Sex-related differences in vascular remodeling of sodium overloaded normotensive mice

Juliane Cristina de Souza Silva[1A], Katia Aparecida da Silva Viegas[2*1], Rariane Silva de Lima[1], Cintia Taniguti Lima[1], Natalia Nunes Peron[1], Maikon Barbosa da Silva[3], Maria Claudia Irigoyen[3], Silvia Lacchini[1]

1 – Department of Anatomy, Institute of Biomedical Sciences, University of Sao Paulo (USP), Sao Paulo, Brazil;
2 – Post-Doctoral Student at the Department of Structural and Functional Biology, Institute of Biosciences, Sao Paulo State University (UNESP), Botucatu, Brazil;
3 – Hypertension Unit, Heart Institute, University of Sao Paulo Medical School (FMUSP), Sao Paulo, Brazil.

Corresponding Author: [2*A] Katia Aparecida da Silva Viegas - e-mail: katia.viegas@unesp.br
Department of Structural and Functional Biology, Institute of Biosciences, Sao Paulo State University (UNESP), R. Prof. Dr. Antônio Celso Wagner Zanin, 250 - Distrito de Rubião Junior - Botucatu/SP, Brazil - CODE 18618-689.

ABSTRACT

Primary hypertension affects about 20% of adults in developed societies and is linked to high salt intake, leading to vascular remodeling. This remodeling results from repair, inflammation, or cell growth and is an adaptive physiological response of blood vessels. However, over time, it can contribute to vascular diseases. Studies indicate sex-related differences in cardiac and vascular remodeling. This study aimed to characterize vascular remodeling in the aorta and cardiac arteries, and to examine gender differences in response to sodium overload. Adult male and female C57Bl/6 mice were divided into six groups: control with filtered water (Cont M; Cont F), 1% NaCl for two weeks (Salt-2 M; Salt-2 F), and 1% NaCl for twelve weeks (Salt-12 M; Salt-12 F). Blood pressure and heart rate were measured using tail plethysmography, and water intake and urine output were recorded over 24 hours in metabolic cages. Morphometric analysis of the aorta and cardiac arteries was performed to identify vascular changes. Elastic laminae and collagen fiber deposition were

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assessed by Weigert van Gieson and Picrosirius staining, respectively. No changes in blood pressure or heart rate were observed. After two weeks of treatment, water intake increased in both genders, but urine production only increased in men. Vascular changes differed between genders: males showed a later increase in aortic elastic lamellae, while females had an earlier increase after two weeks. The number of elastic lamellae did not change in cardiac arteries. Collagen deposition increased in the aorta for both genders but decreased by 50% in the cardiac arteries in the male group. Males also showed an increase in intermediary and mature (thick) collagen fibers in the aorta, while females had younger (thin) as well as mature collagen fibers. Thus, high sodium intake induced different vascular responses in males (elastic arteries) and females (resistance arteries), causing arterial stiffness even in normotensive animals.

Key words: Sodium overload, gender, vascular collagen deposition, arterial stiffening.

1. INTRODUCTION

Cardiovascular diseases are the main cause of morbidity and mortality in the world. According to the World Health Organization, 17.3 million people died in 2008 from diseases related to heart and blood vessels which represents two-thirds of all deaths worldwide. In addition, around 16.5% of deaths that affect the cardiovascular system have been associated with arterial hypertension, including 51% from stroke and 45% from coronary heart disease deaths [1].

Primary hypertension is certainly one of the most common cardiovascular diseases affecting approximately 20% of the adult population in developed societies [2]. It is a multifactorial disorder resulting from the interaction between genetic and environmental factors such as stress, sedentary lifestyle, obesity and the consumption of high salt diet. It is well known that primary hypertension may lead to the development of coronary artery diseases by accelerating the atherosclerotic process [3–5].

Several studies have shown a direct relationship between salt intake and higher levels of blood pressure. The increasing salt intake is detrimental to health and presents different consequences depending on the population heterogeneity and experimental design [6]. The World Health Organization recommends a diet based on a salt consumption up to 2 g sodium/day (equivalent to 5 g salt/day) [1], although recent data on sodium intake show that populations around

the world are consuming much more sodium than is physiologically necessary, there are regional variations in sodium intake [7, 8]. The global average sodium intake is estimated at 3.95g/day, based on a meta-analysis of research from 187 countries using 24-hour urine collections [9].

Variations in the vascular microenvironment or arterial injuries, which can be caused, for example, by excess sodium, can induce changes in the structure and function of the cardiovascular system, triggering a complex and dynamic process described as vascular remodeling that aims to meet the physiological needs of local blood supply as occurs, for example, in the mesenteric arteries [10–13]. with the endothelium also playing a crucial role in vascular homeostasis [14–16].

Research with rats subjected to sodium overload for 2 and 12 weeks demonstrated that it modifies perivascular collagen deposition, increasing thick fibers and decreasing thin fibers [17]. The vascular remodeling emerges from situations of repair, inflammation, development or cell growth [18–21]. The extracellular matrix (ECM) is a three-dimensional network formed by numerous proteins such as proteoglycans, glycosaminoglycans, elastin, laminin and matrix metalloproteases although collagen has been considered the main component [22–26]. The ECM is responsible for maintaining the tissue integrity in the vessel besides regulating the cell migration, and also acting as a reservoir of cytokines and growth factors[18, 19, 25, 27–29].

The tissue perfusion is promoted by microcirculation where vessels, especially arteries, are capable to change the diameter, structure, and composition of the vascular wall in order to increase the surface area needed for blood exchange. This response is essential for tissue integrity and organ function. The perfusion of tissue varying on the metabolic requirements and depending on the stimulus it may lead to remodeling for the purpose of maintain the stability between blood flow and shear stress [30, 31].

Vascular remodeling is an adaptive physiological response of vessels but may contribute to the development of vascular diseases in the long term. Previous studies have shown that women are more likely to develop cardiovascular diseases when compared to men. However, when we compare men with postmenopausal women, these differences tend to decrease [32–35]. Moreover, it has been observed that women have different cardiac and vascular remodeling when compared to men. Thus, many studies have been conducted to understand the possible cardioprotective effect of female hormones but, even today, these data still needed to be clarified [36–38].

Considering that cardiovascular diseases are the main cause of morbidity and mortality, that the rates of arterial hypertension have increased every year in several countries, and also that a large part of the world population has increased the consumption of sodium in their diet, we can question whether these factors would promote a harmful effect on the vascular system, even in normotensive individuals. Thus, our hypothesis is that the amount of sodium from the diet could influence the capacity of the cardiovascular system to respond and adapt to different situations, regardless of blood pressure, and the response could vary depending on exposure time to the diet as well as the gender. The aim of this study was to characterize the vascular remodeling in large and small arteries (aorta and cardiac arteries) and determine the different responses from male and female mice when submitted to sodium overload.

2. MATERIAL AND METHODS

2.1 Animals

The present study used adult male and female C57Bl/6 mice, obtained from the animal care unit of the Department of Anatomy of the Institute of Biomedical Sciences at the University of Sao Paulo. The animals received standard laboratory chow and water *ad libitum* and were distributed in cages in a temperature-controlled room with a 12-h dark-light cycle. The animals were randomly assigned to one of six groups: control, receiving filtered water (Cont M; Cont F); 1% NaCl solution to drink for two weeks (Salt-2 M; Salt-2 F); 1% NaCl solution to drink for twelve weeks (Salt-12 M; Salt-12 F), according to the previous study [17]. The treatments started on the 4-week-old mice and were continued until they reached 16 weeks of age although the salt-2 group received 1% NaCl solution in the drinking water in the last 2 weeks of protocol, therefore, all animals were of the same age at the end of treatment (Figure 1). Experiments were conducted according to the ethical principles established by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of Sao Paulo, research protocol 049/2011.

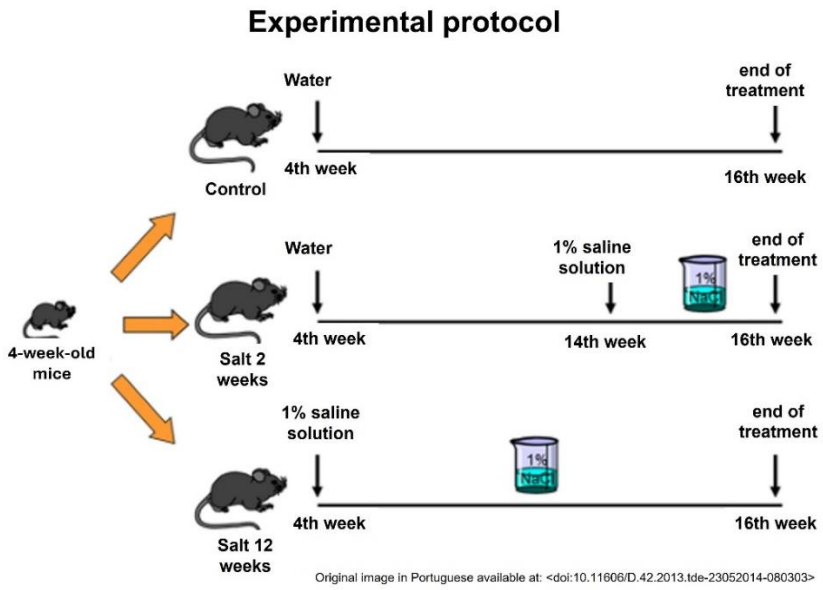

Figure 1 - Experimental protocol diagram

2.2 Blood Pressure and Heart Rate assessment

In the last week of treatment, pulse blood pressure (BP) was measured by tail plethysmography. The animals were adapted to the system during the week prior to the measurement. BP and heart rate (HR) were measured 10 times for two consecutive days. Peak systolic BP was captured by the BP-2000 series II system, according to the previous study [17].

2.3 Water intake and urine output measurements

On the last week (2nd and 12th week) of treatment, mice were individually housed in metabolic cages for a single period of 24 hours and provided with tap water (Cont group) or 1% NaCl water (Salt-2; Salt-12 groups) and regular chow *ad libitum*. During one day prior to the measurement, the animals were adapted to the system. Water intake and urine output were measured within the 24 hours in the metabolic cage.

2.4 Histological Evaluation

2.4.1 Tissue collection and preparation

At the end of treatment, the animals were euthanized with an anesthetic overdose of ketamine (180 mg/kg) supplemented with xylazine (20 mg/kg) and were subsequently perfused

with 0.9% NaCl solution followed by a buffered 4% formalin solution. After that, the tissues were harvested and maintained in formalin during a period of 24h. Following the fixation process, tissues were processed and paraplast embedded for histological evaluation.

2.4.2 Morphometry

Elastic laminae and collagen fibers were analyzed in aorta and cardiac arteries to evaluate the components of the extracellular matrix. These ECM components are responsible for vascular compliance and resistance, respectively. Elastic laminae were identified by using Weigert van Gieson staining and Picrosirius staining was used to identify collagen fiber deposition. Blinded morphometric analyses were performed; the slides containing 5-µm tissue sections of aorta or cardiac arteries were coded and analyzed using an optical microscope (Zeiss Axio Scope II) equipped with a 20x and 40x objective lenses coupled to an image analyzer (Axio Vision 4.8 System). The intima and media area, jointly, were calculated for each section. To calculate the intima area, the difference between the internal elastic layer and the luminal area was used. The media layer area was calculated by the difference between the outer elastic lamina and the inner elastic lamina area. To evaluate the collagen fiber deposition, slides were stained with Picrosirius red solution and analyzed by polychromatic and polarized light. Percent area of mature (red) and intermediary (yellow) fibers was compared to immature (green) fibers under polarized light.

2.5 Statistical Analysis

All values are expressed as means ± SD. These mean values of each animal were compared by Analysis of Variance (ANOVA) of two ways (gender and time of treatment), complemented by the Bonferroni's test. For all statistical analyses, $p \leq 0.05$ were considered statistically significant. The program GraphPad Prism, version 5.01 (GraphPad Software, Inc., 2007) was used to perform the statistical analysis.

3. RESULTS

3.1 Blood Pressure and Heart Rate Measurement

The treatment with 1% NaCl solution did not promote any change in the hemodynamic parameters evaluated. There was no difference in values for systolic arterial pressure and heart rate in sodium overloaded mice compared to the control group (Figure 2A and B).

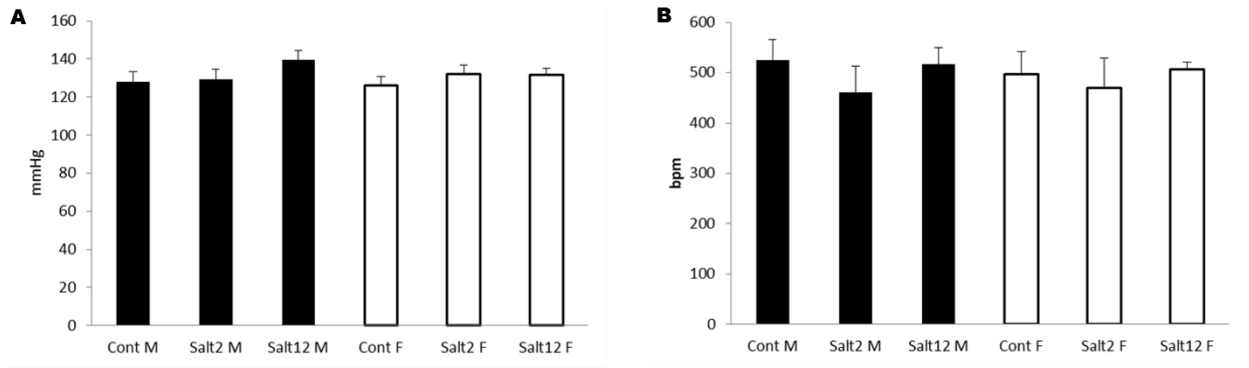

Figure 2 – Tail cuff plethysmography showing mean values for systolic arterial pressure (A) and heart rate (B). N=10/group. No difference was observed between gender or treatments.

3.2 Water intake and urine output measurements

Treated mice showed an increase in water consumption. As can be seen in Figure 3, after two weeks of treatment, the water intake was increased in both male and female groups and remained flat after 12 weeks of treatment. A similar response is shown for urine output in treated male mice, although there was no important difference in urine output in the female group throughout the treatment period. Interestingly, females presented a more reduced increase than males in both analysis and periods of treatment.

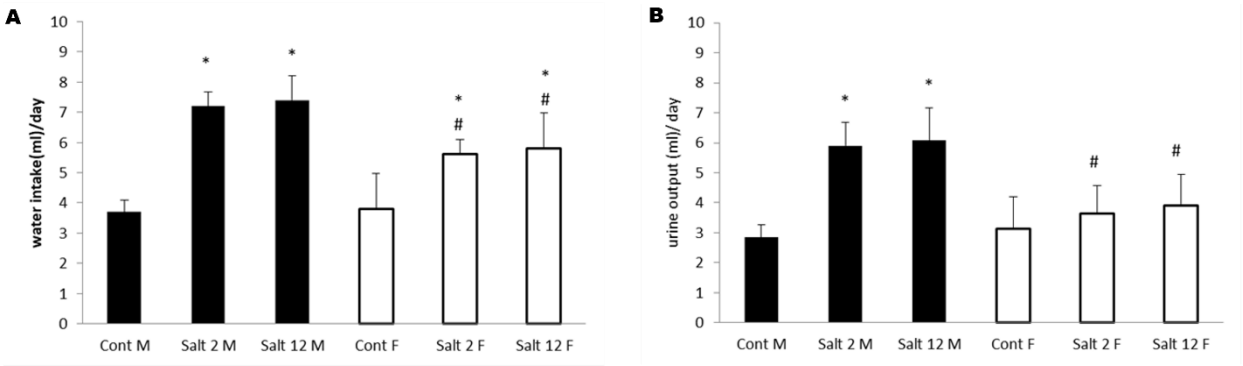

Figure 3 – Analysis for water intake and urine output in the groups treated with 1% NaCl. The chart shows the increase in water consumption (A) after two weeks in both male (M) and female (F), and the increase in urine output (B) in the male group. N=10/group; *p≤0.05 compared to control group; #p≤0.05 compared to respective treatment in male.

3.3 Elastic lamellae analysis

The morphometric evaluation in aorta shows that the treatment with 1% NaCl in the drinking water promoted changes in the vascular structure in both male and female groups. Analyzing the vascular wall in the aorta of males, we found an increase in the number of elastic lamellae after 12 weeks of treatment. However, the female group showed an early response to the treatment, which increased the number of lamellae after 2 weeks and almost doubled during the 12 weeks of treatment (Figure 4).

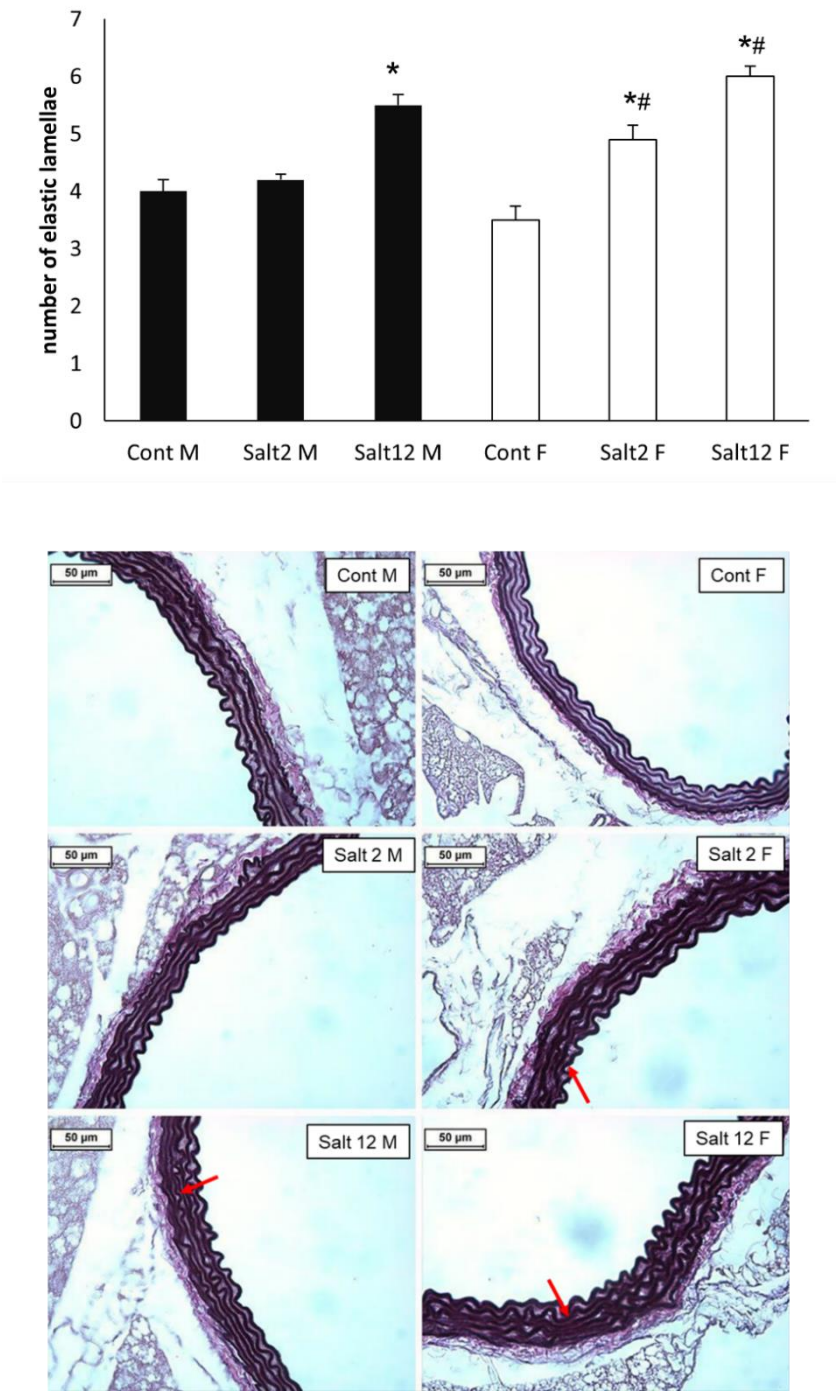

Figure 4 – Number of elastic lamellae in aorta, showing higher increase in the number of elastic lamellae (red arrows). Saline overload increased the number of elastic lamellae in male (M) after chronic treatment, while female (F) increased also after shorter time. N=10/group; * $p \leq 0.05$ compared to control group; # $p \leq 0.05$ compared to respective male group. Staining: Weigert van Gieson.

Differently from the aorta, cardiac arteries did not show any change in the number of elastic lamellae neither for the time of treatment nor gender. As cardiac arteries in mice are small arteries, all groups of males and females presented only the inner elastic laminae and no other elastic laminae. Despite that, the results are according to the expected ones since cardiac arteries are responsible for vascular resistance; therefore, it may respond by modifying the cell content of smooth muscle cells or the ECM production, especially collagen fibers.

3.4 Collagen fibers deposition

To verify if the treatment would be able to modify the vessel structure and, consequently, change the capacity of causing vascular resistance, we analyzed the deposition of perivascular collagen fibers in the aorta and cardiac arteries.

In the aorta, the content of collagen fibers increased gradually in males, increasing +30.2% after two weeks, and +36% after twelve weeks of saline overload. Likewise, females showed a similar response after twelve weeks of saline treatment (+54.5%), although this was more noticeable in males. Collagen content in females after chronic sodium intake was higher than in males (+51.4%), suggesting the propension to vascular stiffness (Figure 5).

On the other hand, analyzing collagen fibers deposition in cardiac arteries, we found differences regarding the treatment as well as gender. Males reduced their collagen fiber content 47.8% after 2 weeks of treatment, and even more after 12 weeks (-52.1%). Interestingly, females seemed to have a fluctuation in collagen fiber deposition, presenting a small increase after 2 weeks (+17.4%) and a small reduction (-11.7%) at the end of treatment; however, these changes were not significant (Figure 5).

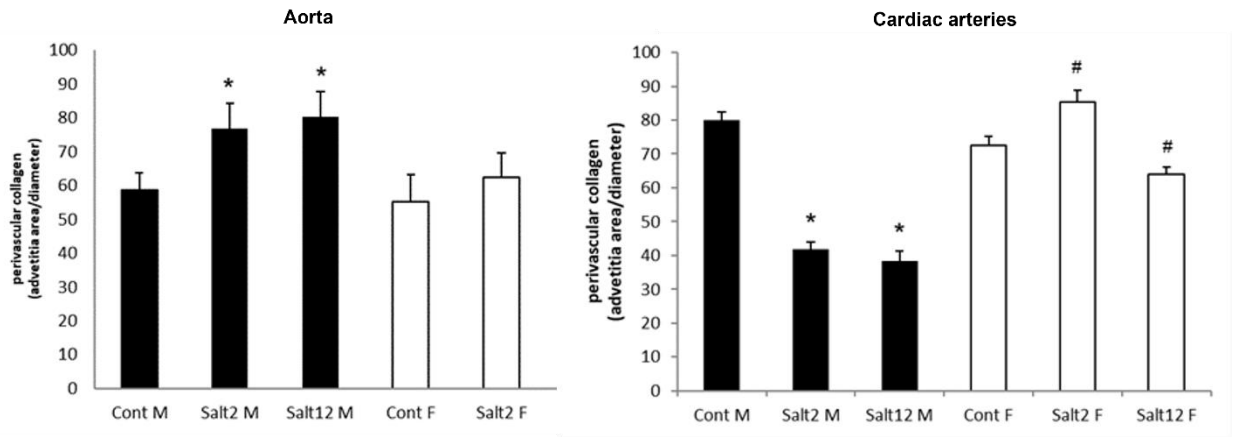

Figure 5 – Perivascular collagen deposition in the aorta and cardiac arteries. The aorta showed an increase in collagen deposition in both saline group males (M), while females (F) presented similar increase only in the chronic treatment. Cardiac arteries of male remodels, reducing perivascular collagens proportion; however, females did not reduce collagen deposition. N=10/group; * $p \leq 0.05$ compared to control group; # $p \leq 0.05$ compared to respective male group.

Collagen fibers deposition was also studied under polarized light since it is possible to identify collagen fibers by their refringence. As it can be seen in figure 6, the number of intermediary collagen fibers doubled in the aorta of the male group treated for 12 weeks. Likewise, there was also an increase in the deposition of mature (thick) collagen fibers (Figure 6). The female group showed a significant increase in the content of intermediary collagen fibers in the aorta when compared to the male group at the same time of the treatment, as well as in young (thin) and mature (thick) collagen fibers after 2 and 12 weeks of treatment, respectively, when compared to the control.

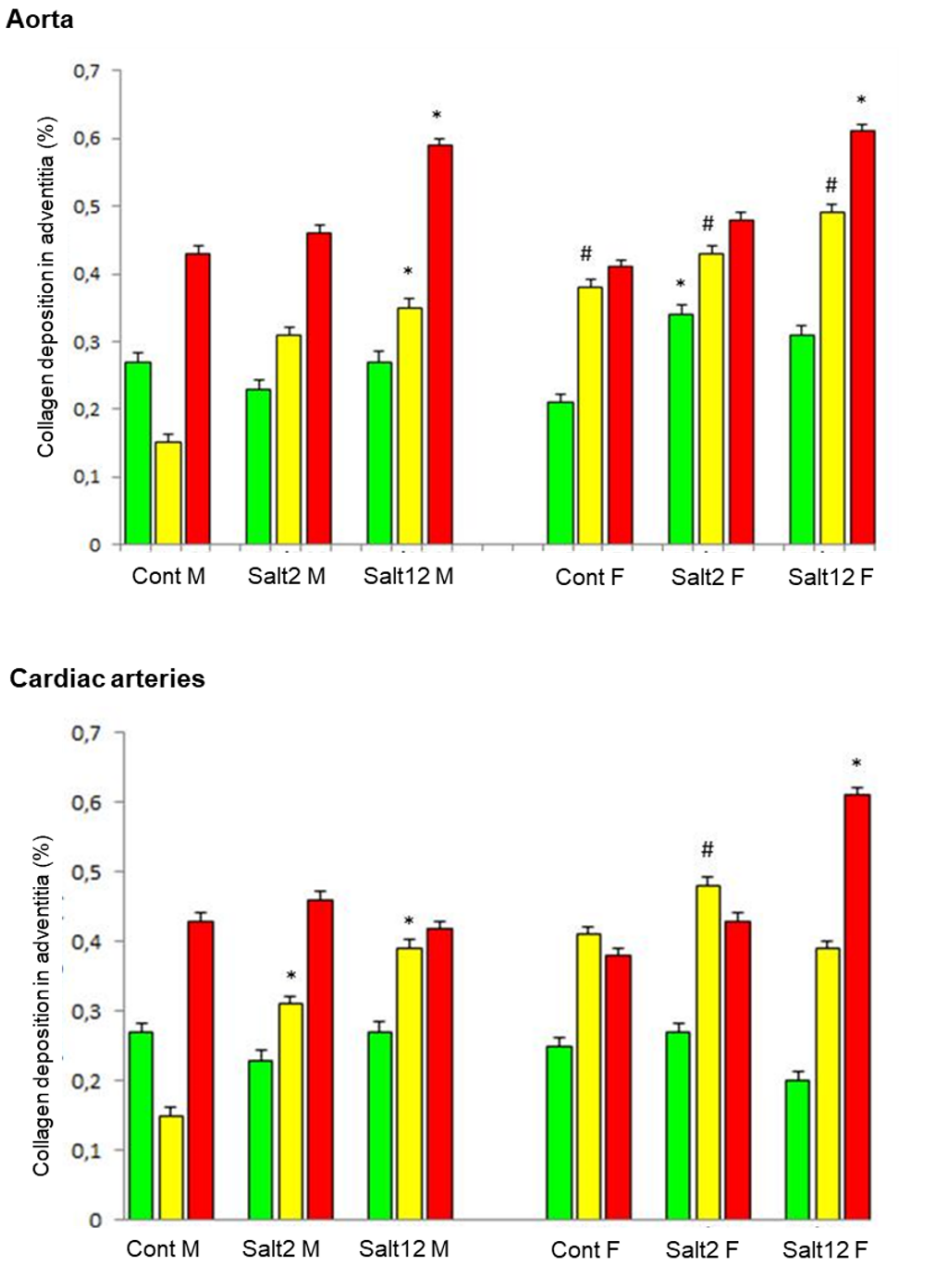

Figure 6 – Collagen deposition in the aorta and cardiac arteries showing the distribution of thin (green bar), intermediary (yellow bar) and thick (red bar) collagen fibers. In the aorta, males (M) increased intermediary and thick fibers after chronic saline treatment, while females (F) increased in both saline treatments. Cardiac arteries showed reduced response in males; however, females increased intermediary (after 2 weeks of saline – Salt2) and thick fibers after chronic saline overload (Salt12). N=10/group; *p≤0.05 compared to control group; #p≤0.05 compared respective male group.

The analysis under polarized light of cardiac arteries showed that, although the perivascular collagen fibers decreased in male, the intermediary collagen fibers (yellow bar) increased significantly with sodium intake during the period of treatment (Figures 6, 7 and 8). However, in females there was a greater increase in the mature (thick) collagen fibers when compared to their control, although a later response showed after 12 weeks of treatment. Otherwise, there was an early and important increase, after 2 weeks of treatment, in the deposition of intermediary collagen fibers when compared to males at the same time of treatment (Figures 6 and 9).

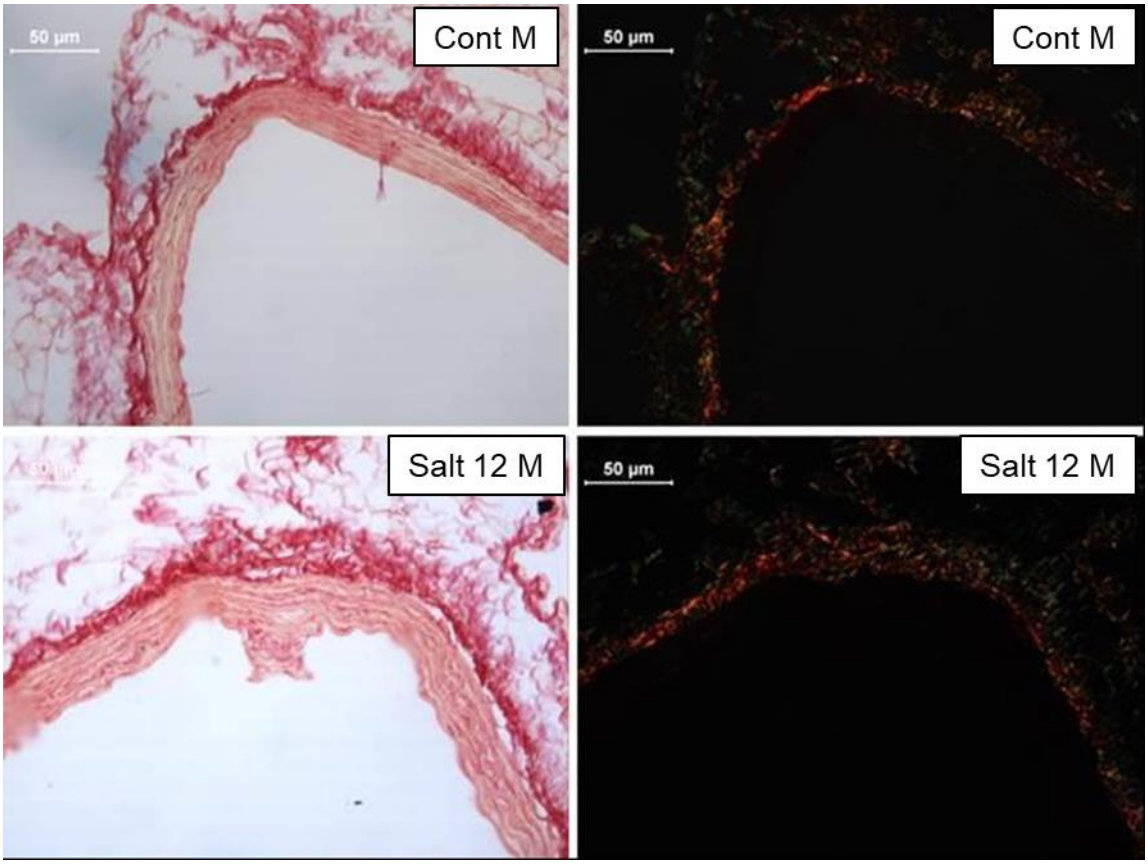

Figure 7 – Representative photomicrographs of collagen deposition in the aorta, comparing male (M) and female (F) mice after 12 weeks of high sodium intake. Picrosirius red-stained sections assessed by bright field (left column) and polarized light (right column) in the aorta.

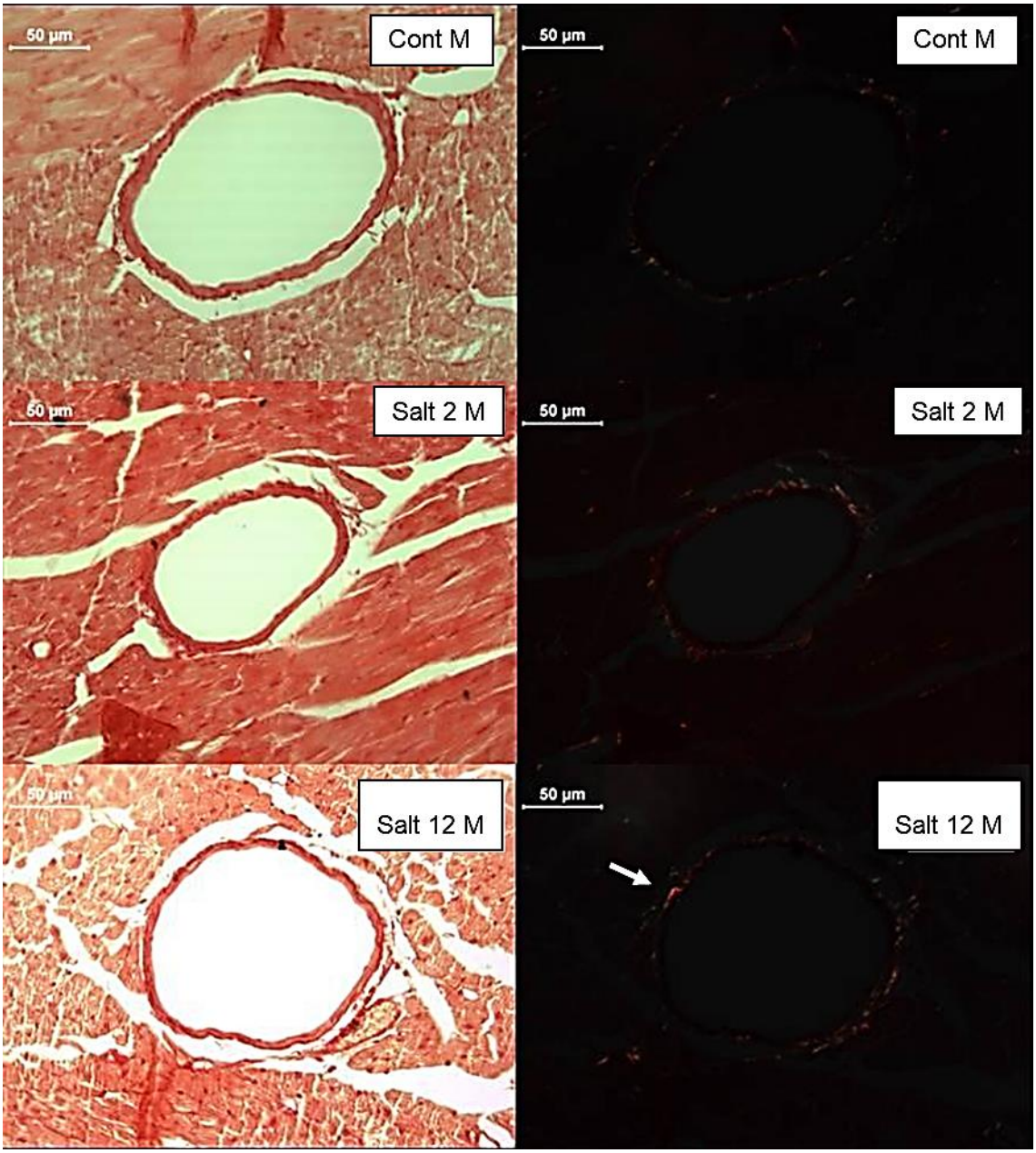

Figure 8 – Collagen deposition in cardiac arteries of male mice. Sirius red-stained sections assessed by bright field (left column) and polarized light (right column). Under polarized light, it was possible to observe an increase of thick collagen fibers (white arrow) after 12 weeks of saline treatment. Staining: Sirius red

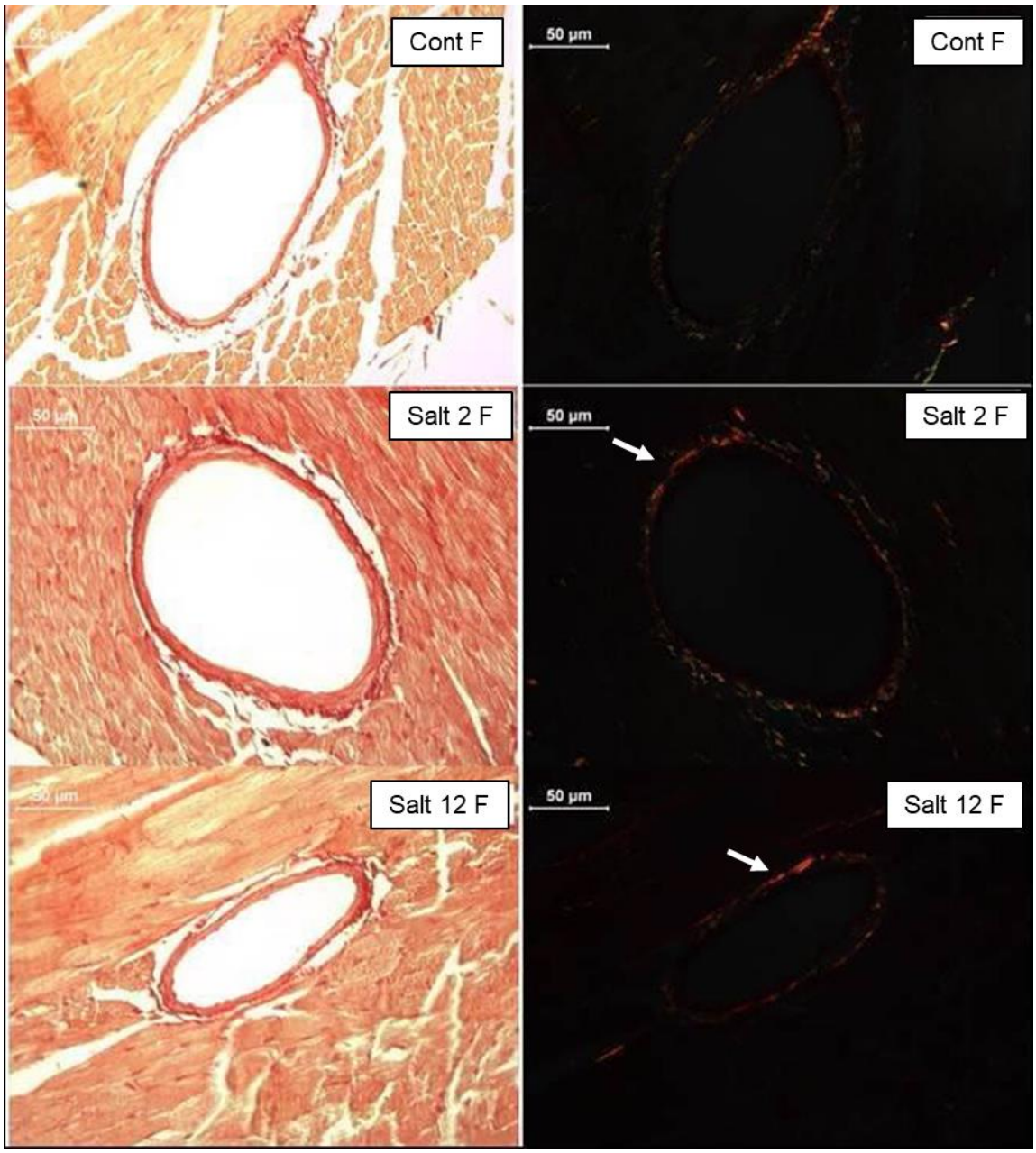

Figure 9 – Collagen deposition in cardiac arteries of female mice. Sirius red-stained sections assessed by bright field (left column) and polarized light (right column). Under polarized light, it was possible to observe an increase of thick collagen fibers in both saline groups. Staining: Sirius red

4. Discussion

During the last decades, several studies have related sodium intake with the onset of cardiovascular diseases. The higher the sodium amount in the diet is, the higher the probability to develop cardiovascular diseases is the opposite has also been confirmed [39–41]. Researches have

also found an increase in the mortality index in rats treated with high salt diet [42] and increased blood pressure and endothelial dysfunction [43, 44]. Paradoxically, the relationship between sodium intake and cardiovascular disease in some studies has not necessarily been associated with increased blood pressure [45, 46].

In this study, we aimed to verify whether the increase in sodium intake would induce morphological vascular changes related or not to variations of arterial pressure as well as the gender. The use of 1% NaCl added to the normal diet was chosen based on previous studies of our laboratory, which showed that a small increase in the salt intake was enough to stimulate the production of different vascular factors [17, 46, 47]. Moreover, higher levels of salt intake will promote gastrointestinal problems that influence the body adaptation, and also the expression of inflammatory markers [48–50]. In this pattern, the age of the animals was also chosen based on studies that showed that the age would influence salt resistance: the older the animal was, the higher the propensity to be salt-sensitive was [51, 52]. At the end of treatment, the animals reached 4 months of age; therefore, they were still considered young.

Hence, as described in previous studies, we did not observe any change in blood pressure [17]. However, fewer studies have evaluated the effect of sodium intake in mice, although it is well known that the intake of NaCl 1% in the drinking water did not promote changes in blood pressure of rats after 1 [46] or 6 weeks [47], as well as after 12 months of treatment [53].

The results obtained in the metabolic cage showed that the increase in sodium consumption promoted a significant increase (corresponding to 95% and 100%) in the water intake in males after 2 and 12 weeks, respectively. On the other hand, the females presented a smaller increase of 47% and 53% for the same period. These results are according to other studies that have shown an increase in water intake in animals treated with high salt diet for a short period and also chronically [54].

The animals also increased the urine output; however, only in the male group, an increase of 107% and 114% was observed after 2 and 12 weeks of treatment, respectively. On the other hand, females had a slight increase of 21% and 30% in the volume of urine for the same period. A smaller increase in water intake in females when compared to males may be related to metabolic differences promoted by sexual hormones [55–57]. Although both males and females had increased water

intake as well as urine volume excretion, males increased the volume retention by 51% and 53% after 2 and 12 weeks, respectively, while females had a greater increase in the volume retention of 145% and 138% after 2 and 12 weeks, respectively.

The treatment with 1% NaCl also promoted important vascular changes in both males and females, even though it depended on the time of exposure to the diet. These results are according to previous studies which have confirmed that the high salt diet can promote vascular and renal alterations in normotensive or hypertensive subjects [58, 59].

The observed changes occurred in elastic and resistance vessels, for example, the aorta, well known for being an elastic artery, increased its elasticity in males treated with 1% NaCl after 12 weeks. Nevertheless, in females, this increase is more precocious at just 2 weeks of treatment. Moreover, analyzing the perivascular collagen fibers in the aorta, we found that males tend to have an increase in these fibers, especially mature (thick) collagen fibers which may promote stiffness as shown in previous studies [45]. Thus, the aorta seems to increase its elasticity in order to counterbalance the higher stiffness. A similar response is shown in females that present not only a greater deposition of mature, but also intermediary collagen fibers suggesting even more stiffness in this vessel.

On the other hand, resistance vessels such as cardiac arteries have a different response pattern from the one shown in elastic vessels. We did not observe any change in the number of elastic laminae as expected since these are resistance vessels. Furthermore, there was a decrease in the deposition of collagen fibers in males while the proportion of intermediary collagen fibers increased which may represent a compensatory mechanism to keep the vascular wall stable. This pattern has been shown in previous studies that analyzed carotid arteries [60]. However, differently from males, females increased the deposition of mature (thick) and intermediary collagen fibers during the treatment, promoting more stiffness.

In fact, the literature describes sex differences in appetite for salt, where women exhibit a greater preference, especially during pregnancy [56, 57, 61, 62]. Additionally, evidence indicates that sex chromosomes and hormones influence blood pressure regulation, cardiovascular risk factors, and comorbidities differently in men and women [63, 64]. Our results suggested a heightened predisposition and poorer vascular response in women under stress factors, such as a high-salt diet.

5. CONCLUSION

The results of this study suggest that a 1% increase in NaCl in drinking water of mice did not significantly change blood pressure levels after 2 or 12 weeks of treatment. Therefore, the results did not indicate the hypertension onset. However, the diet may have induced different adaptive vascular responses in males (elastic arteries) and females (resistance arteries), leading to arterial stiffness even in normotensive animals.

Therefore, elucidating the mechanisms underlying these sexual differences in vascular response to salt intake may contribute to the development of gender-specific prevention and therapeutic strategies for cardiovascular diseases.

6. Acknowledgments

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