

Magnesium orotate elicits acute cardioprotection at reperfusion in isolated and in vivo rat hearts

Silvia N. Mirica, Oana M. Duicu, Simona L. Trancota, Ovidiu Fira-Mladinescu, Denis Angoulvant, and Danina M. Muntean

Abstract: Orotic acid and its salts chronically administered have been shown to significantly improve cardiac function in pathological settings associated with ischemia-reperfusion (I/R) injury. The aim of our study was to investigate the effect of magnesium orotate (Mg-Or) administration at the onset of post-ischemic reperfusion on myocardial function and infarct size (IS). Ex-vivo experiments performed on isolated perfused rat hearts were used to compare Mg-Or administration with a control group (buffer treated), ischemic post-conditioning, orotic acid treatment, and MgCl₂ treatment. Mg-Or administration was also investigated in an in-vivo model of regional I/R performed in rats undergoing reversible coronary ligation. The effect of Mg-Or on mitochondrial permeability transition pore (mPTP) opening after I/R was investigated in vitro to gain mechanistic insights. Both ex-vivo and in-vivo experiments showed a beneficial effect from Mg-Or administration at the onset of reperfusion on myocardial function and IS. In-vitro assays showed that Mg-Or significantly delayed mPTP opening after I/R. Our data suggest that Mg-Or administered at the very onset of reperfusion may preserve myocardial function and reduce IS. This beneficial effect may be related to a significant reduction of mPTP opening, a usual trigger of cardiac cell death following I/R.

Key words: magnesium orotate, cardioprotection, ischemia-reperfusion, ischemic post-conditioning, in vivo.

Résumé : L'administration au long cours d'acide orotique ou de ses sels a montré un effet bénéfique sur la fonction cardiaque dans plusieurs situations pathologiques associées aux lésions d'ischémie-reperfusion (I/R). L'objectif de notre étude était d'évaluer l'effet de l'orotate de magnésium (Mg-Or) administré en tout début de reperfusion myocardique post ischémique sur la taille d'infarctus (TI) et la fonction cardiaque. Un modèle ex vivo d'I/R sur cœur isolé perfusé de rats a comparé l'administration de Mg-Or avec les groupes expérimentaux control (tampon), post conditionnement ischémique, acide orotique, et MgCl₂. L'effet de Mg-Or a également été évalué sur un modèle in vivo d'I/R myocardique régional par ligature coronaire chez le rat. Une étude mécanistique a évalué l'effet de Mg-Or sur l'ouverture du port de perméabilité de transition mitochondriale (mPTP). L'administration de Mg-Or en début de reperfusion sur les modèles ex vivo et in vivo entraîne une diminution de la TI et une préservation de la fonction cardiaque. L'étude mécanistique montre que Mg-Or retarde l'ouverture du mPTP après I/R myocardique. Nos résultats suggèrent que Mg-Or administré en début de reperfusion est susceptible de diminuer la TI et de préserver la fonction cardiaque. Cet effet est vraisemblablement lié à son action inhibitrice sur l'ouverture du mPTP, phénomène impliqué dans les processus de mort cellulaire post I/R myocardique.

Mots-clés : orotate de magnésium, cardioprotection, ischémie-reperfusion, post conditionnement ischémique, in vivo.

Introduction

Myocardial infarction represents a major cause of morbidity and mortality worldwide. The cornerstone of treatment is represented by timely restoration of coronary reperfusion achieved either pharmacologically by thrombolysis and (or) mechanically by percutaneous revascularization (Keeley et al. 2003). Infarct size (IS) reduction and the subsequent preservation of ventricular pump function is a major therapeutic goal as it is a critical determinant of mortality. Paradoxically, restoration of blood flow to a previously ischemic myocardium is associated with a series of deleterious events collectively known as reperfusion injury that are responsible for additional cell death (Braunwald and Kloner 1985; Yellon and Hausenloy 2007). Over the past few decades, cardioprotection, defined as the totality of mechanical and pharmacological interventions aimed at reducing cell death at reper-

fusion, has become the focus of substantial research effort to decipher the underlying mechanisms (Murphy and Steenbergen 2008) and, to translate experimental findings from bench to bedside (Garcia-Dorado et al. 2009). Among these strategies, cardiac post-conditioning represents a highly investigated approach whereby either repeated brief ischemic stimulus (ischemic post-conditioning) or a pharmacological agent (pharmacological post-conditioning) when administered at the onset of reperfusion following lethal ischemia are associated with significant reduction of myocardial injury. Post-conditioning and its signal transduction pathways are nowadays areas of intensive research, with a view to examining their promising therapeutic potential in the clinical settings (Ovize et al. 2010). However, despite a wealth of preclinical data supporting adjunctive reperfusion strategies that showed great promise in animal models and proof-of-concept

Received 26 June 2012. Accepted 26 November 2012.

Abbreviations: 28'I, 28 minutes of ischemia; 3'R, 3 minutes of reperfusion; CF, coronary flow; CRC, calcium retention capacity; CsA, cyclosporine A; dLVP/dt_{max}, maximal first derivative of LVP; dLVP/dt_{min}, minimal first derivative of LVP; HR, heart rate; I/R, ischemia-reperfusion; IPostC, ischemic post-conditioning; IS, infarct size; KH, Krebs-Henseleit; LV, left ventricle; LVDP, left ventricular developed pressure (systolic minus diastolic pressure); LVP, left ventricular pressure; MBP, mean blood pressure; MgCl₂, magnesium chloride; Mg-Or, magnesium orotate; OA, orotic acid; RPP, rate pressure product; SEM, standard error of the mean.

S.N. Mirica,¹ O.M. Duicu,¹ S.L. Trancota, O. Fira-Mladinescu, and D.M. Muntean. Department of Pathophysiology, University of Medicine and Pharmacy, Timisoara, Romania.

D. Angoulvant. Intensive Cardiac-Care Unit and Interventional Cardiology, CHRU de Tours, Hôpital Trousseau, F-37044 Tours, France; EA4245 Université François-Rabelais de Tours, PRES Centre-Val de Loire Université, Tours, France.

Corresponding author: Denis Angoulvant (e-mail: d.angoulvant@chu-tours.fr).

¹These authors contributed equally to the manuscript.



clinical studies (Gerczuk and Kloner 2012), there is currently no routinely and clinically available therapeutic intervention to further reduce infarct size in association with revascularization procedures (Ivanov et al. 2010). It is therefore worthwhile to develop novel cardioprotective strategies that when coupled with reperfusion, further preserve ventricular function and reduce infarct size.

Orotic acid (OA) is the first pyrimidine formed in the de-novo pathway of nucleic acid synthesis (Lieberman et al. 1955). Early pioneering studies performed in the 1970s in the former Soviet Union reported the protective effects of chronically administered OA salts (mainly potassium and magnesium orotate) on the cardiovascular system in both experimental and clinical settings (Rosenfeldt 1998; Muntean et al. 2010). Several mechanisms were postulated as being responsible for this cardioprotection (Meerson 1991; Rosenfeldt 1998; Rosenfeldt et al. 1998). It has been suggested that orotic-acid treatment improved tolerance to global ischemia in infarcted (but not normal) myocardium by preventing adenine nucleotide depletion in the surviving myocardium (Rosenfeldt et al. 1998).

We hypothesized that magnesium orotate administered at the onset of reperfusion following myocardial ischemic assault could reduce IS and preserve myocardial function. Ex-vivo experiments performed in isolated rat hearts compared the effect of magnesium orotate (Mg-Or) with ischemic post-conditioning (IPostC). The effects of magnesium chloride and OA were also quantified. Moreover, since the first minutes of reperfusion are known to be critical for both pharmacological and IPostC-associated cardioprotection, the effects of timing on magnesium orotate administration at reperfusion were further analyzed. In-vivo validation of magnesium orotate cardioprotective effects was performed in a model of myocardial regional ischemia–reperfusion (I/R) injury.

Since the recovery of mitochondrial function is mandatory for cardioprotection (Di Lisa et al. 2007), and to get preliminary insights into the mechanism of protection, we investigated whether Mg-Or has an effect at the mitochondrial level. To this aim we isolated mitochondria from rat hearts subjected to global I/R and assessed calcium induced mitochondrial permeability transition in the presence compared with the absence of Mg-Or, OA, and MgCl₂. Their effect was compared with that elicited by cyclosporine A (CsA), classically reported to desensitize the mitochondrial permeability transition pore (mPTP), and thus, to protect against myocardial reperfusion injury in both experimental and clinical settings (Hausenloy et al. 2012).

Materials and methods

All experimental procedures used in this study were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Ethics Committee of the University for Medicine and Pharmacy of Timișoara, Romania.

Animals (Sprague–Dawley rats) were fed ad libitum and housed under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 ± 5%, 12 h (light) – 12 h (dark) cycle). Animals were fasted but provided with water ad libitum 24 h prior to the start of the experiment.

Most reagents were from Sigma–Aldrich (cyclosporine A was from Novartis). Mg-Or was generously provided by Wörwag Pharma GmbH (Germany). All tested pharmacological agents were dissolved in the Krebs–Henseleit (K-H) buffer before being added into the perfusion buffer.

Ischemia–reperfusion experiments

Detailed protocols for the heart preparations, cardiac function measurements, and infarct size analysis are presented in the supplementary data (cjpp-2012-0216suppl.doc).²

Global ischemia–reperfusion (protocol I)

After stabilization, hearts ($n = 6–8$ per group) were randomly divided among the following experimental groups: control group treated with K-H buffer; IPostC, ischemic post-conditioning with 3 cycles of 30 s of reperfusion per 30 s of ischemia started at the very onset of reperfusion; Mg-Or, magnesium orotate treatment (1 mmol/L); OA, orotic acid treatment (1 mmol/L); and, MgCl₂, magnesium chloride treatment (1 mmol/L). All of the pharmacological agents, including the K-H buffer used on the control group, were administered in a brief bolus of 15 s administered 2 min (28'I) before the onset of reperfusion, to be present at the very onset of reperfusion. The infusions were continued for the duration of the reperfusion period (120 min).

In a separate group of animals ($n = 4–5$) the effects of a high dose of Mg-Or (5 mmol/L, the highest dose that we were able to test because of solubility problems) and MgCl₂ (5 mmol/L) were investigated with respect to post-ischemic functional recovery and infarct size.

To further investigate the effects of timing, in an additional group of animals ($n = 6$), hearts were subjected to 30 min of global ischemia followed by a delayed administration of Mg-Or, 3 min after the onset of reperfusion (Mg-Or, 3'R). The results of this group were compared with those of early drug administration (Mg-Or, 28'I) and the control (treated with K-H buffer) hearts.

Regional ischemia–reperfusion (protocol II)

Detailed protocols for animal preparation and infarct size analysis are presented in the supplementary data.²

Regional ischemia was performed as follows: after performing a left-sided lateral thoracotomy in the 4th intercostal space, the pericardium was opened and the heart was gently exteriorized. A 5.0 (Prolene) suture was placed under the left coronary artery between the base of pulmonary artery and the left atrial appendage. The ends of the suture were threaded through a propylene tube to form a snare. Coronary artery occlusion was obtained by tightening the ends of the suture with a pair of clamps. Ischemia was confirmed by visualizing hypokinesia and pallor distal to the occlusion, and by ST elevation (electrocardiographic monitoring). Reperfusion of the artery was initiated by loosening the snare and was confirmed by visualizing epicardial hyperemia and ST segment normalization on the ECG. At the end of the surgical preparation, rats were allowed to stabilize for 20 min. All animals underwent 30 min of ischemia followed by 120 min of reperfusion. Coronary occlusion was confirmed by the hemodynamic response (typical fall in mean blood pressure), the ECG aspect (ST elevation), and the appearance of ventricular arrhythmias after the first 8–10 minutes of occlusion. Mg-Or (1 mL, 0.1 mg/kg of body mass) was administered intravenously (femoral vein) 2 min before reperfusion (28 min of ischemia) in the early administration group and 3 min after the onset of reperfusion in the delayed administration group.

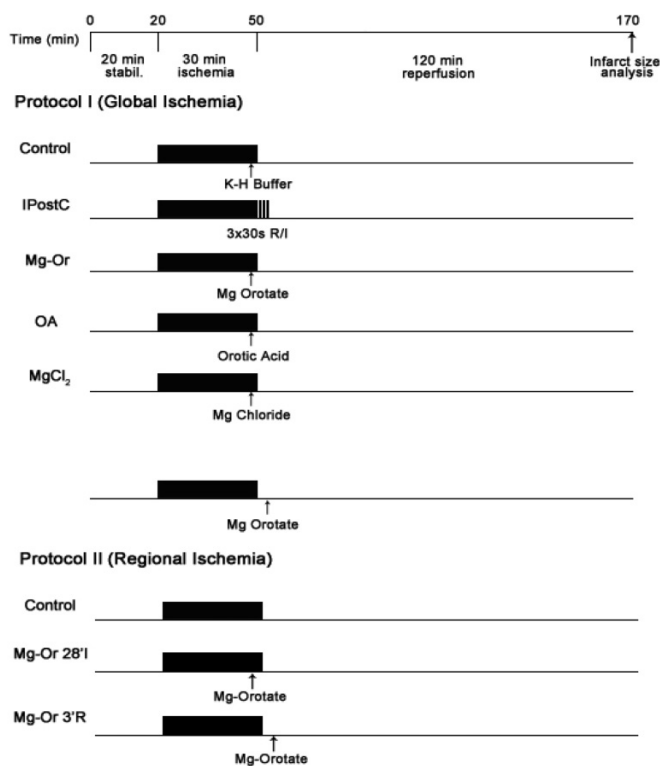
The experimental protocols of global and regional ischemia are depicted in Fig. 1.

Mitochondria isolation and CRC experiments

Cardiac mitochondria were prepared from hearts subjected to global I/R as follows: after 15 min of reperfusion, hearts were rapidly removed and immersed in 20 mL of ice-cold buffer (100 mmol/L sucrose, 50 mmol/L KCl, 20 mmol/L 2-[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES),

²Supplementary data for this article are available on the journal Web site (<http://www.nrcresearchpress.com/doi/cjpp-2012-0216>).

Fig. 1. Ischemia–reperfusion protocols for adult Sprague–Dawley rat hearts. (Protocol I) Isolated hearts subjected to 30 min global ischemia per 120 min reperfusion were randomly assigned to the following groups: Control, IPostC-, Mg-Or-, OA-, or MgCl₂-treated. Drug treatment was a brief bolus of 15 s at 28 min of ischemia and was then continued throughout the reperfusion period. Functional parameters were analysed at 30 min of reperfusion and infarct size at 120 min, respectively. In an additional group, delayed administration of Mg-Or, starting from the 3rd min of reperfusion was designed and compared with the early administration. (Protocol II) In-situ hearts were subjects to 30 min regional ischemia per 120 min reperfusion and delayed administration of Mg-Or, 3 min after the onset of reperfusion (Mg-Or, 3'R) and the results were compared with the early administration of the drug at the end of the ischemic period (Mg-Or, 28'I) and control hearts. Control hearts, hearts treated with Krebs–Henseleit buffer; IPost, ischemic post-conditioned hearts; Mg-Or, treated with magnesium orotate; OA, treated with orotic acid; MgCl₂, treated with magnesium chloride.



1 mmol/L EDTA, and 0.2% fatty acid free bovine serum albumin (BSA), pH 7.2). Tissue was minced with sharp scissors, treated with a protease (nagarse, 5 mg/g wet mass of cardiac tissue), and homogenized in 25 mL of isolation buffer using a Polytron homogenizer with a Teflon pestle (ES VELP Scientifica). The resulting homogenate was centrifuged (Rotina centrifuge 38 R) for 10 minutes at 8500g (4 °C); the pellet was resuspended in 25 mL of isolation buffer and centrifuged for 10 minutes at 8500g (4 °C). The supernatant was further centrifuged at 8500g for 10 min (4 °C), to increase mitochondria purification. The mitochondria pellet was kept on ice and used within 4 h after isolation. Mitochondrial protein concentration was determined by the Biuret method (Gornall et al. 1949).

The calcium retention capacity (CRC) assay is the pedigree method used to measure isolated mitochondria resistance to the phenomenon of mitochondrial permeability transition that occurs in the presence of matrix calcium overload. The change in extramitochondrial Ca²⁺ concentration was monitored using a fluorescent probe Ca-green at 5 N (1 μmol/L; excitation–emission,

500–530 nm) according to a method adapted from Gomez et al. (2008). Isolated mitochondria (0.25 mg/mL) were suspended in an incubation buffer (150 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L KH₂PO₄, 5 mmol/L succinic acid, in 20 mmol/L Tris–HCl, pH 7.4) at 37 °C in the cuvette of a spectrofluorometer (Hitachi F-7000). CaCl₂·2H₂O pulses (20 nmols/pulse) were added every 2 min until opening of the permeability transition pore occurred. This was recorded as a large increase in fluorescence due to the sudden release of accumulated Ca²⁺ from mitochondria in the cuvette. CRC was calculated as the amount of calcium required to trigger mPTP opening and the subsequent maximal calcium release by isolated mitochondria. Data are expressed in nanomoles of CaCl₂ per milligram of mitochondrial proteins. All of the drugs tested were added at a concentration of 5 mmol/L except for CsA (1 μmol/L).

Statistical analysis

All measurements are presented as the mean ± SEM. Group comparisons were performed by one-way analysis of variance (ANOVA) and Tukey's post-hoc multiple comparison test (GraphPad Prism version 5.0). Values for *p* < 0.05 were considered statistically significant.

Results

Mg-Or (1 mmol/L) administered at the onset of reperfusion after ex vivo global ischemia significantly preserved left ventricle (LV) function and reduced infarct size

Baseline hemodynamic parameters for all experimental groups are shown in Table 1. There were no statistically significant differences between groups with respect to body mass and the functional parameters at the end of the stabilization period.

IPostC elicited a significant increase of systolic function parameters measured at 30 min of reperfusion and expressed as a percentage of their pre-ischemic values: LVDP (68.4 ± 2.3) and +dLVP/dt_{max} (70.3 ± 1) compared with the control group (39.2 ± 2.6 and 38.7 ± 3.7, respectively; *p* < 0.001). Administration of Mg-Or (1 mmol/L) and OA elicited a similar degree of protection compared with the controls (LVDP: 68.8 ± 2.08 and 58 ± 5.6 compared with 39.2 ± 2.6, respectively, *p* < 0.01; +dLVP/dt_{max}: 60.1 ± 2.5 and 57.2 ± 5.2 compared with 38.6 ± 3.7, *p* < 0.01; –dLVP/dt_{max}: 66.4 ± 3.2 and 62.5 ± 7.4 compared with 46 ± 4.1, *p* < 0.01), whereas MgCl₂ had no beneficial effects (LVDP: 44.3 ± 3.1 compared with 39.2 ± 2.6, *p* = NS; +dLVP/dt_{max}: 45.3 ± 2.6 compared with 38.6 ± 3.7 and –dLVP/dt_{max} 45.1 ± 3.4 compared with 46 ± 4.1, *p* = not significant (NS)) (Fig. 2).

Infarct size (IS) was measured in all groups at the end of the 120 min reperfusion period (Fig. 3). A significant decrease in IS was observed in Mg-Or (1 mmol/L) group that clearly outperformed the one elicited by OA (32.1 ± 1.8 compared with 46.7 ± 3, *p* < 0.01). No anti-necrotic protection was associated with the administration of MgCl₂ (69.2 ± 1.7 compared with 70 ± 3.5 for the controls, *p* = NS). As expected, IPostC elicited powerful anti-infarct protection (32.3 ± 1.9, *p* < 0.001 compared with the controls).

When investigating the effects of higher doses, we observed that the cardioprotective effects were recapitulated for Mg-Or (5 mmol/L), whereas no protection was elicited by the same dose of MgCl₂ (data not shown).

Delayed administration of Mg-Or (1 mmol/L) at reperfusion after global ischemia was less efficient than early administration on LV function

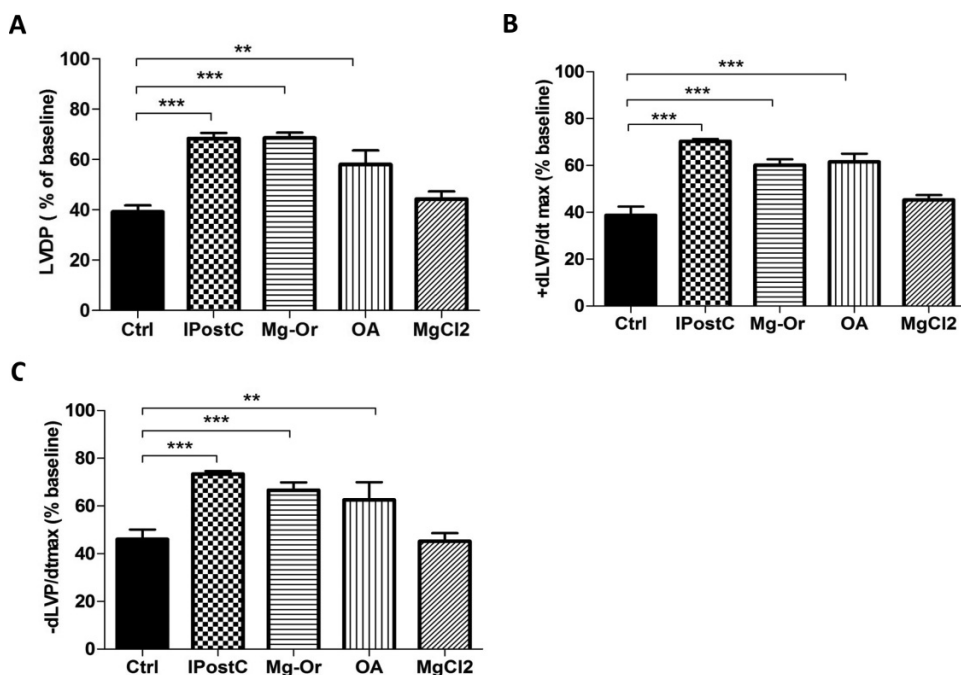
The effects of delayed administration of Mg-Or (1 mmol/L) were tested, and both functional parameters and infarct size measurements were compared with the early administration of the drug and the control group, respectively. In the Mg-Or 3'R treatment group, an important albeit less significant protection of contractile function: LVDP (51.3 ± 1.6 compared with 39.2 ± 2.6, *p* < 0.01), +dLVP/dt_{max} (55.6 ± 2.2 compared with 38.7 ± 3.7, *p* < 0.01) respec-

Table 1. Baseline values of hemodynamic and contractile parameters at the end of the 20 min stabilizing period in the ex-vivo model of ischemia–reperfusion.

Time (min)	Hemodynamic parameters					
	MBP (mm Hg)			HR (bpm)		
	Ctrl	Mg-Or 28'I	Mg-Or 3'R	Ctrl	Mg-Or 28'I	Mg-Or 3'R
20 (stabilized)	95±9	92±14	98±11	273±19	261±25	250±23
5 (reperfusion)	69±8	74±17	75±9	286±21	281±21	256±17
30 (reperfusion)	74±11	84±15	80±13	243±18	259±23	248±12

Note: No significant difference was found between experimental groups (Ctrl, control; Mg-Or, magnesium orotate; 28'I, early administration of drug in the 28 min ischemia; 3'R, delayed administration of drug at the 3rd min of reperfusion) for any of the parameters (MBP, mean blood pressure; 1 mm Hg = 133.322 Pa; HR, heart rate; bpm, beats per minute; LVDP, left ventricular developed pressure; RPP, rate pressure product; CF, coronary flow). Values are the mean ± SEM.

Fig. 2. Left ventricle (LV) function recovery in the ex vivo global ischemia–reperfusion protocol. (A) Recovery of left ventricular developed pressure (LVDP) at 30 min of reperfusion. A significant recovery of LVDP was observed for all of the experimental groups (IPostC, Mg-Or, and OA) compared with the control group (Ctrl), except for the MgCl₂ group ($p = \text{NS}$); ***, $p < 0.001$; **, $p < 0.01$. (B) Recovery of the contractility index $+d\text{LVDP}/dt_{\text{max}}$ at 30 min of reperfusion. Except for the MgCl₂ group ($p = \text{NS}$), a significant recovery of $+d\text{LVDP}/dt_{\text{max}}$ was recorded in all experimental groups (IPostC, Mg-Or, and OA) compared with Ctrl; ***, $p < 0.001$; **, $p < 0.01$. (C) Recovery of the relaxation index $-d\text{LVDP}/dt_{\text{min}}$ at 30 min of reperfusion. Except for the MgCl₂-treated group ($p = \text{NS}$), all experimental groups (IPostC, Mg-Or, and OA) showed a significant recovery of $-d\text{LVDP}/dt_{\text{max}}$ compared with Ctrl; ***, $p < 0.001$; **, $p < 0.01$. Control hearts, hearts treated with Krebs–Henseleit buffer; IPost, ischemic post-conditioned hearts; Mg-Or, treated with magnesium orotate; OA, treated with orotic acid; MgCl₂, treated with magnesium chloride.



tively was present, whereas no protection was observed for the relaxation index $-d\text{LVDP}/dt_{\text{max}}$ ($52.6\% \pm 1.3\%$ compared with $46\% \pm 4.1\%$, $p = \text{NS}$) (Fig. 4). However, when considering the anti-necrotic protection (Fig. 5A), both early and delayed application of Mg-Or (1 mmol/L) induced significant reduction of IS as compared with the non-treated groups (Mg-Or 3'R, 35.4 ± 1.8 ; Mg-Or 28'I, 32.1 ± 1.8 ; compared with the controls, 70 ± 3.5 , $p < 0.001$).

Mg-Or administered at the onset of reperfusion after regional ischemia in the in-vivo model significantly reduced infarct size

There were no significant differences between groups in baseline blood pressure and heart rate. Blood pressure decreased during ischemia and recovered progressively at the end of the reperfusion period (Table 2). Heart rate remained without significant changes throughout the experiments.

As observed in the isolated heart model, in the in-vivo model of myocardial regional ischemia Mg-Or induced a significant

reduction of infarct size, regardless the timing of administration (Fig. 5B).

Mg-Or increased resistance of the mPTP to opening after calcium overload

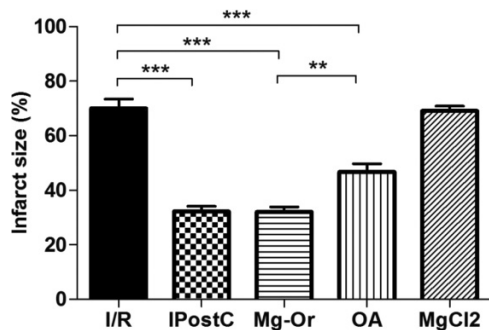
In rat heart mitochondria, the presence of Mg-Or in the suspension buffer increased the resistance of isolated mitochondria to calcium overload, an effect that surprisingly outperformed the one of CsA, the classical pore desensitizer. This effect cannot be attributed to the presence of magnesium ions, since the same concentration of MgCl₂ had no such effect (Fig. 6).

Discussion

The major findings of this study can be summarized as follows:

(i) Ex-vivo experiments showed that (a) acute administration of magnesium orotate at the very onset of reperfusion following global ischemia was associated with both an impressive recovery of

Fig. 3. Infarct size evaluation in the ex vivo global ischemia–reperfusion protocol for adult Sprague–Dawley rat hearts. Both ischemic and pharmacological post-conditioning significantly reduced infarct size compared with the Krebs–Henseleit treated control hearts (Ctrl). However, administration of magnesium orotate (Mg-Or) was superior in terms of anti-necrotic protection compared with orotic acid (OA); ***, $p < 0.001$; **, $p < 0.01$.



LV contractile function and anti-necrotic protection in the isolated rat heart model of I/R injury; (b) for both end-points, the magnitude of cardioprotection was comparable with the one elicited by IPostC; (c) administration of OA elicited an identical protective effect regarding functional recovery, whereas the anti-infarct protection was significantly better with Mg-Or; (d) no protection was observed with the same dose and timing of administration of MgCl₂; (e) delaying the administration of Mg-Or had a negative effect on functional recovery parameters, yet the anti-infarct protection remained unchanged; (f) the protective effects were recapitulated in the case of early administration of a higher dose (5 mmol/L) of Mg-Or, but not with a higher dose of MgCl₂.

(ii) In-vivo experiments showed that both early and delayed administration of Mg-Or at reperfusion following regional ischemia elicited significant infarct size reduction.

(iii) Mechanistic investigations showed that Mg-Or significantly delayed mPTP opening suggesting a cardioprotective effect against ischemia–reperfusion injury at the mitochondrial level.

The presence of Mg-Or from the very beginning of the post-ischemic reperfusion provided both a rapid and significant improvement of LV function parameters, i.e., contractility and relaxation. The degree of protection was similar to that elicited by IPostC. The anti-necrotic effect of post-conditioning in isolated rat hearts was reported early on by Tsang et al. (2004), and was later confirmed by Bopassa et al. (2006) and van Vuuren et al. (2008). However, it has been established that although it can be induced in rat hearts, post-conditioning-related protection, at variance with the robust cardioprotection associated with preconditioning, is more sensitive to several confounders (number of cycles, duration of index ischemia, temperature, type of perfusion). In this experiment, we chose the post-conditioning protocol that we found to be the most protective (i.e., 3 × 30 s cycles of R/I) as a positive control to evaluate the cardioprotection elicited by pharmacological interventions performed at reperfusion.

Kaljusto et al. (2006) have reported the lack of post-conditioning-associated cardioprotection in the isolated rat heart model of global ischemia, regardless of the type of protocol (3 × 10 s R/I; 3 × 30 s R/I; 2 × 60 s R/I) when the Wistar strain was used; however, since our protocol was associated with cardioprotection in adult Sprague–Dawley rats, it is likely that the efficacy of IPostC depends not only on the algorithm (number of cycles, duration of R/I episodes) and species, as previously stated (Vinten-Johansen et al. 2011), but also on the animal strain.

To identify the compound responsible for protection in the case of early application of the drug, we compared the effects of Mg-Or with the ones associated to the administration of the same dose of

OA and, of another magnesium salt (MgCl₂), respectively. The degree of recovery of contractile function assessed at 30 min of reperfusion was comparable for 1 mmol/L Mg-Or and OA. However, the anti-infarct protection afforded by Mg-Or was superior to the one elicited by OA. Similar results were obtained with the early administration of 5 mmol/L of Mg-Or (the highest concentration possible in our hands because of solubility issues). It is important to mention that the equivalent concentration of magnesium (i.e., 5 mmol/L of MgCl₂) did not protect the hearts. Of note, Kirkels et al. (1989) reported that intracellular magnesium deficiency due to leakage of the cation to the extracellular space does not play a role in the poor post-ischemic recovery in the isolated rat heart model. Alternatively, one may speculate that the combined administration of OA and magnesium may potentiate each other with respect to the gold standard end-point of reperfusion injury: the reduction of myocardial cell death.

These observations are suggestive of 2 aspects: (i) in isolated hearts, different mechanisms modulate infarct size and functional recovery and, (ii) the presence of both magnesium and OA is necessary for the anti-infarct protection at reperfusion following global ischemia. With respect to the former aspect, Xi et al. (1998) reported that in the isolated mouse heart subjected to global ischemia, there was a lack of association between the anti-necrotic cardioprotection and the amelioration of post-ischemic ventricular dysfunction after ischemic preconditioning. As for the latter issue, it is well established that magnesium, the second-most abundant intracellular cation after potassium, is a cofactor for many enzymes using nucleotides as cofactors or substrates (Saris et al. 2000). Magnesium also influences a wide range of cellular functions such as trans-membrane ion transport, glycolysis, respiration, excitation–contraction coupling, and phosphorylation of ion channels (Alvarez-Leefmans et al. 1987). In isolated rats hearts Bazargan et al. (2008) addressed the role of timing in cardioprotection associated with the administration of magnesium; they demonstrated that a high dose of a magnesium salt MgSO₄ (8 mmol/L), given after ischemia, did not improve cardiac function, but decreased infarct size. Matsusaka et al. (2002) were the first to report on the mechanism of the infarct size-limiting effect of magnesium in acute myocardial infarction in rabbits. These authors attributed the reduction of infarct size following magnesium administration, at least in part, to the augmentation of adenosine cardioprotective effect, since administration of 8-phenyltheophylline (8PT), an adenosine receptor blocker, abolished this protective effect.

In a similar experimental model, administration of an even higher dose of MgSO₄ (15 mmol/L) prior to reperfusion (but not prior to ischemia) was associated with marked recovery of mechanical function; an effect that was accompanied by the attenuation in the decrease of myocardial level of ATP induced by ischemia–reperfusion (Hara et al. 1990). In our study, neither 1 mmol/L nor 5 mmol/L of MgCl₂, albeit present at the very onset of reperfusion, could elicit an improvement of cardiac contractility.

The first evidence of the beneficial effects of orotate and its salts when given in chronic administration in cardiovascular pathology can be traced back to the late 1960s in the former Soviet Union (Simonson and Berman 1973; Meerson 1991). Early pioneering studies reported the efficacy of orotate potassium salt when added to standard therapy for acute myocardial infarction in patients. They suggested that orotate potassium induced faster recovery of contractile function and decreased incidence of cardiac arrhythmias and post-infarction deaths (Lukomskii et al. 1967; Kheinonen and Makeeva 1970). Several mechanisms were postulated as being responsible for this cardioprotection (Meerson 1991; Rosenfeldt 1998; Rosenfeldt et al. 1998). One of these suggested that OA treatment improves tolerance to global ischemia in infarcted (but not normal) hearts by preventing depletion of adenine nucleotides in the surviving myocardium (Rosenfeldt et al. 1998). OA is a key intermediate in the de-novo pathway of pyrimidine biosynthesis

Fig. 4. Left ventricle (LV) function recovery during reperfusion in the ex vivo global ischemia reperfusion and delayed intervention protocol in adult Sprague–Dawley rat hearts. (A) Recovery of left ventricular developed pressure (LVDP) during reperfusion. Early administration of magnesium orotate (Mg-Or) at 28 min of ischemia (28'I) elicited a more important recovery of LVDP at reperfusion ($p < 0.001$ vs. the control hearts) compared with the delayed administration ($p < 0.01$ vs. the control hearts). (B) Recovery of the contractility index $+dLVDP/dt_{max}$ during reperfusion. Similar to the other contractility index, LVDP, Mg-Or at 28'I elicited a more important recovery of $+dLVDP/dt_{max}$ at reperfusion ($p < 0.001$ vs. control hearts) when compared with the delayed administration ($p < 0.01$ vs. control hearts). (C) Recovery of the relaxation index $dLVDP/dt_{min}$ during reperfusion. Early administration of Mg-Or elicited a significant recovery of diastolic function ($p < 0.01$ vs. control hearts), whereas no recovery of $-dLVDP/dt_{max}$ was recorded in the Mg-Or 3'R group. Control hearts, hearts treated with Krebs–Henseleit buffer; Mg-Or 3'R group, hearts treated with magnesium orotate at 3 min of reperfusion.

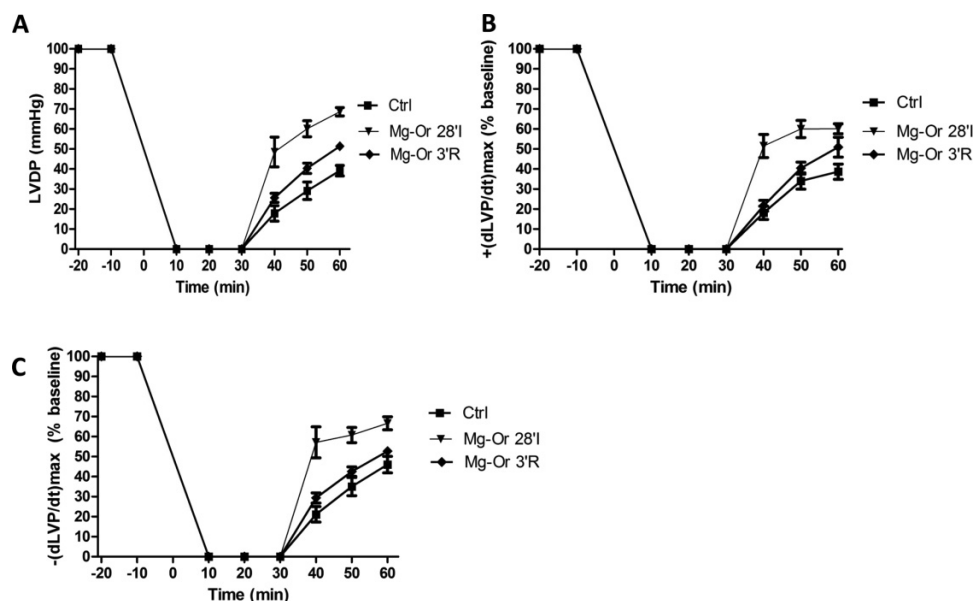


Fig. 5. Infarct size evaluation in (A) the ex vivo global ischemia–reperfusion and (B) the in vivo regional ischemia–reperfusion protocols. In isolated adult Sprague–Dawley rat hearts, Mg-Or elicited an important anti-necrotic protection regardless of the timing of administration: early, at 28 min of ischemia (Mg-Or 28'I); or delayed, at 3 min of reperfusion (Mg-Or 3'R). A similar effect was found for the in-vivo administration, when Mg-Or was associated with both early and delayed administration; ***, $p < 0.001$.

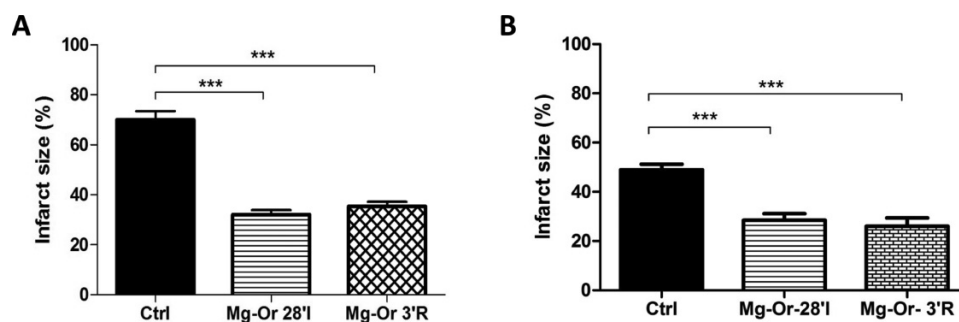
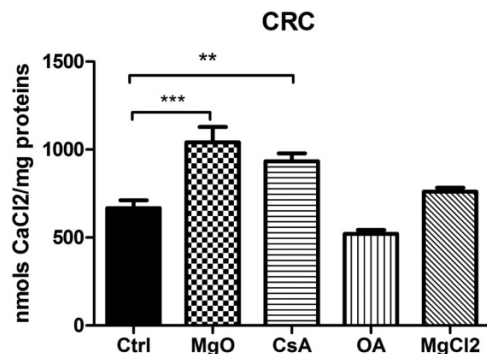


Table 2. Hemodynamic parameters in the in-vivo model of regional ischemia–reperfusion.

Experimental group	n	Body mass (g)	HR (bpm)	LVDP (mm Hg)	RPP = LVDP × HR (mm Hg × bpm)	CF (mL/min)
Control	6	275.0±14	273±13	117±16	29452±1278	11±1
IPostC	8	299.4±19	272±13	120±5	31289±1966	12±0.3
Mg-Or 28'I	8	287.8±13	305±3	124±1.6	37832±525	11±1.6
Mg-Or 3'R	6	332.7±24	309±5	119±9	36615±2585	14±0.6
OA	6	275±9	313±15	90±4	28288±2347	14±1.1
MgCl ₂	6	333±19	329±7	109±7	37339±2157	16±0.5

Note: No significant difference was found between experimental groups (IPostC, ischemic postconditioning; Mg-Or 28'I, early administration of drug in the 28 min of ischemia; Mg-Or 3'R, delayed administration of drug at the 3rd min of reperfusion; OA, orotic acid) for the recorded parameters (HR, heart rate; bpm, beats per minute; LVDP, left ventricular developed pressure; 1 mm Hg = 133.322 Pa; RPP, rate pressure product; CF, coronary flow). Values are the mean ± SEM.

Fig. 6. Calcium retention capacity assessment in isolated mitochondria from adult Sprague–Dawley rat hearts. Calcium load required to induce the opening of the mitochondrial permeability transition pore was higher in the presence of magnesium orotate (Mg-Or; $p < 0.001$ vs. the controls (Ctrl)) compared with the cyclosporin A (CsA)-treatment ($p < 0.01$ vs. Ctrl). No significant difference from the controls was found when orotic acid and magnesium chloride were added to the mitochondrial suspension.



where the formation of orotidine 5'-monophosphate (OMP) from OA and 5-phosphoribosyl-1-pyrophosphate (PRPP) occurs and is further used as a substrate for uridine nucleotides (uridine mono- and tri-phosphate) synthesis. On the other hand, uridine-5'-monophosphate (UMP) has been reported to prevent myocardial stunning during post-ischemic reperfusion of isolated rat heart (Sapronov et al. 2000) and to elicit anti-ischemic and anti-arrhythmic effects when acutely given before coronary occlusion in a rat model of in vivo I/R (Krylova et al. 2006). We could speculate that enhanced formation of UMP may be responsible for cardioprotection in our model of acute administration of Mg-Or at reperfusion.

A second hypothesized mechanism was the prevention of a catecholamine concentration drop in the non-ischemic zone of the myocardium (Meerson 1991; Rosenfeldt et al. 1998). However, in a more recent paper, it was shown that inhibition of norepinephrine reuptake in the early reperfusion period after ischemia in rats increased myocardial injury and abolished ischemic post-conditioning protective effect (Naumenko et al. 2011). In addition, Ferdinandy et al. (1998) reported that chronic administration of OA improved cardiac performance of the ischemic/reperfused rat hearts via the elevation of myocardial glycogen content.

Mechanical post-conditioning has been shown to be a powerful protective strategy when applied in the first minutes of reperfusion (Kin et al. 2004). Therefore, we thought to investigate whether Mg-Or-induced cardioprotection also depends on the timing of intervention. To this aim we delayed the administration of Mg-Or to the 3rd minute of reperfusion after global ischemia (Mg-Or-3'R), and compared the functional and structural effects with the ones associated with early administration of the drug (Mg-Or-28'I). Administration of Mg-Or beyond the time delay assumed to be protective of the post-conditioning-type effect was associated with a degree of anti-necrotic protection similar to the early administration. However, delayed application of the drug elicited a less significant recovery of contractile function when compared with early administration.

To exclude any interference with the experimental model, we recapitulated the protective effect of Mg-Or at reperfusion in the in-vivo model of regional myocardial ischemia in rat, thus clearly confirming the anti-necrotic beneficial effect of the drug during post-ischemic reperfusion.

To get insights into the molecular mechanisms underlying magnesium-orotate-related cardioprotection, we investigated the effects of the pharmacological compounds on CRC in mitochondria isolated from Langendorff-perfused hearts subjected to ischemia-

reperfusion. We compared these effects with the one elicited by CsA, since it has been previously reported that specific inhibition of mPTP prevents lethal reperfusion injury (Argaud et al. 2005). Interestingly, Mg-Or (5 mmol/L) was associated with the highest protective effect in preventing calcium-induced-mitochondrial permeability transition, whereas no significant effect was recorded for the same concentrations of OA and MgCl₂. To our knowledge this is the first study showing a beneficial effect of Mg-Or on infarct size and myocardial function following acute administration at reperfusion with a significant protective effect observed at the mitochondrial level. Future experiments investigating the mechanistic aspect of Mg-Or inhibition on mPTP opening after I/R are warranted.

Protecting the heart from I/R injury with classical or innovative pharmacological agents represents one of the greatest challenges of modern cardiology. On the basis of current understanding of the pathogenesis of reperfusion injury, numerous adjuncts to reperfusion have shown a reduction in infarct size and improved ventricular function in animal models. Indeed, both pharmacological and ischemic post-conditioning induced cardioprotection in basic studies, but new strategies for cardioprotection are therefore required to improve clinical outcomes in the settings of acute I/R injury. Gerczuk and Kloner recently reviewed the cardioprotective adjunctive therapies that failed to induce infarct size reduction in clinical trials (Gerczuk and Kloner 2012). These data suggest that there is still a need for experimental and translational research to reduce infarct size at the time of reperfusion to improve patient's short and long term prognosis. Magnesium orotate is already a long time used medication in chronic administration in the clinical settings making it a potential candidate for future clinical trials.

Conclusions

Our results show that early administration of Mg-Or before the onset of reperfusion reduces infarct size in the same magnitude as delayed administration but induces significantly better recovery of myocardial function. These observations suggest that early administration of Mg-Or during the post-ischemic reperfusion may also have beneficial effects on non-infarcted dysfunctional cardiomyocytes (stunned myocardium). In addition, the observation that Mg-Or may act on mitochondrial function opens new research avenues in the highly investigated field of I/R injuries and cardioprotection.

Conflict of interest

The authors declare that there are no conflicts of interest or other disclosures associated with this work.

Acknowledgments

The authors are thankful to Andreea Privistirescu and Mircea Hancu for their excellent technical assistance. This research is supported by the Hungarian–Romanian cross-border cooperation project HU-RO/0901/137/2.2.2 and the Ph.D. fellowship POSDRU/88/1.5/S/63117 (O.D.).

References

- Alvarez-Leefmans, F.J., Giraldez, F., and Gamino, S.M. 1987. Intracellular free magnesium in excitable cells: its measurement and its biologic significance. *Can. J. Physiol. Pharmacol.* 65(5): 915–925. doi:10.1139/y87-147. PMID:3113706.
- Argaud, L., Gateau-Roesch, O., Muntean, D., Chalabreysse, L., Loufouat, J., Robert, D., and Ovize, M. 2005. Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. *J. Mol. Cell. Cardiol.* 38(2): 367–374. doi:10.1016/j.yjmcc.2004.12.001. PMID:15698843.
- Bazargan, M., Faghihi, M., and Chitsaz, M. 2008. Importance of timing of magnesium administration in the isolated ischemic-reperfused rat heart: role of K(ATP) channels. *Physiol. Res.* 57(6): 839–846. PMID:18052684.
- Bopassa, J.C., Ferrera, R., Gateau-Roesch, O., Couture-Lepetit, E., and Ovize, M. 2006. PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. *Cardiovasc. Res.* 69(1): 178–185. doi:10.1016/j.cardiores.2005.07.014. PMID:16216231.

- Braunwald, E., and Kloner, R.A. 1985. Myocardial reperfusion: a double-edged sword? *J. Clin. Invest.* **76**(5): 1713–1719. doi:10.1172/JCI112160. PMID:4056048.
- Di Lisa, F., Canton, M., Menabo, R., Kaludercic, N., and Bernardi, P. 2007. Mitochondria and cardioprotection. *Heart Fail. Rev.* **12**(3–4): 249–260. doi:10.1007/s10741-007-9028-z. PMID:17516167.
- Ferdinandy, P., Fazekas, T., and Kadar, E. 1998. Effects of orotic acid on ischaemic/reperfused myocardial function and glycogen content in isolated working rat hearts. *Pharmacol. Res.* **37**(2): 111–114. doi:10.1006/phrs.1997.0274. PMID:9572065.
- Garcia-Dorado, D., Ruiz-Meana, M., and Piper, H.M. 2009. Lethal reperfusion injury in acute myocardial infarction: facts and unresolved issues. *Cardiovasc. Res.* **83**(2): 165–168. doi:10.1093/cvr/cvp185. PMID:19502282.
- Gerczuk, P.Z., and Kloner, R.A. 2012. An update on cardioprotection: a review of the latest adjunctive therapies to limit myocardial infarction size in clinical trials. *J. Am. Coll. Cardiol.* **59**(11): 969–978. doi:10.1016/j.jacc.2011.07.054. PMID:22402067.
- Gomez, L., Paillard, M., Thibault, H., Derumeaux, G., and Ovize, M. 2008. Inhibition of GSK3beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. *Circulation*, **117**(21): 2761–2768. doi:10.1161/CIRCULATIONAHA.107.755066. PMID:18490522.
- Gornall, A.G., Bardawill, C.J., and David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**(2): 751–766. PMID:18110453.
- Hara, A., Matsumura, H., and Abiko, Y. 1990. Beneficial effect of magnesium on the isolated perfused rat heart during reperfusion after ischaemia: comparison between pre-ischaemic and post-ischaemic administration of magnesium. *Naunyn Schmiedeberg's Arch. Pharmacol.* **342**(1): 100–106. doi:10.1007/BF00178980. PMID:2402298.
- Hausenloy, D.J., Boston-Griffiths, E.A., and Yellon, D.M. 2012. Cyclosporin A and cardioprotection: from investigative tool to therapeutic agent. *Br. J. Pharmacol.* **165**(5): 1235–1245. doi:10.1111/j.1476-5381.2011.01700.x. PMID:21955136.
- Ivanov, F., Mewton, N., Rioufol, G., Piot, C., Elbaz, M., Revel, D., et al. 2010. Cardioprotection in the clinical setting. *Cardiovasc. Drugs Ther.* **24**(3): 281–287. doi:10.1007/s10557-010-6243-y. PMID:20589423.
- Kaljusto, M.L., Mori, T., Mohammad Husain Rizvi, S., Galagudza, M., Frantzen, M.L., Valen, G., and Vaage, J. 2006. Postconditioning in rats and mice. *Scand. Cardiovasc. J.* **40**(6): 334–341. doi:10.1080/14017430601007587. PMID:17118823.
- Keeley, E.C., Boura, J.A., and Grines, C.L. 2003. Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials. *Lancet*, **361**(9351): 13–20. doi:10.1016/S0140-6736(03)12113-7. PMID:12517460.
- Kheinonen, I.M., and Makeeva, G.K. 1970. [Effect of potassium orotate on the course of myocardial infarct]. *Kardiologia*, **10**(2): 31–35. PMID:5428115.
- Kin, H., Zhao, Z.Q., Sun, H.Y., Wang, N.P., Corvera, J.S., Halkos, M.E., et al. 2004. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. *Cardiovasc. Res.* **62**(1): 74–85. doi:10.1016/j.cardiores.2004.01.006. PMID:15023554.
- Kirkels, J.H., van Echteld, C.J., and Ruigrok, T.J. 1989. Intracellular magnesium during myocardial ischemia and reperfusion: possible consequences for postischemic recovery. *J. Mol. Cell. Cardiol.* **21**(11): 1209–1218. doi:10.1016/0022-2828(89)90697-4. PMID:2607549.
- Krylova, I.B., Kachaeva, E.V., Rodionova, O.M., Negoda, A.E., Evdokimova, N.R., Balina, M.I., et al. 2006. The cardioprotective effect of uridine and uridine-5'-monophosphate: the role of the mitochondrial ATP-dependent potassium channel. *Exp. Gerontol.* **41**(7): 697–703. doi:10.1016/j.exger.2006.03.005. PMID:16621391.
- Lieberman, I., Kornberg, A., and Simms, E.S. 1955. Enzymatic synthesis of pyrimidine nucleotides; orotidine-5'-phosphate and uridine-5'-phosphate. *J. Biol. Chem.* **215**(1): 403–451. PMID:14392174.
- Lukomskii, P.E., Meerson, F.Z., Solov'ev, V.V., Shenderov, S.M., Zharov, E.I., Markovskaia, G.I., et al. 1967. [Disturbances of contractile function of the heart in myocardial infarct and the therapeutic use of cofactors of synthesis and precursors of nucleic acids]. *Kardiologia*, **7**(1): 3–11. PMID:5614645.
- Matsusaka, T., Hasebe, N., Jin, Y.T., Kawabe, J., and Kikuchi, K. 2002. Magnesium reduces myocardial infarct size via enhancement of adenosine mechanism in rabbits. *Cardiovasc. Res.* **54**(3): 568–575. doi:10.1016/S0008-6363(02)00253-5. PMID:12031702.
- Meerson, F.Z. 1991. Influence of orotic acid on adaptive reactions of healthy and sick organisms: cardioprotective effects. Paper presented at the Orotic Acid in Cardiology International Symposium on Orotic Acid and Magnesium-orotate.
- Muntean, D.M., Fira-Mladinescu, O., Mirica, N.S., Duicu, O.M., Trancota, S.L., and Sturza, A. 2010. Metabolic therapy: cardioprotective effects of orotic acid and its derivatives. *Biomed. Rev.* **21**: 47–55.
- Murphy, E., and Steenbergen, C. 2008. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol. Rev.* **88**(2): 581–609. doi:10.1152/physrev.00024.2007. PMID:18391174.
- Naumenko, S.E., Latysheva, T.V., and Gilinsky, M.A. 2011. Ischemic postconditioning and size of myocardial infarction during inhibition of norepinephrine reuptake. *Bull. Exp. Biol. Med.* **152**(2): 192–194. doi:10.1007/s10517-011-1485-7. PMID:22808457.
- Ovize, M., Baxter, G.F., Di Lisa, F., Ferdinandy, P., Garcia-Dorado, D., Hausenloy, D.J., et al. 2010. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovasc. Res.* **87**(3): 406–423. doi:10.1093/cvr/cvq129. PMID:20448097.
- Rosenfeldt, F.L. 1998. Metabolic supplementation with orotic acid and magnesium orotate. *Cardiovasc. Drugs Ther.* **12**(Suppl. 2): 147–152. PMID:9794088.
- Rosenfeldt, F.L., Richards, S.M., Lin, Z., Pepe, S., and Conyers, R.A. 1998. Mechanism of cardioprotective effect of orotic acid. *Cardiovasc. Drugs Ther.* **12**(Suppl. 2): 159–170. doi:10.1023/A:1007700716866. PMID:9794090.
- Sapronov, N.S., Eliseev, V.V., and Rodionova, O.M. 2000. Effect of uridine derivatives on myocardial stunning during postischemic reperfusion of rat heart. *Bull. Exp. Biol. Med.* **130**(10): 964–966. doi:10.1007/BF02682039. PMID:11177294.
- Saris, N.E., Mervaala, E., Karppanen, H., Khawaja, J.A., and Lewenstam, A. 2000. Magnesium. An update on physiological, clinical and analytical aspects. *Clin. Chim. Acta*, **294**(1–2): 1–26. doi:10.1016/S0009-8981(99)00258-2. PMID:10727669.
- Simonson, E., and Berman, R. 1973. New approach in treatment of cardiac decompensation in U.S.S.R. *Am. Heart J.* **86**(1): 117–123. doi:10.1016/0002-8703(73)90017-3. PMID:4268303.
- Tsang, A., Hausenloy, D.J., Mocanu, M.M., and Yellon, D.M. 2004. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ. Res.* **95**(3): 230–232. doi:10.1161/01.RES.0000138303.76488.fe. PMID:15242972.
- van Vuuren, D., Genis, A., Genade, S., and Lochner, A. 2008. Postconditioning the isolated working rat heart. *Cardiovasc. Drugs Ther.* **22**(5): 391–397. doi:10.1007/s10557-008-6119-6. PMID:18563544.
- Vinten-Johansen, J., Granfeldt, A., Mykytenko, J., Undyala, V.V., Dong, Y., and Przyklenk, K. 2011. The multidimensional physiological responses to postconditioning. *Antioxid. Redox Signal.* **14**(5): 791–810. doi:10.1089/ars.2010.3396. PMID:20618066.
- Xi, L., Hess, M.L., and Kukreja, R.C. 1998. Ischemic preconditioning in isolated perfused mouse heart: reduction in infarct size without improvement of post-ischemic ventricular function. *Mol. Cell. Biochem.* **186**(1–2): 69–77. doi:10.1023/A:1006811128561. PMID:9774187.
- Yellon, D.M., and Hausenloy, D.J. 2007. Myocardial reperfusion injury. *N. Engl. J. Med.* **357**(11), 1121–1135. doi:10.1056/NEJMra071667. PMID:17855673.