Introduction

There is evidence of the association of high sodium intake with the development of chronic non-communicable diseases (NCDs). High dietary sodium intake can adversely affect various organs and target tissues, such as the vasculature and heart, and some studies in rodents have demonstrated impaired endothelial function in chronic sodium intake, even without changes in blood pressure.

High serum sodium increases endothelial stiffness and impairs endothelium-dependent vasodilation, and small changes in serum sodium concentration can induce endothelial stiffness in a matter of minutes. Sodium reduces the size of endothelial cells and their surface area, volume, cytoskeleton, deformability and flexibility. In addition, sodium also reduces nitric oxide (NO) endothelial synthase (eNOS) and NO production and increases transforming growth factor β (TGF-β), thus increasing arterial stiffness.

Although sodium intake required to maintain homeostasis in adults is extremely low (<500 mg), the amount consumed by the world population is nearly 3,950 mg sodium / day. Reduction of salt intake has been identified as one of the most cost-effective interventions to reduce the load of NCDs, with the potential to save
into two groups (n=12 per group), following the order that the female rats gave birth: the first rat to give birth was allocated into one group, the second was allocated to the other, successively. A control group (CG) was fed a control diet and filtered water, and the flaxseed group (FG) received the diet added with 25% brown flaxseed flour, and filtered water. Diets were manufactured and stored as pellets at 4 °C in agreement with the American Institute of Nutrition (AIN-93G) recommendations for rodent diets. A concentration of 25% of flaxseed flour in the diet (25g/100g diet) would meet the recommended fiber intake. The addition of oil was not necessary, as the seed itself is a source of oil.

At weaning, two male offspring from each mother started to receive commercial rodent chow with a normal lipid and protein content (Nuvilab®, Nuvital Ltda, Paraná, Brazil). Along with proper eating habits, regular exercise training has been considered one of the main mechanisms to protect against the onset and progression of predisposing risk factors to cardiovascular diseases and other NCDs.

Studies have shown that diets containing food with functional properties, such as the flaxseed, during pregnancy and lactation can induce metabolic programming in the offspring due to its interference in the hormonal system and the ability of omega-3, present in this oleaginous, crossing the placenta and incorporating itself into the cellular membrane, altering its properties. Regarding vascular health, some studies using flaxseed during pregnancy, lactation and post-weaning, reported cardioprotective effect, showing decreased aortic thickness.

Flaxseed is the main plant source of n-3 fatty acid and its use has become popular due to its health benefits. It is also a source of soluble and insoluble fiber, lignan and protein, and contains 41% lipids, of which 50-55% are alpha-linolenic acid (n-3) and 15-18% linoleic acid (n-6). The study aimed to evaluate the effects of exercise training and chronic sodium intake on the aorta morphology of male offspring of rat dams fed a flaxseed flour diet during lactation.

### Material and Methods

#### Animals

The animal protocol was approved by the Animal Ethics Committee of the Fluminense Federal University (Protocol Number CEA 882/2016), and the procedures were in accordance with the guidelines for experimentation with animals (NIH Publication N°, 85-23, revised 1996). Female wistar rats (3 months old) from the Centre of Laboratory Animals of Fluminense Federal University were housed under a controlled temperature (21 ± 1°C), humidity (60 ± 10%) and 12 h light / dark cycle, with free access to water and food.

#### Experimental Design

Initially, 24 female rats were mated with 12 males (2:1 ratio) and fed a commercial rodent chow (Nuvilab®, Nuvital Ltda, Paraná, Brazil). During pregnancy, all rats were kept in individual cages. After the offspring was born, for the lactation period, the rat dams were divided into two groups (n=12 per group), following the order that the female rats gave birth: the first rat to give birth was allocated into one group, the second was allocated to the other, successively. A control group (CG) was fed a control diet and filtered water, and the flaxseed group (FG) received the diet added with 25% brown flaxseed flour, and filtered water. Diets were manufactured and stored as pellets at 4 °C in agreement with the American Institute of Nutrition (AIN-93G) recommendations for rodent diets. A concentration of 25% of flaxseed flour in the diet (25g/100g diet) would meet the recommended fiber intake. The addition of oil was not necessary, as the seed itself is a source of oil.

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Ltda, Paraná, Brazil), containing 22% protein (main protein sources are meat, fish, soy and amino acids), 66% carbohydrate and 11% lipid and allocated into one of the eight experimental groups with six animals each one (n=6) until they were 180 days old:

- Control group (CG), offspring of rat dams who received control diet during lactation;
- Exercise CG (ECG), offspring of rat dams who received control diet during lactation and underwent exercise training;
- Sodium chloride CG (NaClCG), offspring of rat dams who received control diet during lactation and were hydrated with 1% NaCl solution;
- Exercise sodium chloride CG (ENaClCG), offspring of rat dams who received control diet during lactation and were hydrated with 1% NaCl solution and underwent exercise training;
- Flaxseed group (FG), offspring of rat dams who received flaxseed flour diet during lactation;
- Exercise FG (EFG), offspring of rat dams who received flaxseed flour diet during lactation and underwent exercise training;
- Sodium chloride FG (NaClFG), offspring of rat dams who received flaxseed flour diet during lactation and were hydrated with 1% NaCl solution; and
- Exercise sodium chloride FG (ENaClFG), offspring of rat dams who received flaxseed flour diet during lactation and were hydrated with 1% NaCl solution and underwent exercise training.

The number of six animals per group followed the 3 Rs principle, which aims to reduce the number of animals used in the experiment. According to Damy et al., the reduced number of animals in biomedical research does not affect the detection of biological effects.

**Body Mass, Feed and Sodium Intake, Water and 1% NaCl Solution Intake**

For analysis of body mass gain throughout the study, body mass of the groups was measured once a week on a digital scale (precision 0.01g) (Filizola®). Food intake of the groups was measured three times a week. For feed intake calculation, individual intake was obtained by subtracting the remaining feed from the amount offered. Water and 1% NaCl solution intake were measured three times a week with the aid of a graduated test tube with 0.5mL precision. The amount supplied and the amount remained were quantified individually to determine water and NaCl intake. Sodium intake calculation was made adding the sodium intake from the 1% NaCl solution with the sodium contained in the commercial rat chow given to the animals (Nuvilab®, Nuvital Ltda, Paraná, Brazil) (2,700 mg Na / kg of rat chow).

**Exercise Training**

At 90 days of life, exercise training was performed on a motorized treadmill (AvsProjetos®, Brazil) for 12 weeks, 5 times a week for 60 min each session, in individual lanes. Exercise training was started at a speed of 0.3 km/h in the first session, increasing progressively to a final speed of 1.1 km / h, according to each animal’s performance. This exercise intensity corresponded to 70% to 80% of the maximum VO$_2$.³⁹

**Blood Pressure Measurement**

When the animals were three months of age, systolic blood pressure (SBP) was measured (in mmHg) once a month until six months of age, using the non-invasive method of tail-cuff plethysmography (tail plethysmograph V1.10-Insight, Brazil).

After preconditioning in a chamber, the animals were pre-heated in the chamber to 35±2°C for five minutes. The official SBP of each animal was calculated by averaging three consecutive stable measurements (with a difference of about one minute among them).

**Histomorphometric Analysis of the Aorta**

At the end of the experimental protocol (180 days), after a 6-hour fasting period, the animals were anesthetized with a solution containing 80mg of ketamine (10% ketamine hydrochloride, Syntec Tecnologia Farmacêutica Aplicada à Medicina Veterinária, Brazil) and 10mg of xylazine (2% xylazine hydrochloride, Syntec Tecnologia Farmacêutica Aplicada à Medicina Veterinária, Brazil); a thoracotomy was performed and the heart and the aorta were removed. The artery was dissected from the aortic arch, and the aorta was sectioned transversely with transverse cuts in the distal end of the sections. Later, the pieces of aorta were fixed in buffered formalin at 10% (Millonig formalin) for 24 hours, and processed with a standard technique of paraffin inclusion, as described by Pereira et al.³⁵ Subsequently, the paraffin blocks containing the pieces of aorta were cut using a CUT 4050 microtome (Microtec®), in sections of 5µm, stained with hematoxylin and eosin, and Weigert’s Resorcin-Fuchsin for later evaluation.
The measurements were made using the ImageJ® software, with digital images obtained by the cellSens program with an optronics digital video camera and BX51 Olympus microscope. The images were captured at 4x magnification for measurement of the area, at 40x for analysis of thickness, and at 20x for quantification (%) of elastic lamellae and elastic fiber. All images were digitalized in .tiff format. The aortic wall area was measured by the difference between the external and internal areas of the arterial wall. For intima-media thickness, four different regions of the same diametrically opposite cut were analyzed for better precision. The number of lamellae was quantified in four different regions of the same cut, and the aortic elastin content (%) was determined by analysis of Weigert’s Resorcin-Fuchsin slides using the ImageJ® software with a 20x magnification using the plugin Color Segmentation.

**Statistical Analysis**

Data were presented as mean ± standard deviation. Data were tested for normality and homogeneity of variances (Kolmogorov-Smirnov test) and the differences among groups were tested, when appropriated, with one-way analysis of variance (ANOVA), followed by a Holm-Sidak post hoc test or non-parametric Kruskal-Wallis test, followed by Dunn’s post hoc test. Variables that did not present normal distribution were presented as median and interquartile range (box plot graphs). Regarding body mass and blood pressure, the two-way ANOVA test was performed, followed by the Bonferroni post hoc test. P value < 0.05 was considered statistically significant (GraphPad Prism v. 6.01 for Windows, GraphPad Software, San Diego, CA, USA).

**Results**

**Body Mass, Food Intake, Water and 1% NaCl Solution Intake**

At weaning (21 days of age), no difference was found in body mass between the groups, however, there was a tendency of the offspring of the dams of the flaxseed group to be smaller than control offspring (-16.7%, p=0.0686). Regarding body mass gain, it was noted that the ENaClFG showed smaller body mass than the NaClCG between weeks 18 and 21 (-10.3%, p<0.05) (Figure 1). Daily feed intake was not different between the groups (p=0.1330) (Table 2). During the study period, the animals receiving 1% NaCl solution had a higher fluid intake than animals receiving water (p<0.0001) (Table 2). Regarding daily sodium intake, the animals of the groups NaClCG, ENaClCG, NaClFG and ENaClFG consumed a higher amount of sodium than their respective groups that consumed water (+935.5%, +845.3%, +833.4%, +795.2%, respectively, p<0.0001) (Table 2).

**Systolic Blood Pressure**

Chronic consumption of 1% NaCl solution did not increase blood pressure in the study groups, and neither exercise training nor the flaxseed diet affected blood pressure during the lactation period (p=0.5016) (Figure 2).

**Histomorphometric Analysis of the Aorta**

At the end of the study, no difference was found in aortic wall area (p=0.9364) or the aortic lumen area between the groups (p=0.8817) (Figures 3 and 4). However, the chronic use of 1% NaCl solution and exercise training increased the aortic intima-media thickness in the CG (10.4% and 13.3%, respectively, p<0.0001) either alone or combined (+17.7%, p<0.0001). In male offspring of female rats who consumed flaxseed during lactation, the chronic use of 1% NaCl alone did not increase the aortic intima-media thickness (which was not different from the CG and the FG). Nevertheless, exercise training increased the aortic intima-media thickness in the EFG compared to the CG and FG (12.8% and 9.7%, respectively, p<0.0001), and the use of the 1% NaCl solution combined with exercise training also increased aortic intima-media thickness when compared to CG and FG (+17.8% and 14.5%, respectively, p<0.0001) (Figures: 3 and 4). Regarding the elastic component of the arterial wall, the chronic use of 1% NaCl solution by the CG caused a reduction in the number of elastic lamellae (-8.1%, p<0.0001), the FG showed a lower number of elastic lamellae than all CGs (p<0.0001), despite that, no difference amongst the groups were observed regarding the amount of elastin in the aortic intima-media (p=0.1629, Figures 3 and 4).

**Discussion**

The present study showed that maternal intake of a flaxseed diet during lactation associated with exercise
Table 2 - Consumption of feed, water, 1% sodium chloride (NaCl) solution and sodium (Na)

| Parameters / Groups | CG                  | NaClCG              | ECG                  | ENaClCG              | FG                  | NaClFG              | EFG                  | ENaClFG              | p-value   |
|---------------------|---------------------|---------------------|----------------------|----------------------|---------------------|---------------------|----------------------|----------------------|-----------|
| Diet (g/day/animal) | 21.5±3.4            | 22.6±1.4            | 23.7±0.9             | 23.2±1.6             | 22.7±1.6           | 22.3±1.6           | 22.5±1.5             | 20.5±1.5             | 0.1330    |
| Water or 1% NaCl solution (mL/day/animal) | 31.7±5.8            | 54.0±2.6*           | 35.3±2.0             | 54.3±6.4*†           | 33.5±4.2*†         | 51.2±6.9*†          | 33.2±2.9*†          | 48.9±3.9*†          | <0.0001  |
| Sodium (Na)(mg/day/animal) | 58.0±9.3            | 60.1±30.0*          | 64.1±2.5†            | 60.6±69.0*†          | 61.4±5.4*†         | 573.1±72.7*         | 60.8±4.3*†          | 545.1±40.7*†         | <0.0001  |

Data were presented as mean ± SD, with significance level of p<0.05 (one-way ANOVA, Holm-Sidak post-hoc test). * ≠ CG; † ≠ NaClCG; ‡ ≠ ECG; § ≠ ENaClCG; †† ≠ FG; †‡ ≠ NaClFG; †§ ≠ EFG; CG: control group; NaClCG: Sodium Chloride control group; ECG: Exercise training control group; ENaClCG: Sodium Chloride exercise training control group; FG: Flaxseed group; NaClFG: Sodium Chloride flaxseed group; EFG: Exercise training flaxseed group; ENaClFG: Sodium Chloride exercise training flaxseed group.

Figure 1 – Body mass evolution throughout the study. The data were presented as mean ± SD, with significance level of p≤0.05 (two-way ANOVA, Bonferroni post-hoc test). CG: control group; NaClCG: Sodium Chloride control group; ECG: Exercise training control group; ENaClCG: Sodium Chloride exercise training control group; FG: Flaxseed group; NaClFG: Sodium Chloride flaxseed group; EFG: Exercise training flaxseed group; ENaClFG: Sodium Chloride exercise training flaxseed group.
Figure 2 – Monthly systolic blood pressure of study groups. The data were presented as mean ± SD, with significance level of p≤0.05 (two-way ANOVA, Bonferroni post-hoc test). CG: control group; NaClCG: Sodium Chloride control group; ECG: Exercise training control group; ENaClCG: Sodium Chloride exercise training control group; FG: Flaxseed group; NaClFG: Sodium Chloride flaxseed group; EFG: Exercise training flaxseed group; ENaClFG: Sodium Chloride exercise training flaxseed group.

Figure 3 – Aorta histomorphometry of the groups studied at 180 days of age. The data were presented as median and interquartile (Boxplot), with significance level of p<0.05 (one-way ANOVA, Holm-Sidak post-hoc test and Kruskal-Wallis test, Dunn’s post-hoc test). A) Aorta wall area, B) Aorta lumen area, C) Aorta intima-media layer thickness, D) Elastic lamellae and E) Elastin. a ≠ CG; b ≠ NaClCG; c ≠ ECG; d ≠ ENaClCG; e ≠ FG; f ≠ NaClFG. CG: Control group; NaClCG: Sodium Chloride control group; ECG: Exercise training control group; ENaClCG: Sodium Chloride exercise training control group; FG: Flaxseed group; NaClFG: Sodium Chloride flaxseed group; EFG: Exercise training flaxseed group; ENaClFG: Sodium Chloride exercise training flaxseed group.
training of the offspring resulted in structural changes of the aorta of the offspring in adulthood, regardless of sodium intake.

In a review study on the effects of flaxseed flour intake during pregnancy and lactation on the offspring’s body mass at 21 days, it was observed that the consumption of flaxseed flour in a concentration of 25% (25g flour/100g of feed) in the diet did not lead to changes in the animals’ body mass at weaning, showing the same body mass as the animals of control rat dams. In our study, although no statistically difference was found, a trend to lower body mass in animals of rat dams that consumed flaxseed diet during lactation was found. Other studies reported lighter body mass of male offspring of rat dams who consumed flaxseed diet during lactation was found. The lower fat mass of these animals is probably the responsible component for the lower body mass at weaning, since animals did not show changes in other compartments of the body. The flaxseed phytoestrogen, secoisolariciresinol diglucoside (SDG), can be responsible for this effect. The SDG antiobesity effect may be due to the suppression of genes involved in the synthesis of fatty acids and triglycerides, through the sterol regulatory element binding protein-1c (SREBP-1c) activity. It is well known that estrogen directly inhibits fat deposition by reducing lipoprotein lipase (LPL) activity, an enzyme that regulates adipocyte lipid reabsorption; thus, the lower body mass in the flaxseed group can also be explained, somewhat, by the estrogenic action of the SDG in adipocytes.

Flaxseed flour intake by mother during lactation, the practice of regular exercise training after three months of age and consumption of 1% NaCl solution did not lead to changes in feed intake during study time and, hence, the offspring’s body mass was similar in most groups throughout the study. Only from the 12th week, the ENaClFG had lower body mass compared to some of the groups. Despite not significantly different, feed intake in this group decreased by 9.4% compared to the other groups, which, in addition to the combination – maternal flaxseed intake during lactation, chronic 1% NaCl solution intake and exercise training – may have contributed to the lower body mass.

Concerning water and 1% NaCl solution intake, the animals given only saline solution drank more fluid than those given only water, resulting in a higher sodium intake in the former groups. Small increases of 1-2% in the effective osmotic pressure of plasma result in stimulation of thirst in mammals. It has been shown in both human subjects and other mammals, that plasma osmolality is increased experimentally in response to increased concentrations of solutes, such as NaCl, which does not readily pass across cell membranes. Consequently, thirst is stimulated to avoid possible dehydration, which may have occurred in our animals.

Although salt intake in the diet is recognized as a contributing factor to the pathogenesis of hypertension, recent evidence suggests that salt intake in the diet may increase the risk of adverse cardiovascular events regardless of blood pressure. This is in accordance with our results, showing that the offspring that received...
1% NaCl solution, both of control rat dams and mothers who received the flaxseed diet, showed no increase in blood pressure throughout the study. Similar findings were obtained by offering oral NaCl at 1% or a diet with 1.3% NaCl, which did not result in an increase in systolic blood pressure in normotensive rats. Few studies have investigated the effects of maternal intake of flaxseed flour during lactation on blood pressure in adult offspring. The intake of a high-fat diet added with flaxseed flour during pregnancy and lactation did not lead to changes in systolic blood pressure in male offspring of diabetic rats at 100 days of life, corroborating our results; however, another study reported that the intake of flaxseed flour during pregnancy and lactation led to a blood pressure decrease in the offspring of healthy rat dams. In addition, our study showed that exercise training did not lead to differences in blood pressure between sedentary and trained animals. Studies with sedentary animals and trained animals also presented similar blood pressure levels between groups, yet another study showed that chronic exercise training (treadmill) led to a decrease in blood pressure in normotensive rats.

Several lines of evidence suggest that excessive dietary salt intake (NaCl) negatively affects cardiovascular function, regardless of changes in blood pressure. First, population studies have indicated that normotensive humans with a higher level of salt intake are at increased risk for an adverse cardiovascular event. Second, evidence in humans and animal models indicates that excessive dietary salt intake promotes endothelial and microvascular dysfunction. Endothelium has multiple and important roles in physiological and pathophysiological events, and changes in the structure of large arteries, including abnormalities in endothelial function, arterial elasticity, structure and arterial wall thickness, can trigger the onset of cardiovascular disease. Thereby, assessment of large arteries, such as aorta, of lumen area, elasticity changes and intima-media thickness have become important in the investigation of atherosclerosis, since they are indicators of endothelial damage.

In this study, the aorta wall area and the aortic lumen area were similar between groups, showing that the interventions did not affect these results. In relation to flaxseed, a study using a diet with 25% of flaxseed flour during pregnancy and lactation of diabetic rats showed no difference in the aorta lumen area of male offspring at 100 days of life. In our study, chronic administration of 1% NaCl solution to offspring of mothers who received control diet during lactation led to an increase in aortic intima-media thickness, and to a smaller number of elastic lamellae and, although not significant, the aorta elastin amount was also lower than the control group (-17.2%). These results were not found in the offspring of mothers who consumed flaxseed during lactation, once the aortic intima-media thickness of the FG was similar to CG; also, chronic salt overload did not increase aortic intima-media thickness, suggesting a protective effect of flaxseed. Sodium’s deleterious effects on endothelial function of offspring of the CG may be associated with the action of reactive oxygen species, such as superoxide, resulting in reduced nitric oxide bioavailability. Cell culture studies support that high sodium exposure stiffens endothelial cells and damages the glyocalyx. Besides that, changes in elastin deposition in the arteries lead to elasticity loss and consequent increased stiffness, promoting aortic remodeling by thickening of the intima-media layer. Concerning the use of flaxseed during lactation on aortic morphology, different from our results, in a study where flaxseed was given to healthy rats from lactation to adult life, intima-media thickness was smaller than the group that received the control diet. Also, studies in which flaxseed diet was given to diabetic mothers during pregnancy and lactation reported that male and female offspring as adults showed smaller aortic intima-media thickness than the offspring of healthy mothers.

Flaxseed is a rich vegetable source of n-3 fatty acid, which is known for its ability of preventing aortic remodeling due to its capability to be transferred by the placental route and incorporated into the cell membrane modifying its properties. Omega-3 fatty acids are also important anti-inflammatory agents able to decrease endothelial activation and generation of inflammatory cytokines, preventing cardiovascular diseases.

It was observed that exercise training (treadmill), as observed with the chronic use of NaCl, increased aortic intima-media thickness, both in the offspring of control group and in the flaxseed group when compared to their respective sedentary groups. Coura et al., using swimming as exercise training (5 times/week, 30 minutes/day, for eight weeks), also showed that training increased aortic intima-media thickness of elderly Wistar rats. Despite the increase, Mulvany et al. reported that resistance arteries suffer an internal eutrophic remodeling, with no changes in the tissue constituent materials, which is noticed in our results related to the number of elastic lamellae and elastin amount that were similar or greater than the respective sedentary group.
Conclusion

In summary, despite unaltered blood pressure, chronic salt overload caused adverse effects on the aorta of rats, with decreased aortic elasticity. This was prevented by the consumption of a flaxseed diet during lactation, suggesting a protective effect of flaxseed. Exercise training alone increased aortic intima-media thickness but did not affect its elastic components.

Author Contributions

Conception and design of the research: Vicente GC, Correia-Santos AM and Boaventura GT. Acquisition of data: Silva-Couto S, Castro CLC, Barreto VLM, Martins JEC and Lenzi Q. Analysis and interpretation of the data: Correia-Santos AM, Vicente GC, Chagas MA and Boaventura GT. Writing of the manuscript: Silva-Couto S, Correia-Santos AM, Vicente GC and Castro CLC. Critical revision of the manuscript for intellectual content: Silva-Couto S, Correia-Santos AM, Chagas MA and Boaventura GT. Writing of the manuscript: Silva-Couto S, Castro CLC, Barreto VLM, Martins JEC and Lenzi Q. Analysis and interpretation of the data: Correia-Santos AM, Vicente GC, Chagas MA and Boaventura GT. Critical revision of the manuscript for intellectual content: Silva-Couto S, Correia-Santos AM, Chagas MA and Boaventura GT.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

This article is part of the thesis of master submitted by Simoni Silva-Couto, from Universidade Federal Fluminense.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee on Animal Experiments of the Universidade Federal Fluminense under the protocol number 882/2016.

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