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Sex differences in vanadium inhalation effects in non-ciliated bronchiolar cells

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Summary. The non-ciliated bronchiolar cell (NCBC) is responsible for the defense of the lung and responds to negative stimuli such as exposure to toxic pro-oxidant substances, which triggers the hyperproduction and hypersecretion of mucins and CC16 protein. The literature demonstrates that physiological and pathological responses in the lung can be influenced by the organism's sex. The objective of this report was to evaluate response differences to vanadium inhalation in male and female CD-1 mice. Mice were exposed to vanadium for four weeks. Hyperplasia of bronchiolar epithelium, small inflammatory foci and sloughing of the NCBC were observed, without changes between sexes and throughout the exposure time. Mucosecretory metaplasia was found in both males and females, however it was more drastic in males. The expression of CC16 increased in both sexes. This study demonstrated a different susceptibility between male and female mice exposed to vanadium inhalation regarding mucosecretory metaplasia.

Key words: Vanadium, Bronchiole, Mucosecretory, Lung, Sex differences, Non-ciliated bronchiolar cell,

Introduction

The non-ciliated bronchiolar cell (NCBC), also known as club cell or Clara cell, is the main epithelial cell type in conducting airways in rodents (Zhai et al., 2018). The percentage of Clara or non-ciliated bronchiolar cells in the conducting airways of mice, especially in trachea, bronchi, and bronchioles, is predominant compared to other types. In the case of terminal bronchioles up to 80% of the epithelial cells are non-ciliated bronchiolar and 20% are ciliated. In humans this cell type is located only in the bronchioles and increases (up to 80%) as the bronchioles decrease in lumina, while mucosecretory and ciliated cells decrease (Suárez et al., 2012).

NCBC is cuboidal with a basal spherical nucleus and has an apical dome of variable length with secretory granules (Plopper and Hyde, 2015). This cell is multifunctional and performs critical roles such as: barrier maintenance, secretion, and metabolism, especially in the bronchiolar epithelium (Reynolds and Malkinson 2010; Rokicki et al., 2016; Zuo et al., 2018).

The main secretion product of NCBC is the Clara cell secretory protein (CCSP), which is a small homodimer consisting of 70 to 77 amino acids (10-16 kDa), also known as CC16 (Clara cell secretory protein 16kDa) or CC10 (Clara cell secretory 10kDa protein) (Zuo et al., 2018). This protein is inside the secretory granules stored at the apex of NCBC. In addition to being secreted to the broncho-alveolar fluid, it participates in the maintenance of the integrity of the bronchiolar epithelium and its repair (Broeckaert and Bernard, 2000; Stripp et al., 2002; Wong et al., 2009).

The most important identified biological activity of CC16 is the inhibition of the phospholipase A2 that participates in the generation of inflammatory lipid mediators, and the main way in which this protein acts is by modulating the inflammatory response in the lungs (De Luca et al., 2011; Shiyu et al., 2011; Laucho-Contreras et al., 2015). On the other hand, it has been reported that CC16 protein can function as a molecule with antioxidant properties through the interaction with metallothionines (Pilon et al., 2016).

It has been identified that, under negative stimuli, such as exposure to toxic pro-oxidant substances like ultrafine particles and ozone, the hyperproduction and hypersecretion of CC16 occur specially in relation to epithelium repair (Xiao et al., 2013; Ji et al., 2019). In

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addition, NCBC can synthesize, store, and secrete mucins at high levels, a phenomenon known as mucoid metaplasia, which can be detected by special stains (Schiff- PAS for neutral mucins, Alcian Blue/Schiff's Periodic Acid for acid mucins and Alcian Blue/Schiff's Periodic Acid for both) (Davis and Dickey, 2008; Curran and Cohn, 2010). According to the literature, it has been reported that in mouse lungs, non-ciliated bronchiolar cells synthesize and secrete minimal amounts of mucins, whose expression, storage, and secretion increase under conditions of lung inflammation (Young et al., 2007; Curran and Cohn, 2010). According to Alessandrini, quote, "in S/OVA mice, the bronchioli featured goblet cell metaplasia: the dense homogenous secretory granules of the Clara cells converted to mucus containing vesicles, some of them containing dark residues of the secret" (Alessandrini et al., 2010). This evidence indicates that non-ciliated bronchiolar cells showed mucoid metaplasia when it is challenged with OVA. Other evidence clearly demonstrates that in distal conducting airways such as terminal bronchioles, it is the non-ciliated bronchiolar cell that undergoes mucoid metaplasia (Reader et al., 2003).

Recently, our working group published a study about the effects of vanadium inhalation on NCBCs in a murine model of tolerance development. The results showed that vanadium induces mucoid metaplasia as well as an overproduction of CC10 in male mice lungs (López-Valdez et al., 2019).

Vanadium is a transition metal whose presence in the atmosphere is a result of the burning of fossil fuels and the main routes of exposure to this metal for the general population are the oral and aerial routes. It is estimated that about 71,000 to 120,000 tons of vanadium are emitted annually into the atmosphere and two thirds come from anthropogenic activities such as the burning of fossil fuels (Ress et al., 2003). In addition to this, vanadium is used in other applications such as semiconductor manufacturing, in the photographic industry and coloring agents for paints and ceramics (Rojas-Lemus et al., 2021).

The airways are the main route of absorption of vanadium, especially oxides; approximately 25% of the inhaled metal is absorbed into the bloodstream (Rodríguez-Mercado et al., 2006). It is known that vanadium oxides are readily absorbed in the lungs and enter the bloodstream after solubilization in the form of vanadate (Rehdher, 2013) and it is proposed that it enters into the cells through a phosphate transport mechanism, surely facilitated by the strong structural analogies between vanadate and phosphate (Baran, 2008; Rehdher, 2013).

Once vanadium has been absorbed, it is transported to the tissues through the bloodstream attached to proteins such as albumin and transferrin. The vanadium complexes are transformed, through oxidoreduction reactions, in the vanadyl and vanadate ions within the tissues and both forms (cationic and anionic) are distributed in the body, concentrating on bone, lungs, kidneys, spleen and liver (Goc, 2006). *In vivo*, it has been reported that vanadium produces hematotoxicity, hepatotoxicity, neurotoxicity, and pulmonary toxicity (López-Valdez et al., 2019; Fortoul et al., 2011, 2014; Plopper and Hyde, 2015).

It is important to mention that most of the studies that have been done to explore the systemic effects of this metal are in male mice; however, epidemiological evidence in human and animal models has shown that the lung is a dimorphic organ, and different studies demonstrate sex differences in the development of pathological responses to toxic substances (Townsend et al., 2012; Harms et al., 2015). Specifically, it has been reported that the NCBCs in rodents present differential susceptibility between males and females to the exposure of diverse substances as naphthalene (van Winkle et al., 1995) and lead acetate (Fortoul et al., 2005), although these differences have not been studied diligently.

The objective of this study was to evaluate if female mice exposed to vanadium inhalation present the same susceptibility to damage by this metal as male mice and determine the differences between them.

Materials and methods

8-week-old male and female mice of strain CD-1 weighing 33±2 g were, obtained from the vivarium of the Faculty of Medicine of the National Autonomous University of Mexico were used. The CD1 strain is one of the most widely used strains, especially in the field of toxicology and pharmacology, and one of the most relevant advantages they offer is the wide genetic diversity they have, which can be similar to that observed in human populations (Aldinger et al., 2009). In terms of handling, these mice are relatively docile and easy to manipulate (Criver, 2018).

In separate, males and females were randomly distributed in special plastic boxes and maintained in light-dark cycles (12:12h), with water and food (Purina rodent chow) *ad libitum*. The mice were handled in accordance with the Laboratory Animal Care and Use Guide (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council) and the Official Mexican Standard (NOM-062-ZOO-1999), for the production, care and use of laboratory animals. This study was approved by the Research and Ethics Commissions of the Faculty of Medicine of the National Autonomous University of Mexico UNAM (FM/DI/071/2017). The weights of male and female mice were recorded at the beginning and throughout the experiment and there were no significant changes between sexes (data not shown).

Inhalation protocol

The inhalation protocol was performed according to Fortoul et al. (2014). A total of 40 male and 40 female mice were used and distributed randomly by sex in 2

groups: control group (n=20) and exposed group (n=20). The control group was exposed to 0.9% saline inhalation for one hour, twice a week, for four weeks, while the exposed group was exposed to the inhalation of V_2O_5 (99.99% purity, Sigma-Aldrich, St. Louis, MO) one hour, twice a week, for four weeks. The concentration of vanadium in the inhalation chamber was of 1.56 mg/m^3, in accordance with those noted by the World Health Organization (WHO, 2001) (0.01-60mg/m^3). Based on previous determinations of the diameter of the vanadium particles (Cervantes-Yépez et al., 2018) and the specifications from the nebulizer manufacturer, the diameter range of the aerosolized particles was 1 to 5 mm. These particles can be deposited in different areas of the respiratory system. Those particles with a diameter greater than 2.5 µm are deposited in the upper airways, while those smaller than 2.5 are deposited in the deep lung zone, even reaching the alveolar zone (Wei and Tang, 2018; Deng et al., 2019).

Exposure in both groups was performed in a transparent acrylic chamber (45x21x35 cm) that could house 20 mice/ session connected to an ultra Yuehua WH 2000® nebulizer (Guangdong Yuehua Medical Instrument Factory, Shantou, China), with particles of 1 to 5 µm aerodynamic diameters and a maximum nebulization rate of 4 mL/minute, according to the manufacturer.

At each inhalation per week, the 20 mice corresponding to the control group were placed inside the acrylic chamber and whole-body exposure was performed. A volume of 125 ml saline solution was placed in the nebulizer and once in operation, the aerosolized solution was conducted through a hose to the chamber inlet, which was placed in the upper central part of the acrylic box to ensure uniform distribution of the particles. This same procedure was replicated in the case of the exposed group to V_2O_5 with particles of vanadium from 1 to 5 µm aerodynamic diameters, according to the nebulizer manufacturer. Each week the number of mice in the acrylic chamber was reduced by 5 because of sacrifice.

The determination of vanadium concentrations in the inhalation chamber was made by analyzing the filters placed at the nebulizer outlet, with a nebulization rate of 4 mL/min by mass spectrometry of induction-coupled plasma (ICP-MS) according to López-Valdez et al., 2019. The average concentration of vanadium in the inhalation chamber during exposure was 1.56 mg/m^3 of vanadium.

Euthanasia and lung collection

A total of 10 mice per sex were sacrificed per week of exposure (5 controls, 5 exposed). The euthanasia of the animals was performed by a lethal dose of sodium pentobarbital (Pisabental, PiSA Agropecuaria, S.A. de C.V., Guadalajara, Jal., Mexico) administered intraperitoneally in a concentration of 35 mg/kg of weight, and subsequently perfused intracardially with 0.9% saline (PiSA Agropecuaria, S.A. de C.V., Guadalajara, Jal., Mexico) and 4% of paraformaldehyde (MilliporeSigma, St. Louis, Missouri, USA) solution buffered in PBS (pH 7.4). The lungs were extracted and insufflated intratracheally to near physiologic volume (total lung capacity=1mL) with buffered paraformaldehyde to be processed by the conventional histological technique in paraffin.

Histopathological analysis of the lung tissue

Slices 5 µm thick were obtained from the right lungs and were processed for the histopathological analysis and immunohistochemistry. The histopathological evaluation of the lung tissue was performed using the hematoxylin-eosin (HE) stain to analyze the general structure of the lung tissue and the periodic acid-Schiff (PAS) stain was used to identify mucosecretory bronchiolar cells in terminal bronchioles. The observation of slices was performed with the 40x objective of an Olympus microscope (model BH2-RFCA, Tokyo, Japan).

To carry out the quantification of PAS+ cells, 10 photomicrographs of 10 terminal bronchioles were taken for each mouse in each group at 40x magnification using the Olympus microscope model BH2-RFCA and a camera of the same brand. Once the images were obtained, they were analyzed using Image Pro Plus 4.0 software (Media Cybernetics) in which the nuclei located above the basement membrane of PAS-positive mucosecretory cells were identified in a length of 100 micrometers in the terminal bronchioles. Subsequently, the percentage of PAS+ cells over all cells in the aforementioned area was calculated.

Immunohistochemistry for CC16 in the NCBC

An immunohistochemical staining for CC16 in the NCBC was performed by using a streptavidin-biotin-peroxidase complex method. The tissues were dewaxed with xylol and dehydrated with ethylic alcohols in increasing order (70%, 96%, 100%). Subsequently, the antigen retrieval was performed by incubation in a 2% Diva Decloacker recovery solution (Diva Decloaker 20x, BioCare Medical, Pacheco, California, USA) at 15 psi for 3 minutes and then, the endogenous peroxidase was inhibited with 3% H_20_2 (JT Baker, Phillipsburg, New Jersey, USA) for 15 minutes at room temperature. Nonspecific antigens were blocked with a 5% bovine albumin solution (MP Biomedicals, Santa Ana, California, USA). CC16 primary antibody (anti-CC16 antibody, 1:1000 dilution, Santa Cruz Biotechnology Inc., Dallas, Texas, USA) was incubated at 4°C overnight. The slices were incubated with secondary anti-goat antibody (Goat on rodent HRP Polymer, BioCare Medical, Pacheco, California, USA) for 30 minutes at room temperature and thereafter streptavidin-horseradish peroxidase (HRP) complex (Goat on rodent HRP Polymer, BioCare Medical, Pacheco, California, USA) for 30 minutes at room temperature. The peroxidase reaction was revealed by incubation with

0.05% diaminobenzidine tetrachloride (Invitrogen, Camarillo, California, USA). Finally, the sections were counterstained with hematoxylin. Some samples were only incubated with the secondary antibody and served as negative controls.

Image processing

An Olympus microscope (model BH2-RFCA, Tokyo, Japan) was used to observe the slides. The photomicrographs of terminal bronchioles were taken at 40X, with a digital camera (Evolution MP Color, Media Cybernetics Inc., Rockville, Maryland USA).

For the evaluation of the immunoreactivity, the photomicrographs were processed in a color model CMYK (Cyan, Magenta, Yellow and Key) to detect the density of the mark obtained in the immunohistochemistry, using the yellow channel.

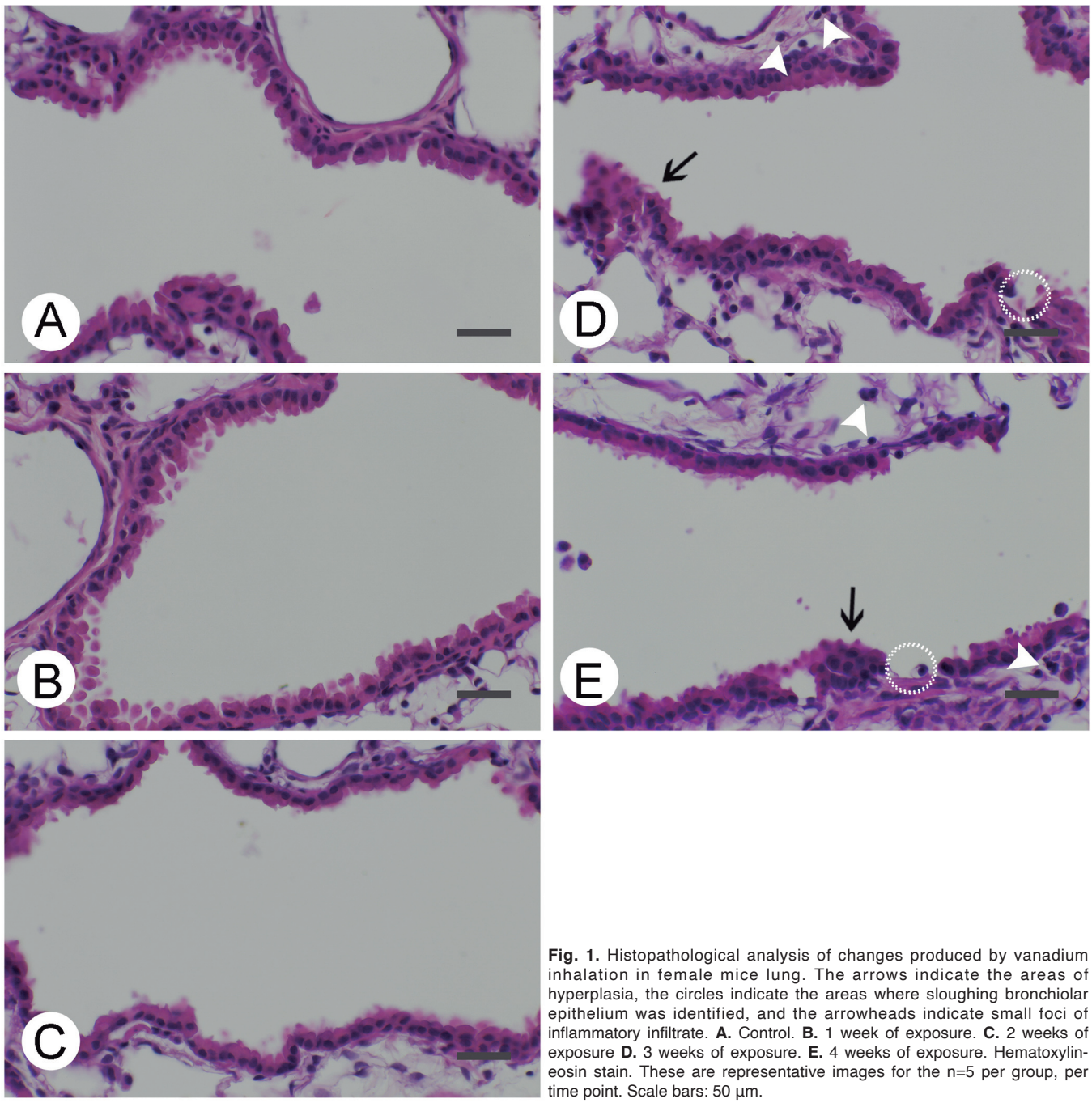

Fig. 1. Histopathological analysis of changes produced by vanadium inhalation in female mice lung. The arrows indicate the areas of hyperplasia, the circles indicate the areas where sloughing bronchiolar epithelium was identified, and the arrowheads indicate small foci of inflammatory infiltrate. **A.** Control. **B.** 1 week of exposure. **C.** 2 weeks of exposure **D.** 3 weeks of exposure. **E.** 4 weeks of exposure. Hematoxylin-eosin stain. These are representative images for the n=5 per group, per time point. Scale bars: 50 µm.

Densitometric analysis

The immunohistochemical analysis was performed with MathLab DensiFe software 1.0.0.0. We analyzed 5 mice per group; for each mouse, 10 fields of 0.088 mm^2 were chosen randomly and the photomicrographs were taken with the 40x objective. The color intensity was reported in pixel units. The program has different thresholds and the one that eliminates the unspecific mark is selected.

Statistical analysis

The data were analyzed with one-way analysis of variance (ANOVA), and the differences between the groups were identified with a Tukey *post hoc* test, considering $p \leq 0.05$ as statistically significant. All the data were reported as mean ± standard error. All the

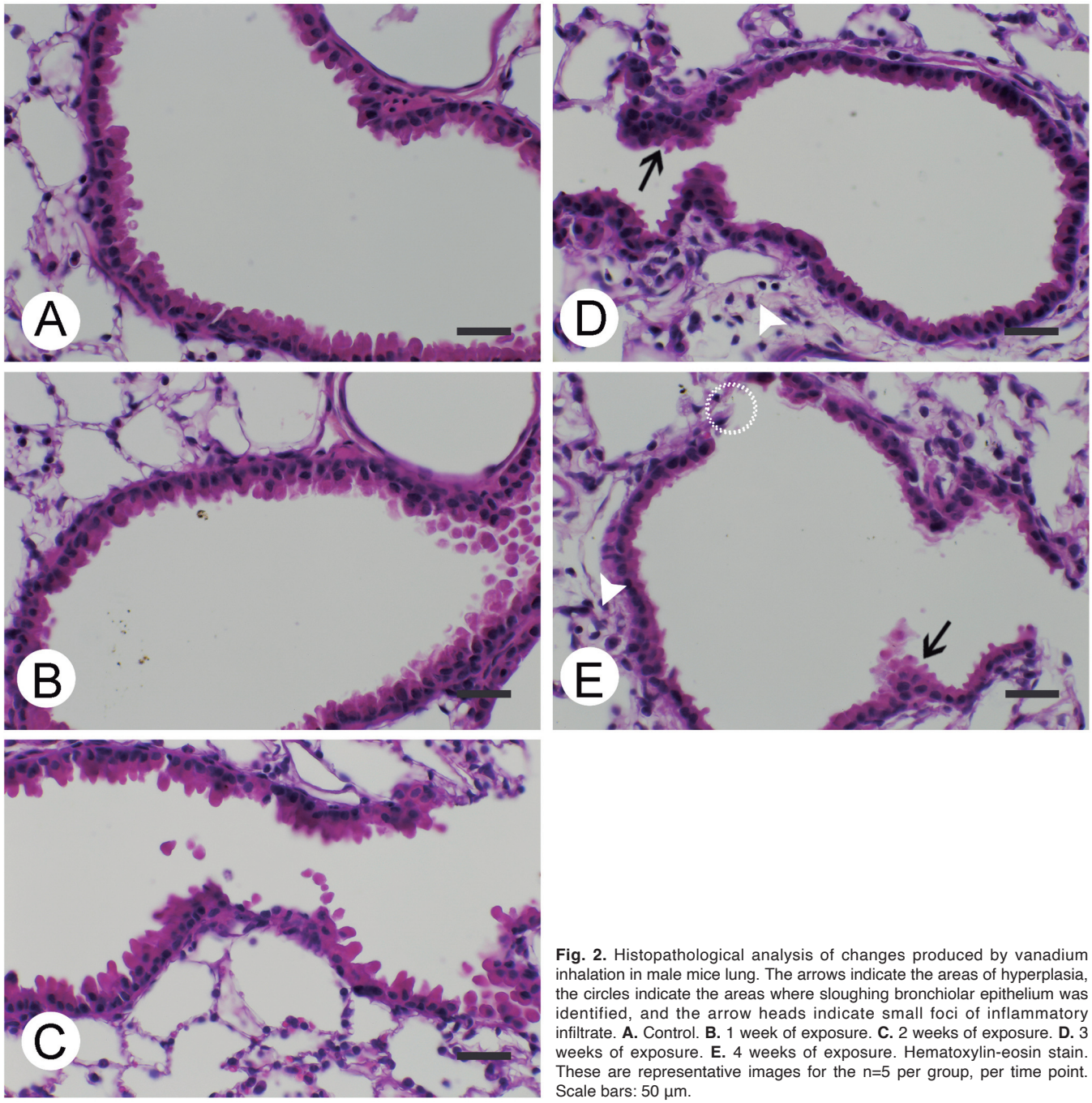

Fig. 2. Histopathological analysis of changes produced by vanadium inhalation in male mice lung. The arrows indicate the areas of hyperplasia, the circles indicate the areas where sloughing bronchiolar epithelium was identified, and the arrow heads indicate small foci of inflammatory infiltrate. **A.** Control. **B.** 1 week of exposure. **C.** 2 weeks of exposure. **D.** 3 weeks of exposure. **E.** 4 weeks of exposure. Hematoxylin-eosin stain. These are representative images for the n=5 per group, per time point. Scale bars: 50 µm.

analyses were performed with Prisma v. 6.0c (GraphPad, San Diego, CA).

Results

Histological analysis

The histological analysis of the lungs of the control mice shows the typical structure of the tissue. Figure 1A shows a bronchiole in which we observed the simple cuboidal epithelium formed mainly by NCBCs and scarce ciliated cells in female mice lungs. The NCBCs presented their typical cubic shape with its characteristic apical protrusion. Intact alveolar sacs, constituted by type I pneumocytes (flat cells) and type II pneumocytes (cubic), were observed adjacent to the bronchioles. No relevant changes in tissue structure were identified (Fig. 1A). The same characteristics described above were also

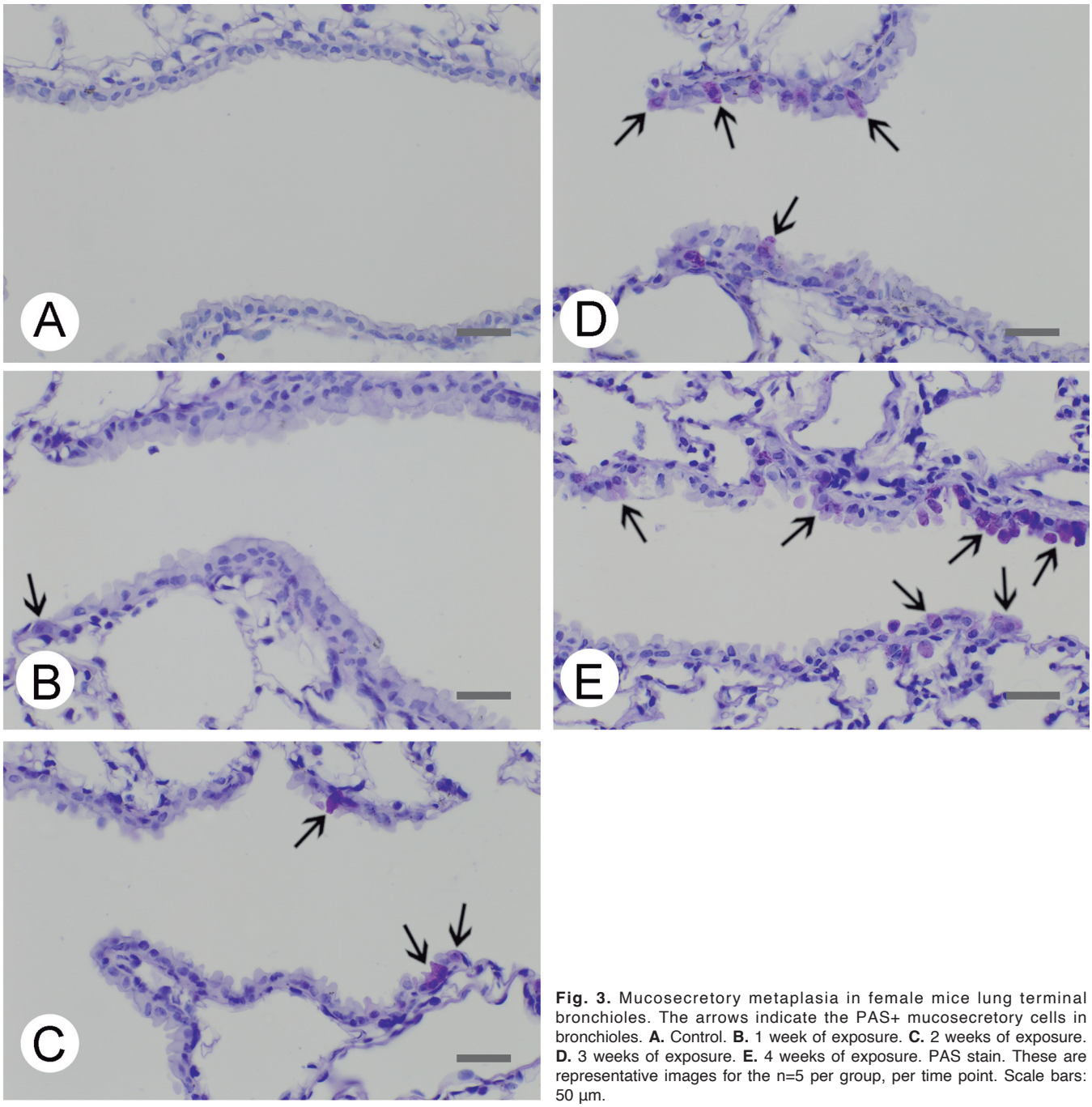

Fig. 3. Mucosecretory metaplasia in female mice lung terminal bronchioles. The arrows indicate the PAS+ mucosecretory cells in bronchioles. **A.** Control. **B.** 1 week of exposure. **C.** 2 weeks of exposure. **D.** 3 weeks of exposure. **E.** 4 weeks of exposure. PAS stain. These are representative images for the n=5 per group, per time point. Scale bars: 50 µm.

found in male control mice (Fig. 2A).

The histopathological analysis of the lung tissue in the female exposed mice presents several alterations that are shown in representative photomicrographies in Figure 1. On the first (Fig. 1B) and the second week (Fig. 1C) no relevant changes in tissue structure were identified, meanwhile in the third (Fig. 1D) and the fourth week (Fig. 1E) female mice lungs presented small areas of peri-vascular and peri-bronchiolar inflammatory infiltrate identified by the morphology of inflammatory cells (mainly lymphocytes with their classic spherical heterochromatic nuclei), areas of bronchiolar epithelial hyperplasia and sloughing of the bronchiolar epithelium. The evaluation of these alterations was qualitative.

Regarding the histopathological analysis of the lung tissue in the male exposed mice, as in females, no relevant changes were found on the first and second weeks of exposure (Fig. 2B,C). On the third week mice

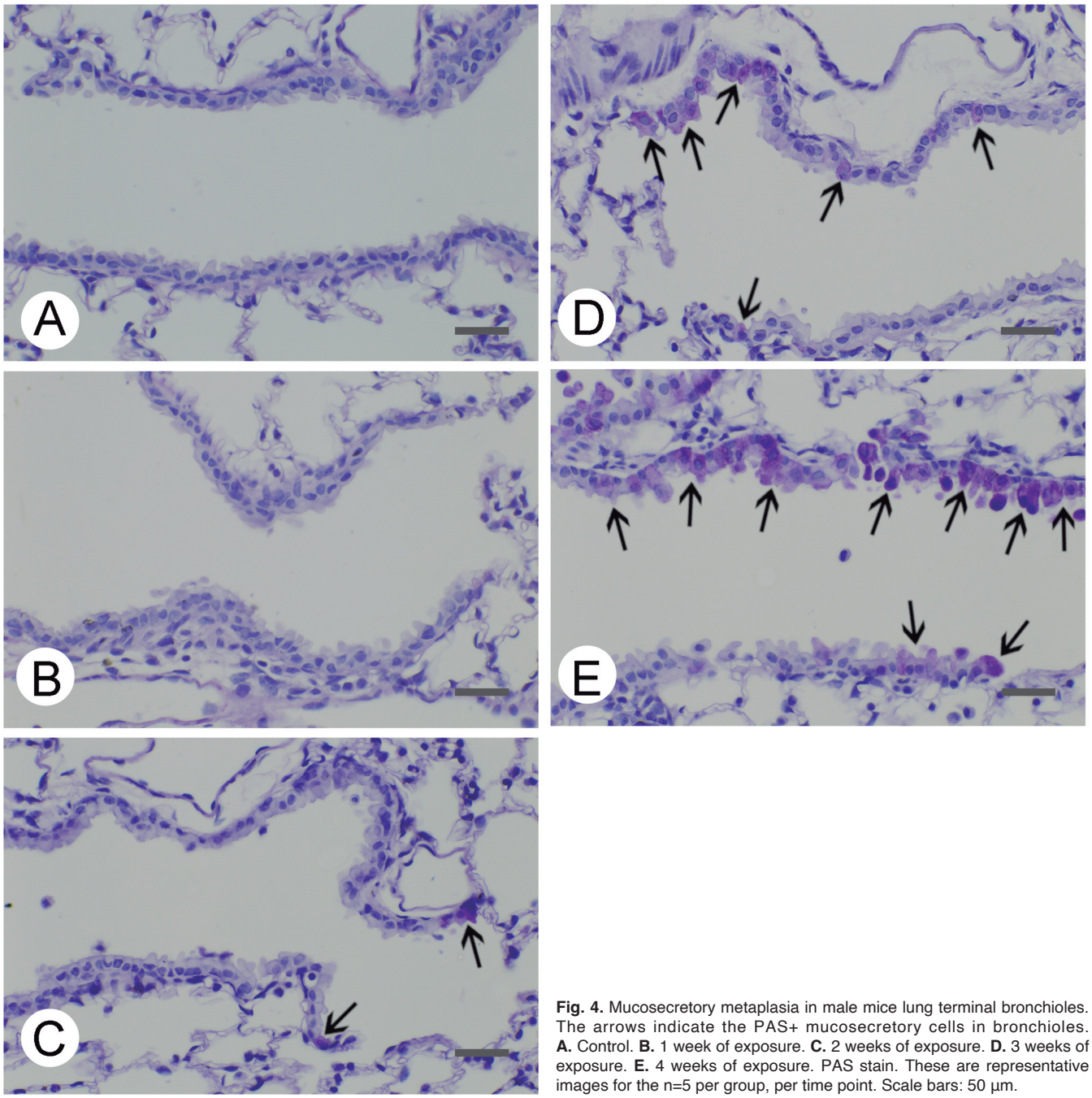

Fig. 4. Mucosecretory metaplasia in male mice lung terminal bronchioles. The arrows indicate the PAS+ mucosecretory cells in bronchioles. **A.** Control. **B.** 1 week of exposure. **C.** 2 weeks of exposure. **D.** 3 weeks of exposure. **E.** 4 weeks of exposure. PAS stain. These are representative images for the n=5 per group, per time point. Scale bars: 50 µm.

lungs presented small areas of peri-vascular and peri-bronchiolar inflammatory infiltrate and bronchiolar hyperplasia (Fig. 2D). On the fourth week we observed the same changes as in the third week in addition to the sloughing of the bronchiolar epithelium (Fig. 2E). The evaluation of these alterations was qualitative.

Mucoid Metaplasia

The analysis regarding the PAS method representative photomicrographies are shown in Figures 3, 4. The bronchioles of the female (Fig. 3A) and male (Fig. 4A) control mice presented scarce ciliated cells, NCBCs and non-mucosecretory cells. During treatment with vanadium, in females, mucoid metaplasia was presented since the first week (Fig. 3B) of the treatment and it increased during the rest of the exposure weeks (Fig. 3C-E). In males mucoid metaplasia was identified on the second week of inhalation (Fig. 4C), which increased on the third (Fig. 4D) and fourth weeks (Fig. 4E). No PAS positive cells were identified on the first week of treatment (Fig. 4B).

Figure 5 corresponds to the analysis of the mucosecretory cell percent in lung terminal bronchioles in female and male mice. The statistical analysis demonstrated that the phenomenon of metaplasia occurred earlier in females, in week 1 (W1), compared to males who showed mucosecretory phenotype until week 2 (W2), although no significant difference was observed. The percentage of mucosecretory cells in male mice was statistically higher compared to female mice in weeks three (W3) and four (W4).

CC16 imunohistochemistry detection

Figures 6, 7 show the presence of CC16, a NCBC marker in the bronchioles of female and male mice, and the modifications produced by the inhalation of vanadium. In female (Fig. 6A) and male (Fig. 7A) control lungs the presence of CC16 was relatively homogeneous in the cytoplasm of NCBCs, concentrating on the apex of some cells. During treatment with vanadium, female mice presented an increase in the presence of CC16 with respect to controls throughout the weeks of treatment (Fig. 6B-E). In male mice the protein increased at the second and the third weeks (Fig. 7C,D). At the end of treatment, in the fourth week, both male (Fig. 7E) and female (Fig. 7E) mice lungs showed that the presence of the protein was higher.

Figure 8 shows the densitometric analysis of CC16 immunohistochemistry detection. In male mice the significant increase of the protein was observed at W2, W3 and W4, while in females the significant increase was presented from W1 and continued until the end of the experiment. The comparative statistical analysis between male and female mice regarding the densitometric analysis of CC16 in NCBC showed significant differences only at week 3 of exposure.

Discussion

Exposure to several atmospheric pollutants exerts changes in the respiratory epithelium that have been the subject of numerous reports. Some working groups, including ours, have reported the morphological changes described in male mice associated with vanadium-inhalation. It has been described that both the inhalation of different vanadium concentrations (National Toxicology Program, 2002; Ress et al., 2003; Fortoul et al., 2014; López-Valdez et al., 2019) as well as intratracheal instillation of this metal (Toya et al., 2001; Wang et al., 2003) produce the activation of the inflammatory lung response, as well as bronchiolar epithelial hyperplasia. In female mice, these same changes occur in a model of vanadium-inhalation, without apparent or important differences related to hyperplasia and inflammation compared with males (National Toxicoloy Program, 2002; Ress et al., 2003).

Regarding the specific changes in NCBCs, we observed a distinct mucosecretory phenotype, especially in the terminal bronchioles in both mice sexes. Normally NCBCs synthesize distinct mucins, neutral and acid; this

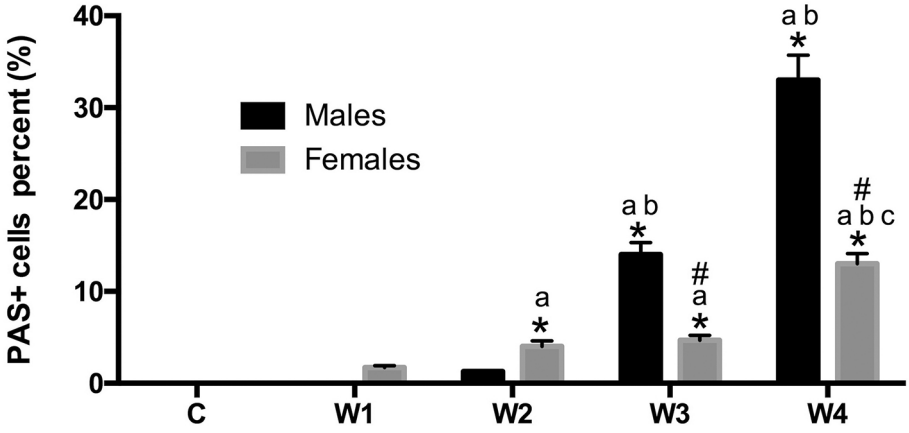

Fig. 5. Mucosecretory metaplasia in mice lung terminal bronchioles. Comparative analysis of percentage of PAS+ cells of exposed and unexposed male and female mice. The values are expressed as the percentage of PAS positive cells ± standard error, ANOVA p≤0.05 post hoc (Tukey). *Difference vs control. a. Difference vs W1. b. Difference vs W2. c. Difference vs W3. # Difference males vs females.

occurs at very low levels and is undetectable through staining techniques. However, when the lung is exposed to toxic agents or pathogens that produce inflammation, the cells synthesize, store, and secrete mucins at high amount, which can be detected through special stains (Davis and Dickey, 2008; Curran and Cohn, 2010; Alessandrini et al., 2010). It has been reported that PAS stain is perhaps the most widely used for the demonstration of glycoproteins and mucins. This stain is particularly sensitive to the detection of neutral mucins as well as acid mucins that contain significant quantities of sialic acid (Ali et al., 2012). Our results demonstrate the hyperproduction of mucins detected with PAS stain which suggests the increase in both, neutral and acid mucins. Previously, in the same inhalation model, we also observed this effect in the terminal bronchioles in

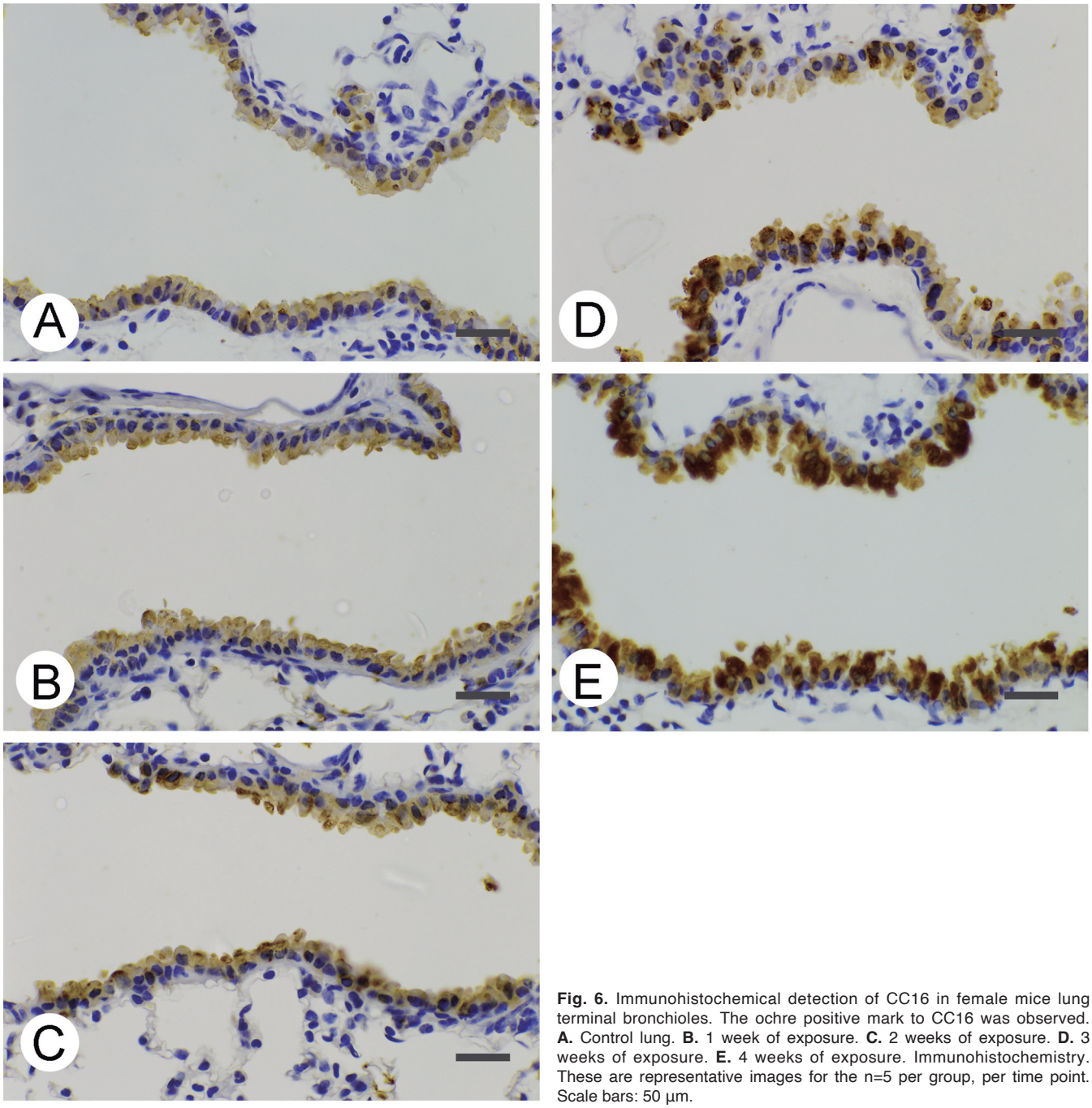

Fig. 6. Immunohistochemical detection of CC16 in female mice lung terminal bronchioles. The ochre positive mark to CC16 was observed. **A.** Control lung. **B.** 1 week of exposure. **C.** 2 weeks of exposure. **D.** 3 weeks of exposure. **E.** 4 weeks of exposure. Immunohistochemistry. These are representative images for the n=5 per group, per time point. Scale bars: 50 µm.

male mice lungs (López-Valdez et al., 2019); in this study we confirmed the same modification in female mice. Further detection could be made to detect small amounts of acid mucins with the Alcian blue-PAS stain that differentiates with more specificity between acid and neutral mucins.

The mucoid metaplasia that is reported in this study can de directly related to the inflammation observed and by the production of pro-inflammatory factors such as IL-6 and TNFα as it is reported in the literature (Bonner et al., 2000; Toya et al., 2001) and is also one of our findings in the bronchiolar epithelium after vanadium exposure in male mice (Fortoul et al., 2014). The inflammation could explain the hyperplasia and mucoid metaplasia in the lungs observed which probably share the same mechanism that exerts inflammation and metaplasia in female mice. It would be interesting to identify the inflammatory factors that contribute to the

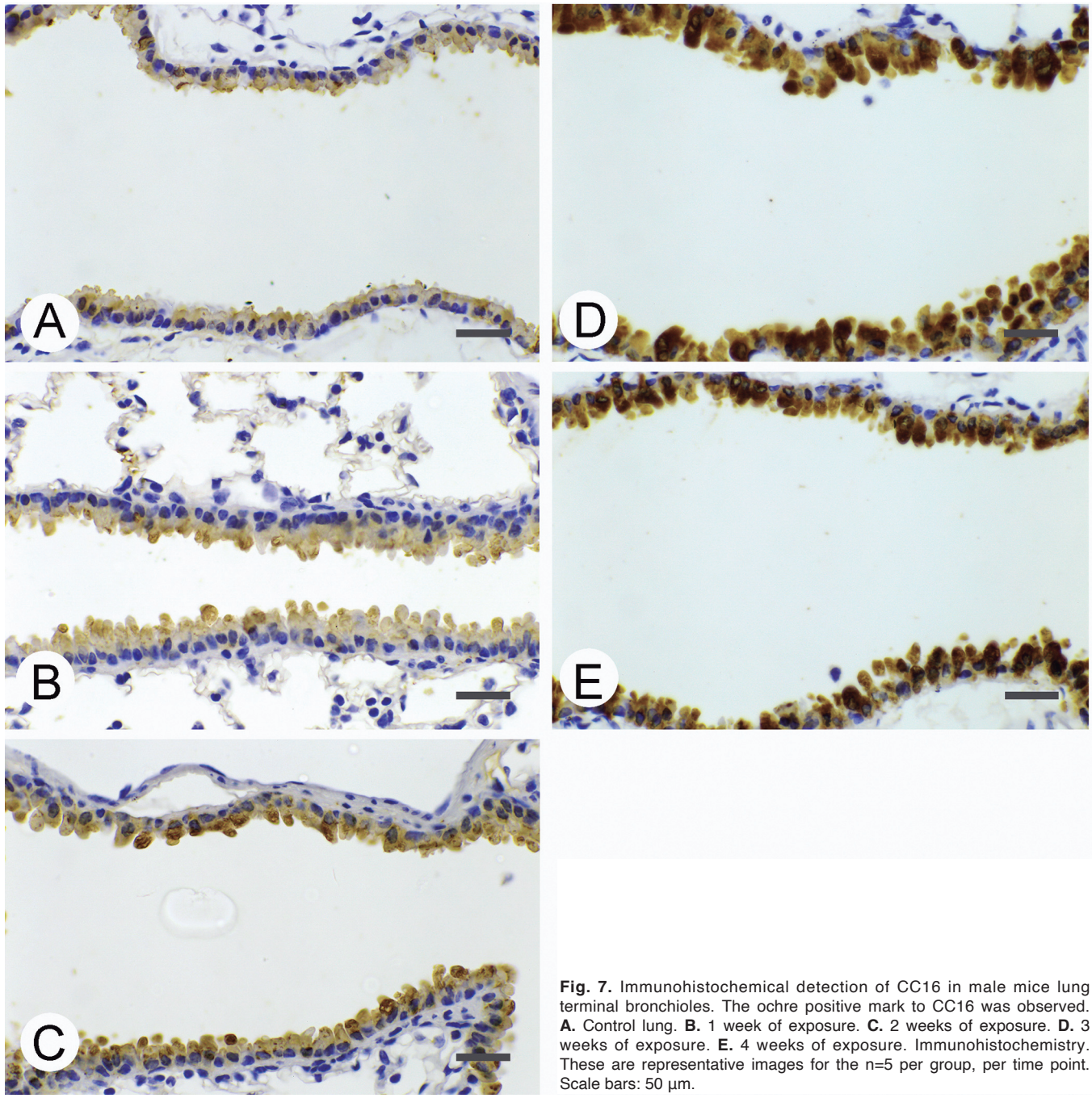

Fig. 7. Immunohistochemical detection of CC16 in male mice lung terminal bronchioles. The ochre positive mark to CC16 was observed. **A.** Control lung. **B.** 1 week of exposure. **C.** 2 weeks of exposure. **D.** 3 weeks of exposure. **E.** 4 weeks of exposure. Immunohistochemistry. These are representative images for the n=5 per group, per time point. Scale bars: 50 µm.

differences observed in the metaplasia between sexes. The phenotype change of NCBC to mucosecretory can provide some protection to the epithelium as it acts as a physical barrier that interacts with xenobiotics and pathogens before they gain access to the cells. It is important to mention that NCBCs keep their identity by retaining their original characteristics such as the synthesis of CC16 (Evans et al., 2004), and once inflammation ceases the expression of mucins is reverted (Roth et al., 2013). Future studies with emphasis on recovery following vanadium inhalation may help to confirm whether the mucosecretory phenotype reverses.

Regarding the expression of CC16 in NCBCs, the increase of the marker in the cytoplasm of these cells is observed by immunohistochemistry in both male and female mice. CC16 has been identified as one of the main proteins present in bronchoalveolar lavage and can be found in the secretory granules stored at the apex of NCBCs. As mentioned above, this protein participates in the maintenance and repair of the bronchiolar epithelium, mainly when the lung is exposed to toxic substances (Broeckaert and Bernard, 2000; Stripp et al., 2002; Wong et al., 2009). It has been identified that under negative stimuli hyperproduction and hypersecretion of CC16 occurs (Xiao et al., 2013; Ji et al., 2019), such as with exposure to pro-oxidant toxic agents like ozone. In addition, it is known that this protein has anti-inflammatory, immunosuppressive (Chen et al., 2001; Snyder et al., 2010; Liu et al., 2013; Tokita et al., 2014) and antioxidant properties (Pilon et al., 2016).

This protection by CC16 in vanadium-exposure may play an important role in modulating the adverse effects of this element, especially in the last two weeks of exposure, when inflammation and hyperplasia were at their highest expression, which coincides with the sharpest increase in CC16 in both sexes. It should be noted that although hyperplasia, inflammation and metaplasia were present in both sexes, the latter was especially exacerbated in males (López-Valdez, 2019).

Our findings denote that the damage differs according to whether the sex is male or female. Numerous studies in both human and animal models have demonstrated that the changes observed in normal and pathological responses in lungs have a close relationship with male or female sex hormones (Carey et al., 2007; Townsend et al., 2012). It has been shown that estrogen, progesterone, and testosterone receptors are expressed in lung tissue, influencing the physiology of this organ (Kimura et al., 2003).

The effects of sex hormones have been explored in different studies regarding exposure to pollutants and the differences between sexes in response to these substances. Some of these studies suggest that females are more susceptible to damage than males. Van Winkle and colleagues reported that during exposure to naphthalene, damage that occurred mainly in NCBC occurred earlier in females than in males and that the damage was more extensive in males (van Winkle et al., 1995). On the other hand, Cabello and coworkers found more severe inflammation triggered by ozone exposure in females (Cabello et al., 2015). The mechanisms that determine these differences between sexes have not yet been well identified.

Almost all of our results suggest that males were more susceptible to vanadium-inhalation, showing a greater effect on the development of mucoid metaplasia, contrary to the reported evidence that states that females are more susceptible. The literature shows that in females the phenomenon of metaplasia occurs with more intensity, change that has been associated with the effect 17-B estradiol which is capable to increase the expression of mucins in the mucosecretory cells (Dammann et al., 2000; Tam et al., 2014), although this phenomenon has not yet been connected to NCBCs. Meléndez-García et al. (2020) reported a decrease of 17-B estradiol and progesterone in female mice exposed to vanadium-inhalation change that could be connected with lower mucoid metaplasia of NCBC observed in female mice. If 17-B estradiol and progesterone are decreased in females exposed to vanadium, this may help to explain why a more dramatic response is found

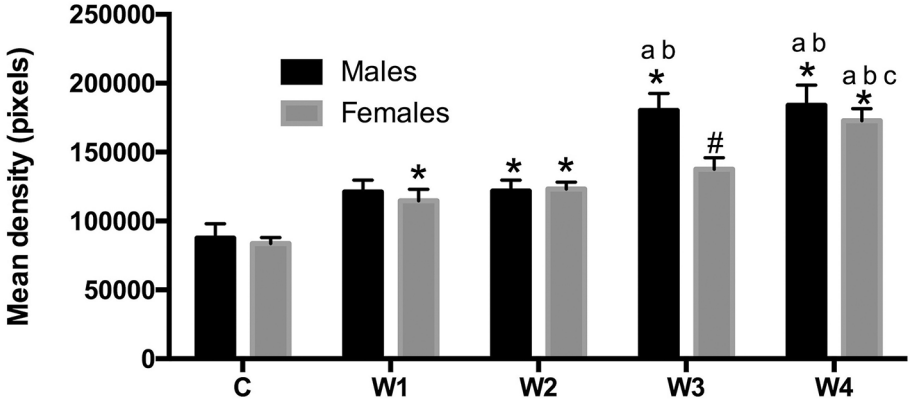

Fig. 8. Analysis of immunohistochemical detection for CC16 in mice lung terminal bronchioles. Comparative analysis of CC16 densitometry label in NCBC of the exposed and unexposed male and female mice. The values are expressed as the average density in pixels ± standard error, ANOVA p≤0.05 post hoc (Tukey). *Difference vs control. a. Difference vs W1. b. Difference vs W2. c. Difference vs W3. #Difference males vs females.

in males than in females.

In conclusion, this study demonstrates that males and females have a different susceptibility to vanadium inhalation, especially in relation to the NCBC mucosecretory phenotype change. These findings confirm the dimorphic response that can occur with different toxicants, although further studies are required to explore the precise mechanisms underlying these outcomes.

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