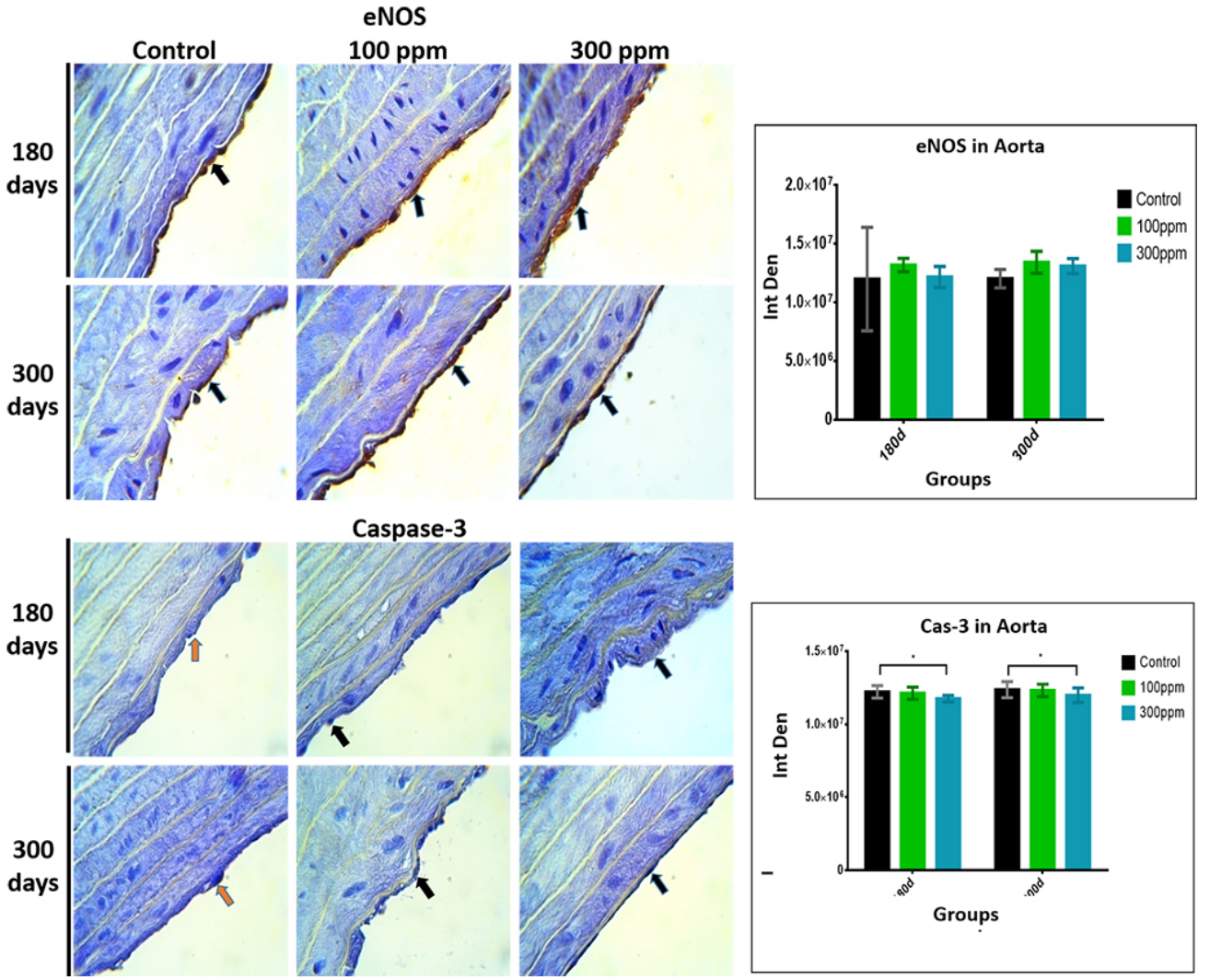

Fig. 2. Exposure to copper induces overexpression of eNOS in aortic endothelium. Observe endothelium positivity (black arrows), IHC, 100X magnification. Exposure to copper induces the expression of active Caspase-3 in the aortic endothelium. Note positivity in the endothelial cell cytoplasm (black arrows), IHC, 100X magnification. Quantitative analysis of the expression of eNOS and active Caspase-3 in the aorta. In the quantitative analysis, a significant difference was only observed in the expression of active Caspase-3 in the aorta at 180 and 300 days at 300 ppm. * $p < 0.05$.

noteworthy that in these groups a greater amount of interalveolar macrophages is observed. The control groups showed normal histological characteristics for this organ (Fig. 3).

Exposure to copper induces increased expression of eNOS in the lung. In this study, eNOS was also detected in the endothelium of the interalveolar capillaries and arterioles, as well as in the lung macrophages of the groups chronically exposed to copper. In the immunohistochemical analysis, a slight increase in the expression of this protein was observed in the groups treated at 100 ppm compared to the control groups, this positivity subsequently decreased in the groups treated with 300 ppm (Fig. 4). When performing the morphometric analysis and the contrasting of the results, no statistically significant difference was observed (Fig. 4).

Chronic exposure to copper causes Caspase-3 expression in the endothelium of the capillaries and arterioles of the lung. In the analysis of active Caspase-3, expression was observed in the endothelium of the capillaries and arterioles of the lung, highlighting a greater positivity at 100 ppm at 180 and even higher in the group treated at 300 ppm. Positivity decreased in the 300 ppm group at 300 exposure days (Fig. 3). When statistically comparing the treated groups, a statistically significant difference was observed between the control group vs 300 ppm (Fig. 4).

Statistical analysis. As we previously mentioned, the parameters evaluated for each of the study groups were statistically compared, and it was analyzed whether there was a significant statistical difference with the results already described (Fig. 4).

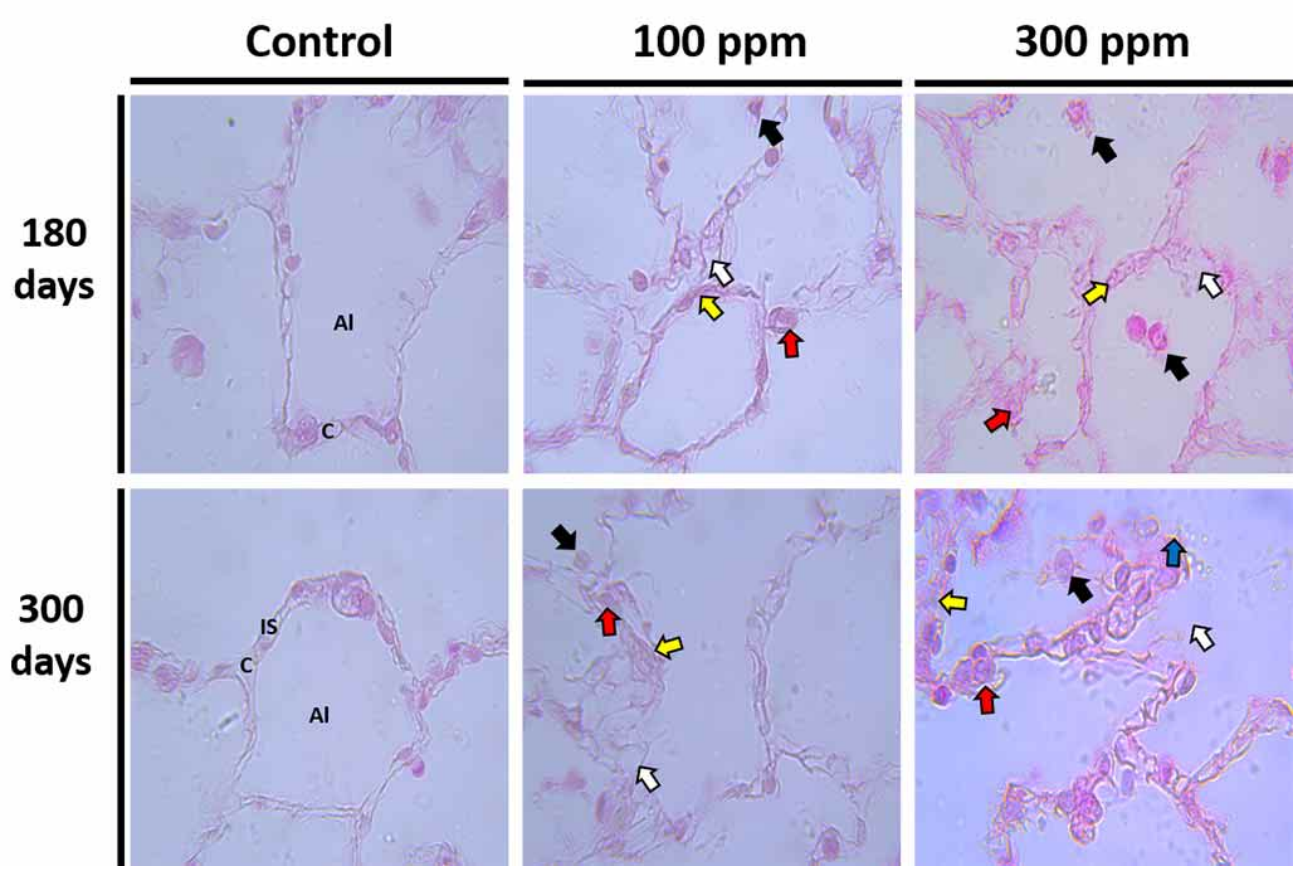

Fig. 3. Damage induced by exposure to copper in the lung. Interstitial infiltrates (red arrows), capillary dilation (white arrows), signs of fibrosis (yellow arrows), interalveolar macrophages (black arrows). Alveoli (Al), interalveolar septum (IS), capillaries (C). H&E, 100X magnification.

DISCUSSION

Endothelial dysfunction is one of the first precursor disorders such as atherosclerosis, as well as metabolic disorders that start in insulin resistance and leads to diabetes mellitus. It is also known as a precursor for the loss of relaxation of the vascular tone and precursor of arterial hypertension; this by mean of altered morphology and

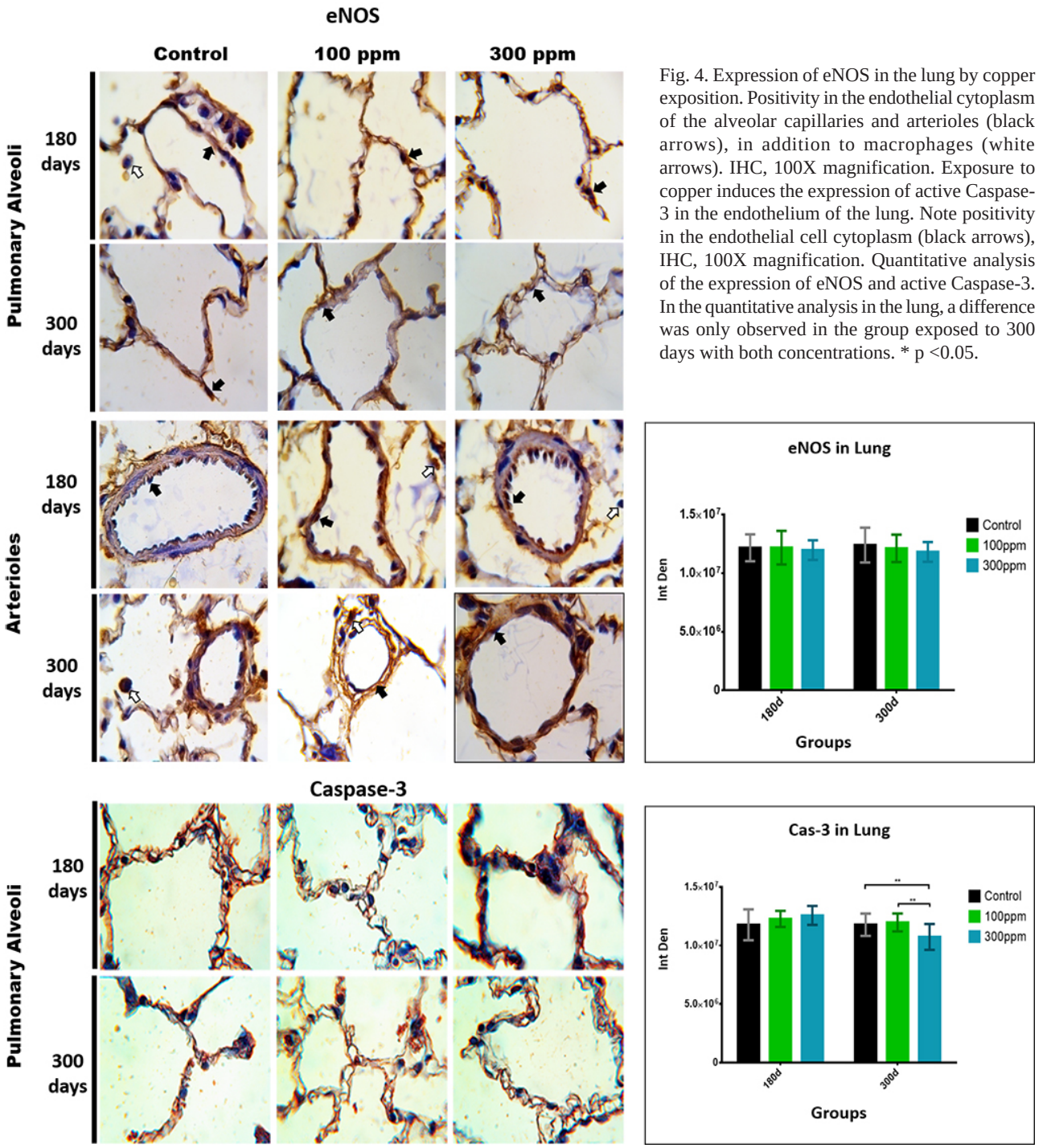

Fig. 4. Expression of eNOS in the lung by copper exposition. Positivity in the endothelial cytoplasm of the alveolar capillaries and arterioles (black arrows), in addition to macrophages (white arrows). IHC, 100X magnification. Exposure to copper induces the expression of active Caspase-3 in the endothelium of the lung. Note positivity in the endothelial cell cytoplasm (black arrows), IHC, 100X magnification. Quantitative analysis of the expression of eNOS and active Caspase-3. In the quantitative analysis in the lung, a difference was only observed in the group exposed to 300 days with both concentrations. * p <0.05.

functions of the endothelium due to a proinflammatory state induced by an imbalance in the presence of free radicals (Endemann & Schiffrin, 2004). It has been described that copper toxicity is associated with tissue damage, expressing itself as a disease by oxidative damage through free radicals. Copper ions tend to form ROS, capable of modifying the structure or function of essential biomolecules, leading to toxicity (Valko *et al*., 2016). Our study shows histological changes in the walls of the great vessels of intoxicated rats with copper. These differences correspond to disorganization signs and fragmentation of collagen, elastic and muscular fibers within the tunica media of the aorta. The changes observed may be a result of the oxidative process that alter the tissue and cellular structure, also alterations in the

structure of the elastic fibers can culminate with the stiffness of the walls, as a consequence of endothelial dysfunction. Similarly, it has been observed that nanoparticles of oxidate metals can generate inflammation in vascular endothelial cells (Gojova *et al*., 2007). Although this study did not evaluate the role of copper in inflammation of the vessels, it is important to consider because these metals can be highly reactive in the body (Shi *et al*., 2020).

A study with copper nanoparticles demonstrates the effect of copper ions on endothelial cells, which is dose-proportional, observed an increase in ROS levels, DNA damage, and apoptosis of endothelial cells (Karlsson *et al*., 2008). In our study we observed the presence of eNOS and active Caspase- 3 in endothelial cells of the aorta of the study groups.

Disorders of copper metabolism like the Wilson disease include damage-mediated by copper-induced oxygen radicals. The disease is a consequence of lipid peroxidation in the liver and decreased levels of vitamin E are found in the blood (Song *et al*., 2011). In the present study, we analyzed the alterations observed at the endothelial level both in the aorta and lung tissue *in vivo.*

Recent studies have shown that exposure to copper as environmental pollution, can promote oxidative stress and generate degenerative diseases due to cellular apoptosis phenomena (Shi *et al*.). Also, the effect of environmental factors when are inhaled can also generate cellular alterations by oxidative stress in the lung (Niemann *et al*., 2017); the imbalance in favor of oxidants is the generator of a variety of responses ranging from direct damage to an increase in ROS production, having an inflammatory response as a common factor (MacNee, 2001). Our results show increase cellularity of inflammatory cells in the alveoli of the study model, mainly at a high dose of copper exposition. Moreover, the increasing of alveolar macrophage could be related to a time-depending activation process; it has been described in individuals exposed to copper an impact of the time-dose mainly in mRNA level of inflammation mediating proteins (interleukin-8, the cell adhesion proteins VCAM-1 and ICAM- 1 and macrophage cationic peptide-1) (Sun *et al*., 2011).

Furthermore, chronic exposure to copper can generate apoptosis and autophagy in organs like the kidney tissue in time/dose-dependent controlled groups (Wan *et al*., 2020). In our study, we observed induction of apoptosis by chronic copper intoxication through the positivity to active Caspase-3 in the lung. Currently, we are evaluating if chronic intoxication with copper induces changes in levels of malondialdehyde or other products derived from lipid peroxidation, or over mechanisms of cell damage that can culminate in apoptosis phenomena. Besides these findings in the aorta and lung resulting from chronic copper exposure, we are evaluating if other alterations in homeostasis may occur that determine a progressive impairment in the functioning at cellular or metabolic level in other organs like the central nervous system.

FUNDING

This study was supported by a grant from PAICYT-UANL. SA227-15 and with internal resources of Departamento de Anatomía Humana, Fac. Medicina. UANL.

ACKNOWLEDGMENTS

We acknowledge the work of Luis I. Botello Soto and Iván A. Mesta R. for technical assistance involved in processing the samples for morphological analysis. Thanks to Dr. med. Alejandro Quiroga for the English review and correction of the manuscript.

FERNÁNDEZ-RODARTE, B. A.; SOTO-DOMÍNGUEZ, A.; GONZÁLEZ-NAVARRO, A.; MELLADO-AYALA, A.; PIÑA-MENDOZA, E. I.; RODRÍGUEZ-ROCHA, H.; MONTES-DE-OCA-LUNA, R.; GUERRA-LEAL, J. D.; GUZMÁN-LÓPEZ, S. & ELIZONDO-OMAÑA, R. E. El cobre induce daño, estrés oxidativo y muerte celular en el endotelio de ratas Wistar intoxicadas de forma crónica. *Int. J. Morphol., 40(1)*:10-17, 2022.

RESUMEN: Las Especies Reactivas de Oxígeno (ROS) son parte del equilibrio funcional de varios sistemas, pueden generar daño celular por estrés oxidativo asociado a procesos patológicos como aterosclerosis, enfermedades cardiovasculares, diabetes y envejecimiento. Algunos estudios informan que el cobre induce daños en el endotelio, lo que podría estar asociado a patologías cardiovasculares. Este estudio fue un ensayo clínico experimental comparativo, prospectivo, longitudinal y controlado en un modelo animal murino. Se incluyeron veinticuatro ratas Wistar macho, la distribución de los grupos fue la exposición crónica al cobre en función del tiempo y un grupo de control. Los resultados muestran alteraciones graduales en los grupos tratados con cobre: áreas con pérdida del endotelio, signos de desorganización de las fibras musculares lisas en la túnica media, así como áreas con la fragmentación de las láminas elásticas. Se observó una diferencia estadística significativa en la expresión del análisis de caspasa-3 activa en el endotelio aórtico y el endotelio de los capilares y arteriolas del pulmón entre el grupo de control frente a 300 ppm de cobre. Se detectó expresión de eNOS en el endotelio de la aorta y los vasos del pulmón. Nuestro estudio muestra cambios histológicos en las paredes de los grandes vasos de ratas intoxicadas con cobre, y el

incremento de células inflamatorias en los alvéolos del modelo de estudio, principalmente a una alta dosis de exposición de cobre. Estos resultados serán útiles para comprender más sobre los mediadores involucrados en el efecto del cobre sobre el endotelio y las enfermedades cardiovasculares en la intoxicación crónica en humanos.

PALABRAS CLAVE: Estrés oxidativo; Cobre; Intoxicación crónica; Daño; eNOS; Muerte celular.

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Received: 17-08-2021
Accepted: 12-10-2021