Differential Effects of Ivabradine and Metoprolol on Cardiovascular Remodeling and Myocardial Infarction Induced by Isoprenaline in Chronic N-nitro-L-arginine Methyl Ester (L-NAME) Treated Rats

Nevein Hendawy*, Amany Helmy*, Hala Salah Abdel Kawy*, Ghada Karouk Mohamed#, Ahmed El Sayed Badawy*, Hoda Sallam*
*Department of Pharmacology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
#Department of Histology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

A B S T R A C T

Background: Elevated heart rate is associated with cardiovascular morbidity and mortality in the general population and in patients with cardiovascular disease.

Aim: This study was designed to investigate the effects of the two bradycardiac agents ivabradine and metoprolol on cardiovascular changes and the infarction size induced by isoprenaline in chronic N-nitro-L-arginine methyl ester (L-NAME) treated rats.

Methods: Four groups of male Wistar rats were studied: the 1st group served as a normal control, the 2nd received L-NAME (100 mg/kg), the 3rd group was treated with the same dose of L-NAME plus ivabradine (10 mg/kg) and the 4th group was treated with the same dose of L-NAME plus metoprolol (150 mg/kg). All treatments were administered daily by gastric gavage. After 6 weeks of L-NAME and drug treatment myocardial infarction was induced by isoprenaline injection (11 mg/100g/day for 2 consecutive days). The following parameters were assessed; systolic blood pressure, electrocardiographic changes, cardiac enzymes, and histopathological examination of heart tissues, aorta & coronary vessels. Moreover, vascular reactivity of the isolated aortic rings to phenylephrine and acetylcholine was tested.

Results: Ivabradine and metoprolol administration to L-NAME/isoprenaline treated rats significantly reduced heart rate, microvascular remodeling, infarct size, serum lactate dehydrogenase, serum creatine kinase and attenuated the mortality resulting from isoprenaline-induced infarction. Pretreatment with ivabradine had non-significant effect on L-NAME-induced hypertension and cardiac hypertrophy, in contrast to metoprolol pretreatment. Selective heart rate reduction with ivabradine improved endothelial dysfunction, and reduced atherosclerotic plaque formation in L-NAME treated rats. On the contrary, metoprolol showed insignificant improvement of endothelial dysfunction as evidenced by assessment of mean EC_{50} and E_{max} of phenylephrine-induced contraction and by considering the mean percent of acetylcholine-induced relaxation in comparison to the L-NAME/isoprenaline treated group.

Conclusion, these results suggest that ivabradine has a significant protective effect against isoprenaline-induced myocardial infarction with improvement of endothelial dysfunction in chronic L-NAME-treated rats.

Key Words: Ivabradine, Metoprolol, NG-nitro-L-arginine methyl ester (L-NAME), myocardial infarction, isoprenaline

Corresponding Author: Amany Helmy Email: helmy_amany@yahoo.com

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1. INTRODUCTION

Heart rate is a major determinant of myocardial oxygen demand, coronary blood flow and myocardial performance. Increased heart rate, irrespective of the underlying trigger, plays a direct role in the pathophysiology of ischemic events, such as myocardial ischemia, clinical manifestations of coronary artery disease, atherosclerosis and
ventricular dysrhythmias. Several clinical trials support an association between increased resting heart rate and a broad range of maladaptive effects on the function and structure of the cardiovascular system, independently of other risk factors or other potentially confounding demographic and physiological characteristics (Reil et al., 2010).

Early experimental studies established the causal connection between heart rate and lipid-induced atherogenesis in primates (Beere et al., 1992). Moreover, high resting heart rate is a strong predictor for total cardiovascular mortality and morbidity in patients with coronary artery disease (CAD). In the acute stage of myocardial infarction (MI), a high heart rate at the time of hospital admission has been found to correlate with mortality. Indeed, heart rate is a critical determinant of myocardial oxygen demand. Since ~80–90% of myocardial perfusion occurs during diastole, prolonging diastolic duration by heart rate reduction (HRR) may optimize coronary blood flow and myocardial perfusion. Therefore, HRR is likely to have anti-ischemic effects that are beneficial for both angina pectoris and MI (Christensen et al., 2009). Also, it is an independent risk factor for development of plaque rupture (Feldman et al., 2010).

ß-Blockade reduces heart rate and attenuates myocardial ischemia, resulting in improved blood flow and contractile function and reduced infarct size. However, ß-blockade does not achieve the full potential that might be expected from the increase in the duration of diastole. This is because of the negative lysitropic (reduction in the rate of isovolumic ventricular relaxation) action of ß-blockade. When the diastolic pressure–time integral was considered as the driving force for coronary blood flow, any slowing of isovolumic ventricular relaxation at any given diastolic duration will impede coronary blood flow, and this is in fact a true problem for ß-blockade. Moreover, ß-blockade increases or unmasks α-vasoconstriction. Unmasking of α-adrenergic coronary vasoconstriction in situations of increased sympathetic activity such as stress and exercise then actively reduces coronary blood flow and contributes to the precipitation of acute myocardial ischaemia (Heusch, 2008). These undesired effects of ß-blockade have prompted the development of drugs which reduce heart rate more selectively.

The only selective bradycardic agent which is currently available for clinical use is ivabradine which inhibits the hyperpolarization-activated pacemaker current (I_{f}) selectively in the sinoatrial node. I_{f} is responsible for the early phase of diastolic depolarization of sinoatrial cells via both sodium and potassium ions during hyperpolarization. It slows the diastolic depolarization by blocking the ionic channel from the inside (Suli & Timmis, 2006).

Ivabradine, in contrast to ß-blockers, has been shown to improve myocardial perfusion without negative inotropic effects while maintaining cardiac contractility (Fox et al., 2006). It has pronounced anti-anginal and anti-ischemic efficacious that are at least equal to those of currently available drugs for the treatment of ischemic angina, such as ß-blockers and calcium channel antagonists. Moreover, it is better tolerated than ß-blockers or calcium channel antagonists, which have more side effects and contraindications in some patients with CAD (Christensen et al., 2009).

Data for ivabradine effects on infarct size and cardiovascular morbidity in hypertensive patients are not available. Whether the beneficial effects of heart rate reduction with ivabradine are applicable to patients with hypertension with cardiovascular remodeling is not known. Therefore, the aim of the present study was to investigate the effects of ivabradine on cardiovascular changes and the infarction size induced by isoprenaline in chronic nitric oxide synthase inhibited rats by N-nitro-L-arginine methyl ester (L-NAME) for 6 weeks. The main features of this model in cardiovascular system are myocardial remodeling (hypertrophy/fibrosis), vascular remodeling (medial thickening/perivascular fibrosis), and hypertension (Numaguchi et al., 1995). Some aspects of vascular pathophysiological and pathobiological events occurring after L-NAME administration are similar to those seen in the course of human arteriosclerosis (Kataoka et al., 2004). In addition, the pathophysiological and morphologic alterations in the heart of this non-coronary myocardial necrotic rat model induced by isoprenaline are comparable with those taking place in human myocardial infarction (Karthikeyan et al., 2007).

2. MATERIALS AND METHODS

2.1. Drugs and chemicals:

Ivabradine and metoprolol were generously provided by Servier (France) and Astra Zeneca (Canada) respectively. L-Nitro Arginine Methyl Ester (L-NAME), isoprenaline, urethane, L-phenylephrine hydrochloride and acetylcholine were purchased from Sigma Chemicals (USA).

2.2. Experimental Animals:

All animal procedures were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine. Male Wistar rats
(weighing 200 to 250 g) purchased from National Research Institute (Cairo, Egypt) were housed in an animal room with a temperature (22 °C) and lighting (12 h light–dark cycle) control. An adaptation period of 1 week for vehicle (tap water) administration, blood pressure measurements and body weight assessment was allowed before initiation of the experimental protocol.

2.3. Experimental Procedures

The aim of the present study was to create a model simulating what happens in humans. The scenario could be started with a hypertensive state with endothelial dysfunction which could extend to the coronaries with an increased incidence of development of coronary artery disease.

Accordingly, a model of chronic inhibition of NO synthesis in male Wistar rats was induced by daily administration of L-NAME (100 mg/kg/day by gavage) for six weeks. L-NAME was given orally to ensure that the whole daily dose was delivered. Myocardial infarction was induced at the end of six weeks by intra-peritoneal administration of isoprenaline (11mg/100g/day) for two successive days. Treatment with either ivabradine or metoprolol lasted for all the 6 weeks (L-NAME) and 2 days (isoprenaline).

2.3.1. Study Design:

Four groups of rats were studied (10 rats each). The normal control group received untreated chow and drinking water. L-NAME group received the NO synthase inhibitor L-NAME (100 mg/kg) for a period of 6 weeks. The ivabradine group received same dose and protocol of L-NAME plus ivabradine (10 mg/kg) (Ulu et al., 2009). The metoprolol group received same dose and protocol of L-NAME plus 150 mg/kg/day metoprolol (Ulu et al., 2009). All treatments were dissolved in water and administered daily by gavage.

2.3.2. Animal Model of Chronic Inhibition of NO Synthesis

NG-nitro-L-arginine methyl ester (L-NAME) was given to rats by gavage (100 mg/kg/day) for a period of 6 weeks guided by (Niumaguchi et al., 1995). A pilot study was planned to determine the duration of L-NAME administration. We found that after 4 weeks of administration, there was a non-significant media thickening in coronary vessels. Yet, at the 8th week there was a very high incidence of mortality especially when isoprenaline was injected later for induction of myocardial infarction. So in this study, L-NAME was given for 6 weeks (At this time there was significant media thickening in coronary vessels.)

2.3.3. Induction of myocardial infarction

Myocardial infarction was induced in all the L-NAME treated groups of rats (6 weeks) by injecting isoprenaline (11 mg/100g/day) intraperitoneally for 2 successive days after the 6 weeks treatment with L-NAME (Kumar & Anandan, 2007). Mortality rate after isoprenaline administration was determined.

2.3.4. Parameters measured

2.3.4.1. Systolic Blood Pressure Measurement

Systolic blood pressure of rats was indirectly measured from the tail of conscious rats at the baseline and by the end of the 6th week, before isoprenaline injection to show the effect of the tested drugs against L-NAME induced hypertension. This was done by non-invasive blood pressure monitor by the tail cuff technique (ML 125 NIBP, AD Instruments, Australia). The inflated cuff pressure was computed using power lab/85p (ML 785 software program). The average of at least three measurements was taken at each occasion.

2.3.4.2. Heart Rate & Electrocardiographic (ECG) Changes:

After 24 hours of the second isoprenaline injection, the rats were anesthetized with urethane 1.2g/kg intraperitoneally. Each of the four limbs was connected to an electrode of ECG apparatus (Siemens Cardiostat) by mean of a needle. The ECG apparatus was switched on to standard limb lead II and run at a paper of speed 25 mm/sec and ECG was recorded for calculating HR and ECG changes.

2.3.4.3. Body weight: Body weights of animals were recorded at the beginning of the study and at the end of the 6th week plus 2 days.

2.3.4.4. Ventricular Weight & Histopathological Studies:

- Tissue preparation: At the end of the experimental period, the chest of the rats in all groups was rapidly opened. The heart was excised. The descending thoracic aorta was removed and divided into two parts, one for isolated organ bath study and the other for histological examination. The excised heart was washed off blood with physiological saline. The ventricles were separated from the atria and great vessels, to be weighed and the ventricle weight/body weight (VW/BW) ratio was calculated. The ventricles, the cardiac apex, the small coronary arteries and the thoracic aorta were taken and fixed in 10% formaldehyde and dehydrated with graded concentrations of alcohol for embedding in paraffin.

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Blocks were done from which 5 µm thick sections were cut and stained for light microscopic examination by Hematoxylin-Eosin stain (H&E) and Masson’s trichome stain.

- Morphometric study: The cross-sectional area of ventricular myocytes; the infarction area size and the intima/media ratio of thoracic aorta were assessed. Signs of atherosclerotic changes were assessed by relative change in the thickening of the coronary arteries and perivascular fibrosis surrounding the blood vessel. N.B: It was studied in small coronary arteries (with internal diameters <200 µm). These were done in 10 fields in 6 sections from all rats of each group, using image analyzer (Leica Q500MC programat X1000).

2.3.4.5. Isolated rat aortic ring preparation

The descending thoracic aorta was removed to a Petri dish containing modified Krebs solution continuously gassed with 95% O₂ and 5% CO₂ and was carefully cleaned of adherent connective tissue to avoid injury of the endothelial layer. Rings about 4-5mm width were prepared and mounted between 2 parallel hooks made of stainless steel wire in a 20ml water jacketed organ bath filled with modified Krebs’ solution consisting of (mmol/l) NaCl, 118.2; KCl, 4.6; CaCl₂,2H₂O, 1.6; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 24.8 and glucose, 10.0) continuously gassed with 95% O₂ and 5% CO₂ and temperature was adjusted to 37°C (Bhattacharya et al., 2001). An initial resting tension of 2 grams was set and the rings were then allowed to equilibrate for 1 - 2hours. Incubation solution was routinely changed every 15 min as a precaution against interfering metabolites. Isometric responses were measured with a force transducer (K30, Hugo Sacks Electronics, Freiburg, Germany) connected to a bridge coupler type 570 and the trace was displayed on a two channel recorder (Lineacorder, HSE, WR3310).

After the equilibration period, a cumulative dose response curve of phenylephrine ((0.5×10⁻³M–1.6×10⁻⁸M) was constructed by addition of phenylephrine to the bathing fluid in gradually increasing doses, then the mean effective concentration 50 (EC₅₀) and the maximal contractile response (Eₘₐₓ) to phenylephrine were determined. Endothelium-dependent relaxation was tested by applying acetylcholine (10⁻⁸M–3.2×10⁻⁷M) after adding the dose of phenylephrine that causes sub-maximal contraction. Percent of acetylcholine induced relaxation in isolated aortic rings were determined.

2.3.4.6. Estimation of serum Creatine Kinase MB isoenzyme (Ck-MB) and lactate dehydrogenase (LDH)

By the end of the study (6 weeks plus 2 days) blood samples were collected from all groups under urethane anesthesia from the ophthalmic venous plexus through retro-orbital approach according to Timm (1979). Blood samples were allowed to clot and centrifuged (5 min, 5000 rpm) and the serum was separated. CK-MB level was determined by ELISA (Enzyme-linked immunosorbent assay) and LDH level was determined spectrophotometrically according to method described by Wroblewski and La Due (1955), using diagnostic kits Signiosis Inc, USA and Randox Laboratories, UK respectively.

2.3.5. Statistical Analysis:

Statistical analysis was carried out using Graph pad prism, software program, version 5.0 (2007), Inc., CA, USA. All values in the results will be expressed as means ± SEM. For all parameters, statistical difference among groups will be determined using one way analysis of variance; ANOVA followed by Benferroni’s multiple comparisons test. P values < 0.05 will be considered statistically significant.

In the experiment of phenylephrine-induced contraction, all doses were transformed into Log values and the contractile responses for each preparation were expressed as a percentage of the maximum response achieved by each ring separately, which is considered in this case, the 100% response of that particular ring; the next step was to plot the log concentration against the responses expressed as percentages, in a linear regression curve, then the mean effective concentration 50 (EC₅₀) was determined.

3. RESULTS

Five of the ten rats of the L-NAME/Isoprenaline group died spontaneously between day 5 and week 6 of treatment. The myocardium of 3 rats that died before 4th week of treatment showed no significant structural changes and the myocardium of 2 rats that died between 4th and 6th weeks of treatment had structural changes comparable to those in rats that survived for 6 weeks. Two and three rats died in the ivabradine and metoprolol groups during the period of treatment respectively.

3.1. Systolic blood Pressure:

It is clear from table (1) that L-NAME administration produced significant increase of the
systolic blood pressure by 62.3% by the end of 6th week in comparison to control group. Metoprolol administration showed significant decrease of the systolic blood pressure by the end of 6th week in comparison to L-NAME/Isoprenaline group. Meanwhile ivabradine had insignificant effect against L-NAME induced hypertension.

3.2. Heart Rate & Electrocardiographic changes:

Isoprenaline administration (11 mg /100 kg/day i.p. for 2 days) at the end of the 6th week induced significant (P <0.05) increase of heart rate by 30.9% compared to the control group. In contrast the ivabradine (10mg/kg/day) and metoprolol (150 mg/kg) treated groups produced significant (P <0.05) decrease of heart rate by 26.3% &24.4% respectively compared to the L-NAME/Isoprenaline group. ECG records of rats in control group showed a normal ECG pattern. Rats treated with L-NAME and isoprenaline showed a marked elevation in ST segments. These changes were restored to near normal in ivabradine and metoprolol pretreated isoprenaline-induced myocardial infarction rats when compared to L-NAME isoprenaline-alone induced myocardial infarction (Table 1& Fig. 1).

3.3. Ventricle Weight / Body Weight ratio

Table (1) shows that animals’ ventricle weight/body weight ratio increased significantly (P <0.05) in the L-NAME/Isoprenaline group by 38.4% compared to the control group. Treatment with ivabradine produced non-significant (P >0.05) decrease of ventricle weight/body weight ratio by 3.28%. In contrast, metoprolol treatment produced significant (P<0.05) decrease of ventricle weight/body weight ratio by 14.3% compared to the L-NAME/Isoprenaline group. Animals in the L-NAME/Isoprenaline group lost body weight during the course of this study.

3.4. Cardiovascular Histopathological Changes

3.4.1. The Heart and Small Coronary Arteries:

As shown in figures 2&3, H&E stained cardiac sections of the L-NAME /isoprenaline treated rats, showed areas of degeneration and mononuclear cellular infiltration in the wall of the left ventricle. The cardiac muscle fibers showed marked vacuolation with pale acidophilic sarcoplasm with deeply stained, small and pyknotic nuclei. Scattered focal areas of myocardial damage in the form of fragmented fibers and interstitial hemorrhages were also detected with congested markedly thickened coronaries. Examination of different sections stained with Masson trichrome revealed that collagen fibers contents were increased with marked coronary perivascular fibrosis.

H&E stained sections in ivabradine treated rats showed small areas of degeneration and mononuclear cellular infiltration, with vacuolated cardiomyocytes and deeply stained small pyknotic nuclei in the wall of the left ventricle, with less congested thickened small coronaries. Using Masson trichrome stain, there was mild interstitial and coronary perivascular fibrosis. In metoprolol treated rats, H&E stained sections showed small areas of degeneration and signs of infarction. The small coronaries were less thickened and congested. Masson trichrome stain revealed that there was mild interstitial fibrosis and coronary perivascular fibrosis

3.4.2. Cardiomyocyte diameter and infarction area size

L-NAME (100mg/kg/day) followed by isoprenaline (11 mg/100g/d for 2 days) produced 67% significant (p<0.05) increase of the mean value of cardiomyocyte diameter (µm), in comparison to control group. Ivabradine (10 mg/kg/day) produced 2.4% non-significant (P >0.05) change of the mean value of the cardiomyocyte diameter (µm). Metoprolol (150 mg/kg/day) produced 28.3% significant (P<0.05) decrease of the mean value of the cardiomyocyte diameter (µm), compared to L-NAME/isoprenaline group. The mean size of infarct area in L-NAME/isoprenaline group was 450.3±2.3 (µm). Ivabradine and metoprolol produced significant (P <0.05) decrease of the mean value of the mean size of infarct area (µm) by 55.1% and 56% respectively compared to L-NAME/isoprenaline group, with no significant difference between the two treated groups (Table 2).

3.4.3. The Thoracic Aorta:

Table 2 & Figure 4 (H&E stained sections of the L-NAME /isoprenaline treated rats) revealed irregularity in the wall of the aorta with increased wall thickness. Apparent increase in the intima media ratio with many areas of vacuolation was seen under the intima and marked intramural fibrosis in Masson trichrome stained sections. On the other hand, H&E stained sections in ivabradine pretreated rats showed that the wall of the aorta was more or less regular with an apparent decrease in the intima media ratio. No areas of vacuolation were noted with little intramural fibrosis in Masson trichrome stained sections. Meanwhile, in metoprolol pretreated rats, H&E stained sections showed that the aorta had slight wall irregularity with mild apparent increase in the intima media ratio. Small areas of vacuolation were also noticed with areas of intramural fibrosis in Masson trichrome stained sections.
L-NAME/isoprenaline group produced significant (p<0.05) increase of the mean value of aortic intima/media ratio by 255% from control group. Ivabradine and metoprolol pretreated groups showed significant (p<0.05) decrease of the mean value of aortic intima/media ratio by 56.2% and 43.8% compared to L-NAME/isoprenaline group respectively. Ivabradine group showed significant (p<0.05) decrease compared to the metoprolol group.

3.5. Isolated aortic ring preparation

Table 3 and Figure 5a&b show that, in the isolated aortic ring preparation, L-NAME administration resulted in a significant (P<0.05) decrease in the mean EC50 of phenylephrine-induced contraction and a significant (P<0.05) increase of the maximal contractile response to phenylephrine (E_max) by 72.4 % and 116.1 % compared to the control group respectively. On the other hand, the mean % relaxation produced by acetylcholine significantly (P<0.05) decreased by 57.8% compared to control group.

Ivabradine-treated group displayed a significant (P<0.05) increase of the mean EC50 of phenylephrine-induced contraction and a significant (P<0.05) decrease of the maximal contractile response (E_max) by 209.7 % and 44% compared to the L-NAME group respectively. Besides, ivabradine produced significant (P<0.05) increase in the mean % value of acetylcholine-induced relaxation by 124.2 % in comparison to L-NAME/Isoprenaline group.

On the other hand, the isolated aortic rings preparation of metoprolol treated group showed non-significant (P>0.05) decrease in the mean EC50 of phenylephrine induced contraction and non-significant (P>0.05) decrease of the Emax by 12.5% and 1.65% compared to the L-NAME group respectively. The mean % value of acetylcholine induced relaxation of the isolated aortic ring preparation was insignificantly (P >0.05) decreased in the metoprolol group by 4.5% compared to L-NAME/Isoprenaline group.

3.6. Serum Creatine Kinase MB isoenzyme (CK-MB) and Lactate Dehydrogenase (LDH)

There was a significant (P < 0.05) increase in the levels of CK-MB and LDH in the serum by 198.7% & 63.4% in the L-NAME/Isoprenaline group compared to the control group respectively. Treatment with ivabradine or metoprolol produced significant (P<0.05) decrease in serum CK-MB by 60.7% & 57.9% respectively compared to the L-NAME/Isoprenaline group. Also ivabradine and metoprolol produced significant (P<0.05) decrease in serum LDH (U/L) by 33.3 % and 30% respectively compared to the L/NAME/Isoprenaline group (Figure 6, A&B).

Table: (1). The effect of tested drugs on systolic blood pressure, heart rate and ventricle weight /body weight ratio in L-NAME induced hypertension in rats

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Timing</th>
<th>Control Group (n=10)</th>
<th>LNAME/Isoprenaline Group (n=5)</th>
<th>Ivabradine Group (n=8)</th>
<th>Metoprolol Group (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic Blood Pressure (mm Hg)</td>
<td>Day 0</td>
<td>118±0.5</td>
<td>116.6±0.6</td>
<td>119.2±0.6</td>
<td>120.4±0.5</td>
</tr>
<tr>
<td></td>
<td>6th week</td>
<td>121.4±1.2</td>
<td>196.8±2.7*</td>
<td>187.8±3.1</td>
<td>139.7±2**</td>
</tr>
<tr>
<td></td>
<td>2 days</td>
<td></td>
<td>(+62.3%)</td>
<td>(-9%)</td>
<td>(-57%)</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>6th week plus 2 days</td>
<td>369.3±2</td>
<td>483.8±2.2*</td>
<td>356.4±2.1**</td>
<td>365.3±1.8**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(+31%)</td>
<td>(-26.3%)</td>
<td>(-24.5%)</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>6th week plus 2 days</td>
<td>222±4.3</td>
<td>220±4.8</td>
<td>217±4.39</td>
<td>219±4.5</td>
</tr>
<tr>
<td>Ventricular Weight (mg)</td>
<td>800±0.05</td>
<td>982±0.03</td>
<td>986±0.01</td>
<td>998±0.01</td>
<td></td>
</tr>
<tr>
<td>Ventricular Weight (mg)/Body Weight (g) Ratio</td>
<td>2.42±0.01</td>
<td>3.35±0.3*</td>
<td>3.24±0.14</td>
<td>2.87±0.09**</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean± SEM; n=number of rats. The control group received chow and drinking water. The L-NAME group received (L-NAME) (100mg/kg). The ivabradine group received L-NAME and 10 mg/kg ivabradine. The metoprolol group received L-NAME and 150 mg/kg metoprolol. All treatments were administered daily by gavage for 6 weeks. Statistical analysis was done using ANOVA followed by Bonferroni’s test.*P <0.05 versus control group; **P < 0.05 versus L-NAME/Isoprenaline Group. a= mean % change from control group, b= mean % change from L-NAME/isoprenaline.
### Table 2: Effect of ivabradine versus metoprolol on cardiomyocyte diameter, size of infarct area and aorta intima/media ratio in L-NAME/isoprenaline treated rats

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Cardiomyocyte Diameter (μm)</th>
<th>Size of Infarct Area (μm)</th>
<th>Thoracic Aorta Intima/media ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>7.6±0.08</td>
<td>0.09±0.001</td>
<td></td>
</tr>
<tr>
<td>L-NAME/Isoprenaline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group n=5</td>
<td>12.7±0.08*</td>
<td>450.3±2.3</td>
<td>0.32±0.005*</td>
</tr>
<tr>
<td>(67%)a</td>
<td></td>
<td></td>
<td>(+225.6%)a</td>
</tr>
<tr>
<td>Ivabradine Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=8</td>
<td>12.4±0.02</td>
<td>202±2.2**</td>
<td>0.14±0.001**</td>
</tr>
<tr>
<td>(-2.4%)b</td>
<td></td>
<td></td>
<td>(-55.1%)b</td>
</tr>
<tr>
<td>Metoprolol Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=7</td>
<td>9.1±0.05*</td>
<td>194.7±2.3*</td>
<td>0.18±0.001*</td>
</tr>
<tr>
<td>(-28.3%)b</td>
<td></td>
<td></td>
<td>(-56.2%)b</td>
</tr>
</tbody>
</table>

Data presented as means± SEM; n =number of rats. The control group received chow and drinking water. The L-NAME/isoprenaline group received N-nitro-L-arginine methyl ester (L-NAME) (100mg/kg/day) for 6 weeks followed by isoprenaline (11mg/100g/d/ i.p for two successive days). The Ivabradine group received L-NAME for 6 weeks followed by isoprenaline and 10 mg/kg/d ivabradine. The Metoprolol group received L-NAME followed by isoprenaline and 150 mg/kg/day metoprolol. All treatments were administered daily by gavage for 6 weeks plus 2 days. Statistical analysis was done using ANOVA followed by Bonferroni’s test. *P < 0.05 versus. Control group; **P < 0.05 versus. L-NAME/Isoprenaline group.  a= mean % change from control group. b= mean % change from L-NAME/Isoprenaline group.

### Table 3: Effect of ivabradine versus metoprolol on vascular reactivity of the isolated aortic ring preparation in L-NAME/isoprenaline treated rats

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Effective concentration 50 (EC_{50}) of PE (x10^{-5}M)</th>
<th>Maximal contraction (E_{max}) of PE (g)</th>
<th>Percent relaxation induced by Ach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group n=10</td>
<td>1.13±0.14</td>
<td>0.56±0.017</td>
<td>88.28±0.5</td>
</tr>
<tr>
<td>L-NAME/Isoprenaline</td>
<td>0.73±0.19*</td>
<td>1.21±0.021*</td>
<td>37.17±0.7*</td>
</tr>
<tr>
<td>Group n=5</td>
<td>(-35.4%)a</td>
<td>(+116.1%)a</td>
<td>(-57.8%)</td>
</tr>
<tr>
<td>Ivabradine Group</td>
<td>0.97±0.08*</td>
<td>0.68±0.005*</td>
<td>83.24±0.4*</td>
</tr>
<tr>
<td>n=8</td>
<td>(+32.8%)b</td>
<td>(-44%)b</td>
<td>(+124.2%)b</td>
</tr>
<tr>
<td>Metoprolol Group</td>
<td>0.78±0.07</td>
<td>1.19±0.005</td>
<td>35.47±0.5</td>
</tr>
<tr>
<td>n=7</td>
<td>(+68%)b</td>
<td>(-1.65%)b</td>
<td>(-4.5%)b</td>
</tr>
</tbody>
</table>

Data are presented as means± SEM; n =number of rats. The control group received chow and drinking water. L-NAME/isoprenaline group received L-NAME (100mg/kg/d) followed by isoprenaline (11mg/100g/d/ i.p for two successive days). Ivabradine group received L-NAME followed by isoprenaline and 10 mg/kg/d ivabradine. Metoprolol group received L-NAME followed by isoprenaline and 150 mg/kg/day metoprolol. All treatments were administered daily by gavage for 6 weeks plus 2 days. *P<0.05 versus. control normal group; **P<0.05 versus L-NAME/isoprenaline group. Using ANOVA followed by Bonferroni’s multiple comparison test. Phenylephrine (PE) concentrations (0.5x10^{-5}M-1.60x10^{-4}M), Acetylcholine (ACH) concentrations (10^{-3}M-32x10^{-6}M). a= mean % change from control group. b= mean % change from L-NAME/Isoprenaline group.
**Figure 1:** Effect of ivabradine and metoprolol on changes in electrocardiographic (ECG) patterns in isoprenaline (ISO)-induced myocardial infarction in L/NAME treated rats. (A) **Control Group** showing normal ECG patterns (HR=375 beats/min); (B) **L-NAME/Isoprenaline Group** treated with L-NAME for 6 weeks and ISO (11mg/100mg/day i.p. for two successive days) - showing a marked ST-segment elevation (HR=495 beats/min); (C) **Ivabradine Group** (10 mg/kg) + ISO showing a moderate ST-segment elevation (HR=358 beats/min); (D) **Metoprolol Group** (150 mg/kg) + ISO showing a near normal ECG patterns (HR=370 beats/min). All treatments were administered daily by gavage for 6 weeks plus 2 days. Paper speed 25 mm/sec.

**Figure 2:** Photomicrographs of sections in rat's left ventricle isolated from: **Control Group** (A) **H&E X 125 (A1):** Shows branching and anastomosing cardiac muscle fibers with uniform diameter. **Masson trichrome stain X 125 (A2):** Little content of collagenous fibers in between the cardiac muscle. **H&E X 250 (A3):** Transverse cut muscle fibers with acidophilic cytoplasm and central vesicular nuclei (↑). Notice the diameter of the cardiomyocytes. **L-NAME/Isoprenaline Group (B) H&E X 125 (B1):** Shows large areas of mononuclear cellular infiltration (↑) due to infarction. **Masson trichrome stain X 125 (B2):** Marked increase in the collagen fiber content in the infract area (↑). **H&E X 250 (B3):** Transverse muscle fibers having marked vacuolation of the sarcoplasm (↑) with deeply stained small and pyknotic nuclei (◄) and areas of interstitial hemorrhage are clearly identified (*). Notice the apparent increase in the cardiomyocyte diameter in relation to the control group. **Ivabradine Group (C) H&E X 125 (C1):** Shows small areas of mononuclear cellular infiltration (↑). **Masson trichrome stain X 125 (C2):** Mild increase in the collagen fiber content in the infract area (↑). **H&E X 250 (C3):** Transverse muscle fibers having vacuolation of the sarcoplasm (↑) with some deeply stained small and pyknotic nuclei (◄) and areas of mononuclear cellular infiltration (*). Notice the apparent increase in the cardiomyocyte diameter in relation to L-NAME Group. **Metoprolol group (D) H &E X 125 (D1):** Shows small areas of mononuclear cellular infiltration (↑). **Masson trichrome stain X 125 (D2):** Moderate increase in the collagen fiber content in the infract area (↑). **H &E X 250 (D3):** Transverse muscle fibers having little vacuolation of the sarcoplasm (↑) with few small and pyknotic nuclei (◄) and areas of mononuclear cellular infiltration (*). Notice the apparent increase in the cardiomyocyte diameter in relation to the L-NAME/Isoprenaline Group.
Figure 3: Photomicrographs of sections in rat’s small intra-cardiac coronary arteries isolated from:

(A) **Control Group.**
(B) **L-NAME/Isoprenaline Group.** Notice the apparent increase in the wall to lumen ratio and perivascular fibrosis in relation to the control group.
(C) **Ivabradine Group.** Showing an apparent decrease in the wall to lumen ratio and perivascular fibrosis in relation to the L-NAME/Isoprenaline Group.
(D) **Metoprolol Group.** Showing an apparent decrease in the wall to lumen ratio and perivascular fibrosis in relation to the L-NAME/Isoprenaline Group.

Masson trichrome stain  X 125.

Figure 4: Photomicrographs of sections in rats’ thoracic aorta isolated from **Control Group** (A) **Masson trichrome stain** X 250(A1): Shows collagenous fibers content in the wall of the aorta. **H&E X 250 (A2):** Shows The three tunics of the wall of the aorta from inside outward are: tunica intima (1), tunica media (2) and tunica adventitia (3); **H&E X 35 (A3)** More or less round regular wall of the thoracic aorta. (B) **L-NAME/Isoprenaline Group, Masson trichrome stain** X 250 (B1): Shows marked perivascular fibrosis (↑) and many areas of intramural collagen deposition (●). Many vacuolated areas of fat deposition in the wall of the aorta (↑). **H&E X 250 (B2):** Notice apparent increase in the intima media ratio. **H&E X 35 (B3):** Shows irregular thickening in the wall of the aorta. (C) **Ivabradine Group, Masson trichrome stain** X 250(C1): shows mild perivascular fibrosis (↑) and few areas of intramural collagen deposition (●). Few small vacuolated areas of fat deposition in the wall of the aorta (↑). **H&E X 250(C2):** Notice the decreased intima media ratio in comparison to L-NAME group **H&E X 35(C3):** Mild thickening in the wall of the aorta. (D) **Metoprolol Group, Masson trichrome stain** X 250(D1): Moderate perivascular fibrosis (↑) and areas of intramural collagen deposition (●). Some vacuolated areas of fat deposition in the wall of the aorta (↑). **H&E X 250(D2):** Notice the intima media ratio. **H&E X 35(D3):** Shows moderate thickening in the wall of the aorta.
Differential Effects of Ivabradine and Metoprolol on Cardiovascular Remodeling

Figure (5a): Cumulative response curves for phenylephrine (0.5×10^{-5} M-1.6×10^{-4} M) on isolated rats’ aortic rings. Numbers on the arrows indicate, (1)=0.5×10^{-5} M, (2)=1×10^{-5} M, (3)=2×10^{-5} M, (4)=4×10^{-5} M, (5)=8×10^{-5} M, (6)=1.6×10^{-4} M).

Figure (5b): Endothelial-dependent relaxation induced by acetylcholine (1×10^{-7} M-3.2×10^{-6} M) on isolated rats’ aortic rings precontracted by phenylephrine * (4×10^{-5} M): Numbers on the arrows indicate, (1)=1×10^{-7} M, (2)=2×10^{-7} M, (3)=4×10^{-7} M, (4)=8×10^{-7} M, (5)=1.6×10^{-6} M, (6)=3.2×10^{-6} M (Acetylcholine)

(A) Control group. (B) L-NAME/Isoprenaline group. (C) Ivabradine Group. (D) Metoprolol Group.

Chart speed = 5mm/min, Tension= 2g.
Figure 6 (A&B): The effect of ivabradine versus metoprolol on serum cardiac markers in L-NAME/isoprenaline treated animals. (A) Serum CK-mb (ng/ml). (B) Serum LDH (U/L).

Control group received chow and drinking water. L-NAME/Isoprenaline (ISO) Group: received N-nitro-L-arginine methyl ester (L-NAME) (100mg/kg). Ivabradine group received L-NAME and 10 mg/kg ivabradine. Metoprolol group received L-NAME and 150 mg/kg/day metoprolol. Data are presented as mean±SEM. All treatments were administered daily by gavage for 6 weeks plus 2 days. *P <0.05 versus Control group; #P < 0.05 versus L-NAME/Isoprenaline group using ANOVA followed by Bonferroni’s multiple comparison test.

4. DISCUSSION

In the current work, chronic inhibition of NO synthesis with L-NAME in normotensive male Wistar rats produced significant increase of systolic blood pressure (SBP). Treatment with ivabradine elicited non-significant effect on SBP. This agrees with the work done by Albaladejo et al. (2003) who demonstrated that spontaneously hypertensive rats (SHRs), receiving ivabradine for 28 days, showed a non-significant decrease in SBP. On the other hand, the present work illustrated that metoprolol produced significant decrease in SBP. A previous study showed that metoprolol attenuated the arterial hypertension induced by prolonged ingestion of L-NAME in rats (Erley et al., 1995). This also coincides with Fasullo et al. (2009) who proved that patients treated with metoprolol had a significantly reduced SBP in comparison to ivabradine that has no effect on blood pressure.

Twenty four hours after the last injection of isoprenaline to L-NAME treated rats in the present work, there was a significant increase of heart rate with electrocardiographic signs of infarction (in the form of elevated ST segment and widened QRS complex). Both ivabradine and metoprolol administration produced significant decrease in heart rate as well as mortality rate in comparison to L-NAME/isoprenaline group which showed high mortality rate. The results of the present study go parallel with Mączewski and Mączewski (2008) who proved that ivabradine and metoprolol attenuated heart rate increase in a rat model of myocardial infarction (MI) and also decreased mortality rate. Also Ulu et al. (2009) showed that both metoprolol and ivabradine produced nearly comparable heart rate reduction either in early or late treatment in a model of MI.

Langenbach et al. (2006) assessed the effects of either metoprolol or ivabradine once 15 min after experimental occlusion of coronary artery (CAO) and another time after 28 days of treatment in a rabbit model of MI. They showed that the ST segment displacement and the Q waves that appeared in MI group had disappeared in ivabradine and metoprolol groups with significant reduction of heart rate.

In the present study, there was significant increase in the ventricular weight/body weight ratio in L-NAME treated rats. Ivabradine produced non-significant change in ventricular weight/body weight ratio. Mulder et al. (2004) demonstrated that in a model of congestive heart failure in rat induced by coronary artery ligation, the increased of LV weight was not modified by ivabradine treatment; as ivabradine improved the increased LV collagen density at the expense of increased LV capillary density. However, Dedkov et al. (2007) explained that the increase in VW/BW ratio associating ivabradine treatment in a rat model of MI was likely due to decrease in animal’s body weight. Ulu et al. (2009) correlated the lower body weight in ivabradine treated rats with the decrease in water intake in treated animals; explaining that the rat disliked the taste of the drugs.

On the other hand, the present study showed that metoprolol significantly decreased the ventricular weight/body weight ratio. Mustonen et al. (2010) proved that metoprolol attenuated the elevated relative ventricular weight in a rat model of hypertensive
cardiac hypertrophy. Mączewski and Mączewski (2008) reported that metoprolol and ivabradine similarly improve LV function, although metoprolol prevented LV dilation and hypertrophy in the post-infarction rat heart.

Ivabradine and metoprolol treatment reduced areas of degeneration and mononuclear cellular infiltration with mild interstitial and coronary perivascular fibrosis in comparison to L-NAME/isoprenaline group with no significant difference between the two groups. Metoprolol reduced the cardiomyocyte diameter and infarct area compared with L-NAME/isoprenaline group. However, ivabradine did not reduce the cardiomyocyte diameter but reduced the infarct area. The wall of aorta of ivabradine pretreated rats was more or less regular with an apparent decrease in the intima media ratio and no areas of vacuolation were noted with little intramural fibrosis. However in the metoprolol treated rats the aortae had slight wall irregularity with mild apparent increase in the intima media ratio. Small areas of vacuolation were also noticed with areas of intramural fibrosis.

Heusch et al. (2008) demonstrated that ivabradine pre-treatment in anaesthetized pigs subjected to 90 min controlled left anterior descending coronary artery hypoperfusion and 120 min reperfusion significantly reduced the infarct size. Albaladejo et al. (2003) demonstrated that the thoracic aortic wall thickness and medial cross sectional area were significantly lowered and even reached the normal range in spontaneously hypertensive rats treated with ivabradine for 28 days.

Baumhäkel et al. (2010) found that ivabradine prevented aortic atherosclerotic lesions and reduced plaques when given simultaneously with a high cholesterol diet, and also when given to animals for 4 wks after initiation of a high-cholesterol diet. This was referred to the antioxidative effects of ivabradine. The in-vivo effects of ivabradine were absent at a dose that did not lower heart rate (Custodis et al., 2008) that correlates the HR reduction with corresponding anti-atherosclerotic effects and decreased oxidative stress. These results may, in part, explain the significant decrease of myocardial infarction (MI) observed in patients treated with ivabradine (Fox et al., 2008). Walcher et al. (2010) demonstrated that ivabradine reduces the atherosclerotic plaque formation as it inhibits chemokine-induced migration of CD4-positive lymphocytes which is a critical step in atherogenesis by interfering with its signaling pathway.

In a model of hypertensive cardiovascular remodeling in rats pretreated with metoprolol for 7 weeks, it partially prevented the development of LV hypertrophy attenuating both increased cardiomyocyte diameter and cardiac fibrosis induced in the hypertensive rats (Kobayashi et al., 2004). This effect was related to the direct antitrophic effect of metoprolol by directly inhibiting the hypertrophic mediators including the adrenergic (Brítow, 1997), renin-angiotensin-aldosterone (Blumenfeld et al., 1999) and endothelin (Krum et al., 1996) systems as well as various inflammatory cytokines (Prabhhu et al., 2000). In addition, Chan et al. (2011) found that chronic metoprolol treatment decreased the increased thoracic aortic wall thickness, without affecting endothelium dependent relaxation in the spontaneously hypertensive rats. The antiatherogenic effects of metoprolol could be due to β-blockade in different systems as the central nervous system leading to a reduction in peripheral sympathetic nerve discharge; the heart leading to hemodynamic changes caused by reduced heart rate, blood pressure, and contractility; and biochemical systems leading to reduced atherogenic activity (i.e) increased production of prostacyclins (Pettersson et al., 1991). Inhibition of platelet accumulation (Pettersson & Björk, 1992) and decreased affinity of LDL to proteoglycans in the vessel wall as the atherogenic effect of sympathetic activation can be assumed to result from a complex interaction of hemodynamic factors and an array of biochemical processes (Lindén et al., 1990).

Results of the present work showed that ivabradine treatment improved the aortic vascular reactivity manifested by significant increase of the mean EC_{50}, decrease of E_{max} and increase in the mean % of acetylcholine induced relaxation of the isolated aortic ring. On the contrary metoprolol produced non-significant effect on the vascular reactivity in comparison to the untreated group. These results were consistent with those obtained by Drouin et al. (2008) who showed that 3-months treatment of young dyslipidemic mice with ivabradine had a protective effect on endothelial function with no effect on lipid profile. One of the possible explanations is that chronic treatment with ivabradine potently inhibited vascular oxidative stress. It induced marked inhibition of nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase) activity and superoxide release. In addition, vascular lipid peroxidation as a global marker of oxidative stress was significantly decreased.

Baumhäkel et al. (2010) demonstrated that chronic ivabradine treatment prevented the endothelial dysfunction in isolated aortic ring of high-fat cholesterol-rich diet -induced atherosclerosis in ApoE/mice. The authors proved that ivabradine restored eNOS and prevented the increased reactive oxygen species (ROS).
Recently, the protective role of ivabradine in an animal model of chronic stress regarding the endothelial function and ischemic brain injury was evaluated. Mice were randomized to the I_{1}-channel inhibitor underwent a chronic stress protocol for 28 days. Ivabradine proved to restore endothelial function. Ivabradine improved the vascular lipid hydroperoxides and NADPH oxidase activity which were upregulated in the stressed mice. Also the stress reduced aortic endothelial nitric oxide synthase mRNA and the increased AT1 receptor mRNA were both attenuated by ivabradine (Custodis et al., 2011).

The chemokine monocyte chemotactic protein-1 (MCP-1) provides a link between endothelial dysfunction and atherosclerotic lesion formation by inducing leukocyte arrest and trans-endothelial migration (Wung et al., 2005). Ivabradine treatment resulted in a potent down-regulation of MCP-1 expression (Custodis et al., 2008). Drouin et al. (2008) excluded the endothelial protective effects of ivabradine to be secondary to the reduction of heart rate. As metoprolol treatment had no effect on endothelial function. This was explained by the fact that the inhibitory effect of β-adrenoceptor antagonist on endothelial cell β-adrenoceptor mediated activation of endothelial NO synthase, is a β_{2}-mediated process. Metoprolol has only six fold selectivity for β_{1} over β_{2}-adrenoceptors (Smith & Teitler, 1999).

5. CONCLUSION

The present study showed that chronic administration of ivabradine significantly reduced the structural and the functional cardiovascular changes induced by L-NAME without significant decrease in blood pressure. Indeed, ivabradine treatment decreased the perivascular fibrosis, the wall thickness of aortae and small coronaries in parallel with improvement of the endothelium dysfunction induced by L-NAME. In addition, ivabradine decreased myocardial fibrosis, even though it did not affect LV hypertrophy. It reduces the serum cardiac enzymes, electrocardiographic signs and infarct size associated with isoprenaline induced infarction. On the other hand chronic treatment with metoprolol induced a significant decrease of systolic blood pressure. Metoprolol treatment decreased the perivascular fibrosis, thickness of the wall of small coronaries and aortae.

Recommendations

Future clinical research may be directed towards investigating the use of ivabradine in treatment of endothelial dysfunction and atherosclerosis. Our results provide impetus for testing the impact of ivabradine in association of classic medication in treatment of myocardial infarction.

6. REFERENCES


Custodis, F., Baumhäkel, M., Schlimmer, N., List, F., Gensch, C., Böhm, M., Laufs, U., 2008. Heart rate reduction by ivabradine reduces oxidative stress, improves endothelial function, and prevents...


