Effects of Iron Nanoparticles Administration on Ischemia/Reperfusion Injury in Isolated Hearts of Male Wistar Rats

Lucia KINDERNAY[1]*, Kristina FERENCZYOVÁ[1]*, Veronika FARKAŠOVA[1], Monika BARTEKOVÁ[1], Iveta BERNÁTOVÁ[2], Táňa RAVINGEROVÁ[1]

** These authors contributed equally to this work.*

[1]Centre of Experimental Medicine, Slovak Academy of Sciences, Institute for Heart Research, Bratislava, Slovak Republic, [2]Centre of Experimental Medicine, Slovak Academy of Sciences, Institute of Normal and Pathological Physiology, Bratislava, Slovak Republic

Received November 11, 2022
Accepted April 14, 2023

Summary

Iron is an essential mineral participating in numerous biological processes in the organism under physiological conditions. However, it may be also involved in the pathological mechanisms activated in various cardiovascular diseases including myocardial ischemia/reperfusion (I/R) injury, due to its involvement in reactive oxygen species (ROS) production. Furthermore, iron has been reported to participate in the mechanisms of iron-dependent cell death defined as "ferroptosis". On the other hand, iron may be also involved in the adaptive processes of ischemic preconditioning (IPC). This study aimed to elucidate whether small amounts of iron may modify the cardiac response to I/R in isolated perfused rat hearts and their protection by IPC. Pretreatment of the hearts with iron nanoparticles 15 min prior to sustained ischemia (iron preconditioning, Fe-PC) did not attenuate post-I/R contractile dysfunction. Recovery of left ventricular developed pressure (LVDP) was significantly improved only in the group with combined pretreatment with iron and IPC. Similarly, the rates of contraction and relaxation [+/-$(dP/dt)_{max}$] were almost completely restored in the group preconditioned with a combination of iron and IPC but not with iron alone. In addition, the severity of reperfusion arrhythmias was reduced only in the iron+IPC group. No changes in protein levels of "survival" kinases of the RISK pathway (Reperfusion Injury Salvage Kinase) were found except for reduced caspase 3 levels in both preconditioned groups. The results indicate that a failure to precondition rat hearts with iron may be associated with the absent upregulation of RISK proteins and the pro-ferroptotic effect manifested by reduced glutathione peroxidase 4 (GPX4) levels. However, combination with IPC suppressed the negative effects of iron resulting in cardioprotection.

Key words

Iron nanoparticles • Ischemia/reperfusion injury • Preconditioning • Protective cell signaling

Corresponding author

T. Ravingerová, Institute for Heart Research, Centre of Experimental Medicine Slovak Academy of Sciences, Dúbravská cesta 9, 84104 Bratislava, Slovak republic. E-mail: usrdravi@savba.sk

Introduction

Iron as an important component in all living organisms is intimately involved in a wide range of biological processes, such as oxygen transport, DNA synthesis, cellular respiration, electron transfer, and others [1-3]. Moreover, recently developed preparation of metal nanoparticles (NPs), including superparamagnetic iron oxide NPs, have shown their potential in various biomedical applications, from magnetic resonance imaging (MRI)/positron emission tomography (PET) contrast agents used for the identification of tumor metastases, detection of bacteria/viruses, or drug delivery systems and the usage of magnetic hyperthermia in cancer treatment [4,5]. NPs are also widely used in the investigation of their effects on cardiovascular function and various forms of stress [6,7]. To reduce their cytotoxicity, different coating, e.g. polyethylene glycol (PEG) polymer, is used [7,8]. It was found that PEGylated NPs did not alter blood pressure regulation or red blood cells parameters in SHR rats [9].

© 2023 by the authors. Published by the Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic

However, iron also exerts potentially deleterious effects that may be manifested under conditions of ischemia/reperfusion (I/R) injury, myocardial infarction, heart failure, and other pathological conditions, due to its involvement in reactive oxygen species (ROS) production facilitating iron-mediated Fenton chemistry with conversion of the less potent oxidants, superoxide and hydrogen peroxide, to the highly reactive strong oxidant, hydroxyl radical [10]. Both, ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron mediate lipid peroxidation, resulting in the formation of alkoxyl (RO•) and peroxyl (RO_2•) radicals [11]. Moreover, iron has been recently reported to participate in the mechanisms of iron-dependent regulated cell death defined as "ferroptosis", which is a form of cell death that is distinct from apoptosis, necrosis, necroptosis, and other types of cell death [12,13]. Ferroptosis has been documented to underlie lethal injury of the heart due to both acute and chronic I/R, and various types of cardiomyopathies have been shown to involve inhibition of its negative regulator, glutathione peroxidase 4 (GPX4), leading to lipid peroxidation and oxidative stress [14,15]. Thus, ferroptosis plays an important role in molecular mechanisms of cardiac dysfunction [16,17].

On the other hand, some studies have demonstrated that iron may play a dual role in myocardial injury [17,18]. Responses to small/high amounts of iron NPs depend on ROS (RNS) formation, experimental settings, cell type, duration (or number of cycles) of NPs administrations, or tissue-specificity. For an example, 20 μM iron induced cell hypertrophy and survival in cultured rat cardiomyocytes *via* inducible nitric oxide synthase upregulation, while 50-100 μM iron induced necrosis of cardiomyocytes [19]. On the other hand, Galleano *et al.* [20] found that transient oxidative stress induced in the rat liver following sub-chronic iron administration (50 mg Fe/kg i.p. every second day during 10 days) protected liver against I/R injury associated with suppression of the pro-inflammatory and pro-oxidative responses and recovery of NF-κB, which was lost during I/R.

While high levels of iron mobilized following sustained ischemia are detrimental, increasing susceptibility of cardiac tissue to oxidative damage, small, nontoxic amounts of iron produced during short episodes of ischemic preconditioning (IPC) play a signaling role in the mechanisms of IPC [19]. This iron is not involved in cardiac injury, but rather prepares the heart for the forthcoming action of high levels of "free" iron after prolonged ischemia, the phenomenon termed "iron-preconditioning" activating cellular pathways of cardioprotection [20]. Thus, it was proposed that ischemic preconditioning may be induced *via* novel pathway initiated by iron and mediated by ferritin [21].

Cardioprotection afforded by IPC and by other novel forms of "conditioning" is manifested by a delay of necrotic and apoptotic processes in cardiomyocytes [22], reduction of lethal arrhythmias [23], and an improved post-I/R functional recovery [24]. Although molecular mechanisms of "conditioning" are not completely elucidated, it has been revealed that brief episodes of I/R-induced ROS production related to mitochondrial K(ATP) channels opening [25] lead to activation of the cell survival RISK (Reperfusion Injury Salvage Kinase) pathway and upregulation of kinases such as phosphoinositol 3-kinase (PI3K)/protein kinase B (Akt), extracellular signal-regulated kinases 1/2 (ERK1/2), protein kinase Cε (PKCε), increased expression of endothelial nitric oxide synthase (eNOS) [26], and inactivation of glycogen synthase kinase-3β (GSK-3β) [27]. The latter is considered a crucial step leading to the inhibition of mitochondrial permeability transition pore (MPTP) opening during reperfusion followed by disruption of mitochondrial membranes integrity and release of proapoptotic molecules activating cell death mechanisms [28].

Although it has been proposed already decades ago that iron may represent a further risk factor of cardiovascular diseases [29], this issue is still not completely resolved. Moreover, it has been reported that iron in relationship with ferritin accumulation might be also involved in the mechanisms of IPC [18,21]. We have hypothesized that small amount of iron applied prior to ischemic insult in the isolated rat heart could induce preconditioning-like protection in the heart against sustained I/R injury and exaggerate resistance of the heart to ischemia. The present study was designed to unravel: i), whether short-term administration of iron nano-particles in the isolated Langendorff-perfused rat hearts given alone or in combination with IPC may exert either deleterious effects in the heart after prolonged ischemia or to confer protection against sustained I/R; ii), to explore potential molecular mechanisms of such cardioprotection through examination of the activity and levels of pro-survival proteins under post-ischemic conditions.

Materials and Methods

Animals

Adult male Wistar rats were employed. Animals (250-500 g body weight) were fed a standard pellet diet and had access to drinking water *ad libitum*. All experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH) (Guide, NRC 2011) and approved by the Animal Health and Welfare Division of the State Veterinary and Food Administration of the Slovak Republic and Ethics Committee of the CEM SAS (protocol code 3754/ 18-221/3, 11.1.2019).

Materials and chemicals

Iron oxide (II, III) magnetic nanoparticles [30 nm avg. part. size (TEM)] were purchased from Sigma-Aldrich (cat. No 747408-10ML; St. Louis, MO, USA). A specific anti-Akt kinase (Santa Cruz Biotechnology, sc-81434), anti-phospho-Akt kinase (Ser473, Cell Signaling Technology, #4058), anti-eNOS (Abcam, ab5589), anti-phospho-eNOS (phospho S1177, Abcam, ab184154), anti-GSK-3β (Santa Cruz Biotechnology, sc-7291), anti-pGSK-3β (Ser 9, Santa Cruz Biotechnology, sc-11757), anti-PKCε (Santa Cruz Biotechnology, sc-1681), anti-Bcl-2 (Santa Cruz Biotechnology, sc-783), anti-Bax (Santa Cruz Biotechnology, sc-7480), anti-caspase-3 (Cell Signaling Technology, #9664), anti-cleaved-caspase 3 (Cell Signaling Technology, #14220), anti-GPX4 (Abcam, ab125066) and GAPDH (anti-GAPDH antibody, Santa Cruz Biotechnology, sc-32233) antibodies were used for the primary immunodetection. Peroxidase-labeled anti-rabbit (Cell Signaling Technology 7070S) and anti-mouse (Cell Signaling Technology 7076S) immunoglobulin were used as the secondary antibody.

Perfusion technique

Isolated heart procedures were performed as described before [30]. Rats were anesthetized with thiopental (50-60 mg/kg) administered intraperitoneally together with heparin (500 IU). The hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated *via* the aorta, and perfused in the Langendorff mode at a constant perfusion pressure of 73 mm Hg and constant temperature of 37 °C. The perfusion solution was a modified Krebs-Henseleit buffer gassed with O_2 and CO_2 (pH 7.4) containing (in mM): glucose 11.0; $CaCl_2$ 1.6; NaCl 118.0; $NaHCO_3$ 25.0; $MgSO_4$ 1.18; KH_2PO_4 1.28; KCl 3.0.

An epicardial electrogram (EG) was registered using two stainless steel electrodes attached to the apex of the heart and the aortic cannula and continuously recorded. Heart rate was calculated from the EG. Left ventricular (LV) pressure was measured by means of a non-elastic water-filled balloon inserted into the left ventricle via the left atrium and connected to a pressure transducer (MLP844, ADInstruments, Germany). LV systolic pressure (LVSP), LV diastolic pressure (LVEDP), LV developed pressure (LVDP, systolic minus diastolic pressure), maximal rates of pressure development $[+(dP/dt)_{max}]$ and fall $[-(dP/dt)_{max}]$ as the indexes of contraction and relaxation, as well as the heart rate (HR) and coronary flow (CF), were used to assess cardiac function that was analyzed using PowerLab/8SP Chart 7 software (ADInstruments, Germany).

Experimental protocols

The hearts of all experimental groups (n=7-9 hearts per group) were randomly assigned to the following protocols (Fig. 1). In the protocol of control ischemia and reperfusion (A; C-I/R), hearts underwent 15-20 min of stabilization period followed by 30 min of global ischemia and 40 min of reperfusion. The protocol of iron preconditioning (B; Fe-PC) consisted of a 20-min stabilization, 15 min of infusion by Krebs-Henseleit solution enriched with iron nanoparticles, and 30 min of global ischemia followed by 40 min reperfusion. The protocol of iron preconditioning combined with classical ischemic preconditioning (C; Fe+IPC) consisted of 20-min stabilization period followed by 5 min of iron nanoparticles infusion, 5 min of global ischemia and 5 min of short reperfusion with iron nanoparticles, then 30 min of global ischemia and 40 min of reperfusion. Iron nanoparticles were dissolved in Krebs-Henseleit solution (from stock solution with 1 mg/ml iron nanoparticles in water) to

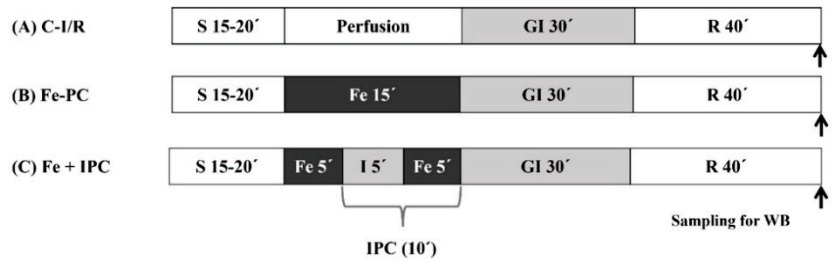

Fig. 1. Experimental protocols. C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC – preconditioned with iron and ischemic PC group. S – stabilization. GI – global ischemia. R – reperfusion. Fe – iron infusion.

a final concentration of 13.3 μg/ml and administered by infusion pump (AL-1000, World Precision Instruments, 941-371-1003, Sarasota Florida) at a rate of 0.1 ml/min.

We have chosen the dosage considered as small (1 mg Fe/kg b.w., i.v.), in accordance with the experiments of Líšková *et al.* [8], Oleksa *et al.* [7], and Laubertová *et al.* [9] in rats *in vivo* testing cardiovascular function regulation under normal conditions and after exposure to different forms of stress [7-9]. Detailed preparation, dosage and properties of NPs (including their superparamagnetic properties) are described in details in those studies. In our study, the mode of administration was adjusted to our experimental conditions (isolated perfused rat heart).

Induction of ischemia/reperfusion

Global ischemia was induced by clamping of aortic inflow for 30 min followed by its unclamping for the evaluation of postischemic recovery of contractile function after 40-min reperfusion [LVDP and $+/-(dP/dt)_{max}$] expressed in percentage of pre-ischemic values.

Quantification of arrhythmias

Susceptibility to reperfusion-induced malignant ventricular tachyarrhythmias was evaluated during 10-min reperfusion from the electrogram recording based on Lambeth´s Conventions [31], the Guidelines for the investigation and classification of different forms of tachyarrhythmias induced by ischemia, infarction, and reperfusion. Quantification of arrhythmias was performed using arrhythmia score (AS) ranging from 0 points given to the hearts with no arrhythmias up to 5 points given to the hearts with sustained ventricular fibrillation (0 points = basic sinus rhythm without arrhythmias; 1 point = rare extrasystoles (ES); 2 points = bigeminy (BG)/salvos; 3 points = ventricular tachycardia (VT), no time difference; 4 points = ventricular fibrillation (VF) with duration <120 s; 5 points = sustained ventricular fibrillation with duration >120 s) [32].

Preparation of tissue protein fractions

The tissue samples used for Western blot analysis were obtained from the left ventricles of hearts from all experimental groups after 40 min of reperfusion. The tissues were wiped in liquid nitrogen, resuspended in ice-cold buffer A containing (in mmol/l): 20 Tris-HCl, 250 sucrose, 1.0 EGTA, 1.0 dithiothreitol (DTT), 1.0 phenylmethylsulphonylfluoride (PMSF), and 0.5 sodium orthovanadate (pH 7 4), and homogenized with a Teflon homogenizer. The homogenates were centrifuged at 800× g for 5 min at 4 °C, and the resulting supernatants were centrifuged again at 16100× g for 30 min. The supernatants from the second centrifugation, termed cytosolic fraction, were used for further analysis. Protein concentration was estimated by the method of Bradford [33].

Electrophoresis and Western blot analysis

Samples of the protein fractions containing equivalent amounts of proteins per lane (54.3 μg) were separated by 10 % or 12 % SDS-PAGE gel electrophoresis (according to the molecular weight of specific protein) (n=3-4 per group). For Western blot assays, proteins were transferred to a nitrocellulose membrane. A specific anti-Akt kinase (Santa Cruz Biotechnology, sc-81434), anti-phospho-Akt kinase (Ser473, Cell Signaling Technology, #4058), anti-eNOS (Abcam, ab5589), anti-phospho-eNOS (phospho S1177, Abcam, ab184154), anti-GSK3β (Santa Cruz Biotechnology, sc-7291), anti-pGSK3β (Ser 9, Santa Cruz Biotechnology, sc-11757), anti-PKCε (Santa Cruz Biotechnology, sc-1681), anti-Bcl-2 (Santa Cruz Biotechnology, sc-783), anti-Bax (Santa Cruz Biotechnology, sc-7480), anti-caspase-3 (Cell Signaling Technology, #9664), anti-cleaved-caspase 3 (Cell Signaling Technology, #14220) and anti-GPX4 (Abcam, ab125066) antibodies were used for the primary immunodetection. Peroxidase-labeled anti-rabbit (Cell Signaling Technology 7074S) and anti-mouse (Cell Signaling Technology 7076S) immunoglobulin were used as the secondary antibody. Bound antibodies were detected using the enhanced chemiluminescence (ECL) method. The optical density of individual bands was analyzed with PCBAS 2.08e software and normalized to GAPDH (anti-GAPDH antibody, Santa Cruz Biotechnology, sc-32233) as the internal control.

Statistical evaluation

The data were expressed as means ± S.E.M. One-way ANOVA and subsequent Tuckey test (results from Western Blots) or Newman Keuls test, as well as Mann-Whitney U test using GraphPad Prism version 6.00 (GraphPad 9 Software, San Diego, USA) for Windows (Microsoft Corporation, USA), were used where appropriate. Differences were considered as significant at $p<0.05$.

Results

Characteristics of isolated hearts: Effect of iron nanoparticles on functional parameters of the isolated hearts

The application of iron nanoparticles had no effect on the baseline functional parameters. The values of post-stabilization and baseline pre-ischemic hemodynamic parameters in all groups are summarized in Table 1. No significant differences in the values of these parameters among the groups after stabilization and at the baseline after 15 min administration of nanoparticles prior to ischemia were observed. However, the values of $+(dP/dt)_{max}$ and $-(dP/dt)_{max}$ were significantly lower in the Fe+IPC group after 15-min perfusion with iron than in the control iron-free group and in the Fe-PC group.

Table 1. Values of hemodynamic parameters of isolated rat hearts.

Groups	**HR (beats/min)**	**LVSP (mm Hg)**	**LVEDP (mm Hg)**	**LVDP (mm Hg)**	**$+(dP/dt)_{max}$ (mm Hg/s)**	**$-(dP/dt)_{max}$ (mm Hg/s)**
After stabilization						
C-I/R	268 ± 6.5	91.8 ± 4.2	4.7 ± 0.3	87.2 ± 4.1	2366 ± 95	1565 ± 81
Fe-PC	262 ± 13.6	80.6 ± 4.0	4.7 ± 1.2	75.8 ± 5.1	1619 ± 115	1232 ± 128
Fe+IPC	258 ± 8.8	79.5 ± 3.7	3.9 ± 0.8	75.6 ± 4.2	1569 ± 88	1230 ± 84
Prior to ischemia						
C-I/R		85.4 ± 7.1	3.1 ± 1.6	82.3 ± 6.7	1758 ± 177	1426 ± 150
Fe-PC		81.9 ± 4.9	4.5 ± 1.0	76.7 ± 4.7	1576 ± 117	1232 ± 116
Fe+IPC		73.8 ± 5.0	7.0 ± 5.3	66.9 ± 4.6	1283 ± 83*	863 ± 62*

C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC – preconditioned with iron and ischemic PC group. LVSP – left ventricular systolic pressure, LVEDP – left ventricular end-diastolic pressure, LVDP – left ventricular developed pressure (LV systolic minus LV diastolic pressure), $+(dP/dt)_{max}$, $-(dP/dt)_{max}$ – maximal rates of pressure development and fall, respectively, HR – heart rate. Data are means ± S.E.M., n=7-9 per group. * $p<0.05$, Fe+IPC vs. C-I/R group.

Effect of iron preconditioning on ischemia/reperfusion injury

Recovery of contractile function in nanoparticles-treated hearts

Recovery of LVDP after I/R did not differ significantly between the control group and that subjected to iron preconditioning prior to sustained ischemia/reperfusion (perfusion with iron-free buffer). On the other hand, in the group where iron pretreatment was combined with IPC, post-ischemic recovery of LVDP was significantly better than in the other groups (Fig. 2).

Similarly, the protocol of preconditioning with iron nanoparticles did not improve post-I/R recovery of either the rate of contraction or the rate of relaxation. On the other hand, a combination of iron nanoparticles with IPC resulted in a significant (nearly 50 %) increase in these parameters as compared with controls and with group preconditioned with iron alone (Fig. 3).

Effect of pretreatment with iron nanoparticles on cardiac susceptibility to reperfusion-induced arrhythmias

No effect of iron preconditioning on the severity of reperfusion-induced arrhythmias was observed. However, AS was significantly lower in the group pretreated with a combination of iron nanoparticles and IPC in comparison with that in the control group and preconditioned with the iron alone group (Fig. 4).

The effect of short-term administration of iron nanoparticles alone or in combination with IPC on the expression of selected RISK pathway proteins in rat myocardium after ischemia and reperfusion

Even though phosphorylated (activated) Akt (p-Akt) levels did not change significantly among the experimental groups, there was a trend of their decrease in both groups with iron nanoparticles pretreatment (Fe-PC; Fe+IPC) compared to the control I/R group (Fig. 5A), without reaching the level of significance. Similarly, the levels of phosphorylated (inactivated) GSK-3β tended to decrease in both groups treated with iron nanoparticles (Fe-PC; Fe+IPC) compared to the control I/R group, however, without reaching the level of significance (Fig. 5B). The PKCε levels did not change significantly among the experimental groups (Fig. 5D). The levels of eNOS tended to decrease in groups treated with iron nanoparticles (Fe-PC; Fe+IPC) compared with the control I/R group, without reaching the level of significance (Fig. 5C).

The effect of short-term administration of iron nanoparticles alone or in combination with IPC on the expression of selected proteins of pro- and anti-apoptotic cascades in rat myocardium after ischemia and reperfusion

Investigation of the expression of pro-apoptotic pro-caspase 3, caspase 3, and the ratio of pro-apoptotic BAX and anti-apoptotic Bcl-2 (BAX/Bcl-2) clearly showed that in Fe-PC and Fe+IPC groups, there was a significant decrease in caspase 3 level compared to that in the control I/R group (Fig. 6C). Levels of pro-caspase 3 and BAX/Bcl-2 ratio did not change significantly among the experimental groups (Fig. 6B and 6A).

The effect of short-term administration of iron nanoparticle alone or in combination with IPC on the expression of GPX4 in rat myocardium after ischemia and reperfusion

Levels of GPX4 decreased significantly in the iron preconditioned group (Fe-PC) as compared to the control group (Fig. 7) indicating a possibility of increased development of ferroptosis. On the other hand, GPX4 levels in the Fe+IPC group did not differ from those in the control non-preconditioned group. The latter might indicate the suppression of pro-ferroptotic effect of iron by IPC.

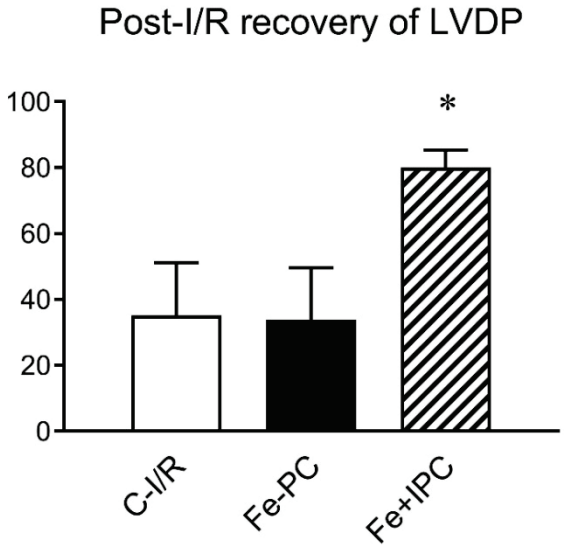

Fig. 2. Recovery of post-ischemic heart function in iron particles-pretreated hearts. C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC– preconditioned with iron and ischemic PC group. LVDP – left ventricular developed pressure (LV systolic minus LV diastolic pressure), Data are means ± S.E.M., n=7-9 per group. * p<0.05, vs. C-I/R and preconditioned groups.

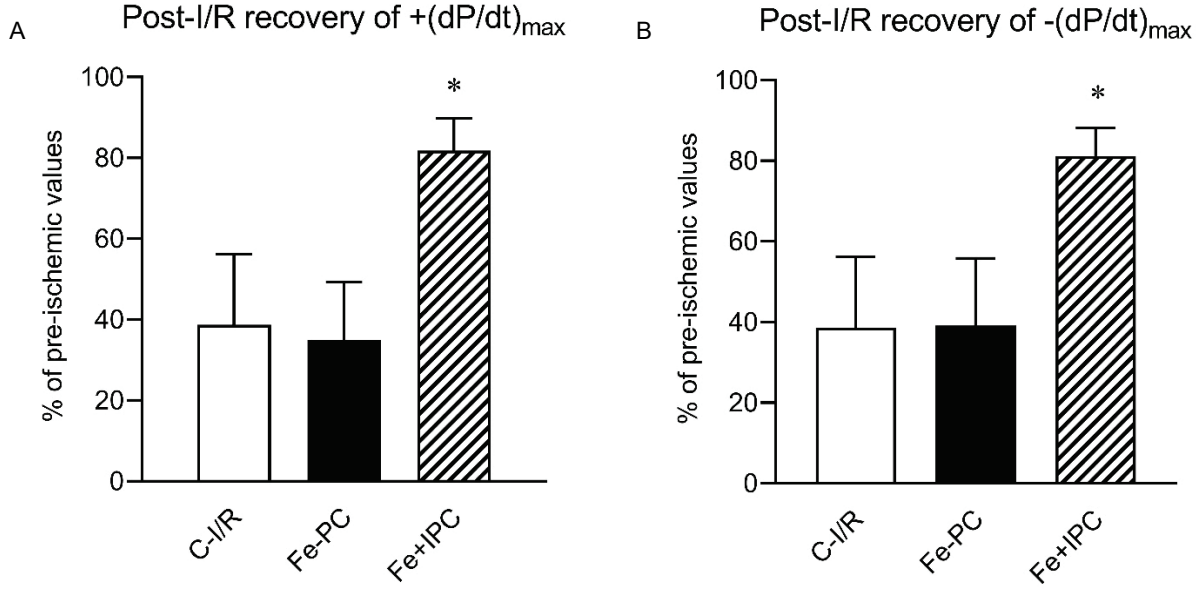

Fig. 3. Recovery of post-ischemic heart function in iron particles-preconditioned hearts. C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC – preconditioned with iron and ischemic PC group. Postischemic recovery of +(dP/dt)max (**A**) and -(dP/dt)max (**B**) – maximal rates of LV pressure development and fall as the indexes of contraction and relaxation. Data are means ± S.E.M., n=7-9 per group.* p<0.05, vs. C-I/R and Fe-PC group.

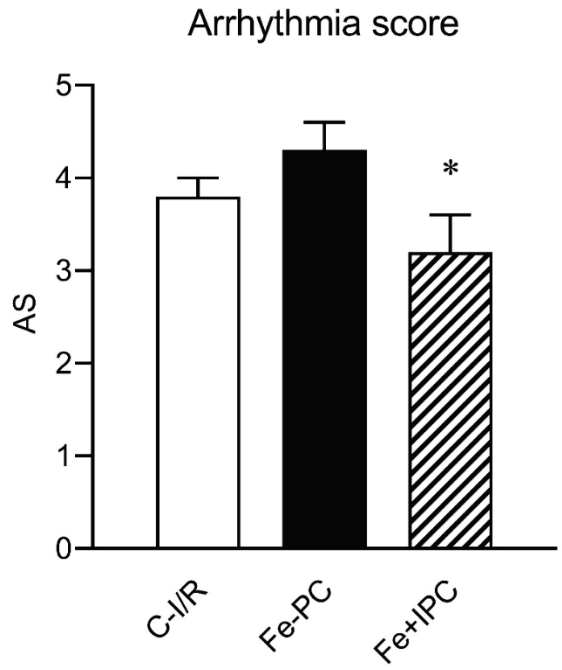

Fig. 4. Severity of reperfusion-induced arrhythmias evaluated by means of arrhythmia score (AS). C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC – preconditioned with iron and ischemic PC group. Data are means ± S.E.M., n=7-9 per group. * p<0.05, vs. C-I/R and Fe-PC group.

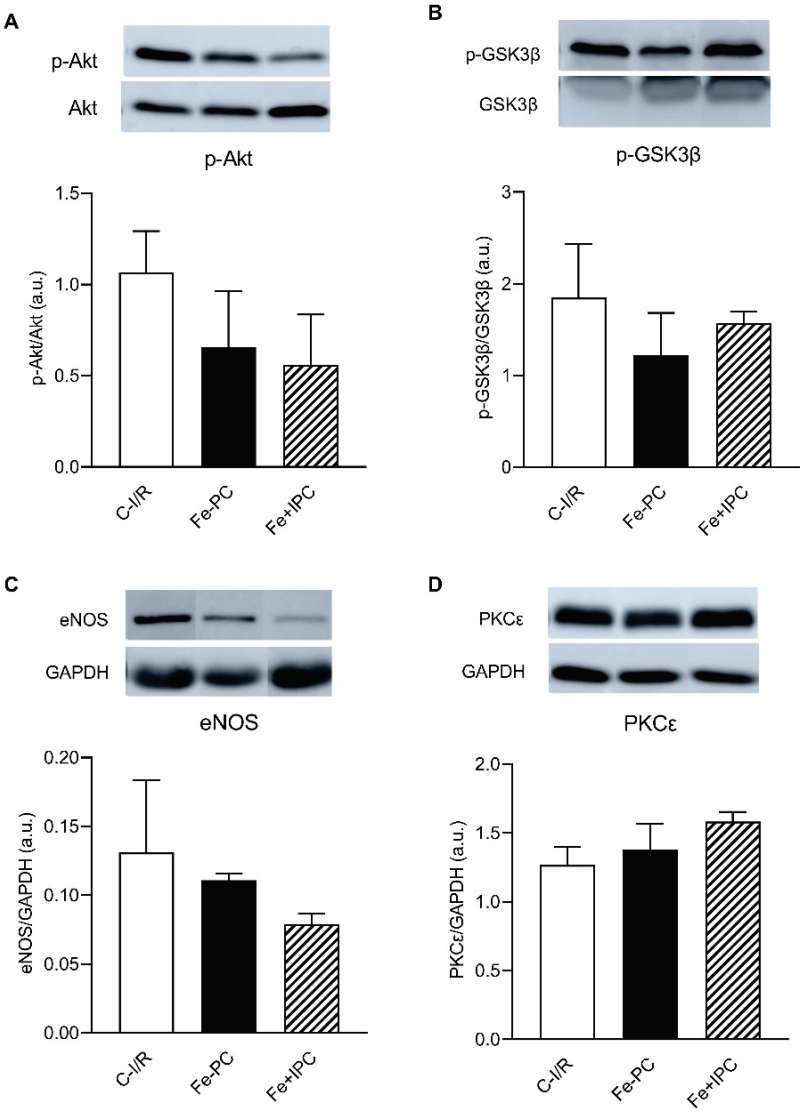

Fig. 5. Expression of selected RISK pathway proteins in rat myocardium after ischemia and reperfusion. C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC – preconditioned with iron and ischemic PC group. (**A**) p-Akt/Akt – phosphorylated/total protein kinase B normalized to GAPDH. (**B**) p-GSK3β/GSK3β – phosphorylated/total glycogen synthase kinase 3 beta normalized to GAPDH. (**C**) eNOS – endothelial nitric oxide synthase normalized to GAPDH. (**D**) PKCε – proteinkinase C epsilon normalized to GAPDH. GAPDH – glyceraldehyde-3-phosphate dehydrogenase. Data are means ± S.E.M., n=3-4 per group.

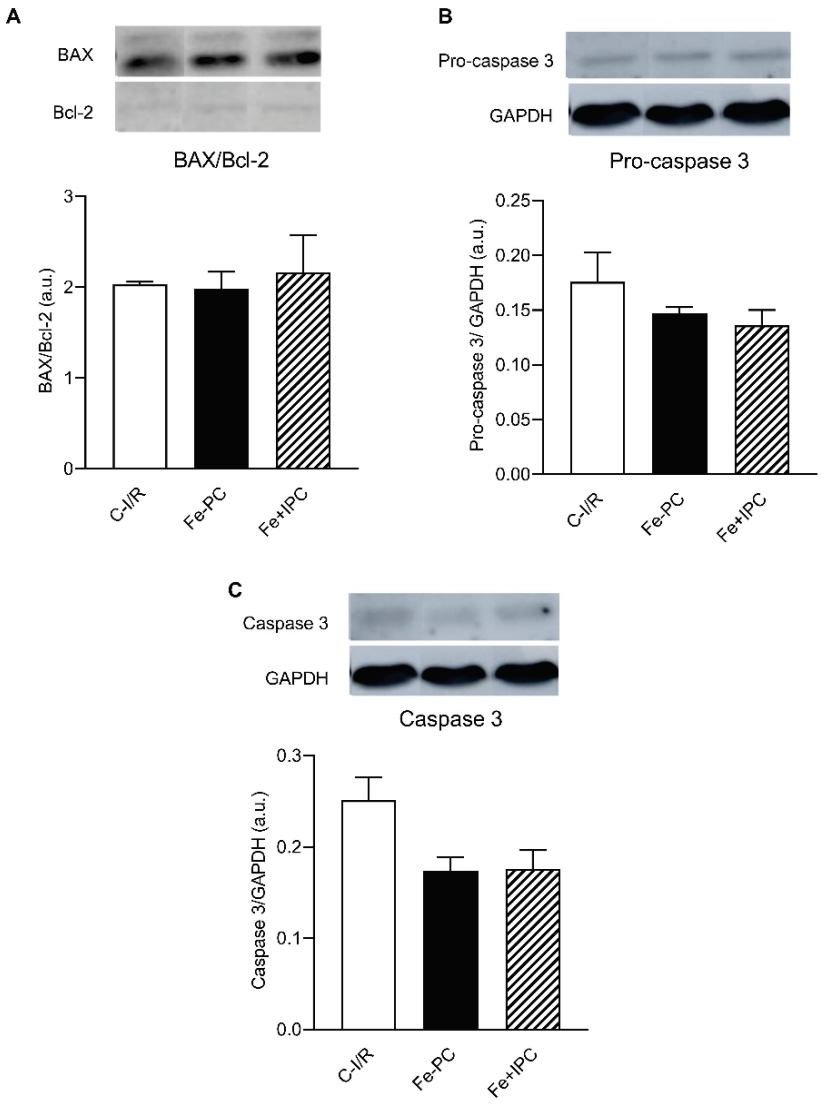

Fig. 6. Expression of selected proteins of pro- and anti-apoptotic cascades in rat myocardium after ischemia and reperfusion. C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC – preconditioned with iron and ischemic PC group. (**A**) BAX – Bcl-2-associated X protein/Bcl-2 – B-cell lymphoma 2 protein normalized to GAPDH. (**B**) Pro-caspase 3 normalized to GAPDH. (**C**) Caspase 3 normalized to GAPDH. GAPDH – glyceraldehyde-3-phosphate dehydrogenase. Data are means ± S.E.M., n=3-4 per group. * $p<0.05$, vs. C-I/R group.

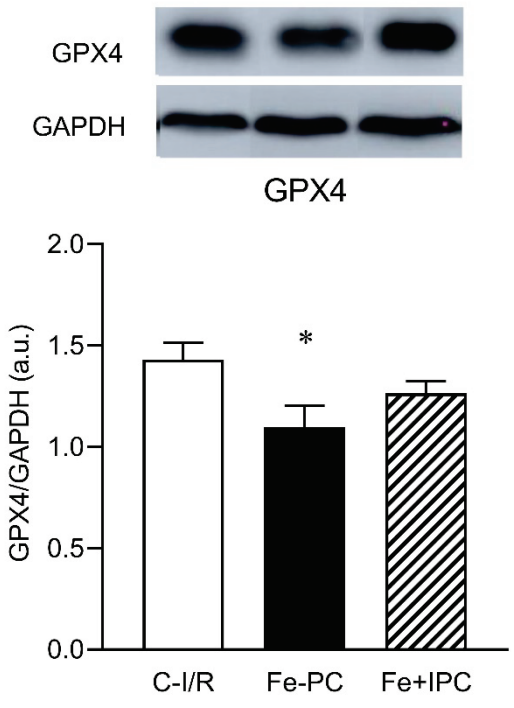

Fig. 7. Expression of GPX4 in rat myocardium after ischemia and reperfusion. C-I/R – control iron-free group. Fe-PC – preconditioned with iron group. Fe+IPC – combined preconditioning with iron and ischemic PC group. GPX4 – glutathione peroxidase 4. GAPDH – glyceraldehyde-3-phosphate dehydrogenase. Data are means ± S.E.M., n=3-4 per group. * $p<0.01$, vs. C-I/R group.

Discussion

Increased iron has been implicated in the pathology of I/R injury in a variety of organs including the heart, and mobilization of myocardial iron caused by ischemia may contribute to the oxidative stress and loss of cardiac function associated with the "reperfusion injury" [34]. Moreover, under conditions of global I/R, small doses of iron (3.0-12.0 mg/ml, i.p.) impaired post-I/R rat heart function and increased free radicals production suggesting that even mild, non-overloading doses of iron can be detrimental to the hearts exposed to I/R stress [35] that might explain the absence of protective effect of Fe-PC in our study.

Mitochondria play a key role in energy production, however, their role in iron metabolism and in the modulation of cardiac damage during ischemia and reperfusion is less elucidated [36]. Iron deficiency could deteriorate mitochondrial energetics, whereas iron overload could result in mitochondrial damage through ROS production. ROS react with iron in mitochondria and produce extremely deleterious hydroxyl radicals followed by the depolarization of the mitochondrial membrane potential, opening of the mitochondrial permeability transition pore, leading to cell rupture and eventually to cardiac dysfunction and cardiomyopathy [37]. Although the exact mechanisms of mitochondrial injury induced by iron overload have not yet been fully elucidated, it cannot be excluded that mitochodrial dysfunction may explain the failure of Fe-PC to improve post-ischemic cardiac recovery.

In our study, a lower dose of iron nanoparticles was applied before ischemia (Fe-PC), and that did not exert neither negative nor positive effect on heart tolerance to I/R-induced damage, which is in contrast with the results of Galleano *et al.* who demonstrated that low-level iron administration protected the rat liver against I/R injury [20]. However, different from our study, sub-chronic mode of administration was applied, and animals received iron preparation in six doses before I/R, each of them associated with short episodes of ROS production that could possibly magnify cardioprotective effect of iron preconditioning.

On the other hand, we found that iron nanoparticles administered during IPC did not affect its protective action on the recovery of cardiac function after I/R injury (Fig. 2,3) as well as its resistance to reperfusion-induced arrhythmias (Fig. 4) supporting the lack of negative effect of iron in the preconditioned myocardium. However, the RISK pathway known to be activated by IPC [26] was not activated when iron nanoparticles were applied alone or simultaneously with IPC, since some key RISK components were unchanged (pAkt, p-GSK-3β, PKCε) or tended to decrease (eNOS) (Fig. 5). On the other hand, caspase 3 levels were significantly decreased in both, Fe-PC and Fe+IPC groups indicating preserved anti-apoptotic activity even under increased iron conditions (Fig. 6C). This anti-apoptotic activity could be attributed to downstream of Survivor Activating Factor Enhancement (SAFE) cascade, which is also crucial to achieve maximal protection in IPC [38]. Moreover, it has been shown that pharmacological preconditioning can be induced without involving classic prosurvival kinases (pAkt and extracellular signal-regulated kinase), through the activation of the SAFE cascade at reperfusion that involves signal transducer and activator of transcription-3 (STAT-3) [39]. Furthermore, Suleman *et al.* demonstrated that besides pAkt, STAT-3 activation is required to trigger ischemic preconditioning [40].

GPX4 converts polyunsaturated fatty acids' hydroperoxides formed in membrane phospholipids to their corresponding, less harmful, lipid alcohols [15]. The direct inhibition or indirect inactivation of GPX4 [14,41] results in oxidative stress as a component of ferroptosis [15,17]. Iron nanoparticles administered before lethal ischemia (Fe-PC) significantly decreased the levels of GPX4 indicating potential oxidative stress and increased lipid peroxidation (Fig. 7). The latter can explain the lack of cardioprotection in a setting of iron preconditioning in our study. Interestingly, GPX4 levels in the Fe+IPC group did not change compared to those in the control non-preconditioned group (Fig. 7) suggesting prevention of the excess radicals generation.

Limitations of the study

In this study, we explored susceptibility to ischemic injury under conditions of iron preconditioning and preconditioning with iron+IPC. While short-term iron administration alone did not show any protective effects, it could be important to elucidate the effect of IPC alone under the same experimental conditions, to clarify whether the application of iron attenuated its cardioprotective effect or contributed to cardioprotection. On the other hand, in our laboratory, for many years we have been using a standard protocol of IPC, where postischemic recovery of function oscillated between

70±8.5 % and 77±5 % of baseline values (Andelová *et al.* [42], Griecsová *et al*. [43], Lonek *et al.* [44], Kindernay *et al.* [45] and others), which does not differ from the values of functional recovery after Fe+IPC in the present study indicating the absence of additional protection/deterioration afforded by co-administration of NPs. Therefore, we assume that administration of NPs, at least in this setting, did not affect the response of the heart to I/R. However, the application of several small doses of iron might have exerted a positive effect on cardiac resistance against I/R. Moreover, in our study, the role of the SAFE pathway was not explored. Since phosphorylation of RISK kinases typically peaks early in reperfusion, the assessment of RISK proteins at 40 min of reperfusion need not reveal the most relevant changes that may have occurred.

Conclusions

Small amounts of iron applied prior to ischemia neither induced preconditioning protection in the isolated rat heart nor facilitated further deleterious effects of ischemia/reperfusion. The failure of iron to precondition the heart may be associated with the absence of the upregulation of cell survival pathways, such as proteins of RISK cascade, as well as with the reduction of GPX4 levels leading to lipid peroxidation and increased oxidative stress. On the other hand, cardioprotection by IPC was effective despite the presence of iron indicating also that other protective pathways and not only RISK cascade (e.g. cascade SAFE) may be involved in the mechanisms of cardioprotection under described experimental conditions.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This research was funded by grants of The Slovak Research and Development Agency (APVV-19-0540, APVV-20-0242 and APVV-16-0263), Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic (VEGA SR) 2/0104/22, 1/0775/21 and IT Monitoring System (ITMS) 26230120009. The authors are thankful to Mrs. I. Formanková for her excellent technical assistance.

References

1. Hirst J. Mitochondrial complex I. Annu Rev Biochem 2013;82:551-575. https://doi.org/10.1146/annurev-biochem-070511-103700
2. Abbaspour N, Hurrell R, Kelishadi R. Review on iron and its importance for human health. J Res Med Sci 2014;19:164-174.
3. von Haehling S, Ebner N, Evertz R, Ponikowski P, Anker SD. Iron deficiency in heart failure: an overview. JACC Heart Fail 2019;7:36-46. https://doi.org/10.1016/j.jchf.2018.07.015
4. González-Gómez MA, Belderbos S, Yañez-Vilar S, Piñeiro Y, Cleeren F, Bormans G, Deroose CM, Gsell W, Himmelreich U, Rivas J. Development of superparamagnetic nanoparticles coated with polyacrylic acid and aluminum hydroxide as an efficient contrast agent for multimodal imaging. Nanomaterials (Basel) 2019;9:1626. https://doi.org/10.3390/nano9111626
5. Rego GNA, Nucci MP, Mamani JB, Oliveira FA, Marti LC, Filgueiras IS, Ferreira JM, ET AL. Therapeutic efficiency of multiple applications of magnetic hyperthermia technique in glioblastoma using aminosilane coated iron oxide nanoparticles: in vitro and in vivo study. Int J Mol Sci 2020;21:958. https://doi.org/10.3390/ijms21030958
6. Suk JS, Xu Q, Kim N, Hanes J, Ensign LM. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. Adv Drug Deliv Rev 2016;99(Pt A):28-51. https://doi.org/10.1016/j.addr.2015.09.012
7. Oleksa V, Bernátová I, Patsula V, Líšková S, Bališ P, Radošinská J, ET AL. Poly(ethylene glycol)-alendronate-coated magnetite nanoparticles do not alter cardiovascular functions and red blood cells' properties in hypertensive rats. Nanomaterials (Basel) 2021;11:1238. https://doi.org/10.3390/nano11051238
8. Líšková S, Bališ P, Mičurová A, Kluknavský M, Okuliarová M, Puzserová A, Škrátek M, ET AL. Effect of iron oxide nanoparticles on vascular function and nitric oxide production in acute stress-exposed rats. Physiol Res 2020;69:1067-1083. https://doi.org/10.33549/physiolres.934567

9. Laubertova L, Dvorakova M, Balis P, Puzserova A, Zitnanova I, Bernatova I. preliminary findings on the effect of ultrasmall superparamagnetic iron oxide nanoparticles and acute stress on selected markers of oxidative stress in normotensive and hypertensive rats. Antioxidants (Basel) 2022;11:751. https://doi.org/10.3390/antiox11040751
10. Kruszewski M. Labile iron pool: the main determinant of cellular response to oxidative stress. Mutat Res 2003;531:81-92. https://doi.org/10.1016/j.mrfmmm.2003.08.004
11. Valko M, Jomova K, Rhodes CJ, Kuča K, Musílek K. Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. Arch Toxicol 2016;90:1-37. https://doi.org/10.1007/s00204-015-1579-5
12. Li J, Cao F, Yin HL, Huang ZJ, Lin ZT, Mao N, Sun B, Wang G. Ferroptosis: past, present and future. Cell Death Dis 2020;11:88. https://doi.org/10.1038/s41419-020-2298-2
13. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, Morrison B 3rd, Stockwell BR. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 2012;149:1060-1072. https://doi.org/10.1016/j.cell.2012.03.042
14. Xie Y, Hou W, Song X, Yu Y, Huang J, Sun X, Kang R, Tang D. Ferroptosis: process and function. Cell Death Differ 2016;23:369-79. https://doi.org/10.1038/cdd.2015.158
15. Forcina GC, Dixon SJ. GPX4 at the crossroads of lipid homeostasis and ferroptosis. Proteomics 2019;19:e1800311. https://doi.org/10.1002/pmic.201800311
16. Fang X, Wang H, Han D, Xie E, Yang X, Wei J, Gu S, ET AL. Ferroptosis as a target for protection against cardiomyopathy. Proc Natl Acad Sci U S A 2019;116:2672-2680. https://doi.org/10.1073/pnas.1821022116
17. Ravingerová T, Kindernay L, Barteková M, Ferko M, Adameová A, Zohdi V, Bernátová I, Ferenczyová K, Lazou A. The molecular mechanisms of iron metabolism and its role in cardiac dysfunction and cardioprotection. Int J Mol Sci 2020;21:7889. https://doi.org/10.3390/ijms21217889
18. Berenshtein E, Vaisman B, Goldberg-Langerman C, Kitrossky N, Konijn AM, Chevion M. Roles of ferritin and iron in ischemic preconditioning of the heart. Mol Cell Biochem 2002;234-235:283-292. https://doi.org/10.1023/A:1015923202082
19. Munoz JP, Chiong M, García L, Troncoso R, Toro B, Pedrozo Z, Diaz-Elizondo J, Salas D, ET AL. Iron induces protection and necrosis in cultured cardiomyocytes: Role of reactive oxygen species and nitric oxide. Free Radic Biol Med 2010;48:526-534. https://doi.org/10.1016/j.freeradbiomed.2009.11.017
20. Galleano M, Tapia G, Puntarulo S, Varela P, Videla LA, Fernández V. Liver preconditioning induced by iron in a rat model of ischemia/reperfusion. Life Sci 2011;89:221-228. https://doi.org/10.1016/j.lfs.2011.06.005
21. Chevion M, Leibowitz S, Aye NN, Novogrodsky O, Singer A, Avizemer O, Bulvik B, Konijn AM, Berenshtein E. Heart protection by ischemic preconditioning: a novel pathway initiated by iron and mediated by ferritin. J Mol Cell Cardiol 2008;45:839-845. https://doi.org/10.1016/j.yjmcc.2008.08.011
22. Bulluck H, Yellon DM, Hausenloy DJ. Reducing myocardial infarct size: challenges and future opportunities. Heart 2016;102:341-348. https://doi.org/10.1136/heartjnl-2015-307855
23. Végh A, Parratt JR. The role of mitochondrial K(ATP) channels in antiarrhythmic effects of ischaemic preconditioning in dogs. Br J Pharmacol 2002;137:1107-1115. https://doi.org/10.1038/sj.bjp.0704966
24. Cave AC. Preconditioning induced protection against post-ischaemic contractile dysfunction: characteristics and mechanisms. J Mol Cell Cardiol 1995;27:969-979. https://doi.org/10.1016/0022-2828(95)90066-7
25. Matejíková J, Kucharská J, Pintérová M, Pancza D, Ravingerová T. Protection against ischemia-induced ventricular arrhythmias and myocardial dysfunction conferred by preconditioning in the rat heart: involvement of mitochondrial K(ATP) channels and reactive oxygen species. Physiol Res 2009;58:9-19. https://doi.org/10.33549/physiolres.931317
26. Hausenloy DJ, Yellon DM. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. Heart Fail Rev 2007;12:217-234. https://doi.org/10.1007/s10741-007-9026-1
27. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, ET AL. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 2004;113:1535-1549. https://doi.org/10.1172/JCI19906
28. Heusch G. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. Circ Res 2015;116:674-699. https://doi.org/10.1161/CIRCRESAHA.116.305348
29. Bell RM, Mocanu MM, Yellon DM. Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. J Mol Cell Cardiol 2011;50:940-950. https://doi.org/10.1016/j.yjmcc.2011.02.018

30. Watanabe M, Okada T. Langendorff perfusion method as an ex vivo model to evaluate heart function in rats. Methods Mol Biol 2018;1816:107-116. https://doi.org/10.1007/978-1-4939-8597-5_8
31. Walker MJ, Curtis MJ, Hearse DJ, Campbell RW, Janse MJ, Yellon DM, Cobbe SM, ET AL. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. Cardiovasc Res 1988;22:447-455. https://doi.org/10.1093/cvr/22.7.447
32. Curtis MJ, Walker MJ. Quantification of arrhythmias using scoring systems: an examination of seven scores in an in vivo model of regional myocardial ischaemia. Cardiovasc Res 1988;22:656-665. https://doi.org/10.1093/cvr/22.9.656
33. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254. https://doi.org/10.1016/0003-2697(76)90527-3
34. Chevion M, Jiang Y, Har-El R, Berenshtein E, Uretzky G, Kitrossky N. Copper and iron are mobilized following myocardial ischemia: possible predictive criteria for tissue injury. Proc Natl Acad Sci U S A 1993;90:1102-1106. https://doi.org/10.1073/pnas.90.3.1102
35. Kramer JH, Lightfoot FG, Weglicki WB. Cardiac tissue iron: effects on post-ischemic function and free radical production, and its possible role during preconditioning. Cell Mol Biol (Noisy-le-grand) 2000;46:1313-1327.
36. Lesnefsky EJ, Chen Q, Tandler B, Hoppel CL. Mitochondrial dysfunction and myocardial ischemia-reperfusion: implications for novel therapies. Annu Rev Pharmacol Toxicol 2017;57:535-565. https://doi.org/10.1146/annurev-pharmtox-010715-103335
37. Chan S, Lian Q, Chen M-P, Jiang D, Ho JTK, Cheung Y-F, Chan GC-F. Deferiprone inhibits iron overload-induced tissue factor bearing endothelial microparticle generation by inhibition oxidative stress induced mitochondrial injury, and apoptosis. Toxicol Appl Pharmacol 2018;338:148-158. https://doi.org/10.1016/j.taap.2017.11.005
38. Lecour S. Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? J Mol Cell Cardiol 2009;47:32-40. https://doi.org/10.1016/j.yjmcc.2009.03.019
39. Lecour S, Suleman N, Deuchar GA, Somers S, Lacerda L, Huisamen B, Opie LH. Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). Circulation 2005;112:3911-3918. https://doi.org/10.1161/CIRCULATIONAHA.105.581058
40. Suleman N, Somers S, Smith R, Opie LH, Lecour SC. Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning. Cardiovasc Res 2008;79:127-133. https://doi.org/10.1093/cvr/cvn067
41. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, Fulda S, ET AL. Ferroptosis: A regulated cell death nexus linking metabolism, redox biology, and disease. Cell 2017;171:273-285. https://doi.org/10.1016/j.cell.2017.09.021
42. Andelová E, Barteková M, Pancza D, Styk J, Ravingerová T. The role of NO in ischemia/reperfusion injury in isolated rat heart. Gen Physiol Biophys 2005;24:411-426.
43. Griecsová L, Farkašová V, Gáblovský I, Khandelwal VK, Bernátová I, Tatarková Z, Kaplan P, Ravingerová T. Effect of maturation on the resistance of rat hearts against ischemia. Study of potential molecular mechanisms. Physiol Res 2015;64(Suppl 5):S685-S696. https://doi.org/10.33549/physiolres.933222
44. Lonek L, Puhova A, Griecsova-Kindernay L, Patel SP, Zohdi V, Jezova D, Ravingerova T. Voluntary exercise may activate components of pro-survival risk pathway in the rat heart and potentially modify cell proliferation in the myocardium. Physiol Res 2019;68:581-588. https://doi.org/10.33549/physiolres.934182
45. Kindernay L, Farkasova V, Neckar J, Hrdlicka J, Ytrehus K, Ravingerova T. Impact of maturation on myocardial response to ischemia and the effectiveness of remote preconditioning in male rats. Int J Mol Sci 2021;22:11009. https://doi.org/10.3390/ijms222011009