

## Comparison of the effect of nebivolol, metoprolol and diltiazem on tissue nitric oxide synthase activity in internal mammary artery

*Nebivolol, metoprolol ve diltiazemin internal mammaryan arter greftindeki doku nitrik oksit sentaz aktivitesine etkisinin karşılaştırılması*

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**Background:** This study aims to evaluate the effects of nebivolol on nitric oxide synthase activity in human internal mammary artery compared to metoprolol and diltiazem.

**Methods:** Seventy-five patients (43 males, 32 females; mean age 61.6±5.8 years; range 41 to 76 years) who were scheduled for coronary artery bypass grafting were enrolled in this study. Patients were assigned randomly to three groups. In group 1, 50 mg metoprolol, in group 2 5 mg nebivolol, and in group 3 90 mg diltiazem therapy was initiated five days before surgery. Samples of internal mammary artery were obtained intraoperatively. Nitric oxide synthase activity was measured by the quantitative sandwich enzyme-linked immunosorbent assay and graft free flow was calculated.

**Results:** The mean tissue nitric oxide synthase activity was found to be 0.4±0.0 U/mg in group 1, 2.4±0.3 U/mg in group 2 and 0.4±0.0 U/mg in group 3. There was a significant difference in the mean tissue nitric oxide synthase activity among the groups (p<0.05) with a higher rate in group 2. The mean graft free flow was significantly higher in group 2 compared to other groups (p<0.01).

**Conclusion:** Our study results suggest that preoperative nebivolol therapy increases tissue nitric oxide synthase concentration and graft free flow in the human internal mammary artery compared to metoprolol and diltiazem.

**Keywords:** Coronary artery bypass grafting; internal mammary artery; nebivolol; nitric oxide; nitric oxide synthase.

**Amaç:** Bu çalışmada metoprolol ve diltiazeme kıyasla, insan internal mammaryan arterinde nitrik oksit sentaz aktivite düzeyine nebivololün etkisi araştırıldı.

**Çalışma planı:** Çalışmaya koroner arter bypass greftleme planlanan 75 hasta (43 erkek, 32 kadın; ort. yaş 61.6±5.8 yıl; dağılım 41-76 yıl) dahil edildi. Hastalar randomize olarak üç gruba ayrıldı. Ameliyattan beş gün önce grup 1'de 50 mg metoprolol, grup 2'de 5 mg nebivolol ve grup 3'de 90 mg diltiazem tedavisi başlandı. Ameliyat sırasında internal mammaryan arterinden doku örnekleri alındı. Kantitatif sandwich enzim bağlı immünosorbent assay tekniği ile nitrik oksit sentaz aktivitesi ölçüldü ve serbest greft akımı hesaplandı.

**Bulgular:** Ortalama doku nitrik oksit sentaz aktivitesi grup 1'de 0.4±0.0 U/mg, grup 2'de 2.4±0.3 U/mg ve grup 3'de 0.4±0.0 U/mg bulundu. Grup 2'de daha yüksek olmakla birlikte, gruplar arasında nitrik oksit sentaz aktivitesi açısından istatistiksel olarak anlamlı farklılık saptandı (p<0.05). Ortalama serbest greft akımı, diğer gruplara kıyasla, grup 2'de anlamlı düzeyde daha yüksekti (p<0.01).

**Sonuç:** Çalışmamızın sonuçları, ameliyat öncesi nebivolol tedavisinin insan internal mammaryan arterinde, metoprolol ve diltiazeme kıyasla, doku nitrik oksit sentaz konsantrasyonunu ve serbest greft akımını artırdığını göstermektedir.

**Anahtar sözcükler:** Koroner arter baypas greftleme; internal mammaryan arter; nebivolol; nitrik oksit; nitrik oksit sentaz.



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The internal mammary artery (IMA) graft is the preferred choice over other conduits for coronary artery bypass grafting (CABG) operations because it has superior long-term patency rates and offers symptomatic control.<sup>[1,2]</sup> However, IMA graft spasms can occur during mobilization and anastomosis. Surgical stimulus is an important factor that is associated with graft spasm and can lead to myocardial ischemia and the deterioration of myocardial viability.<sup>[3,4]</sup> Fortunately, the incidence rate of IMA graft spasms can be reduced via the use of vasodilators such as papaverine.<sup>[5]</sup>

The effects of topical vasodilators, which are commonly used during CABG, have been extensively analyzed,<sup>[6-8]</sup> but to the best of our knowledge, no comparative analysis has been published regarding the vasodilatory effects of systemic medications which patients begin taking preoperatively. These medications are primarily used for treating angina, hypertension (HT), and arrhythmia and include the use of nebivolol, metoprolol, and diltiazem on arterial conduits like the IMA graft.

Nitric oxide (NO), which is produced by the endothelium of the arterial wall, has a wide range of biological properties that help maintain vascular homeostasis, including modulating vasodilatation, regulating local cell growth, and protecting the vessel from the injurious consequences of platelets. In fact, the enhanced release of NO into the arterial wall may prevent the constriction of coronary arteries and bypass grafts in patients with coronary artery disease (CAD).<sup>[9,10]</sup>

Nebivolol, a third generation beta ( $\beta$ )-blocker with high selectivity for  $\beta_1$ -adrenergic receptors and NO-dependent vasodilatory and antioxidant properties, is able to stimulate the endogenous production of NO by inducing the phosphorylation of the endothelial nitric oxide synthase (eNOS).<sup>[11]</sup>

In this prospective, randomized clinical study, we aimed to compare the eNOS levels and IMA graft free blood flows in patients who were systematically administered nebivolol preoperative in conjunction with the commonly used antihypertensive agents metoprolol and diltiazem.

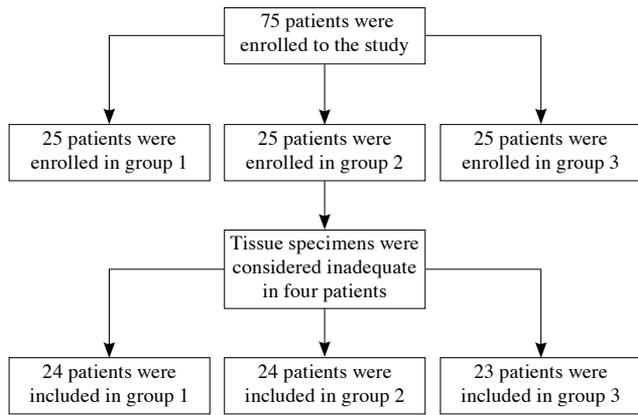
## PATIENTS AND METHODS

Among the patients who underwent elective CABG between May 2012 and February 2013, those for whom antihypertensive therapy had been initiated were enrolled in this study, which was designed in accordance with the Consolidated Standards of

Reporting Trials (CONSORT) Statement. In addition, the institutional ethics committee approved the study, and informed consent was obtained from each patient.

We consulted with a statistician who advised us to maintain 60 samples to achieve statistical significance, and in the end, 75 patients (43 males, 32 females; mean age  $61.6 \pm 5.8$  years; range 41 to 76 years) were included in our study. They were randomly assigned to three groups consecutively, and preoperative antihypertensive therapy was initiated five days before surgery with standard initial doses started at the cardiology unit of our institution. Group 1 received 50 mg metoprolol succinate (Beloc-Zok<sup>®</sup>, AstraZeneca, Istanbul, Turkey) while group 2 received 5 mg nebivolol (Vasoxen, Abdi İbrahim, Istanbul, Turkey) in a single dose and group 3 was prescribed 90 mg diltiazem (Mustafa Nevzat, Istanbul, Turkey) in three doses, all of which were given as tablets for oral administration on a daily basis. The average duration of the preoperative period was between one and five days. In addition, the patients who did not receive any antihypertensive therapy before coronary angiography also began to receive medications, with this therapy being initiated to those subjects with a systolic blood pressure of  $\geq 140$  mmHg and/or a diastolic blood pressure of  $\geq 90$  mmHg. These measurements were taken twice after at least a five-minute rest during the preoperative period. To avoid controversy, the study participants were operated on after five days as a standard protocol since the therapeutic effect of metoprolol, nebivolol, and diltiazem begins within 12-24 hours. In all three groups, the patients were given no other antihypertensive drugs, and all received the same antihyperlipidemic medication (atorvastatin). Five days prior to the procedure, the antiplatelet and antithrombotic therapies were withdrawn, and the vasodilatory drugs, including nitrates and sodium nitroprusside, were discontinued to avoid any effects they might have on the results of the study.

First, the patients' demographic data, tissue eNOS activity, and graft free flow (GFF) rate were compared statistically. Then a median sternotomy was performed under general anesthesia, and a standard IMA harvesting retractor was inserted into each patient. Next, the left IMA graft was dissected along with a pedicle originating from the subclavian artery and extending to the bifurcation of the distal branches via electrocautery (Force Fx, Valleylab, a division of Tyco Healthcare, New Brunswick, NJ, USA) at a setting of 20 watts. Hemoclips were used to clamp the left internal mammary artery (LIMA)



**Figure 1.** Flow diagram of the study.

branches, and the IMA graft was wrapped with warm protective gauze and papaverine. No other topical solution was applied in order to not hinder the effects of the nebivolol, diltiazem, and metoprolol. All of the patients then underwent on-pump CABG, and after heparin administration (150 mg/kg), the aorta and right atrium were cannulated for cardiopulmonary bypass (CPB). Before the initiation of CPB, the IMA was transected just before the bifurcation, and the distal 1.5 cm artery section was obtained along with the perivascular tissue and stored at -80 °C. The harvesting of all grafts as well as the flow measurements and tissue sampling were all performed by the same surgeon.

The tissue samples were exposed to hemolysis via centrifuge, but adequate tissue homogenates were not achieved in four patients. This resulted in their exclusion, leaving 71 patients [mean age 66.6±6.7 years; 46 males (61.9% and 25 females (38.1%) in the study (Figure 1). The eNOS activity was measured by the quantitative sandwich human eNOS enzyme-linked immunosorbent assay (ELISA) Kit (Cusabio

Biotech Co., LTD, Wuhan, Hubei Province, P.R. China).

The GFF of the left IMA was measured after obtaining the distal tissue sample, and the distal cut end was allowed to bleed into a special tube for 60 seconds under controlled hemodynamic conditions [mean arterial blood pressure (MAP) between 60 and 90 mmHg and heart rate (HR) of 60-100 beats/min]. This was expressed as mL/minute, and the HR, MAP, and central venous pressure (CVP) was recorded synchronously during each IMA flow measurement.

**Statistical analysis**

The statistical analyses were performed with the SPSS for Windows version 16.0 software program (SPSS, Inc., Chicago, IL, USA), and all data was expressed as mean ± standard deviation (SD). The results were analyzed using Student’s t-test or the Mann-Whitney U test for the quantitative data and a Chi-square or Fisher’s exact test for the categorical data. In addition, a between-group comparison was performed using the Kruskal-Wallis test, and a *p* value of 0.05 was considered to be statistically significant. Furthermore, the Mann-Whitney U test was used for the post-hoc analysis for which the *p* value was accepted as 0.0167 with a Bonferroni correction.

**RESULTS**

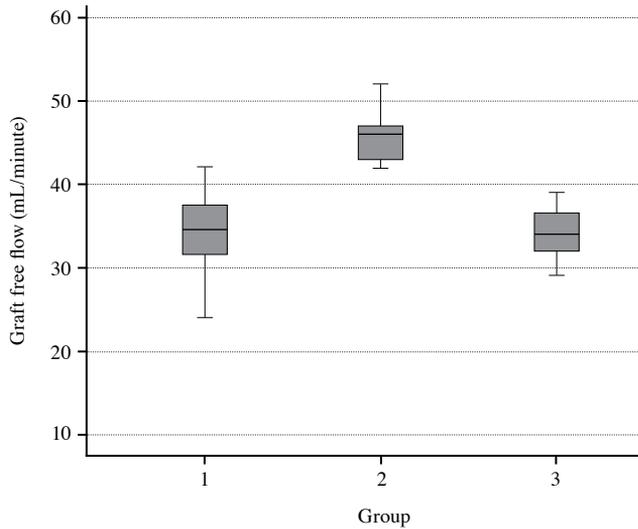
The demographic data was similar between the three groups with no significant differences (Table 1). No perioperative myocardial infarction or serious complications requiring postoperative exploration occurred, and the mean values of HR, MAP, CVP, and the temperature did not differ significantly between the groups during the IMA GFF measurements.

The patients’ mean GFF of the left IMA graft was 33.8±6.5 mL/minute in group 1, 46.2±3.6 mL/minute

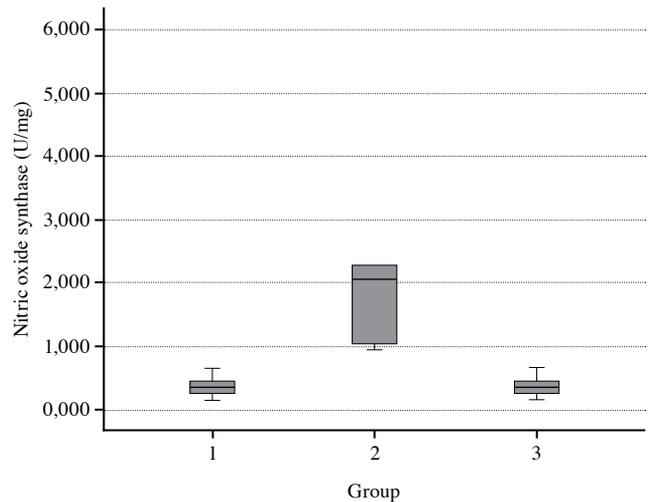
**Table 1. Demographic and clinical characteristics of the patients**

	Group 1		Group 2		Group 3		Difference
	n	Mean±SD	n	Mean±SD	n	Mean±SD	
Patients	24		24		23		
Age in years		67.5±7.4		65.4±7.1		66.8±5.4	NS
Sex							
Male	15		15		14		NS
Female	9		9		9		
Diabetes mellitus	5		6		4		NS
Smokers	16		14		13		NS
Hyperlipidemia	7		5		5		NS

SD: Standard deviation; NS: Non significant.



**Figure 2.** Box plot of the graft free flow.



**Figure 3.** Box plot of nitric oxide synthase.

in group 2, and  $33.7 \pm 3.9$  mL/minute in group 3 (Figure 2), and the difference between the groups was markedly significant ( $p < 0.05$ ). Furthermore, the post-hoc analysis was performed to compare groups with each other in pairs and we found no difference between groups 1 and 3, but the mean GFF for group 2 was significantly higher than the other two groups ( $p < 0.01$ ).

The highest values were encountered in the group 2 samples. The patients' mean tissue eNOS activity was  $0.4 \pm 0.0$  U/mg in group 1,  $2.4 \pm 0.3$  U/mg in group 2, and  $0.4 \pm 0.0$  U/mg in group 3 (Figure 3), with the difference being strongly significant between groups 1 vs. 2 and 2 vs. 3 ( $p < 0.05$ ). Again, a post-hoc analysis was performed between groups in doubles. No difference was found between groups 1 and 3, but the mean tissue eNOS activity was significantly higher in group 2 ( $p < 0.01$ ) (Table 2).

## DISCUSSION

In the present study, we investigated the effect of nebivolol, a third generation  $\beta_1$ -adrenoreceptor antagonist in human IMA samples, and to the best of our knowledge, we made the initial discovery that pretreatment with nebivolol enhances tissue eNOS activity and that it also effectively increases the GFF when compared with metoprolol and diltiazem. These results present new insights regarding the preservation of the endothelium in CABG surgery performed to improve the long-term patency of these conduits since endothelial function is one of the most important factors that influences their long-term patency.

Nitric oxide has a fundamental role in the endothelium-mediated regulation of vascular equilibrium and has been accepted as the key mediator of endothelium-dependent vasorelaxation and cellular

**Table 2. Nitric oxide synthase levels, graft free flow, and hemodynamic data of the groups recorded during the measurements**

	Group 1	Group 2	Group 3	<i>p</i>
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Nitric oxide synthase (U/mg)	$0.4 \pm 0.0$	$2.4 \pm 0.3$	$0.4 \pm 0.0$	$< 0.01$ (1 vs. 2 and 2 vs. 3)
Graft free flow (mL/minute)	$33.8 \pm 6.5$	$46.2 \pm 3.6$	$33.7 \pm 3.9$	$< 0.01$ (1 vs. 2 and 2 vs. 3)
Mean arterial pressure (mmHg)	$80.8 \pm 6.5$	$82.2 \pm 5.2$	$83.5 \pm 7.2$	0.421 (1 vs. 2) 0.783 (2 vs. 3)
Heart rate (beats/minute)	$74.7 \pm 11.5$	$73.6 \pm 8.4$	$75.9 \pm 10.8$	0.783 (1 vs. 2) 0.192 (2 vs. 3)
Central venous pressure (mmHg)	$7.2 \pm 1.2$	$7.9 \pm 1.9$	$7.5 \pm 1.5$	0.089 (1 vs. 2) 0.236 (2 vs. 3)

SD: Standard deviation.

interactions in the vessel wall.<sup>[10]</sup> Recent studies have also demonstrated that asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of endothelial NOS, is responsible for endothelial dysfunction which in turn leads to a reduction in NO. Moreover, elevated plasma ADMA levels have been found in patients with CAD, chronic heart failure, and DM, and these are widely recognized as being a biomarker for cardiovascular disease risk and adverse cardiovascular and renal outcomes.<sup>[12-14]</sup>

Jiang et al.<sup>[15]</sup> reported that the production of reactive oxygen species (ROS), for example superoxide anions in the vascular endothelium, is induced by ADMA via the uncoupling of eNOS and other eNOS independent pathways, and this may trigger apoptosis in endothelial cells. Additionally, superoxide anions may damage the eNOS directly by inducing the transformation of the eNOS dimer into a monomer.<sup>[16]</sup> In another study, Jiang et al.<sup>[17]</sup> showed that the reduction in NO production might be related to a decrease in eNOS expression due to the elevation of ADMA levels in cultured endothelial cells.

Beta blockers are widely used in the treatment of HT and other cardiovascular diseases, and their antihypertensive effectiveness is similar to that of calcium antagonists and angiotensin-converting enzyme (ACE) inhibitors.<sup>[18,19]</sup> Moreover, several new  $\beta$ -blockers have been developed with pharmacodynamic and endothelium-dependent, vasodilating properties comparable to the existing  $\beta$ -adrenoreceptor antagonists.<sup>[11]</sup>

Nebivolol is a  $\beta_1$ -adrenoreceptor antagonist as well as a  $\beta_3$ -adrenoreceptor agonist, and the stimulation of the  $\beta_3$ -adrenoreceptor results in an increase in NO production.<sup>[20]</sup> In cultured human umbilical vein cells, nebivolol has been demonstrated to decrease ADMA, but this did not occur when carvedilol or metoprolol were used.<sup>[21]</sup>

Asymmetric dimethylarginine is formed by the methylation of arginine residues via the enzyme protein arginine methyltransferase-1 (PRMT-1) and is metabolized by dimethylarginine dimethylaminohydrolase (DDAH). The stimulation of DDAH by the l-enantiomer of nebivolol may be responsible of the decrease of ADMA,<sup>[22]</sup> and it is also responsible for the  $\beta_3$ -adrenoreceptor agonism. In addition, the d-enantiomer is responsible for the  $\beta_1$ -adrenoreceptor antagonism of nebivolol.

Kandavar et al.<sup>[23]</sup> analyzed the effects of nebivolol and metoprolol on plasma ADMA concentration levels in HT patients and determined that the ADMA

increased by 44.78% and 72% in the metoprolol group at weeks four and eight, respectively. However, there was no increase in the nebivolol group. This increase in ADMA with metoprolol suggests that the  $\beta_1$ -adrenoreceptor antagonism alone may not produce optimal benefits for the treatment of some diseases, for instance HT. Even though there is a lack of comparisons between nebivolol and diltiazem, there is evidence that a calcium channel blockade enhances eNOS expression. Ding and Vaziri<sup>[24]</sup> showed that both diltiazem and nifedipine significantly increased NO production and eNOS protein expression as well as the activity in human coronary artery endothelial cells *in vitro*.

In addition to the increased eNOS activity, we found that the IMA GFF also increased significantly when patients received nebivolol therapy preoperatively. We measured the GFF after the distal tissue sample was obtained and before the use of topical vasodilator agent and determined that the spasm, which occurred after the dissection of the distal IMA, may have been the reason for the less than average GFF values of the study groups. However, since all of the measurements were done at the same interval, the significant statistical difference was still meaningful. After the measurement was completed, the topical papaverin was then applied. All of the grafts were bleeding briskly and were anastomosed to the left anterior descending artery (LADA). Moreover, no major adverse events were noted in the postoperative period.

In general practice, topical vasodilatory agents, such as papaverine, are commonly applied to adventitia directly or with gauze soaked with this drug. Intraluminal injections and systemic administration methods are other options, but for the IMA, it is still not clear which drug and administration choice can provide acceptable effectiveness, and the exact mechanism is unknown. Intraluminal injection seems logical considering that the IMA has little vasorum and taking into account that 80% of the blood in the vessel wall is supplied by the blood in the lumen. However, intraluminal injection may damage the vascular integrity and cause ultrastructural changes in the endothelium due to the mechanical disturbance of the injection and chemical properties of the solution. Preoperative nebivolol therapy can reduce the necessity and amount of vasodilatory agents during the CABG procedure. However, the *in vivo* protective effect of nebivolol on the endothelial function in the IMA and its potential clinical impact on the long-term patency of the graft have yet to be defined.

The primary limitation of our study was that there were no flow measurements made after applying the papaverin. In a recently published paper, Sarikaya et al.<sup>[25]</sup> reported that when compared with other antihypertensive drugs, pretreatment with nebivolol increases the IMA GFF even after papaverine usage. The second limitation was that the medication was only used for a short time in the study groups. Although there was a statistical difference in our study, longer times of pretreatment will undoubtedly strengthen our results.

In conclusion, the results of our study suggest that preoperative nebivolol therapy increases tissue eNOS concentration and GFF in the human IMA when compared with metoprolol and diltiazem. These findings provide new insights into the protection of the endothelium of the CABG conduit to achieve superior long-term patency of grafts. Hence, nebivolol therapy in standard doses can be recommended for the preoperative management of patients who will undergo CABG surgery.

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#### Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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