Gender differences in hearts subjected to decreased NO-production and elevated blood pressure

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Abstract

Background

Hypertension is one of the most important risk factors for heart failure in the general population. Hypertensive heart failure is associated with heart failure with preserved ejection fraction (HFpEF). Women have an increase proportion of HFpEF compared to men. Endothelial dysfunction thereby reduced NO production is proposed to play an important role in the pathophysiology of HFpEF. A suggested aetiology is related to cGMP´s important roles in myocardial cells. Females normally have higher constitutively activity of NOS and thereby NO production, an important stimulator of sGC and thereby cGMP. PKG1 is a cGMP-regulated protein that has cardio protective effects in rodents.

Aim

In this experimental study we wanted to investigate gender differences when NOS was blocked.

Methods

Adult rats, males, females and ovariectomized females were treated with L-NAME in drinking water for 4 weeks. Blood pressure was measured, and hearts investigated by echocardiography, histology and gene expression analysis at endpoint.

Results

MAP increased in all treatment groups; the increase was significantly larger in Males and Females Ovariectomized (OVX) compared to Females. Histological analysis of collagen showed no increase in interstitial or perivascular collagen. Gene expression analysis showed an increase in fibrosis genes, ANF and BNP, most pronounced in Females OVX and intact Females. There was also an isoform shift of MHC, more pronounced in Females OVX. Echocardiography showed a higher relative increase in LV mass in intact Females than Males. There was an increase in LV mass in all treatment groups, but no changes in diastolic or systolic diameter, suggesting concentric remodeling. There were no clinical signs of heart failure in treatment groups and cardiac output was maintained.

Conclusion

This study confirm that with loss of NO production females developed more hypertrophy than males independent of blood pressure. Females also tended to have more extreme changes in expression of genes related to heart failure compared to males.

Introduction

Hypertension is one of the most important risk factors for heart failure in the general population(1,2). Hypertensive heart disease is also characterized by an increased prevalence of heart failure with preserved ejection fraction (HFpEF) compared to heart failure with reduced ejection fraction (HFrEF)(3). According to some studies women have a higher prevalence of HFpEF than men, and they more often have other comorbidities such as hypertension and diabetes, whilst men more often have ischemic heart disease(4,5). Both patients with HFpEF and HFrEF have clinical heart failure (reduced cardiac output (CO)); ejection fraction (EF) is used in the clinic to decide which phenotype the patient has(6,7). Unfortunately, treatment for HFpEF is less effective compared to HFrEF. Knowledge of the pathophysiology of the hypertensive heart is therefore important.

In the present study, the aim was to study heart remodeling and differences between male and female hearts in an experimental setting. There are several models available for studying development of heart failure in rats(8). In previous studies done in rats, oestrogen has been shown to protect against salt-sensitive hypertension, but not against essential hypertension in spontaneous hypertensive rats(9). The volume overload model with an AV-fistula has been used to study gender differences. They found that females developed more hypertrophy than males, but heart function, measured by an invasive catheter, was better preserved in females compared to males. Females that were ovariectomized (OVX) had even more hypertrophy than intact females(10,11). The same model has been used to show that estrogen administered to male rats seemed to protect them against fibrosis(12). The spontaneous hypertensive rat (SHR) has also been used to study gender differences in heart failure. In this model, males develop symptoms of heart failure earlier than females, and echocardiographic changes related to heart failure also occur earlier in males than females(13,14). Studies using aorta banding have demonstrated that females get more hypertrophy than males when measured as left ventricular mass (LV mass)/body mass (BM). Females also had an increase tendency of concentric remodeling of the left ventricle. But the females seemed to tolerate this better than males, who developed signs of heart failure earlier than females(15).

Nitric oxide (NO) is a well known vasodilator(16), and is produced by the enzyme nitric oxide synthase (NOS). NOS exists as three isoforms, neuronal-NOS (nNOS, NOS1), inducible-NOS (iNOS, NOS2) and endothelial-NOS (eNOS, NOS3)(17,18). Chronic blockage of NOS with the chemical agent L-NG-nitroarginine methyl ester (L-NAME) in rats increases peripheral resistance, and results in a significant hypertension(19–24). Previous studies also found that chronic L-NAME treatment induces remodeling of the heart, with increased fibrosis in the left ventricle (LV)(20,23,24). Some studies have found that chronic L-NAME treatment induces cardiac hypertrophy(20–22,24), while other studies have results indicating no hypertrophy, only hypertension(23,25). L-NAME inhibits all three isoforms of the NOS enzyme(26,27). Measurements done with invasive catheter method demonstrate that chronic L-NAME treatment for 8 weeks was followed by a decrease in cardiac output (CO), stroke volume (SV) and heart rate (HR)(21). Echocardiographic findings supported these findings, but also showed an increase in LV mass, decrease in end diastolic volume and decrease in cardiac index (CI)(CO/body weight), suggesting concentric remodeling(28). Blockage of NOS is also suggested to have direct effects on the myocardium. NO is through activation of guanylate cyclase an important factor in production of cyclic guanosine monophosphate (cGMP), which regulates the activity of protein kinase G-1 (PKG1), an enzyme with

reported cardio protective effects(29,30). Endothelial dysfunction is suggested to have an important role in the pathophysiology of HFpEF(31). Lack of flow-induced vasodilatation is one of the main problems in endothelial dysfunction. Damage to the cardiac endothelium has been shown to have a direct effect on the myocardial cells, altering their contractility and relaxation(32). On this background we decided to use an experimental model of NOS inhibition.

In spite of multiple studies of the hypertensive rat heart, there are still a very small proportion of studies that investigated differences between male and female hearts regarding heart failure and remodeling. The primary goal of this study was to investigate functional, structural and molecular differences in the male and female heart when subjected to NOS inhibition. Since loss of hormonal influence with aging is so marked in the female heart and heart failure in general is an age dependent condition, it was also a goal to study how ovariectomy (OVX) influenced the female heart when NOS was inhibited.

Material and methods

Animals and experimental design

Wistar rats where bought from Charles River Laboratories inc. (Margate, United Kingdom) and allocated to the following groups: Male L-NAME (Male, n=11), Female L-NAME (Female, n=8), Female ovariectomized L-NAME (F OVX, n=7) and Female ovariectomized control (F OVX C, n=7). The rats were 10 weeks old at arrival, ovariectomization were performed when they were 11 weeks old. The treatment groups were given 1g/L L-NAME in their drinking water. In addition we also included 12 weeks old Male (Male C, n=8) and Female control group (Female C, n=6). All groups had unlimited availability of food, normal diet. The protocol started when the rats where 12 weeks old and the cardiomyocytes had reached adult size. The experimental protocol was finished when the rats were 16 weeks old. At the endpoint of the study, echocardiographic examination was performed, and blood samples and tissue were obtained. The study was performed according to regulations of the Norwegian Animal Welfare Act, and approved by the National Animal Research Authority (FOTS ID 4652).

Blood pressure measurements

Blood pressure was measured weekly from start to endpoint. All animals also had two rounds of measurements at 11 weeks of age (the week before we started the protocol) to adapt them to the procedure. Blood pressure was measured using the non-invasive tail cuff method, using Coda™ Standard (Kent Scientific Corporation, Connecticut, USA). Rats need to be in warm surroundings to have well perfused tails, so they were placed in a 40˚C heating chamber for 20 minutes before being put into cylinders, that were placed on a 37°C heating plate, and covered with blankets to keep them warm and dark. Four cycles of blood pressure measurements were performed for acclimatization, and then 10 cycles were done and the average value of these was used.

Echocardiography

The animals were placed in a 3% isoflurane chamber for induction of anaesthesia. After that they were placed in the supine position with all four legs taped to ECG electrodes. Thereafter isoflurane was administered on a nose cone and reduced to 1,5%. Fur was removed from the chest area, and contact gel was used on the probe. Body temperature was monitored with a rectal probe, and with use of a heated plate and a heating lamp, we were able to keep the body temperature around 37°C. Baseline echocardiographic

measurements of left ventricular (LV) function and dimensions were done as standard procedure in all animals at baseline and at week 4. Images were acquired with a VEVO 2100 with a MS201 18MHz transducer (Fujifilm Visualsonics Inc., Toronto, Canada). M-mode images of the left ventricle were acquired from the parasternal short-axis view at the level of the papillary muscles. Data were analysed by a blinded observer using the Visualsonic software following current European Society of Cardiology guidelines(33,34).

Blood and tissue sampling

After echocardiography animals were euthanized with sodium pentobarbital 100mg/kg intraperitoneally. Blood was collected by cardiac puncture using a syringe through the skin; approximately 3mL of blood was collected. Heart, lung, kidney, liver and aorta were weighed and stored in RNAlater, McDowels fix, formaldehyde and liquid nitrogen. The right tibia was also stored for later adjustment of values to the animal's size.

Gene expression

After blood was sampled, the heart was dissected out, and the apex was immediately stored in RNAlater (Qiagen, Hilden, Germany) for later gene expression analysis using quantitative RT-PCR. Twenty-seven genes related to heart function, apoptosis, interstitial fibrosis, angiogenesis, inflammation plus estrogen associated genes were tested. The following genes were chosen for gene analysis: atrial natriuretic peptide (ANF), brain natriuretic peptide (BNP), myosin heavy chain α and β (MHCα and MHCβ), collagen type I and III (Col1a1 and Col3a1), nitric oxide synthase 2 and 3 (NOS2 and NOS3), fibronectin (Fn-1), protein kinase C a, d and e (PKCa, PKCd and PKCe), tissue inhibitor of matrix metalloproteinase (Timp-1), early growth response protein (EGR1), caspase 2 (Casp2), prostaglandin endoperoxide synthase 2 (Ptgs2), solute carrier family 2 member 4 and 9 (Slc2a4 and Slc2a9), protein kinase CGMP-dependent type 1 (Prkg1), peroxisome proliferator activated receptor gamma (Pparg), peroxisome proliferator activated receptor gamma coactivator 1 a and b (Ppargc1a and Ppargc1b), pyruvate dehydrogenase kinase 4 (PDK4), natriuretic peptide receptor 1 and 2 (NPR1 and NPR 2) and vascular endothelial growth factor A and B (VegfA and VegfB). Samples were homogenized and lysed. Total RNA was isolated according to the RNeasyFibrous Tissue protocol (Qiagen). RNA concentration was measured spectrophotometrically (NanoDrop, Witec, Switzerland) and stored at -70°C before use.

Reverse transcription of RNA was carried out using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed in an ABI PRISM 7900 HT Fast real-time thermal cycler using the SYBR green master mix (Applied Biosystems). Primers were obtained from Sigma-Aldrich (St Louis, Mo, USA). The relative expression ratio of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. The expression of the target genes were normalized to the stably expressed reference genes (hypoxanthine-guanine phosphoribosyltransferase (HPRT) and hydroxymethylbilane synthase (HMBS) based on testing by Normfinder(35).

Collagen content

Transverse sections of the left ventricle were fixed in paraffin and sliced with a Leica Ultracut S (Vienna, Austria). The slices were stained in Sirius Red solution as previously described (Junqueira et al. 1979). A digital microscope was used to take 20 pictures of each heart. Image J (National Institutes of Health, Bethesda, MD, USA) was used to analyse the collagen content in % in each picture. The pictures were taken from

randomly selected areas on the slice, including myocardium close to endocard and epicard as well as the mid myocardial area. Areas with blood vessels larger than capillaries were not included to avoid interference with perivascular collagen.

Perivascular collagen was semi quantified by a grading (1-5) using light microscopy of the sections stained with Sirius Red. Then 10 arteries in each heart were identified, and they were graded in the following way: 1 = diameter of collagen-ring-wall < 25% in comparison to diameter of media-wall. 2 = 25-50%. 3 = 50-75%. 4 = 75-100%. 5 = >100%. Mean values for each heart were used to indicate degree of perivascular collagen.

Statistics

Data analysis was performed in SigmaPlot 13.0 (Systat Software Inc, San Jose, California, USA). In the tables data is presented as group average ± SD (standard deviation). In Figure 1 data is presented as group average ± SEM (Standard Error of the Mean). Gene expression, echocardiographic parameters, weights and collagen data were examined with One Way ANOVA and Two Way ANOVA comparing the effect of gender and treatment. The Holm-Sidak post hoc test was used for multiple comparisons of groups. Repeated measures ANOVA was used when analysing blood pressure data.

Results

Body mass (BM) and heart weight

There was a significant increase in BM ($p<0.001$) from start of the study at 12 weeks of age to endpoint in Males, Females OVX and Females OVX Control, but not in intact Females. There was significantly higher BM ($p<0.001$) in the Females OVX and Females OVX Control groups compared to the normal female group (table 1). Heart weight normalized to tibia length did not differ significantly between the groups (Table 1).

Blood pressure

Mean Arterial Pressure (MAP) increased significantly in all groups during the study ($p<0.001$). With repeated measurements statistics there was significantly ($p<0.001$) higher MAP during the study in Males and F OVX compared to intact Females (Figure 1). With respect to acute response to L-NAME, the MAP 12 hour after administration begun showed a significant increase in Males and Females OVX, but no significant increase in intact Females (Table 1). The pulse pressure (week 4 – week 0) did not change significantly during the study when comparing week 4 with corresponding value in week 0. But when change in Males was compared to Females OVX there was a significant decrease in Males (Table 1).

Collagen

Sirius Red staining for measuring of interstitial collagen showed no significant differences between groups (Table 1). Scoring of perivascular fibrosis around the arteries in the heart also did not show any significant differences between the groups (Table 1).

Gene expression

All groups were compared to the Female control group (value set to 1). There was a significant effect of L-NAME in 17 of the 27 genes that were tested in this study (Table 2).

BNP was significantly increased in all treatment groups. ANF was increased in Females and Females OVX compared to control group, but was not significantly increased in Males. Thus, there seem to be a tendency of a greater increase in both ANF and BNP in intact Females and Females OVX compared to Males, but the difference between these groups did not reach statistical significance in this study.

MHCb was significantly increased in all treatment groups, while MHCa was only significantly decreased in Females OVX. There was tendency towards an isoform shift in all treatment groups, resulting in less MHCa and more MHCb. When tested with Two Way ANOVA, there was a significant difference between Females OVX and Males and intact Females with respect to MHCb.

Col1a1 showed a significant difference between males and Females OVX tested with Two Way ANOVA. In general there is a tendency of a greater increase in fibrosis genes in Females and Females OVX, but no statistic significant differences between genders in this study.

VegfA showed a significant difference between Males and Females OVX, but that was depending of treatment, and required that treatment was present.

PDK 4 is a gene that is regulated by oestrogen, and in this study we see that the control group of Males and Females OVX are almost the same, and both are lower than the Female group. This is a good indicator that the ovariectomization was done properly, and the Females OVX group's oestrogen production is reduced.

Echocardiography

Males had a significant higher CO, diastolic diameter, fractional shortening (FS) and SV than Females and F OVX, but there was no effect of treatment. Systolic diameter and EF was significantly higher in Males than in Females. LV Mass showed a significant increase in all treatment groups compared to control groups. When LV mass was adjusted to tibia length, there was still a significant increase from baseline to 4 weeks, but no significant differences between the genders. Relative increase in Heart weight/tibia ratio showed a significant ($p<0.05$) larger increase in intact Females than in Males. Heart Rate (HR) showed no significant differences between groups (Table 3).

Discussion

The present study confirms what previous studies have showed, that chronic treatment with L-NAME are making rats hypertensive(19–24). It also confirms what other hypertensive models have showed before, that Males and Females OVX have a larger increase in blood pressure compared to intact Females (Figure 1)(36–39). The effect of OVX on blood pressure seems to be rapid, since the 12-hour effect of L-NAME was significantly more pronounced in Males and Females OVX compared to intact Females (Table 1). Interestingly, in spite of less pronounced blood pressure response, intact Females had a significantly larger increase in left ventricular hypertrophy measured as echocardiography determined relative LV mass/tibia ratio (Table 3) compared to Males and Females OVX.

Increase in fibrosis with chronic L-NAME treatment has been reported repeatedly(19,22,24), but in the present study, there was no significant differences in myocardial collagen or perivascular collagen in histological sections (Table 1). The reason for this discrepancy is that the L-NAME treatment was given for only 4 weeks as discussed more in detail later in this paper.

In 17 of the 27 genes that were tested there was a significant change with L-NAME treatment compared to controls (Table 2). Gene expression analysis showed an

increase in both ANF and BNP in all treatment groups, the increase was largest in Females OVX and intact Females. The results for fibrosis related genes (Col1a1, Col3a1, Fn-1 and Timp1) had the same pattern, with an increase in all treatment groups, but the largest increase was in Female OVX and intact Females (Table 2). In a heart failure model with Angiotensin II, the same pattern with a more pronounced increase in fibrosis genes in females than in males was observed(40). In the present study there were also signs of an isoform shift of MHC genes; MHCb was significantly increased in all treatment groups, and the increase in Females OVX was significantly higher than in Males and intact Females. There was a significant decrease in MHCa in Females OVX, and there was a tendency to decrease in Males and intact Females, but no significance (Table 2). The b-isomer of MHC is slower than the a-isomer(41) and this isoform shift has been showed to be an early sign of cardiomyopathy(42,43). This isoform shift has also been shown to reduce the contractility of the cardiomyocytes(44). The gender difference in MHC pattern in general adds to the changes observed with remodelling.

In the present study compensatory hypertrophy and remodelling of the hearts have taken place. Echocardiographic examination revealed no significant changes in CO, systolic/diastolic diameter, EF%, FS%, SV or HR from baseline to endpoint. But there was sign of hypertrophy of the left ventricle, because the LV mass was increased in all groups after 4 weeks of treatment (Table 3). When we adjusted LV mass to tibia length, there were no significant differences between the treatment groups. However, the relative increase in LV mass/tibia ratio from baseline to endpoint was significantly larger in intact Females than in Males. Previous studies have also found a greater relative development of hypertrophy in females(11,13,15,40), and our results indicate the same. All groups had significant hypertrophy of the left ventricle, with no increase in diastolic or systolic diameter of the left ventricle; this indicates that there is a concentric remodelling of the left ventricle. There are no signs of heart failure in the present study, indicating that it is too early in the disease to detect any gender differences with respect to heart failure development. Our echocardiographic findings, with no significant changes in diameter or heart function is similar to what Rossi et al 2003(20) found when they treated rats with L-NAME for 4 weeks. For signs of heart failure to develop, treatment with L-NAME for more than 4 weeks is needed.

The length of treatment time is also the likely reason for the lack of histological fibrosis in this study, since there is a marked increase in gene expression of fibrosis genes, but it has not resulted in structural fibrosis or collagen remodelling, measured by histological methods. The gender related pattern indicated that the response is more marked in female hearts.

LV mass measured with echocardiography was increased in all treatment groups, but when the heart was weighed at endpoint before tissue harvesting, there were no significant differences between same age controls and treatment groups in Female OVX. We have no clear explanation for this, bet the echo data is based on LV dimensions, and small changes might be masked when weighing the whole heart.

In the present study we wanted to investigate the effects of lack of NO on cGMP and downstream effects of that. NO is one of the main pathways for cGMP production(45), the other main pathway is through ANF and BNP(46). Interestingly, in the present study we see a large increase in gene expression for these two natriuretic peptides. This could be a preferable mechanism for keeping the cGMP levels up when NO levels are decreasing. One of the most studied, and probably most important targets of cGMP is PKG1. Increased activity of PKG1 has shown to be cardio protective in rodent experiments, and is a promising therapeutic target for heart failure(47). We tested

several genes that are linked to cGMP (Table 2). The NOS2 and NOS3 genes are genes that encode the NOS enzyme. There is a significant increase in both genes in all treatment groups, but no gender differences. NPR1 and NPR2 are genes that encodes the receptor that ANF and C-type natriuretic peptide (CNP) binds to create cGMP(46). We found no signs of relevant changes in these genes. We also wanted to see how genes downstream of cGMP were inflicted by the lack of NO. The genes Prkg1, ppargc1a, ppargc1b, pparg and egr1 are all regulated by cGMP(45). Prkg1 and Egr1 were both increased in all treatment groups, but there were no significant differences between the genders. It is quite interesting that the gene expression of these genes is increasing with NO-blockage, because NO-blockage should theoretically give less cGMP activity since NO is an important positive regulator. There is a possibility that these proteins are regulated by negative feedback mechanisms, and theoretically since there is less NO, leading to less cGMP, and therefore less Prkg1 and Egr1 protein, there is less negative feedback regulating the gene expression, leading to increased gene expression.

There are several limitations to this study. A limited number of animals in each group in most of the variables measured are one of them. Another limitation is the short exposure time, only 4 weeks, which gives less time for heart remodelling related to gender differences to develop. The echocardiographic examinations were only successful in a subset of animals, and therefore the data is too small to draw any conclusions. In the gene expression analysis, there are also a relatively small number of animals, so the statistical power in the analysis is maybe too weak to detect more marked gender differences this early in the heart failure process. To be able to observe gender differences in function in failing hearts, a higher number of animals and a prolonged treatment would be needed. There is also a limitation that for animal ethical reasons the Female and Male control groups used in gene expression part of the study were 12 weeks old when they were sacrificed, compared to the 16 weeks old treatment animals. Theoretically, this could be a problem with the gene expression analysis if any genes naturally change their expression level with this aging. Since L-NAME not only inhibits eNOS, but also iNOS and nNOS, this model is not a perfect model of endothelial dysfunction. The model also results in acute inhibition of NOS, the natural development of endothelial dysfunction probably happens over a longer period.

Due to higher constitutive activity of NOS in female heart, there are differences at the biochemical level with respect to sGC-cGMP signalling. We have with this study found, in agreement with other studies that chronic L-NAME treatment is leading to hypertension, but less pronounced in intact Females than in Males and Females OVX. With respect to changes at the molecular lever, the findings were opposite, with more pronounced findings in Females and Females OVX than Males. We found no histological fibrosis, perivascular or interstitial, but in gene expression analysis, there were significant increase of fibrotic genes in all treatment groups. This increase was most pronounced in Females OVX and intact Females. The gene expression analysis found that there had been an isoform shift of MHC from MHCa to MHCb. This isoform shift was more pronounced in the Females OVX group. There was also a significant increase in ANF and BNP in intact Females and Females OVX. Prkg1 and Egr1 were increased in all treatment groups. There were no signs of heart failure in the echocardiographic examinations, but we found a significant increase in LV mass in all groups from baseline to endpoint. The relative increase from baseline to endpoint was significantly higher in intact Females than in Males. All groups show signs of hypertrophy, but no increase in diastolic or systolic diameter of the left ventricle, this indicates that there is a concentric remodelling of the left ventricle. We conclude that there is a significant difference

between the male and female heart during development of hypertensive heart remodelling caused by reduced NO production. Correlation between blood pressure and subsequent molecular changes in the heart differ, the female response is more marked at a lower blood pressure. More studies are needed to understand the interaction between BNP and ANF related signalling as source of cGMP and NO as source of cGMP in the heart and how this relates to heart failure development.

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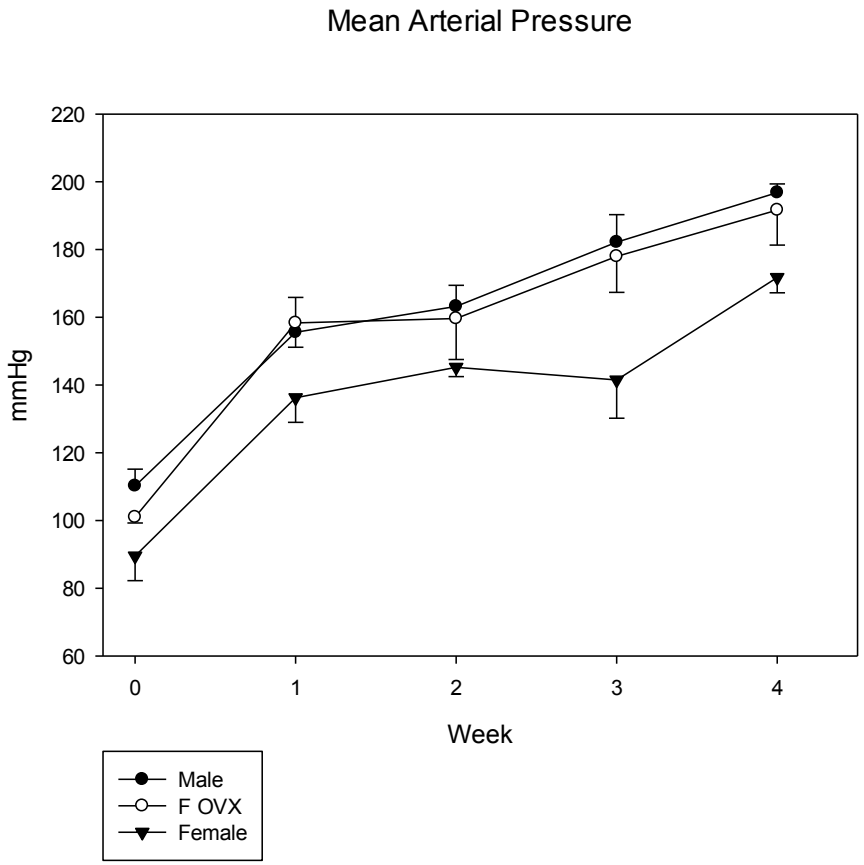
Figure 1: Mean Arterial Pressure (MAP) in each treatment group during the study. Values are presented as mean value ± SEM.



Females have a significant ($p<0.001$) lower MAP compared to males and F OVX with repeated measures statistics.

Table 1: Body mass, heart weight/tibia length, blood pressure (week 0 and 4), 12-hour effect of L-NAME, change in pulse pressure (week 4-week 0), histology. Values are presented as average ± SD (standard deviation)

	Female C N=6	Female L-NAME N=9		F OVX C N=7	F OVX L-NAME N=7		Male C N=8	Male L-NAME N=11		Effect of L-NAME and/or OVX
BM week 0 (g)	234±9	212±7		215±10	220±21		357±39	332±14		
BM week 4 (g)		221±3		271±22 +	247±10 +			359±13 +		*
MAP week 0 (mmHg)		90±14			101±4			110±11		
MAP week 4 (mmHg)		172±9			192±21			197±6		* &
MAP 12 hour after L-NAME (mmHg)		102±16			135±8 +			135±19 +		*
Change in pulse pressure (4-0) (mmHg)		-0.8±4.2			1.8±9.2			-10.8±4.2 #		
Heart weight/tibia (mg/mm)		27.9±1.7		27.2±1.8	30.0±2.2			35.2±3.8		
Collagen %	5.1±1.7	5.4±1.6		7.4±1.9	5.2±1.6		3.3±0.3	4.5±2.3		
Perivascular collagen	2.9±0.5	2.5±0.6		2.7±0.4	2.9±0.4		2.8±0.3	3.0±0.6		

* = $p<0.05$ significant effect of OVX compared to intact Female
& = $p<0.05$ significant effect of L-NAME treatment
+ = $p<0.05$ significant increase compared to baseline measurements
# = $p<0.05$ significant difference from Female OVX
Empty cells: Blood pressure measurements were not performed in the 3 control groups.

Table 2: mRNA expression in heart tissue

	Female C	Female L-NAME	F OVX C	F OVX L-NAME	Male C	Male L-NAME
Gene	n=6	n=7	n=7	n=7	n=7	n=7
Heart function related genes						
ANF *	1±0.36	8.92±2.35 ↑	1.46±0.52	10.18±2.31 ↑	1.12±0.33	4.29±1.77
BNP *	1±0.13	4.65±1.06 ↑	1.11±0.33	5.01±0.71 ↑	0.99±0.21	3.24±0.58 ↑
MHCa	1±0.03	0.94±0.07	1.10±0.05	0.69±0.09 ↓	1.03±0.03	0.81±0.04
MHCb * #	1±0.14	5.22±1.00 ↑	2.25±0.78	12.23±1.99 ↑	1.08±0.17	9.54±0.92 ↑
PKCa *	1±0.05	0.97±0.07	0.87±0.03	1.07±0.01	1.01±0.10	1.10±0.05
PKCd *	1±0.08	1.11±0.10	0.79±0.05	1.31±0.14	1.04±0.14	1.09±0.11
PKCe	1±0.02	0.72±0.05	0.73±0.05	0.71±0.06	0.59±0.03	0.77±0.04
Fibrosis related genes						
Col1a1 * +	1±0.05	2.04±0.64	1.17±0.08	2.57±0.65	1.71±0.24	1.62±0.19
Col3a1 *	1±0.05	2.18±0.51	1.28±0.15	2.77±0.55	1.66±0.31	1.97±0.25
Fn-1 *	1±0.11	3.56±2.01	1.22±0.15	6.41±4.43	1.51±0.15	1.68±0.23
Timp1 *	1±0.09	5.07±2.79	0.90±0.11	7.83±4.31	1.28±0.15	2.84±0.53
cGMP related genes						
NOS3 *	1±0.02	1.05±0.07	0.81±0.04	1.01±0.04	0.86±0.06	1.07±0.06 ↑
NOS2	1±0.06	1.37±0.26	1.40±0.19	1.34±0.14	1.54±0.10	1.51±0.13
NPR1 *	1±0.04	0.98±0.06	0.78±0.06	0.94±0.06	0.81±0.09	0.91±0.03
NPR2	1±0.03	0.95±0.05	0.87±0.04	0.97±0.03	0.82±0.03	0.96±0.03
Prkg1 *	1±0.14	1.50±0.23	1.04±0.07	1.71±0.13	1.23±0.15	1.73±0.20
Ppargc1a	1±0.04	0.80±0.04	0.78±0.06	0.73±0.05	1.00±0.10	0.86±0.06
Ppargc1b	1±0.02	0.85±0.04	0.72±0.04	0.78±0.04	0.84±0.06	0.84±0.04
Pparg *	1±0.05	0.96±0.06	0.77±0.03	0.95±0.03	0.77±0.09	0.99±0.03 ↑
Egr1 *	1±0.13	6.47±1.66 ↑	1.45±0.29	3.95±1.19 ↑	1.50±0.29	3.50±0.78 ↑
Other heart related genes						
Casp2	1±0.05	1.10±0.12	1.03±0.06	1.18±0.10	0.89±0.05	1.11±0.04
Ptgs2 *	1±0.10	5.96±3.25	0.74±0.09	3.87±0.56 ↑	1.28±0.19	2.59±0.57
Slc2a4	1±0.03	0.94±0.07	0.91±0.07	0.85±0.08	0.84±0.04	0.90±0.02
Slc2a9 *	1±0.15	1.26±0.18	0.67±0.09	1.55±0.29 ↑	0.73±0.10	1.18±0.11
PDK4 *	1±0.15	1.20±0.22	0.61±0.05	1.43±0.29	0.58±0.12	1.09±0.17 ↑
VegfA ¤	1±0.03	0.83±0.08	0.63±0.04	0.71±0.04	0.55±0.02	0.77±0.04
VegfB	1±0.04	0.89±0.10	0.77±0.04	0.76±0.05	0.73±0.02	0.84±0.04

Two Way ANOVA:

* = Significant difference between treatments $p<0.05$

= Significant difference from F OVX when compared to males and females $p<0.05$

+ = Significant difference between males and F OVX, $p<0.05$

¤ = Significant difference between males and F OVX, when treatment is present $p<0.05$

One Way ANOVA

↑ = $p<0.05$ significant increase compared to same gender control group

Table 3: Echocardiographic measures, values are presented as average±SD. Echo examination was performed only in L-NAME treated groups, the baseline examination is used as a control and compared to the 4-week examination.

	Female B (n=4)	Female 4w (n=4)		F OVX B (n=3)	F OVX 4w (n=3)		Male B (n=6)	Male 4w (n=6)
CO (mL/min) *	44.3 ± 6.9	51.8 ± 10.0		46.2 ± 4.6	47.9 ± 8.1		64.4 ± 13.6	62.6 ± 4.8
Diameter diastole (mm) *	5.39 ± 0.39	5.37 ± 0.76		5.79 ± 0.28	5.59 ± 0.28		6.44 ± 0.52	6.51 ± 0.59
Diameter systole (mm) +	2.84 ± 0.46	2.62 ± 0.68		3.59 ± 0.25	3.11 ± 0.24		3.65 ± 0.40	3.96 ± 0.49
EF (%) +	77.8 ± 6.3	81.9 ± 6.1		67.2 ± 3.7	74.8 ± 2.5		72.3 ± 7.7	68.2 ± 4.4
FS (%) *	47.6 ± 6.4	51.9 ± 7.1		38.0 ± 3.1	44.5 ± 2.3		43.1 ± 7.6	39.2 ± 3.5
LV mass (mg) & #	512 ± 77	741 ± 57		476 ± 94	605 ± 49		661 ± 116	853 ± 100
LV mass/tibia (mg/mm) &	20.2 ± 3.5	29.2 ± 2.6		18.2 ± 4.4	23.2 ± 2.2		23.1 ± 4.5	26.2 ± 4.9
Relative LV mass increase (Baseline/4 week-Baseline)*100		46.6 ± 14.7 $			30.0 ± 18.5			13.6 ± 10.6
SV (uL) *	110.0 ± 16.5	117.2 ± 28.3		111.7 ± 12.9	114.8 ± 11.8		156.5 ± 40.0	149.0 ± 26.2
HR (beats/min)	379 ± 21.6	447 ± 29.5		415 ± 8.7	417 ± 60.4		420 ± 46.4	440 ± 17.7

CO=Cardiac output, EF=Ejection fraction, FS=Fractional shortening, LV mass= Left ventricular mass,
SV=Stroke volume. HR=Heart Rate
Two Way ANOVA effect of gender and treatment time:
* = Male significant different from OVX and Female, $p<0.05$
+ = Male significant different from Female, $p<0.05$
# = Male significant different from F OVX, $p<0.05$
& = Significant different from baseline to 4 week, $p<0.05$
One Way ANOVA:
$ = $p<0.05$ significant difference compared to Male 4 week