Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

MODULATION OF N-METHYL-D-ASPARTATE RECEPTORS IN ISOLATED RAT HEART

Journal:	*Canadian Journal of Physiology and Pharmacology*
Manuscript ID	cjpp-2017-0056.R1
Manuscript Type:	Article
Date Submitted by the Author:	28-Jun-2017
Complete List of Authors:	Srejovic, Ivan; University of Kragujevac, Faculty of Medical Sciences, Department of Physiology Zivkovic, Vladimir; University of Kragujevac, Faculty of Medical Sciences, Department of Physiology Nikolic, Tamara; University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy Jeremic, Nevena; University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy Stojic, Isidora; University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy Jeremic, Jovana; University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy Djuric, Dragan; University of Belgrade, Medical Faculty, Institute of Medical Physiology "Richard Burian" Jakovljevic, Vladimir; University of Kragujevac, Faculty of Medical Sciences, Department of Physiology; First Moscow State University I.M. Sechenov, Department of Human Pathology
Is the invited manuscript for consideration in a Special Issue?:	IACS European Section 2016
Keyword:	N-methyl-D-aspartate receptors, homocysteine, memantine, ifenprodil, oxidative stress

MODULATION OF N-METHYL-D-ASPARTATE RECEPTORS IN ISOLATED RAT HEART

Ivan Srejovic[1], Vladimir Zivkovic[1], Tamara Nikolic[2], Nevena Jeremic[2], Isidora Stojic[2], Jovana Jeremic[2], Dragan Djuric[3], Vladimir Jakovljevic[1,4]

[1]*University of Kragujevac, Faculty of Medical Sciences, Department of Physiology, Kragujevac, Serbia,*

[2]*University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy, Kragujevac, Serbia,*

[3]*University of Belgrade, Faculty of Medicine, Institute of Medical Physiology "Richard Burian," Belgrade, Serbia,*

[4]*First Moscow State University I.M. Sechenov, Department of Human Pathology, Moscow, Russian Federation*

Correspondence should be addressed to:

Prof. Vladimir Lj. Jakovljevic, MD, PhD

Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, SERBIA

Phone: +381 34 342944; Fax: +381 34 306800/ext 112; E-mail: drvladakgbg@yahoo.com

LIST OF ABBREVIATIONS

NMDA-R - N-methyl-D-aspartate receptor

NMDA - N-methyl-D-aspartate

DL-Hcy TLHC - DL-homocysteine thiolacotne

CF - coronary flow

NO_2^- - nitrites

O_2^- - superoxide anion radical

H_2O_2 – hydrogen peroxide

AMPA-R - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

HHcy – hyperhomocysteinemia

CNS - central nervous system

NO - nitric-oxide

NOS - nitric-oxide synthase

MMP - matrix metalloproteinase

ROS - reactive oxygen species

CPP - constant perfusion pressure

dp/dt max - maximum rate of pressure development in the left ventricle

dp/dt min - minimum rate of pressure development in the left ventricle

SLVP - systolic left ventricular pressure

DLVP - diastolic left ventricular pressure

HR - heart rate

MPT - mitochondrial permeability transition

RVLM - rostral ventrolateral medulla

CVLM - caudal ventrolateral medulla

RyR2 - ryanodine receptor 2

ABSTRACT

Considering the limited data about the role of N-methyl-D-aspartate receptors (NMDA-R) in cardiovascular system and heart, the aim of the present study was to examine the effects of NMDA and DL-homocysteine thiolacotne (DL-Hcy TLHC), alone and in combinations with glycine, memantine and ifenprodil, in the isolated rat heart. The hearts of Wistar albino rats were retrogradely perfused according to the Langendorff technique at a constant perfusion pressure. The experimental protocol for all experimental groups included the stabilization period, application of estimated substance in duration of 5 minutes, followed by wash-out period in duration of 10 minutes. Using sensor in the left ventricle we registered the next parameters of myocardial function: dp/dt max, dp/dt min, systolic and diastolic left ventricular pressure, and heart rate, and coronary flow (CF) was measured flowmetrically. In the coronary venous effluent spectrophotometrically were estimated following oxidative stress biomarkers: TBARS, NO_2^-, O_2^-, and H_2O_2. NMDA alone did not induce any change in any observed parameter, while DL-Hcy TLHC alone, as well as combined application of NMDA and DL-Hcy TLHC with glycine, induced reduction of most cardiodynamic parameters. Memantine and ifenprodil induced reduction of cardiodynamic parameters and coronary flow, as well as some oxidative stress biomarkers.

Key words: N-methyl-D-aspartate receptors, homocysteine, memantine, ifenprodil, oxidative stress, cardiodynamics, isolated rat heart

INTRODUCTION

The N-methyl-D-aspartate receptors (NMDA-R) belong to the ionotropic glutamate receptor family together with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R), kainite receptor and delta (δ) receptor. NMDA-R are heterotetramers, composed of four subunits, two obligatory, glycine binding GluN1 subunits, and two glutamate binding GluN2 subunits, or one GluN2 subunit and one GluN3 subunit (Vyklicky et al. 2014). NMDA-R subunits are assembled to form a central channel through the cell membrane that functions as an ion channel. The specificities of the NMDA-R, compared to other receptors of the same group, include necessity of the presence of the two co-agonists, glutamate and glycine, for receptor activation, the entry of considerable quantities of calcium following receptor activation, and dual control of receptor function, by ligands (glutamate and glycine) and by changing the value of the membrane potential (Traynelis et al. 2010; Wollmuth et al. 1998). Depending on the subtypes of subunits which build NMDA-R vary their characteristics regarding calcium permeability, channel conductance, opening rate, voltage dependent magnesium blocking (Perez-Otano et al. 2001; Qian and Johnson 2006).

N-methyl-D-aspartate (NMDA) binds to a glutamate binding place and exerts the same effect as glutamate. Homocysteine (Hcy) also binds to glutamate binding site, and it is considered that detrimental effects of hyperhomocysteinemia (HHcy, plasma values of Hcy above 15 μmol/l) in various conditions arise from excessive activation of the NMDA receptors (Hankey and Eikelboom 1999; Moshal et al. 2008; Snyder et al. 2005). Use of memantine, as an NMDA-R antagonist, in the case of Alzheimer's disease has a neuroprotective effect, bearing in mind the fact that amyloid beta exerts its negative effect by increasing the concentration of glutamate in extrasynaptic space (Ota et al. 2015). On the other hand, substances, which are

denoted as allosteric modulators of NMDA-R, are attracting more attention due to the possibility of fine regulation of receptor function and higher selectivity depending on the composition of the subunits that make up the receptor. Ifenprodil causes noncompetitive inhibition of NMDA-R that contains the GluN2B subunit and it has been shown its neuroprotective effect during cerebral ischemia (Carter et al. 1988; Hess et al. 1998).

Despite relatively well-known roles of NMDA-R in central nervous system (CNS), there are growing data considering localization of NMDA-R in other organs and tissues, including the cardiovascular system (Morhenn et al. 1994; Bozic and Valdivielso 2015). Investigation of time and spatial distribution of radiolabelled antagonists of NMDA-R showed widespread acceptance of these receptors in a number of organs and tissues, including heart (Näsström et al. 1993). NMDA-Rs are found in different cell types that exist in the cardiovascular system, particularly in endothelial cells, vascular smooth muscle cells and cardiomyocytes (Chen et al. 2005; Gao et al. 2007).

Since the activation of the NMDA-Rs is associated with entry of calcium into the cytoplasm, their overstimulation causes an increase intracellular calcium content and consequent imbalance in the production and elimination of free radicals and oxidative stress (Kamel et al. 2008; Gao et al. 2007). Inactivation of NMDA-Rs by deletion of GluN1 subunit induced decrease of production of reactive oxygen species (ROS) during HHcy in the heart, as well as decreased content of nitric-oxide (NO) and matrix metalloproteinase 9 (MMP9) in cardiomyocytes mitochondria (Tyagi et al. 2010; Moshal et al. 2008).

There are many doubts related to physiological function of NMDA-R in cardiovascular system, as well as their role in certain pathophysiological conditions. Accordingly, the aim of this study was to investigate the acute effects of N-methyl-D-aspartate (NMDA), DL-

homocysteine thiolactone (DL-Hcy TLHC), as well as their combinations with glycine, memantine and ifenprodil on cardiac function, coronary flow and oxidative stress, or to assess the effects of acute modulation of NMDA-R in heart by mentioned substances, and possible role of oxidative stress in that sense.

MATERIAL AND METHODS

Isolated rat heart preparation

Seventy two animals were included in study, 12 animals per experimental group. All experiments were conducted on male Wistar albino rats (8 weeks old, body mass 180-200 g), obtained from Military Medical Academy, Belgrade, Serbia. After anesthesia induced by ketamine (10 mg/kg) and xylazine (5 mg/kg), the animals were euthanized via cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986, UK). After euthanasia and prompt thoracotomy, the rapid cardiac arrest was induced by superfusion with ice-cold isotonic saline and hearts then were quickly excised and attached to the Langendorff apparatus (Experimetria Ltd, 1062 Budapest, Hungary) via aortic cannulation. This was followed by removal of the left auricula and making the incision in the left atria, through which the sensor (transducer BS4 73-0184, Experimetria Ltd, Budapest, Hungary) is placed in the left ventricle, for continuous measurement of cardiac function parameters. The hearts were retrogradely perfused under a constant perfusion pressure (CPP) of 70 cmH_2O with complex Krebs-Henseleit solution composed of the following (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.7, $NaHCO_3$ 25, KH2PO4 1.2, glucose 11, pyruvate 2, equilibrated with 95% O_2 plus 5% CO_2 at 37 ºC (pH 7.4).

Experimental protocol

The hearts from all experimental groups were undergone to a 25 minutes stabilization period. During this period, each of the hearts was subjected to short term occlusion (20 s) followed by simultaneous bolus injections of 5 mmol/l adenosine (60 µl at a flow of 10 ml/min to elicit maximal coronary flow) to test coronary vascular reactivity. If coronary flow (CF) did

not increase by 100% compared with control values, the hearts were disposed of. Coronary flow was determined flowmetricaly. When the CF was stabilised (three repeated measurements of the same value), samples of coronary effluent were collected (control value - C), and the experimental protocol was initiated. Hearts were perfused with:

1. 100 µmol/l N-methyl-D-aspartate (NMDA)
2. 100 µmol/l N-methyl-D-aspartate (NMDA) + 100 µmol/l glycine
3. 10 µmol/l DL-homocysteine thiolactone (DL-Hcy TLHC)
4. 10 µmol/l DL-homocysteine thiolactone (DL-Hcy TLHC) + 100 µmol/l glycine
5. 100 µmol/l memantine
6. 1 µmol/l ifenprodil

Each of the applied substances was administered for 5 minutes, followed by a wash-out period lasting for 10 minutes. In the last minute of substance application (effect - E) and in the last minute of the wash-out period (wash-out - W) the samples of coronary venous effluent were collected. Using the sensor within the left ventricle, the following parameters of myocardial function were determined:

1. The maximum rate of pressure development in the left ventricle (dp/dt max)
2. The minimum rate of pressure development in the left ventricle (dp/dt min)
3. The systolic left ventricular pressure (SLVP)
4. The diastolic left ventricular pressure (DLVP)
5. The heart rate (HR)

All research procedures were carried out in accordance with European Directive for welfare of laboratory animals N^{o} 86/609/EEC and principles of Good Laboratory Practice (GLP),

approved by Ethical committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Biochemical assays

The following oxidative stress parameters were determined spectrophotometrically (Shimadzu UV 1800, Japan) using collected samples of the coronary venous effluent:

1. The index of lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS)
2. The level of nitrite (NO_2^-)
3. The level of the superoxide anion radical (O_2^-) and
4. The level of hydrogen peroxide (H_2O_2)

TBARS determination (index of lipid peroxidation)

The degree of lipid peroxidation in the coronary venous effluent was estimated by measuring TBARS, using 1% thiobarbituric acid in 0.05 NaOH, which was incubated with the coronary effluent at 100°C for 15 min and measured at 530 nm. Krebs–Henseleit solution was used as a blank probe (Ohkawa et al. 1979).

Determination of the nitrite level

Nitric-oxide decomposes rapidly to form stable nitrite/nitrate products. The nitrite level (NO_2^-) was measured and used as an index of nitric oxide (NO) production, using Griess's reagent. A total of 0.5 ml of perfusate was precipitated with 200 µl of 30% sulpho-salicylic acid, vortexed for 30 min, and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess's reagent,

containing 1% sulphanilamide in 5% phosphoric acid/0.1% naphthalene ethylenediamine-di hydrochloride were added and incubated for 10 min in the dark and measured at 543 nm. The nitrite levels were calculated using sodium nitrite as the standard (Green et al. 1982).

Determination of the level of the superoxide anion radical

The level of the superoxide anion radical (O_2^-) was measured via a nitro blue tetrazolium (NBT) reaction in TRIS buffer with coronary venous effluent, at 530 nm. Krebs–Henseleit solution was used as a blank probe (Auclair and Voisin 1985).

.

Determination of the hydrogen peroxide level

The measurement of the level of hydrogen peroxide (H_2O_2) was based on the oxidation of phenol red by hydrogen peroxide in a reaction catalysed by horseradish peroxidase (HRPO) (Pick and Keisari 1980). Two hundred microliters of perfusate was precipitated using 800 ml of freshly prepared phenol red solution; 10 µl of (1:20) HRPO (made ex tempore) was subsequently added. For the blank probe, an adequate volume of Krebs–Henseleit solution was used instead of coronary venous effluent. The level of H_2O_2 was measured at 610 nm.

Drugs

All drugs used in this experimental protocol were provided by Sigma-Aldrich.

Statistical analysis

All values are expressed as mean ± SE. Paired t test was used in statistical analysis and p values less than 0.05 were considered to be statistically significant.

RESULTS

The effects of N-methyl-D-aspartate on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Application of N-methyl-D-aspartate (NMDA) did not induce any significant change of the observed cardiodynamic parameters and coronary flow (Figure 1).

The effects of combined application of N-methyl-D-aspartate and glycine on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

The simultaneous administration of N-methyl-D-aspartate (NMDA) and glycine induced significant decrease of dp/dt max, dp/dr min, SLVP, HR and CF (Figure 1a, 1c, 1d, 1e and 1f). During the wash-out period values of all mentioned parameters were significantly increased, and reached values that were insignificantly different compared to control.

The effects of DL-homocysteine thiolactone on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Application of DL-homocysteine thiolactone (DL-Hcy TLHC) induced significant decrease of dp/dt max, SLVP, HR and CF (Figure 1a, 1c, 1d, 1e and 1f). DLVP was insignificantly decreased, but this decrease continued during wash-out period, so that the control value and the value after the wash-out period differed significantly (Figure 1d). Flowing wash-out period the values of dp/dt max and CF were insignificantly increased, so that the control values and the values after the wash-out period did not differ significantly (Figure 1a and 1f). Values of SLVP and HR after wash-out period were significantly lower compared to the control (Figure 1c and 1e).

The effects of combined application of DL-homocysteine thiolactone and glycine on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Combined administration of DL-homocysteine thiolactone (DL-Hcy TLHC) and glycine induced significant decrease of all observed cardiodynamic parameters (dp/dt max, dp/dt min, SLVP, DLVP and HR), as well as CF (Figure 1a, 1b, 1c, 1e and 1f). Values of all mentioned parameters were significantly increased during wash-out period, and reached values similar to control, except HR, which was significantly lower after wash-out period.

The effects of memantine on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Memantine induced significant decrease of dp/dt max, dp/dt min, SLVP, HR and CF. During wash-out period all mentioned parameters were significantly increased, and all of them, except HR, reached the initial values (Figure 1a, 1b, 1c, 1e and 1f). The value of DLVP was not significantly changed during the experiment (Figure 1d).

The effects of ifenprodil on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Ifenprodil induced significant decrease of CF and HR compared with control conditions. Following the wash-out period both mentioned parameters were significantly increased and reached values approximate to control (Figure 1e and 1f). Furthermore, dp/dt max was significantly increased during wash-out period (Figure 1a). Other cardiodynamic did not change significantly.

The effects of N-methyl-D-aspartate on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Administration of N-methyl-D-aspartate (NMDA) did not induce any significant change of the observed biomarkers of oxidative stress (Figure 2).

The effects of combined application of N-methyl-D-aspartate and glycine on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Combined administration of N-methyl-D-aspartate and glycine induced significant increase of TBARS, nitrites and superoxide anion radical, which were decreased significantly following wash-out period, to values similar to control (Figure 2a, 2b and 2c).

The effects of DL-homocysteine thiolactone on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

DL-homocysteine thiolactone (DL-Hcy TLHC) did not induce any significant change of the observed biomarkers of oxidative stress (Figure 2).

The effects of combined application of DL-homocysteine thiolactone and glycine on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Combined administration DL-homocysteine thiolactone (DL-Hcy TLHC) and glycine induced significant increase of TBARS and superoxide anion radical, which were decreased significantly during wash-out period, to values similar to control (Figure 2a and 2c).

The effects of memantine on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Memantine induced statistically significant reduction of production of superoxide anion radical (Figure 2c). Following wash-out period this reduction was even more pronounced, but on the other hand values of TBARS and hydrogen peroxide were significantly increased compared to the effect of memantine (Figure 2a, 2c and 2d).

The effects of ifenprodil on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Application of ifenprodil induced significant decrease of hydrogen peroxide levels. On the other hand production of nitrites was increased during wash-out period (Figure 2b and 2d).

DISCUSSION

The aim of the present study was to estimate the acute effects of N-methyl-D-aspartate (NMDA), combination of NMDA and glycine, DL-homocysteine thiolactone (DL-Hcy TLHC), combination of DL-Hcy TLHC and glycine, memantine and ifenprodil, as agonists and antagonists of NMDA receptors, on cardiac function, coronary flow and oxidative stress, or to assess the effects of acute modulation of NMDA-R in heart by mentioned substances, as well as possible role of oxidative stress in the resultant changes.

Application of NMDA in concentration of 100 μmol/l did not induce any significant change on cardiodynamic parameters, while on the other hand combination of NMDA and glycine induced significant changes in dp/dt max, dp/dt min, SLVP, HR and CF (Figure 1). Bearing in mind the fact that NMDA exerts its effects by binding to the glutamate binding site on NMDA-R and has the same effects as glutamate, these results are in correlation with our previous results where application of glutamate alone in same concentration also did not cause changes in cardiodynamic parameters nor coronary flow (Srejovic et al. 2015b). In the same research only combined application of both subunits agonists, glutamate and glycine, induced changes in heart function. Having all this in mind, we can conclude that, in this experimental model, it is necessary to apply both NMDA-R co-agonists for their activation (Cheriyan et al. 2015).

DL-Hcy TLHC in concentration of 10 μmol/l induced significant decrease of all observed cardiodynamic parameters and coronary flow, except dp/dt min (Figure 1). Simultaneous administration of DL-Hcy TLHC and glycine induced significant decrease of all observed cardiodynamic parameters (Figure 1). Moshal and co-authors in their investigation induced HHcy in mice and shoved that increased levels of Hcy have resulted in decrease of parameters of

cardiac contractility (Moshal et al. 2008). Since the use of inhibitors of NMDA-R, matrix metalloproteinase activity (MMP) and mitochondrial permeability transition (MPT) induced mitigation of the mentioned changes of myocardial contractility, these authors concluded that Hcy via NMDA-R activates MMP and induces MPT which results in a decrease in myocardial contractility.

DL-Hcy TLHC alone, as well as combination of DL-Hcy TLHC and glycine, induced significant reduction of SLVP and insignificant decrease of DLVP. During wash-out period value of DLVP in both mentioned experimental groups continued to decrease so that the value of this parameter was significantly lower after wash-out period compare to control (Figure 1c and 1d). Takemoto in his research examined the effects of the application of microinjection of L-Hcy in rostral ventrolateral medulla (RVLM) and caudal ventrolateral medulla (CVLM) (Takemoto 2016). Injection of L-Hcy to RVLM and CVLM induced pressor and depressor effects, respectively, which were abolished by previous microinjection of MK-801, as NMDA-R antagonist. On the other hand, Walker and co-workers showed that HHcy induces early disturbances in myocardial structure, without significant changes in function (Walker et al. 2004). Similar conclusions were performed by Joseph and colleagues based on results of their research (Joseph et al. 2003). Discrepancies in the results of these studies and our research could be the consequence of different experimental protocols. Reductions of SLVP and DLVP in our research represent direct effect of DL-Hcy TLHC on heart, since the effects of Hcy on heart via autonomic nervous system, as well as effects of chronic HHcy are excluded. Possible mechanisms that mediate in direct effects of DL-Hcy TLHC could include disturbances in calcium currents or nitric-oxide (NO) production (Rosenberger et al. 2006; Moshal et al. 2008). Differences in effects of NMDA alone and DL-Hcy TLHC alone might be the result of different

affinity of NMDA-R for these compounds, or eventually the existence of another mechanism through which the Hcy may exert its effect on the cardiovascular system (Flores-Soto et al. 2013). On the other hand, combinations of NMDA and glycine and DL-Hcy TLHC and glycine had similar effects on SLVP (Figure 1c).

DL-Hcy TLHC alone, as well as combined applications of DL-Hcy TLHC and NMDA with glycine, induced significant decrease of HR (Figure 1e). Muntzel and co-authors presumed that Hcy may cause increases in sympathetic nerve activity, what could be one of the mechanisms through which occur damage of cardiovascular system in HHcy, but their results showed that infusion of Hcy have no effects on heart rate and blood pressure (Muntzel et al. 2006). In previous results from our laboratory DL-Hcy TLHC has had similar effect on HR (Srejovic et al. 2015a). On the other hand, Resstel and coworkers showed increase of HR in rats with induced mild HHcy (Resstel et al. 2008). Different effects of Hcy on HR in these studies may be due to the different structures involved in regulating heart rate, keeping in mind that in chronic and *in vivo* experiments, the primacy in the regulation of HR might have influence of Hcy on the NMDA-R (and maybe other glutamate receptors) in certain structures in the CNS, whereas the experimental protocol used in this study focused on the direct effects on the heart and coronary circulation.

Similarly to HR, application of DL-Hcy TLHC alone, DL-Hcy TLHC and glycine and NMDA and glycine, induced significant decrease of CF. In groups treated with combinations of DL-Hcy TLHC and NMDA with glycine, values of CF were significantly increased during wash-out period, while in group treated with DL-Hcy TLHC value of this parameter was slightly increased during wash-out period, due to which the control values of CF and values after wash-out period did not differ significantly (Figure 1f). This decrease of CF is in accordance with

results of other studies, whereby the effects of Hcy on nitric-oxide synthase (NOS) and disturbances in NO production are most commonly mentioned as possible mechanism for this action (McCully 2016; Toda and Okamura 2016). Abahji and colleagues assessed the effects of HHcy induced by oral methionine supplementation on endothelial function in healthy subjects (Abahji et al. 2007). HHcy induced significant decrease in flow-mediated vasodilatation of the brachial artery, parameter which reflects endothelial function and NO synthesis.

Memantine as a noncompetitive NMDA-R antagonist, induced decrease of all observed cardiodynamic and coronary flow, except DLVP, whereby all these parameters returned to values approximate to control values, with the exception of HR (Figure 1). In the study conducted by the Makhro and co-workers, intracoronary application of memantine, as well as other NMDA-R antagonists (eliprodil, Ro25-6981, ketamine, and MK-801) exerted negative inotropic and chronotropic effect on autonomous heart function (Makhro et al. 2016). Seeber and co-authors pointed out the complex formation between NMDA-R subunit GluN2B and ryanodine receptor 2 (RyR2) in neonatal rat myocardium, so that negative inotropic effect induced by memantine probably occurs because of changes in the concentrations of Ca^{2+} and complies with the effects of other NMDA receptor antagonists, as it is not excluded existence of this complexes in older age (Seeber et al. 2004).

Memantine is in clinical use for the treatment of Alzheimer's disease, and most of its effects on cardiovascular system were noticed as side effects in the treatment of these patients (Takehara et al. 2015). Following the systemic effects of memantine as local anaesthetic, Chen and colleagues found that memantine induces decrease in mean arterial pressure and heart rate (Chen et al. 2012).

Beside the above mentioned bradycardic effect of NMDA-R antagonists, they have antiarrhythmic property also (Makhro et al. 2016). Both of these actions could be associated with prolongation of QT interval induced by memantine (Takehara et al. 2015; Howes 2014). Since memantine is used in therapy of Alzheimer's disease, the mostly examined were its effects on cerebral blood vessels and blood flow in the brain. Intravenous administration of memantine in anesthetized rats induced decrease of blood flow in brain by 15% in average within 10 minutes, and further reduction of blood flow reached 53% (Mirzoyan et al. 2014). In the human population of patients suffering from Parkinson's disease, memantine caused decrease of blood flow in basal ganglia and several frontal cortical areas (Borghammer et al. 2008).

Ifenprodil in concentration of 1 μmol/l induced significant reduction of HR and CF values, which were returned to values similar to initial after wash-out period (Figure 1). Furthermore, value of dp/dt max was significantly increased during wash-out period. Absence of effects of ifenprodil on myocardial contractility and systolic and diastolic pressure could be due to relatively modest applied dose. Also there are limited data dealing with effects of ifenprodil on cardiovascular system. Monassier and co-authors assessed the effects of ifenprodil on baroreceptor heart rate reflex in rats, whereby did not significantly change the basal values of hemodynamic parameters (Monassier et al. 1999). These authors indicated pretty complex action of ifenprodil, taking into account the possibility of its action on adrenergic, serotonergic and sigma receptors (Chenard et al. 1991, Hashimoto and London 1995). Furthermore, the research data indicated the ifenprodil action on G protein-activated inwardly rectifying K^+ channels, tetrodotoxin-resistant Na^+ channels, N and P-type voltage-dependent Ca^{2+} channels and Na^+/Ca^{2+} exchanger (Kobayashi et al. 2006; Bath et al. 1996, Brittain et al. 2012). Başkaya and co-workers investigated the neuroprotective properties of ifenprodil which have been confirmed, wherein the

cerebral blood flow was not affected (Başkaya et al. 1997). Reduction of HR and CF induced by ifenprodil in this research is consequence of direct effect of this substance on myocardium, excluding its effect on brain structures that can modulate heart function. The reduction in CF correlates with concentration of nitrites that reflect NO production (Figure 2b).

The next part of experimental protocol deals with dynamic of oxidative stress biomarkers during administration of tested substances.

Neither NMDA nor DL-Hcy TLHC caused significant changes in values of biomarkers of oxidative stress (Figure 2). On the other hand combined administration of NMDA and glycine induced significant increase of TBARS values, as well as nitrites and superoxide anion radical (O_2^-) production (Figure 2a, 2b and 2c). Concomitant application of DL-Hcy TLHC and glycine also caused significant increase of TBARS and O_2^-. Values of all mentioned parameters decreased significantly during wash-out period. McGee and Abdel-Rahman investigated the effects of ethanol on peripheral NMDA-R, and showed that bolus of NMDA exhibited significant increases in vascular NOx and ROS (McGee and Abdel-Rahman 2015). Increased activity of NMDA-Rs leads to increased content of intracellular Ca^{2+}, resulting in increment of NOS activity and NO production (Pall 2013). Similar study of the same research group indicated that activation of nNOS and increased production of NO have crucial role in increased ROS production due to NMDA-R activation in vasculature (McGee and Abdel-Rahman 2012). Similarly, a number of authors indicate that Hcy also cause an increase in production of ROS and oxidative stress in tissues of cardiovascular system. Tyagi and colleagues pointed out the role of NMDA-R and increased production of ROS in deleterious effects of Hcy on cardiovascular system (Tyagi et al. 2005). Increased production of ROS by Hcy was abolished by MK-801 as NMDA-R antagonist. The truancy of effects of NMDA and DL-Hcy TLHC alone on ROS

production in this research could be due to the lack of activation of the NMDA receptors. The effects of combined application of NMDA and DL-Hcy TLHC with glycine support this view.

Memantine induced decrease in production of superoxide anion radical (O_2^-), and during the wash-out period the values of TBARS and hydrogen peroxide (H_2O_2) were significantly increased (Figure 2). Liu and co-authors in their study showed protective role of memantine on changes in neurons induced by methylmercury, and concluded that underlining mechanisms are based on NMDA-Rs blockade and maintaining of Ca^{2+} homeostasis, and indirect antioxidative action (Liu et al. 2016). Namely, methylmercury exhibits its deleterious effects on nervous system by over-activation of NMDA-Rs, imbalance in intracellular Ca^{2+} concentration and ROS production (Xu et al. 2012). Memantine also reduced the effects of diabetes on kidneys, suggesting the role of NMDA-R in developing of diabetic nephropathy (Roshanravan et al. 2016). Antioxidative effect of memantine had pivotal role having in mind the role of ROS and oxidative stress in pathogenesis of this disorder (Bhattacharjee et al. 2016). Increased values of TBARS and H_2O_2 during wash-out period can possibly be due to the termination of the inhibitory effects of memantine and the entry of certain quantities of Ca^{2+}, enough to temporarily increase the production of ROS.

In study conducted by Di Maio and others ifenprodil significantly reduced the oxidation of the thiol induced by pilocarpine in experimental model of temporal lobe epilepsy (Di Maio et al. 2013). Based on their results these authors concluded that ifenprodil could prevent glutamate-induced aberrant calcium influx and over-activation of NMDA-R. Similar mechanism, based on the impact of memantine and ifenprodil on Ca^{2+} flux, probably mediates the effects of these compounds in cardiovascular system.

CONCLUSIONS

Absence of effects of NMDA in this investigation is probably due to the lack of coagonist for the other subunit of NMDA-R, as in the previous research on this experimental model it was shown that it is necessary synergy action of both coagonists, glutamate and glycine, for NMDA-Rs activation. In support of this view are results of combined application of NMDA and glycine. Taking into account the overall effect of DL-Hcy TLHC in this investigation, alone and in combination with glycine, as well as in other studies from our laboratory, there is the possibility that homocysteine does not act only through the NMDA-Rs. Effects of memantine and ifenprodil, as NMDA-Rs antagonists, have indicated the fact that blockade of NMDA-Rs causes a decrease of cardiodynamic parameters and parameters of oxidative stress, emphasizing the importance of NMDA-Rs in the regulation of function of cardiovascular system in physiological conditions. On the basis of all the above it can be concluded that modulation of the NMDA-Rs in any direction significantly affects the function of the cardiovascular system, and their role should be further clarified in future research, which should include the different structures of the cardiovascular system.

ACKNOWLEDGMENTS

This project was supported by Grant no. 175043 from the Ministry of Science and Technical Development of the Republic of Serbia, and the Junior Project 04/2011, Faculty of Medical Sciences, University of Kragujevac, Serbia.

CONFLICT OF INTERESTS

None of the authors of the present study has any actual or potential conflicts of interest to disclose, including financial, personal, or other relationships with specific persons or organisations.

REFERENCES

Abahji, T.N., Nill, L., Ide, N., Keller, C., Hoffmann, U., and Weiss, N. 2007. Acute hyperhomocysteinemia induces microvascular and macrovascular endothelial dysfunction. Arch. Med. Res. **38**(4): 411-416. doi:10.1016/j.arcmed.2007.01.004 PMID:17416288

Auclair, C., and Voisin, E. 1985. Nitroblue tetrazolium reduction. In: Greenvvald RA (ed) Handbook of Methods for Oxygen Radical Research. Boca Raton, CRC Press. Une. 123-132.

Başkaya, M.K., Rao, A.M., Donaldson, D., Prasad, M.R., and Dempsey, R.J. 1997. Protective effects of ifenprodil on ischemic injury size, blood-brain barrier breakdown, and edema formation in focal cerebral ischemia. Neurosurgery. **40**(2): 364-371. PMID:9007871

Bath, C.P., Farrell, L.N., Gilmore, J., Ward, M.A., Hicks, C.A., O'Neill, M.J., and Bleakman, D. 1996. The effects of ifenprodil and eliprodil on voltage-dependent Ca2+ channels and in gerbil global cerebral ischaemia. Eur. J. Pharmacol. **299**(1-3): 103-112. PMID:8901012

Bhattacharjee, N., Barma, S., Konwar, N., Dewanjee, S., and Manna, P. 2016. Mechanistic insight of diabetic nephropathy and its pharmacotherapeutic targets: An update. Eur. J. Pharmacol. **791**: 8-24. doi:10.1016/j.ejphar.2016.08.022 PMID:27568833

Borghammer, P., Vafaee, M., Ostergaard, K., Rodell, A., Bailey, C., and Cumming, P. 2008. Effect of memantine on CBF and CMRO2 in patients with early Parkinson's disease. Acta. Neurol. Scand. **117**(5): 317-323. doi:10.1111/j.1600-0404.2007.00943.x PMID:17927800

Bozic, M., and Valdivielso, J.M. 2015. The potential of targeting NMDA receptors outside the CNS. Expert. Opin. Ther. Targets. **19**(3): 399-413. doi:10.1517/14728222.2014.983900 PMID:25495517

Brittain, M.K., Brustovetsky, T., Brittain, J.M., Khanna, R., Cummins, T.R., and Brustovetsky, N. 2012. Ifenprodil, a NR2B-selective antagonist of NMDA receptor, inhibits reverse Na+/Ca2+ exchanger in neurons. Neuropharmacology. **63**(6): 974-982. doi:10.1016/j.neuropharm.2012.07.012 PMID:22820271

Carter, C., Benavides, J., Legendre, P., Vincent, J.D., Noel, F., Thuret, F., Lloyd, K.G., Arbilla, S., Zivkovic, B., and MacKenzie, E.T. 1988. Ifenprodil and SL 82.0715 as cerebral anti-ischemic agents. II. Evidence for N-methyl-D-aspartate receptor antagonist properties. J. Pharmacol. Exp. Ther. **247**(3): 1222-1232. PMID:2849669

Chen, H., Fitzgerald, R., Brown, A.T., Qureshi, I., Breckenridge, J., Kazi, R., Wang, Y., Wu, Y., Zhang, X., Mukunyadzi, P., Eidt, J., and Moursi, M.M. 2005. Identification of a homocysteine receptor in the peripheral endothelium and its role in proliferation. J. Vasc. Surg. **41**(5): 853-860. doi:10.1016/j.jvs.2005.02.021 PMID:15886671

Chen, Y.W., Shieh, J.P., Chen, Y.C., Leung, Y.M., Hung, C.H., and Wang, J.J. 2012. Cutaneous analgesia after subcutaneous injection of memantine and amantadine and their systemic toxicity in rats. Eur. J. Pharmacol. **693**(1-3): 25-30. doi:10.1016/j.ejphar.2012.07.037 PMID:22935636

Chenard, B.L., Shalaby, I.A., Koe, B.K., Ronau, R.T., Butler, T.W., Prochniak, M.A., Schmidt, A.W., and Fox, C.B. 1991. Separation of alpha 1 adrenergic and N-methyl-D-aspartate antagonist activity in a series of ifenprodil compounds. J. Med. Chem. **34**(10): 3085-3090. PMID:1681106

Cheriyan, J., Mezes, C., Zhou, N., Balsara, R.D., and Castellino, F.J. 2015. Heteromerization of ligand binding domains of N-methyl-D-aspartate receptor requires both coagonists, L-glutamate and glycine. Biochemistry. **54**(3): 787-794. doi:10.1021/bi501437s PMID:25544544

Di Maio, R., Mastroberardino, P.G., Hu, X., Montero, L.M., and Greenamyre, J.T. 2013. Thiol oxidation and altered NR2B/NMDA receptor functions in in vitro and in vivo pilocarpine models: implications for epileptogenesis. Neurobiol. Dis. **49**: 87-98. doi:10.1016/j.nbd.2012.07.013 PMID:22824136

Flores-Soto, M.E., Chaparro-Huerta, V., Escoto-Delgadillo, M., Ureña-Guerrero, M.E., Camins, A., and Beas-Zarate, C. 2013. Receptor to glutamate NMDA-type: the functional diversity of the nr1 isoforms and pharmacological properties. Curr. Pharm. Des. **19**(38): 6709-6719. doi:10.2174/1381612811319380003 PMID:23530508

Gao, X., Xu, X., Pang, J., Zhang, C., Ding, J.M., Peng, X., Liu, Y., and Cao, J.M. 2007. NMDA receptor activation induces mitochondrial dysfunction, oxidative stress and apoptosis in cultured neonatal rat cardiomyocytes. Physiol. Res. **56**(5): 559-569. PMID:16925458

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., and Tannenbaum, S.R. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. **126**(1): 131-138. PMID:7181105

Hankey, G.J., and Eikelboom, J.W. 1999. Homocysteine and vascular disease. Lancet. **354**(9176): 407-413. doi:10.1016/S0140-6736(98)11058-9 PMID:10437885

Hashimoto, K., and London, E.D. 1995. Interactions of erythro-ifenprodil, threo-ifenprodil, erythro-iodoifenprodil, and eliprodil with subtypes of sigma receptors. Eur. J. Pharmacol. **273**(3): 307-310. doi:org/10.1016/0014-2999(94)00763-W PMID:7737340

Hess, S.D., Daggett, L.P., Deal, C., Lu, C.C., Johnson, E.C., and Veliçelebi, G. 1998. Functional characterization of human N-methyl-D-aspartate subtype 1A/2D receptors. J. Neurochem. **70**(3): 1269-1279. doi:10.1046/j.1471-4159.1998.70031269.x PMID:9489750

Howes, L.G. 2014. Cardiovascular effects of drugs used to treat Alzheimer's disease. Drug. Saf. **37**(6): 391-395. doi:10.1007/s40264-014-0161-z PMID:24777654

Joseph, J., Joseph, L., Shekhawat, N.S., Devi, S., Wang, J., Melchert, R.B., Hauer-Jensen, M., and Kennedy, R.H. 2003. Hyperhomocysteinemia leads to pathological ventricular hypertrophy in normotensive rats. Am. J. Physiol. Heart. Circ. Physiol. **285**(2): 679-686. doi:10.1152/ajpheart.00145.2003 PMID:12730062

Kamel, I.R., Wendling, W.W., Chen, D., Wendling, K.S., Harakal, C., and Carlsson, C. 2008. N-methyl-D-aspartate (NMDA) antagonists--S(+)-ketamine, dextrorphan, and dextromethorphan--act as calcium antagonists on bovine cerebral arteries. J. Neurosurg. Anesthesiol. **20**(4): 241-248. doi:10.1097/ANA.0b013e31817f523f PMID:18812887

Kobayashi, T., Washiyama, K., and Ikeda, K. 2006. Inhibition of G protein-activated inwardly rectifying K+ channels by ifenprodil. Neuropsychopharmacology. **31**(3): 516-524. doi:10.1038/sj.npp.1300844 PMID:16123769

Liu, W., Xu, Z., Yang, T., Xu, B., Deng, Y., and Feng, S. 2016. Memantine, a Low-Affinity NMDA Receptor Antagonist, Protects against Methylmercury-Induced Cytotoxicity of Rat Primary Cultured Cortical Neurons, Involvement of Ca(2+)

Dyshomeostasis Antagonism, and Indirect Antioxidation Effects. Mol. Neurobiol. doi:10.1007/s12035-016-0020-2 PMID:27538940

Makhro, A., Tian, Q., Kaestner, L., Kosenkov, D., Faggian, G., Gassmann, M., Schwarzwald, C., and Bogdanova, A. 2016. Cardiac N-methyl D-aspartate Receptors as a Pharmacological Target. J. Cardiovasc. Pharmacol. **68**(5): 356-373. doi:10.1097/FJC.0000000000000424 PMID:27525575

McCully, K.S. 2016. Homocysteine, Infections, Polyamines, Oxidative Metabolism, and the Pathogenesis of Dementia and Atherosclerosis. J. Alzheimers. Dis. **54**(4): 1283-1290. doi:10.3233/JAD-160549 PMID:27567876

McGee, M.A., and Abdel-Rahman, A.A. 2012. Enhanced vascular neuronal nitric-oxide synthase-derived nitric-oxide production underlies the pressor response caused by peripheral N-methyl-D-aspartate receptor activation in conscious rats. J. Pharmacol. Exp. Ther. **342**(2): 461-471. doi:10.1124/jpet.112.194464 PMID:22580349

McGee, M.A., and Abdel-Rahman, A.A. 2015. Ethanol attenuates peripheral NMDAR-mediated vascular oxidative stress and pressor response. Alcohol. **49**(5): 499-506. doi:10.1016/j.alcohol.2015.03.004 PMID:25986731

Mirzoyan, R.S., Gan'shina, T.S., Maslennikov, D.V., Kovalev, G.I., Zimin, I.A., Pyatin, B.M., Avdyunina, N.I., Kukhtarova, A.M., Khostikyan, N.G., Meliksetyan, V.S., Alikhanyan, C.B., and Mirzoyan, N.R. 2014. Cerebrovascular and neuroprotective effects of adamantane derivative. Biomed. Res. Int. **2014**:586501. doi:10.1155/2014/586501 PMID:24772429

Monassier, L., Riehl, V., Lienhard, J.P., Tibiriça, E., Feldman, J., and Bousquet, P. 1999. Effects of ifenprodil and baclofen on exercise-induced increase of myocardial oxygen

demand in normotensive rats. J. Pharmacol. Exp. Ther. **290**(3): 1188-1194. PMID:10454494

Morhenn, V.B., Waleh, N.S., Mansbridge, J.N., Unson, D., Zolotorev, A., Cline, P., and Toll, L. 1994. Evidence for an NMDA receptor subunit in human keratinocytes and rat cardiocytes. Eur. J. Pharmacol. **268**(3): 409-414. PMID:7805765

Moshal, K.S., Tipparaju, S.M., Vacek, T.P., Kumar, M., Singh, M., Frank, I.E., Patibandla, P.K., Tyagi, N., Rai, J., Metreveli, N., Rodriguez, W.E., Tseng, M.T., and Tyagi, S.C. 2008. Mitochondrial matrix metalloproteinase activation decreases myocyte contractility in hyperhomocysteinemia. Am. J. Physiol. Heart. Circ. Physiol. **295**(2): 890-897. doi:10.1152/ajpheart.00099.2008 PMID:18567713

Muntzel, M.S., Joseph, T., and Onwumere, O. 2006. Acute homocysteine administration does not elevate sympathetic nerve activity in rats. Atherosclerosis. **184**(2): 290-294. doi:10.1016/j.atherosclerosis.2005.04.009 PMID:15927186

Näsström, J., Böö , E., Ståhlberg, M., and Berge, O.G. 1993. Tissue distribution of two NMDA receptor antagonists, [3H]CGS 19755 and [3H]MK-801, after intrathecal injection in mice. Pharmacol. Biochem. Behav. **44**(1): 9-15. doi:org/10.1016/0091-3057(93)90275-X PMID:8430132

Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. **95**(2) :351-358. PMID:36810

Ota, H., Ogawa, S., Ouchi, Y., and Akishita, M. 2015. Protective effects of NMDA receptor antagonist, memantine, against senescence of PC12 cells: A possible role of nNOS and combined effects with donepezil. Exp. Gerontol. **72**: 109-16. doi:10.1016/j.exger.2015.09.016 PMID:26408226

Pall, M.L. 2013. The NO/ONOO-cycle as the central cause of heart failure. Int. J. Mol. Sci. **14**(11): 22274-22330. doi:10.3390/ijms141122274 PMID:24232452

Perez-Otano, I., Schulteis, C.T., Contractor, A., Lipton, S.A., Trimmer, J.S., Sucher, N.J., and Heinemann, S.F. 2001. Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. J. Neurosci. **21**(4): 1228-1237. PMID:11160393

Pick, E., and Keisari, Y. 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Methods. **38**(1-2): 161-170. doi:org/10.1016/0022-1759(80)90340-3 PMID:6778929

Qian, A., and Johnson, J.W. 2006. Permeant ion effects on external Mg2+ block of NR1/2D NMDA receptors. J. Neurosci. **26**(42): 10899-10910. doi:10.1523/JNEUROSCI.3453-06.2006 PMID:17050728

Resstel, L.B., de Andrade, C.R., Haddad, R., Eberlin, M.N., de Oliveira, A.M., and Corrêa, F.M. 2008. Hyperhomocysteinaemia-induced cardiovascular changes in rats. Clin. Exp. Pharmacol. Physiol. **35**(8): 949-956. doi:10.1111/j.1440-1681.2008.04940.x PMID:18430058

Rosenberger, D., Moshal, K.S., Kartha, G.K., Tyagi, N., Sen, U., Lominadze, D., Maldonado, C., Roberts, A.M., and Tyagi, S.C. 2006. Arrhythmia and neuronal/endothelial myocyte uncoupling in hyperhomocysteinemia. Arch. Physiol. Biochem. **112**(4-5): 219-227. doi:10.1080/13813450601093443 PMID:17178594

Roshanravan, H., Kim, E.Y., and Dryer, S.E. 2016. NMDA receptors as potential therapeutic targets in diabetic nephropathy: Increased renal NMDA receptor subunit expression in Akita mice and reduced nephropathy following sustained treatment with

memantine or MK-801. Diabetes. **65**(10): 3139-3150. doi: 10.2337/db16-0209 PMID:27388219

Seeber, S., Humeny, A., Herkert, M., Rau, T., Eschenhagen, T., and Becker, C.M. 2004. Formation of molecular complexes by N-methyl-D-aspartate receptor subunit NR2B and ryanodine receptor 2 in neonatal rat myocard. J. Biol. Chem. **279**(20):21062-21068. doi:10.1074/jbc.M313009200 PMID:15010472

Snyder, E.M., Nong, Y., Almeida, C.G., Paul, S., Moran, T., Choi, E.Y., Nairn, A.C., Salter, M.W., Lombroso, P.J., Gouras, G.K., and Greengard, P. 2005. Regulation of NMDA receptor trafficking by amyloid-beta. Nat. Neurosci. **8**(8):1051-1058. doi:10.1038/nn1503 PMID:16025111

Srejovic, I., Jakovljevic, V., Zivkovic, V., Barudzic, N., Radovanovic, A., Stanojlovic, O., and Djuric, D.M. 2015. The effects of the modulation of NMDA receptors by homocysteine thiolactone and dizocilpine on cardiodynamics and oxidative stress in isolated rat heart. Mol. Cell. Biochem. **401**(1-2): 97-105. doi:10.1007/s11010-014-2296-8 PMID:25467376

Srejovic, I., Jakovljevic, V., Zivkovic, V., Jeremic, N., Jevdjevic, M., Stojic, I., and Djuric, D. 2015. The effects of glycine, glutamate and their combination on cardiodynamics, coronary flow and oxidative stress in isolated rat heart. Curr. Res. Cardiol. **2**:63-68 doi:10.4172/2368-0512.1000031

Takehara, H., Suzuki, Y., and Someya, T. 2015. QT prolongation associated with memantine in Alzheimer's disease. Psychiatry. Clin. Neurosci. **69**(4): 239-240. doi:10.1111/pcn.12236 PMID:25186635

Takemoto, Y. 2016. Central cardiovascular actions of L-homocysteine microinjected into ventrolateral medullary autonomic areas of the rat. Amino. Acids. **48**(9): 2215-2225. doi:10.1007/s00726-016-2259-3 PMID:27178024

Toda, N., and Okamura, T. 2016. Hyperhomocysteinemia impairs regional blood flow: involvements of endothelial and neuronal nitric oxide. Pflugers. Arch. **468**(9): 1517-1525. doi:10.1007/s00424-016-1849-y PMID:27417104

Traynelis, S.F., Wollmuth, L.P., McBain, C.J., Menniti, F.S., Vance, K.M., Ogden, K.K., Hansen, K.B., Yuan, H., Myers, S.J., and Dingledine, R. 2010. Glutamate receptor ion channels: structure, regulation, and function. Pharmacol. Rev. **62**(3): 405-496. doi:10.1124/pr.109.002451 PMID:20716669

Tyagi, N., Sedoris, K.C., Steed, M., Ovechkin, A.V., Moshal, K.S., and Tyagi, S.C. 2005. Mechanisms of homocysteine-induced oxidative stress. Am. J. Physiol. Heart. Circ. Physiol. **89**: 2649-2656. doi:10.1152/ajpheart.00548.2005 PMID:16085680

Tyagi, N., Vacek, J.C., Givvimani, S., Sen, U., and Tyagi, S.C. 2010. Cardiac specific deletion of N-methyl-d-aspartate receptor 1 ameliorates mtMMP-9 mediated autophagy/mitophagy in hyperhomocysteinemia. J. Recept. Signal. Transduct. Res. **30**(2): 78-87. doi:10.3109/10799891003614808 PMID:20170426

Vyklicky, V., Korinek, M., Smejkalova, T., Balik, A., Krausova, B., Kaniakova, M., Lichnerova, K., Cerny, J., Krusek, J., Dittert, I., Horak, M., and Vyklicky, L. 2014. Structure, function, and pharmacology of NMDA receptor channels. Physiol. Res. **63**(Suppl 1): 191-203. PMID:24564659

Walker, E., Black, J., Parris, C., Bryda, E.C., Cansino, S., Hunt, L., Chappell, J., Wehner, P., Studeny, M., and Wright, G.L. 2004. Effect of experimental hyperhomocysteinemia

on cardiac structure and function in the rat. Ann. Clin. Lab. Sci. **34**(2): 175-180. PMID:15228230

Wollmuth, L.P., Kuner, T., and Sakmann, B. 1998. Adjacent asparagines in the NR2-subunit of the NMDA receptor channel control the voltage-dependent block by extracellular Mg2+. J. Physiol. **506**(1): 13-32. doi:10.1111/j.1469-7793.1998.013bx.x PMID:9481670

Xu, F., Farkas, S., Kortbeek, S., Zhang, F.X., Chen, L., Zamponi, G.W., and Syed, N.I. 2012. Mercury-induced toxicity of rat cortical neurons is mediated through N-Methyl-D-Aspartate receptors. Mol. Brain. **5**:30. doi:10.1186/1756-6606-5-30 PMID:22980357

FIGURE CAPTIONS

Figure 1. Effects of N-methyl-D-aspartate (NMDA), combination of N-methyl-D-aspartate (NMDA) and glycine, DL-homocisteine thiolactone (DL-Hcy TLHC), combination of DL-homocisteine thiolactone (DL-Hcy TLHC) and glycine, memantine and ifenprodil on cardiodynamic parameters and coronary flow in isolated rat heart. Data are expressed as mean ± SE (Standard Error).

The values were measured in three period times (C - control, E – effect, W – wash-out). *Statistical significance compared with previous value ($p<0.05$)

Figure 2. Effects of N-methyl-D-aspartate (NMDA), combination of N-methyl-D-aspartate (NMDA) and glycine, DL-homocisteine thiolactone (DL-Hcy TLHC), combination of DL-homocisteine thiolactone (DL-Hcy TLHC) and glycine, memantine and ifenprodil on the biomarkers of oxidative stress in coronary venous effluent. Data are expressed as mean ± SE (Standard Error).

The values were measured in three period times (C - control, E – effect, W – wash-out). *Statistical significance compared with previous value ($p<0.05$)

MODULATION OF N-METHYL-D-ASPARTATE RECEPTORS IN ISOLATED RAT HEART

Ivan Srejovic[1], Vladimir Zivkovic[1], Tamara Nikolic[2], Nevena Jeremic[2], Isidora Stojic[2], Jovana Jeremic[2], Dragan Djuric[3], Vladimir Jakovljevic[1,4]

[1]*University of Kragujevac, Faculty of Medical Sciences, Department of Physiology, Kragujevac, Serbia,*

[2]*University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy, Kragujevac, Serbia,*

[3]*University of Belgrade, Faculty of Medicine, Institute of Medical Physiology "Richard Burian," Belgrade, Serbia,*

[4]*First Moscow State University I.M. Sechenov, Department of Human Pathology, Moscow, Russian Federation*

Correspondence should be addressed to:

Prof. Vladimir Lj. Jakovljevic, MD, PhD

Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, SERBIA

Phone: +381 34 342944; Fax: +381 34 306800/ext 112; E-mail: drvladakgbg@yahoo.com

LIST OF ABBREVIATIONS

NMDA-R - N-methyl-D-aspartate receptor

NMDA - N-methyl-D-aspartate

DL-Hcy TLHC - DL-homocysteine thiolacotne

CF - coronary flow

NO_2^- - nitrites

O_2^- - superoxide anion radical

H_2O_2 – hydrogen peroxide

AMPA-R - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

HHcy – hyperhomocysteinemia

CNS - central nervous system

NO - nitric-oxide

NOS - nitric-oxide synthase

MMP - matrix metalloproteinase

ROS - reactive oxygen species

CPP - constant perfusion pressure

dp/dt max - maximum rate of pressure development in the left ventricle

dp/dt min - minimum rate of pressure development in the left ventricle

SLVP - systolic left ventricular pressure

DLVP - diastolic left ventricular pressure

HR - heart rate

MPT - mitochondrial permeability transition

RVLM - rostral ventrolateral medulla

CVLM - caudal ventrolateral medulla

RyR2 - ryanodine receptor 2

ABSTRACT

Considering the limited data about the role of N-methyl-D-aspartate receptors (NMDA-R) in cardiovascular system and heart, the aim of the present study was to examine the effects of NMDA and DL-homocysteine thiolacotne (DL-Hcy TLHC), alone and in combinations with glycine, memantine and ifenprodil, in the isolated rat heart. The hearts of Wistar albino rats were retrogradely perfused according to the Langendorff technique at a constant perfusion pressure. The experimental protocol for all experimental groups included the stabilization period, application of estimated substance in duration of 5 minutes, followed by wash-out period in duration of 10 minutes. Using sensor in the left ventricle we registered the next parameters of myocardial function: dp/dt max, dp/dt min, systolic and diastolic left ventricular pressure, and heart rate, and coronary flow (CF) was measured flowmetrically. In the coronary venous effluent spectrophotometrically were estimated following oxidative stress biomarkers: TBARS, NO_2^-, O_2^-, and H_2O_2. NMDA alone did not induce any change in any observed parameter, while DL-Hcy TLHC alone, as well as combined application of NMDA and DL-Hcy TLHC with glycine, induced reduction of most cardiodynamic parameters. Memantine and ifenprodil induced reduction of cardiodynamic parameters and coronary flow, as well as some oxidative stress biomarkers.

Key words: N-methyl-D-aspartate receptors, homocysteine, memantine, ifenprodil, oxidative stress, cardiodynamics, isolated rat heart

INTRODUCTION

The N-methyl-D-aspartate receptors (NMDA-R) belong to the ionotropic glutamate receptor family together with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R), kainite receptor and delta (δ) receptor. NMDA-R are heterotetramers, composed of four subunits, two obligatory, glycine binding GluN1 subunits, and two glutamate binding GluN2 subunits, or one GluN2 subunit and one GluN3 subunit (Vyklicky et al. 2014). NMDA-R subunits are assembled to form a central channel through the cell membrane that functions as an ion channel. The specificities of the NMDA-R, compared to other receptors of the same group, include necessity of the presence of the two co-agonists, glutamate and glycine, for receptor activation, the entry of considerable quantities of calcium following receptor activation, and dual control of receptor function, by ligands (glutamate and glycine) and by changing the value of the membrane potential (Traynelis et al. 2010, Wollmuth et al. 1998). Depending on the subtypes of subunits which build NMDA-R vary their characteristics regarding calcium permeability, channel conductance, opening rate, voltage dependent magnesium blocking (Perez-Otano et al. 2001, Qian and Johnson 2006).

N-methyl-D-aspartate (NMDA) binds to a glutamate binding place and exerts the same effect as glutamate. Homocysteine (Hcy) also binds to glutamate binding site, and it is considered that detrimental effects of hyperhomocysteinemia (HHcy, plasma values of Hcy above 15 μmol/l) in various conditions arise from excessive activation of the NMDA receptors (Hankey and Eikelboom 1999, Moshal et al. 2008, Snyder et al. 2005). Use of memantine, as an NMDA-R antagonist, in the case of Alzheimer's disease has a neuroprotective effect, bearing in mind the fact that amyloid beta exerts its negative effect by increasing the concentration of glutamate in extrasynaptic space (Ota et al. 2015). On the other hand, substances, which are

denoted as allosteric modulators of NMDA-R, are attracting more attention due to the possibility of fine regulation of receptor function and higher selectivity depending on the composition of the subunits that make up the receptor. Ifenprodil causes noncompetitive inhibition of NMDA-R that contains the GluN2B subunit and it has been shown its neuroprotective effect during cerebral ischemia (Carter et al. 1988, Hess et al. 1998).

Despite relatively well-known roles of NMDA-R in central nervous system (CNS), there are growing data considering localization of NMDA-R in other organs and tissues, including the cardiovascular system (Morhenn et al. 1994, Bozic and Valdivielso 2015). Investigation of time and spatial distribution of radiolabelled antagonists of NMDA-R showed widespread acceptance of these receptors in a number of organs and tissues, including heart (Näsström et al. 1993). NMDA-Rs are found in different cell types that exist in the cardiovascular system, particularly in endothelial cells, vascular smooth muscle cells and cardiomyocytes (Chen et al. 2005, Gao et al. 2007).

Since the activation of the NMDA-Rs is associated with entry of calcium into the cytoplasm, their overstimulation causes an increase intracellular calcium content and consequent imbalance in the production and elimination of free radicals and oxidative stress (Kamel et al. 2008, Gao et al. 2007). Inactivation of NMDA-Rs by deletion of GluN1 subunit induced decrease of production of reactive oxygen species (ROS) during HHcy in the heart, as well as decreased content of nitric-oxide (NO) and matrix metalloproteinase 9 (MMP9) in cardiomyocytes mitochondria (Tyagi et al. 2010, Moshal et al. 2008).

There are many doubts related to physiological function of NMDA-R in cardiovascular system, as well as their role in certain pathophysiological conditions. Accordingly, the aim of this study was to investigate the acute effects of N-methyl-D-aspartate (NMDA), DL-

homocysteine thiolactone (DL-Hcy TLHC), as well as their combinations with glycine, memantine and ifenprodil on cardiac function, coronary flow and oxidative stress, or to assess the effects of acute modulation of NMDA-R in heart by mentioned substances, and possible role of oxidative stress in that sense.

MATERIAL AND METHODS

Isolated rat heart preparation

Seventy two animals were included in study, 12 animals per experimental group. All experiments were conducted on male Wistar albino rats (8 weeks old, body mass 180-200 g), obtained from Military Medical Academy, Belgrade, Serbia. After anesthesia induced by ketamine (10 mg/kg) and xylazine (5 mg/kg), the animals were euthanized via cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986, UK). After euthanasia and prompt thoracotomy, the rapid cardiac arrest was induced by superfusion with ice-cold isotonic saline and hearts then were quickly excised and attached to the Langendorff apparatus (Experimetria Ltd, 1062 Budapest, Hungary) via aortic cannulation. This was followed by removal of the left auricula and making the incision in the left atria, through which the sensor (transducer BS4 73-0184, Experimetria Ltd, Budapest, Hungary) is placed in the left ventricle, for continuous measurement of cardiac function parameters. The hearts were retrogradely perfused under a constant perfusion pressure (CPP) of 70 cmH_2O with complex Krebs-Henseleit solution composed of the following (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.7, $NaHCO_3$ 25, KH2PO4 1.2, glucose 11, pyruvate 2, equilibrated with 95% O_2 plus 5% CO_2 at 37 ºC (pH 7.4).

Experimental protocol

The hearts from all experimental groups were undergone to a 25 minutes stabilization period. During this period, each of the hearts was subjected to short term occlusion (20 s) followed by simultaneous bolus injections of 5 mmol/l adenosine (60 µl at a flow of 10 ml/min to elicit maximal coronary flow) to test coronary vascular reactivity. If coronary flow (CF) did

not increase by 100% compared with control values, the hearts were disposed of. Coronary flow was determined flowmetricaly. When the CF was stabilised (three repeated measurements of the same value), samples of coronary effluent were collected (control value - C), and the experimental protocol was initiated. Hearts were perfused with:

1. 100 µmol/l N-methyl-D-aspartate (NMDA)
2. 100 µmol/l N-methyl-D-aspartate (NMDA) + 100 µmol/l glycine
3. 10 µmol/l DL-homocysteine thiolactone (DL-Hcy TLHC)
4. 10 µmol/l DL-homocysteine thiolactone (DL-Hcy TLHC) + 100 µmol/l glycine
5. 100 µmol/l memantine
6. 1 µmol/l ifenprodil

Each of the applied substances was administered for 5 minutes, followed by a wash-out period lasting for 10 minutes. In the last minute of substance application (effect - E) and in the last minute of the wash-out period (wash-out - W) the samples of coronary venous effluent were collected. Using the sensor within the left ventricle, the following parameters of myocardial function were determined:

1. The maximum rate of pressure development in the left ventricle (dp/dt max)
2. The minimum rate of pressure development in the left ventricle (dp/dt min)
3. The systolic left ventricular pressure (SLVP)
4. The diastolic left ventricular pressure (DLVP)
5. The heart rate (HR)

All research procedures were carried out in accordance with European Directive for welfare of laboratory animals N^{o} 86/609/EEC and principles of Good Laboratory Practice (GLP),

approved by Ethical committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Biochemical assays

The following oxidative stress parameters were determined spectrophotometrically (Shimadzu UV 1800, Japan) using collected samples of the coronary venous effluent:

1. The index of lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS)
2. The level of nitrite (NO_2^-)
3. The level of the superoxide anion radical (O_2^-) and
4. The level of hydrogen peroxide (H_2O_2)

TBARS determination (index of lipid peroxidation)

The degree of lipid peroxidation in the coronary venous effluent was estimated by measuring TBARS, using 1% thiobarbituric acid in 0.05 NaOH, which was incubated with the coronary effluent at 100°C for 15 min and measured at 530 nm. Krebs–Henseleit solution was used as a blank probe (Ohkawa et al. 1979).

Determination of the nitrite level

Nitric-oxide decomposes rapidly to form stable nitrite/nitrate products. The nitrite level (NO_2^-) was measured and used as an index of nitric oxide (NO) production, using Griess's reagent. A total of 0.5 ml of perfusate was precipitated with 200 µl of 30% sulpho-salicylic acid, vortexed for 30 min, and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess's reagent,

containing 1% sulphanilamide in 5% phosphoric acid/0.1% naphthalene ethylenediamine-di hydrochloride were added and incubated for 10 min in the dark and measured at 543 nm. The nitrite levels were calculated using sodium nitrite as the standard (Green et al. 1982).

Determination of the level of the superoxide anion radical

The level of the superoxide anion radical (O_2^-) was measured via a nitro blue tetrazolium (NBT) reaction in TRIS buffer with coronary venous effluent, at 530 nm. Krebs–Henseleit solution was used as a blank probe (Auclair and Voisin 1985).

.

Determination of the hydrogen peroxide level

The measurement of the level of hydrogen peroxide (H_2O_2) was based on the oxidation of phenol red by hydrogen peroxide in a reaction catalysed by horseradish peroxidase (HRPO) (Pick and Keisari 1980). Two hundred microliters of perfusate was precipitated using 800 ml of freshly prepared phenol red solution; 10 µl of (1:20) HRPO (made ex tempore) was subsequently added. For the blank probe, an adequate volume of Krebs–Henseleit solution was used instead of coronary venous effluent. The level of H_2O_2 was measured at 610 nm.

Drugs

All drugs used in this experimental protocol were provided by Sigma-Aldrich.

Statistical analysis

All values are expressed as mean ± SE. Paired t test was used in statistical analysis and p values less than 0.05 were considered to be statistically significant.

RESULTS

The effects of N-methyl-D-aspartate on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Application of N-methyl-D-aspartate (NMDA) did not induce any significant change of the observed cardiodynamic parameters and coronary flow (Figure 1).

The effects of combined application of N-methyl-D-aspartate and glycine on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

The simultaneous administration of N-methyl-D-aspartate (NMDA) and glycine induced significant decrease of dp/dt max, dp/dr min, SLVP, HR and CF (Figure 1a, 1c, 1d, 1e and 1f). During the wash-out period values of all mentioned parameters were significantly increased, and reached values that were insignificantly different compared to control.

The effects of DL-homocysteine thiolactone on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Application of DL-homocysteine thiolactone (DL-Hcy TLHC) induced significant decrease of dp/dt max, SLVP, HR and CF (Figure 1a, 1c, 1d, 1e and 1f). DLVP was insignificantly decreased, but this decrease continued during wash-out period, so that the control value and the value after the wash-out period differed significantly (Figure 1d). Flowing wash-out period the values of dp/dt max and CF were insignificantly increased, so that the control values and the values after the wash-out period did not differ significantly (Figure 1a and 1f). Values of SLVP and HR after wash-out period were significantly lower compared to the control (Figure 1c and 1e).

The effects of combined application of DL-homocysteine thiolactone and glycine on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Combined administration of DL-homocysteine thiolactone (DL-Hcy TLHC) and glycine induced significant decrease of all observed cardiodynamic parameters (dp/dt max, dp/dt min, SLVP, DLVP and HR), as well as CF (Figure 1a, 1b, 1c, 1e and 1f). Values of all mentioned parameters were significantly increased during wash-out period, and reached values similar to control, except HR, which was significantly lower after wash-out period.

The effects of memantine on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Memantine induced significant decrease of dp/dt max, dp/dt min, SLVP, HR and CF. During wash-out period all mentioned parameters were significantly increased, and all of them, except HR, reached the initial values (Figure 1a, 1b, 1c, 1e and 1f). The value of DLVP was not significantly changed during the experiment (Figure 1d).

The effects of ifenprodil on the cardiodynamic parameters and coronary flow in isolated rat heart (Figure 1)

Ifenprodil induced significant decrease of CF and HR compared with control conditions. Following the wash-out period both mentioned parameters were significantly increased and reached values approximate to control (Figure 1e and 1f). Furthermore, dp/dt max was significantly increased during wash-out period (Figure 1a). Other cardiodynamic did not change significantly.

The effects of N-methyl-D-aspartate on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Administration of N-methyl-D-aspartate (NMDA) did not induce any significant change of the observed biomarkers of oxidative stress (Figure 2).

The effects of combined application of N-methyl-D-aspartate and glycine on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Combined administration of N-methyl-D-aspartate and glycine induced significant increase of TBARS, nitrites and superoxide anion radical, which were decreased significantly following wash-out period, to values similar to control (Figure 2a, 2b and 2c).

The effects of DL-homocysteine thiolactone on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

DL-homocysteine thiolactone (DL-Hcy TLHC) did not induce any significant change of the observed biomarkers of oxidative stress (Figure 2).

The effects of combined application of DL-homocysteine thiolactone and glycine on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Combined administration DL-homocysteine thiolactone (DL-Hcy TLHC) and glycine induced significant increase of TBARS and superoxide anion radical, which were decreased significantly during wash-out period, to values similar to control (Figure 2a and 2c).

The effects of memantine on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Memantine induced statistically significant reduction of production of superoxide anion radical (Figure 2c). Following wash-out period this reduction was even more pronounced, but on the other hand values of TBARS and hydrogen peroxide were significantly increased compared to the effect of memantine (Figure 2a, 2c and 2d).

The effects of ifenprodil on the biomarkers of oxidative stress in isolated rat heart (Figure 2)

Application of ifenprodil induced significant decrease of hydrogen peroxide levels. On the other hand production of nitrites was increased during wash-out period (Figure 2b and 2d).

DISCUSSION

The aim of the present study was to estimate the acute effects of N-methyl-D-aspartate (NMDA), combination of NMDA and glycine, DL-homocysteine thiolactone (DL-Hcy TLHC), combination of DL-Hcy TLHC and glycine, memantine and ifenprodil, as agonists and antagonists of NMDA receptors, on cardiac function, coronary flow and oxidative stress, or to assess the effects of acute modulation of NMDA-R in heart by mentioned substances, as well as possible role of oxidative stress in the resultant changes.

Application of NMDA in concentration of 100 μmol/l did not induce any significant change on cardiodynamic parameters, while on the other hand combination of NMDA and glycine induced significant changes in dp/dt max, dp/dt min, SLVP, HR and CF (Figure 1). Bearing in mind the fact that NMDA exerts its effects by binding to the glutamate binding site on NMDA-R and has the same effects as glutamate, these results are in correlation with our previous results where application of glutamate alone in same concentration also did not cause changes in cardiodynamic parameters nor coronary flow (Srejovic et al. 2015b). In the same research only combined application of both subunits agonists, glutamate and glycine, induced changes in heart function. Having all this in mind, we can conclude that, in this experimental model, it is necessary to apply both NMDA-R co-agonists for their activation (Cheriyan et al. 2015).

DL-Hcy TLHC in concentration of 10 μmol/l induced significant decrease of all observed cardiodynamic parameters and coronary flow, except dp/dt min (Figure 1). Simultaneous administration of DL-Hcy TLHC and glycine induced significant decrease of all observed cardiodynamic parameters (Figure 1). Moshal and co-authors in their investigation induced HHcy in mice and shoved that increased levels of Hcy have resulted in decrease of parameters of

cardiac contractility (Moshal et al. 2008). Since the use of inhibitors of NMDA-R, matrix metalloproteinase activity (MMP) and mitochondrial permeability transition (MPT) induced mitigation of the mentioned changes of myocardial contractility, these authors concluded that Hcy via NMDA-R activates MMP and induces MPT which results in a decrease in myocardial contractility.

DL-Hcy TLHC alone, as well as combination of DL-Hcy TLHC and glycine, induced significant reduction of SLVP and insignificant decrease of DLVP. During wash-out period value of DLVP in both mentioned experimental groups continued to decrease so that the value of this parameter was significantly lower after wash-out period compare to control (Figure 1c and 1d). Takemoto in his research examined the effects of the application of microinjection of L-Hcy in rostral ventrolateral medulla (RVLM) and caudal ventrolateral medulla (CVLM) (Takemoto 2016). Injection of L-Hcy to RVLM and CVLM induced pressor and depressor effects, respectively, which were abolished by previous microinjection of MK-801, as NMDA-R antagonist. On the other hand, Walker and co-workers showed that HHcy induces early disturbances in myocardial structure, without significant changes in function (Walker et al. 2004). Similar conclusions were performed by Joseph and colleagues based on results of their research (Joseph et al. 2003). Discrepancies in the results of these studies and our research could be the consequence of different experimental protocols. Reductions of SLVP and DLVP in our research represent direct effect of DL-Hcy TLHC on heart, since the effects of Hcy on heart via autonomic nervous system, as well as effects of chronic HHcy are excluded. Possible mechanisms that mediate in direct effects of DL-Hcy TLHC could include disturbances in calcium currents or nitric-oxide (NO) production (Rosenberger et al. 2006, Moshal et al. 2008). Differences in effects of NMDA alone and DL-Hcy TLHC alone might be the result of different

affinity of NMDA-R for these compounds, or eventually the existence of another mechanism through which the Hcy may exert its effect on the cardiovascular system (Flores-Soto et al. 2013). On the other hand, combinations of NMDA and glycine and DL-Hcy TLHC and glycine had similar effects on SLVP (Figure 1c).

DL-Hcy TLHC alone, as well as combined applications of DL-Hcy TLHC and NMDA with glycine, induced significant decrease of HR (Figure 1e). Muntzel and co-authors presumed that Hcy may cause increases in sympathetic nerve activity, what could be one of the mechanisms through which occur damage of cardiovascular system in HHcy, but their results showed that infusion of Hcy have no effects on heart rate and blood pressure (Muntzel et al. 2006). In previous results from our laboratory DL-Hcy TLHC has had similar effect on HR (Srejovic et al. 2015a). On the other hand, Resstel and coworkers showed increase of HR in rats with induced mild HHcy (Resstel et al. 2008). Different effects of Hcy on HR in these studies may be due to the different structures involved in regulating heart rate, keeping in mind that in chronic and *in vivo* experiments, the primacy in the regulation of HR might have influence of Hcy on the NMDA-R (and maybe other glutamate receptors) in certain structures in the CNS, whereas the experimental protocol used in this study focused on the direct effects on the heart and coronary circulation.

Similarly to HR, application of DL-Hcy TLHC alone, DL-Hcy TLHC and glycine and NMDA and glycine, induced significant decrease of CF. In groups treated with combinations of DL-Hcy TLHC and NMDA with glycine, values of CF were significantly increased during wash-out period, while in group treated with DL-Hcy TLHC value of this parameter was slightly increased during wash-out period, due to which the control values of CF and values after wash-out period did not differ significantly (Figure 1f). This decrease of CF is in accordance with

results of other studies, whereby the effects of Hcy on nitric-oxide synthase (NOS) and disturbances in NO production are most commonly mentioned as possible mechanism for this action (McCully 2016, Toda and Okamura 2016). Abahji and colleagues assessed the effects of HHcy induced by oral methionine supplementation on endothelial function in healthy subjects (Abahji et al. 2007). HHcy induced significant decrease in flow-mediated vasodilatation of the brachial artery, parameter which reflects endothelial function and NO synthesis.

Memantine as a noncompetitive NMDA-R antagonist, induced decrease of all observed cardiodynamic and coronary flow, except DLVP, whereby all these parameters returned to values approximate to control values, with the exception of HR (Figure 1). In the study conducted by the Makhro and co-workers, intracoronary application of memantine, as well as other NMDA-R antagonists (eliprodil, Ro25-6981, ketamine, and MK-801) exerted negative inotropic and chronotropic effect on autonomous heart function (Makhro et al. 2016). Seeber and co-authors pointed out the complex formation between NMDA-R subunit GluN2B and ryanodine receptor 2 (RyR2) in neonatal rat myocardium, so that negative inotropic effect induced by memantine probably occurs because of changes in the concentrations of Ca^{2+} and complies with the effects of other NMDA receptor antagonists, as it is not excluded existence of this complexes in older age (Seeber et al. 2004).

Memantine is in clinical use for the treatment of Alzheimer's disease, and most of its effects on cardiovascular system were noticed as side effects in the treatment of these patients (Takehara et al. 2015). Following the systemic effects of memantine as local anaesthetic, Chen and colleagues found that memantine induces decrease in mean arterial pressure and heart rate (Chen et al. 2012).

Beside the above mentioned bradycardic effect of NMDA-R antagonists, they have antiarrhythmic property also (Makhro et al. 2016). Both of these actions could be associated with prolongation of QT interval induced by memantine (Takehara et al. 2015, Howes 2014). Since memantine is used in therapy of Alzheimer's disease, the mostly examined were its effects on cerebral blood vessels and blood flow in the brain. Intravenous administration of memantine in anesthetized rats induced decrease of blood flow in brain by 15% in average within 10 minutes, and further reduction of blood flow reached 53% (Mirzoyan et al. 2014). In the human population of patients suffering from Parkinson's disease, memantine caused decrease of blood flow in basal ganglia and several frontal cortical areas (Borghammer et al. 2008).

Ifenprodil in concentration of 1 μmol/l induced significant reduction of HR and CF values, which were returned to values similar to initial after wash-out period (Figure 1). Furthermore, value of dp/dt max was significantly increased during wash-out period. Absence of effects of ifenprodil on myocardial contractility and systolic and diastolic pressure could be due to relatively modest applied dose. Also there are limited data dealing with effects of ifenprodil on cardiovascular system. Monassier and co-authors assessed the effects of ifenprodil on baroreceptor heart rate reflex in rats, whereby did not significantly change the basal values of hemodynamic parameters (Monassier et al. 1999). These authors indicated pretty complex action of ifenprodil, taking into account the possibility of its action on adrenergic, serotonergic and sigma receptors (Chenard et al. 1991, Hashimoto and London 1995). Furthermore, the research data indicated the ifenprodil action on G protein-activated inwardly rectifying K^+ channels, tetrodotoxin-resistant Na^+ channels, N and P-type voltage-dependent Ca^{2+} channels and Na^+/Ca^{2+} exchanger (Kobayashi et al. 2006, Bath et al. 1996, Brittain et al. 2012). Başkaya and co-workers investigated the neuroprotective properties of ifenprodil which have been confirmed, wherein the

cerebral blood flow was not affected (Başkaya et al. 1997). Reduction of HR and CF induced by ifenprodil in this research is consequence of direct effect of this substance on myocardium, excluding its effect on brain structures that can modulate heart function. The reduction in CF correlates with concentration of nitrites that reflect NO production (Figure 2b).

The next part of experimental protocol deals with dynamic of oxidative stress biomarkers during administration of tested substances.

Neither NMDA nor DL-Hcy TLHC caused significant changes in values of biomarkers of oxidative stress (Figure 2). On the other hand combined administration of NMDA and glycine induced significant increase of TBARS values, as well as nitrites and superoxide anion radical (O_2^-) production (Figure 2a, 2b and 2c). Concomitant application of DL-Hcy TLHC and glycine also caused significant increase of TBARS and O_2^-. Values of all mentioned parameters decreased significantly during wash-out period. McGee and Abdel-Rahman investigated the effects of ethanol on peripheral NMDA-R, and showed that bolus of NMDA exhibited significant increases in vascular NOx and ROS (McGee and Abdel-Rahman 2015). Increased activity of NMDA-Rs leads to increased content of intracellular Ca^{2+}, resulting in increment of NOS activity and NO production (Pall 2013). Similar study of the same research group indicated that activation of nNOS and increased production of NO have crucial role in increased ROS production due to NMDA-R activation in vasculature (McGee and Abdel-Rahman 2012). Similarly, a number of authors indicate that Hcy also cause an increase in production of ROS and oxidative stress in tissues of cardiovascular system. Tyagi and colleagues pointed out the role of NMDA-R and increased production of ROS in deleterious effects of Hcy on cardiovascular system (Tyagi et al. 2005). Increased production of ROS by Hcy was abolished by MK-801 as NMDA-R antagonist. The truancy of effects of NMDA and DL-Hcy TLHC alone on ROS

production in this research could be due to the lack of activation of the NMDA receptors. The effects of combined application of NMDA and DL-Hcy TLHC with glycine support this view.

Memantine induced decrease in production of superoxide anion radical (O_2^-), and during the wash-out period the values of TBARS and hydrogen peroxide (H_2O_2) were significantly increased (Figure 2). Liu and co-authors in their study showed protective role of memantine on changes in neurons induced by methylmercury, and concluded that underlining mechanisms are based on NMDA-Rs blockade and maintaining of Ca^{2+} homeostasis, and indirect antioxidative action (Liu et al. 2016). Namely, methylmercury exhibits its deleterious effects on nervous system by over-activation of NMDA-Rs, imbalance in intracellular Ca^{2+} concentration and ROS production (Xu et al. 2012). Memantine also reduced the effects of diabetes on kidneys, suggesting the role of NMDA-R in developing of diabetic nephropathy (Roshanravan et al. 2016). Antioxidative effect of memantine had pivotal role having in mind the role of ROS and oxidative stress in pathogenesis of this disorder (Bhattacharjee et al. 2016). Increased values of TBARS and H_2O_2 during wash-out period can possibly be due to the termination of the inhibitory effects of memantine and the entry of certain quantities of Ca^{2+}, enough to temporarily increase the production of ROS.

In study conducted by Di Maio and others ifenprodil significantly reduced the oxidation of the thiol induced by pilocarpine in experimental model of temporal lobe epilepsy (Di Maio et al. 2013). Based on their results these authors concluded that ifenprodil could prevent glutamate-induced aberrant calcium influx and over-activation of NMDA-R. Similar mechanism, based on the impact of memantine and ifenprodil on Ca^{2+} flux, probably mediates the effects of these compounds in cardiovascular system.

CONCLUSIONS

Absence of effects of NMDA in this investigation is probably due to the lack of coagonist for the other subunit of NMDA-R, as in the previous research on this experimental model it was shown that it is necessary synergy action of both coagonists, glutamate and glycine, for NMDA-Rs activation. In support of this view are results of combined application of NMDA and glycine. Taking into account the overall effect of DL-Hcy TLHC in this investigation, alone and in combination with glycine, as well as in other studies from our laboratory, there is the possibility that homocysteine does not act only through the NMDA-Rs. Effects of memantine and ifenprodil, as NMDA-Rs antagonists, have indicated the fact that blockade of NMDA-Rs causes a decrease of cardiodynamic parameters and parameters of oxidative stress, emphasizing the importance of NMDA-Rs in the regulation of function of cardiovascular system in physiological conditions. On the basis of all the above it can be concluded that modulation of the NMDA-Rs in any direction significantly affects the function of the cardiovascular system, and their role should be further clarified in future research, which should include the different structures of the cardiovascular system.

ACKNOWLEDGMENTS

This project was supported by Grant no. 175043 from the Ministry of Science and Technical Development of the Republic of Serbia, and the Junior Project 04/2011, Faculty of Medical Sciences, University of Kragujevac, Serbia.

CONFLICT OF INTERESTS

None of the authors of the present study has any actual or potential conflicts of interest to disclose, including financial, personal, or other relationships with specific persons or organisations.

REFERENCES

1. Abahji, T.N., Nill, L., Ide, N., Keller, C., Hoffmann, U., and Weiss, N. 2007. Acute hyperhomocysteinemia induces microvascular and macrovascular endothelial dysfunction. Arch. Med. Res. **38**(4): 411-416. doi:10.1016/j.arcmed.2007.01.004 PMID:17416288
2. Auclair, C., and Voisin, E. 1985. Nitroblue tetrazolium reduction. In: Greenvvald RA (ed) Handbook of Methods for Oxygen Radical Research. Boca Raton, CRC Press. Une. 123-132.
3. Başkaya, M.K., Rao, A.M., Donaldson, D., Prasad, M.R., and Dempsey, R.J. 1997. Protective effects of ifenprodil on ischemic injury size, blood-brain barrier breakdown, and edema formation in focal cerebral ischemia. Neurosurgery. **40**(2): 364-371. PMID:9007871
4. Bath, C.P., Farrell, L.N., Gilmore, J., Ward, M.A., Hicks, C.A., O'Neill, M.J., and Bleakman, D. 1996. The effects of ifenprodil and eliprodil on voltage-dependent Ca2+ channels and in gerbil global cerebral ischaemia. Eur. J. Pharmacol. **299**(1-3): 103-112. PMID:8901012
5. Bhattacharjee, N., Barma, S., Konwar, N., Dewanjee, S., and Manna, P. 2016. Mechanistic insight of diabetic nephropathy and its pharmacotherapeutic targets: An update. Eur. J. Pharmacol. **791**: 8-24. doi:10.1016/j.ejphar.2016.08.022 PMID:27568833
6. Borghammer, P., Vafaee, M., Ostergaard, K., Rodell, A., Bailey, C., and Cumming, P. 2008. Effect of memantine on CBF and CMRO2 in patients with early Parkinson's disease. Acta. Neurol. Scand. **117**(5): 317-323. doi:10.1111/j.1600-0404.2007.00943.x PMID:17927800

7. Bozic, M., and Valdivielso, J.M. 2015. The potential of targeting NMDA receptors outside the CNS. Expert. Opin. Ther. Targets. **19**(3): 399-413. doi:10.1517/14728222.2014.983900 PMID:25495517

8. Brittain, M.K., Brustovetsky, T., Brittain, J.M., Khanna, R., Cummins, T.R., and Brustovetsky, N. 2012. Ifenprodil, a NR2B-selective antagonist of NMDA receptor, inhibits reverse Na+/Ca2+ exchanger in neurons. Neuropharmacology. **63**(6): 974-982. doi:10.1016/j.neuropharm.2012.07.012 PMID:22820271

9. Carter, C., Benavides, J., Legendre, P., Vincent, J.D., Noel, F., Thuret, F., Lloyd, K.G., Arbilla, S., Zivkovic, B., and MacKenzie, E.T. 1988. Ifenprodil and SL 82.0715 as cerebral anti-ischemic agents. II. Evidence for N-methyl-D-aspartate receptor antagonist properties. J. Pharmacol. Exp. Ther. **247**(3): 1222-1232. PMID:2849669

10. Chen, H., Fitzgerald, R., Brown, A.T., Qureshi, I., Breckenridge, J., Kazi, R., Wang, Y., Wu, Y., Zhang, X., Mukunyadzi, P., Eidt, J., and Moursi, M.M. 2005. Identification of a homocysteine receptor in the peripheral endothelium and its role in proliferation. J. Vasc. Surg. **41**(5): 853-860. doi:10.1016/j.jvs.2005.02.021 PMID:15886671

11. Chen, Y.W., Shieh, J.P., Chen, Y.C., Leung, Y.M., Hung, C.H., and Wang, J.J. 2012. Cutaneous analgesia after subcutaneous injection of memantine and amantadine and their systemic toxicity in rats. Eur. J. Pharmacol. **693**(1-3): 25-30. doi:10.1016/j.ejphar.2012.07.037 PMID:22935636

12. Chenard, B.L., Shalaby, I.A., Koe, B.K., Ronau, R.T., Butler, T.W., Prochniak, M.A., Schmidt, A.W., and Fox, C.B. 1991. Separation of alpha 1 adrenergic and N-methyl-D-aspartate antagonist activity in a series of ifenprodil compounds. J. Med. Chem. **34**(10): 3085-3090. PMID:1681106

13. Cheriyan, J., Mezes, C., Zhou, N., Balsara, R.D., and Castellino, F.J. 2015. Heteromerization of ligand binding domains of N-methyl-D-aspartate receptor requires both coagonists, L-glutamate and glycine. Biochemistry. **54**(3): 787-794. doi:10.1021/bi501437s PMID:25544544

14. Di Maio, R., Mastroberardino, P.G., Hu, X., Montero, L.M., and Greenamyre, J.T. 2013. Thiol oxidation and altered NR2B/NMDA receptor functions in in vitro and in vivo pilocarpine models: implications for epileptogenesis. Neurobiol. Dis. **49**: 87-98. doi:10.1016/j.nbd.2012.07.013 PMID:22824136

15. Flores-Soto, M.E., Chaparro-Huerta, V., Escoto-Delgadillo, M., Ureña-Guerrero, M.E., Camins, A., and Beas-Zarate, C. 2013. Receptor to glutamate NMDA-type: the functional diversity of the nr1 isoforms and pharmacological properties. Curr. Pharm. Des. **19**(38): 6709-6719. doi:10.2174/1381612811319380003 PMID:23530508

16. Gao, X., Xu, X., Pang, J., Zhang, C., Ding, J.M., Peng, X., Liu, Y., and Cao, J.M. 2007. NMDA receptor activation induces mitochondrial dysfunction, oxidative stress and apoptosis in cultured neonatal rat cardiomyocytes. Physiol. Res. **56**(5): 559-569. PMID:16925458

17. Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., and Tannenbaum, S.R. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. **126**(1): 131-138. PMID:7181105

18. Hankey, G.J., and Eikelboom, J.W. 1999. Homocysteine and vascular disease. Lancet. **354**(9176): 407-413. doi:10.1016/S0140-6736(98)11058-9 PMID:10437885

19. Hashimoto, K., and London, E.D. 1995. Interactions of erythro-ifenprodil, threo-ifenprodil, erythro-iodoifenprodil, and eliprodil with subtypes of sigma receptors. Eur. J. Pharmacol. **273**(3): 307-310. doi:org/10.1016/0014-2999(94)00763-W PMID:7737340

20. Hess, S.D., Daggett, L.P., Deal, C., Lu, C.C., Johnson, E.C., and Veliçelebi, G. 1998. Functional characterization of human N-methyl-D-aspartate subtype 1A/2D receptors. J. Neurochem. **70**(3): 1269-1279. doi:10.1046/j.1471-4159.1998.70031269.x PMID:9489750

21. Howes, L.G. 2014. Cardiovascular effects of drugs used to treat Alzheimer's disease. Drug. Saf. **37**(6): 391-395. doi:10.1007/s40264-014-0161-z PMID:24777654

22. Joseph, J., Joseph, L., Shekhawat, N.S., Devi, S., Wang, J., Melchert, R.B., Hauer-Jensen, M., and Kennedy, R.H. 2003. Hyperhomocysteinemia leads to pathological ventricular hypertrophy in normotensive rats. Am. J. Physiol. Heart. Circ. Physiol. **285**(2): 679-686. doi:10.1152/ajpheart.00145.2003 PMID:12730062

23. Kamel, I.R., Wendling, W.W., Chen, D., Wendling, K.S., Harakal, C., and Carlsson, C. 2008. N-methyl-D-aspartate (NMDA) antagonists--S(+)-ketamine, dextrorphan, and dextromethorphan--act as calcium antagonists on bovine cerebral arteries. J. Neurosurg. Anesthesiol. **20**(4): 241-248. doi:10.1097/ANA.0b013e31817f523f PMID:18812887

24. Kobayashi, T., Washiyama, K., and Ikeda, K. 2006. Inhibition of G protein-activated inwardly rectifying K+ channels by ifenprodil. Neuropsychopharmacology. **31**(3): 516-524. doi:10.1038/sj.npp.1300844 PMID:16123769

25. Liu, W., Xu, Z., Yang, T., Xu, B., Deng, Y., and Feng, S. 2016. Memantine, a Low-Affinity NMDA Receptor Antagonist, Protects against Methylmercury-Induced Cytotoxicity of Rat Primary Cultured Cortical Neurons, Involvement of Ca(2+)

Dyshomeostasis Antagonism, and Indirect Antioxidation Effects. Mol. Neurobiol. doi:10.1007/s12035-016-0020-2 PMID:27538940

26. Makhro, A., Tian, Q., Kaestner, L., Kosenkov, D., Faggian, G., Gassmann, M., Schwarzwald, C., and Bogdanova, A. 2016. Cardiac N-methyl D-aspartate Receptors as a Pharmacological Target. J. Cardiovasc. Pharmacol. **68**(5): 356-373. doi:10.1097/FJC.0000000000000424 PMID:27525575
27. McCully, K.S. 2016. Homocysteine, Infections, Polyamines, Oxidative Metabolism, and the Pathogenesis of Dementia and Atherosclerosis. J. Alzheimers. Dis. **54**(4): 1283-1290. doi:10.3233/JAD-160549 PMID:27567876
28. McGee, M.A., and Abdel-Rahman, A.A. 2012. Enhanced vascular neuronal nitric-oxide synthase-derived nitric-oxide production underlies the pressor response caused by peripheral N-methyl-D-aspartate receptor activation in conscious rats. J. Pharmacol. Exp. Ther. **342**(2): 461-471. doi:10.1124/jpet.112.194464 PMID:22580349
29. McGee, M.A., and Abdel-Rahman, A.A. 2015. Ethanol attenuates peripheral NMDAR-mediated vascular oxidative stress and pressor response. Alcohol. **49**(5): 499-506. doi:10.1016/j.alcohol.2015.03.004 PMID:25986731
30. Mirzoyan, R.S., Gan'shina, T.S., Maslennikov, D.V., Kovalev, G.I., Zimin, I.A., Pyatin, B.M., Avdyunina, N.I., Kukhtarova, A.M., Khostikyan, N.G., Meliksetyan, V.S., Alikhanyan, C.B., and Mirzoyan, N.R. 2014. Cerebrovascular and neuroprotective effects of adamantane derivative. Biomed. Res. Int. **2014**:586501. doi:10.1155/2014/586501 PMID:24772429
31. Monassier, L., Riehl, V., Lienhard, J.P., Tibiriça, E., Feldman, J., and Bousquet, P. 1999. Effects of ifenprodil and baclofen on exercise-induced increase of myocardial oxygen

demand in normotensive rats. J. Pharmacol. Exp. Ther. **290**(3): 1188-1194. PMID:10454494

32. Morhenn, V.B., Waleh, N.S., Mansbridge, J.N., Unson, D., Zolotorev, A., Cline, P., and Toll, L. 1994. Evidence for an NMDA receptor subunit in human keratinocytes and rat cardiocytes. Eur. J. Pharmacol. **268**(3): 409-414. PMID:7805765
33. Moshal, K.S., Tipparaju, S.M., Vacek, T.P., Kumar, M., Singh, M., Frank, I.E., Patibandla, P.K., Tyagi, N., Rai, J., Metreveli, N., Rodriguez, W.E., Tseng, M.T., and Tyagi, S.C. 2008. Mitochondrial matrix metalloproteinase activation decreases myocyte contractility in hyperhomocysteinemia. Am. J. Physiol. Heart. Circ. Physiol. **295**(2): 890-897. doi:10.1152/ajpheart.00099.2008 PMID:18567713
34. Muntzel, M.S., Joseph, T., and Onwumere, O. 2006. Acute homocysteine administration does not elevate sympathetic nerve activity in rats. Atherosclerosis. **184**(2): 290-294. doi:10.1016/j.atherosclerosis.2005.04.009 PMID:15927186
35. Näsström, J., Böö , E., Ståhlberg, M., and Berge, O.G. 1993. Tissue distribution of two NMDA receptor antagonists, [3H]CGS 19755 and [3H]MK-801, after intrathecal injection in mice. Pharmacol. Biochem. Behav. **44**(1): 9-15. doi:org/10.1016/0091-3057(93)90275-X PMID:8430132
36. Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. **95**(2) :351-358. PMID:36810
37. Ota, H., Ogawa, S., Ouchi, Y., and Akishita, M. 2015. Protective effects of NMDA receptor antagonist, memantine, against senescence of PC12 cells: A possible role of nNOS and combined effects with donepezil. Exp. Gerontol. **72**: 109-16. doi:10.1016/j.exger.2015.09.016 PMID:26408226

38. Pall, M.L. 2013. The NO/ONOO-cycle as the central cause of heart failure. Int. J. Mol. Sci. **14**(11): 22274-22330. doi:10.3390/ijms141122274 PMID:24232452

39. Perez-Otano, I., Schulteis, C.T., Contractor, A., Lipton, S.A., Trimmer, J.S., Sucher, N.J., and Heinemann, S.F. 2001. Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. J. Neurosci. **21**(4): 1228-1237. PMID:11160393

40. Pick, E., and Keisari, Y. 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Methods. **38**(1-2): 161-170. doi:org/10.1016/0022-1759(80)90340-3 PMID:6778929

41. Qian, A., and Johnson, J.W. 2006. Permeant ion effects on external Mg2+ block of NR1/2D NMDA receptors. J. Neurosci. **26**(42): 10899-10910. doi:10.1523/JNEUROSCI.3453-06.2006 PMID:17050728

42. Resstel, L.B., de Andrade, C.R., Haddad, R., Eberlin, M.N., de Oliveira, A.M., and Corrêa, F.M. 2008. Hyperhomocysteinaemia-induced cardiovascular changes in rats. Clin. Exp. Pharmacol. Physiol. **35**(8): 949-956. doi:10.1111/j.1440-1681.2008.04940.x PMID:18430058

43. Rosenberger, D., Moshal, K.S., Kartha, G.K., Tyagi, N., Sen, U., Lominadze, D., Maldonado, C., Roberts, A.M., and Tyagi, S.C. 2006. Arrhythmia and neuronal/endothelial myocyte uncoupling in hyperhomocysteinemia. Arch. Physiol. Biochem. **112**(4-5): 219-227. doi:10.1080/13813450601093443 PMID:17178594

44. Roshanravan, H., Kim, E.Y., and Dryer, S.E. 2016. NMDA receptors as potential therapeutic targets in diabetic nephropathy: Increased renal NMDA receptor subunit expression in Akita mice and reduced nephropathy following sustained treatment with

memantine or MK-801. Diabetes. **65**(10): 3139-3150. doi: 10.2337/db16-0209 PMID:27388219

45. Seeber, S., Humeny, A., Herkert, M., Rau, T., Eschenhagen, T., and Becker, C.M. 2004. Formation of molecular complexes by N-methyl-D-aspartate receptor subunit NR2B and ryanodine receptor 2 in neonatal rat myocard. J. Biol. Chem. **279**(20):21062-21068. doi:10.1074/jbc.M313009200 PMID:15010472
46. Snyder, E.M., Nong, Y., Almeida, C.G., Paul, S., Moran, T., Choi, E.Y., Nairn, A.C., Salter, M.W., Lombroso, P.J., Gouras, G.K., and Greengard, P. 2005. Regulation of NMDA receptor trafficking by amyloid-beta. Nat. Neurosci. **8**(8):1051-1058. doi:10.1038/nn1503 PMID:16025111
47. Srejovic, I., Jakovljevic, V., Zivkovic, V., Barudzic, N., Radovanovic, A., Stanojlovic, O., and Djuric, D.M. 2015. The effects of the modulation of NMDA receptors by homocysteine thiolactone and dizocilpine on cardiodynamics and oxidative stress in isolated rat heart. Mol. Cell. Biochem. **401**(1-2): 97-105. doi:10.1007/s11010-014-2296-8 PMID:25467376
48. Srejovic, I., Jakovljevic, V., Zivkovic, V., Jeremic, N., Jevdjevic, M., Stojic, I., and Djuric, D. 2015. The effects of glycine, glutamate and their combination on cardiodynamics, coronary flow and oxidative stress in isolated rat heart. Curr. Res. Cardiol. **2**:63-68 doi:10.4172/2368-0512.1000031
49. Takehara, H., Suzuki, Y., and Someya, T. 2015. QT prolongation associated with memantine in Alzheimer's disease. Psychiatry. Clin. Neurosci. **69**(4): 239-240. doi:10.1111/pcn.12236 PMID:25186635

50. Takemoto, Y. 2016. Central cardiovascular actions of L-homocysteine microinjected into ventrolateral medullary autonomic areas of the rat. Amino. Acids. **48**(9): 2215-2225. doi:10.1007/s00726-016-2259-3 PMID:27178024

51. Toda, N., and Okamura, T. 2016. Hyperhomocysteinemia impairs regional blood flow: involvements of endothelial and neuronal nitric oxide. Pflugers. Arch. **468**(9): 1517-1525. doi:10.1007/s00424-016-1849-y PMID:27417104

52. Traynelis, S.F., Wollmuth, L.P., McBain, C.J., Menniti, F.S., Vance, K.M., Ogden, K.K., Hansen, K.B., Yuan, H., Myers, S.J., and Dingledine, R. 2010. Glutamate receptor ion channels: structure, regulation, and function. Pharmacol. Rev. **62**(3): 405-496. doi:10.1124/pr.109.002451 PMID:20716669

53. Tyagi, N., Sedoris, K.C., Steed, M., Ovechkin, A.V., Moshal, K.S., and Tyagi, S.C. 2005. Mechanisms of homocysteine-induced oxidative stress. Am. J. Physiol. Heart. Circ. Physiol. **89**: 2649-2656. doi:10.1152/ajpheart.00548.2005 PMID:16085680

54. Tyagi, N., Vacek, J.C., Givvimani, S., Sen, U., and Tyagi, S.C. 2010. Cardiac specific deletion of N-methyl-d-aspartate receptor 1 ameliorates mtMMP-9 mediated autophagy/mitophagy in hyperhomocysteinemia. J. Recept. Signal. Transduct. Res. **30**(2): 78-87. doi:10.3109/10799891003614808 PMID:20170426

55. Vyklicky, V., Korinek, M., Smejkalova, T., Balik, A., Krausova, B., Kaniakova, M., Lichnerova, K., Cerny, J., Krusek, J., Dittert, I., Horak, M., and Vyklicky, L. 2014. Structure, function, and pharmacology of NMDA receptor channels. Physiol. Res. **63**(Suppl 1): 191-203. PMID:24564659

56. Walker, E., Black, J., Parris, C., Bryda, E.C., Cansino, S., Hunt, L., Chappell, J., Wehner, P., Studeny, M., and Wright, G.L. 2004. Effect of experimental hyperhomocysteinemia

on cardiac structure and function in the rat. Ann. Clin. Lab. Sci. **34**(2): 175-180. PMID:15228230

57. Wollmuth, L.P., Kuner, T., and Sakmann, B. 1998. Adjacent asparagines in the NR2-subunit of the NMDA receptor channel control the voltage-dependent block by extracellular Mg2+. J. Physiol. **506**(1): 13-32. doi:10.1111/j.1469-7793.1998.013bx.x PMID:9481670
58. Xu, F., Farkas, S., Kortbeek, S., Zhang, F.X., Chen, L., Zamponi, G.W., and Syed, N.I. 2012. Mercury-induced toxicity of rat cortical neurons is mediated through N-Methyl-D-Aspartate receptors. Mol. Brain. **5**:30. doi:10.1186/1756-6606-5-30 PMID:22980357

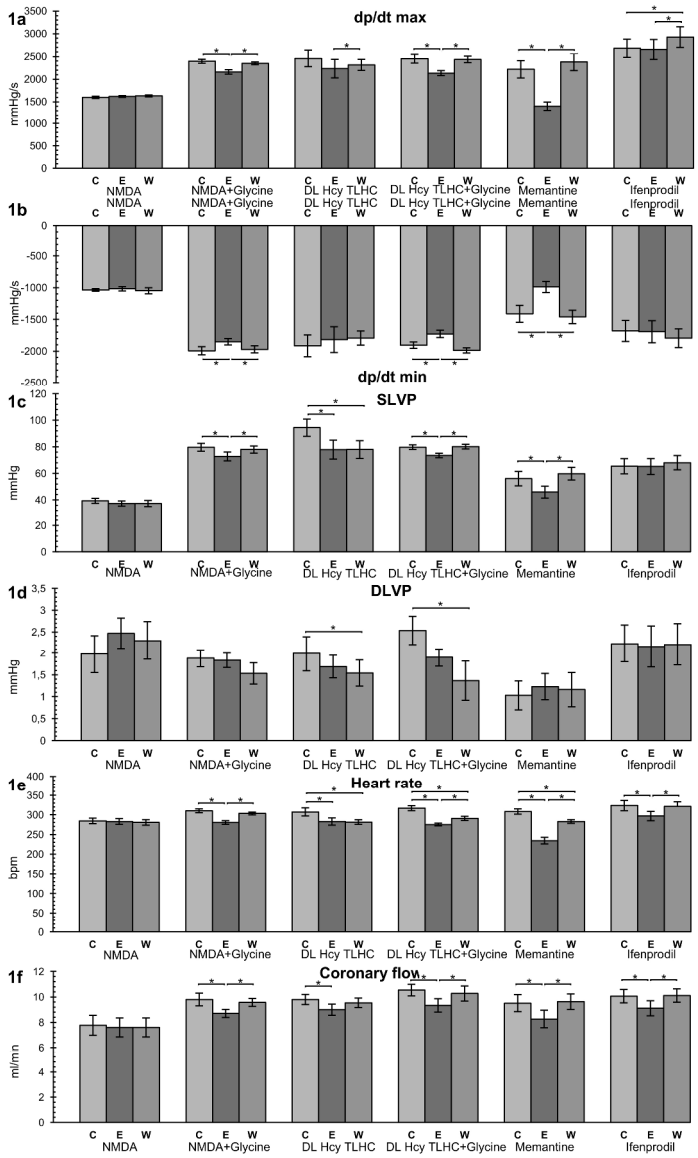
FIGURE CAPTIONS

Figure 1. Effects of N-methyl-D-aspartate (NMDA), combination of N-methyl-D-aspartate (NMDA) and glycine, DL-homocisteine thiolactone (DL-Hcy TLHC), combination of DL-homocisteine thiolactone (DL-Hcy TLHC) and glycine, memantine and ifenprodil on cardiodynamic parameters and coronary flow in isolated rat heart. Data are expressed as mean ± SE (Standard Error).

The values were measured in three period times (C - control, E – effect, W – wash-out). *Statistical significance compared with previous value ($p<0.05$)

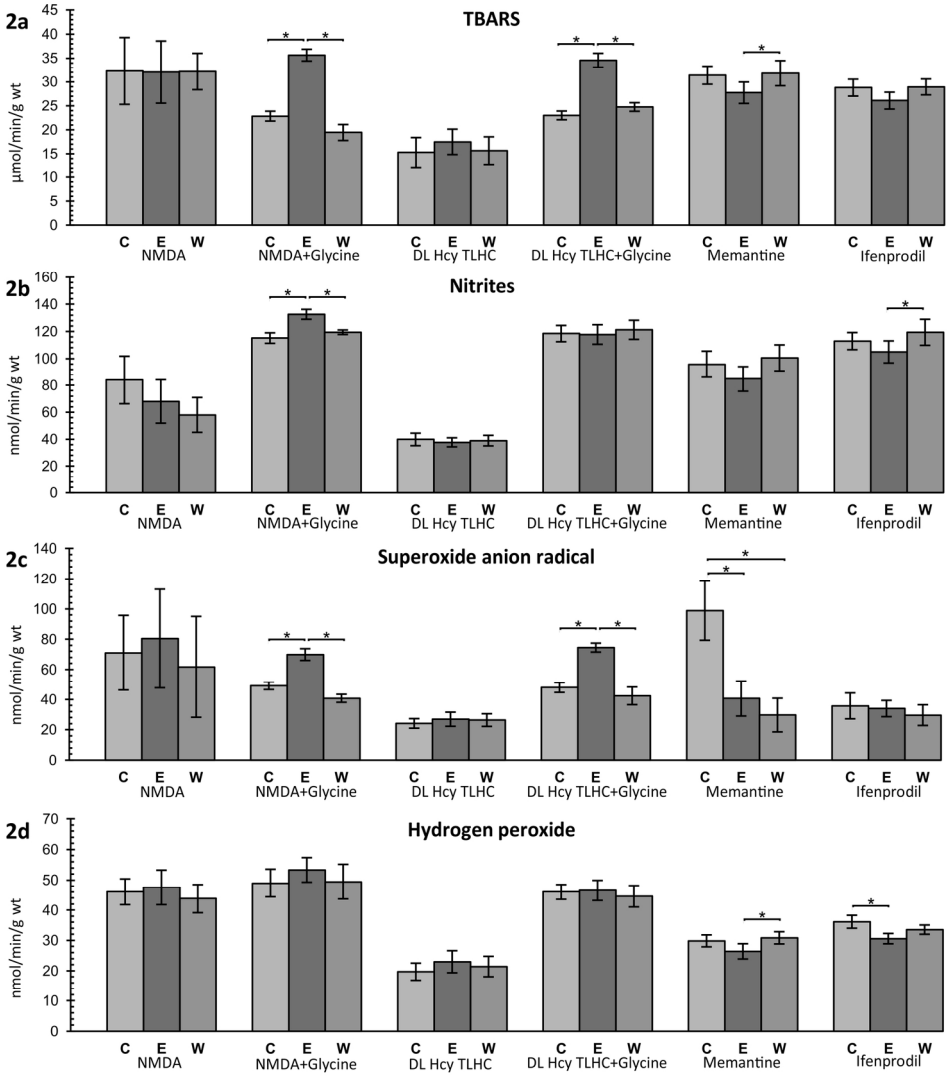
Figure 2. Effects of N-methyl-D-aspartate (NMDA), combination of N-methyl-D-aspartate (NMDA) and glycine, DL-homocisteine thiolactone (DL-Hcy TLHC), combination of DL-homocisteine thiolactone (DL-Hcy TLHC) and glycine, memantine and ifenprodil on the biomarkers of oxidative stress in coronary venous effluent. Data are expressed as mean ± SE (Standard Error).

The values were measured in three period times (C - control, E – effect, W – wash-out). *Statistical significance compared with previous value ($p<0.05$)



Effects of N-methyl-D-aspartate (NMDA), combination of N-methyl-D-aspartate (NMDA) and glycine, DL-homocisteine thiolactone (DL-Hcy TLHC), combination of DL-homocisteine thiolactone (DL-Hcy TLHC) and glycine, memantine and ifenprodil on cardiodynamic parameters and coronary flow in isolated rat heart. Data are expressed as mean ± SE (Standard Error).

250x419mm (300 x 300 DPI)



Effects of N-methyl-D-aspartate (NMDA), combination of N-methyl-D-aspartate (NMDA) and glycine, DL-homocisteine thiolactone (DL-Hcy TLHC), combination of DL-homocisteine thiolactone (DL-Hcy TLHC) and glycine, memantine and ifenprodil on the biomarkers of oxidative stress in coronary venous effluent. Data are expressed as mean ± SE (Standard Error).

168x190mm (300 x 300 DPI)