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Histological and Endocrine Effects of Chronic Exposure to MP2.5 Derived from Wood Smoke in the Uterus of Nulliparous Adult Rats

Efectos Histológicos y Endocrinos de la Exposición Crónica a MP2.5 Derivada del Humo de Leña en el Útero de Ratas Adultas Nulípara

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SUMMARY: The study explores the relationship between chronic exposure to fine particulate matter (PM2.5), sourced from wood smoke, and the histological structure and endocrine function of the uterus in nulliparous adult rats. It assesses potential structural changes in the uterus that could impact reproductive health, viewing PM2.5 exposure as a possible risk factor. A controlled experiment was conducted in a city known for high air pollution levels, exposing rats to filtered and unfiltered air conditions, thus mimicking human PM2.5 exposure. Histological findings indicated a significant increase in collagen density and uterine wall thickness in PM2.5 exposed subjects, suggesting a reproductive function risk. However, no significant differences were observed in progesterone and estradiol hormone levels, pointing to the complex relationship between PM2.5 exposure and its endocrine impact, and emphasizing the need for further studies for a deeper understanding. This work highlights the importance of thoroughly investigating the long-term effects of PM2.5 pollution on reproductive health, underlining the significance of considering environmental exposure as a critical factor in reproductive health research.

KEY WORDS: Pollution; Wood smoke; Uterus; Reproductive system; Particulate matter 2.5, PM2.5

INTRODUCTION

Air pollution, a global phenomenon, is emerging as a challenge with multiple and profound repercussions that transcend geographic and socioeconomic borders (Garcia *et al*., 2023). Exposure to airborne particulate matter (PM), particularly fine particles such as PM2.5 and PM10, as well as nitrogen oxides, has been consistently and worryingly linked to a wide range of adverse health effects, that manifest from the early stages of human development until adulthood (Lim *et al*., 2022). PM2.5, due to its minuscule size, has the ability to penetrate deep into the respiratory tract, reaching the lung alveoli and, in some cases, crossing the alveolar-capillary barrier to enter the bloodstream (Jia *et al*., 2022). This penetration capacity gives PM2.5 a lethal potential to cause systemic damage, not only to the lungs but also to the cardiovascular, brain and other vital organs. Epidemiological and experimental research has associated exposure to PM2.5 with an increase in the incidence and mortality of cardiovascular and respiratory diseases, exacerbation of asthmatic conditions, and neurotoxic effects that can manifest in cognitive impairment and an increased risk of neurodegenerative disorders (Pope 3rd *et al*., 2002; Brook *et al*., 2010). Furthermore, recent studies have shed light on the relationship between prenatal exposure to PM2.5 and a number of unfavorable perinatal outcomes, such as low birth weight, prematurity, and decreased head circumference. These findings underscore the impact of these pollutants on optimal fetal growth and development, demonstrating the ability of PM2.5 to transcend the placental barrier and pose significant risks to intrauterine development. Exposure to these pollutants during critical stages of fetal development not only affects perinatal outcomes, but may also predispose individuals to long-term health complications, including alterations in neurodevelopment and increased susceptibility to chronic diseases in later life adult (Lee *et al*., 2014; Sun *et al*., 2016; Woodward *et al*., 2017; Salinas *et al*., 2020).

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Air pollution resulting from the burning of firewood for indoor heating presents a significant problem in various regions, including Chile, Argentina, Canada. This practice contributes substantially to the release of PM2.5 and other harmful substances, affecting air quality both inside and outside homes, leading to public health and environmental issues, contributing to the degradation of air quality (Naeher *et al*., 2007). This situation is particularly critical in areas such as southern Chile, where wood burning in homes has been identified as a major contributor to air pollution, with alarming consequences for public health and the environment (Díaz-Robles *et al*., 2014; Jorquera *et al*., 2018). In Temuco, a representative city of southern Chile, it was reported that wood burning is a primary source of PM2.5 emissions. A study in homes of this city showed that the average concentrations of PM2.5 indoors and outdoors were 44.4 and 41.8 mg/m^3, respectively. It was estimated that 68 % of indoor PM2.5 came from infiltration from the outside, reflecting the high outdoor pollution and the high rates of air exchange in homes. It is suggested that stove replacement programs and improvements in home energy efficiency could be beneficial for improving indoor air quality in southern Chile (Jorquera *et al*., 2018). In another study in Temuco, it was found that wood burning was responsible for 84.6 % of ambient PM2.5, attributed to widespread poverty and the lack of efficient heating methods in homes. Despite emission mitigation policies implemented by the government, meeting air quality standards for PM2.5 in southern Chile remains a challenge (Villalobos *et al*., 2017).

In this context, our study focuses on elucidating the impact of chronic exposure, from birth to reproductive maturity, to PM2.5 specifically originating from wood smoke, on the structure of the uterus. We hypothesize about the potential effect of PM2.5 on the uterine structure in adult nulliparous females exposed from birth to reproductive maturity. Our aim was to describe potential structural changes in the uterus of nulliparous females directly exposed to PM2.5, originating from wood burning, which may lead to reduced reproductive outcomes during gestation. Here, we report findings that reveal the severity of PM2.5 exposure and its disruptive influence on reproductive health, providing concrete evidence of the endocrine and structural effects of air pollution on the female reproductive system and laying the groundwork for further research.

MATERIAL AND METHOD

Exposure Site. The study was conducted in Temuco, a city in southern Chile (38°44'59.4"S 72°37'07.8"W), identified as the sixth most polluted in Chile (IQAir, 2022). The predominant source of air pollution in this locale has historically been attributed to residential wood-burning stoves (Sanhueza *et al*., 2009; Díaz-Robles *et al*., 2014; Jorquera *et al*., 2018; Boso *et al*., 2019). The study was carried out during the austral winter, specifically from June 15 to September 30, 2021. Throughout this period, Sprague-Dawley rats were housed under controlled conditions, maintaining a temperature range of 20 - 25ºC and adhering to a 12/12-hour light/dark cycle. No other industrial pollution sources were detected in the area.

Exposure Conditions. Based on Veras *et al*. (2009), the exposure was simulated in two 2.1m x 2.0m x 2.1m chambers with a PM2.5 gradient, using urban air from a polluted area near a monitoring station. Air, regulated by a Zepol fan, was introduced at 20 m^3/min, supporting up to 50 cages per chamber, with internal pressure maintained below 33 mmH_2O. One chamber uniquely featured a triple filtration system: the first two filters trapped large and medium-sized particles (metal and pleated filters; 24 x 24 x 2 cm; featuring MERV8 particle filters and a final HEPA PH97 filter that eliminated 99.97 % of particles larger than 0.3 microns). The third filter was a Purafil PSA 102 device, with a capacity of 500 cfm (Purafil Inc., USA), fitted with a Purafil Select filtering medium in PK12 modules (Purafil Inc., USA; Figs. 1A y 1B).

Air analysis. The PM2.5 concentration inside the chambers was monitored daily with a digital analyzer, and outside using a beta attenuation monitor BAM 1020 (Met One Instruments, Inc., Grants Pass, OR, USA; Beta Source: 4C (carbon-14), 60 mCi ±15 mCi (2.22 MBq), equipped with a photomultiplier tube Beta Detector with an organic plastic scintillator at a flow rate of 16.7 liters per minute. Results were expressed in μgm^3. The data was provided by "Algoritmos y Mediciones Ambientales SpA" from the "Las Encinas Monitoring Station", located 200 meters from the chambers and available online at the National Air Quality Information System (https://sinca.mma.gob.cl). NO_2 and CO gas concentrations were consistent in both chambers, as the filtering system did not retain these pollutants.

Study design. This study examined PM2.5 exposure and histological changes in the uterus using a cross-sectional design. In compliance with Chilean Law 20.380 and the Guide for the Care and Use of Laboratory Animals (National Research Council (US) & Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011), and with approval from the Scientific Ethical Committee of Universidad de La Frontera (Record 122/20) and the Bioethics and Biosafety Committee of the Pontificia Universidad Católica de Valparaíso (BIOEPUCV-BA 373-2020). We strictly adhered to current regulations and guidelines for animal research. Adherence to the ARRIVE guidelines (Animals in Research: Reporting In Vivo Experiments)

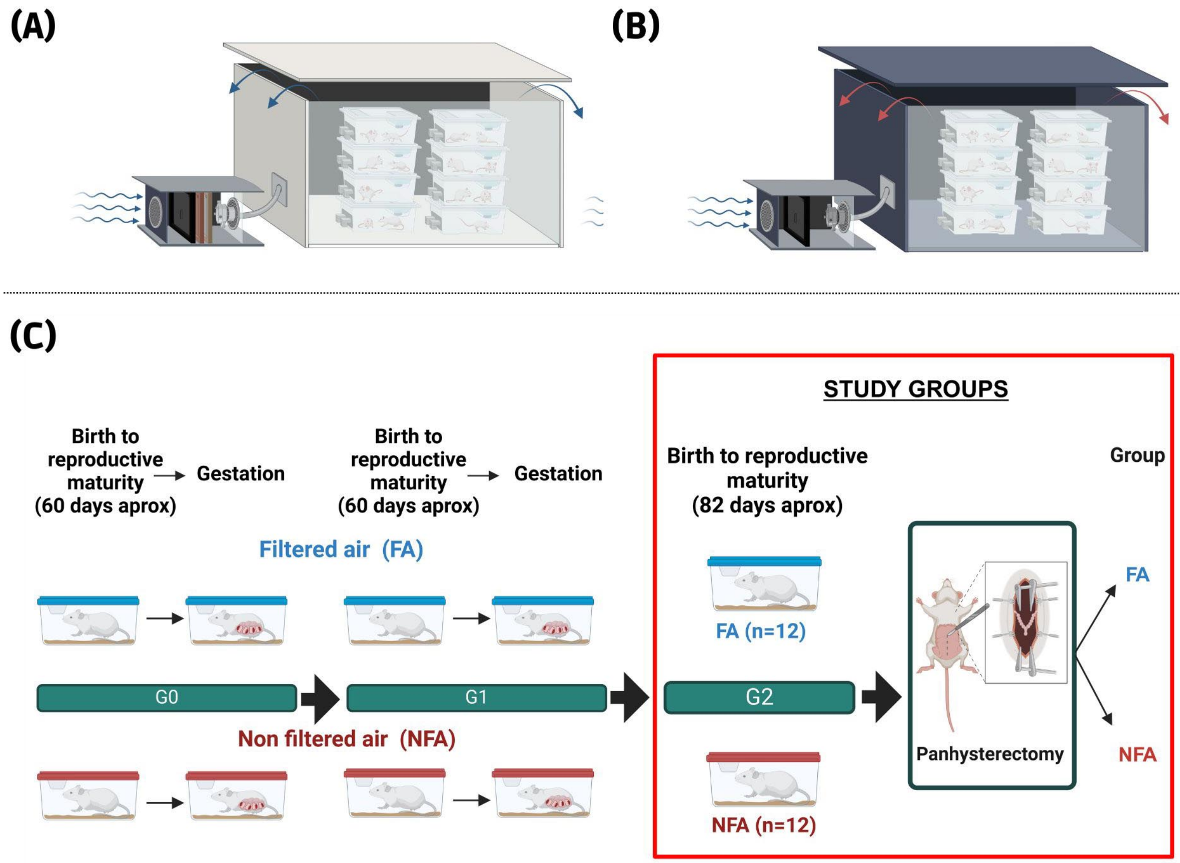

Fig. 1. Experimental design. (A) Filtered air chamber. (B) Non-filtered air chamber. (C) Exposure groups.

(Percie du Sert *et al*., 2020) was ensured for research transparency and ethics. Two generations of rats were subjected to PM2.5 exposure. The focus was on the second generation (G2) to examine the effects of PM2.5 exposure. The first-generation (G1) rats were conceived under both exposure conditions. Upon reaching maturity, 24 G1 females were mated to produce the second-generation (G2) rats. These G2 rats, upon reaching reproductive maturity (around 60 days), underwent serial vaginal cytology to determine the phase of the estrous cycle and confirm their reproductive maturity. Subsequently, these specimens, continuously exposed to both conditions (filtered air chamber and unfiltered air chamber), were assessed at 82 days of age. The study period spanned from the day of birth, June 30, 2021, to the day when the uterine tissue samples were collected, September 20, 2021. Those that had exhibited at least two complete estrous cycles were subjected to hysterectomy, for the purpose of conducting histological analysis of the uterine tissue.

Groups. We utilized two groups of adult nulliparous G2 rats, continuously exposed from birth until reproductive maturity (82 days old), forming two exposure groups. The Filtered Air group (FA) consisted of 12 Sprague-Dawley rats exposed to air that had been pre-filtered to remove PM2.5 particles and other impurities related to air pollution. This group of rats was designated as the control group to compare the effects of PM2.5 exposure with an unfiltered environment. The second group, the No-filtered Air group (NFA), comprised 12 Sprague-Dawley rats exposed to unfiltered air, meaning they were subjected to PM2.5 and other contaminant particles present in the environment. This group allowed for the assessment of the actual effects of PM2.5 exposure during development (Fig. 1C).

Histology and histomorphometry. The fresh uteri were separated from the ovaries and uterine tube. The broad ligament was dissected. Three 1-cm sections were cut from each uterus (body and horn) and uterine tube (ampulla) and fixed in in 4 % formaldehyde v/v for 72 h at room temperature. Samples were processed to obtain paraplast sections for microscopic evaluation (Paraplast Plus embedding medium; melting point: 54 ºC; Sigma-Aldrich Chemical Co., St Louis, MO, USA). Uterine sections were processed through a series of alcohols with increasing concentration and cleared in xylene. The paraplast block was cooled and stored at 4 ºC until cutting. For microscopy, uterine tissue was cut into 5-µm-thick sections using a microtome (Leica RM2265) and then washed 3 times for 10 minutes in Tris-phosphate buffer pH 7.8 (Tris 10 mM, NaCl 120 mM, Na_2HPO_4, 8.4 mM, KH_2PO_4, 3.5 mM). Consequently, cross-histological section of uteri were stained with hematoxylin and eosin, Mallory trichrome stain and Picrosirius Red (Rittié *et al*., 2017), and scanned using a Motic Easy Scan® Pro Digital slide scanner (Motic Instrument Inc, Canada) to produce fully digital panoramic views. Each histological analysis was estimated by examining 5 microscopic fields per slide, providing a 10 % non-random sample. All terms used in this study were according to the *Nomina Histologica Veterinaria* (International Committee on Veterinary Histological Nomenclature, 2017). For the planimetric study, digital photomicrographs of each tissue sample were obtained using a Leica® DM750 optical microscope equipped with a Leica® MC170HD digital camera. The proportion of the average thickness of uterine wall components was calculated using optical microscopy with a 2x magnification (%; area fraction of endometrium, circular layer, vascular layer, and longitudinal layer).

Blood sampling and hormone analysis in nulliparous rats. Blood was obtained from nulliparous rats (82 days old) via lateral tail vein puncture and collected in heparinized tubes. Following centrifugation, plasma was extracted, immediately frozen at -20°C, and later analyzed. Post-collection, rats were monitored for hemostasis and stability before cage return. Serum progesterone and estradiol levels were quantitatively measured using MAGLUMI® assays (Snibe Co., Ltd.) with specific ranges (progesterone: 0.13-80 ng/ml, estradiol: 8-6000 pg/ml). Both assays demonstrated high precision (intra-assay CV < 8 %, inter-assay CV < 9 %). Values below detection limits were recorded as half the limit value. The measurements were standardized and conducted using chemiluminescence immunoassay on the MAGLUMI 600 analyzer.

Determination of estrous cycle stage. Vaginal cell samples were collected through lavage prior to uterine retrieval. The samples were analyzed on slides using a Leica DM750 optical microscope. Since differences in the proportion of epithelial cells occur during proestrus compared to other stages of the estrous cycle (Warren & Juraska, 2000; Rummel *et al*., 2010), we distinguished between proestrus and non-proestrus phases. Proestrus was defined by the presence of approximately 70 % round nucleated epithelial cells, in accordance with Marcondes *et al*. (2002), as well as based on the histological characteristics of the uterus and the serum levels of steroid hormones.

Statistical analysis. All data were expressed as mean ± standard deviation. The D'Agostino-Pearson Normality Test was conducted to assess the data distribution. To detect differences between groups, either the Student's t-test or the Mann-Whitney U test was employed, depending on the distribution of the data. All tests were performed with a 95 % confidence interval and a significance threshold set at $p < 0.05$. Data analysis was conducted using GraphPad Prism 9.0 for Windows (GraphPad Software, San Diego, CA).

RESULTS

The data presented in Table I illustrate the average concentrations of particulate matter (PM2.5 and PM10) and carbon monoxide (CO) recorded during the specified study period and on an annual basis. For the PM2.5 measurements, the average concentration during the study period was 48.8 µg/m³ with a standard deviation of 36.1 µg/m³, resulting in a high coefficient of variation (CV) of 74.0 %. Annually, the average concentration of PM2.5 was significantly lower at 26.2 µg/m³, albeit with a comparable standard deviation of 30.6 µg/m³, leading to a higher CV of 117 %. This indicates a greater relative variability in annual PM2.5 concentrations compared to the study period. In the case of PM10, the average concentration during the study period was 56.9 µg/m³ with a standard deviation of 38.3 µg/m³, and a CV of 67.3 %. On an annual basis, the average PM10 concentration was 36.6 µg/m³, with a standard deviation of 30.8 µg/m³ and a CV of 84.2 %. Similar to PM2.5, PM10 concentrations showed higher relative variability annually compared to the study period. For CO measurements, the average concentration during the study period was 0.78 ppm with a standard deviation of 0.49 ppm, resulting in a CV of 61.5 %. Annually, the average concentration was lower at 0.44 ppm with a standard deviation of 0.43 ppm and a higher CV of 97.7 %. The elevated CV values, especially in the annual measurements for both particulate matter and CO, indicate considerable fluctuation in concentration levels, highlighting potential sporadic pollution events or seasonal variations affecting air quality. During the exposure period at the site, the daily average concentrations of PM2.5, PM10, and CO in the ambient air were measured as follows: PM2.5 at 48.8 µg/m³ (±36.1; CV: 74 %), PM10 at

Table I. Averages of particulate matter (MP) and CO recordings during the study period (June 15 - September 30, 2021; southern hemisphere) and annually.

	Study period	Annually
PM2.5 [ug/m³]	48.8 ± 36.1 (CV: 74.0 %)	26.2 ± 30.6 (CV: 117 %)
MP_{10} [ug/m³]	56.9 ± 38.3 (CV: 67.3 %)	36.6 ± 30.8 (CV: 84.2 %)
CO [ppm]	0.78 ± 0.49 (CV: 61.5 %)	0.44 ±0.43 (CV: 97.7 %)

56.9 µg/m^3 (±38.3; CV: 67.3 %), and CO at 0.78 ppm (±0.49; CV: 61.5 %). The mean concentration of PM2.5 within the NFA chamber was recorded at 44.6 µg/m^3 (±9.8; CV: 21.9 %), mirroring the ambient environmental levels closely. Conversely, in the FA chamber, the mean concentration was substantially reduced to 3.0 µg/m^3 (±1.3; CV: 34.3 %; $p<0.001$), marking a significant 94 % decrease in PM2.5 levels. The PM2.5 exposure levels of the female rats were estimated by multiplying the total volume of air inhaled during the study period (4.841 liters = 82 days x 1440 minutes x an estimated inhalation rate of 0,041 l/min) and multiplying it by the 24-hour average concentration of PM2.5. The resulting estimated intake of PM2.5 for NF group was 215921.088 µg/m^3 throughout the entire gestation period, averaging 2633,184 µg/m^3 daily. For FA group, the estimated intake figures were significantly lower, with total exposure calculated at 14523.88 µg/m3 and a daily average of 177.12 µg/m^3.

In general, in both groups, the endometrium (epithelium and lamina propria), the myometrium (circular, vascular and longitudinal layer), and a perimetrium were well-defined (Figs. 2A and 2B). Additionally, the deep, compact, circular, vascular and longitudinal layers were identifiable (Figs. 2C and 2D). Regarding the distribution and proportion of collagen that constitutes the extracellular matrix of the uterus, a greater presence of collagen was

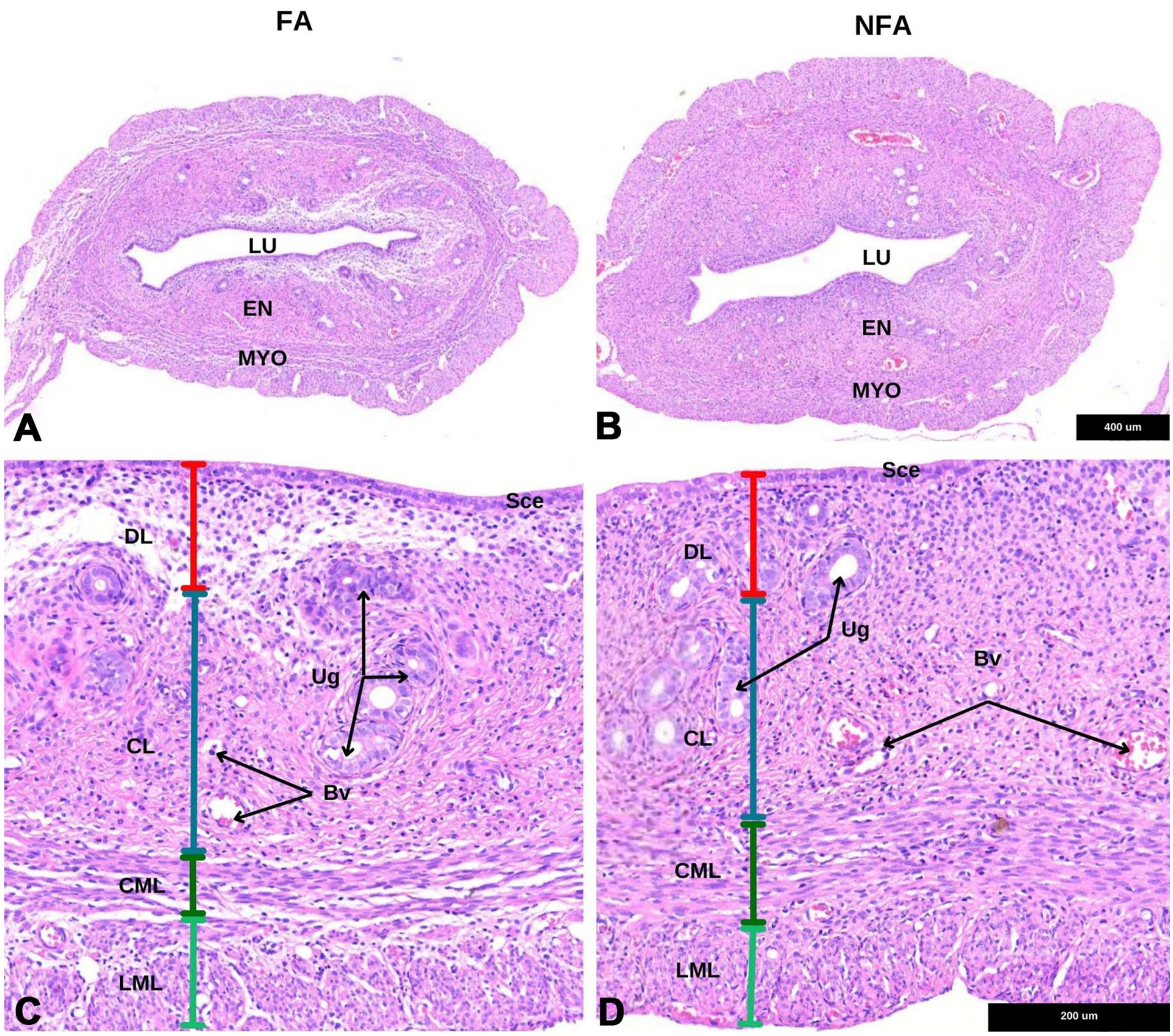

Fig. 2. Cross-sectional view of the uterine horn of nulliparous adult Sprague Dawley rats (82 days old). Presence of endometrial folds along the basal epithelium FA (A) and NFA (B). Endometrium (EN), myometrium (MYO), and lumen (LU). Hematoxylin-eosin staining (4X; Bar: 400 µm). Layers of the uterine horn AF (C) and NFA (D). Presence of uterine glands (Ug) and blood vessels (Bv) in the endometrium in both exposure groups. Deep layer of the endometrium (DL). Compact layer of the endometrium (CL). Circular muscular layer (CML). Vascular layer (VL). Longitudinal muscular layer (LML). Hematoxylin-eosin staining (10X; Bar: 200 µm).

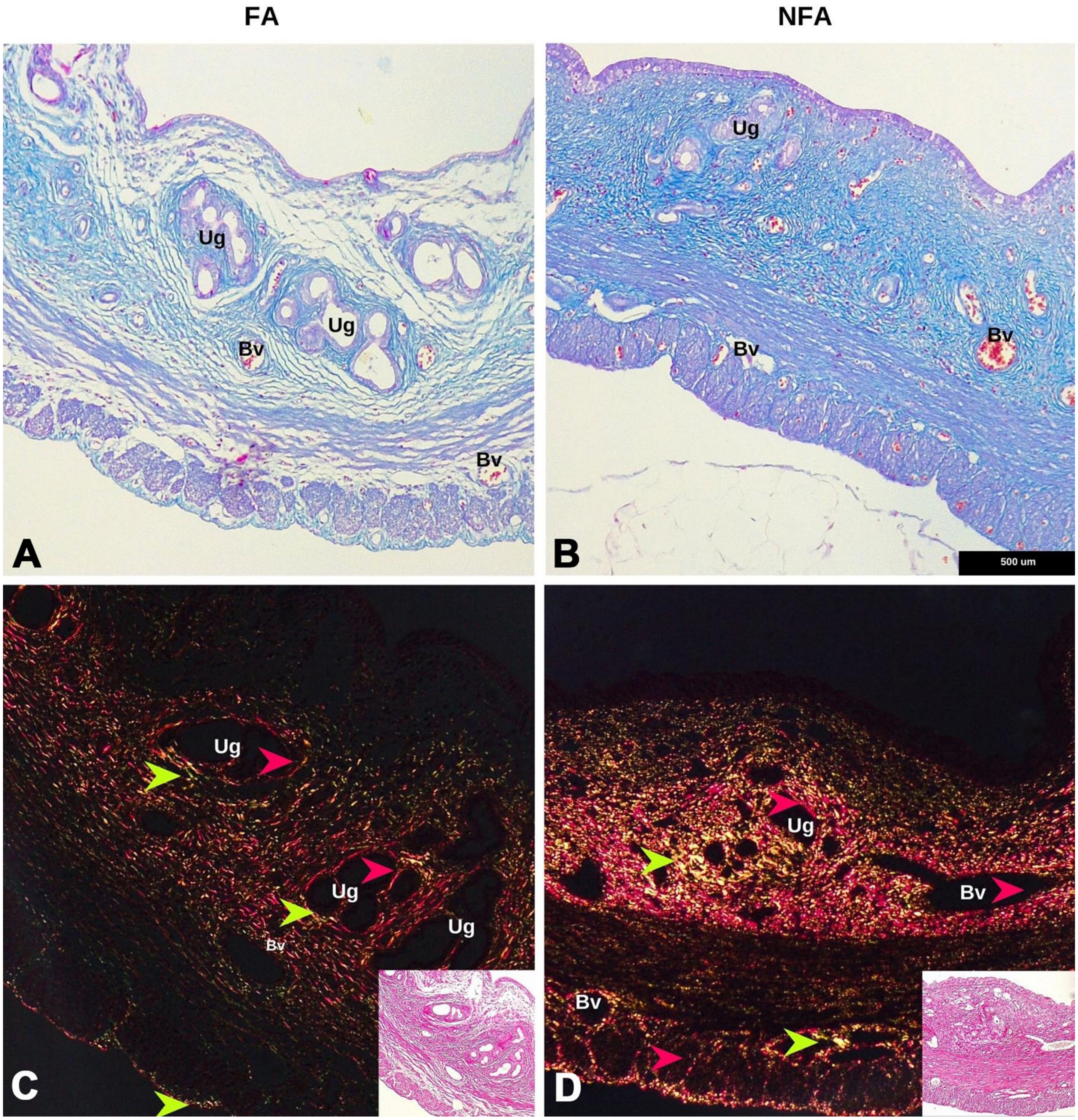

Fig. 3. Uterus of nulliparous adult Sprague Dawley rats (82 days old). Distribution of collagen fibers of the extracellular matrix. Mallory's trichrome staining (A and B; 10X; Bar: 500 µm). Picrosirius Red staining under polarized light (C and D; 10X; Bar: 500 µm). Increased density of total collagen fibers in the uterine tissue in the NFA group (C and D) compared to the FA group (A and C). Increase in the density of type I collagen fibers in the NFA group (D) compared to the FA group (C). Aligned type I collagen fibers (pink arrow). Not-aligned type III collagen fibers (green arrow). Uterine glands (Ug). Blood vessels (Bv).

observed in the NFA group compared to the FA group (control; Figs. 3A and 3B). This pattern was confirmed in endometrium and myometrium. Furthermore, using picrosirius red staining, it was noted that the NFA group exhibited a higher density of aligned type I collagen fibers (Figs. 3C and 3D).

The endometrium of both groups was characterized by longitudinal folds covered by a luminal epithelium, varying from a simple columnar to a pseudostratified type. In the epithelial cells, rounded nuclei in a basal position were identified (Figs. 4A and 4B). The epithelial cells of the NFA group (exposed to PM2.5) exhibited a more elongated

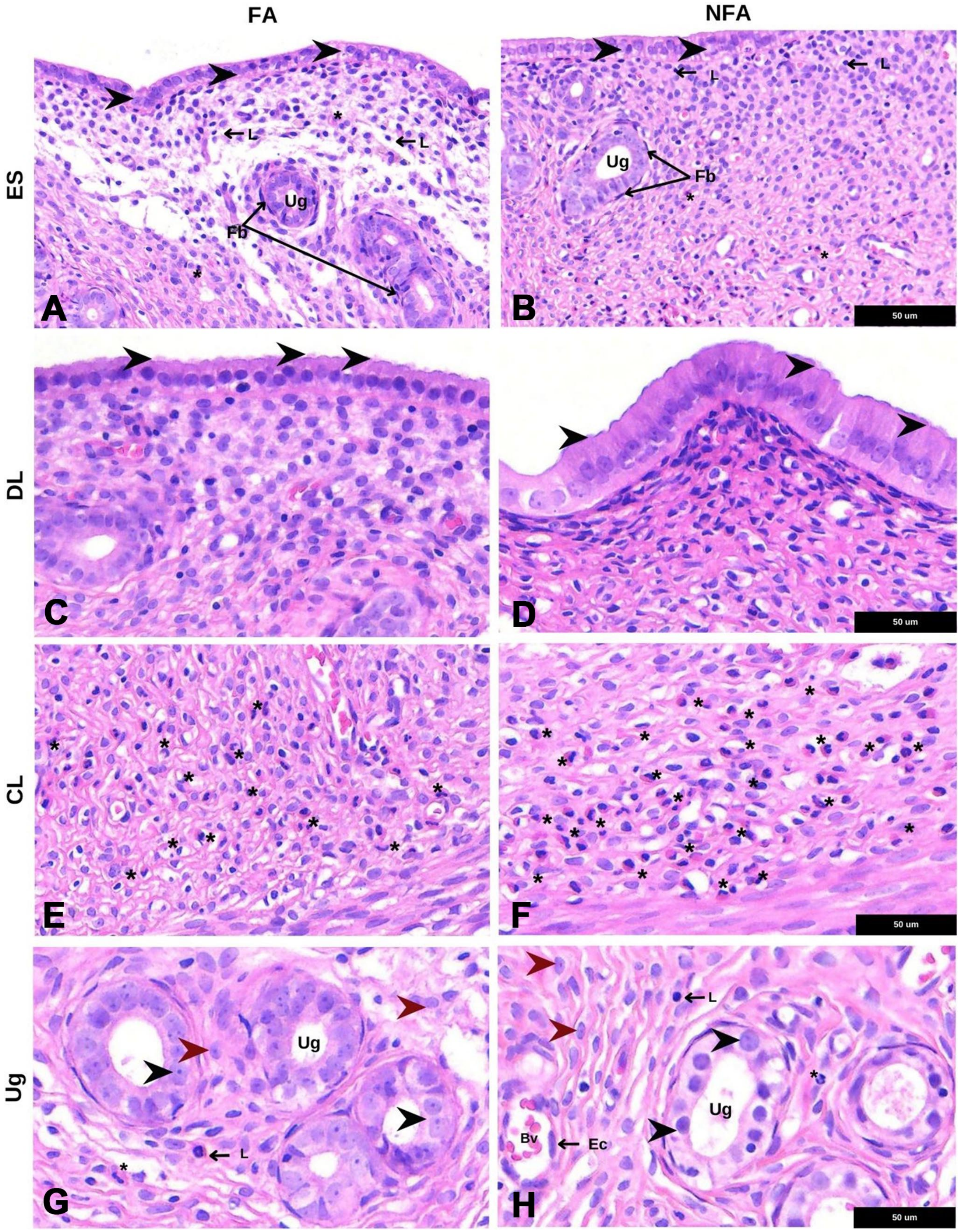

Fig. 4. Uterine endometrium of nulliparous adult Sprague-Dawley rats (82 days old) (Hematoxylin-eosin staining; Bar: 50µm). Endometrial stroma (A and B), Increased cell density in the superficial loose connective tissue in the NFA group. Luminal epithelium (black arrowhead). Lymphocytes (L). Fibroblasts (Fb). Uterine glands (Ug). Macrophages (*) (20X). Deep layer of the endometrium (C and D), cylindrical epithelium with basal nuclei and presence of ciliated basal cells (black arrowhead) (40X). Compact layer of the endometrium (E and F), increased macrophages in the NFA group (40X). Uterine glands (H and G), cells with eosinophilic cytoplasm (black arrowhead), stromal cells (red arrowhead), lymphocytes (L), blood vessels (Bv), endothelial cells (Ec), endometrial glands (Ug), and macrophages (*) (40X).

(cylindrical) cytoplasm compared to the epithelial cells of the FA group. Additionally, the presence of cilia in the epithelial cells was confirmed in both groups (Figs. 4C and 4D). The lamina propria of the endometrium consisted of loose connective tissue. In the *stratum internum* of the FA group, a higher cell density was observed in contrast to the NFA group. In the latter, an increase in the lymphocyte population near the epithelial barrier was noted (Figs. 4C and 4D). In the *stratum externum*, loose connective tissue and an abundant amount of macrophages adjacent to the myometrium were observed. Specifically, in the NFA group, a higher density of phagocytic cells was detected (Figs. 4E and 4F). In both study groups, blood vessels were observed near the uterine glands of the *stratum externum*. The vascularized loose connective tissue was seen in both the *stratum externum* and *internum*. Regarding the *uterine glands*, they exhibited a branched tubular structure, composed of cylindrical epithelial cells with eosinophilic cytoplasm. In the glands of the FA group, a higher density of epithelial cells and a lower density of cells with vacuolated cytoplasm were evidenced compared to the NFA group (Figs. 4G and 4H). In the NFA group, an increased density of collagen fibers was observed both in the superficial stratum of the lamina propria (Figs. 5A and 5B) and around the uterine glands (Figs. 5D and 5F), compared to the FA group (Figs. 5C and 5E). Specifically, regarding the aligned type I collagen fibers located around the uterine glands, it was noted that the NFA group exhibited a significantly higher density of aligned type I collagen fibers compared to the control FA group (Fig. 5G).

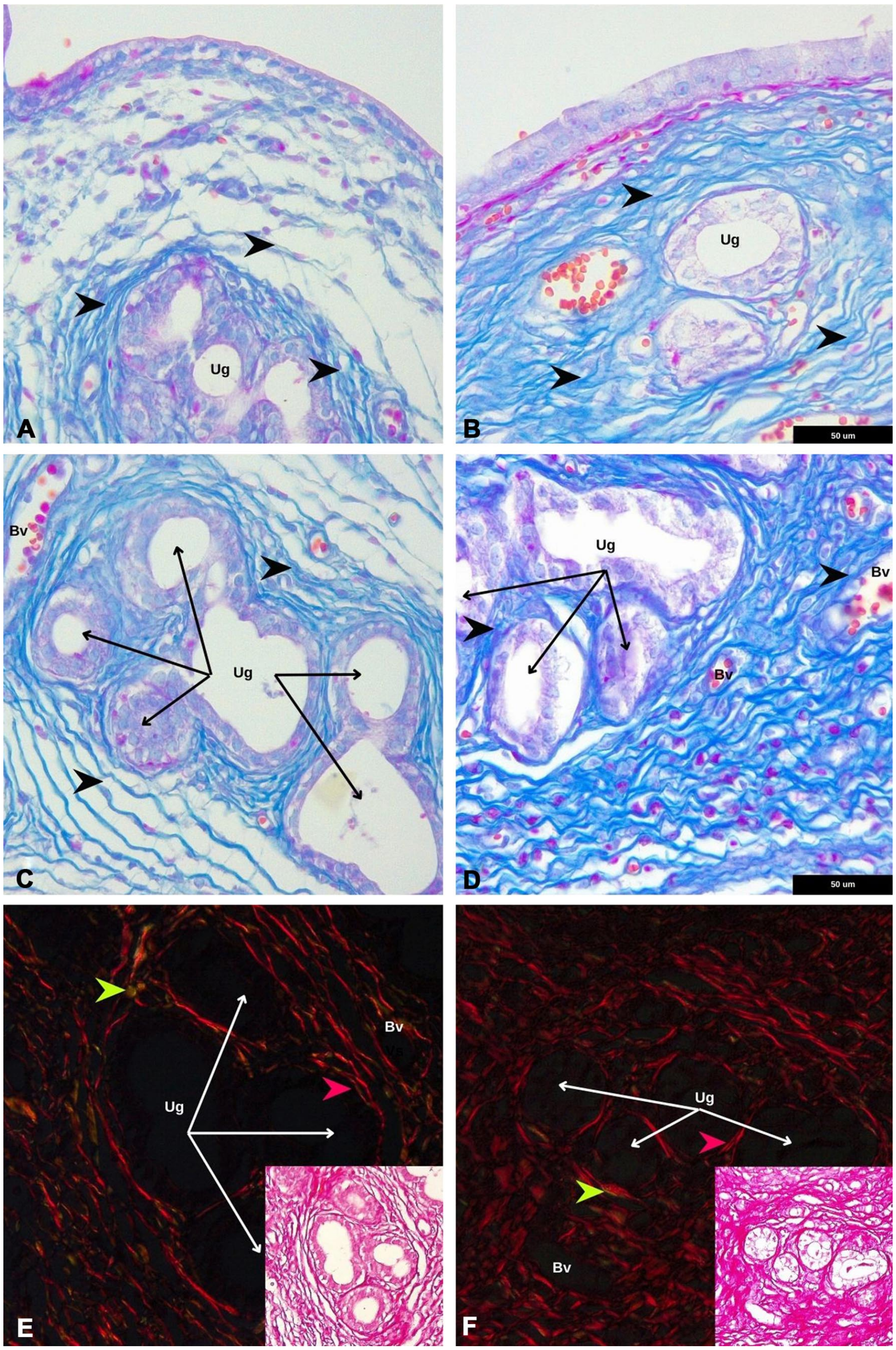

Fig. 5. Uterine endometrium in nulliparous adult Sprague-Dawley rats (82 days old). Deep layer of the endometrium (A and B), in NFA a higher density of collagen fibers is observed in the deep layer of the endometrium near the epithelium (A and B) and uterine glands (C, D; Mallory's trichrome staining; 40X; Bar: 50µm). Furthermore, a higher density of aligned type I collagen fibers surrounding the endometrial glands was observed in the NFA group (F) compared to the FA group (E; Picrosirius Red staining; 40X; Bar: 50µm). Aligned type I collagen fibers (pink arrow). Not-aligned type III collagen fibers (green arrow). Uterine glands (Ug). Blood vessels (Bv).

The myometrium exhibited an inner stratum with circularly arranged cells and an outer stratum arranged longitudinally. The control FA group exhibited a lower density of muscle fibers in the circular stratum (Figs. 6A and 6B) and a reduced density of connective tissue between both muscular layers compared to the NFA group, primarily surrounding the vascular stratum where blood vessels (arteries and arterioles) are located (Figs. 6C and 6D). In both study groups, the myometrium presented a defined structural organization, with an inner stratum composed of circularly aligned cells and an outer stratum organized longitudinally. It was observed that the control FA group exhibited a reduced density of muscle fibers in the circular stratum (Figs. 6A and 6B). Additionally, this group showed a lower density of connective tissue interspersed between the muscular layers compared to the NFA group. This feature was particularly evident around the vascular stratum, which houses the blood vessels, including arteries and arterioles (Figs. 6C and 6D). Regarding the density and distribution of collagen fibers, it was observed that the FA group exhibited a reduced density in the three examined strata: circular, vascular, and longitudinal (Figs. 7B and 7D). In contrast, the NFA group showed a higher density of these fibers (Figs. 7A and 7C). Specifically, the FA group exhibited a reduced density of aligned type I collagen fibers around the vascular stratum and the perimetrium (Fig. 7F), compared to a higher density of fibers in these areas in the NFA group (Fig. 7E).

Regarding the histomorphometric analysis of the uterus body, the total height of the uterus showed statistically significant differences (p=0.0116) between the exposure groups, being greater in the NFA group compared to the FA group. No differences were detected between groups in the mean height of the endometrium (p=0.1027) or the vascular stratum (p=0.1027). However, statistically significant differences were established in the mean height of the circular (p<0.0001) and longitudinal (p=0.0041) strata between both groups (Table II). When evaluating the proportion of each stratum relative to the total of the uterus body, statistically significant differences were found in the circular (p=0.0204) and vascular (p=0.4044) strata between the groups. The area fraction of the circular stratum increased and that of the vascular stratum decreased in the NFA group, compared to the FA group. Meanwhile, the percentage of the endometrium (p=0.1653) and the longitudinal stratum ((p=0.1027) did not show a difference between groups (Table II). The uterine gland density per exposure group was higher in the FA group; however, no differences were detected between exposure groups (p=0.5617). The glands present in the FA group were of larger diameter compared to those observed in the NFA group (Table I).

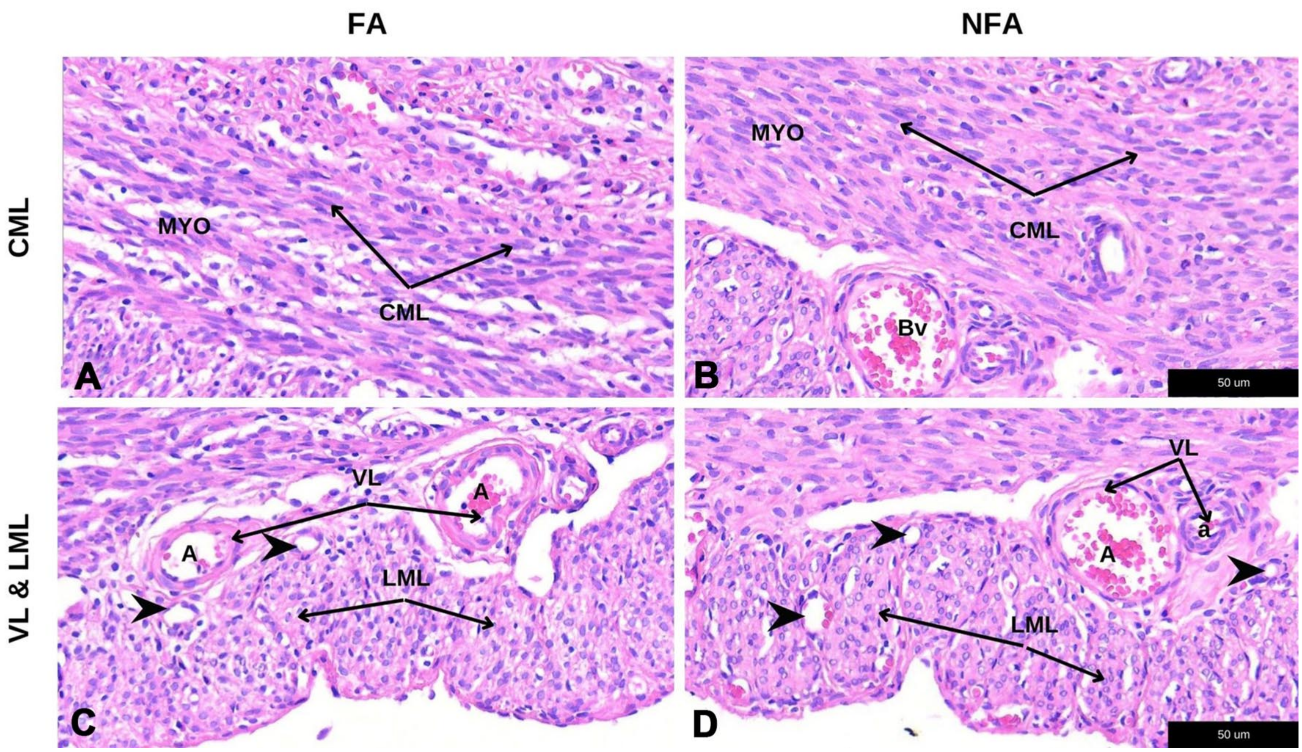

Fig. 6. Uterine myometrium (MYO) of nulliparous adult Sprague-Dawley rats (82 days old). Hematoxylin-eosin staining 40X; Bar: 50 μm. Circular muscular layer (CML). A higher density of smooth muscle fibers was observed in the NFA group (B and D) compared to the FA group (A and C). Vascular layer (VL). Longitudinal muscular layer (LML). Arteries (A). Arterioles (a). Capillaries (black arrowhead).

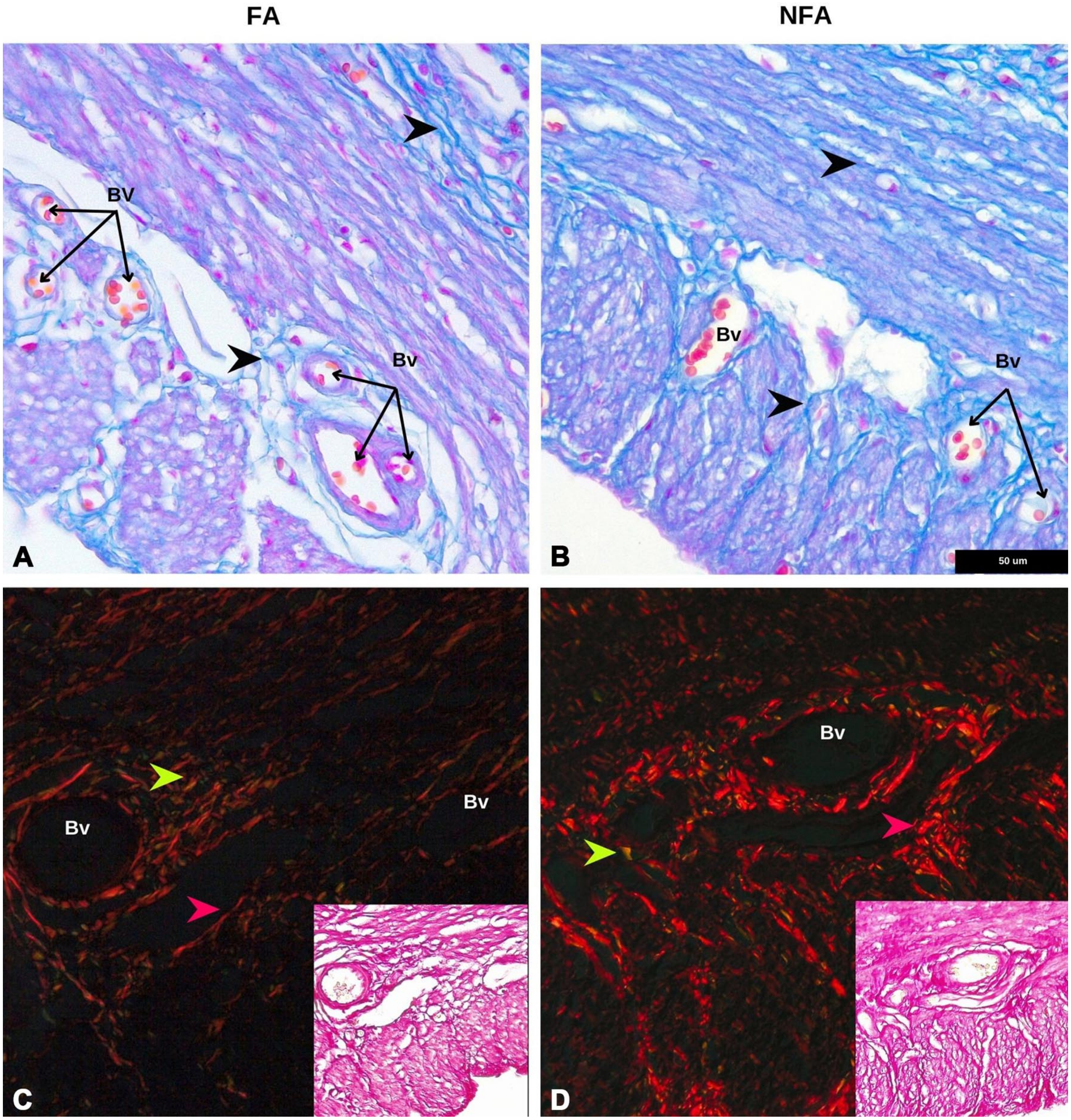

Fig. 7. Uterine myometrium in nulliparous adult Sprague-Dawley rats (82 days old). A higher density of collagen fibers surrounding blood vessels was observed in the NFA group (B) compared to the FA group (A; Mallory's trichrome staining; 40X; Bar: 50 μm). Specifically, these collagen fibers were identified as aligned type I (C and D; Picrosirius Red staining; 40X).

Figure 8 displays the serum levels of progesterone and estradiol in 82-day-old nulliparous Sprague Dawley female rats. No differences were detected in the serum levels of estradiol (p= 0.831) or progesterone (p= 0.341) between the groups. Regarding the stage of the estrous cycle, based on the study by Marcondes *et al*. (2002), and considering the characteristics of the endometrial tissue, as well as the predominance of 70 % nucleated round epithelial cells and the observed serum levels of progesterone and estradiol, it was determined that the rats examined were in the proestrus phase.

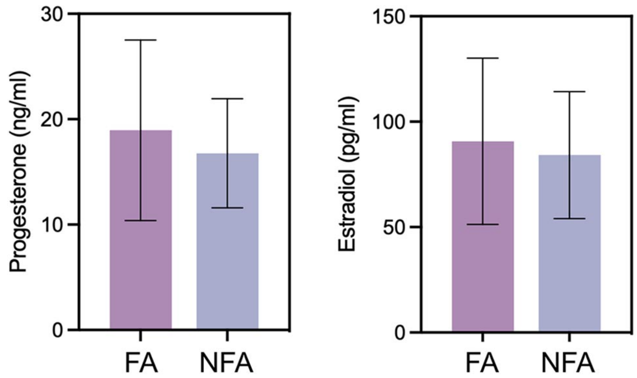

Fig. 8. Serum progesterone and estradiol levels in nulliparous Sprague-Dawley female rats (n=6; 81 days old).

Table II. Histomorphometric comparison of the uterus in nulliparous adult sprague dawley rats (82 days old). effects of PM2.5 particulate matter exposure on uterine metric characteristics (n=12/group; h: height).

	FA	NFA	p-value
Total *h* (µm)	699.2 ± 143.0	807.6 ± 82.7	0.0116
h Endometrium	442.8 ± 100.2	495.7 ± 42.6	0.1027
Myometrium			
h Circular muscle layer	107.0 ± 38.1	144.3 ± 30.0	<0.0001
h Vascular layer	69.0 ± 31.5	61.2 ± 20.9	0.4044
h Longitudinal muscle layer	80.3 ± 19.0	106.4 ± 35.3	0.0041
% Fraction area			
% Endometrium	63.0 ± 3.5	61.4 ± 3.7	0.1653
Myometrium			
% Circular muscle layer	15.2 ± 3.6	17.8 ± 3.3	0.0204
% Vascular layer	10.0 ± 4.0	7.6 ± 2.5	0.0272
% Longitudinal muscle layer	11.4 ± 2.0	12.9 ± 3.5	0.1027
Uterine gland./mm^2	2.7 ± 0.2	2.3 ± 0.5	0.5617

DISCUSSION

This study establishes the importance of PM2.5, primarily sourced from wood smoke, as a public health concern. It emphasizes the widespread use of wood for heating and cooking in certain regions of the world, including southern Chile, leading to indoor and outdoor air pollution. The findings presented here highlight the urgency of addressing this issue due to the substantial public health risks posed by PM2.5. We employed an experimental design with two groups of Sprague-Dawley rats exposed to filtered and unfiltered air in chambers, respectively, to simulate direct and chronic exposure to PM2.5 wood smoke. Histological and endocrine analyses were conducted to detect morphophysiological effects on the uterus. Significant histomorphometric differences in uterine structure were observed between the exposed (NFA) and control (FA) groups. Findings include an increase in uterine wall thickness and collagen density in the group exposed to PM2.5 (NFA), suggesting potential adverse effects on reproductive health.

Regarding our methodological approach, uterine histomorphometry was a robust and reliable technique for studying the structure of the uterus, particularly in our model of Sprague-Dawley rats. This technique was chosen as it has enabled precise quantitative assessment in studies of uterine architecture, offering essential insights into the impact of various physiological and pathological conditions on uterine tissue (Salinas *et al*., 2017; García-Vázquez *et al*., 2019; da Silva *et al*., 2022; Sánchez-Caycho *et al*., 2023). These studies demonstrate the versatility and precision of histomorphometry, highlighting its value in biomedical research for understanding the complex dynamics of uterine tissue under diverse experimental or therapeutic conditions, which is crucial for advancing our understanding of reproductive and pathological processes. On the other hand, Mallory and Picrosirius Red staining have been widely used and recognized for their effectiveness in identifying and analyzing collagen in tissues, particularly in the uterus (Borges *et al*., 2007; Salinas *et al*., 2016; Rittié *et al*., 2017). The latter is valuable due to its ability to enhance the natural birefringence of collagen fibers when exposed to polarized light, allowing us a detailed assessment of the organization and distribution of these fibers in the uterine tissue.

Endocrine implications of PM2.5. Regarding the serum levels of progesterone and estradiol, they were within the parameters reported in the literature (Ward *et al*., 1977; Faccio *et al*., 2013). Findings in the uterine tissue and the concentrations of both hormones suggest that the rats were in the proestrus stage. Moreover, the absence of differences in both hormones implies that exposure to PM2.5 did not exert a differential effect on the serum levels of progesterone and estradiol measured in both rat groups. These findings are not consistent with those reported in specialized literature. The exposure to PM2.5 in air pollution has been the subject of research due to its potential effects on human and animal health. Therefore, understanding how these particles might influence progesterone and estradiol, especially in rats, is crucial. Studies have provided mixed evidence, with some showing alterations in serum hormonal levels and others not. For instance, a study conducted by Nääv *et al*. (2020), indicated that exposure to PM2.5 from oil combustion could significantly decrease progesterone levels in rats, suggesting interference in hormonal synthesis or regulation. Although Wang *et al*. (2021) describe effects of PM2.5 on hormonal levels; they note that understanding the exact mechanisms being altered is necessary. Our seemingly contradictory findings suggest that the relationship between PM2.5

exposure and complex endocrine function may be influenced by factors such as the type of emission source, duration of exposure, particle dosage, and specific susceptibilities of the animal model used. Therefore, the absence of differences in serum levels of progesterone and estradiol in our study does not rule out the possibility of subclinical effects or compensatory mechanisms that temporarily maintain hormonal homeostasis in the face of exposure to environmental pollutants.

The interaction of PM2.5 with the endocrine system at the molecular and cellular levels is intricate, involving a series of mechanisms that disrupt hormonal homeostasis, representing a critical area for future research. PM2.5 particles can exert direct and indirect effects on the endocrine function of the reproductive system. Directly, the particles and their adsorbed components, such as heavy metals and polycyclic aromatic hydrocarbons, can penetrate cell membranes and interact with nuclear receptors, like estrogen and progesterone receptors, thereby altering hormonal signaling and gene expression (Kim *et al*., 2018; Plunk & Richards, 2020). This interaction can lead to alterations in the synthesis, secretion, and metabolism of steroid hormones, affecting the regulation of critical physiological processes. Indirectly, PM2.5 induces systemic inflammation and oxidative stress, as evidenced by the increase in the production of pro-inflammatory cytokines and reactive oxygen species (Yang *et al*., 2016; Garcia *et al*., 2023). This inflammatory state can disrupt endocrine function by influencing stromal cells and granulosa cells in the ovaries, altering the production of estradiol and progesterone (Darbre, 2018). Moreover, oxidative stress can directly damage hormone-producing cells, triggering a cellular response that includes the activation of stress-related signaling pathways, such as the nuclear factor kappa B (NF-kB) pathway, potentially leading to further disruption of hormone synthesis and action (Yang *et al*., 2016). Based on the discussion, it is hypothesized that the concentrations of PM2.5 in the air, as well as the presence of additional pollutants such as heavy metals and polycyclic aromatic hydrocarbons (not addressed in this study), might not have been sufficient to trigger a physiological response capable of altering the synthesis of progesterone and estradiol in adult nulliparous females. Consequently, exposure to PM2.5 from wood smoke does not appear to pose a substantial threat to endocrine homeostasis, at least under the reported exposure conditions, that could lead to significant changes in hormonal levels or reproductive function.

Histological implications in the uterus of PM2.5. Overall, the uterine structure presents distinctive components such as the mucosa (endometrium), the muscular layer (myometrium), and the serous layer (perimetrium). Studies have outlined these layers and identified specific features in each, like the presence of collagen and the organization of muscle fibers and connective tissue. The histological findings in the endometrium reflect significant alterations in the structure and morphology of the endometrium due to PM2.5 exposure, suggesting an impact of particulate matter on endometrial biology. The change in the morphology of the luminal epithelium from simple columnar to stratified and the presence of rounded nuclei in a basal position indicate tissue remodeling, a phenomenon documented in previous studies as a response to disruptive environmental stimuli (Wang *et al*., 2021). The observation of a more elongated (cylindrical) cytoplasm in the epithelial cells of the NFA group exposed to PM2.5 might reflect a cellular adaptation to an inflammatory or stressful environment in epithelial tissues subjected to potential oxidative stress. The increased presence of lymphocytes near the epithelial barrier and a higher density of phagocytic cells, specifically in the NFA group, suggests an active immune response, likely as a reaction to inflammation induced by PM2.5 exposure. This finding is consistent with literature linking air pollution to the activation of inflammatory pathways and the mobilization of immune cells to sites of inflammation (Nachman *et al*., 2016). The increased density of collagen fibers in the NFA group, especially around the uterine glands, could indicate a process of fibrosis or scarring, as observed in conditions of chronic tissue stress (Pryor *et al*., 2022). Endometrial fibrosis can alter the normal function of the tissue, potentially affecting fertility and other endocrine functions. Moreover, the difference in the density of epithelial cells and the presence of cells with vacuolated cytoplasm between the FA and NFA groups points to a possible alteration in glandular function and structure, which could have implications on the secretion of essential paracrine and autocrine factors for endometrial homeostasis. These findings underscore the need for more in-depth research to understand the specific pathways through which exposure to PM2.5 affects the morphology and function of the endometrium, and how these changes might impact overall reproductive and endocrine health.

The effects of PM2.5 on the endometrium of rats could have significant implications for reproductive health, both in animal models and potentially in humans. The remodeling of the luminal epithelium and the presence of cells with elongated cytoplasm suggest a change in endometrial architecture, which could directly impact embryonic implantation and gestational success. The structure and function of the luminal epithelium are crucial for embryo reception, and any disturbance in this delicate balance could compromise fertility (Singh & Aplin, 2009). The increased infiltration of immune cells, such as lymphocytes and macrophages, in the endometrium could indicate a chronic inflammatory response. Although inflammation is a normal component of the menstrual cycle and embryonic implantation, chronic or excessive inflammation has been associated with pathological conditions such as endometriosis, infertility, and spontaneous abortion (Kwak-Kim *et al*., 2009; Donnez & Cacciottola, 2022). Therefore, prolonged exposure to PM2.5 and the consequent inflammatory response could have adverse effects on female reproductive health.

The presence of increased collagen fiber density and signs of fibrosis in the endometrial tissue is also concerning. Fibrosis can alter the elasticity and functionality of the tissue, potentially hindering embryonic implantation or causing complications during pregnancy. Furthermore, endometrial fibrosis has been implicated in the pathogenesis of disorders such as adenomyosis, associated with chronic pelvic pain, abnormal menstruations, and infertility problems (Huang *et al*., 2022). Alterations in the structure and density of glandular epithelial cells could affect the secretion of essential factors for the maintenance of the endometrium and embryonic implantation. Changes in the composition and function of the uterine glands could compromise the release of nutrients and growth factors, crucial for the embryo in the early stages of development (Luke, 1994).

The histological findings in the myometrium of the rats, particularly in relation to the density of muscle fibers and the presence of aligned type I collagen, provide valuable insights into the potential effects of exposure to environmental factors such as PM2.5. The observed decrease in muscle fiber density in the circular stratum of the control group (AF) compared to the NFA group might indicate a change in muscle structure with potential repercussions on myometrial functionality. Previous studies have linked alterations in the density and organization of muscle fibers with changes in uterine contractility, which could have implications for menstruation and the childbirth process (Garay *et al*., 2021). Moreover, the increased density of connective tissue between muscle layers observed in the group exposed to PM2.5 might reflect a change in the extracellular matrix affecting the structural integrity and elasticity of the myometrium. Connective tissue, primarily composed of collagen fibers, plays a critical role in providing support and mechanical resistance to tissues; therefore, a decrease in connective tissue density could compromise myometrial function, particularly during gestation and labor (Anum *et al*., 2009). The increased density of aligned type I collagen fibers observed in the group exposed to PM2.5 (NFA), particularly around the vascular stratum and perimetrium, is a notable finding. Aligned type I collagen is a key component of the extracellular matrix, and its altered density may indicate a process of tissue remodeling or fibrosis. The latter has been associated with disorders such as uterine fibroids, which can affect fertility and cause symptoms like heavy menstrual bleeding and pelvic pain (Uimari *et al*., 2022; Bhat *et al*., 2023). These findings highlight the complexity of tissue responses to environmental factors and suggest that exposure to PM2.5 might have direct or indirect effects on the structure and function of the myometrium. However, further studies are essential to better understand these mechanisms and determine their clinical relevance in human reproductive health.

The changes observed in both cellular fibers and collagen probably involve biochemical signaling pathways and complex regulatory factors. For example, the alteration in muscle fiber density could be related to disruption in the signaling pathways that regulate muscle differentiation and proliferation, such as JAK/STAT (Xu *et al*., 2015), MAPK (Xu *et al*., 2019), ERK1/2 (Zang *et al*., 2021), and NFATc1 (Tong *et al*., 2015). For instance, growth factors like TGF-b have been implicated in regulating muscle tissue growth and repair. Pollutants such as PM2.5 could interfere with these pathways, thereby altering muscle homeostasis (Delaney *et al*., 2017). As for the connective tissue and extracellular matrix, the remodeling observed in the NFA group suggests an activation of fibroblasts and increased synthesis of matrix components like t aligned type I collagen. This process could be induced by the TGF-β pathway, known for its role in tissue fibrosis. Overexpression of TGF-β can lead to excessive accumulation of extracellular matrix and fibrosis, affecting tissue functionality (Meng *et al*., 2016). Therefore, it is imperative to delve deeper into the complex interaction between TGF-β, collagen synthesis, and the potential development of fibrosis as a repair mechanism and tissue damage in response to PM2.5. Additionally, oxidative stress induced by exposure to PM2.5 may play a crucial role in altering the myometrial structure. Excessive production of reactive oxygen species (ROS) can damage muscle cells and activate pro-fibrotic pathways, leading to an alteration in the composition and organization of the extracellular matrix (Llanos & Palomero, 2022). The chronic inflammation, a well-documented effect of PM2.5 exposure, could contribute to the disruption of myometrial tissue. Inflammation can induce the secretion of cytokines and chemokines that recruit immune cells to the tissue, thereby enhancing the pro-fibrotic response and altering the architecture and function of the myometrium (Elhamouly *et al*., 2018). Lastly, a regulatory factor implicated in the pathophysiology of uterine fibroids and their symptoms is TNF-α. The presence of an "inflammation-like state" in women with uterine fibroids suggests that TNF-α is a potent inducer of inflammation

(Ciebiera *et al*., 2018). Moreover, it was found that the expression of TNF-α, a pro-inflammatory cytokine, was higher in uterine fibroids than in the adjacent myometrium, supporting its role in the pathophysiology of uterine fibroids (Luddi *et al*., 2019). Investigating these molecular mechanisms to assess their potential role in the changes observed in the myometrium exposed to PM2.5 is crucial for understanding how air pollution impacts reproductive health.

Study Limitations. The current research, utilizing Sprague-Dawley rats as an animal model, encounters limitations regarding the direct extrapolation of findings to humans, due to intrinsic species differences in physiology, metabolism, and responses to environmental stimuli. Although the exposure to PM2.5 was simulated under controlled conditions, the inherent complexity of the actual environment introduces additional variables, such as the presence of other pollutants and variability in the composition of wood smoke, which could influence the interpretation of the results and their applicability. Furthermore, the absence of significant differences in the levels of progesterone and estradiol between the exposed and control groups suggests a potential limitation in the study's ability to detect subtle endocrine alterations. This, combined with the complexity of the interaction between PM2.5 and the endocrine system, could be influenced by the duration of exposure, the dosage of particles, and specific susceptibilities of the animal model. Finally, while the study provides valuable insights into the immediate effects of PM2.5 on uterine structure, it does not consider the potential long-term consequences nor assesses the clinical relevance of these findings for human reproductive health, thus limiting the understanding of the effect of wood smoke on reproduction.

In conclusion, exposure to PM2.5, originating from wood smoke, demonstrates an impact on uterine tissue. Histologically, an effect on the uterine mucosal epithelium and an increased density of collagen fibers were observed, suggesting potential adverse effects on reproductive health. Endocrinologically, despite no significant differences being detected in the levels of progesterone and estradiol, the complexity of the interaction between PM2.5 and the endocrine system underscores the need for further research. These findings highlight the importance of more thoroughly evaluating the long-term effects and clinical relevance of PM2.5 exposure on human reproductive health.

Ethics approval. All procedures were performed in accordance with the approval of the Bioethics Committee of the Universidad de La Frontera (nº122/20; October 26, 2020) and the Bioethics and Biosafety Committee of the Pontificia Universidad Católica de Valparaíso (BIOEPUCV-BA 373-2020).

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VILLARROEL, F. & SALINAS, P. Efectos histológicos y endocrinos de la exposición crónica a MP2.5 derivada del humo de leña en el útero de ratas adultas nulíparas. *Int. J. Morphol., 42(3)*:647-662, 2024.

RESUMEN: El estudio explora la relación entre la exposición crónica a partículas finas (PM2,5), procedentes del humo de leña, y la estructura histológica y la función endocrina del útero en ratas adultas nulíparas. Evalúa posibles cambios estructurales en el útero que podrían afectar la salud reproductiva, considerando la exposición a PM2,5 como un posible factor de riesgo. Se llevó a cabo un experimento controlado en una ciudad conocida por sus altos niveles de contaminación del aire, exponiendo ratas a condiciones de aire filtrado y sin filtrar, imitando así la exposición humana a PM2,5. Los hallazgos histológicos indicaron un aumento significativo en la densidad del colágeno y el grosor de la pared uterina en sujetos expuestos a PM2,5, lo que sugiere un riesgo para la función reproductiva. Sin embargo, no se observaron diferencias significativas en los niveles de las hormonas progesterona y estradiol, lo que apunta a la compleja relación entre la exposición a PM2,5 y su impacto endocrino, y enfatiza la necesidad de realizar más estudios para una comprensión más profunda. Este trabajo destaca la importancia de investigar a fondo los efectos a largo plazo de la contaminación por PM2,5 en la salud reproductiva, subrayando la importancia de considerar la exposición ambiental como un factor crítico en la investigación de la salud reproductiva.

PALABRAS CLAVE. Contaminación; Humo de leña; Útero; Sistema reproductivo; Partículas 2.5, PM2.5.

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