Cardiac Hypertrophy and Brain Natriuretic Peptide Levels in an Ovariectomized Rat Model Fed a High-Fat Diet

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Heart failure in women increases around the time of menopause when high-fat diets may result in obesity. The heart produces brain natriuretic peptide (*BNP*), also known as B-type natriuretic peptide. This aims of this study were to assess cardiac hypertrophy and *BNP* levels in ovariectomized rats fed a high-fat diet.

Material/Methods: Forty-eight female Wistar rats were divided into four groups: sham-operated rats fed a control diet (SC) (n=12); ovariectomized rats fed a control diet (OC) (n=12); sham-operated rats fed a high-fat diet (SF) (n=12); and ovariectomized rats fed a high-fat diet (OF) (n=12). Body weight and blood pressure were measured weekly for 24 weeks. Rats were then euthanized, and plasma samples and heart tissue were studied for gene expression, hydroxyproline levels, and histological examination.

Results: A high-fat diet and ovariectomy (group OF) increased the weight body and the systolic blood pressure after three months and five months, respectively. Cardiomyocyte hypertrophy was associated with increased expression of ventricular *BNP*, decreased natriuretic peptide receptor (*NPR)-A* and increased levels of hydroxyproline and transforming growth factor (*TGF*)-β. The plasma levels of *BNP* and estradiol were inversely correlated; expression of estrogen receptor (*ER*)β and *ER*α were reduced.

Conclusions: The findings of this study showed that, in the ovariectomized rats fed a high-fat diet, the *BNP-NPR-A* receptor complex was involved in cardiac remodeling. *BNP* may be a marker of cardiac hypertrophy in this animal model.

MeSH Keywords: Estradiol • Hypertension • Natriuretic Peptide, Brain • Obesity, Abdominal

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Background

Obesity is clinically associated with the development of cardiovascular disease, but the mechanisms involved remain poorly understood. Obesity is a risk factor for the development of hypertension, associated with endothelial dysfunction [1–3]. High blood pressure, associated with expression of inflammatory cytokines and fibrinolytic factors, are associated with obesity [4–6]. High blood pressure and cardiac hypertrophy can be associated with atherogenesis and oxidative stress, which contributes to heart failure [7].

Brain natriuretic peptide (*BNP*), which is also known as B-type natriuretic peptide, has been reported to be associated with cardiac overload and shows antiproliferative and antifibrinolytic activity [8]. Also, *BNP* modulates the expression of the transforming growth factor (*TGF*)-β gene that mainly promotes fibrosis and cardiac hypertrophy [9]. Therefore, *BNP* has been proposed as a potential marker for heart disease and to evaluate the progression of cardiovascular disease, including cardiac hypertrophy [10,11]. The effects of *BNP* are by binding to the A-receptor natriuretic peptide (*NPR-A*), leading to increased synthesis of cyclic guanosine monophosphate (cGMP) [12]. *BNP* as a mediator of cardiac disease may participate in the regulation of several hypertrophic pathways, including via cytokines and growth factors that can stimulate the production of cardiac natriuretic hormones, and some authors have proposed a link between endocrine function and remodeling in myocardial and smooth muscle cells [13–15].

Ovarian hormones have an important role in the cardiovascular system as premenopausal women have a lower risk of developing cardiovascular disease when compared with men at the same age; this gender difference disappears after menopause [16–19]. Also, the contribution of ovarian hormones on the pattern of cardiac hypertrophy has been demonstrated by studies showing that the physiological replacement with 17β-estradiol (E2) in female mice limits left ventricular hypertrophy induced by pressure overload [20]. A previous study in our laboratory, using a rat model of obesity, has shown that a high-fat diet and ovariectomy increased blood pressure as well as inducing the development of cardiac hypertrophy [21]. This established animal model was used in the present study.

This aims of this study were to assess cardiac hypertrophy, and *BNP* levels in ovariectomized rats fed a high-fat diet. Estradiol levels, and gene expression for estrogen receptor alpha (*ERα*) and beta (*ERβ*), measurement blood pressure, cardiac hypertrophy, and fibrosis were evaluated.

Material and Methods

Animals studied

Forty-eight female Wistar rats were maintained in an environment with controlled light (12 hours) and dark (12 hours), and controlled temperature (23±1ºC), in the Institute of Multidisciplinary Health, University Federal of Bahia, BA, Brazil. The animals received food and water *ad libitum*. The study was conducted for 24 weeks.

The study was approved by the Ethics Committee in Animal Experimentation of the State University of Feira de Santana and carried out according to the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

To simulate the post-menopausal state, female rats were ovariectomized at ten weeks of age. The animals were ovariectomized or underwent sham operation using general anesthesia with a tribromoethanol solution consisting of 2.5% (0.025 g/ml) tribromoethanol diluted in 0.9% normal saline, administered at a dose of 1 ml of solution per 100 gm of animal body weight.

Animal diets

Animals were fed a standardized laboratory rat diet from the American Institute of Nutrition 93 (AIN 93) (Pragsoluções Biosciences, São Paulo, SP, Brazil) for a week of acclimatization. After the procedure of ovariectomy, the rats received the following diets: a diet containing 54.4% of total calories from fat (Pragsoluções Biosciences, São Paulo, SP, Brazil) or a control diet or standard diet AIN 93 (Pragsoluções Biosciences, São Paulo, SP, Brazil). The specific compositions of the diets have been reported in a previous study [21].

The 48 female Wistar rats were divided into four groups: sham-operated rats fed a control diet (SC) (n=12); ovariectomized rats fed a control diet (OC) (n=12); sham-operated rats fed a high-fat diet (SF) (n=12); and ovariectomized rats fed a high-fat diet (OF) (n=12).

Rat cardiac and plasma samples

Measurements of systolic blood pressure were made by the tail plethysmography method using an electronic sphygmomanometer LE5001 (Panlab, Barcelona, Spain). After five minutes of pre-heating, the animal tail was placed in contact with a cuff, and a pulse transducer was used to measure the heart rate, and the mean systolic and diastolic blood pressures, calculated from five consecutive data readings each day.

Body weight and blood pressure were measured weekly for 24 weeks. The rats were then euthanized by decapitation, and the heart was removed, weighed, and used for further analysis of gene expression, histology, immunohistochemistry, and determination of hydroxyproline levels (a total of four hearts per group for each of three techniques).

Blood was collected in heparinized tubes containing protease inhibitors. After centrifugation (10,000×G) for 15 minutes, plasma was separated for measurement of estradiol, transforming growth factor (*TGF*)-β and brain natriuretic peptide (*BNP*), which is also known as B-type natriuretic peptide. RNA extraction and polymerase chain reaction (PCR) were performed to determine the gene expression of *BNP* and the natriuretic peptide receptor (*NPR)-A* gene, *NPR-A,* and levels of estrogen receptor (*ER*) genes in the ventricles.

Plasma measurements using ELISA

The plasma measurement of *BNP* levels was made by an enzyme-linked immunosorbent assay (ELISA) (RayBio® *BNP* EIA Kit) (Cat. No. EIA-*BNP*-1) (RayBiotech, Inc. Norcross, GA, USA).

The plasma levels of estradiol were determined using an ELISA kit (Cat. No. KAQ0621) (Biosource, CA, USA). The determination of plasma levels of transforming growth factor (*TGF*)-β was performed using an ELISA kit for rat *TGF*-β (Cat. No. E0131Ra) (Uscn Wuhan Life Science Inc.).

Gene expression analysis

Rat cardiac RNA was extracted using Brazol® reagent, 1 mL/100 mg, following the manufacturer's protocol. Moloney murine leukemia virus reverse transcriptase (M-MLV RT) was used under standard conditions in a 20 µl reaction, in which 1 µg of RNA was reverse transcribed.

The following oligonucleotides were used for amplification: *BNP*, 258 bp, 5'-TCTGCTCCTGCTTTTCCTTA and GAACTATGTGCCATCTTGGA-3' as forward and reverse primers, respectively; *NPR-A*, 454 bp, 5'-CTCAACATCACAGTAAATCACC and CCTGAAGGCACCTGTCTCG-3' as forward and reverse primers, respectively; *ER*α, 623 bp, 5'-TAAGAACCGGAGGAAGAGTTG and TCATGCGGAATCGACTTG-3' as forward and reverse primers, respectively and
*ER*β, 159 bp, 5'-GCTCCTCTATGCAGAACCTCAAA and CAGAAGTGAGCATCCCTCTTTG-3' as forward and reverse primers, respectively.

The conditions of the polymerase chain reaction (PCR) were standardized and defined after testing [22]. Primers were manufactured by Prodimol Biotechnology (Belo Horizonte, Brazil). The PCR amplifications for *BNP* were performed at 95°C for 5 min,

followed by 35 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s. The PCR amplifications for *NPR-A* were performed at 95ºC for 5 min followed by 40 cycles at 94ºC for 15 s, 58ºC for 30 s, and 72ºC for 30 s. The PCR amplifications for *ER*α were performed at 95 ºC for 5 min followed by 25 cycles at 94ºC for 30 s, 45ºC for 30 s, and at 72ºC for 1 min. The PCR amplifications for *ER*β were performed at 95 ºC for 5 min followed by 30 cycles at 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 40 s. The final extension was for 10 min. All reaction products were separated on a 1.0–1.5% agarose gel and stained with ethidium bromide. β-Actin was amplified as an internal control in each PCR test using specific primers to provide a semi-quantitative assessment. All PCR reactions were performed in triplicate.

Rat cardiac morphology and histology

The rat hearts were removed and fixed in 4% formaldehyde. Transmural slices were taken from the middle region of the ventricular myocardium, and longitudinal and transverse sections were taken. The rat heart tissues were embedded in paraffin wax and processed using conventional histological techniques. Tissue sections of 5 mm thickness were cut onto glass slides, and the sections were stained with histochemically with hematoxylin and eosin (H&E). Morphometric measurements were performed using computerized interactive image analysis with Image Pro Plus 4.1 imaging software (Media Cybernetics, Silver Springs, MD, USA), a method previously shown to reduce interobserver variability. For the linear measurements (diameters) of cardiomyocytes, an imaging magnification of ×400 objective was used. Sixty cells were measured by region, in at least ten different fields, with the smallest cardiomyocyte diameter measured as the line a line that intersected the cell nucleus. Photomicrographs were taken of the rat cardiac histological fields with a magnification of ×200.

Immunohistochemistry for rat cardiac α-smooth muscle actin (*ASMA*)

The unstained 5 mm rat cardiac tissue sections were deparaffinized prior to immunohistochemistry. The tissue sections were incubated with primary monoclonal anti-mouse α-smooth muscle actin (*ASMA*) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1: 500 in PBS solution and 1% bovine serum albumin (BSA) at 8ºC overnight in a humid chamber. The reaction product was detected with an avidin-biotin-peroxidase complex (Leica Biosystems, Newcastle, United Kingdom). Antigen-antibody binding was detected using the chromogen, diaminobenzidine (DAB) (Cat. No. K3468) (DakoCytomation, CA, USA), one drop diluted in 2 ml of buffer and the tissue sections were incubated for 1 minute, then washed in distilled water to remove any remaining DAB. Counterstaining was carried out in Harris hematoxylin for 5 minutes. A negative control was included that omitted the use of the primary antibody.

Positive immunohistochemical staining (brown) was localized by the primary antibody used and was quantified by the histological evaluation of 30 fields per slide with positive immunostaining attributed to the marked ventricular area by assigning a score of 0–4. A score of 0 was equivalent to 0–5% of the set; a score of 1 was equivalent to 5–25%; a score of 2 was equivalent to 25–50%; a score 3 was equivalent to 50–75%; and a score of 4 was equivalent to 75–100% [26]. Immunohistochemical staining scoring analysis was performed with a ×400 objective, and photomicrographs were taken with an objective magnification of ×200.

Rat cardiac hydroxyproline levels as an indirect measure of collagen content

The measurement of rat cardiac hydroxyproline, as an indirect measure of collagen content, was performed using a modified version of the colorimetric assay, as described previously [24]. Briefly, whole hearts from four animals per group were selected at random, and the ventricles were separated from the atria. The samples of rat cardiac ventricles were then lyophilized overnight using the Labconco 3 freeze dryer (Labconco, Kansas City, MI, USA). The samples were weighed and hydrolyzed with HCl solution overnight at 120°C. For the generation of the standard curve, 20–70 mg of hydroxyproline were added to individual wells in a 96-well plate. The hydrolyzed samples were then separately added to the wells and incubated with Ehrlich's reagent (p-dimethylamino benzaldehyde) for 30 minutes at 80°C. Colorimetric results were read using a Bio-Rad Benchmark Microplate Reader at a wavelength of 557 nm. The calculation of the levels of hydroxyproline was made by linear regression analysis and compared with known standards. The total hydroxyproline levels were calculated in milligram per gram of rat cardiac tissue.

Statistical analysis

The values of two variables, diet and ovariectomy, and the means between groups were determined from the individual variation or variation of error (s^2), using two-way analysis of variance (ANOVA) followed by the Bonferroni method of analysis. Correlations were calculated using the two-tailed Pearson's correlation coefficient (r) (r=0.10–0.30, a weak correlation; r=0.40–0.60, a moderate correlation; r=0.70–1.0, a strong correlation). The results were expressed as mean ± the standard deviation (SD) using the GraphPad Software®. A p-value ≤0.05 was considered to be statistically significant.

Results

Ovariectomized rats fed a high-fat diet and hypertension associated with cardiac hypertrophy

Forty-eight female Wistar rats were divided into four groups: sham-operated rats fed a control diet (SC) (n=12); ovariectomized rats fed a control diet (OC) (n=12); sham-operated rats fed a high-fat diet (SF) (n=12); and ovariectomized rats fed a high-fat diet (OF) (n=12).

In this study, ovariectomy associated with a high-fat diet (OF group) induced a significant increase ($p<0.05$) in body weight (Figure 1A). The high-fat diet (SF group) or ovariectomy (OC group) increased the body weight of animals when compared with the control group (SC group). This difference was noted from the third month of the study and remained until the end of the sixth month. Also, the OF group showed an increase in weight gain that started at the third month, with a greater weight gain when compared with all other groups by the fifth and sixth month of the study (Figure 1A).

The heart weight was measured after the sixth month of a high-fat diet and ovariectomy. Ovariectomy or isolated fat diet alone did not alter the heart weight. However, the association of these variables in the ovariectomized rats fed a high-fat diet (OF group) resulted in a significant increase ($p<0.05$) in heart weight (Figure 1B). The same result was observed regarding the diameter of cardiomyocytes. Ovariectomized rats fed a high-fat diet (OF group) showed a significant ($p<0.05$) increase in the diameter of cardiomyocytes when compared with the other groups (Figure 1C).

Previous studies on the morphological development of cardiac hypertrophy in this model have been shown previously by our group [21]. With the onset of cardiac remodeling, both cells and the macroscopic structure of the myocardium undergo functional and morphological changes. There was thickening of the walls of the ventricles of in ovariectomized rats fed a high-fat diet (OF group). Also, a reduction in lumen diameter of the cardiac chambers was found in these animals, shown by concentric hypertrophy.

Morphometric analysis showed that the increase in myocardial wall thickness occurred as a result of an increase in the diameter of the cardiomyocytes in ovariectomized rats fed a high-fat diet (OF group) when compared with the other groups (Figure 1C). The systolic blood pressure measurements were made by tail plethysmography, and only the ovariectomized rats fed a high-fat diet (OF group) showed a significant increase ($p<0.05$) in the systolic blood pressure, and this difference appeared at the fifth month until the end of the study protocol (Figure 1D).
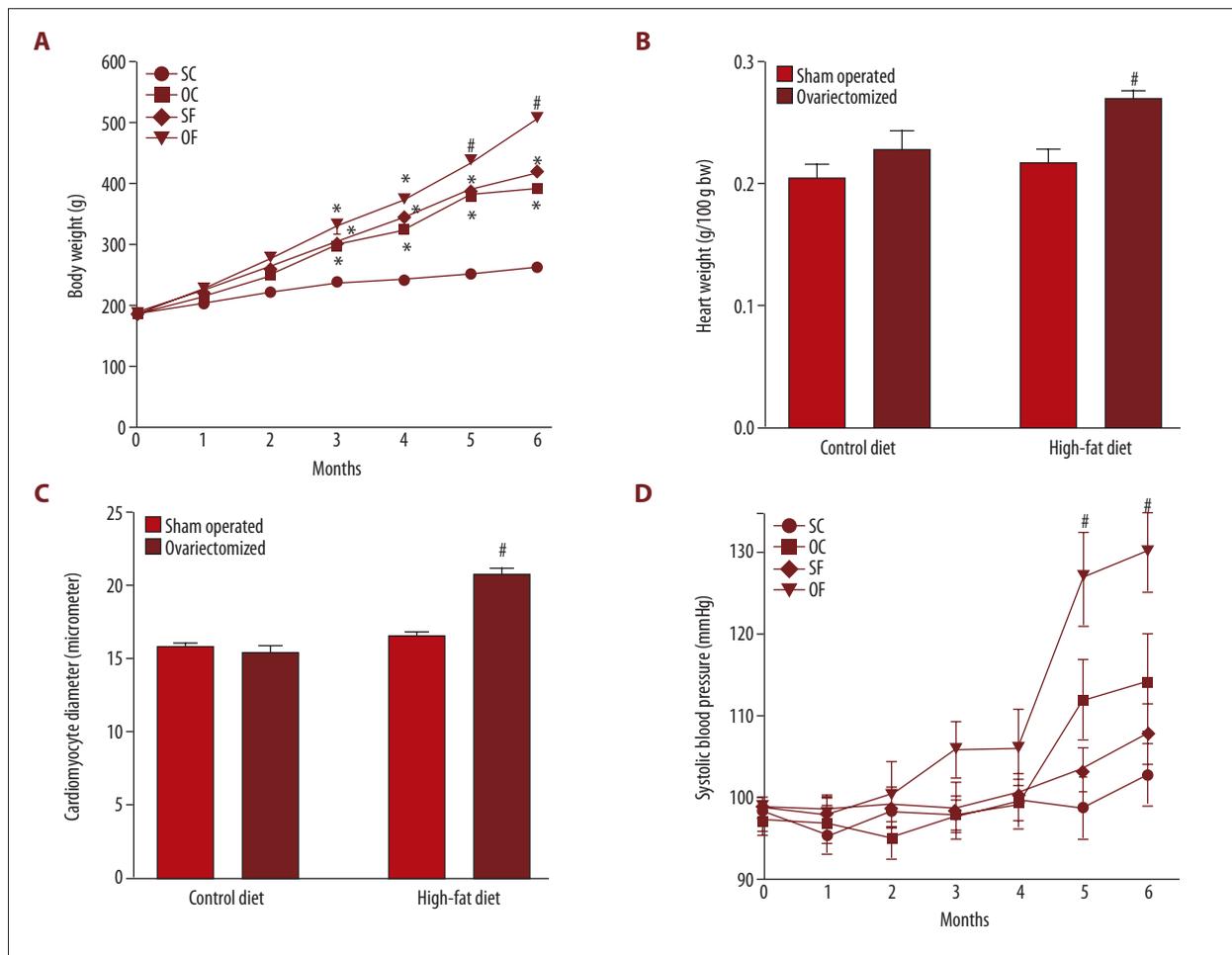
Figure 1. Ovariectomy associated with high-fat diet (the OF group) induces weight gain, increase in cardiac weight and increase in systolic blood pressure (SBP). (**A**) Body weight gain. The body weight gain was measured weekly. The results show an average of each animal group per month. N=12 for each group. (**B**) This figure shows the results of the heart weight of the animals. After euthanasia, the whole hearts were removed and weighed immediately. The results were normalized by body weight and expressed as gram per 100 grams. N=12 for each group. (**C**) Cardiomyocyte diameter. For the linear measurements (diameters) of cardiomyocytes, ×400 objective was used. Sixty cells were measured by region in at least ten different fields. As a parameter, the smallest diameter measured in cardiomyocytes is in a line that intersects the nuclei. N=4 for each group. (**D**) Systolic blood pressure (SBP). The SBP was measured weekly by tail plethysmography, and the results indicate an average of each animal group per month. Measurements were made by tail plethysmography using the electro-sphygmomanometer LE5001. After five minutes of pre-heating at 37°C, the rat tail was placed in contact with a cuff, and a pulse transducer to register data from mean cardiac rate, and mean systolic and diastolic blood pressure. The mean from five consecutive data each day was used as an individual result. N=8 for each group. SC – sham-operated fed a control diet; OC – ovariectomized fed a control diet; SF – sham-operated fed a high-fat diet; OF – ovariectomized fed a high-fat diet. p<0.05 *vs.* SC; # p<0.05 *vs.* SC, OC and SF. The results were analyzed by two-way ANOVA followed by Bonferroni's test.

Ovariectomy associated with a high-fat diet increased the parameters involved in cardiac fibrosis

The detection of α-smooth muscle actin (*ASMA*) in this rat model indicated that the influence of both diet and ovariectomy in the activation of myofibroblasts, and demonstrated the presence of myofibroblasts in the myocardial tissue. The cardiac tissue sections from the SC group were almost negative for *ASMA* (Figure 2A). However, *ASMA*-positive cells were observed in rats subjected to ovariectomy or fat diet alone (Figure 2B, 2C). A negative control was tested where the primary antibody was omitted, and no unspecific labeling was found (Figure 2A – upper right corner).

There was an increase in expression of *ASMA* in the ovariectomized rats fed a high-fat diet (OF group, Figure 2D), although there was no difference when compared with the SF group (Figure 2E). Compared with the control group (Figure 2A)
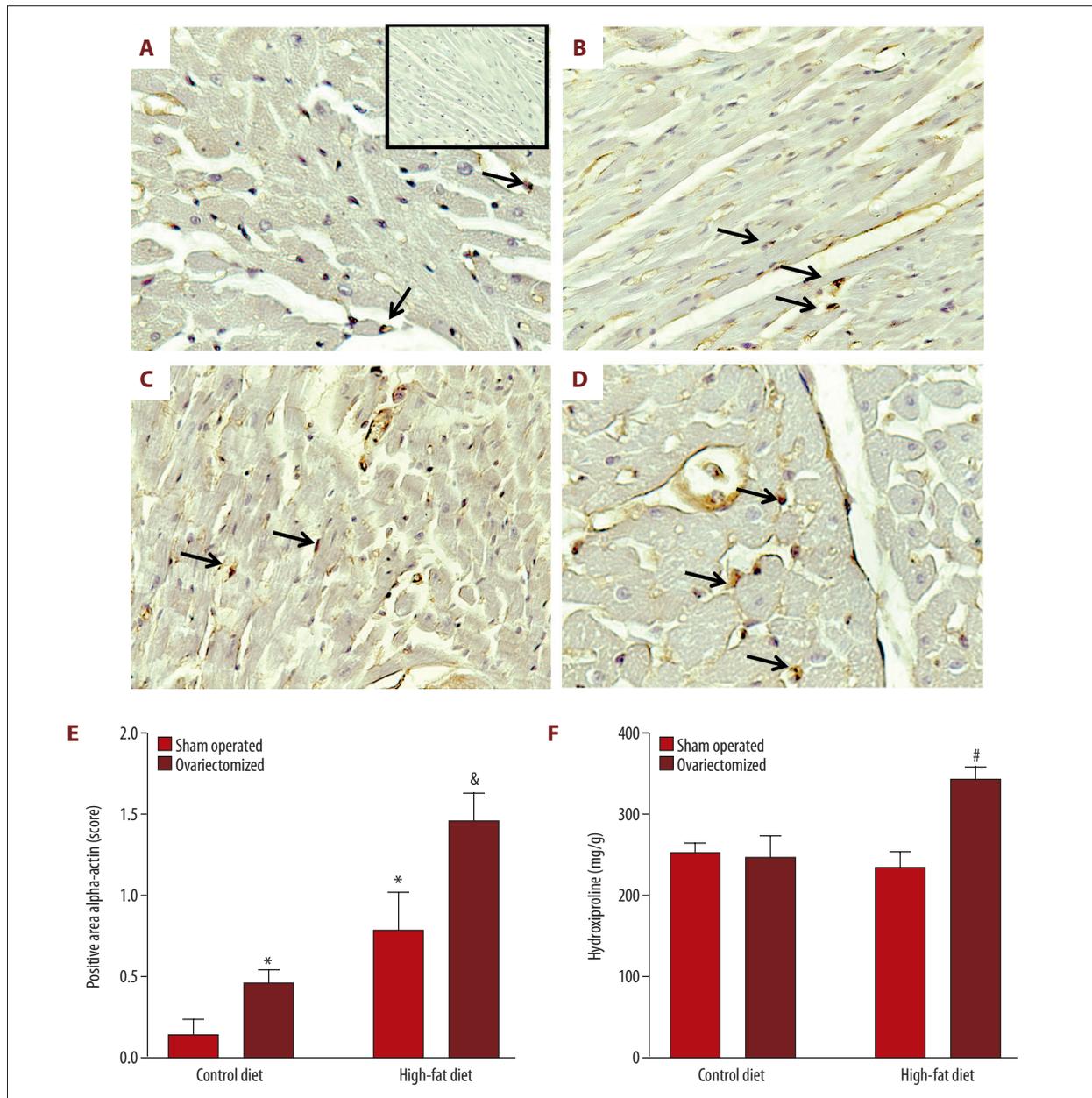
Figure 2. Ovariectomy associated with high-fat diet (the OF group) increases the parameters involved in rat cardiac fibrosis. **A–D** are photomicrographs showing the immunohistochemistry for α-smooth muscle actin (*ASMA*) in the ventricles of sham-operated rats fed control diet (SC); ovariectomized rats fed control diet (OC); sham-operated rats fed a high-fat diet (SF) and ovariectomized rats fed a high-fat diet (OF), respectively. Analysis of the images shows a gradual increase in *ASMA* expression. The arrows illustrate the presence of activated myofibroblasts in the interstitium of the myocardium of animals under study. Objective magnification ×200. Ovariectomy accounts for 19.66% of the variation (p=0.0077), diet accounts for 45.72% of the variation (p=0.0003). The negative control (omission of the primary antibody) is presented on the upper right corner in **A**. (**E**) Positive immunohistochemical staining (brown) for α-smooth muscle actin (*ASMA*) was localized by the primary antibody used and was quantified by the histological evaluation of 30 fields per slide with positive immunostaining attributed to the marked ventricular area by assigning a score of 0–4. A score of 0 was equivalent to 0–5% of the set; a score of 1 was equivalent to 5–25%; a score of 2 was equivalent to 25–50%; a score 3 was equivalent to 50–75%; and a score of 4 was equivalent to 75–100% [26]. Immunohistochemical staining scoring analysis was performed with ×400 objective, and photomicrographs were taken at an objective magnification of ×200. (**F**) Hydroxyproline concentrations. Data analyzed by two-way ANOVA followed by Bonferroni's test. * p<0.05 *vs.* SC group; # p<0.05 *vs.* SC, OC and SF; and p<0.05 *vs.* SC and OC; (n=4, for each group).
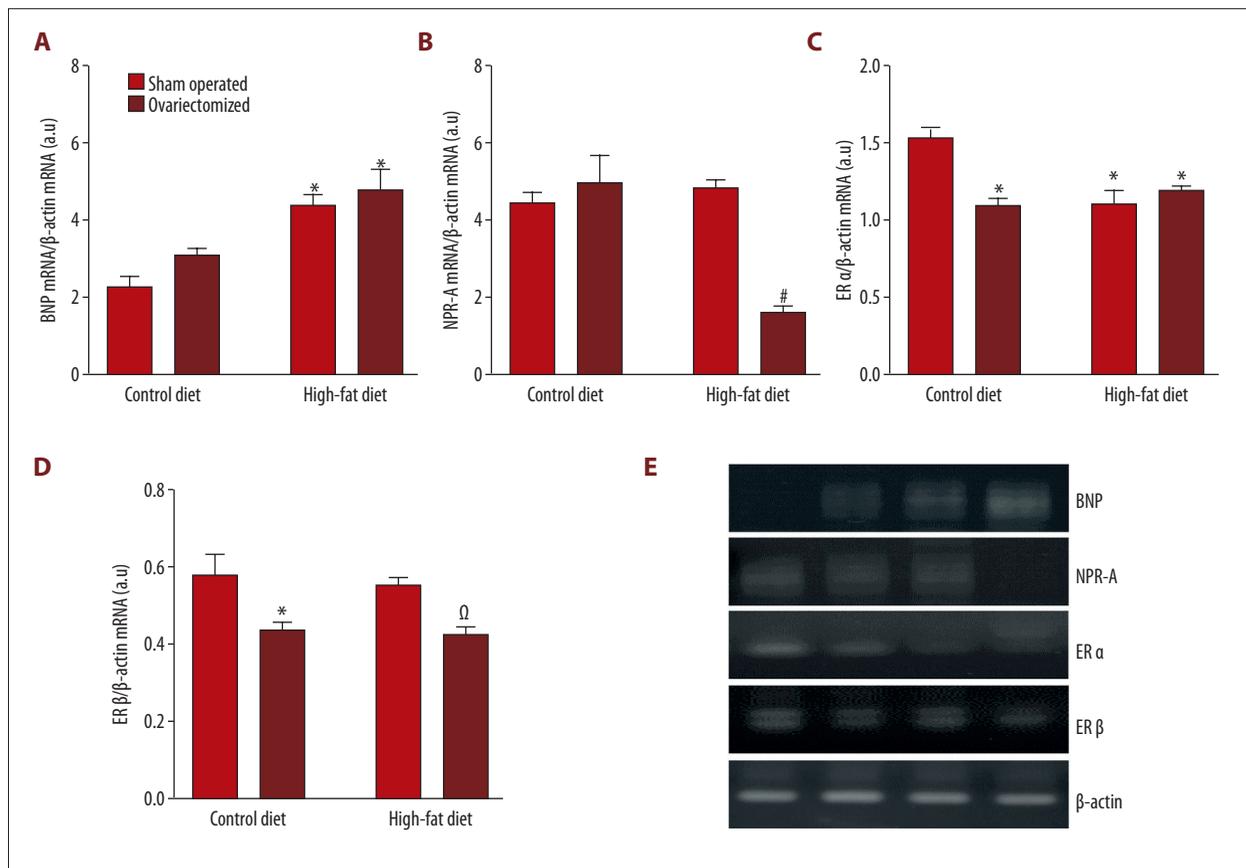
Figure 3. Expression of estrogen receptor genes are associated with cardiac hypertrophy and expression of *BNP* in ovariectomized rats fed a high-fat diet (the OF group). Gene expression in rat ventricle showing the mRNA detection of *BNP, NPR-A, Er*α, and *ER*β. The mRNA determination was made by polymerase chain reaction (PCR) using the whole hearts (n=4 for each group) from sham-operated rats fed control diet (SC); ovariectomized rats fed control diet (OC); sham-operated rats fed a high-fat diet (SF); and ovariectomized rats fed a high-fat diet (OF). mRNA values have been normalized with mRNA β-actin values. **A–D** are the ratio of *BNP, NPR-A, ER*α and *ER*β expressed in arbitrary units respectively. (**E**) Representative ethidium bromide visualization of polymerase chain reaction (PCR) products. N=4 each group. The results were analyzed by two-way ANOVA followed by Bonferroni's test. * p <0.05 *vs.* SC group; # p<0.05 *vs.* SC, OC and SF; Ω p<0.05 *vs.* SC and SF; (n=4, for each group).

only rare myofibroblasts and *ASMA* was expressed by smooth muscle cells in blood vessels.

Figure 2F shows the presence of hydroxyproline, an indirect measure of collagen. Only the combined effect of ovariectomized rats fed a high-fat diet (OF group) showed an increase in the levels of hydroxyproline in the myocardial tissue in ovariectomized rats fed a high-fat diet (OF group).

Gene expression for *BNP, NPR-A, ER*α, and *ER*β

The gene expression analysis performed in the ventricle of the animals showed that mRNA levels of expression of brain natriuretic peptide (*BNP*), which is also known as B-type natriuretic peptide, was increased in ovariectomized rats fed a high-fat diet (OF group) when compared with the SC group (Figure 3A).

Also, the ovariectomized rats fed a high-fat diet (OF group) showed a significant reduction (*p*<0.05) in expression of the *NPR-A* gene in the rat myocardium (Figure 3B). It is possible that downregulation of *NPR-A* can explain how low estradiol levels interact to promote cardiac hypertrophy.

The expression of *ER*α and *ER*β differed between the experimental groups. Figure 3C shows the pattern of expression of mRNA for *ER*α. All ovariectomized rats (the OC and OF groups) or rats fed a high-fat diet (the OF group) showed a significant reduction (*p*<0.05) in expression of *ER*α compared with the control group. However, analysis of the mRNA for *ER*β (Figure 3D) showed a reduction in expression only in the ovariectomized groups (the OC and OF groups) when compared with the sham-operated groups.
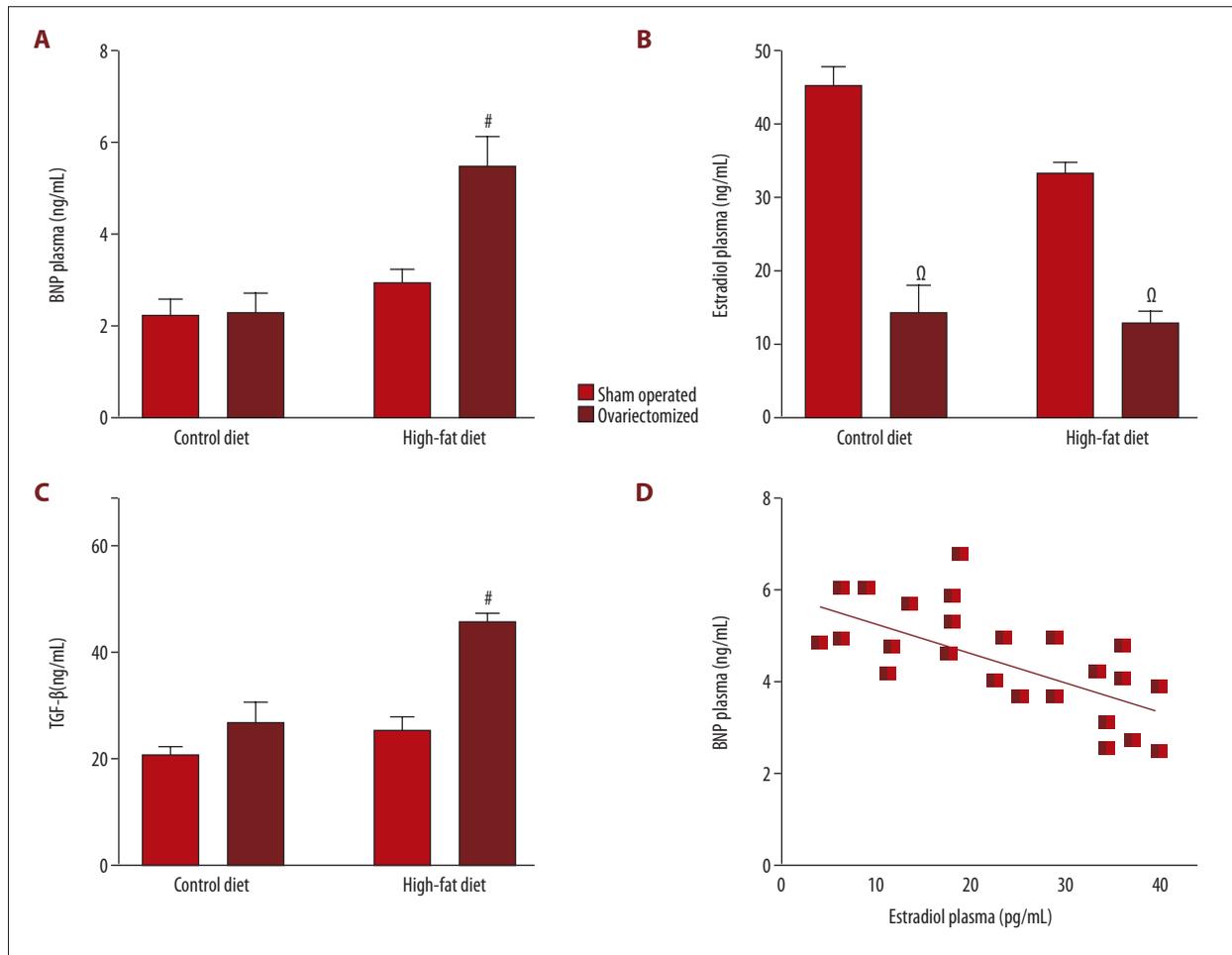
Figure 4. Expression of brain natriuretic peptide (*BNP*) correlates with plasma estradiol levels in the ovariectomized rats fed a high-fat diet (the OF group), and with transforming growth factor (*TGF*)-β levels. Plasma levels of brain natriuretic peptide (*BNP*), which is also known as B-type natriuretic peptide, estradiol and *TGF*-β represented in **A**, **B** and **D**, respectively were measured by the enzyme-linked immunosorbent assay (ELISA) using plasma samples (n=12 for each group) from sham-operated rats fed control diet (SC); ovariectomized rats fed control diet (OC); sham-operated rats fed a high-fat diet (SF); and ovariectomized rats fed a high-fat diet (OF). **C** represents the correlation between *BNP* and estradiol in ovariectomized rats (n=12) fed a control diet (OC) or a high-fat diet (OF) for six months. The results were analyzed by two-way ANOVA followed by Bonferroni's test, and correlations were analyzed by Pearson's coefficient. # p<0.05 *vs.* SC, OC and SF; Ω p<0.05 *vs.* SC and SF.

Plasma levels of *BNP*, estradiol, and transforming growth factor (*TGF*)-β

The plasma levels of *BNP*, estradiol and transforming growth factor (*TGF*)-β were measured by enzyme-linked immunosorbent assay (ELISA). Plasma levels of *BNP* in the sham-operated animals were very low. However, *BNP* levels were significantly increased (*p*<0.05) in ovariectomized rats fed a high-fat diet (OF group) (Figure 4A). This result indicates that the production and release of *BNP* were associated with increased weight gain and cardiac hypertrophy in the rat model. Also, estradiol levels were reduced in ovariectomized animals (Figure 4B), demonstrating that ovariectomy was effective in reducing plasma levels of estradiol.

There was an inverse relationship between *BNP* and estradiol in the rat model (Figure 4D). This finding showed that a reduction in estrogen levels was correlated with increased plasma levels of *BNP*, which is not common in models that do not use diet associated with ovariectomy. Also, *TGF*-β levels were increased in ovariectomized rats fed a high-fat diet (OF group) (Figure 4C), which may be a possible factor involved in the activation of cardiac remodeling.

Plasma *BNP* and body weight, blood pressure and diameter of the cardiomyocytes

The relationship between plasma *BNP* levels and cardiac hypertrophy were analyzed with other variables involved in the

Table 1. Brain natriuretic peptide (*BNP*) (also known as B-type natriuretic peptide) expression correlates with the diameter of cardiomyocytes and body weight but not with blood pressure in the ovariectomized rats fed a high-fat diet (the OF group).

Variable	Control diet		High-fat diet	
	Pearson (r)	Statistical significance	Pearson (r)	Statistical significance
Plasma levels of BNP *vs.* diameter of cardiomyocytes	0.461	*p*=0.357 (ns)	0.914	*p*=0.004
Plasma levels of BNP *vs.* body weight	0.71	*p*=0.746 (ns)	0.890	*p*=0.007
Plasma levels of BNP *vs.* systolic blood pressure	−0.84	*p*=0.223 (ns)	0.726	*p*=0.065 (ns)

The correlations were made using Pearson's correlation coefficient (two-tailed) among the entire population of Wistar rats fed control diet, and the whole population of Wistar rats fed a high-fat diet (n=24 for each variable). This type of correlation indicates trends or associations between variables. Statistical significance was determined to be a p-value ≤0.05. ns – not significant. r=0.10–0.30 (weak correlation), 0.40–0.60 (moderate correlation), 0.70–1.0 (strong correlation) [50].

development of cardiac remodeling, as shown in Table 1. Plasma *BNP* levels were significantly correlated (91%) (p=0.004) with the diameter of cardiomyocytes in the groups with a high-fat diet. There was also a significant correlation (89%) (p=0.007) between plasma *BNP* and body weight but again only in the ovariectomized rats fed a high-fat diet (OF group). However, there was no significant correlation between plasma *BNP* and systolic blood pressure in the groups fed a control diet and a high-fat diet (p=0.223 and p=0.065, respectively).

Discussion

The findings of this study, using ovariectomized rats fed a high-fat diet, showed a new aspect of the biology of brain natriuretic peptide (*BNP*), which is also known as B-type natriuretic peptide, in relation to obesity, reduced estradiol, and cardiac hypertrophy. Forty-eight female Wistar rats were divided into four groups, which included sham-operated rats fed a control diet (SC), ovariectomized rats fed a control diet (OC), sham-operated rats fed a high-fat diet (SF), and ovariectomized rats fed a high-fat diet (OF).

The findings of this study were that in ovariectomized rats fed a high-fat diet (the OF group) there was an increase in body weight, an increase in blood pressure, and cardiac hypertrophy, characterized by an increase in heart weight, increase in diameter of cardiomyocytes and cardiac fibrosis. In ovariectomized rats fed a high-fat diet (the OF group), there was an increase in plasma *BNP* levels and cardiac *BNP* expression, a decrease in ventricular synthesis of *NPR-A*, *ERα* and *ERβ* and an increase in expression of transforming growth factor (*TGF*)-β. The study also showed that the increase in plasma *BNP* levels was associated with increased body weight and increased the diameter of cardiomyocytes in the ovariectomized rats fed a high-fat diet.

Ventricular hypertrophy is reported to be more common in obese subjects [25]. The probable mechanisms involved include an increase in total blood volume and cardiac output and the increased cardiac burden imposed on the myocardium due to cardiac compression [26,27]. Obesity also favors a state of imbalance between pro-inflammatory and anti-inflammatory factors that affect cardiac remodeling, which may contribute to inflammation and fibrosis. The chronic cardiac overload eventually leads to contractile dysfunction and heart failure [28], since weight loss by either bariatric surgery or diet has been found to promote a significant improvement in cardiac function [29].

In this study, the OC and SF rats showed increased body weight beginning on the third month of the high-fat diet. However, the ovariectomized rats fed a high-fat diet (the OF group) had a greater gain in body weight in the last two months of the study. Systolic blood pressure was associated with body weight, which was increased in the last two months of the study for the ovariectomized rats fed a high-fat diet (the OF group). The weight gain could be a cause of the changes found in the myocardium of the experimental animals.

The ovariectomized rats fed a high-fat diet (the OF group) demonstrated increased systolic blood pressure and increased heart weight and diameter of cardiomyocytes. These results could indicate a direct relationship between the development of cardiac overload and cardiac hypertrophy. This pattern of hypertrophy was probably due to increased pressure on the cardiac structures together with overproduction of inflammatory cytokines by adipose tissue. Also, an unfavorable hormonal state due to estrogen deficiency could be responsible for reducing the production of mediators of vasodilation [30] which can lead to an increase in peripheral resistance. Reduced estrogen levels may result in an inflammatory profile in the

ovariectomized rat groups [31], especially in the ovariecto-mized rats fed a high-fat diet (the OF group) that showed an increase in *TGF*-β expression. Also, the findings of the increase in blood pressure in the ovariectomized rats fed a high-fat diet (the OF group) suggest that estrogen deficiency and a high-fat diet may create the environment for the development of hypertension. Previously published studies have shown that CYP1B1 (cytochrome P450) protects against Ang II-induced hypertension and associated cardiovascular changes in female mice [32]. The findings of this study also indicate that cardiac remodeling and an increase in peripheral vascular resistance may begin at an early stage when obesity is associated with a lack of estrogen.

In this study, using a rat model, the influence of the biological peptide, *BNP*, on cardiac hypertrophy was studied. The clinical importance of *BNP*-guided treatment for heart failure may lie in its ability to indicate an imbalance in cardiac overload [33]. Hemodynamic changes and the activity of the neurohormonal system may activate the synthesis and release of *BNP*, as small changes in the levels of renin, aldosterone, cortisol, epinephrine, norepinephrine, as well as cytokines such as IL-6, can promote the wide variation in *BNP* levels [34]. There may be a relationship between the production and release of *BNP* with the hemodynamic and neurohormonal state [34]. Research on the hormones produced by the adrenal cortex and components of the renin-angiotensin system may explain the increase in *BNP* and may be considered in future studies using this animal model. From the findings of this study, it may be possible to propose that *TGF*-β associated with inflammation secondary to obesity is a key factor in cardiac remodeling and cardiac hypertrophy.

In this study, ovariectomized animals fed a high-fat diet showed higher levels of expression of *BNP* mRNA in the ventricles as well as higher circulating levels of *BNP*. In previously published studies, the relationship between body mass index (BMI) and plasma *BNP* levels has been controversial [35–37]. The controversial relationship between natriuretic peptides and obesity may indicate the existence of different mechanisms for clearance or synthesis of *BNP* in obesity. Some studies have shown that an increase in expression of NPR-C in adipose tissue and reduced expression of *BNP* by the ventricle could be the main mechanism to explain the low *BNP* levels found in obese individuals [38,39]. However, in this study, increased expression of *BNP* mRNA and protein, in the ventricle and plasma of the rat model was associated with the greatest gain in body weight. These findings are supported by previous studies that have found an increase in the expression of the *BNP* precursor, NT-pro*BNP*, in patients with severe obesity [40]. However, there was no correlation between *BNP* expression levels and obesity in these patients [40]. It is possible that, in the present study, mechanisms that stimulate increased production of *BNP*, such as local stimulators on the rat heart, combined to increase the clearance of this peptide, resulting in increased plasma concentrations.

Increased *BNP* levels found in women may be explained by the stimulating effect on *BNP* production by female steroid hormones [41]. However, with cardiac overload or cardiovascular disease, levels of *BNP* rise regardless of gender but is associated with an increased cardiac clinical risk in women [42]. In this study, increased plasma levels of *BNP* in ovariectomized animals fed a high-fat diet (the OF group), were inversely correlated with plasma estradiol levels, indicating that even in the absence of hormonal stimulation, the production and release of *BNP* was increased due to the state of cardiac remodeling [43]. Therefore, *BNP* may also be a marker of cardiac hypertrophy. Resistance to the biological effects of *BNP* has been reported and can be explained by changes in the synthesis or function of the *NPR-A* receptor, as shown in this study by the reduction in expression of the *NPR-A* gene in the ventricles of the ovariectomized rats fed a high-fat diet (the OF group). This form of resistance to *BNP* has been reported in patients with heart failure [44].

The major structural changes that occur during the progression of cardiac remodeling are the conversion of fibroblasts to myofibroblasts and excessive deposition of extracellular matrix. Myofibroblasts may then express markers such as α-smooth muscle actin (*ASMA*). In this study, an increase in *ASMA* in myocardial tissue was found in ovariectomized rats fed a high-fat diet (the OF group), indicating the presence of cardiac remodeling in this group; this was supported by the increase in hydroxyproline expression and increased levels of *TGF*-β in this group. *TGF*-β is a cytokine that may work by activating the transition of myofibroblasts to fibroblasts as part of the fibrotic process involving *ASMA* and collagen synthesis [45].

The effects on estrogen receptors (*ER*) mediated by estradiol have been associated with modulation of cardiac hypertrophy in animal models, and in humans as estradiol stimulates the release of cardioprotective factors and contribute to the regulation of metabolic diseases such as obesity [20,46]. The findings of this study demonstrated that the *ER*α gene underwent downregulation in ovariectomized rats fed a high-fat diet (the OF group), while the *ER*β gene was also downregulated. The marked reduction in *ER* gene expression and reduced plasma levels of this hormone in the OF group may partly explain the development of cardiac hypertrophy in these animals. However, in this rat model, a high-fat diet without ovariectomy was also associated with reduced expression of *ER*α. As yet, this finding cannot be explained, but a hypothesis is that obesity may lead to a modification of the constitution of the cardiomyocyte cell membrane, including the type of phospholipids, and also the density of receptors expressed
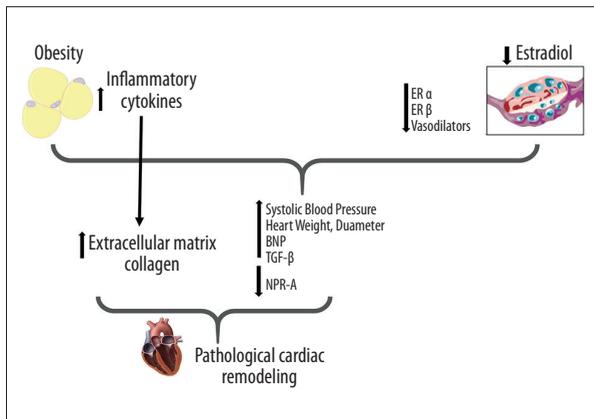
Figure 5. Summary of pathways involved in ovariectomized rats fed a high-fat diet (the OF group): the role of brain natriuretic peptide (*BNP*). Obesity, characterized by an increase in adipocytes, leads to a high level of inflammatory cytokines, which, together with the effects of estradiol reduction contribute to the elevation of blood pressure in these animals. Such events initiate the stimulus for cardiac remodeling, characterized by cardiomyocyte hypertrophy, and increased brain natriuretic peptide (*BNP*) production. Obesity may still represent a risk factor for cardiac remodeling because it stimulates the production of extracellular matrix (ECM) and inflammatory factors such as *TGF*-β. These factors, associated with the reduction of the biological activity of *BNP* (lower expression of *NPR-A*) may intensify cardiac hypertrophy and reduce the anti-hypertrophic activity of *BNP*.

in the membrane may be altered [47]. An excess of adipose tissue may, by itself, explain the modification of gene expression observed in our study.

Additionally, previous studies have shown that estradiol can modulate the process of fibrosis in cardiac tissue as a selective agonist for *ER*β preventing the differentiation of myofibroblasts and collagen deposition in cardiac tissue of mice treated with Ang II [20,46,48]. However, this finding has not been reported in *ER*β knockout mice, and the anti-hypertrophic and antifibrotic effect has not been shown to occur with the use of a selective agonist for *ER*α [48,49]. Therefore, this study may demonstrate the involvement of *ER*β receptor in the anti-hypertrophic effect of estradiol which may regulate the metabolism of fat distribution and also through the *ER*β gene [49]. A summary of the possible pathways involved in ovariectomized rats fed a high-fat diet (the OF group) and the role of *BNP* are summarized in Figure 5.

Conclusions

The findings of this study have shown that measurement of plasma brain natriuretic peptide (*BNP*), which is also known as B-type natriuretic peptide, was associated with cardiac hypertrophy in ovariectomized rats fed a high-fat diet. The *BNP-NPR-A* receptor complex may be involved in cardiac remodeling and *BNP* may be a marker of cardiac hypertrophy in this animal model. Future clinical studies on the role of *BNP* in the heart in obesity and the menopause are indicated.

Conflict of interest

None.

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