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The Effects of Nebivolol on Apoptosis in a Rat Infarct Model

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Background In the present study, nitric oxide (NO) was investigated to see if it mediated effects of nebivolol on apoptosis in the rat myocardial infarction (MI) model.

Methods and Results Rats were divided into 3 groups: sham operated (sham-control), MI-induced (MI-control) and nebivolol treated (MI-nebivolol). The initial dose of nebivolol was administrated intravenously (iv) within 10 min of post-MI reperfusion and continued orally for 28 days. NO mediated effects of nebivolol were assessed either in the early (2nd day) or sub-acute (28th day) period of MI by histologic, hemodynamic and biologic studies. Left ventricular (LV) pressure changes were prevented with nebivolol (the increase in LV end-diastolic pressure and the decrease in maximum rise and fall rate of LV pressure (+dp/dt and –dp/dt) was significantly less in MI-nebivolol). Total and regional apoptotic indexes were significantly lower in the MI-nebivolol group (10.2 vs 7.1%, respectively on the 2nd day; p=0.004). Although plasma nitrite/nitrate, cyclic guanylate cyclase and peroxynitrite concentrations were high both in MI-control and MI-nebivolol groups on the 2nd day, these concentrations were decreased to the basal value on the 28th day in the MI-nebivolol group.

Conclusion As a result, nebivolol treatment (initially by iv within 10 min of reperfusion and continued orally) reduced the myocardial apoptosis after MI. This beneficial effect of nebivolol is mediated by NO regulation. (*Circ J* 2008; **72:** 660–670)

Key Words: Apoptosis; Myocardial infarction; Nebivolol; Nitric oxide

After myocardial infarction (MI), a series of structural changes (expansion and thinning of the infracted area) occur[1,2] Although these changes help to maintain the cardiac function at the early phase of MI, when they are not controlled, it might lead to left ventricular (LV) dysfunction[3]

Cellular and molecular mechanisms contributing to the LV dysfunction are not fully understood, apoptosis, both in the infarct and healthy myocardium has been shown as an important role[4,5]

Nebivolol, a selective β-blocker[6] was shown to improve the ventricular function and hemodynamic parameters in patients with LV dysfunction. In addition to β-blocker activity, nebivolol regulates the releasing nitric oxide (NO) by the activation of endothelial NO synthase (e-NOS) in endothelial cells. NO is related not only to the maintenance of healthy endothelial function, but also to apoptosis.

In the present study our aim is to evaluate the NO mediated effects of nebivolol after MI in rats and the role of antiapoptotic mechanisms in this effect.

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Methods

Animal Groups, Dose Selection and Study Duration

Animals were housed in a temperature-controlled animal facility with a 12-h light/dark cycle, with tap water and rodent chow ad libitum and handled in accordance with the Guide for the Care and the Use of Laboratory Animals published by the US National Institutes of Health. All experimental procedures were approved by the Institutional Animal Ethic Committee of Istanbul University Experimental Medical Research Institute.

Twelve-week-old male Sprague Dawley rats, with a mean weight of 250–300 g, were divided in 3 groups of 12 each: sham operated control (sham-control), MI induced (MI-control) and nebivolol treated (MI-nebivolol).

Nebivolol was administrated within 10 min of reperfusion at dose of 0.1 mg/kg iv and continued orally at dose of 2.0 mg/kg, by gastric gavage once daily for 28 days. The iv dose of nebivolol was selected as the minimum β-blocker dose (absence of significant effect on blood pressure and heart rate (HR)), after a preliminary dose-response study, (using 0.1-0.5-1 mg/kg of nebivolol iv) according to Sacco et al (Fig 1)[7] The oral dose was selected according to Sanchez et al[8] Hemodynamic effects of these 2 dose regimes were compared with metoprolol, a β_1-selective adrenoreceptor antagonist (Tables 1,2).

To evaluate the effects of nebivolol on the apoptosis both in acute and sub-acute period of MI, time intervals were selected as 2 days (for acute) and 28 days (for sub-acute) of

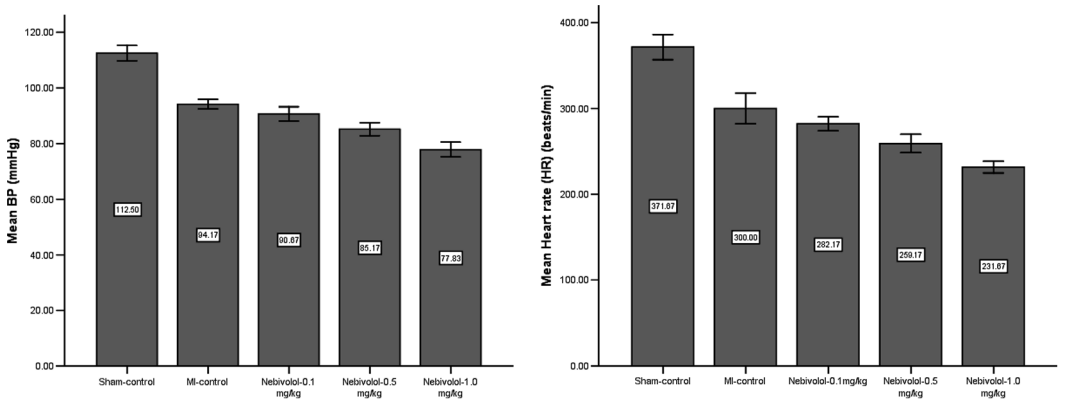

Fig 1. Blood pressure (BP) and heart rates (HRs) recorded during 60 min in the preliminary dose-response study. Although BP and HRs were decreased with the increasing nebivolol dose, this decrease was only statistically significant in the 1.0 mg/kg treated group (p=0.012 and p=0.002, respectively).

Table 1 Comparison of Hemodynamic Effects of Initiation Dose of Nebivolol With Metoprolol in the Preliminary Dose-Response Study

Group	*MBP*	*HR*	*LVEDP*
Sham-control	*120±10*	*355±18*	*5.3±2.3*
Sham-metoprolol 0.1	*112±8*	*338±12*	*6.0±2.2*
Sham-metoprolol 1.0	*106±11**	*325±14**	*4.9±0.9*
Sham-nebivolol 0.1	*115±12*	*345±10*	*5.7±1.3*
Sham-nebivolol 1.0	*108±7**	*332±17**	*4.5±1.1*
MI-control	*102±10**	*310±11**	*15±3.1**
MI-metoprolol 0.1	*89±9**	*286±16**	*12±2.7**
MI-metoprolol 1.0	*80±11*,&*	*220±12*,&*	*7.4±1.1*,&*
MI-nebivolol 0.1	*97±14**	*292±18**	*9.5±0.8*,&*
MI-nebivolol 1.0	*85±12*,&*	*245±22*,&*	*6.8±2.2*,&*

**p<0.05 compared to sham-control group, &p<0.05 compared to MI-control group. Ischemia for 30 min and followed by reperfusion for 60 min. Metoprolol and/or nebivolol was given intravenously at the onset of reperfusion at 2 different doses: 0.1 mg/kg and 1.0 mg/kg.*
MBP, mean blood pressure; HR, heart rate; LVEDP, left ventricular end diastolic pressure; MI, myocardial infarction.

Table 2 Comparison of Hemodynamic Effects of Continuation Dose of Nebivolol With Metoprolol in the Preliminary Dose-Response Study

	Sham-control		*MI-control*		*MI-metoprolol*		*MI-nebivolol*	
	Day 2	*Day 28*	*Day 2*	*Day 28*	*Day 2*	*Day 28*	*Day 2*	*Day 28*
HR	*350±15*	*362±12*	*324±18#*	*318±14*,#*	*306±11#,&*	*284±17*,#,&*	*315±15#*	*298±23#*
LVEDP	*3.5±2.8*	*4.2±3.2*	*25.2±4.7#*	*28.6±5.3*,#*	*13.9±4.2#,&*	*15±3.6#,&*	*13.2±5.1#,&*	*15.8±2.8#,&*
+dp/dt	*6,176±153*	*6,219±187*	*4,314±169#*	*4,070±144*,#*	*4,870±132#,&*	*4,624±176#,&*	*4,723±189#,&*	*4,665±167#,&*

*p<0.05 *compared to day 2, #compared to sham-control group at same point in time, &compared to MI-control group at same point in time. Continuation dose of metoprolol and nebivolol (2 mg/kg) was given orally by gastric gavage during the 28 days after MI.*
+dp/dt, maximum rise of of left ventricle pressure. Other abbreviations see in Table 1.

MI.

Induction of MI

MI was induced by the ligation of the left anterior descending coronary artery (LAD) as described previously[9] After anesthesing with ketamine and xylazine combination, rats were intubated through a tracheotomy and ventilated with a volume-cycled small-animal ventilator (TOPO ventilator, Kent Scientific, Torrington, CT, USA). An anterior thoracotomy was performed, the heart was rapidly exteriorized and LAD was ligated approximately 2 mm from its origin with 6-0 prolene suture, which was released after 30 min. MI was confirmed by regional cyanosis, ST elevation on electrocardiogram, and elevation of serum creatin kinase MB band and troponin T concentrations. After releasing of the ligature, reperfusion was confirmed by myocardial blush over the risk area. Positive end-expiratory pressure was applied to fully inflate the lungs and then the chest was closed in layers. The sham-control rats underwent the same procedure except ligation. All surgical procedures were done under aseptic conditions.

Evaluation of LV Functions

LV functions were evaluated echocardiographically. Animals were lightly anesthetized with ketamine and xylazine combination. Transthoracic echocardiography was performed as described previously[10] Two-dimensionally-guided M-mode echocardiography and pulse-wave Doppler echocardiography were performed with an echocardiographic system equipped with a 10 MHz sector probe (General

Table 3 Left Ventricular Geometry, Volume and Function Infarct Sizes

Parameter/time	Sham-control (n=12)		MI-control (n=12)		MI-nebivolol (n=12)	
	Day 2	Day 28	Day 2	Day 28	Day 2	Day 28
LVEDd (cm)	0.67±0.09	0.68±0.04	0.67±0.07	0.74±0.10#	0.65±0.06	0.69±0.02&
LVEDv (ml)	0.70±0.04	0.66±0.05	0.78±0.06#	0.81±0.04#	0.74±0.12	0.77±0.06#
LVEF (%)	70.17±6.20	69.50±6.20	58.17±7.90#	56.00±6.79#	66.17±3.98#,&	65.60±2.79#,&

p<0.05 #compared to sham-control group at same point in time, &compared to MI-control group at same point in time. LVEDd, left ventricular end-diastolic dimension; LVEDv, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction. Other abbreviation see in Table 1.

Electric, System Five, Horten, Norway). All measurements and calculations were done in accordance with the American Society of Echocardiography.[11]

In Vivo Hemodynamic Parameters

After echocardiographic evaluation, hemodynamic measurements was done as previously described in detail.[12] In brief, LV pressure was recorded by inserting a heparinized water-filled polyethylene-tubing catheter (PE-50) in to the right carotid artery and advanced to the left ventricle. The catheter was connected to the pressure transducer (MLT 0699, PowerLab, ADI Instruments, Oxfordshire, UK). The pressures were recorded on a physiological recorder (10T Hardware System, PowerLab). LV systolic, diastolic, end-diastolic (LVED) and the maximum rise (+dp/dt) and fall (–dp/dt) rate of LV pressure were recorded.

Perfusion-Fixation and Histological Assessments

After the measurements of ventricular pressure, the catheter was pulled back to the aorta and the heart was arrested in diastole with iv KCl injection (3cc, 10% solution). The thorax was opened quickly and the right atrium was cut for the drainage of the blood. The heart was perfused-fixed with 10% phosphate-buffered formalin at a pressure of 7.5 cm H_2O for 1 h. After fixation, the heart was excised quickly and weighed; left ventricle was dissected, weighed, dehydrated and embedded in paraffin.

Infarct Size The 10-μm thin sections were serially cut from the apex to the base at 1-mm intervals, deparaffinized and stained with hematoxylin-eosin (HE) and Masson's trichrome (MS). Samples were evaluated under optic microscope. MI size was average of all slices and expressed as:

Infarct size % = total infarct length/[(epicardial + endocardial lenght)/2] × 100.

Assesment of Apoptosis Apoptotic cardiomyocytes were detected with a terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay in accordance with the manufacturer's instruction (In-situ Cell Death Detection Kit POD, Roche, Basel, Switzeland). Apoptotic cardiomyocytes were counted in the infarct area, in the border zone and in the remote non-infarct area.

Selection of number of sample slices was based on stereological technique.[13] In the preliminary study, 1 whole LV was cut serially in 5 μm thickness and the number of slices determined for 1 left ventricle, and then the sampling rate was determined as 1/10. According to the sampling rate, the first sample was randomly selected within the first 10 slices, then the others were every 10-fold. The apoptotic nuclei were counted in whole slices and for each experimental group 6 hearts were evaluated.

The amount of apoptotic cardiomyocytes were calculated as the percentage of the TUNEL positive cardiomyocyte nuclei from the total number of cardiomyocyte nuclei and were expressed as apoptotic index (AI).[14]

AI was determined in the infarct area (core necrotic area), border zone (the myocardium extending 0.5–1.0 mm from the infarcted area) and remote myocardium. Myocardial area extending 1–2 mm from the border zone was not included in the analysis to avoid contamination.

Electron Microscopy Electron microscopic evaluation was done in 2 rats of each group. Hearts were perfused-fixed with 10% phosphate-buffered formalin containing 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through a passage in a series of graded ethanol, and embedded in Epon. Ultrathin sections were cut with a diamond knife and collected on bare 300-mesh nickel grids. They were stained with uranyl acetate and lead citrate, and examined by an electron microscope.

Biological Assessments In all experimental groups, plasma nitrate/nitrite (NO_x), cyclic guanilate cyclase (cGMP) and nitrotyrosine that the fingerprint of peroxynitritte ($ONOO^-$), concentrations were measured.

Plasma NO_x, cGMP and $ONOO^-$ measurements were done using the commercially available kits as manufacturer's instruction (Nitric Oxide Colorimetric Assay, Roche; Zymed Laboratories Inc, South San Francisco, CA, USA and HBT, HyCuit Biotechnology, USA, respectively).

Statistical Analysis

All variables are expressed as mean ± SD. Differences among groups were assessed by ANOVA with a Scheffe's test. The differences from basal values were assessed by Mann–Whitney U test. A value of $p<0.05$ in a 2-tailed distribution was considered statistically significance. Correlation coefficiency was calculated by Pearson correlation analysis.

Results

A total of 78 animals were included in the study. MI was confirmed by the increase in trophonin T concentrations. There was no difference in trophonin concentrations between groups (2.13 and 2.35 ng/ml for MI-control and MI-nebivolol groups, respectively; $p>0.05$).

There was no death in the sham-control group. In MI groups, mortality were 15% and 10% for MI-control and MI-nebivolol groups, respectively. In the infarct groups death was seen within the first 24 h of infarction.

LV Functions

Echocardiograhic assessments of LV geometry and function are shown in Table 3. When compared to sham-control rats, MI-control animals exhibited significant LV structural changes as thickening of the remote non-infarct myocardial wall. These changes were statistically significant as early as

Table 4 Hemodynamics, Ventricular Weights

Time/parameter	Sham-control (n=12)		MI-control (n=12)		MI-nebivolol (n=12)	
	Day 2	Day 28	Day 2	Day 28	Day 2	Day 28
HR (beats/min)	331±23	333±21	306±27#	295±22#	313±19#	287±34#
LVEDP (mmHg)	2.0±0.23	2.3±0.3	28.3±4.1#	33.7±1.7#	12.5±4.0#,&	15.2±3.6#,&
MBP (mmHg)	109.1±7.4	110.1±6.7	96.5±8.8#	102.8±8.3#	90.3±10.0#	95±8.1#
Δ+dp/dt (mmHg/min)	6,716±574	6,600±352	4,078±411#	3,831±398*,#	4,613±291#,&	4,765±278#,&
Δ-dp/dt (mmHg/min)	5,308±595	5,058±618	2,691±346#	2,861±268*,#	3,354±339*,&	3,410±258#,&
LVW (mg)	609±33	701±42*	822±48#	912±68*,#	658±23#	723±36*,&
LVW/HW (mg/g)	0.76±0.02	0.73±0.03	0.94±0.07#	0.83±0.04#	0.79±0.06&	0.80±0.03
Infarct size (%)	–	–	43.66±5.3	52.33±4.9	44.21±6.5	47.66±3.5

*p<0.05 *compared to day 2, #compard to sham-control group at same point in time, &compared to MI-control group at same point in time.*

Δ+dp/dt, maximum rise of left ventricle pressure; Δ-dp/dt, maximum fall of left ventricle pressure; LVW/HW, left ventricle weight to heart weight ratio. Other abbreviations see in Table 1.

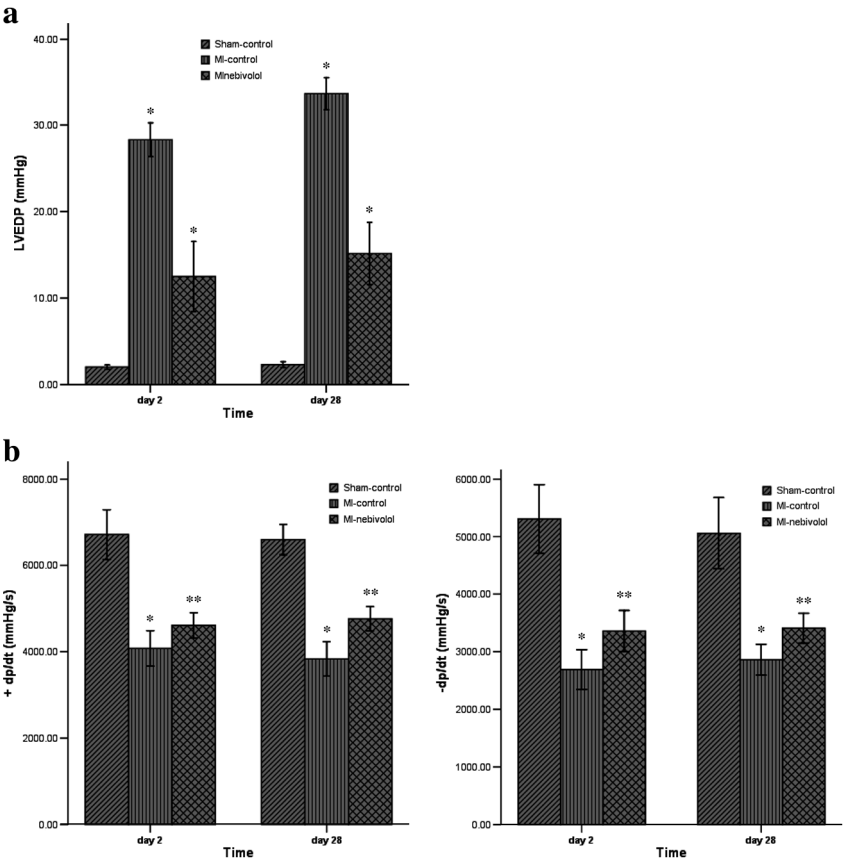

Fig 2. (a) Left ventricle end-diastolic pressure (LVEDP) of the 3 groups. Significant increase was seen in the myocardial infarction (MI)-control group soon after MI, this trend was observed throughout the study period (*p<0.005 compared to sham-control). (b) The +dp/dt and –dp/dt values measured in the 3 groups. Although both +dp/dt and –dp/dt were decreased significantly in the MI-control group as early as 2 days, this decraese was significantly less in the MI-nebivolol group. Furthermore, although these values were further decreased to the 28th day in MI-control group, this decrease was prevented in MI-nebivololgroup (*p<0.05 and **p<0.01 compared to the sham-control group).

day 2 post-MI. Functional abnormalities in MI-control rats were in accordance with the structural changes: ejection fraction (EF) was significantly decreased immediately after MI (2nd day). This trend continued for 28 days when compared with the sham-control group. LV structure and functions were maintained in MI-nebivolol group both in acute (on day 2 after MI) and sub-acute (on day 28 after MI) period of MI. In contrast to MI-control, LV dimensions and functional parameters (EF) were not significantly changed in the MI-nebivolol group during the study period.

In Vivo Hemodynamic Parameters

Hemodynamic parameters measured in the 3 groups of rats are shown in Table 4. Compared with the sham-control animals although mean blood pressure (MBP) was significantly decreased as early as 2 days; this trend was not continued to the 28th day. Most dramatic changes were seen in LVED pressure (LVEDP), +dp/dt and –dp/dt values of MI-control rats. Although LVEDP was significantly increased, +dp/dt and –dp/dt values were decreased soon after MI. This trend was continued throughout the study (Fig 2).

As shown in Table 4, nebivolol treatment slightly, but not significantly, lowered MBP in rats with MI (on the 2nd day: 96.5±8.8 and 90.3±10.0 for the MI-control and MI-nebivolol groups; p>0.05). The increase in LVEDP and the decrease both in +dp/dt and –dp/dt was also prevented in the MI-nebivolol group (Fig 2).

Histological Assessment

Body weights and postmortem cardiac chamber weights

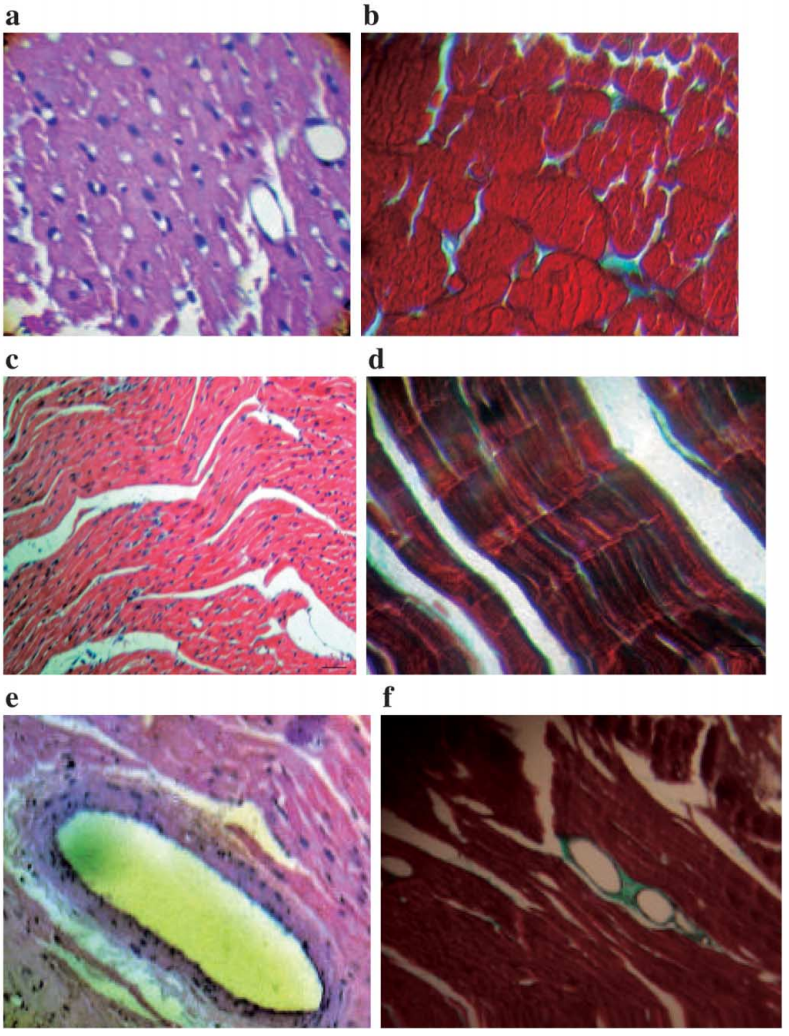

Fig 3. Light microscopic features of all groups in acute period of myocardial infarction (MI). Light micrographs of adjacent normal fibers of sham-control rats with (a) hematoxylin-eosin (HE), (b) Masson's trichrome (MS), (c) wavy fibers with elongation and narrowing of MI-control rats MI with HE, (d) MS mostly prevented muscle fibers of MI-nebivolol group polymorphonuclear leukocyte infiltration in the vascular bed can be seen easily with (e) HE, (f) MS (×400).

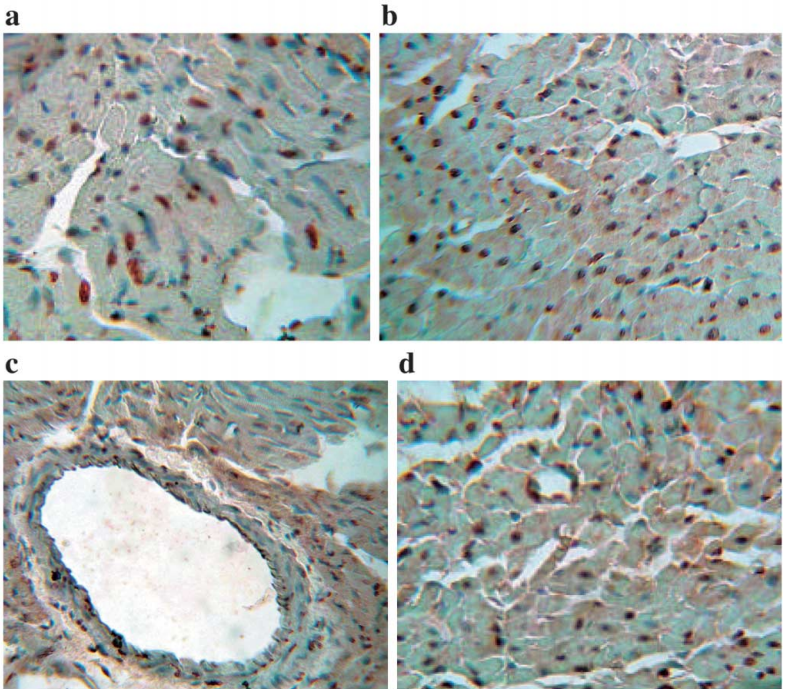

Fig 4. Microscopic features of normal and apoptotic nuclei. Light micrographs of terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL)-positive nuclei stain red, and TUNEL-negative nuclei stain blue. Counterstain was done by hematoxylin. TUNEL positive cell of myocardial infarction (MI)-control group in (a) acute period, (b) sub-acute period of MI, (c) TUNE positive cell of MI-nebivolol group in acute period, (d) sub-acute period of MI. Apoptotic leukocytes in the vascular bed is consistent with the hematoxylin-eosin stain. Prevention of muscle fibers and less apoptotic nuclei is more pronounced.

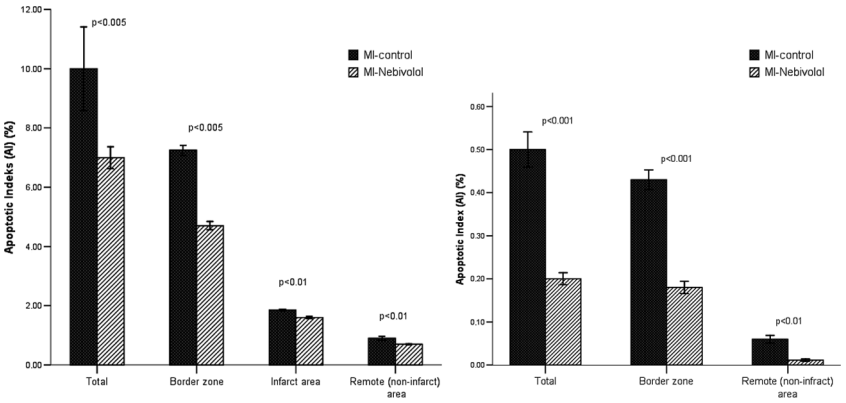

Fig 5. (a) Total and local apoptotic indexes (AI) on day 2 of myocardial infarction (MI). AI in all 3 areas were lower in the MI-nebivolol group in comparison with the MI-control. Total AI was also lower too. (b) AI on day 28 of MI. No apoptotic cell was observed in the infarct area. Total AI and AI in 2 areas were significantly lower in the MI-nebivolol group.

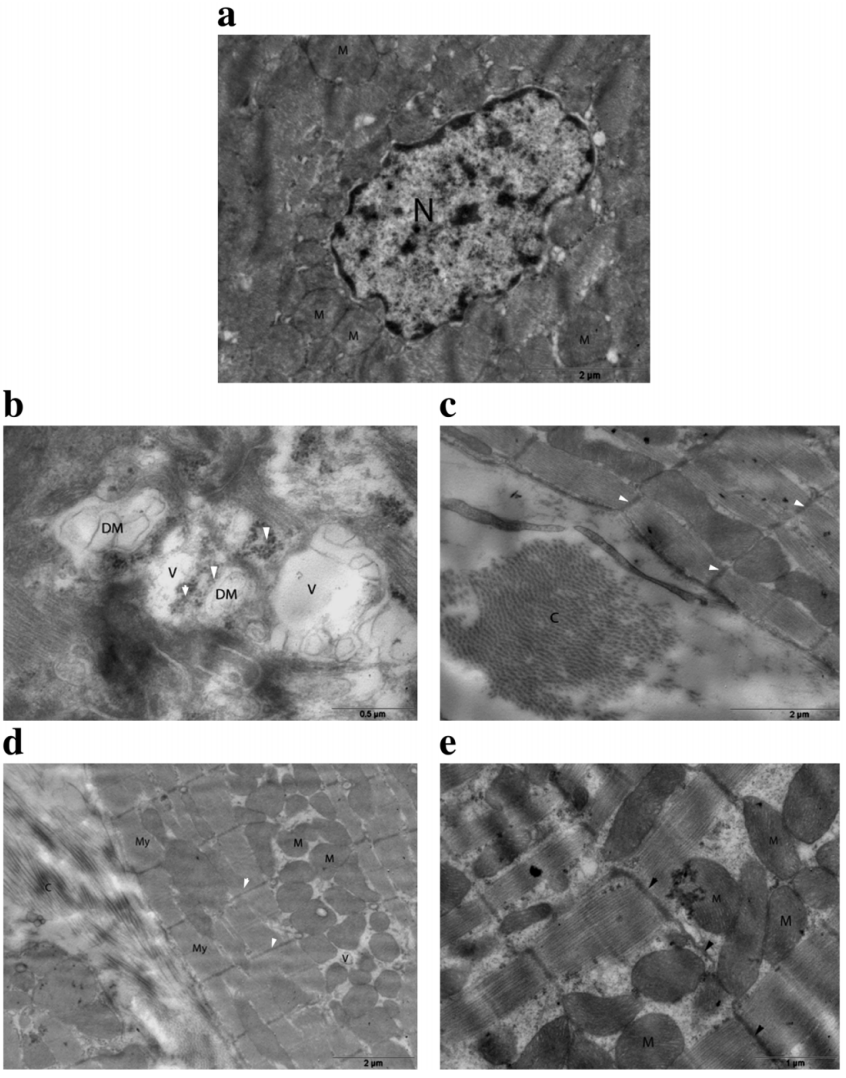

Fig 6. Electron microscopic features of all 3 groups. Electron micrographs of: (a) normal myocytes, with characterised by intact nucleus (N) and mitochondria (M); (b–d) cardiomyocytes of myocardial infarction (MI)-control rats contain vacuoles (V), numerous damaged mitochondria (DM) with loss of electrodense matrix and amorphous matrix densities in the mitochondria (white arrow) early and late collagen accumulation (C); (e) small extend of injury MI-nebivolol group cardiomyocytes where majority of M are intact and mostly preserved Z lines (black arrows).

for 3 groups of rats are shown in Table 4. Although LV weight (LVW) was significantly higher both on the 2nd and 28th days in the MI-control group in comparison with the sham-control rats (609±33 to 822±48 mg on day 2 and 701±42 to 912±68 mg on day 28, respectively), this increase was only significant on the 2nd day in the MI-nebivolol group (609±33 and 658±23 for sham-control and MI-nebivolol groups, respectively). Left ventricle weight to body weight ratio (LVW/BW) was also significantly less in MI-nebivolol group compared with the MI-control group.

The presence of either signs of acute (eosinophilia, caryolysis, leukocyte infiltration) or old (collagen scars) infarction was analyzed either by HE and MS stain in LV transverse sections. In the MI groups, infarct was located in the anterolateral area of the LV (from sub-endocardial to endocardial area). Collagen accumulation was seen in the vascular structure, together with cardiomyocytes both in early and sub-acute phase of MI. In the sub-acute phase of MI colla-

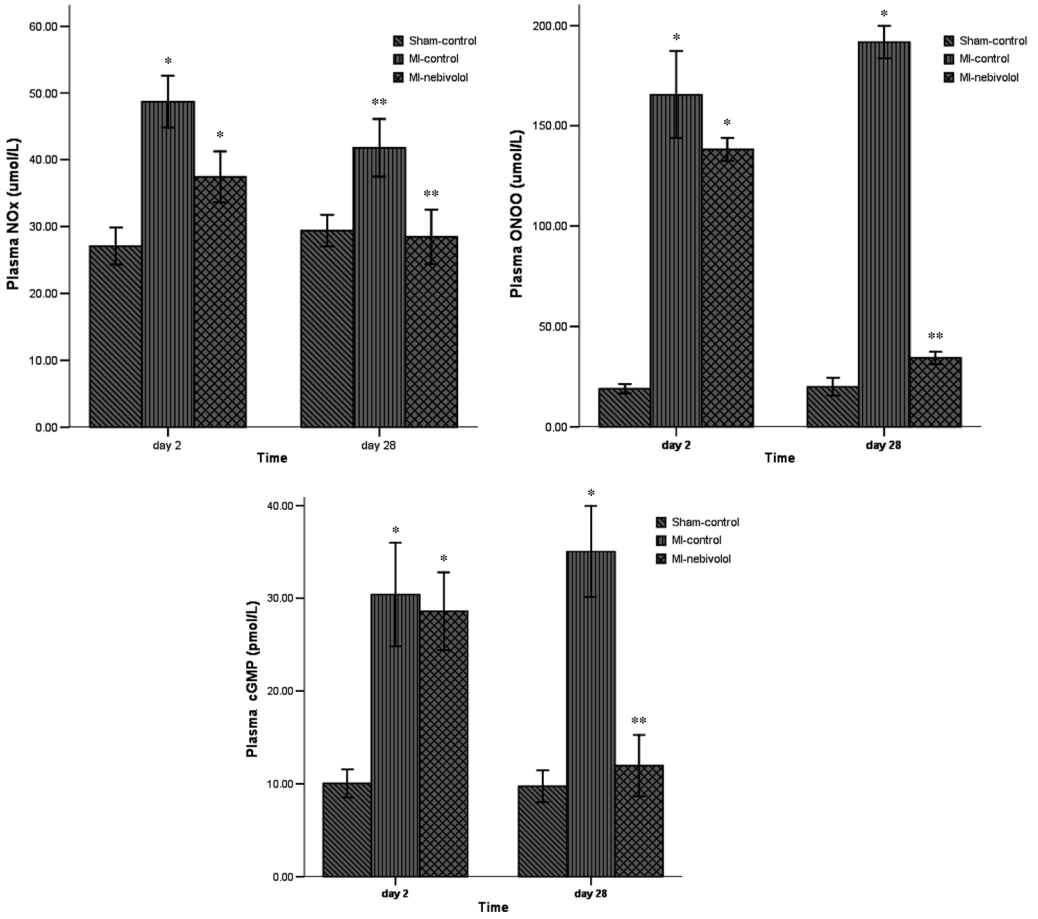

Fig 7. Plasma nitrite/nitrate (NO_x), cyclic guanylate cyclase (cGMP) and peroxynitrite ($ONOO^-$) concentrations. In myocardial infarction (MI)-control rats; plasma NO_x, cGMP and $ONOO^-$ concentrations were significantly increased in the 2nd day. Although cGMP and $ONOO^-$ concentrations were further increased, NO_x concentration was decreased in the 28th day. In the MI-nebivolol group, although plasma concentrations of NO_x, cGMP and $ONOO^-$ were increased soon after MI, these concentrations were returned to basal values on the 28th day (*p<0.005 compared to sham-control, **p=0.05).

gen accumulation was also seen in the mitral cuspis. Although there was no difference in the MI sizes between the groups, there was a trend to increase in MI-control group throughout the study (Fig 3).

Although apoptotic cardiomyocytes were rarely observed in sham-control animals (AI in the sham-control group was 0.031±0.005% and remained stable during the study), apoptotic nucleus were much more numerous in MI induced rats. The number of apoptotic nuclei was highest in the border zone both in early and sub-acute period of MI. In the infarct area, while apoptotic cardiomyocytes were observed on the 2nd day, they could not observed on the 28th day. In the early phase of MI, together with apoptotic cardiomyocytes, TUNEL-positive inflammatory cells were also observed in the infarct area. In contrast, very few TUNEL positive inflammatory cells were found in the border zone (Fig 4).

On day 2 of MI, AI of all 3 areas (border zone, infarct area and remote myocardium) were significantly lower in the MI-nebivolol group compared with MI-control rats (in the border zone AI was 10.2±1.4% and 7.1±0.4%, for MI-control and MI-nebivolol groups, respectively; Fig 5).

On day 28 of MI, apoptotic cardiomyocytes coul not observed in the infarct area. Total AI was 0.50±0.04% and 0.02±0.02% (p=0.004) for MI-control and MI-nebivolol, respectively. Again AI in the border zone and remote myocardium were lower in the nebivolol treated group compared with the infarcted group (Fig 5).

In the electron microphotographs of sham-control rats, myocardium was compact and consists of darkly stained myocytes and intact microvasculature. Although in the micrographs of MI-control rats, cytoplasm of myocytes has severe edema and the myofibrils and Z-bands were distorted. The mitochondrias were edematous, disrupted with separation of their cristae. Vacuoles probably because of dilated T-tubules, in cytoplasm were also seen. In the MI-control group capillary injury characterised by narrowed lumen, edema and increased transporter cells in the membrane was also seen. In the early period, collagen acumulation and increased capillary damage was seen. Apoptotic body residues cleared by macrophages were also seen. In the MI-nebivolol group smaller extent of injury was observed. Myocytes showed essentially the same ultrastructure as seen in the sham-control group, mithocondrias were intact and capillary damage was limited. Glycogen loss did not occur also (Fig 6).

Biological Assesment

Compared with the sham-control group, although plasma NO_x and cGMP concentrations were significantly higher in

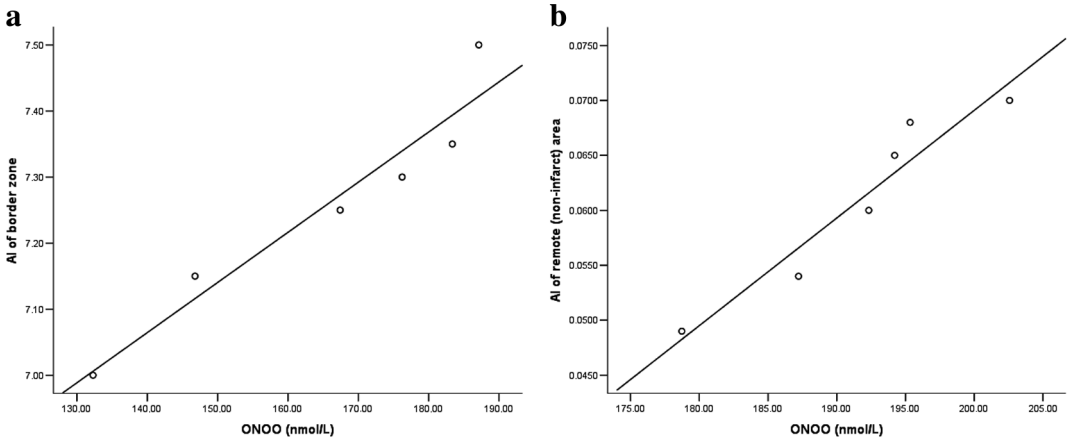

Fig 8. Correlation curves of apoptotic indexes (AI) with the plasma $ONOO^-$ concentrations. Plasma $ONOO^-$ concentrations were correlated with the apoptotic cardiomyocytes (a) in the border zone in the early period of myocardial infarction (day 2) and (b) in the healthy (non-infarct) zone in the sub-acute period (day 28).

the MI-control group both on the 2nd and 28th days, these values were only higher on the 2nd day in the MI-nebivolol group. The most dramatic change was seen in the plasma $ONOO^-$ concentrations in the MI-control group. Compared to the sham-control rats, plasma $ONOO^-$ concentration was significantly high on day 2 (18.9±2.4 and 165.5±21.7 nmol/L for sham-control and MI-control, respectively) and further increased over the study period (19.9±4.5 and 191.7±8.9 nmol/L for sham-control and MI-control, respectively). In contrast to the MI-control group, however, plasma $ONOO^-$ concentrations of MI-nebivolol rats were higher than that of sham-control group on the 2nd day, these concentrations were decreased to the basal value on the 28th day (Fig 7).

Another important finding was the correlation of AI with the plasma $ONOO^-$ value. Although plasma $ONOO^-$ concentrations were correlated with the AIs of border zone in the early period of MI (2nd day) (correlation coefficient was 0.961; $p<0.01$), plasma $ONOO^-$ concentrations were correlated with the AIs of remote (non-infarct) myocardium in the sub-acute period (28th day) (Fig 8).

Discussion

Necrosis is the main cell death type in MI. Although apoptosis is other type of cell loss, it is not measured in clinical practice. It was reported that apoptosis is reached at the maximal level at the first hours of MI.[15] There are various methods for both the inhibition of pro-apoptotic pathways and the activation of anti-apoptotic mechanisms.[16–18]

Beta-blockers, used in the treatment of MI and heart failure, show their useful effect by controlling the neurohormonal activation.[19–21] Furthermore, some of the β-blockers were also shown to prevent apoptosis.[22,23] Although the mechanism has not been fully explained, in vitro studies showed that noradrenaline (via β1 receptors) activates caspases, that are basic apoptotic enzymes.[24,25]

Nebivolol is a 3rd generation β-blocker that has also a NO regulating effect. Although the effects of NO on apoptosis have been evaluated in many studies, the results of these studies were controversial.[26–29] However, NO mediated effects of nebivolol on apoptosis after MI has not been investigated before.

When compared to sham-control rats, HR and MBP were decreased in MI groups. In some animal MI models, independent from drug effect, a decrease in HR and MBP was reported. However these decreases, such as in our study, are either slight or low in significance.[30,31] In the present study, MBP and HR of MI-nebivolol group were similar with the MI-control group. As previously mentioned, the main reason of this is the administration of lowest dose of drug with minimal hemodynamic effect to evaluate the NO mediated effects. In contrast, although the nebivolol dose we used had a minimum β-blocking effect, the hemodynamic parameters in MI-nebivolol group was improved more significantly than MI-control group. The main reason for this is the prevention of ventricular function after MI. Inhibition of increase in LVEDd and LVEDV together with conservation of LVEF in the echocardiographic study support this theory. Moreover, it is well known that cardiomyocytes in the non-infarct region are responsible for the continuation of LV functions after MI so prevention of myocardial cell loss in the healthy myocardium is the most important factor for LV function. In the present study it was shown that the beneficial effects of nebivolol in hemodynamic parameters were inconsistent with the prevention of apoptosis in the remote healthy myocardium, which plays an important role in the prevention of LV functions after MI.

Although apoptotic myocytes were rarely found in the sham-control group, significant apoptosis was found both on day 2 and 28 in the MI groups. Apoptosis reached the maximum level on day 2 and decreased significantly on day 28. Previous studies showed that apoptosis reaches maximum level in 4–12 h after MI and remaines for 3 to 10 days, then decreases gradually.[32–34]

In the sub-acute and long term period, quite different apoptotic rates (0.05–30%) are given in animal studies.[4] The wide difference in these rates is possibly as a result of the differences in determination method of apoptosis and MI size. In the present study, AI rates were 10 and 0.5% on day 2 and day 28 of MI, respectively. Similar rates were given in other experimental MI studies.[27–29] In our study both in the 2nd and 28th day, the most intensive apoptosis was determined in the border zone of infarct. Palojoki et al reported that apoptosis in the border zone continues until the 12th week.[14] In the same study, the authors found a sig-

nificant relationship between border zone apoptosis and LV dimensions. These findings led us to think that apoptosis has a detrimental role in LV functions after MI. In this study finding of trend to increase in the infarct size during the sub-acute period of MI is consistent with the literature. Clinical observations also support this relationship.[35–37]

In the nebivolol treated group, AI was found significantly lower than MI-control group both on day 2 and day 28 of MI. In this group, in addition to total AI, regional AI rates were also significantly lower than the MI-control group. In the previous animal studies some of the β-blockers were shown to decrease apoptosis.[22,23,38,39] It is known that noradrenaline induce apoptosis via β-adrenergic receptors.[24] It is also known that although β-1 adrenergic receptors stimulate apoptosis, β-2 receptors thought to not only stimulate but also inhibits apoptosis.[24] However, prevention of apoptosis by nebivolol might possibly be via β-blockage activity, our findings indicate that NO regulating effect of nebivolol might also be important in this effect. The decrease in apoptosis with lower β-blocker dose, which does not cause any significant change hemodaynamic parameters, supports this idea. Similar findings were reported with carvedilol, but not with metoprolol and propranolol. Feuerstein et al showed that cardioprotective efects of carvedilol might be related to additional pharmacological properties of carvedilol, such as the antioxidant and anti-neutrophil effects rather than its haemodynamic effects.[40] In the other study conducted by Okafor et al the decrease in apoptosis and the improvements of ventricular functions in the animal model of dilated cardiomyopathy is profound in even lower carvedilol dose, which has no haemodynamic effect related to β-blockade.[38] In contrast, Yaoita et al explain the attenuation of LV remodeling with propranolol and metoprolol by reduction of heart rate and systolic blood pressure.[41] Furthermore, Zhu et al have showed that in a rat model ischemia/reperfusion injury, metoprolol produced cardioprotection via hemodynamic effects.[42] More recently, Omerovic et al suggest that metoprolol attenuates post-infarct structural remodeling, without concomitant improvement in myocardial energy metabolism and function in rats with chronic congestive heart failure,[43] but authors could not completely explain which effect is responsible from this improvement.

Determination of apoptosis with TUNEL technique together with electron microscopy is considered the gold standard for qualitative determination of apoptosis.[44] Evaluation of cellular changes with electron microscopy also gives valuable information. In the electron microscopy analysis the most important finding is the protection of capillary cells together with the cardiomyocytes. As it was known, capillary cells plays important role in the oxygen and energy supplementation.

The effect of NO on apoptosis are quite complex. NO is known to have both apoptotic and anti-apoptotic effects.[26–29,45,46] NO, synthesized by iNOS stimulated by the cytokines was shown to stimulate apoptosis.[26–28] The toxic effects of high concentrations of NO have been reported to be mediated by the formation of $ONOO^-$.[29] The NO synthesized by eNOS, however, shows anti-apoptotic effects in the myocardium. The angiotensin converting enzyme inhibitors, with preventive effects on remodeling, have been shown to increase the eNOS activation.[47] Although cGMP mediates the protective effects of NO, it was also reported that NO cause apoptosis through cGMP.[48] Thus, although evaluating the role of NO on apoptosis, determining the NO concentration is not sufficient. In our study together with NO_x concentrations, $ONOO^-$ and cGMP concentrations were also determined. The increase in NO_x level of MI-control group was an expected result. Together with high NO_x concentrations, the increase in $ONOO^-$ concentrations in MI-control group led us to think that NO activation in this group is related to the increase in oxidative stress and mediated by iNOS activation. Likewise, the higher concentrations of apoptosis in the MI-control group support this finding. Furthermore, in the MI-control group plasma $ONOO^-$ concentrations were also high on the 28th day. The increase in $ONOO^-$ concentrations, together with NO_x, actually are the signals of pathological NO concentrations. Correlation of plasma $ONOO^-$ concentrations with the AI of border zone in early period supports the idea that apoptosis plays an important role for the expansion of MI and cytokine induced NO is the major triggering factor for apoptosis. Again correlation of plasma $ONOO^-$ concentrations with AI of healthy myocardium (non-infarct area) in the sub-acute period shows that $ONOO^-$ induced apoptosis is responsible for the reduced LV function. Finding of strong iNOS immunoreactivity in myocytes, together with infiltrating cells in the infarct region and spared myocytes close to infarct region soon after MI and continuation of this immunoreactivity in the non-infarct region in the sub-acute period, supports these findings (unpublished data).

In contrast to these changes in MI-control group, plasma NO_x and $ONOO^-$ values in MI-nebivolol group were lower. On day 28 of MI, $ONOO^-$ concentrations was 5.6 times lower than MI-control group. NO_x and cGMP concentrations were also similar with the sham-control group. In the MI-nebivolol group $ONOO^-$ concentrations were decreased to the concentrations in the sham-control group, which indicates that in this group the source of NO is eNOS. Lower $ONOO^-$ concentrations were also the indicator of the decreased oxidative stress. Likewise, anti-oxidative effects of nebivolol was shown both in vivo and in vitro studies.[49–51] According to Mollnau et al this effect was achieved by the prevention of eNOS uncoupling and the inhibition of neutrophilic NADPH oxidase activity.[47] Again, the finding of decreased iNOS immunoreactivity in the acute period together with sustained eNOS immunoreactivity in the sub-acute period of MI is consistent with the results (unpublished data).

As a result, in this study it was found that nebivolol treatment (initially by iv within the 10 min of reperfusion and continue orally) reduced the myocardial apoptosis by preventing $ONOO^-$ formation after MI. Although prevention of apoptosis in the border zone limited the expansion of MI in the acute period of MI, prevention of apoptotic cell loss in the healthy myocardium helped to maintain of LV functions in sub-acute period. Moreover, preservation of cGMP and NO_x concentrations with nebivolol also helped the maintenance of LV functions in the sub-acute period.

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