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The Effects of L-Citrulline Pretreatment on the Isometric Tension of the Isolated Perfused Rat Aorta

Seyyed Reza Hashemi[1], Hossein Ali Arab[1], Behjat Seifi[2], Samad Muhammadnejad[3]

[1]*Department of Comparative Biological Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

[2]*Department of Physiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[3]*Cell-Based Therapies Research Center, Digestive Disease Research Institute, Tehran University of Medical Sciences, Tehran, Iran*

Abstract:

BACKGROUND: The achievement of an effective compound for prevention/treatment of hypertension with fewer complications has been of interest in recent years.

OBJECTIVES: This study aimed to examine the effects of L-citrulline pretreatment on the tension of isolated rat aortic tissues precontracted by different vasoconstrictors.

METHODS: Sixteen male Wistar rats (300-350g) were randomly divided into two groups of control and test. The control group was injected 1ml distilled water, while the animal in the test group received 200 mg/Kg L-citrulline (CIT) i.p. for 7days. Rats were euthanized, their thoracic aortas were immediately separated and placed into a petridish containing cold Kerebs-Henseleit solution (KHS). The aorta were cleaned of the surrounding tissues and cut into 4 rings in the presence of 95% O_2+5% CO_2. The aortic rings divided into 6 subgroups, were suspended into organ bath containing KHS at 37°C. The isolated rings were contracted by 2×10^{-6} M phenylephrine (Phe) and 60Mm KCl. When the plateau was reached, a cumulative concentration of acetylcholine (Ach) was added into organ bath to induce relaxation. The effects of CIT on relaxation and the role of NO were tested using L-NAME as a pharmacological probe.

RESULTS: The pretreatment of rats by CIT significantly ($P<0.05$) reduced the plateau contraction induced by Phe. CIT also significantly ($P<0.01$) decreased the contraction induced by KCl and L-NAME+Phe. However, cumulative addition of ACh significantly ($P<0.001$) decreased the vasoconstriction induced by Phe but not by KCl and L-NAME+Phe in both control and CIT-treated group.

CONCLUSIONS: It suggests that, CIT can reduce the rat aorta vasoconstriction through releasing NO.

Keywords:

Aortic ring, Hypertension, L-citrulline, NO, Vasorelaxation

Correspondence

Hossein Ali Arab, Department of Comparative Biological Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Tel: +98(21) 88259862, Fax: +98(21) 66933222, Email: harab@ut.ac.ir

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Introduction

Blood pressure (BP) is defined as the force exerted by blood upon any area of the walls of the blood vessels. Slight increase in blood pressure is normal in daily life, but even mild increase of arterial pressure is associated with cardiovascular dysfunction and subsequently reducing the lifespan in animals and human being (Bolívar, 2013; Weber and Lackland, 2016). Hypertension is known as systolic BP level above or equal to 14 mm Hg and/or diastolic BP level above or equal to 90 mm Hg (Mancia et al., 2013). It is now a main public health problem because of its vast outbreak all over the world which causes about 7.5 million deaths yearly in the world (Singh et al., 2017). Uncontrolled raised blood pressure can lead to various disorders including stroke, coronary artery disease, chronic kidney disease, alzheimer's disease and ophthalmologic disturbances (Aronow et al., 2011). Different hormones and mediators including some autacoids and neurotransmitters are involved in constriction or dilation of blood vessels, and thus, any imbalance in the actions of these substances can lead to cardiovascular dysfunctions including hypertension (Delacroix et al., 2014).

Nitric oxide (NO) is an important endogenous mediator which is produced by endothelium, which is initially known as an endothelium-derived relaxing factor (Levine et al., 2012). The previous studies have shown that the endothelial dysfunction can lead to reduction of NO generation, and subsequently causes hypertension (Rajendran et al., 2013). There is evidence that any disturbance in the NO production or its bioavailability is accompanied with other disorders including atherosclerosis and angiogenesis-associated disturbances (Zhao et al., 2015). In an attempt to access any NO donors or precursors, investigators found the L-argentine (ARG) as a potent precursor for NO production (Mcrae, 2016). However, further studies demonstrated that citrulline (CIT) can be another precursor for NO production (Allerton et al., 2018; El-Hattab et al., 2017). CIT as a nonessential amino acid, physiological situation, acts as an intermediate in urea cycle. However, in the disturbed intestinal conditions, it works as an essential amino acid (Papadia et al., 2017). It is made from ARG and glutamine in enterocytes, released into the blood circulation and distributes in different organs including kidneys, vascular endothelium. In these tissues, CIT is converted into ARG and NO, and so, it can enhance the NO production in the body (Romero et al., 2006). Although it is reported that oral supplementation of both CIT and ARG are effective on hypertensive patient, it seems that CIT is more efficient than ARG because of not metabolizing in the intestine and liver. Citrulline is known as a safe supplementation for oral administration in comparison with ARG, which in high doses can cause gastrointestinal adverse effects (Romero et al., 2006; Sanchez-Gonzalez et al., 2012).

The isolated perfused organ system is a classical pharmacological probe widely used to examine the effects and possible mechanisms of different substances on contraction or relaxation of vascular smooth muscles (Jespersen et al., 2015). Using this method, a few studies have tested the direct effects of CIT on rat aorta and have shown that it is able to induce a significant vasorelaxation on isolated tissues (Raghavan and Dikshit, 2001; Ruiz and Tejerina, 1998).

However, there is not any evidence to show the activities of the isolated aorta taken from rats pretreated by CIT. So, the present study was designed to investigate the effects of CIT pretreatment on the isolated rat aortic rings tension that was precontracted by different agents.

Materials and Methods

Animal and experimental groups: Sixteen male Wistar rats weighing 300- 350g were obtained from animal house of Faculty of Veterinary Medicine, University of Tehran. The rats housed at 23±1˚C in a 12-h light/dark cycle; beginning at 08.00 o'clock. All rats received water and standard food ad libitum until the time of experiment. All methods were in accordance with the national institutes of health guide for the care and use of laboratory animals (Council, 2011). Rats were randomly divided into two groups of 8 animals in each. The animals in group 1 were injected by 1ml distilled water i.p. but the animals in group 2 were injected 200 mg/Kg CIT i.p. for seven consecutive days.

Isolated aortic rings preparation: On the day after the final treatment, the rats were anesthetized with diethyl ether and were killed by dislocation of cervical vertebrae in agreement with institutional guidelines. The thoracic aorta was immediately removed and placed into a petridish containing cold Krebs-Henseleit solution of the following composition (g/L): NaCl 6.87, MgSO4 0.14, KCl 0.4, CaCl2 0.28, NaH-2PO4 0.14, D-Glucose 2, NaHCO3 0.5, and pH 7.4. The surrounding fats and connective tissues were removed carefully and each aorta was cut into rings approximately 3-5 mm in length, which were continuously gassed with a mixture of 95% O2+5% CO2. The aortic rings in each group were divided into 3 subgroups (n=6), and then, they were suspended vertically between two stainless steel mini hooks (LEO 145-8, ADInstruments, Australia) into the organ bath chambers (Panlab Four Chamber Organ Baths, Spain) containing 20 ml of Krebs-Henseleit. The upper hook was connected to an isometric force recording transducer (MLT0201/D, ADInstruments, Australia) by a silk string. A Power Lab/4SP (ADInstruments, Australia) system was used for conversion of isolated tissue activities to digital data, which were recorded by Lab Chart v7.3.8 software on Microsoft desktop computer. The Krebs-Henseleit was kept at 37±5 ˚C and gassed continuously with 95%O2+5% CO2 mixture (PH~7.4). The isolated rings were exposed under 1g basic tension and each ring was allowed to equilibrate for 30-45 min.

Experimental design: In an preliminary test, the aortic rings were precontracted with phenylephrine (Phe, Sigma-Alderich Chemical Company) and when the plateau was reached, the integrity of the endothelium was tested by the capability of the acetylcholine (Ach, Sigma-Aldrich) to induce relaxation, and the basic tension for each ring was 1g. The contractions were induced in 4 subgroups of isolated rings taken from both control and CIT-treated groups by adding Phe (M) and KCl (60 mM). When the plateau was reached, cumulative concentrations of ACh from to were added to the organ bath chambers to induce possible relaxation. To test the possible role of NO in the relaxation induced by Ach, the aortic rings of 2 remaining subgroups of both control and treatment groups, were incubated with Nω-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich) for 40 min and,

Table 1. Changes induced by DW (Distilled Water), CIT (Citrulline), Phe (Phenylephrine), L-NAME (Nω-nitro-L-arginine methyl ester) and ACh (Acetylcholine) on vasoactive tone of aortic rings rats. The vasoactive tone of aortic rings is determined by alteration in gram tension induced by treatments. All data expressed as mean ± S.E.M (n=6), and ***= P<0.001, **= P<0.01, *= P<0.05.

Drugs	Control group (DW-treated rats)	Test group (CIT-treated rats)
Phe (2×10^{-6} M)	1.83 ± 0.04	1.35 ± 0.03*
KCl (60 Mm)	2.28 ± 0.10*	1.73 ± 0.07**
L-NAME (2×10^{-4} M) + Phe (2×10^{-6} M)	2.31 ± 0.11*	1.77 ± 0.03**
Phe (2×10^{-6} M) + ACh (2×10^{-10}- 2×10^{-5} M)	1.11 ± 0.03***	0.94 ± 0.01***
KCl (60 Mm) + ACh (2×10^{-10}- 2×10^{-5} M)	2.28 ± 0.13	1.53 ± 0.08
L-NAME (2×10^{-4} M) + Phe (2×10^{-6} M) + ACh (2×10^{-10}- 2×10^{-5} M)	1.92 ± 0.15	1.38 ± 0.06

then, the relaxation procedure was induced by addition of cumulative dose of ACh. The concentrations for each drug or chemical substance were estimated as final concentration in the chamber.

Statistical analysis: All values were expressed as the mean±S.E.M. obtained from 6 tests in each subgroup. Relaxation was expressed as percentage decrease in contraction induced by Phe, KCl or L-NAME. The statistical comparison of the means was performed by a one way analysis of variance (ANOVA) and tukey post hoc test using the SPSS software (version 22). A P value less than <0.05 was considered significant.

Results

Effects of vasoconstrictors on the aortic rings tone: The contractions induced by Phe, KCl and L-NAME-Phe in the isolated aortic rings are displayed in table 1. As this table and figure 1 show all agents used as vasoconstrictors caused significantly contraction in the isolated rings obtained from both control and CIT treated groups. However, the contraction induced by KCl and L-NAME+Phe in control group was significantly (P<0.05) more than those induced by Phe (2.28 ± 0.10 g for KCl vs Phe 1.83 ± 0.04 g and L-NAME + Phe 2.31± 0.11g vs Phe). It was also shown that the vasocontraction induced by KCl and L-NAME was 54.41 ± 12.34 and 58.62 ± 14.03 percent more than that of Phe, respectively (table 1). However, the vasoconstriction induced by Phe was significantly (P<0.05) reduced in aortic rings taken from the animals treated by CIT (1.35±0.03 g vs 1.83 ± 0.04 g). It was estimated that, the CIT pretreatment was able to reduce the Phe-induced contraction by 57.42 ± 4.8% in comparison to the non-treated group (Fig 2). It was also shown that aortic rings contraction in CIT-treated rats were significantly (P<0.01) reduced by addition of KCl and L-NAME + Phe added to the chambers (42.96 ± 6.09% reduction for KCl and 40.83 ± 2.67% for L-NAME + Phe) (Figs 3 & 4).

Relaxation induced by ACh in the aortic rings of control and CIT-treated rats: The relaxation induced by cumulative concentrations of Ach in different subgroups obtained from both control and CIT-treated rats is demonstrated in table 1. It is shown that addition of cumulative doses of ACh to the aortic rings of control group precontracted by Phe, (2×10^{-10} - 2×10^{-5} M) was associated with significant (P<0.001) reduction in

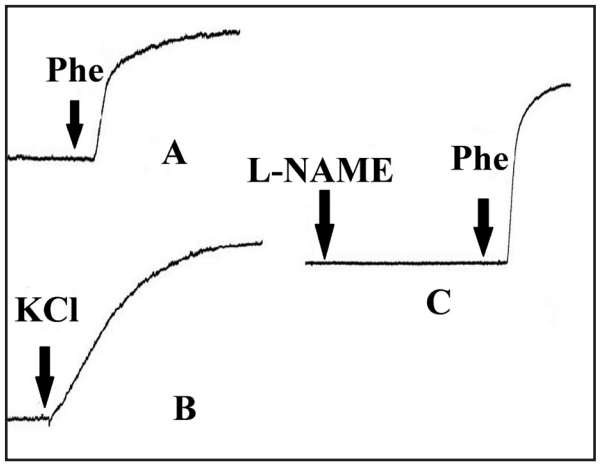

Figure 1. A comparison of vasoconstriction effects induced by phenylephrine Phe (2× 10^{-6} M) (A), KCl (60 mM) (B) and/or L-NAME (2× 10^{-4} M) + Phe (2× 10^{-6} M) (C) on the isolated rat aortic rings in the control (DW-treated) group.

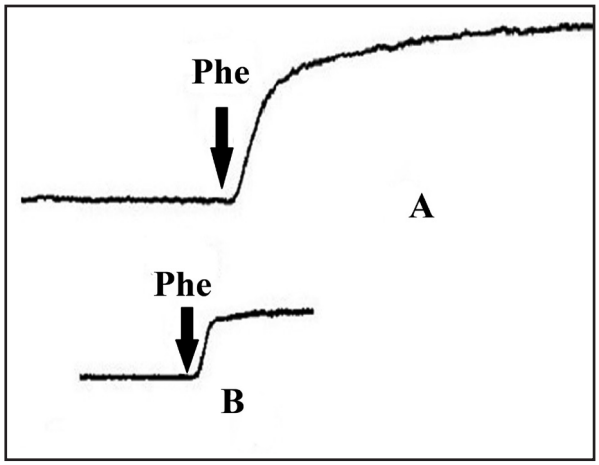

Figure 2. A comparison of the plateau contraction induced by Phe (2× 10^{-6} M) in the isolated aorta rings taken from control (A) and citrulline-treated (B) rats.

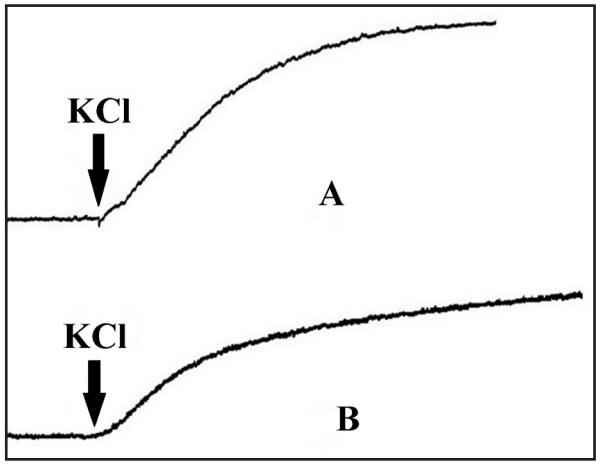

Figure 3. The effect of CIT-pretreatment (B) on the plateau contraction induced by KCl (60 mM) in the isolated rat aorta rings. The trace of A shows the plateau contraction in the isolated tissue taken from control group.

the contraction of isolated tissue from 1.83 ± 0.04 g to 1.11 ± 0.03g. This shows that ACh caused 85.02 ± 12.34% reductions in contraction of aortic rings. However, the use of Ach was not able to cause a significant reduction in contraction induced by KCl and L-NAME+ Phe. It was also showed that the use of cumulative ACh concentrations significantly (P<0.001) reduced the plateau contraction induced by Phe in aortic rings obtained from rats treated by CIT (1.35 ± 0.03 g vs 0.94 ± 0.01 g). A trace of the relaxation effects induced by cumulative concentrations of Ach on Phe-induced plateau contraction in the isolated aorta taken from control (A) and citrulline-treated rats is shown in Fig 5.

Discussion

The present study was designed to investigate the possible inhibitory effects of CIT pretreatment on aortic rings contracted by different vasoconstrictors using an isolated perfused organ system. Our data showed that the contraction induced by KCl and L-NAME+Phe in the isolated aorta rings taken from control group was significantly more than those induced by Phe alone. The use of ACh in a cumulative manner significantly reduced the vasoconstriction induced by Phe in the isolated rings, but not by KCl and L-NAME+Phe. However, in the aortic rings taken from rats pretreated by citrulline for 7 days, the contractile effects of Phe, KCl and L-NAME+Phe were significantly reduced and the reduction of the vasoconstriction induced by cumulative concentrations of ACh was significant in Phe-treated rings, while ACh was not able to decrease the plateau contraction induced by KCl and L-NAME+Phe.

The present study showed that the va-

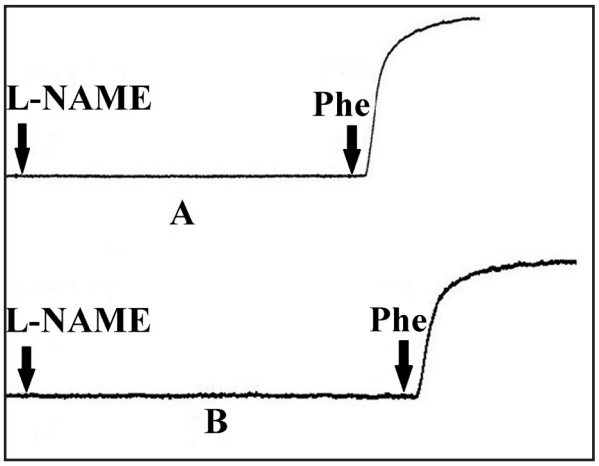

Figure 4. A comparison of the L-NAME (2×10^{-4} M) + Phe (2×10^{-6} M)-induced plateau contraction in the isolated rat aorta rings taken from control group (A) and citrulline-treated group (B).

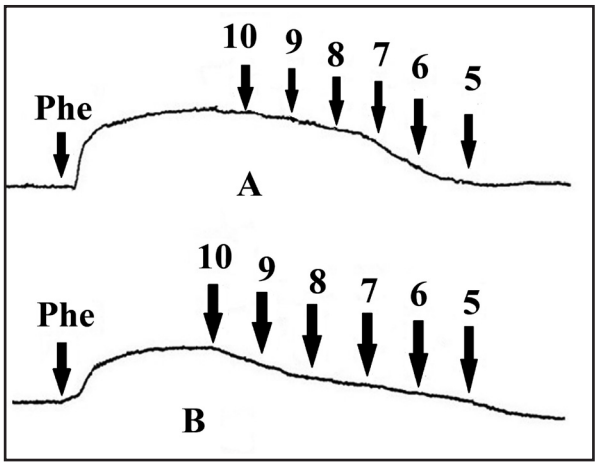

Figure 5. The trace of the relaxation effects induced by cumulative concentrations of acetylcholine (ACh) on Phe (2×10^{-6} M)-induced plateau contraction in the isolated aorta taken from control (A) and citrulline-treated rats. (B) (The numbers indicate the cumulative concentrations of ACh from 2×10^{-10}- 2×10^{-5} M).

soconstriction effect induced by KCl was more potent than that of Phenylephrine. The mechanism by which Phe exerts vasoconstriction effect on smooth muscles is due to the interaction with α1 adrenergic receptors. This leads to activation of phospholipase C, decomposition of certain phospholipids in the cell membrane and releasing inositol triphosphate (IP3) (Syrovatkina et al., 2016). The IP3 as a second messenger causes calcium mobility and consequently induces contraction in the vasoactive smooth muscle cells (Rosenbaum et al., 2009). However, the possible mechanism of action of KCl is through membrane depolarization and causes ca+2 mobility via voltage-operated ca+2 channels (VOCCs). This mechanism will activate myosin light chain kinase (MLCK), and increases in the myosin light chain phosphorilation (Ratz et al., 2005). It was also shown that the simultaneous use of L-NAME with Phe accelerates the contractile effects of Phe. L-NAME as an endothelial nitric oxide synthase (eNOS) inhibitor was used to investigate the possible role of NO in vasodilatation induced by ACh and CIT in rat aorta (Dodd-O et al., 1997). While addition of ACh as stimulator of NO generation in the vicinity of isolated organ reduced vasoconstriction induced by Phe (Woodman and Boujaoude, 2004), it was not able to inhibit the contraction induced by Phe + L-NAME (Dodd-O et al., 1997). This shows that the generation of NO is inhibited by L-NAME and thus, ACh was not able to reduce the vasoconstriction induced by Phe in isolated aortic rings. Our results showed that the plateau contractions induced by Phe, KCl and/or L-NAME+Phe were significantly decreased in the aortic rings taken from the CIT-pretreated rats in comparison with control group. ACh also caused significant vasorelaxation in the CIT-pretreated aortic rings precontracted by Phe. This suggests that NO, as an endothelium-derived mediator is involved in the CIT- and Ach-induced relaxation in the rat aortic rings.

It has been shown that NO has a fundamental role in the ACh-induced relaxation of the rat thoracic aorta (Callera et al., 2000; Woodman et al., 2004). Amino acid L-arginine is converted to NO and CIT by three ni-

tric oxide synthase (NOS) isoforms; eNOS presents in vascular endothelium, neuronal (nNOS) presents in the neurons, and cytokine-inducible NOS (iNOS) presents in different cell types including muscles, and macrophages. The eNOS has a crucial role in the regulation of vascular tone, whilst iNOS induces high generation of NO in the pathological conditions (Förstermann et al., 1994; Villanueva and Giulivi, 2010). There is evidence that NO plays an important role in the cardiovascular system function and, therefore, any disturbances in its generation and/or its bioavailability can lead to different disorders including hypertension and atherosclerosis (Zhao et al., 2015). The vascular endothelium plays a pivotal role in the basic and dynamic regulation of blood vessels diameter by releasing NO (Rajendran et al., 2013). In this tissue, L-arginine is converted to NO and CIT by the eNOS in the presence of tetrahydrobiopterin (BH4), NADPH and oxygen (Zhao et al., 2015). Different substances including acetylcholine, bradykinin and histamine, can trigger the activity of the enzyme (eNOS), and subsequently increase NO generation (Kuchan and Frangos, 1994). It has been shown that the oral consumption of L-arginine was effective to improve the NO-mediated vascular action in different diseases including coronary artery diseases, congestive heart failure and peripheral arterial disease (Mcrae, 2016). L-arginine supplementation can ameliorate the endothelial dysfunction and as a result can improve the NO production in vascular system (Preli et al., 2002). It has been shown that CIT is also involved in the generation of NO in most tissues (Papadia et al., 2017). CIT is productively converted to L-arginine in many cells, and thus, both arginine and CIT represent a backup system for NO generation in different tissues (Oyadomari et al., 2001). Three enzymes are involved for recycling of CIT leading to production of NO. In the kidney and vascular endothelium, argininosuccinate synthase (ASS) converts CIT to argininosuccinate, then argininosuccinate is converted to arginine by argininosuccinate lyase (ASL), and finally the ARG converts to NO by eNOS (Kaore et al., 2013). The released NO diffuses into the smooth muscle cells and activates soluble guanylyl cyclase for production of cyclic Guanosine Mono Phosphate (cGMP) synthesis from Guanosine Triphosphate (GTP). The cGMP attaches and activates protein kinase G (PKG) and depression of calcium influx into the smooth muscle cells and consequently muscle contraction (Mendes-Junior et al., 2015; Morita et al., 2014). Studies show that oral consumption of CIT can lead to an up regulation of the eNOS expression, amelioration of endothelial dysfunction and acts as an anti-atherosclerosis effect in animal models (Hayashi et al., 2005; Romero et al., 2008). It has been suggested that in addition to release of NO, the mechanism of CIT vasodilatation is exerted through the prostaglandin pathways (Mori et al., 2015). This result is in agreement with the present study, because the use of L-NAME could not entirely inhibit the decreasing effect of CIT on L-NAME+Phe-induced plateau contraction in the isolated aortic rings taken from-CIT treated rats.

In conclusion, the results of the present study indicate that the pretreatments of rats by CIT was effective against vasoconstriction induced by different vasoactive agents in the isolated aortic rings. This suggests that the use of CIT can be a promising method to improve the endothelial dysfunction

and NO deficiency in hypertension.

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Conflicts of interest

The author declared no conflict of interest.

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اثرات پیش درمانی سیترولین بر روی فشار ایزومتریک حلقه‌های آئورت ایزوله جدا شده از موش رت

سیدرضا هاشمی[۱] حسینعلی عرب[۱] بهجت سیفی[۲] صمد محمدنژاد[۳]

۱) گروه علوم زیستی مقایسه‌ای، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران
۲) بخش فیزیولوژی، دانشکده پزشکی دانشگاه علوم پزشکی تهران، تهران، ایران
۳) مرکز تحقیق درمان‌های سلولی، انستیتو علمی بیماری گوارشی دانشگاه علوم پزشکی تهران، تهران، ایران

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چکیده

زمینه مطالعه: دستیابی به یک ترکیب موثر ولی با عوارض کمتر، برای پیشگیری و درمان فشار خون بالا در سال های اخیر مورد توجه بوده است.

هدف: هدف از مطالعه حاضر بررسی تأثیر پیش درمانی سیترولین روی انقباض قطعات ایزوله عروقی جدا شده از آئورت موش صحرایی بوده است.

روش کار: شانزده موش صحرایی نر نژاد ویستار (۳۵۰-۳۰۰ g) به صورت تصادفی به دو گروه کنترل و آزمون تقسیم شدند. به گروه شاهد یک میلی لیتر آب مقطر تزریق شد، در حالی که حیوانات گروه آزمون مقدار ۲۰۰ mg/kg سیترولین از راه صفاقی، برای مدت ۷ روز دریافت کردند. آئورت های قفسه سینه آنها بلافاصله بیرون آورده شده و در محلول حاوی Kerebs-Henseleit (KHS) قرار داده شدند. آئورت از بافت های اطراف تمیز شد و در حضور $95\% O_2 + 5\% CO_2$ به چند حلقه ۳ میلی‌متری تقسیم شدند. حلقه‌های آئورت به ۶ زیرگروه تقسیم شدند و در حمام بافت حاوی KHS در دمای ۳۷°C معلق شدند. حلقه‌های جدا شده توسط فنیل افرین (Phe) یا ۶۰ mmol پتاسیم کلراید (KCl) منقبض شدند. پس از ایجاد کفه، غلظت تجمعی از استیل کولین (ACh) برای ایجاد شل شدگی به حمام بافت افزوده شد. اثرات سیترولین بر شل شدگی و نقش نیتریک اکساید (NO) با استفاده از L-NAME به عنوان یک ابزار فارماکولوژیک، مورد بررسی قرار گرفت.

نتایج: تزریق موش‌ها با سیترولین به طور معنی‌داری ($P<0/05$) باعث کاهش انقباض عضلانی ناشی از Phe شد. سیترولین همچنین، به طور معنی‌داری ($P<0/01$) انقباض ناشی از KCl و L-NAME+Phe را کاهش داد. اما، اضافه شدن دوزهای تجمعی از ACh به طور معنی‌داری ($P<0/001$) باعث کاهش انقباض ناشی از Phe، اما نه ناشی از KCl و L-NAME+Phe، در گروه کنترل و دریافت کننده سیترولین شد.

نتیجه گیری نهایی: نتایج این مطالعه نشان می‌دهد که سیترولین می‌تواند سبب کاهش انقباض عروقی از طریق آزاد کردن NO در رگ‌ها شود.

واژه‌های کلیدی:
حلقه آئورت، فشارخون، سیترولین، نیتریک اکساید، شل شدگی عروق

نویسنده مسؤول: تلفن: ۸۸۲۵۹۸۶۲ (۲۱)۹۸+ نمابر: ۶۶۹۳۳۲۲۲ (۲۱)۹۸+ Email: harab@ut.ac.ir