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***Experimental Study** / Deneysel Çalışma*

Cilostazol enhances endothelium-dependent vasodilatation of intact endothelium in isolated rat aortic rings

Silostazol izole sıçan aort halkalarında sağlam endotelin endotel bağımlı vazodilatasyonunu artırır

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Background: The aim of this study was to investigate the effects of chronic cilostozol treatment on vascular reactivity of intact endothelium.

Methods: Thirty adult male Wistar Albino rats were randomly assigned to one of the following three groups: control group (n=10), dimethylsulfoxide (DMSO) group (n=10) or cilostazol (CIL) group (n=10). The rats in the control group received no treatment. In the CIL group, dissolved cilostazol in DMSO was administered at a dose of 10 mg/kg bid via intraperitoneal (IP) route for six weeks. Rats in the DMSO group received an IP injection of DMSO at a volume which was similar to that used in the CIL group with cilostazol bid for the same time period. At the end of six-week treatment, acethylcholine (ACh)-induced (endothelium dependent) relaxation responses of isolated rat aortic rings in organ bath were evaluated.

Results: There was no statistically significant difference in the percent of relaxation in aortic rings between the control and DMSO groups. In the CIL group, relaxation responses to ACh concentrations of $3x10^{-9}$ Moles and higher were significantly higher, compared to the other two groups ($p<0.05$). Cilostazol treatment was observed to result in a response which led to a reduction of vascular tone beyond the basal tonus. This phenomenon became more evident in higher concentrations of ACh, representing a concentration-dependent augmentation of vasodilation in CIL group.

Conclusion: In this study, the sensitivity of endothelium to ACh-induced vasodilatation increased in the rats receiving cilostazol treatment. We suggest that induction nitric oxide (NO) and prostacyclin (PG12) release by cilostazol may be the possible mechanisms of enhanced relaxation response of intact endothelium.

Key words: Cilostozol; endothelium; organ bath; vasodilatation.

Amaç: Bu çalışmada, kronik silostazol tedavisinin sağlam endotelin vasküler reaktifliği üzerindeki etkileri araştırıldı.

Çalışma planı: Otuz adet erişkin erkek Wistar Albino cinsi sıçan, kontrol grubu (n=10), dimetilsülfoksid (DMSO) grubu (n=10) ve silostazol (CIL) grubu (n=10) şeklinde üç gruba ayrıldı. Kontrol grubundaki sıçanlara herhangi bir tedavi verilmedi. Silostazol grubunda altı hafta boyunca günde iki kez olmak üzere DMSO içinde çözünmüş haldeki silostazol 10 mg/kg dozunda intraperitoneal (İP) yoldan verildi. Dimetilsülfoksid grubunda bulunan sıçanlara, CIL grubundaki sıçanlara uygulanan silostazolün içinde çözündüğü hacme eşdeğer DMSO, aynı süre boyunca günde iki kez olacak şekilde İP olarak enjekte edildi. Altı haftalık tedavi sonrasında izole sıçan aort halkaları hazırlanarak organ banyosunda asetil kolin (ACh) aracılı (endotel bağımlı) gevşeme yanıtları incelendi.

Bulgular: Kontrol ve DMSO gruplarındaki aort halkalarının gevşeme yüzdeleri arasında istatistiksel olarak anlamlı bir farklılık tespit edilmedi. Silostazol grubunda $3x10^{-9}$ Mol ve bunun üzerindeki ACh konsantrasyonlarına karşı elde edilen gevşeme yanıtları, diğer iki gruba kıyasla, anlamlı olarak daha yüksekti ($p<0.05$). Silostazol tedavisinin vasküler tonusta bazal tonusun bile altına inen gevşeme ile sonuçlandığı gözlemlendi. Bu fenomenin yüksek ACh konsantrasyonlarında daha belirgin hale geldiği ve CIL grubundaki vazodilatasyonda konsantrasyon bağımlı bir artış olduğu görüldü.

Sonuç: Bu çalışmada silostazol tedavisi uygulanan sıçanlarda endotelin asetilkolin aracılı vazodilatasyona duyarlılığı arttı. Silostazol ile ortaya çıkan sağlam endotelin gevşeme yanıtındaki artışın muhtemel mekanizmasının nitrik oksit (NO) ve prostasiklin (PGI2) salınımı olduğu düşünülmektedir.

Anahtar sözcükler: Silostazol; endotel; organ banyosu; vazodilatasyon.

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The endothelium is the monolayer of cells that lines the vascular system; however, it serves as more than a barrier between the circulating blood and the tissues. The vascular endothelium plays a crucial role in homeostasis by mediating the vasodilatation response to acetylcholine (ACh) in collaboration with smooth muscle cells. Furthermore, it behaves very much like an endocrine organ and acts to release various endothelium-derived vasoactive factors, including nitric oxide (NO) and prostacyclin (PGI2).[1]

Vascular tone is critical for the regulation of hemodynamics and is determined by the counteracting arterial constrictor and dilator activities, which maintain balance so as to match the circulatory needs of the metabolism.[2] Pathophysiological conditions, such as atherosclerosis, can interfere with endothelial functions and lead to a disturbance of this balance. Alterations in vascular tone are a primary factor in vascular disease etiology; therefore, modulation and preservation of endothelial functions is an important focus for vascular research.

Cilostazol (CIL) is a quinolone derivative and a cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase type III (PDE III) inhibitor.[3-5] It has an antiplatelet and antithrombotic activity and is commonly used in the medical treatment of peripheral vascular disease.[6] Cilostazol induces vasodilatation by increasing the cAMP in the vascular smooth muscle cells and decreasing the intracellular calcium concentration.[7,8] Recent studies have indicated that CIL is also associated with increased NO production in vascular cells.[9] Moreover, it has been shown to exert additional effects on the vascular endothelium and offer endothelial protection via the inhibition of apoptosis and neutrophil-endothelial cell adhesions.[10]

The aim of this experimental study was to investigate the effect of chronic CIL treatment on the vascular reactivity of intact endothelium by examining the endothelium-dependent relaxation responses of isolated rat aortic rings in an organ bath.

MATERIALS AND METHODS

The experimental designs and procedures were approved by the Animal Experimental Committee of Adnan Menderes University in Aydın, Turkey. Thirty male Wistar albino rats over 16 weeks old (body weight 270-300 g) were fed a standard laboratory diet, given water ad libitum, and housed at a controlled room temperature (24.5-25 °C) with a 12-hour light-dark cycle before and during the experiment. Tablets of CIL (Pletal® 100 mg tb. Abdi İbrahim İlaç Sanayi, Istanbul, Turkey) were crushed in sterile containers, and each tablet was dissolved in 1.5 ml of dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and centrifuged using an IKA Yellow Line TTS2 low profile vortex mixer (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 2500 rpm. for five minutes to obtain a homogenous mixture.

The rats were randomly assigned to one of the following three groups: the control group (n=10), the DMSO group (n=10) or the CIL group (n=10). The rats in the control group received no treatment. In the CIL group, the drug was dissolved in DMSO and administered at a dose of 10 mg/kg intraperitoneally twice a day for six weeks. The rats in the DMSO group received twice-daily intraperitoneal (IP) injections of DMSO without CIL at a volume which was similar to that used in the CIL group over the same period of time.

At the end of six weeks of treatment, a median sternotomy was performed under high-dose ether anesthesia, the heart and lungs were retracted, and the arcus and thoracic aortic segments adjacent to the thoracic vertebrae were removed. The rats were then sacrificed by decapitation. The aorta was carefully dissected free from the adipose and connective tissue remnants with the help of surgical binocular loupes (x3.5), meticulous attention was paid to avoid damaging the endothelium. The segments of the aortic rings, each 3-4 mm in length, were then quickly prepared, and the isolated rings were suspended in four parallel IOBS 99 isolated tissue bath stand sets (Commat Ltd., Ankara, Turkey), each containing 25 ml. Krebs solution [118.3 mM sodium chloride (NaCl), 4.7 mM potassium chloride (KCl), 1.2 mM magnesium sulfate (MgSO4), 1.22 mM monopotassium phosphate (KH_2PO_4), 2.5 mM calcium chloride (CaCl2), 25.0 mM sodium bicarbonate ($NaHCO_3$), and 11.1 mM glucose] (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) maintained at 37 °C and bubbled with 95% oxygen (O_2) and 5% carbon dioxide (CO_2). This was connected to an MP 100 data acquisition system (BIOPAC Systems Inc., Goleta, CA, USA) for recording tension, and this data was analyzed via Acq*Knowledge* 3.8.2 data acquisition and analysis software (Biopac Systems Inc., Goleta, CA, USA).

After stabilization, the aortic rings were put under a resting tension of 3 g by gradually increasing the tension 1 g every 10 minutes. Then each ring was contracted with the addition of norepinephrine (0.1 mlx10-4 molar concentration) to the baths. Afterwards, a stable plateau level was reached, and the rings were washed with the Krebs solution. The tension was then augmented to 4 g, and the rings were contracted once again by the same concentration

Table 1. Concentrations of acetylcholine doses

Dose	Acetylcholine Molar concentrations
1st	10^{-9}
2nd	$3x10^{-9}$
3rd	10^{-8}
4th	$3x10^{-8}$
5th	10^{-7}
6th	$3x10^{-7}$
7th	10^{-6}
8th	$3x10^{-6}$
9th	10^{-5}
10th	$3x10^{-5}$
11th	10^{-4}

of norepinephrine. This was subsequently followed by the addition of ACh (0.1 ml x 10^{-4} molar concentration) to the baths. The resultant relaxation responses to the ACh were observed, and the rings were washed as a precaution against interfering metabolites and allowed to equilibrate for 45 minutes. The resting vascular tone recorded at the end of the equilibration phase is regarded as the basal tonus for that particular ring. In a previous study the aortic rings which manifested relaxation by ACh were regarded as endothelium-intact aortic rings and those which failed to respond properly were considered to have damaged endothelium [11] Therefore the rings which fail to relax upon Ach application were excluded from the study.

The rings were contracted with norepinephrine (0.1 ml x 10^{-4} molar concentration) after equilibration, and a stable plateau level was reached. The endothelium-dependent relaxation response for each aortic ring was then examined by adding cumulative concentrations of ACh (0.1 ml x 10^{-9} -0.1 ml x 10^{-4} molar concentration) into the baths as depicted in Table 1.

Statistical analysis

The change in the tone of the rings as a response to the given concentrations of ACh were reported as changes in percentage from the contracted levels. The Kolmogorov-Smirnov test was used to examine the normal distribution of the data. The data was then analyzed by one-way analysis of variance (ANOVA) and presented as mean ± standard deviation. The differences were considered to be statistically significant with a p value of <0.05. The analytical results were evaluated using the SPSS version 15.0 for Windows software program (SPSS Inc., Chicago, IL, USA).

RESULTS

The changes in percentage of the vascular tone in all groups for each concentration of ACh are shown in Table 2. The relaxation responses of the aortic rings to any given concentration of ACh in the control and DMSO groups did not significantly differ. However, in the CIL group, except for the initial concentration of ACh, the relaxation responses were significantly increased ($p<0.05$) for each ACh concentration compared with the other two groups (Figure 1). The relaxation responses gradually increased with the elevated concentrations of Ach, and these became more evident with high concentrations ($p<0.001$ for $3x10^{-5}$ and 10^{-4} moles). This represented a concentration-dependent augmentation of vasodilation.

The relaxation response of the aortic rings in the CIL group for each concentration of Ach (other than the first one) resulted in a reduction of vascular tone beyond

Table 2. Relaxation percentage of the aortic rings as a response to the acethylcholine doses

Acetylcholine doses	Control group	Cilostazol group	DMSO group
	Mean±SD	Mean±SD	Mean±SD
1st	52.1±11.9	84.2±52.8	50.2±15.9
2nd	59.7±11.9	103.2±65.1	55.8±18.9
3rd	65.5±18.6	116.1±75.6	64.7±19.0
4th	69.6±16.5	120.6±75.6	60.5±16.4
5th	71.7±15.3	133.5±83.6	66.7±18.0
6th	78.8±11.5	150.1±89.8	65.0±21.2
7th	81.6±18.6	157.5±94.4	74.6±20.2
8th	78.8±18.8	167.2±99.3	83.4±23.5
9th	84.3±14.7	187.4±104.9	94.7±24.2
10th	86.6±13.0	217.3±99.7	101.5±18.0
11th	89.2±9.7	280.4±99.4	90.1±10.0

DMSO: Dimethylsulfoxide; SD: Standard deviation.

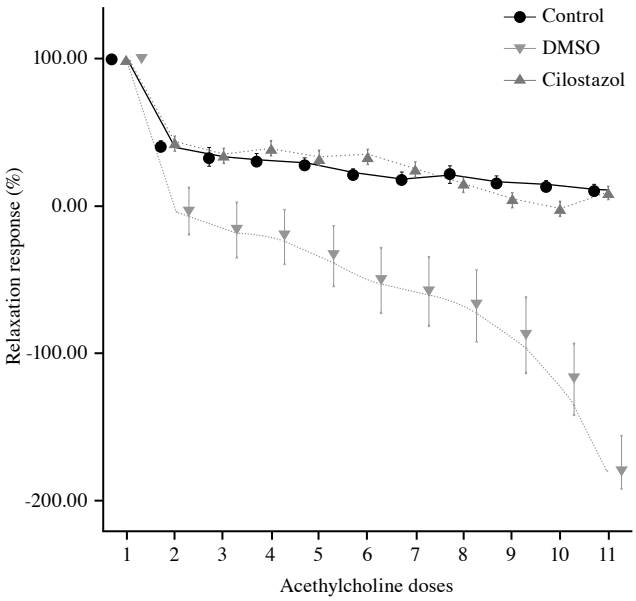

Figure 1. The relationship between the relaxation percentage of the aortic rings for each group and the concentration of acethylcholine in the baths. DMSO: Dimethylsulfoxide.

the basal tonus. The relaxation percentage following the second dose of ACh was 103.23%, which corresponded to a vascular tone that was lower than the basal tonus. This phenomenon became more notable as the ACh doses increased and reached 280.42% relaxation for the maximum dose of ACh used in our study. On the other hand, neither of the rings in the control or DMSO groups exhibited a relaxation response, which resulted in a vascular tone below the level of the basal tonus.

DISCUSSION

In 1980, Furchgott and Zawadzki[12] reported that the removal of the endothelial layer in isolated arteries prevents the dilator response of the endothelium to ACh. This pioneering study showed the presence of a diffusible factor that leads to relaxation in the vascular smooth muscle cells. This became known as the endothelium-derived relaxing factor (EDRF) and was subsequently recognized as NO.[1] Since then, various studies have revealed the importance of the endothelium in maintaining homeostasis since it represents a dynamic group of cells with autocrine and paracrine functions.[13] Both the disruption of endothelial integrity and dysfunction of endothelial cells are now considered to be major factors in the pathogenesis of vascular diseases.[14,15]

Vascular disorders, including hypertension, atherosclerosis, and vasospasm, are associated with a defect in the endothelial cells that produces NO, resulting in the loss of local regulation of the basal arterial tone. This then leads to compromises in blood flow.[16] Panza et al.[17] demonstrated that there is a deficiency in the endothelium-derived NO system that contributes to increased vascular resistance in essential hypertension. In the early phase of atherosclerosis, the ability of the endothelium to release NO is reduced, and the tendency towards production of endothelium-dependent contracting factors is enhanced.[18] Similarly, in diabetes mellitus (DM), the endothelial NO synthase (eNOS) activity and NO production are impaired, thus disturbing the endothelium-dependent vasodilatation.[19]

Cilostazol is a selective phosphodiesterase III enzyme inhibitor used in the pharmacological treatment of intermittent claudication (IC), the main symptom of peripheral arterial disease. It has been proven to lead to increases in claudication-free walking and maximum walking distances.[20] This feature is essentially dependent on the vasodilatation potential.[21] Cilostazol induces vasodilatation by triggering the release of vasodilator agents in the endothelium and by directly relaxing the vascular smooth muscle cells.

The beneficial effects of CIL on the restoration of vascular functions has been extensively studied in various disorders that cause endothelial dysfunction. Our study focused on investigating the effect of chronic CIL administration on the vascular reactivity of the intact endothelium. For this purpose, we isolated the aortic rings from rats that received intraperitoneal CIL for six weeks and examined the endothelium-dependent relaxation responses in an organ bath. In the rats treated with CIL, the aortic rings exhibited a significantly increased vasodilatation response to ACh compared with the control group. This response occurred in a dose-dependent manner that became more apparent with increased doses of ACh and resulted in a relaxation response beyond the level of the basal vascular tonus. There were no statistically significant differences regarding the relaxation responses of the aortic rings between the DMSO and control groups. This suggests that the enhanced vasodilatation response of the aortic rings to ACh obtained in the CIL group was particularly related to CIL and not DMSO.

The vascular protective effect that CIL possesses is substantially associated with its pharmacological action on the NO pathway.[22] Cilostozol induces NO production by eNOS activation via both cAMP/protein kinase A and phosphatidylinositol-3-OH kinase (Pl3K)/ arachidonyl trifluoromethyl ketone (ATK)-dependent mechanisms.[23] The treatment of human umbilical vein endothelial cells (HUVECs) with CIL has resulted in the activation of AMP-activated protein kinase and

caused phosphorylation of eNOS, causing increased production of NO.[24] Oyama et al.[25] demonstrated that in hypertensive rats, five weeks of CIL treatment preserved eNOS phosphorylation and cerebral blood flow. Furthermore, CIL has recently been declared to improve the ischemia/reperfusion-associated defect in ACh-dependent relaxation in rat aortae and increase tissue nitrite and nitrate levels, which has been suggested to be mediated by eNOS phosphorylation.[9]

In addition to the increased production of NO, CIL has also been found to trigger the release of PGI2,[26] which inhibits the release of noradrenaline from adrenergic nerve endings, thereby indirectly causing a vasodilator effect.

Our study involved the utilization of commercial tablets as a source of CIL, which could be viewed as a potential limitation, although the substances used as adjunctives in the tablets were pharmacologically inactive. Another limitation was that this study provided no information related to CIL's mechanism of action on intact endothelium.

In this study, we observed an increased sensitivity of the endothelium to Ach- induced vasodilatation in a concentration-dependent manner in the group of rats treated with CIL. We suggest that CIL-induced NO production and PGI2 release can be one of the possible mechanisms of the enhanced relaxation response of intact endothelium. However, this finding could also be related to several other factors; hence further research is needed to determine the exact mechanisms related to the effect of CIL on the vascular endothelium.

Declaration of conflicting interests

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