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Original Research

Nitric oxide mediated effects of nebivolol on erectile function in rats with heart failure

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Abstract

Background and objective: Heart failure (HF) is a common complication of cardiovascular disease, which leads to functional cardiac abnormalities. Beta-blockers are commonly used to reduce mortality in HF patients; however, they are associated with an increased risk of erectile dysfunction (ED). Nebivolol is a third-generation beta-blocker with also having a Nitric oxide (NO) releasing effect. NO plays a key role in penile erection. The aim of this study was to investigate the NO-mediated effects of nebivolol on ED in HF. **Material and methods**: Twenty-four weeks old rats were divided into three groups: sham-operated control (SC), HF-induced control (HFC), and nebivolol-treated (HFNEB). HF was induced by the ligation of the left anterior descending coronary artery. Eight weeks after the ligation, functional, hemodynamic, biologic, and histologic studies were conducted to assess NO-mediated effects of nebivolol. **Results**: HF rats displayed impaired erectile function represented by decreased intracavernosal/mean arterial pressure ratio (ICP/MAP). Increased nitrosative damage/decreased antioxidant capacity was consistent with decreased endothelial NOS (eNOS) and increased inducible NOS (iNOS) and neuronal NOS (nNOS) immunoreactivity in this group. Nebivolol treated animals were characterized by improved functional capacity, increased antioxidant and decreased oxidant capacity. Prevention of eNOS and an increase in nNOS immunoreactivity was also significant in this group. **Conclusion**: Our study showed the positive effects of nebivolol on erectile function in HF. NO-mediated mechanisms behind this effect can be summarized as eNOS mediated dilation of the cavernous body and nNOS mediated smooth muscle relaxation. To the best of our knowledge, this study is the first in the literature to discuss all three NOS isoforms in order to explain the NO-mediated effects of nebivolol in ED.

Keywords: Erectile dysfunction; Nitric oxide; Heart failure; Nitric oxide synthase; Nebivolol

1. Introduction

Heart failure (HF) is a serious clinical condition in elderly, with high mortality, morbidity, and related costs. The prevalence rises to almost 10% of those over 70 years of age [1]. In addition to being one of the leading causes of mortality in advanced ages, heart failure also significantly impairs the patient's quality of life with different mechanisms. Erectile dysfunction, which occurs with a number of mechanisms, such as neurohumoral changes, low flow rate, depression, and medication, is one of the factors that disrupt the quality of life [1].

The failure to produce or maintain a penile erection during sexual intercourse is referred as erectile dysfunction (ED) [2]. Epidemiological studies show that ED prevalence is high in heart failure (HF) [3]. In men affected by this pathological condition ED is a serious problem since it leads to low quality of life and depression. Several factors may contribute to the development or worsening of ED in men with HF. Among these, psychological, social, lifestyle-related, and therapeutic factors can be listed as the most important [3].

Beta-blockers are commonly used to reduce mortality in patients with HF or a history of myocardial infarction (MI) [3]. However, they are correlated with a higher risk of ED [4]. Beta blockers differ in their mechanism of action, particularly beta-adrenoceptor selectivity and vasoactive effects [5]. While first-generation beta-blockers (e.g., propranolol) are non-beta selective, second generation agents (e.g., metoprolol) are more beta-1 selective. Third-generation agents have additional vasodilating properties in addition to their beta receptor-blocking effects [6]. Nebivolol is a third-generation agent which has nitric oxide (NO) releasing effect via the stimulation of endothelium in addition to its beta-blocking activity. The close functional relationship between the vascular and nervous system of the penis coordinates penile erection [7]. NO plays an important role in this coordination through nitrergic fibers and vascular endothelial cells [7]. Therefore, nebivolol may be favorable to other beta-blockers in the treatment of ED in HF. The beneficial effects of nebivolol were shown in patients with or at risk of developing ED in clinical studies with a small sample size [8]. However, the exact mechanism is still unknown.

In the recent guideline, according to the left ventricular (LV) systolic function HF is categorized as follows: (1) HF with decreased EF (EF <40%); (2) HF with preserved

HF (EF >50%) and (3) HF with mild range EF (EF: 40–49%) [3]. The present study aims to investigate the effect of nebivolol on erectile function in HF through NO-mediated mechanisms.

2. Materials and methods

2.1 Study design

Thirty-six adult Sprague-Dawley rats (400–500 g; 24 weeks old) were randomly assigned to one of the three groups (12 rats in each): Sham controlled control (SC), Heart failure control (HFC), and Nebivolol treated (HFNEB).

The nebivolol dose was chosen as 2 mg/kg, according to our previous study results comparing the beta-blocker effects of nebivolol to metoprolol on the animal myocardial infarction (MI) model [9]. The dose was administered by gastric gavage in 2 mL saline once daily. SC and HFC groups were administered 2 mL of saline in the same route. Animals were acclimatized with free access to water and regular chow, and were kept on a 12 h light-dark period. All experimental procedures were carried out in conjunction with the National Institution of Health's Guide to Care and Use of Laboratory Animals and approval was obtained from the local Animal Ethic Committee.

2.2 Induction of heart failure

Animals were anesthetized with a combination of ketamine-xylazine (50/10 μg/g) and mechanically ventilated with an endotracheal intubation respirator (Topo Small Animal Ventilator, Kent Scientific, Torrington, CT, USA). MI was induced by the ligation of the left anterior descending coronary artery (LAD). A left thoracotomy was performed, the pericardium was opened and the heart was externalized. LAD was ligated 2–3 mm from its origin with 6–0 prolene suture for 30 minutes. Regional cyanosis, ST elevation on electrocardiogram, and plasma troponin T level elevation were used to confirm MI. Thirty minutes after the ligation, the heart was returned to its usual position, lungs were completely infiltrated with positive end-expiratory pressure and the chest was closed in layers. Except for the ligation, the same procedure was followed in the SC group of rats. After the surgery, an analgesic (buprenorphine, 0.05 mg/kg; sc) was administered and animals were housed in the animal care facility for eight weeks before the data was collected. HF was evaluated according to left ventricular (LV) systolic function in accordance with ESC guideline [3].

2.3 Evaluation of cardiac function and ventricular pressures

Cardiac function was evaluated echocardiographically. Transthoracic echocardiography was performed under light anesthesia eight weeks after ligation and/or sham surgery with an echocardiographic system (General Electric, System Five, Horten, Norway) equipped with a 10 MHz sector probe. LV inner and wall diameters were measured from 2D-dimensional guided M-mode images, recorded from the parasternal short axis view. Transmitral inflow Doppler spectra were recorded in an apical 4-chamber view. Ejection fraction (EF) were calculated by modified Simpson method. End-diastolic and end-systolic volumes were assessed on four- and two-chamber apical views. The end-diastolic volume (EDV) was determined by tracing the end-diastolic endocardial borders at the peak of R wave in ECG. End-systolic volume (ESV) was determined by tracing the end-systolic endocardial borders just after opening the mitral valve at the same cycle. EF and cardiac output (CO) were calculated using the measured parameters with the following formulas [10]:

$$EF\ (\%) = (EDV–ESV)/EDV \times 100$$

$$CO = (\text{LV diastolic volume-LV systolic volume}) \times \text{heart rate}$$

The LV pressures were measured according to our previous study [9]. Right carotid artery was dissected in anesthetized animals and a polyethylene catheter filled with heparinized saline was inserted and advanced to the LV. The catheter was attached to a pressure transducer (MLT 0699, PowerLab, ADI Instruments, Oxford, UK) and LV-systolic (LVSP) and end-diastolic (LVEDP) pressures were measured using physiological recorder (10T Hardware System, PowerLab, ADI Instruments, Oxford, UK).

2.4 Cardiac morphological analysis

Rats were weighed and euthanized by intracardiac injection of potassium chloride. Following bilateral thoracotomy, hearts were removed, weighed, fixed in paraformaldehyde (4% in phosphate buffer solution) at 4 °C overnight and embedded in paraffin. The serially cut sections (10-μm thick, from apex to the base at 1-mm intervals) were deparaffinized, stained with hematoxylin-eosin (HE) and Masson's trichrome (MS) and evaluated under an optic microscope. MI size was calculated using the following formula [9]:

$$MI\ (\%) = (\text{infarct length/LV circumference}) \times 100.$$

2.5 Evaluation of erectile function

Erectile function was assessed by electrical stimulation of the cavernosal branch of the pelvic nerve (10 V, 8 ms, 16 Hz for 30 s) [11]. The animals were lightly anesthetized with a mixture of ketamine and xylazine. Intracavernosal pressure (ICP) and mean arterial pressure (MAP) were measured using a transducer (PowerLab, AD Instruments, Oxford, UK) connected 23-gauge polyethylene catheter positioned in the corpus cavernosum and femoral artery respectively. Simultaneous recordings were obtained using a physiological recorder (10 T Hardware System, PowerLab, ADI Instruments, Oxford, UK).

2.6 Immunohistochemical evaluation of NOS expression in penile tissue

Cross sections of penile tissue were fixed in 4% paraformaldehyde (for overnight at 4 °C) and embedded in paraffin. 4-μm-thick sections were cut from the paraffin blocks, deparaffinized and autoclaved in citrate buffer (10 mM, pH: 6). Samples were incubated with 0.3% hydrogen peroxide for blocking endogenous peroxidase activity. Nonspecific binding was prevented with a nonspecific-blocking reagent (Ultra-V-Block Lab Vision Co., Westinghouse, CA, USA) was used to prevent nonspecific bindings. Samples were incubated with eNOS Ab-1 (dilution 1 : 200) and iNOS Ab1 (dilution 1 : 200) antibodies (Labvision, Fremont, CA, USA) for 2 h at room temperature and nNOS (dilution 1 : 100) (Zymed, 61-7700 South San Francisco, CA, USA) for overnight at 4 °C according to the manufacturer instructions. Counterstain was done using HE. Adjacent sections were processed following the same steps with the exception of the primary antibodies for the negative control. Sections were examined with optic microscope and scored as 1+ (weak), 2+ (medium), or 3+ (strong) staining by evaluating the intensity of a total of 100 cells in a blinded manner.

2.7 Penile tissue oxidative status and antioxidant capacity

Superoxide dismutase (SOD) and glutathione (GSH) for the antioxidant capacity, and malondialdehyde (MDA) for the lipid oxidation capacity were measured in tissue homogenates prepared in ice-cold 10% trichloroacetic acid. MDA concentration was spectrophotometrically assessed by examining the presence of thiobarbituric acid reactive substances [12]. SOD enzyme activity was measured on the basis of H_2O_2 production, from xanthine-by-xanthine oxidase and the reduction of nitro blue tetrazolium as previously described [12]. Tissue GSH levels were measured by colorimetric assay using ready-to-use kit according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

2.8 Penile tissue oxidative stress index

Penile tissue total antioxidant capacity (TAC) and total oxidant capacity (TOC) measurements were achieved by using commercially available colorimetric assay kits (Abcam, Cambridge, UK). The results were expressed as nanomole Trolox equivalent/mg protein for TAC and nanomole H_2O_2 equivalent/mg protein for TOC.

The TOC/TAC percentage level was accepted as the oxidative stress index (OSI) and was calculated as the (nanomole H_2O_2 equivalent/milligram protein)/(nanomole Trolox equivalent/mg protein) [13].

2.9 Penile tissue nitric oxide (NO), peroxynitrite (ONO_2^-) and cyclic guanylate cyclase (cGMP) levels

NO was measured as nitrite/nitrate (NOx) concentration in the tissue supernatants via spectrophotometry (Roche, USA). ONO_2^- and cGMP levels were measured by ELISA using commercially available kits as directed by the manufacturer (HBT, Hycult Biotech Inc., Wayne, PA, USA, Zymed Laboratories Inc., San Francisco, CA, USA). Tissue protein levels were calculated following the methodology suggested by Folin-Lowry [14].

2.10 Statistical analysis

Data are presented as mean $\pm$ SD. Data were analyzed by ANOVA, Man-Whitney U tests were used as appropriate. The significance level was set at $p < 0.05$.

3. Results

3.1 Animal characteristics

In this study, the study cohort included a total of thirty-six animals. The general characteristics of the groups are shown in Table 1. Compared to the SC, HF group of rats were characterized by increased left ventricular weight (LVW), heart weight (HW) along with decreased body weight (BW) which resulted in an increased LVW/HW and HW/BW ratios ($p < 0.05$). In HFNEB group, the increase in both two ratios was prevented compared to HF rats ($p < 0.05$). There was no death in the SC group. In HF groups, mortality was 17% and 12% for HFC and HFNEB groups, respectively.

Table 1. General characteristics of the groups.

Parameter	SC	HFC	HFNEB
	(n = 6)	(n = 6)	(n = 6)
LVW (g)	0.69 $\pm$ 0.52	0.93 $\pm$ 0.61	0.81 $\pm$ 0.34
HW (g)	1.63 $\pm$ 0.08	1.75 $\pm$ 0.05*	1.68 $\pm$ 0.10$^{\&}$
BW (kg)	0.508 $\pm$ 0.04	0.436 $\pm$ 0.02*	0.485 $\pm$ 0.01$^{*,\&}$
LVW/HW	0.42 $\pm$ 0.06	0.53 $\pm$ 0.06*	0.48 $\pm$ 0.04$^{*,\&}$
HW/BW (g/kg)	3.21 $\pm$ 0.18	4.07 $\pm$ 0.06*	3.44 $\pm$ 0.22$^{*,\&}$
Infarct size (%)	-	49.2 $\pm$ 4.34	46.9 $\pm$ 5.02$^{\&}$

$p < 0.05$ *compared to SC, $^{\&}$compared to HFC.
BW, Body weigth; HW, Heart weight; LVW, left ventricular weight.

3.2 Cardiac morphology

Histologic slices of the heart of the HF group of animals revealed anterolateral LV infarction. In these groups, MI sizes were between 41–51%, with an average of 48%. HE stained slices of the heart confirmed the congestion, focal necrosis with inflammatory cell infiltration of the HF. Collagen accumulation was also significant in the vascular structure, together with cardiomyocytes (Fig. 1). Infarct sizes were similar in HFC and HFNEB groups.

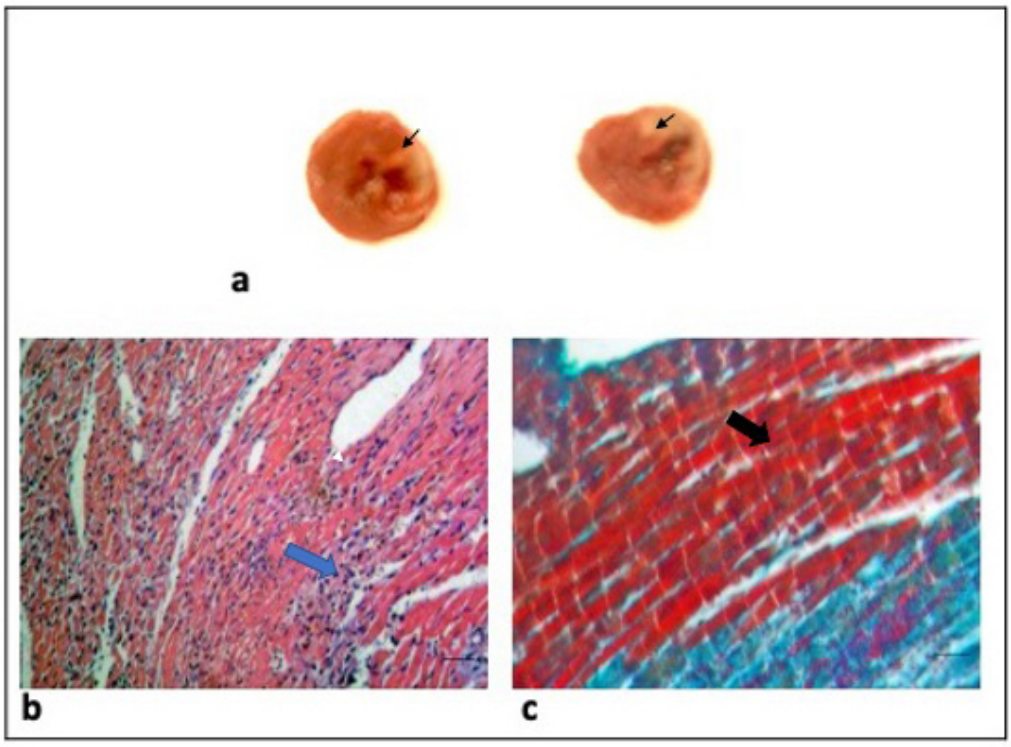

Fig. 1. Representative heart images and histological features of the heart in HF groups (200×). (a) The infarct area was located in the anterolateral region of the left ventricular anterior wall and was extended from the subendocardial region to the endocardium (black arrow); (b) Congestion, focal necrosis with inflammatory cell infiltration in HE stained slices (blue arrow); (c) Necrosis characterized by contraction bands linked by muscle fiber bridges and haemorrhage between them in MS-stained slices (black arrow).

3.3 Cardiac function and ventricular pressures

HF rats were characterized by LV dilatation, LVED pressure and compensatory LV hypertrophy compared to SC group of rats. Fig. 2 displays representative echocardiograms and LV hypertrophy. LV structural changes (characterized by increased LVEDd and LVEDv) were significant in HF rats compared to SC ($p < 0.05$). LVEDP were also significantly increased in the HFC group compared to SC. Although there was a significant decrease in EF in HFC group of rats compared to the SC, the mean EF value was still within normal limits according to the heart failure guideline. In the HFNEB group, in addition to the LV diastolic pressures, LV volumes and EF were also significantly preserved compared to the HFC group (Table 2).

Table 2. Cardiac function and ventricular pressures.

Parameter	SC	HFC	HFNEB
	(n = 18)	(n = 18)	(n = 18)
LVEDd (cm)	0.69 ± 0.05	0.76 ± 0.12*	0.63 ± 0.03&
LVEDv (mL)	0.70 ± 0.09	0.83 ± 0.04*	0.72 ± 0.09&
EF (%)	71.04 ± 6.23	55.6 ± 7.13*	66.23 ± 3.24*,&
CO (mL/min)	742 ± 124	727 ± 136*	735 ± 212*,&
LVSP (mmHg)	134 ± 3.65	118 ± 5.48*	123 ± 4.67*
LVEDP (mmHg)	2.52 ± 0.34	34.1 ± 2.73*	19.3 ± 3.41*,&

$p < 0.05$ *compared to SC, &compared to HFC.

LVEDd, left ventricular end-diastolic diameter; EF, left ventricular ejection fraction;

LVEDv, left ventricular end-diastolic volume; CO, cardiac output;

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure.

3.4 Erectile function

Compared to the SC group, HFC rats were characterized by decreased ICP in response to increased frequency. However, the MAP was similar in all the groups (Table 3). The decreased ICP/MAP ratio suggests ED in HF rats. Nebivolol treatment prevented the impairment in ICP (Fig. 3).

3.5 Penile tissue oxidative status and antioxidant capacity

Compared to the SC group, HF rats were characterized by suppressed SOD and GSH activity and up-regulated MDA levels (Table 4). Parallel to the increased oxidative status, the OSI in HF rats was high, characterized by the increased TOC/TAC ratio (Fig. 4). Nebivolol reduced the

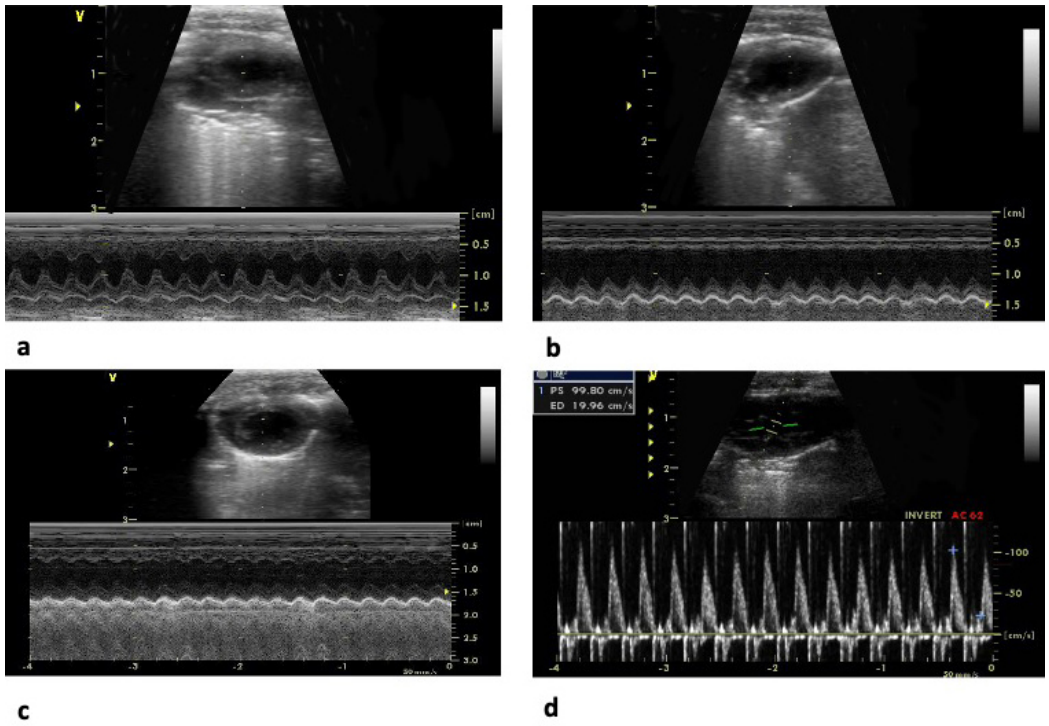

Fig. 2. Representative echocardiograms. (a) Normal anterior and posterior wall motion amplitudes in the SC group; (b) Anterior wall akinesia in the HFC group; (c) Anterior wall dyskinesia in the HFNEB group; (d) Restrictive diastolic dysfunction characterized by increased mitral E wave and decreased mitral E wave flow velocity on pulsed doppler measurement in the HFC group.

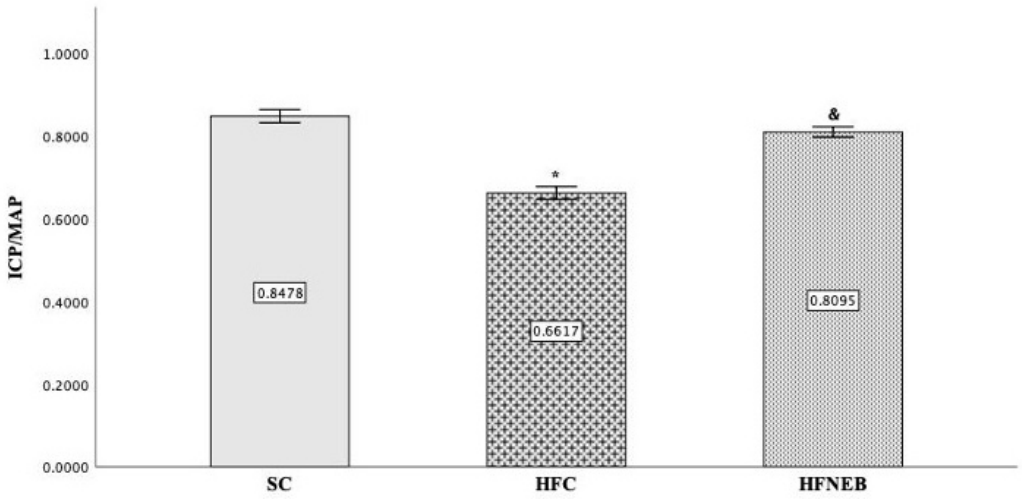

Fig. 3. Erectile functions characterized by ICP/MAP. Compared to SC group, HFC rats were characterized by decreased ICP/MAP ratio. Nebivolol treatment prevent this decrease ($p < 0.05$ *compared to SC, $^{\&}$compared to HFC).

MDA levels and enhanced SOD and GSH activity (Table 4). In this group, OSI was also lower than the HFC group ($p <$ 0.05 for all comparisons) (Fig. 4).

3.6 Penile tissue NO, ONO_2^- and cGMP levels

When compared to the SC, NO and ONO_2^- levels increased and cGMP levels decreased significantly in the HFC group ($p < 0.05$). On the contrary, nebivolol treatment reduced the tissue NO, ONO_2^- levels and prevented the reduction in cGMP levels significantly ($p < 0.05$) (Fig. 5).

3.7 Histological assessment of penile tissue and evaluation of NOS isoforms

Histologic slices of the penis in the SC group were normal morphology with consisting of three cylindrical vascularized erectile tissue masses: corpus spongiosum that surrounds the penile urethra and two lateral corpora cavernosa which are bordered by a layer of dense collagenous. More-

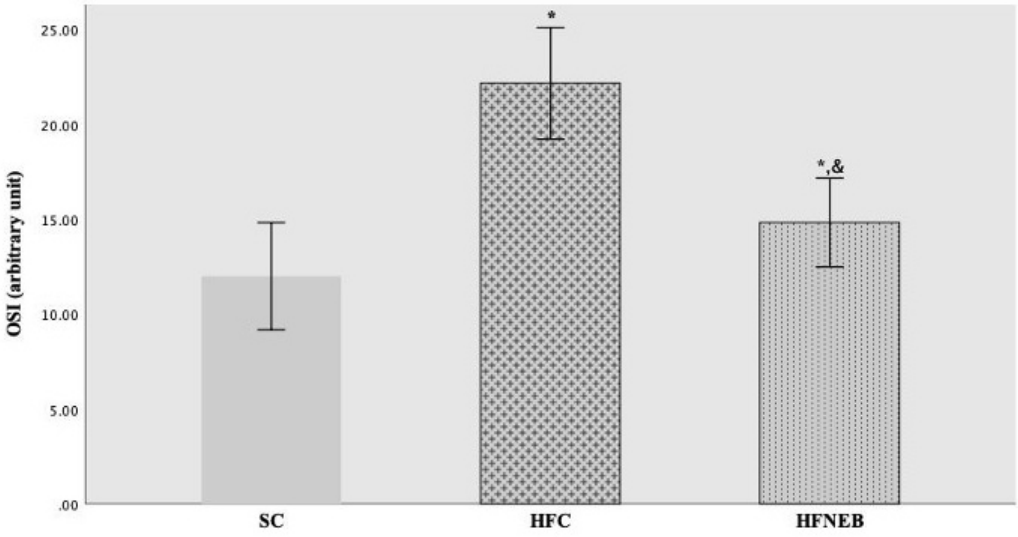

Fig. 4. Oxidative stress status of the groups (OSI). HFC group was characterized by increased OSI. Nebivolol treatment prevented the increase in OSI ($p < 0.05$ *compared to SC, $^{\&}$compared to HFC).

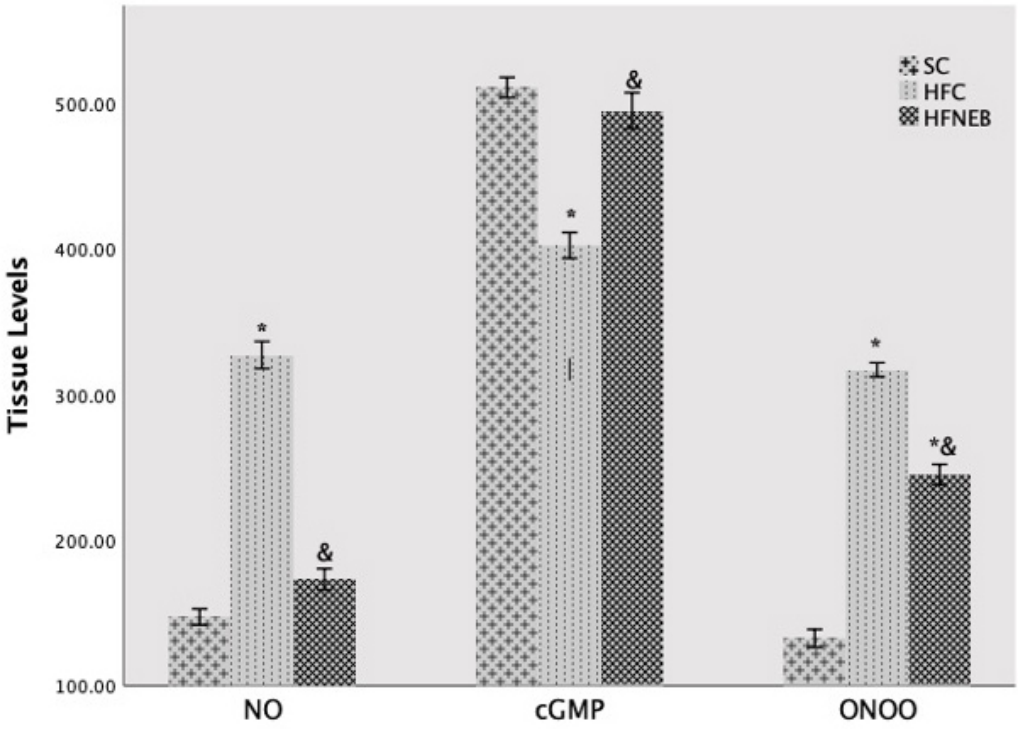

Fig. 5. Tissue NO, ONO_2^- and cGMP levels. Compared to SC group, HFC group of animals were characterized increased NO and ONO_2^- levels together with decreased cGMP. On the contrary, nebivolol treatment reduced the tissue NO, ONO_2^- levels and prevented the reduction in cGMP levels ($p < 0.05$ *compared to SC, $^{\&}$compared to HFC).

over, the corpora are composed of irregular cavernous sinuses lined with endothelial cells and collagenous, elastic smooth muscle fibers, as well as nerves in the trabeculae. Unlike in the SC group, corpus cavernosum was characterized by mild degeneration of endothelial and smooth muscle cells and infiltration of inflammatory cells in the HF group. In the slices taken from the nebivolol treated group, corpus cavernosum was in almost normal morphology. Immunohistochemistry of penile tissue of the SC group showed strong eNOS staining in the endothelium of the cavernosal space, and slight nNOS positive stains along penile cavernosal and dorsal nerves. Slight iNOS immunoreactivity was seen throughout the erectile tissue (Fig. 6). Compared to the SC, the HFC group of rats was characterized by decreased

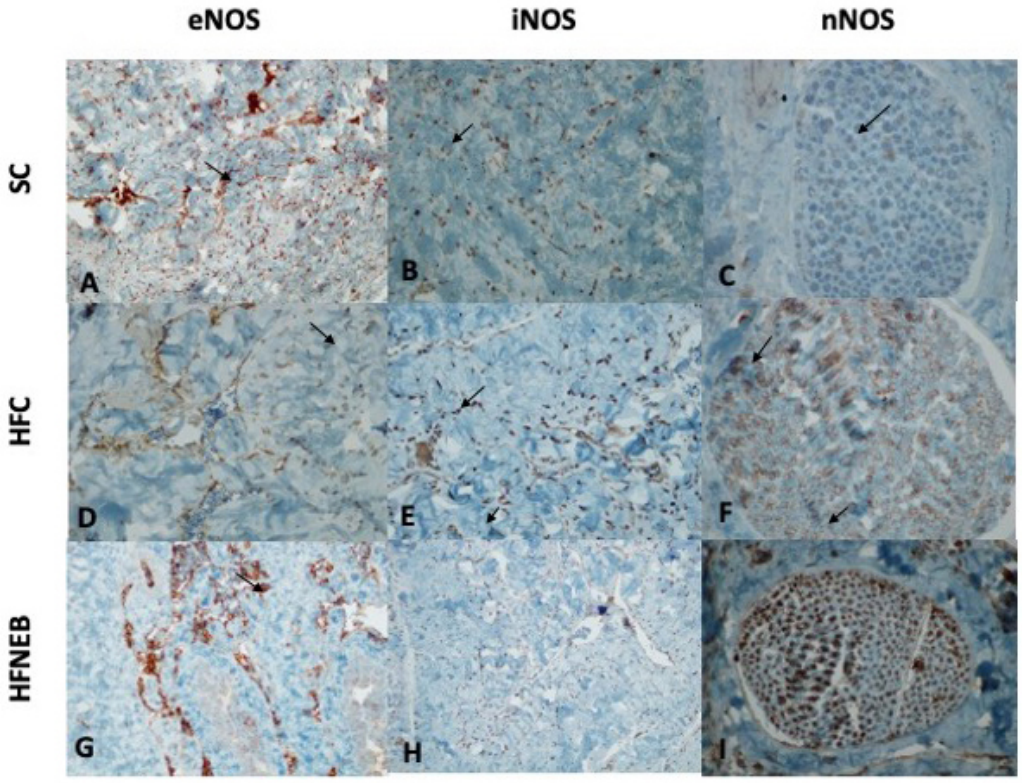

Fig. 6. NOS immunoreactivity of the groups (200x). Red colouring indicates positive immunostaining (black arrows). The individual panels represent penis sections from SC (A, B, C); from HFC (D, E, F) and from HFNEB (G, H, I) group of animals.

Table 3. Erectile functions.

Parameter	SC	HFC	HFNEB
	(n = 6)	(n = 6)	(n = 6)
MAP (mmHg)	109 ± 2.66	112 ± 3.38*	107 ± 1.36&
ICP (mmHg)	92 ± 2.16	74 ± 3.14*	87 ± 2.40*,&

$p < 0.05$ *compared to SC, &compared to HFC.
ICP, Intracavernosal pressure; MAP, Mean arterial pressure.

Table 4. Tissue oxidative status and antioxidant capacity.

Parameter	SC	HFC	HFNEB
	(n = 6)	(n = 6)	(n = 6)
SOD (U/gr tissue)	5.18 ± 0.31	1.95 ± 0.52*	4.86 ± 0.38*,&
GSH (μmole/gr tissue)	2.48 ± 0.17	1.36± 0.26*	2.19± 0.21*,&
MDA (pmole/gr tissue)	0.42 ± 0.35	0.78 ± 0.27*	0.50 ± 0.20 &

$p < 0.05$ *compared to SC, &compared to HFC.
GSH, Glutathione; MDA, Malondialdehyde; SOD, Superoxide dismutase.

eNOS and increased iNOS and nNOS immunoreactivity. Prevention of eNOS, together with an increase in nNOS immunoreactivity was significant in the HFNEB group (Table 5).

4. Discussion

4.1 MI caused heart failure with preserved ejection fraction

HF is caused by cardiovascular disorders such as MI and consists primarily of LV dysfunction, which is characterized by decreased myocardial contractility, increased LVED and decreased CO [15]. LAD ligation causes MI with ventricular tissue loss. The heart starts to collapse if the residual myocardium does not compensate for the loss of contractile tissue [15]. Coronary artery ligation, a widely used small animal HF model [16], is a reliable model to induce tissue damage and HF [17]. Significant HF signs 2–6 weeks after LAD ligation causing MI in more than 40% of the ventricular area have been shown in animal studies [18]. Consistent with these studies, the infraction area was about 49% of the LV in our study.

In rats with HF, a decrease in BW was seen. A loss of more than 7.5% of the normal body weight was shown to be a major indicator of decreased survival in HF [19]. Different causes of weight loss in HF, including reduction in food intake, immune, and neurohormonal activation were previously studied [20]. However, in the present study, we did not investigate the responsible mechanism. In the present study, this group of animals was also characterized by increased LVW/BW which is a sign of pulmonary congestion commonly associated with HF [3]. Moreover, in this

Table 5. Immunolabelling intensities.

Parameter/Time	Staining and localization	Labelling intensity		
		SC	HFC	HFNEB
eNOS	Cytoplasmic staining in the endothelium of the cavernosal space	3+	2+	3+
nNOS	Cytoplasmic and nuclear staining along penile cavernosal and dorsal nerves	1+	2+	3+
iNOS	Cytoplasmic staining throughout the erectile tissue	1+	2+	2+

Labelling intensities: 1+: weak 2+: moderate 3+: strong.

group, anatomical changes, such as increased fibrosis and interstitial proliferation in the histologic slices of the heart were consistent with the functional alterations in echocardiography. In the presented study, significant increase in LVEDP together with protected LV systolic function (characterized with mild reduction in EF) is in line with heart failure with preserved EF [3]. Although we observed a significant decrease in MBP after MI, this decrease is within physiological limits and does not reflect a significant hypotension [21].

4.2 NO played a key role on ED in HF

Close relationship between ED and HF is well known [3]. In-vivo studies have shown decreased ICP/MAP ratio in HF rats [15]. Similar to these studies, in the present study, the HF group of animals developed ED characterized by decreased ICM/MAP. Different mechanisms including hypoperfusion, endothelial dysfunction, increased level of vasoconstrictor substances, medication was studied to contribute to ED in HF [3]. However, more than one mechanism is likely to play a role in this pathology.

Corpora cavernosa, which consists of sinusoidal spaces with trabecular meshwork lined by endothelium are the two corporal bodies of penile erectile tissue [22]. Penile erection develops through two consecutive steps which are coordinated by neurogenic, vascular, and humoral events [7]: (1) the expansion of cavernosal sinusoids with the inflow of the blood results in the enlargement and rigidity of the penis, and (2) the cavernosal veno-occlusion avoids the leakage of blood from the veins until the sexual act is completed. In the first step, as blood flows through the cavernosal arteries into the sinusoids, the corporal sinusoids expand to provide a space for the pooling blood. In the second step, the rise in intracorporal pressure by pooling the blood into enlarged sinusoids shuts the venous channels and reduces the outflow to prevent blood leaking out of the vein. The increase in the inflow of the blood via cavernosal arteries into the corporal bodies and expansion of the sinusoids depends on the relaxation of smooth muscles of both the arterial system and the sinusoids [23].

NO, which is expressed in both nitrergic fibers and endothelial cells, is essential in this coordination [24]. NO, which is formed by nNOS in the terminal axons of the nerve, diffuses into the smooth muscle cells, causing the relaxation of smooth muscles through the activation of cGMP and initiating the penile erection [25]. Erection requires a balance between inflow and the outflow of blood within the sinusoids therefore NO produced by eNOS plays a role both in the initiation and the continuation of the erection [26]. NO produced by eNOS in endothelial cells diffuses into the smooth cells and causes relaxation similar with the nNOS. iNOS mediated NO is also produced in smooth muscle cells and acts on mitochondria. However, its effects are quite different [27]. Although iNOS is generally considered to be expressed in an inflammatory condition and responsible for the nitrosative damage, protecting effect of iNOS either by prevention of apoptosis induced by the oxidative stress or by inhibition of the breakdown of cGMP in the corpus cavernosum has recently been shown [28,29]. Therefore, the role of NO produced by iNOS might be due to the oxidative status of the corpus cavernosum. In our study, while the SC group of animals were characterized by strong eNOS together with weak nNOS and iNOS immunoreactivity, HF induced animals were characterized by decreased eNOS together with increased iNOS and nNOS immunoreactivity. In the HF induced animals, decreased eNOS levels could be the predictor of endothelial injury. Increased ICP/MAP ratio supports this argument. However, NO produced by the nitrergic nerve endings could act as a compensatory mechanism and helps to the smooth muscle relaxation in the corpus cavernosum via activation of cGMP in the HF induced rats. Increased cGMP levels, together with the high NO in the HFC group, support this argument. Furthermore, NO produced by iNOS was the key molecule responsible for the nitrosative damage in the ED induced by HF. Increased $ONOO^-$ and NO levels along with decreased antioxidant capacity (as characterized by decreased SOD and GSH levels) in the HFC group support this argument.

4.3 Nebivolol showed positive effects via NO-mediated mechanisms on ED in HF

Beta-blockers are commonly prescribed to reduce mortality in patients with HF or a history of MI [3]. However, they are associated with the ED. “The Cross-National Survey on Men’s Health Issues” showed that up to 20% of men under the treatment of beta-blocker therapy experience ED [30]. Beta-blockers differ according to their beta-1 receptor selectivity and their additional vasoactive effects. Nebivolol is a third-generation selective beta-1 blocker with additional NO-mediated effects. In our previous studies,

we have shown NO mediated effects of nebivolol in different pathologies, especially in MI [9,12]. Positive effects of nebivolol on ED by enhancing eNOS, reducing oxidative stress at the endothelial level of the corpus cavernosum were shown in animal studies [31]. In line with these preclinical studies, the positive effects of nebivolol were shown in patients with or at risk of developing ED in small sample clinical studies [8]. Conversely, a cross-sectional observational study classifying ED (no ED, mild-moderate-severe ED) in hypertensive patients treated with different beta blockers (atenolol, carvedilol, bisoprolol, metoprolol, and nebivolol) showed that though ED was less common with nebivolol, moderate ED was more common with nebivolol, bisoprolol, and atenolol [32]. Therefore, the NO-mediated effects of nebivolol on ED and the suggested mechanism is controversial. We argue that the main reason for these controversial results is the use of different experimental setups, especially in preclinical studies. In the present study, the MAP levels in nebivolol treated animals were similar to those in the SC group. This may result from the use nebivolol at a minimum beta-blocker dose in this study. Conserved eNOS and ICP/MAP levels in animals treated with nebivolol indicated that the eNOS-mediated effect of nebivolol is the dilatation of the cavernous body in addition to the increasing vasodilation-mediated intracavernosal blood flow. Preserved cGMP levels in this group of animals also support this argument. When compared to the SC and HFC groups, the most prominent increase in the nebivolol-treated group was observed in nNOS immunoreactivity. As discussed earlier, NO produced by nNOS in the terminal axons of the nerve innervating the corporal smooth muscle diffuses into the smooth muscle cells and causes the relaxation of smooth muscles [25]. Therefore, it can be concluded that the positive effect of nebivolol on ED developed after HF is largely accompanied by NO-releasing effect of nebivolol from the nitrogenic nerve endings. Unlike nNOS levels, iNOS levels in the nebivolol treated group were the same as HFC and higher than the SC group. In this group, although the antioxidant levels (SOD, GSH) were higher and the oxidant status (MDA, OSI) were lower than the HFC, these parameters were still significantly different from those of the SC group. Therefore, unlike similar studies [33] in our study, the positive effect of nebivolol was not a result of the reduction in nitrosative/oxidative stress in the corpus cavernosum.

Although molecular techniques (i.e., Western blot analysis) are superior to immunohistochemistry as they allow for quantitative analysis, identifying the distinct cellular localization of protein alterations is the advantage of the immunohistochemical methods. Therefore, since this study aimed to determine the mechanism, immunohistochemistry was preferred.

5. Conclusions

Our study shows that nebivolol has positive effects on ED developed in HF. The NO-mediated mechanisms underlying this effect can be summarized as eNOS mediated dilation of the cavernous body and nNOS mediated smooth muscle relaxation. While NO produced by nNOS in the terminal axons of nitrergic nerves causes the relaxation of smooth muscle cells of the corpus cavernosum and initiates the erection, eNOS mediated dilation of the cavernous body helps to maintain the erection. To the best of our knowledge, this study is the first in the literature to discuss all three NOS isoforms in order to explain the NO-mediated effects of nebivolol in ED.

In conclusion, in addition to the favorable hemodynamic profile, unlike other beta-blockers, NO-mediated effects of nebivolol can help to minimize ED, which is a typical co-morbidity of HF.

Abbreviations

BW, body weight; CO, cardiac output; cGMP, cyclic guanylate cyclase; ED, erectile dysfunction; EF, ejection fraction; eNOS, endothelial nitric oxide synthase; GSH, glutathione; HF, heart failure; HE, hematoxylin eosin; HW, heart weight; ICP, intracavernosal pressure; iNOS, inducible nitric oxide synthase; LAD, left anterior descending coronary artery; LV, left ventricle; LVEDd, left ventricular end diastolic dimension; LVEDV, left ventricular end diastolic volume; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; MAP, mean arterial pressure; MDA, malondialdehyde; MI, myocardial infarction; MS, massons' trichrome; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; SOD, superoxide dismutase; OSI, oxidative stress index; ONO_2^-, peroxynitrite; TAC, total antioxidant capacity; TOC, total oxidant capacity.

Author contributions

GM and CM designed the research study. GM and CM performed the research. GG provided help and advice on the histological and immunohistological experiments. GH analyzed the data. GM, GG and CM wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Istanbul Medipol University Animal Experiments Local Ethics Committee (approval number: 95).

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Conflict of interest

The authors declare no conflict of interest.

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