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NEW IRON CHELATORS AND ANTIOXIDANTS IN A MODEL OF ACUTE MYOCARDIAL INFARCTION AND OXIDATIVE STRESS INDUCED BY CATECHOLAMINES – INFLUENCE ON BASIC BIOCHEMICAL PARAMETERS

Nové chelátory železa a antioxidancia u modelu akutního infarktu myokardu a oxidačního stresu navozeného katecholaminy - vliv na základní biochemické parametry

DOCTORAL THESIS

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I. INTRODUCTION 5

II. THEORETICAL PART 6

1. Acute myocardial infarction (AMI) 6

1.1 Intracellular changes during ischaemia 7

1.2 Changes during reperfusion 9

2. Catecholamines and AMI 11

2.1 Myocardial hypoxia 12

2.2 ROS and catecholamines 13

2.3 Calcium overload 15

3. Laboratory diagnostic findings of AMI and comparison to experimental models 17

3.1 ECG changes 17

3.2 Biochemical markers 18

4 Studied compounds 19

4.1 Iron chelators 19

4.1.1 Deferoxamine (DFO) 21

4.1.2 Aroylhydrazones 24

4.1.3 Lactoferrins 27

4.2 Flavonoids 28

4.2.1 Rutin 29

5 Abbreviations 32

6. References 33

III. ARTICLES RELATED TO DOCTORAL THESIS 39

1. **The fate of iron in the organism and its regulatory pathways 40**
2. **The role of reactive oxygen and nitrogen species in cellular iron metabolism 68**
3. **The effects of lactoferrin in a rat model of catecholamine cardiotoxicity...91**
4. **The Novel Iron Chelator, PCTH, Reduces Catecholamine-Mediated Myocardial Toxicity 111**
5. **Direct administration of rutin does not protect against catecholamine cardiotoxicity 148**

IV. SUMMARY..175

V. SOUHRN...179

VI. LIST OF PUBLISHED WORKS......................................183

I. INTRODUCTION

Coronary heart disease (CHD) is the most common cause of morbidity and mortality in the developed countries. Extension of present knowledge concerning the pathogenesis of CHD as well as development of new approaches and drugs for its treatment are therefore highly desired.

The most serious form of CHD represents the acute myocardial infarction (AMI). Experimental administration of catecholamines may evoke a pathological state in many aspects similar to AMI in man. The mechanisms of catecholamine cardiotoxicity have not yet been fully understood but includes overstimulation of the heart leading to ischaemia and redox-cycling of catecholamines. Both processes are associated with reactive oxygen species (ROS) production catalyzed by free redox-active iron which is released during and after ischaemia.

Iron chelators may therefore act as protective drugs in catecholamine cardiotoxicity. Novel iron chelator from aroylhydrazone group PCTH, endogenous lactoferrin and flavonoid rutin with iron chelating properties were tested in this study and their effects were compared to those of clinically used iron chelator deferoxamine.

II. THEORETICAL PART

1. Acute myocardial infarction (AMI)

Heart diseases, especially coronary heart disease (CHD), are the most common cause of morbidity and mortality in the developed countries. Mortality around 20% associated with coronary heart disease remains important medicinal problem despite some reduction in recent years (Coady *et al.*, 2001; Goldberg *et al.*, 2006).

The most serious syndrome of CHD represents the acute myocardial infarction (AMI). The prominent feature of AMI is acute localized myocytes necrosis which develops as a consequence of insufficient myocardial blood supply. 95% of cases of AMI are associated with atherosclerosis of a coronary artery with the following thrombosis (Widimský and Špaček, 2004).

The therapy of AMI includes immediate symptomatic care

1. pain relief by application of opiates
2. nitrates
3. acetylsalicylic acid
4. oxygen inhalation
5. eventually in specific cases other drugs, e.g. β-blockers, antiarrhythmic drugs

and therapeutic approaches intended for reperfusion of infarcted area

- thrombolytic therapy by use of fibrinolytic drugs

6. interventional cardiologic approaches (percutaneous coronary intervention or bypass surgery) (Widimský and Špaček, 2004)

Traditionally, ischaemia following coronary artery occlusion was considered to be the main cause of heart injury in AMI. Nowadays, there is evidence that restoration of coronary blood flow (reperfusion) either spontaneous or therapeutic by above referenced medical approaches represents an additional risk for further myocardial tissue impairment. Because whole phenomenon cannot be separated in clinical conditions, a term ischaemia-reperfusion injury (I-R) is generally used.

The diagnosis of AMI is based mainly on non-invasive procedures and biochemical parameters in the blood (WHO, 1979). Intracellular derangement has been described only in post-mortem human tissue or in animal models due to ethical principles. The most relevant animal model of AMI has been considered the occlusional model imitating clinical AMI by ligation of a coronary artery and outcomes from such studies are summarized in the following chapters:

1.1 Intracellular changes during ischaemia

Ischaemia converts cellular aerobic metabolism in anaerobic glycolysis. This is associated with lactate production and marked drop in pH with intracellular acidosis (Ambrosio *et al.*, 1987; Ravingerova *et al.*, 2001). Additionally, free fatty acids released by peroxidation of plasmalemmal phospholipids may contribute to acidosis after prolonged ischaemia (Ohmi *et al.*, 1992). Metabolic derangement during ischaemia leads further to a significant drop in high-energy phosphate compounds (creatine phosphate-CP and ATP) and glycogen and to an increase in NADH (Ambrosio *et al.*, 1987; Lesnefsky *et al.*, 1991; Ohmi *et al.*, 1992; Ravingerova *et al.*,

2001; Varadarajan *et al.*, 2001). The decrease in cellular energetic sources changes cellular homeostasis which is maintained under physiological condition mainly by energy-dependent transport ways through plasmalemmal membrane.

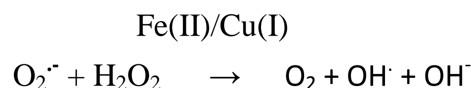
Xanthine dehydrogenase is an enzyme involved in normal purine metabolisms, precisely, it represents the terminal metabolic pathway for adenine and guanosine nucleotides. However, a short period of hypoxia, especially in heart tissue, stimulates its conversion into xanthine oxidase (XO) and, moreover, activates its enzyme transcription (McCord *et al.*, 1985; Terada *et al.*, 1997). In such form, XO in presence of substrates (xanthine and hypoxanthine) and molecular oxygen readily generates reactive oxygen species (ROS) (Chambers *et al.*, 1985). In addition, recently it has been discovered that XO may use accumulated NADH as a substrate instead of oxygen for generation of ROS (Berry and Hare, 2004).

Low weight molecular iron (LWMFe) is usually negligible under physiological condition, but ischaemia augments LWMFe proportionally to ischaemic duration (Holt *et al.*, 1986; Voogd *et al.*, 1992). Further, hypoxia reduces the activity of antioxidant enzymes (SOD or MnSOD and CuZnSOD, GPx) in the heart tissue. These drops progressively increase proportionally to duration of ischaemia (Guarnieri *et al.*, 1980; Roth *et al.*, 1985; Kirshenbaum and Singal, 1992). Similarly, cellular glutathione content and total cellular SH-content reduces progressively in dependence on duration of ischaemia (Guarnieri *et al.*, 1980; Roth *et al.*, 1985). Malonyldialdehyde, generally measured as thiobarbituric acid substances (TBARS), is commonly used as a biomarker of lipid peroxidation caused by ROS (Ceconi *et al.*, 1992). Increased TBARS production was observed generally only after prolonged ischaemia and may

reflect decreased levels of antioxidant enzymes (Guarnieri *et al.*, 1980; Roth *et al.*, 1985; Kirshenbaum and Singal, 1992).

1.2 Changes during reperfusion

Oxygen enters previously ischaemic tissue and is quickly transformed into superoxide and other forms of ROS. This increase of ROS during first 5 minutes of reperfusion has been proven by many authors (Ambrosio *et al.*, 1987; Bolli *et al.*, 1990; Boucher *et al.*, 1992; Prasad *et al.*, 1992). The role of catalytically active metals, especially iron and copper, cannot be omitted. Iron is markedly released from ischaemic tissue during the first minute after reperfusion, and interestingly, elevated levels of LWMFe remained in the heart tissue even during reperfusion (Boucher *et al.*, 1992; Voogd *et al.*, 1992; Chevion *et al.*, 1993; Coudray *et al.*, 1994; Berenshtein *et al.*, 2002). Similar kinetics concerning copper has been observed although it appears to take place only after more severe ischaemic insult (Chevion *et al.*, 1993; Berenshtein *et al.*, 2002). "Free" iron, as well as copper, can catalyze Haber-Weiss reaction, which can be summarized as follows (Gutteridge *et al.*, 1979; Gunther *et al.*, 1995; Halliwell and Gutteridge, 1999; Berenshtein *et al.*, 2002):



$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe(II)/Cu(I)} O_2 + OH^{\cdot} + OH^{-}$$

Hydroxyl radical generated by this reaction is supposed to be the most potent biological oxidant. Further, electron paramagnetic resonance suggested that released ROS may be most likely alkyl peroxyl radicals ($ROO^{\cdot}$) and indeed, such radicals seem to come out from reaction of hydroxyl radical with membrane lipids or other macromolecules (Ambrosio *et al.*, 1987).

Additionally, the whole process may be amplified, because ROS are documented to release free iron (*see chapter III.2*).

The increase in ROS seems to be facilitated by a decrease in antioxidants evoked during ischaemia. Additionally, reperfusion with burst of ROS appears to further decrease intracellular levels of glutathione and total SH-groups and activity of antioxidant enzymes – SOD and GPx after more severe (longer) ischaemia (Guarnieri *et al.*, 1980; Lesnefsky *et al.*, 1991; Kirshenbaum and Singal, 1992; Gonzalez-Flecha *et al.*, 1993). A decrease in catalase activity, probably due to low physiological levels in heart tissue, seems to be significant only after even more sever ischaemic insult (Kirshenbaum and Singal, 1992; Gonzalez-Flecha *et al.*, 1993). On the other hand, some controversy ensues from the study of Coudray et al. who did not found any statistical decrease in SOD, catalase or GPx levels in rat heart tissue after various period of global or partial ischaemia followed by reperfusion (Coudray *et al.*, 1995).

In general, an increase in ROS is reflected by TBARS which reached higher levels after reperfusion as compared to that of ischaemia in most studies (Guarnieri *et al.*, 1980; Roth *et al.*, 1985; Holt *et al.*, 1986; Omar *et al.*, 1989; Ohmi *et al.*, 1992; Prasad *et al.*, 1992; Fantini and Yoshioka, 1993). In addition, ROS lead to oxidation of various macromolecules, e.g. unsaturated fatty lipid of plasmalemmal membrane. And in fact, marked plasmalemmal damage is documented by an augmentation in the membrane resting potential ("functional marker of cell membrane status") and by a substantial release of proteins during reperfusion (Fantini and Yoshioka, 1993; Chevion *et al.*, 1993). Release of lactate dehydrogenase (LDH), a known non-specific marker of myocardial injury, has been well documented even in ischemia but, in addition to that it is markedly enhanced during reperfusion (Kirshenbaum and Singal, 1992).

An increase in myocardial calcium content has been acknowledged as one of crucial feature of myocardial damage many decades ago (Fleckenstein *et al.*, 1973). An increase in myocardial intracellular content leads otherwise to fortification of myocardial contractile force but longer calcium overflow, called “calcium overload”, overstimulates myofilaments, intensifies oxygen demands and is associated with excessive high-energy phosphate cleavage (Fleckenstein *et al.*, 1973; Sulova *et al.*, 1998). Coronary artery ligation leads to an increase in myocardial calcium level as well and this myocardial calcium overload is much more pronounced during reperfusion (Kirshenbaum and Singal, 1992).

It has to be emphasized that only 15 minutes of ischaemia followed by various period of reperfusion can be considered as a model of AMI. Shorter ischaemia does not lead to biochemical changes and necrosis associated with AMI (Bolli *et al.*, 1990; Lesnefsky *et al.*, 1991).

2. Catecholamines and AMI

It has been accepted for many years that excessive release of endogenous catecholamines (adrenaline and noradrenaline) is associated with Western lifestyle and it appears to be an important trigger of AMI in human (von Kanel *et al.*, 2002; Kloner, 2006). Moreover, high concentration of endogenous catecholamines or exogenous application of catecholamines are cardiotoxic (Rona, 1985; Persoon-Rothert *et al.*, 1989; Tan *et al.*, 2003). More recently, studies revealed that ischaemia is associated with progressive increment in endogenous catecholamines levels which are normalized upon reperfusion (Schomig, 1990; Lameris *et al.*, 2000). In light of these data, it is not very surprising that the catecholamine model seems to be a suitable model of

AMI commonly used by researchers. For such purpose, synthetic catecholamine isoprenaline (ISO) with non-selective betaagonistic activity has been widely employed.

Experimentally, administration of catecholamines in relatively large doses induces in animals a pathological state with many similarities to AMI. The whole process of catecholamine cardiotoxicity has not been yet fully elucidated. It seems to be started by excessive stimulation of beta-adrenergic receptors, which leads to myocardial hypoxia or anoxia and to a drop in energy compounds. Additional pathologic mechanisms, especially the production of ROS by oxidation of catecholamines, play unquestionably the role and are discussed in the following paragraphs.

2.1 Myocardial hypoxia

Pharmacological application of ISO results quickly in tachycardia and drop in arterial blood pressures due to stimulation of β_1- and β_2-adrenoreceptors, respectively (Diaz-Munoz *et al.*, 2006). Large doses produce a decrease in myocardial blood flow while small doses the opposite effect, although such increase is associated with a decrease in functional capillary density, especially in the subendocardium (Vetterlein and Schmidt, 1980; Blasig *et al.*, 1985). Moreover, ISO favours coagulation as demonstrated by shortening partial tromboplastine time, and in fact, formation of microthrombi with apparent obstruction of many small vessels was observed after ISO administration (Blasig *et al.*, 1985; von Kanel *et al.*, 2002; Pinelli *et al.*, 2004). Acetylsalicylic acid markedly reduces ISO damage indicating that increased platelet aggregation is involved in the pro-coagulative state induced by ISO. Additionally, enhanced neutrophil activation is suggested (Sumitra *et al.*, 2001). Altogether, tachycardia and the increase in myocardial contractility lead to elevated oxygen demands and this, along with drop in arterial blood pressure and capillary derangement, leads to dysbalance between oxygen demands and

oxygen supply. In fact, decreased myocardial oxygen tension was observed even after relatively small doses of ISO (Winsor *et al.*, 1975).

Catecholamine administration leads to rapid drop in high-energy phosphate compound (ATP, CP) and glycogen. The recovery depends on a dose of ISO used and is much more faster for glycogen and CP than for total adenine nucleotides and ATP (Tsuboi *et al.*, 1974; Singal *et al.*, 1982; Blasig *et al.*, 1985; Kondo *et al.*, 1987; Chagoya de Sanchez *et al.*, 1997) . The ATP-drop in the myocardium is accompanied by a decrease in blood ATP concentration and elevated levels of AMP in heart tissue, as well as in the blood (Tsuboi *et al.*, 1974; Chagoya de Sanchez *et al.*, 1997). In addition, ISO administration caused markedly elevated levels of uric acid in serum (Rajadurai and Prince, 2007). It has not to be emphasized that decomposition of ATP leads to elevated levels of hypoxantine and xanthine, which are substrates for XO.

2.2 ROS and catecholamines

The ROS generation, measured as TBARS or lipid hydroperoxide, has been well documented after ISO application in tissue as well in plasma and has been associated with drop in vitamin C and GSH levels (Sumitra *et al.*, 2001; Tappia *et al.*, 2001; Diaz-Munoz *et al.*, 2006; Padmanabhan and Prince, 2006). Similarly, adrenaline evoked overproduction of superoxide (Mehta and Li, 2001). XO/xanthine dehydrogenase ratio is increased (Diaz-Munoz *et al.*, 2006) and heart tissue antioxidant enzymes (catalase, GPx and SOD) and vitamin E in serum are reduced after ISO administration (Sumitra *et al.*, 2001; Pinelli *et al.*, 2004).

Catecholamines are metabolised via catechol-o-methyltransferase and monoamine oxidase, but these enzymes are saturable and high concentration of catecholamines caused by any

alteration of metabolism, disruption of their transport or exogenous application leads to their oxidation. Their oxidation may proceed non-enzymatically as autooxidation by participation of transient metals – for details see Fig. 1, or, may be mediated by various enzymes (XO, tyrosinase, leucocytes myeloperoxidase, heart muscle cytochrome c oxidase) (Matthews *et al.*, 1985; McCord *et al.*, 1985; Bindoli *et al.*, 1992; Remiao *et al.*, 2001). Importantly, catecholamine oxidation is facilitated by ROS and, besides, it leads alone to production of ROS (Matthews *et al.*, 1985; Bindoli *et al.*, 1992; Remiao *et al.*, 2001). Catecholamine oxidation products oxidize free thiol groups and this leads to reduction in extracellular and intracellular GSH content as well, further they decrease activity of GPx and glutathione reductase (Bindoli *et al.*, 1992; Remiao *et al.*, 2001; Remiao *et al.*, 2002). Based on the role of ROS and transition metals in their production, antioxidants as well as iron chelator razoxane were able markedly reduce damages caused by ISO in cell cultures, while non selective β-blocker propranolol did not (Persoon-Rothert *et al.*, 1989). Moreover, perfusion of isolated rat heart and mitochondria with adrenochrome evoked changes similar to I-R (Takeo *et al.*, 1981; Tappia *et al.*, 2001).

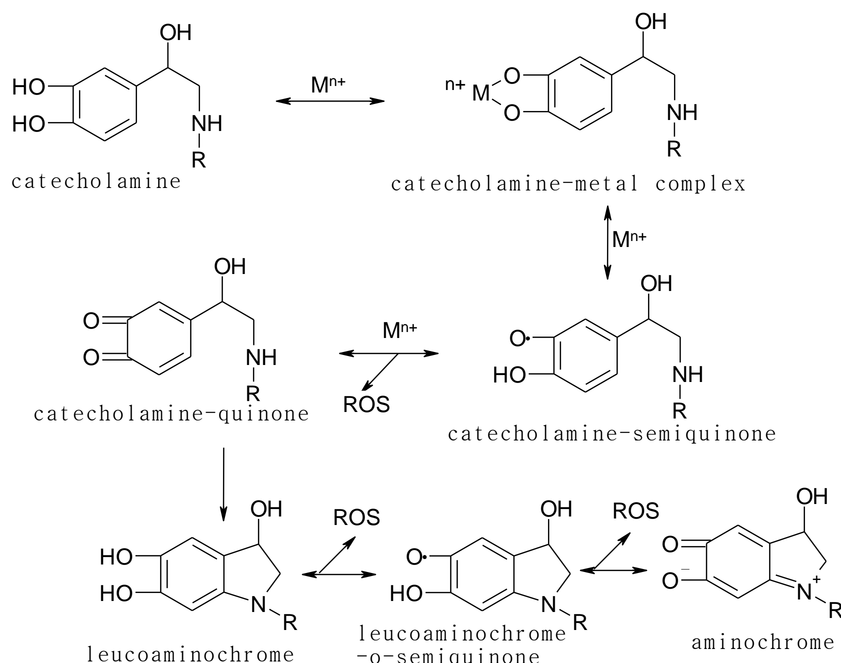


Fig. 1 Catecholamine autooxidation in presence of transition metal ion (M^{n+}) according to published studies (Bindoli *et al.*, 1992; Remiao *et al.*, 2001). The first step is the transformation of catecholamines into o-quinones, which are reactive intermediates that can undergo an irreversible 1,4-intramolecular cyclization leading to the formation of leucoaminochromes (2,3-dihydroindole-5,6-quinones) and finally to aminochromes (2,3-dihydroindole-5,6-diones). Aminochromes can be further transformed by oxidation and/or polymerization into several other compounds, such aminolutins, melanins (not shown). The whole process is associated with production of ROS.

2.3 Calcium overload

Many researchers described calcium overload caused by catecholamines (Rona, 1985; Tappia *et al.*, 2001; Diaz-Munoz *et al.*, 2006), unfortunately, the precise mechanism of this calcium overload remains still very elusive. Calcium influx caused by catecholamines appears to

start with opening of L-type calcium channels in plasmalemmal membrane. It has been well documented that this way of calcium influx is amplified by catecholamines (Zheng *et al.*, 1992). Plasmalemmal Na^+-Ca^{2+}-exchanger appears to play a significant role, as well (Saini *et al.*, 2006). Calcium influx via plasmalemmal membrane leads to an increase in intracellular calcium concentration, which triggers Ca^{2+}-release from sarcoplasmatic reticulum (SR) (Fabiato, 1985; Bers *et al.*, 1990). This process seems to be mediated by phosphorylation of ryanodine receptor 2 which is a calcium channel releasing SR calcium. During repolarization, calcium returns to SR by use of SR Ca^{2+}-ATPase (SERCA) and it is transported outward of the cell via Na^+-Ca^{2+}-exchanger (this transporter is involved in both influx and efflux of calcium) and plasmalemmal calcium ATP-ase (Bers *et al.*, 1990). The whole process seems to take place physiologically, as well. Markedly elevated levels of catecholamines alter this process by several proposed mechanisms:

- A decrease in Na^+-Ca^{2+}-exchanger activity has been documented several hours after ISO application (Tappia *et al.*, 2001; Diaz-Munoz *et al.*, 2006). Additionally, this transporter is voltage dependent, may be therefore associated with calcium overload deterioration under pathological changes in cell homeostasis (Tappia *et al.*, 2001).

- Hyperphosporylation of ryanodine receptor 2, leading to significant calcium leak from SR into the cytosole, is stimulated by beta-adrenergic receptors (Curran *et al.*, 2007; Ellison *et al.*, 2007). ISO induces apoptosis *in vivo* experiments and this apoptosis can be eliminated by inhibiting ryanodine receptor 2 function (Ellison *et al.*, 2007).

The role of other transporters is not clear and results of studies are contradictory: An increase in SERCA activity was described after ISO bolus (Diaz-Munoz *et al.*, 2006), although

continuous application of ISO led to a decrease in SERCA gene expression and this effect could be absolutely inhibited by propranolol (Boluyt *et al.*, 1995). Likewise, raised plasmalemmal Ca-ATPase activity was observed in one study while in another, a marked decrease was observed (Tappia *et al.*, 2001; Diaz-Munoz *et al.*, 2006).

3. Laboratory diagnostic findings of AMI and comparison to experimental models

According to the nomenclature, AMI diagnosis is based particularly on unequivocal ECG changes and/or enzyme changes (WHO, 1979; Widimský and Špaček, 2004). Other laboratory diagnostic approaches, in particular echocardiography and coronarography, carry additional information related especially to the success of AMI-treatment and AMI-complications (Widimský and Špaček, 2004).

3.1 ECG changes

ECG of an AMI-patient shows typically elevations or depression in ST-segment, in case of transmural AMI abnormal, persistent Q wave and, eventually, other abnormalities (negative T wave). Application of ISO to laboratory animals was associated with typical AMI changes on ECG: ST segment elevation/depression, deep Q wave and T wave inversion. More precisely, ST segment derangement returned to normal at 48 hours, while Q wave persisted and negative T wave inversion appeared (Singal *et al.*, 1982; Chagoya de Sanchez *et al.*, 1997; Pinelli *et al.*, 2004).

Arrhythmias, especially ventricular fibrillation, occurring during I-R seems to develop in 2 phases (Bernier *et al.*, 1986; Clements-Jewery *et al.*, 2002). The first phase takes place during ischaemia and is probably based on elevated levels of potassium (Sulova *et al.*, 1998). The second phase appears during reperfusion and is associated with ROS production. The involvement of catecholamines in the second phase cannot be ruled out but remains controversial (Clements-Jewery *et al.*, 2002). Clinically, interventional coronary reperfusion procedures (e.g., primary coronary angioplasty) are accompanied by occurrence incidence of ventricular fibrillations (VF) (Olsson *et al.*, 2002) which could have identical pathophysiological mechanism with second phase arrhythmias. It can be hypothesized that suppression of ROS during reperfusion may decrease the incidence of such arrhythmias. In fact, some experimental studies revealed protective effects of agents diminishing ROS generation or directly scavenging ROS on incidence of VF but there is still large discrepancy (Singal *et al.*, 1982; Chambers *et al.*, 1985; Bernier *et al.*, 1986).

3.2 Biochemical markers

Biochemical markers of AMI have changed during last decades. Formerly used creatine kinase (CK) or even LDH were displaced firstly by MB-isoform of CK (CK-MB) and in the present unambiguously by cardiac troponins. Cardiac troponin T (cTnT) is preferred by most laboratories although cardiac troponin I may be similarly useful (Jaffe *et al.*, 2000). Both AMI models (ISO administration and coronary artery ligation) provoked marked increase in serum CK, CK-MB and LDH concentrations (Wexler and McMurtry, 1981; Badylak *et al.*, 1987; Sumitra *et al.*, 2001).

Clinical studies reported a good correlation among myocardial infarction size, enzymatic assessment and 6-month mortality (Gibbons *et al.*, 2004). Both coronary artery ligation and ISO application lead to myocardial necrosis after sufficient time or dose, respectively. ISO has an advantage that the extent of necrosis is proportional to the dose (Rona, 1985). It is well known fact that tissue necrosis is associated with tissue functional impairment and hence both AMI models documented marked derangement in many parameters of heart function, e.g., in stroke volume, cardiac output, left ventricle developed pressure, increased end-diastolic pressure, alternation in coronary blood flow (Blasig *et al.*, 1985; Ambrosio *et al.*, 1987; DeBoer and Clark, 1992; Tappia *et al.*, 2001).

4 Studied compounds

4.1 Iron chelators

Iron chelators represent a large group of drugs with diverse chemical structure that avidly bind iron (Liu and Hider, 2002; Kalinowski and Richardson, 2005). Traditionally they are used in acute intoxication, in chronic iron overload diseases and as preventive agents hindering the antracycline cardiotoxicity (Olivieri and Brittenham, 1997; Hrdina *et al.*, 2000). Quite recently, new therapeutic application has revealed: some of them may be useful in antitumour therapy, in the treatment of some neurodegenerative disorders and they seem to possess antibacterial, antiprotozoal and antifungal activity, too (Tam *et al.*, 2003; Kalinowski and Richardson, 2005). The idea that iron chelation may be useful in various conditions associated with I-R or oxidative stress has been proposed many years ago. Virtually, oxidative stress is associated with chronic iron overload and plays a role in antracycline cardiotoxicity. Unfortunately, results concerning I-

R are somehow conflicting (see later). Therefore, there is a need to test novel iron chelators, in such pathological conditions.

Coordination number of iron is six. This enables to use various types of iron chelators. Hexadentate chelators possess 6 donor atoms per one molecule, so one molecule of chelator ligates directly all 6 iron coordination sites forming complex chelators-iron 1:1, analogously, chelators with 3 donor atoms are termed tridentate and form complexes 2:1 and chelators with 2 donor atoms bidentate forming complex 3:1, chelator : iron, respectivelly (see Fig.2). In general, hexadentate ligands have higher stability for their ferric complexes as compared to bi- or tridentates (Liu and Hider, 2002). Regrettably, recently developed compounds are mostly bidentates or tridentates mainly due to low oral bioavailability of hexandetate drugs (Tam *et al.*, 2003). For hindering the redox cycling, all 6 coordination sites have to be firmly occupied because “loosely-bound” iron or iron with one coordination site occupied by an easily dissociable ligand can catalyze production of hydroxyl radical (Graf *et al.*, 1984). Unfortunately not all chelators even with relatively high association constant of the chelators-Fe^{III+} complex hamper redox cycling of iron with subsequent formation of hydroxyl radical. Such example represents the hexadentate chelators EDTA which is too small to encompass the iron atom (Liu and Hider, 2002). Indeed, the correlation between the association constant of chelating agent with Fe^{III+} and redox cycling of iron is very poor (Graf *et al.*, 1984). More information than affinity constant may give redox potential of iron-chelator complex. Chelators hindering redox cycling have very low redox potential, e.g., deferoxamine-Fe^{III} complex has E_0 -475 mV, Fe^{III}-complexes with aroylisonicotinoylhydrazines have E_0 -285 ± 30 mV (Spasojevic *et al.*, 1999; Bernhardt *et al.*, 2005). Nevertheless, iron-chelators complexes which can undergo redox cycling

are not suitable for possible usage in I-R condition, but they may be useful as antitumour drugs (Kalinowski and Richardson, 2005).

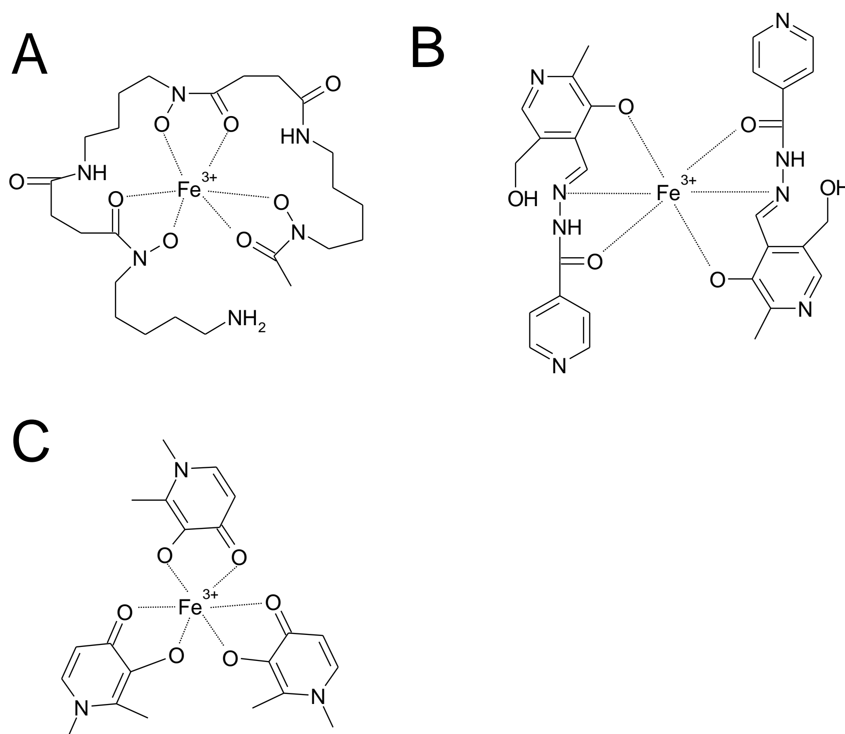


Fig.2. Examples of iron-chelator complexes. **A**: hexadentate (complex deferoxamin:iron, 1:1), **B**: tridentate (complex PIH:iron, 2:1), **C**: bidentate (complex deferiprone:iron, 3:1).

4.1.1 Deferoxamine (DFO)

Deferoxamine (desferrioxamine, **Fig. 3**) is a hexadentate microbial iron chelators extracted from *Streptomyces pilosus*. It is the most widely used iron chelators and has been the drug of choice for clinical treatment of iron overload conditions, especially thalassemia, since 1970s (Olivieri and Brittenham, 1997).

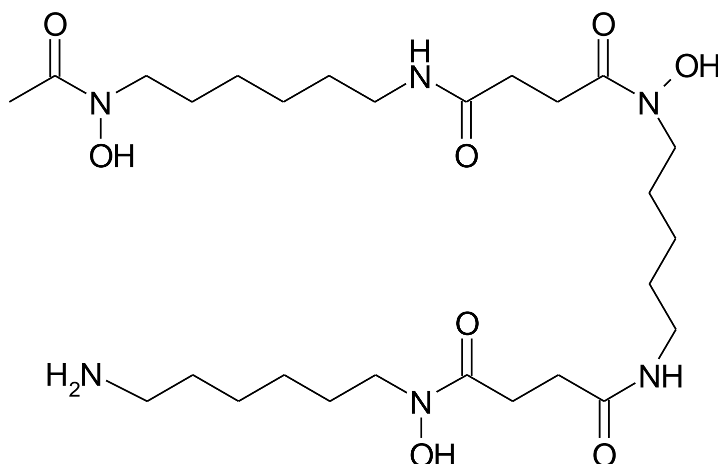


Fig.3. Structure of deferoxamine.

Unfortunately, it has several disadvantages, particularly:

- It is not absorbed by peroral application and its effects are limited to intravascular space with an exception of the liver (Liu and Hider, 2002)
- Long-term infusion (8-12h) i.v. or s.c. 5-7 days weakly are needed to obtain clinical efficacy. This is associated with low patient compliance.
- It has short elimination half-life 5-10 minutes because of rapid renal clearance (Liu and Hider, 2002)
- Swelling and pain at the site of injection is documented in one third of patients (Kalinowski and Richardson, 2005)

Nevertheless, DFO has high affinity for Fe^{3+} and renders chelated iron inactive and therefore hindering ROS propagation (Liu and Hider, 2002; Kalinowski and Richardson, 2005). Iron excretion is clearly augmented in urine as well in faeces after DFO admistration (Pippard *et*

al., 1982). In addition, DFO enters into the liver via a facilitated transport system and may therefore interact not only with extracellular iron but also with iron stored in hepatic cells (Hershko *et al.*, 1978; Liu and Hider, 2002). On the other hand, limited distribution to other organs, especially to the heart, diminishes its clinical effectiveness. Indeed, it has been demonstrated that DFO improves laboratory abnormalities in hepatic function, arrest hepatic fibrosis, reduces significantly mortality and morbidity on cardiac disease although such therapy does not reverse iron-induced cardiac dysfunction in thalassemic patients treated with blood transfusions (Olivieri and Brittenham, 1997).

In contrast to the documented positive effects of DFO in thalassemic patients, the usefulness of DFO in various I-R remains obscure. There are some reports showing positive effects of DFO, however, there are also some reports claiming the opposite results, as well (see Tab.1). It should be taken in the consideration that studies reporting positive results were based mostly on less severe experiemental protocol (e.g., hypoxia instead of anoxia, only 15 minutes of ischaemia) or were obtained from experiments performed on isolated heart or liver. In the latter case, DFO is much more efficient because of its reported better penetration into the liver in comparison to that to other tissues. This is supported by better recovery of hepatic function in thalassemic patients (see previous paragraph). In addition, 15 minutes of ischaemia has been documented not to cause myocardial necrosis (Bolli *et al.*, 1990). Perfusion experiments carried out on isolated heart cannot establish the contribution of leucocytes in this injury. Moreover, continuous infusion of DFO appears to be more efficient probably due to short elimination half-life of DFO. Conclusively, it seems that DFO may have positive effects on impairment caused by catalytical involvement of iron substantially in:

- Liver injury
- Iron over-load condition
- Longer infusion of sufficient dose

4.1.2 Aroylhydrazones

Aroylhydrazones are a large novel group of tridentate chelators containing wide range of analogues (see Fig.4). One of subgroups are **2-pyridylcarboxaldehyde isonicotinoyl hydrazones**. These chelators bind iron through imine and pyridyl nitrogens and a carbonyl oxygen. Based on inclusion of another nitrogen in the ligation site, these chelators were expected to redox-cycle and therefore originally developed as antitumour drugs. Paradoxically, they demonstrated only low antiproliferative activity while they showed potential to replace DFO in the treatment of iron overload pathologies (Kalinowski and Richardson, 2005). 2-pyridylcarboxaldehyde-2-thiophenecarboxyl hydrazone (PCTH) seems to be the most promising agent. Recently, PCTH has been shown to protect iron-loaded cells against oxidative stress caused by hydrogen peroxide (Lim *et al.*, 2008).

Study	Animal	Type	Duration (minutes)	DFO	Outcomes
Ambrosio *et al.*, 1987	rabbit	isolated perfused heart	30/up to 45	B+I	almost total recovery of biochemical and functional parameters

Badylak *et al.*, 1987	rat	isolated perfused heart	60/60	I^{+}	a decrease in LDH release, mitochondrial damage and lesser increase in peripheral resistance
Bolli *et al.*, 1990	dog	open-chest	15/240	B+I	total recovery of functional parameters except for myocardial blood flow
Bolli *et al.*, 1987	dog	ope-chest	15/240	B+I	lower occurence of VF, otherwise no difference in arrhythmias, better recovery of myocardial function
DeBoer and Clark, 1992	rat	isolated perfused heart	25/15	B+I	no amelioration in functional parameters
Myers *et al.*, 1985	rabbit	isolated perfused heart	60*/60	I	almost absolute inhibition of creatine kinase release, amelioration of stroke volume but almost no effect on myocardial blood flow
Myers *et al.*, 1986	rabbit	isolated perfused heart	CS + 120/60	B$^{\nabla}$	no significant improvement of functional parameters except, interestingly, for coronary blood flow
Omar *et al.*, 1989	rat	liver ischaemia	30/up to 24 h	B	marked improvement of liver histopathological injury at 24 h and a significant reduction in TBARS in liver tissue at 2 h
Reddy *et al.*, 1991	dog	open-chest	60/up to 24 h	I	no differences in mortality and necrosis,no amelioration in functional parameters

Table 1 Studies concerning effects of DFO on I-R. Studies differ in strains of animals, type of methodological procedure, duration of I-R (left number is duration of ischemia, the number behind the slash is duration of reperfusion) and administration of DFO. Symbols and abbreviations: open-chest – I-R was carried out by a ligation of the left anterior descending coronary artery, liver ischaemia – I-R caused by ligation of entire hilar pedicle, CS – cardioplegic solution (27°C for 5 min), B (bolus) – DFO was administered before the beginning of ischaemia, B$^{\nabla}$ – DFO was integral part of cardioplegic solution, I (infusion) – DFO was continuously infused during experiment, I^{+} - DFO was infused only during reperfusion, VF – ventricular fibrillation on reperfusion, * - instead of absolute ischaemia, hypoxic solution was used.

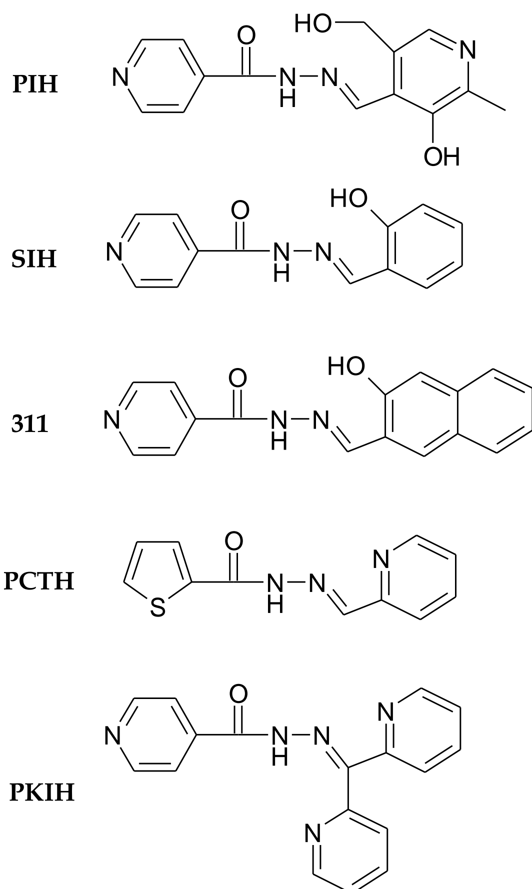


Fig. 4. Aroylhydrazones. Five generation of aroylhydrazone iron chelators are depicted with the representative drug (mostly tested). First generation (series 100) are pyridoxal isonicotinoyl hydrazones and their representative is pyridoxal isonicotinoyl hydrazone (PIH). In series 200 pyridoxal moiety was replaced by salicylaldehyde group and representative is SIH (salicylaldehyde isonicotinoyl hydrazone). The series 300 contains 2-hydroxy-1-naphtaldehyde residue, these compounds are powerful antiproliferative agents – representative is 311. 2-pyridylcarboxaldehyde isonicotinoyl hydrazones generation is represented by PCTH (2-pyridylcarboxaldehyde-2-thiophenecarboxyl hydrazone). The most recent generation (di-2-pyridylketone isonicotynoyl hydrazones) represents PKIH (di-2-pyridylketone thiophenecarboxyl hydrazone) - these drugs possess high antiproliferative activity.

4.1.3 Lactoferrins

Lactoferrins are mammal 80-kDa large iron-binding glycoproteins, which share high-degree of homology with each other and with transferrin at amino acid sequence as well at three dimensional conformation (Metz-Boutigue *et al.*, 1984; Abdallah and El Hage Chahine, 2000; Baker and Baker, 2005; Weinberg, 2006). One molecule of lactoferrin has two binding sites for ferric ions (Fig.5). Its affinity for iron is about 260 times higher than that of transferrin and in contrast to the mentioned iron-carrier, lactoferrin is able to retain iron even under more acidic condition (Mazurier and Spik, 1980). The role of lactoferrin in man is not fully understood. Its presence on mucosal surfaces suggests, together with its iron binding properties, its involvement in the defence against microbes, especially bacteria, who necessitates iron (Weinberg, 2003). Moreover, lactoferrin may scavenge free iron in inflammation and therefore prevent its participation in ROS-generation (Legrand *et al.*, 2004).

Lactoferrin has been shown *in vitro* to inhibit hydroxyl radical formation via Fenton chemistry as well as to protect some food against oxidation (Baldwin *et al.*, 1984; Raghuveer *et al.*, 2002; Nielsen *et al.*, 2004). Moreover, lactoferrin decreased ROS-generation in iron-overloaded mice (Schaible *et al.*, 2002). Based on the mentioned studies, lactoferrin may act protectively in myocardial I-R injuries and may possess special advantage over other iron chelators, especially by its endogenous origin.

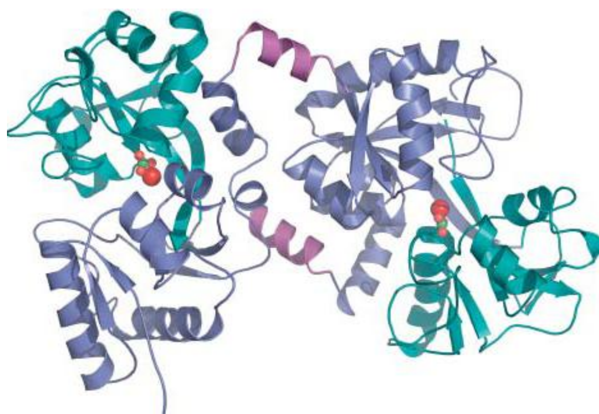


Fig. 5. Molecular structure of human lactoferrin (Baker and Baker, 2005). The N-lobe is on the left, the C-lobe on the right. Ferric ions are synergically bound with CO_3^{2-} (shown by red spherical atoms) in each lobe.

There is a huge amount of other, in this study not tested, known iron chelators, which are described in details in various excellent reviews (Liu and Hider, 2002; Tam *et al.*, 2003; Kalinowski and Richardson, 2005).

4.2 Flavonoids

Flavonoids (Fig. 6A) are the most common and widely distributed class of natural polyphenolic compounds. Intense investigation of their properties demonstrated large spectrum of proposed pharmacological activity (antiallergic, anti-atherogenic, anti-anflammatory, antidiabetic, hepato- and gastroprotective, antiviral and antineoplastic). These properties are probably based mainly on their ROS-scavenging properties and interaction with enzymes (e.g. inhibition of xantine oxidase, lipoxygenase, cyclooxygenase) (Wilcox *et al.*, 1999; Russo *et al.*, 2000; Moridani *et al.*, 2003).

The former is explained by at least 3 mechanisms:

- direct reaction with superoxide ion (Chen *et al.*, 1990; Russo *et al.*, 2000; Moridani *et al.*, 2003)
- iron and copper chelation (Kuo *et al.*, 1998; Mira *et al.*, 2002)
- inhibition of lipid peroxidation by direct reaction with lipid peroxy radicals (Chen *et al.*, 1990; Russo *et al.*, 2000) (Afanas'ev *et al.*, 1989)

These complex ROS-scavenging properties may predispose flavonoids as therapeutically useful agents in conditions associated with I-R, and indeed, their effectivity was documented (Ahlenstiel *et al.*, 2003). Additionally, antracycline cardiotoxicity, in which iron and ROS probably play a significant role, may be reduced by various flavonoids (Kozluca *et al.*, 1996; van Acker *et al.*, 2001; Psotova *et al.*, 2004; Kaiserova *et al.*, 2007). The contribution of iron chelation on scavenging properties of flavonoids is supported by reduction of iron overload tissue damage by these drugs (Zhang *et al.*, 2006). Therefore, these polyphenolic compounds may have some impact on catecholamine cardiotoxicity.

4.2.1 Rutin

Rutin (Fig. 6B), also called rutoside or quercetin-3-rutinoside), is a citrus flavonoid glycoside found substantially in buckwheat, as well as in other sources, e.g., in *Ruta graveolens,* whose genus name gave word origin of rutin (Kreft *et al.*, 1999). Rutin has been extensively investigated like other flavonoids. Its efficiency to scavenge superoxide, to chelate iron/copper

and to inhibit lipid peroxidation is well documented (Chen *et al.*, 1990; Kuo *et al.*, 1998; Russo *et al.*, 2000).

There are more purposes why rutin was chosen as a representative of flavonoids for this study:

- Its ROS-scavenging activity is high and comparable in many studies with other powerful antioxidant flavonoids (Chen *et al.*, 1990; Russo *et al.*, 2000)
- Similarly, its iron binding properties have been well documented. Spectrophotometic analysis revealed that rutin forms a stable complex with ferrous/ferric iron in ratio 2:1 (Afanas'ev *et al.*, 1989). Electrospray mass spectrometry suggested that additional complexes with other stechiometry may be formed in minor scale, as well (Fernandez *et al.*, 2002).
- On the one hand, its aglycone quercetin may be more efficient in inhibition of lipid peroxidation but on the other hand, it is clearly more toxic than rutin, which contrarily did not show any overt signs of cellular toxicity up to 500 μM (Afanas'ev *et al.*, 1989; Saija *et al.*, 1995; Soares *et al.*, 2006).
- Better water solubility in comparison to other flavonoids enables i.v. administration and comparison with iron chelators also administered i.v. in this study.

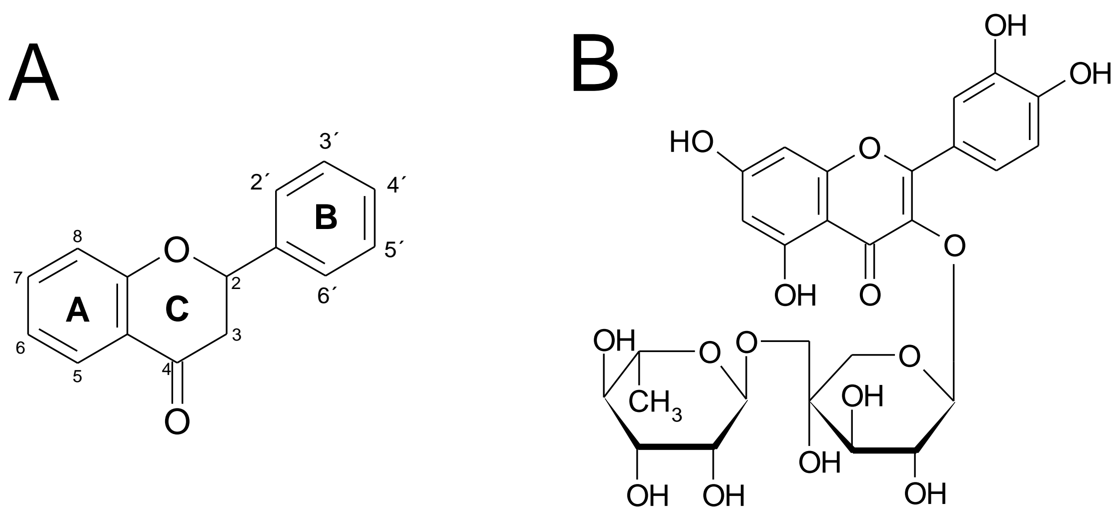


Fig. 6. Structure of flavonoids. **A:** Flavonoids backbone forms 2-phenyl-1,4-benzopyrone. Flavonoid are divided mainly according to the presence of 2,3-double bound, hydroxygroup in position 3 and ketogroup in position 4 into several subclasses (flavanols, flavanons, flavonols, flavons) **B**: Structure of rutin (an example of flavonol). 3-rhamnosyl-glucosyl residue is attached by a glycoside bound to the flavonol aglycon quercetin.

5 Abbreviations

AMI	acute myocardial infarction
CHD	coronary heart disease
CK	creatine kinase
CP	creatine phosphate
DFO	deferoxamine (desferrioxamine)
GPx	glutathione peroxidase
I-R	ischaemia-reperfusion injury
ISO	isoprenaline
LDH	lactate dehydrogenase
LWMFe	low weight molecular iron
PCTH	2-pyridylcarboxaldehyde-2-thiophenecarboxyl hydrazone
ROS	reactive oxygen species
SERCA	sarcoplasmatic reticulum Ca^{2+}-ATPase
SOD	superoxide dismutase
SR	sarcoplasmatic reticulum
TBARS	thiobarbituric acid reactive substances
VF	ventricular fibrillation
XO	xanthine oxidase

6. References

1 Abdallah FB and El Hage Chahine JM. Transferrins: iron release from lactoferrin. *J Mol Biol* 2000; **303**: 255-266.

2 Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, and Potapovitch AI. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* 1989; **38**: 1763-1769.

3 Ahlenstiel T, Burkhardt G, Kohler H, and Kuhlmann MK. Bioflavonoids attenuate renal proximal tubular cell injury during cold preservation in Euro-Collins and University of Wisconsin solutions. *Kidney Int* 2003; **63**: 554-563.

4 Ambrosio G, Zweier JL, Jacobus WE, Weisfeldt ML, and Flaherty JT. Improvement of postischemic myocardial function and metabolism induced by administration of deferoxamine at the time of reflow: the role of iron in the pathogenesis of reperfusion injury. *Circulation* 1987; **76**: 906-915.

5 Badylak SF, Simmons A, Turek J, and Babbs CF. Protection from reperfusion injury in the isolated rat heart by postischaemic deferoxamine and oxypurinol administration. *Cardiovasc Res* 1987; **21**: 500-506.

6 Baker EN and Baker HM. Molecular structure, binding properties and dynamics of lactoferrin. *Cell Mol Life Sci* 2005; **62**: 2531-2539.

7 Baldwin DA, Jenny ER, and Aisen P. The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. *J Biol Chem* 1984; **259**: 13391-13394.

8 Berenshtein E, Vaisman B, Goldberg-Langerman C, Kitrossky N, Konijn AM, and Chevion M. Roles of ferritin and iron in ischemic preconditioning of the heart. *Mol Cell Biochem* 2002; **234-235**: 283-292.

9 Bernhardt PV, Chin P, Sharpe PC, Wang JY, and Richardson DR. Novel diaroylhydrazine ligands as iron chelators: coordination chemistry and biological activity. *J Biol Inorg Chem* 2005; **10**: 761-777.

10 Bernier M, Hearse DJ, and Manning AS. Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. *Circ Res* 1986; **58**: 331-340.

11 Berry CE and Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J Physiol* 2004; **555**: 589-606.

12 Bers DM, Lederer WJ, and Berlin JR. Intracellular Ca transients in rat cardiac myocytes: role of Na-Ca exchange in excitation-contraction coupling. *Am J Physiol* 1990; **258**: C944-954.

13 Bindoli A, Rigobello MP, and Deeble DJ. Biochemical and toxicological properties of the oxidation products of catecholamines. *Free Radic Biol Med* 1992; **13**: 391-405.

14 Blasig IE, Zipper J, Muschick P, Modersohn D, and Lowe H. Absolute and relative myocardial ischemia by isoproterenol overdosage. *Biomed Biochim Acta* 1985; **44**: 1641-1649.

15 Bolli R, Patel BS, Jeroudi MO, Li XY, Triana JF, Lai EK, and McCay PB. Iron-mediated radical reactions upon reperfusion contribute to myocardial "stunning". *Am J Physiol* 1990; **259**: H1901-1911.

16 Bolli R, Patel BS, Zhu WX, O'Neill PG, Hartley CJ, Charlat ML, and Roberts R. The iron chelator desferrioxamine attenuates postischemic ventricular dysfunction. *Am J Physiol* 1987; **253**: H1372-1380.

17 Boluyt MO, Long X, Eschenhagen T, Mende U, Schmitz W, Crow MT, and Lakatta EG. Isoproterenol infusion induces alterations in expression of hypertrophy-associated genes in rat heart. *Am J Physiol* 1995; **269**: H638-647.

18 Boucher F, Pucheu S, Coudray C, Favier A, and de Leiris J. Evidence of cytosolic iron release during post-ischaemic reperfusion of isolated rat hearts. Influence on spin-trapping experiments with DMPO. *FEBS Lett* 1992; **302**: 261-264.

19 Ceconi C, Cargnoni A, Pasini E, Condorelli E, Currello S, and Ferrari R. Lipid peroxidation during myocardial reperfusion. *Mol Cell Biochem* 1992; **111**: 49-54.

20 Clements-Jewery H, Hearse DJ, and Curtis MJ. Independent contribution of catecholamines to arrhythmogenesis during evolving infarction in the isolated rat heart. *Br J Pharmacol* 2002; **135**: 807-815.

21 Coady SA, Sorlie PD, Cooper LS, Folsom AR, Rosamond WD, and Conwill DE. Validation of death certificate diagnosis for coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) Study. *J Clin Epidemiol* 2001; **54**: 40-50.

22 Coudray C, Boucher F, Pucheu S, De Leiris J, and Favier A. Relationship between severity of ischemia and oxidant scavenger enzyme activities in the isolated rat heart. *Int J Biochem Cell Biol* 1995; **27**: 61-69.

23 Coudray C, Pucheu S, Boucher F, Arnaud J, de Leiris J, and Favier A. Effect of ischemia/reperfusion sequence on cytosolic iron status and its release in the coronary effluent in isolated rat hearts. *Biol Trace Elem Res* 1994; **41**: 69-75.

24 Curran J, Hinton MJ, Rios E, Bers DM, and Shannon TR. Beta-adrenergic enhancement of sarcoplasmic reticulum calcium leak in cardiac myocytes is mediated by calcium/calmodulin-dependent protein kinase. *Circ Res* 2007; **100**: 391-398.

25 DeBoer DA and Clark RE. Iron chelation in myocardial preservation after ischemia-reperfusion injury: the importance of pretreatment and toxicity. *Ann Thorac Surg* 1992; **53**: 412-418.

26 Diaz-Munoz M, Alvarez-Perez MA, Yanez L, Vidrio S, Martinez L, Rosas G, Yanez M, Ramirez S, and de Sanchez VC. Correlation between oxidative stress and alteration of intracellular calcium handling in isoproterenol-induced myocardial infarction. *Mol Cell Biochem* 2006; **289**: 125-136.

27 Ellison GM, Torella D, Karakikes I, Purushothaman S, Curcio A, Gasparri C, Indolfi C, Cable NT, Goldspink DF, and Nadal-Ginard B. Acute beta-adrenergic overload produces myocyte damage through calcium leakage from the ryanodine receptor 2 but spares cardiac stem cells. *J Biol Chem* 2007; **282**: 11397-11409.

28 Fabiato A. Rapid ionic modifications during the aequorin-detected calcium transient in a skinned canine cardiac Purkinje cell. *J Gen Physiol* 1985; **85**: 189-246.

29 Fantini GA and Yoshioka T. Deferoxamine prevents lipid peroxidation and attenuates reoxygenation injury in postischemic skeletal muscle. *Am J Physiol* 1993; **264**: H1953-1959.

30 Fernandez MT, Mira ML, Florencio MH, and Jennings KR. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. *J Inorg Biochem* 2002; **92**: 105-111.

31 Fleckenstein A, Janke J, Doring H, and Packinger O. *Calcium overload as the determinant factor in the production of catecholamine-induced myocardial lesions*. University Park Press Baltimore.1973

32 Gibbons RJ, Valeti US, Araoz PA, and Jaffe AS. The quantification of infarct size. *J Am Coll Cardiol* 2004; **44**: 1533-1542.

33 Goldberg RJ, Glatfelter K, Burbank-Schmidt E, Lessard D, and Gore JM. Trends in community mortality due to coronary heart disease. *Am Heart J* 2006; **151**: 501-507.

34 Gonzalez-Flecha B, Cutrin JC, and Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. *J Clin Invest* 1993; **91**: 456-464.

35 Graf E, Mahoney JR, Bryant RG, and Eaton JW. Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. *J Biol Chem* 1984; **259**: 3620-3624.

36 Guarnieri C, Flamigni F, and Caldarera CM. Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. *J Mol Cell Cardiol* 1980; **12**: 797-808.

37 Gunther MR, Hanna PM, Mason RP, and Cohen MS. Hydroxyl radical formation from cuprous ion and hydrogen peroxide: a spin-trapping study. *Arch Biochem Biophys* 1995; **316**: 515-522.

38 Gutteridge JM, Richmond R, and Halliwell B. Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochem J* 1979; **184**: 469-472.

39 Halliwell B and Gutteridge J. *Free radicals in biology and medicine 3rd ed.* . Oxford University Press New York.1999

40 Hershko C, Grady RW, and Cerami A. Mechanism of iron chelation in the hypertransfused rat: definition of two alternative pathways of iron mobilization. *J Lab Clin Med* 1978; **92**: 144-151.

41 Holt S, Gunderson M, Joyce K, Nayini NR, Eyster GF, Garitano AM, Zonia C, Krause GS, Aust SD, and White BC. Myocardial tissue iron delocalization and evidence for lipid peroxidation after two hours of ischemia. *Ann Emerg Med* 1986; **15**: 1155-1159.

42 Hrdina R, Gersl V, Klimtova I, Simunek T, Machackova J, and Adamcova M. Anthracycline-induced cardiotoxicity. *Acta Medica (Hradec Kralove)* 2000; **43**: 75-82.

43 Chagoya de Sanchez V, Hernandez-Munoz R, Lopez-Barrera F, Yanez L, Vidrio S, Suarez J, Cota-Garza MD, Aranda-Fraustro A, and Cruz D. Sequential changes of energy metabolism and mitochondrial function in myocardial infarction induced by isoproterenol in rats: a long-term and integrative study. *Can J Physiol Pharmacol* 1997; **75**: 1300-1311.

44 Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, Parmley LF, and Downey JM. Xanthine oxidase as a source of free radical damage in myocardial ischemia. *J Mol Cell Cardiol* 1985; **17**: 145-152.

45 Chen YT, Zheng RL, Jia ZJ, and Ju Y. Flavonoids as superoxide scavengers and antioxidants. *Free Radic Biol Med* 1990; **9**: 19-21.

46 Chevion M, Jiang Y, Har-El R, Berenshtein E, Uretzky G, and 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.

47 Jaffe AS, Ravkilde J, Roberts R, Naslund U, Apple FS, Galvani M, and Katus H. It's time for a change to a troponin standard. *Circulation* 2000; **102**: 1216-1220.

48 Kaiserova H, Simunek T, van der Vijgh WJ, Bast A, and Kvasnickova E. Flavonoids as protectors against doxorubicin cardiotoxicity: role of iron chelation, antioxidant activity and inhibition of carbonyl reductase. *Biochim Biophys Acta* 2007; **1772**: 1065-1074.

49 Kalinowski DS and Richardson DR. The evolution of iron chelators for the treatment of iron overload disease and cancer. *Pharmacol Rev* 2005; **57**: 547-583.

50 Kirshenbaum LA and Singal PK. Changes in antioxidant enzymes in isolated cardiac myocytes subjected to hypoxia-reoxygenation. *Lab Invest* 1992; **67**: 796-803.

51 Kloner RA. Natural and unnatural triggers of myocardial infarction. *Prog Cardiovasc Dis* 2006; **48**: 285-300.

52 Kondo T, Ogawa Y, Sugiyama S, Ito T, Satake T, and Ozawa T. Mechanism of isoproterenol induced myocardial damage. *Cardiovasc Res* 1987; **21**: 248-254.

53 Kozluca O, Olcay E, Surucu S, Guran Z, Kulaksiz T, and Uskent N. Prevention of doxorubicin induced cardiotoxicity by catechin. *Cancer Lett* 1996; **99**: 1-6.

54 Kreft S, Knapp M, and Kreft I. Extraction of rutin from buckwheat (Fagopyrum esculentumMoench) seeds and determination by capillary electrophoresis. *J Agric Food Chem* 1999; **47**: 4649-4652.

55 Kuo SM, Leavitt PS, and Lin CP. Dietary flavonoids interact with trace metals and affect metallothionein level in human intestinal cells. *Biol Trace Elem Res* 1998; **62**: 135-153.

56 Lameris TW, de Zeeuw S, Alberts G, Boomsma F, Duncker DJ, Verdouw PD, Veld AJ, and van Den Meiracker AH. Time course and mechanism of myocardial catecholamine release during transient ischemia in vivo. *Circulation* 2000; **101**: 2645-2650.

57 Legrand D, Elass E, Pierce A, and Mazurier J. Lactoferrin and host defence: an overview of its immuno-modulating and anti-inflammatory properties. *Biometals* 2004; **17**: 225-229.

58 Lesnefsky EJ, Dauber IM, and Horwitz LD. Myocardial sulfhydryl pool alterations occur during reperfusion after brief and prolonged myocardial ischemia in vivo. *Circ Res* 1991; **68**: 605-613.

59 Lim CK, Kalinowski DS, and Richardson DR. Protection against hydrogen peroxide-mediated cytotoxicity in Friedreich's ataxia fibroblasts using novel iron chelators of the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone class. *Mol Pharmacol* 2008; **74**: 225-235.

60 Liu ZD and Hider RC. Design of clinically useful iron(III)-selective chelators. *Med Res Rev* 2002; **22**: 26-64.

61 Matthews SB, Henderson AH, and Campbell AK. The adrenochrome pathway: the major route for adrenalin catabolism by polymorphonuclear leucocytes. *J Mol Cell Cardiol* 1985; **17**: 339-348.

62 Mazurier J and Spik G. Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. *Biochim Biophys Acta* 1980; **629**: 399-408.

63 McCord JM, Roy RS, and Schaffer SW. Free radicals and myocardial ischemia. The role of xanthine oxidase. *Adv Myocardiol* 1985; **5**: 183-189.

64 Mehta JL and Li D. Epinephrine upregulates superoxide dismutase in human coronary artery endothelial cells. *Free Radic Biol Med* 2001; **30**: 148-153.

65 Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J, and Jolles P. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. *Eur J Biochem* 1984; **145**: 659-676.

66 Mira L, Fernandez MT, Santos M, Rocha R, Florencio MH, and Jennings KR. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radic Res* 2002; **36**: 1199-1208.

67 Moridani MY, Pourahmad J, Bui H, Siraki A, and O'Brien PJ. Dietary flavonoid iron complexes as cytoprotective superoxide radical scavengers. *Free Radic Biol Med* 2003; **34**: 243-253.

68 Myers CL, Weiss SJ, Kirsh MM, Shepard BM, and Shlafer M. Effects of supplementing hypothermic crystalloid cardioplegic solution with catalase, superoxide dismutase, allopurinol, or deferoxamine on functional recovery of globally ischemic and reperfused isolated hearts. *J Thorac Cardiovasc Surg* 1986; **91**: 281-289.

69 Myers CL, Weiss SJ, Kirsh MM, and Shlafer M. Involvement of hydrogen peroxide and hydroxyl radical in the 'oxygen paradox': reduction of creatine kinase release by catalase, allopurinol or deferoxamine, but not by superoxide dismutase. *J Mol Cell Cardiol* 1985; **17**: 675-684.

70 Nielsen NS, Petersen A, Meyer AS, Timm-Heinrich M, and Jacobsen C. Effects of lactoferrin, phytic acid, and EDTA on oxidation in two food emulsions enriched with long-chain polyunsaturated fatty acids. *J Agric Food Chem* 2004; **52**: 7690-7699.

71 Ohmi H, Ichihara K, and Abiko Y. Role of oxygen radicals in canine myocardial metabolic derangement during regional ischemia. *Am J Physiol* 1992; **262**: H553-561.

72 Olivieri NF and Brittenham GM. Iron-chelating therapy and the treatment of thalassemia. *Blood* 1997; **89**: 739-761.

73 Olsson KA, Harnek J, Ohlin AK, Pavlidis N, Thorvinger B, and Ohlin H. No increase of plasma malondialdehyde after primary coronary angioplasty for acute myocardial infarction. *Scand Cardiovasc J* 2002; **36**: 237-240.

74 Omar R, Nomikos I, Piccorelli G, Savino J, and Agarwal N. Prevention of postischaemic lipid peroxidation and liver cell injury by iron chelation. *Gut* 1989; **30**: 510-514.

75 Padmanabhan M and Prince PS. Preventive effect of S-allylcysteine on lipid peroxides and antioxidants in normal and isoproterenol-induced cardiotoxicity in rats: a histopathological study. *Toxicology* 2006; **224**: 128-137.

76 Persoon-Rothert M, van der Valk-Kokshoorn EJ, Egas-Kenniphaas JM, Mauve I, and van der Laarse A. Isoproterenol-induced cytotoxicity in neonatal rat heart cell cultures is mediated by free radical formation. *J Mol Cell Cardiol* 1989; **21**: 1285-1291.

77 Pinelli A, Trivulzio S, Tomasoni L, Brenna S, Bonacina E, and Accinni R. Isoproterenol-induced myocardial infarction in rabbits. Protection by propranolol or labetalol: a proposed non-invasive procedure. *Eur J Pharm Sci* 2004; **23**: 277-285.

78 Pippard MJ, Callender ST, and Finch CA. Ferrioxamine excretion in iron-loaded man. *Blood* 1982; **60**: 288-294.

79 Prasad K, Lee P, Mantha SV, Kalra J, Prasad M, and Gupta JB. Detection of ischemia-reperfusion cardiac injury by cardiac muscle chemiluminescence. *Mol Cell Biochem* 1992; **115**: 49-58.

80 Psotova J, Chlopcikova S, Miketova P, Hrbac J, and Simanek V. Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part III. Apigenin, baicalelin, kaempherol, luteolin and quercetin. *Phytother Res* 2004; **18**: 516-521.

81 Raghuveer TS, McGuire EM, Martin SM, Wagner BA, Rebouche CJ, Buettner GR, and Widness JA. Lactoferrin in the preterm infants' diet attenuates iron-induced oxidation products. *Pediatr Res* 2002; **52**: 964-972.

82 Rajadurai M and Prince PS. Preventive effect of naringin on isoproterenol-induced cardiotoxicity in Wistar rats: an in vivo and in vitro study. *Toxicology* 2007; **232**: 216-225.

83 Ravingerova T, Neckar J, Kolar F, Stetka R, Volkovova K, Ziegelhoffer A, and Styk J. Ventricular arrhythmias following coronary artery occlusion in rats: is the diabetic heart less or more sensitive to ischaemia? *Basic Res Cardiol* 2001; **96**: 160-168.

84 Reddy BR, Wynne J, Kloner RA, and Przyklenk K. Pretreatment with the iron chelator desferrioxamine fails to provide sustained protection against myocardial ischaemia-reperfusion injury. *Cardiovasc Res* 1991; **25**: 711-718.

85 Remiao F, Carmo H, Carvalho F, and Bastos ML. The study of oxidative stress in freshly isolated Ca(2+)-tolerant cardiomyocytes from the adult rat. *Toxicol In Vitro* 2001; **15**: 283-287.

86 Remiao F, Carvalho M, Carmo H, Carvalho F, and Bastos ML. Cu2+-induced isoproterenol oxidation into isoprenochrome in adult rat calcium-tolerant cardiomyocytes. *Chem Res Toxicol* 2002; **15**: 861-869.

87 Rona G. Catecholamine cardiotoxicity. *J Mol Cell Cardiol* 1985; **17**: 291-306.

88 Roth E, Torok B, Zsoldos T, and Matkovics B. Lipid peroxidation and scavenger mechanism in experimentally induced heart infarcts. *Basic Res Cardiol* 1985; **80**: 530-536.

89 Russo A, Acquaviva R, Campisi A, Sorrenti V, Di Giacomo C, Virgata G, Barcellona ML, and Vanella A. Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biol Toxicol* 2000; **16**: 91-98.

90 Saija A, Scalese M, Lanza M, Marzullo D, Bonina F, and Castelli F. Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radic Biol Med* 1995; **19**: 481-486.

91 Saini HK, Tripathi ON, Zhang S, Elimban V, and Dhalla NS. Involvement of Na+/Ca2+ exchanger in catecholamine-induced increase in intracellular calcium in cardiomyocytes. *Am J Physiol Heart Circ Physiol* 2006; **290**: H373-380.

92 Schaible UE, Collins HL, Priem F, and Kaufmann SH. Correction of the iron overload defect in beta-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. *J Exp Med* 2002; **196**: 1507-1513.

93 Schomig A. Catecholamines in myocardial ischemia. Systemic and cardiac release. *Circulation* 1990; **82**: II13-22.

94 Singal PK, Kapur N, Dhillon KS, Beamish RE, and Dhalla NS. Role of free radicals in catecholamine-induced cardiomyopathy. *Can J Physiol Pharmacol* 1982; **60**: 1390-1397.

95 Soares VC, Varanda EA, and Raddi MS. In vitro basal and metabolism-mediated cytotoxicity of flavonoids. *Food Chem Toxicol* 2006; **44**: 835-838.

96 Spasojevic I, Armstrong SK, Brickman TJ, and Crumbliss AL. Electrochemical Behavior of the Fe(III) Complexes of the Cyclic Hydroxamate Siderophores Alcaligin and Desferrioxamine E. *Inorg Chem* 1999; **38**: 449-454.

97 Sulova Z, Vyskocil F, Stankovicova T, and Breier A. Ca(2+)-induced inhibition of sodium pump: effects on energetic metabolism of mouse diaphragm tissue. *Gen Physiol Biophys* 1998; **17**: 271-283.

98 Sumitra M, Manikandan P, Kumar DA, Arutselvan N, Balakrishna K, Manohar BM, and Puvanakrishnan R. Experimental myocardial necrosis in rats: role of arjunolic acid on platelet aggregation, coagulation and antioxidant status. *Mol Cell Biochem* 2001; **224**: 135-142.

99 Takeo S, Taam GM, Beamish RE, and Dhalla NS. Effect of adrenochrome on calcium accumulation by heart mitochondria. *Biochem Pharmacol* 1981; **30**: 157-163.

100 Tam TF, Leung-Toung R, Li W, Wang Y, Karimian K, and Spino M. Iron chelator research: past, present, and future. *Curr Med Chem* 2003; **10**: 983-995.

101 Tan LB, Burniston JG, Clark WA, Ng Y, and Goldspink DF. Characterization of adrenoceptor involvement in skeletal and cardiac myotoxicity Induced by sympathomimetic agents: toward a new bioassay for beta-blockers. *J Cardiovasc Pharmacol* 2003; **41**: 518-525.

102 Tappia PS, Hata T, Hozaima L, Sandhu MS, Panagia V, and Dhalla NS. Role of oxidative stress in catecholamine-induced changes in cardiac sarcolemmal Ca2+ transport. *Arch Biochem Biophys* 2001; **387**: 85-92.

103 Terada LS, Piermattei D, Shibao GN, McManaman JL, and Wright RM. Hypoxia regulates xanthine dehydrogenase activity at pre- and posttranslational levels. *Arch Biochem Biophys* 1997; **348**: 163-168.

104 Tsuboi T, Ishikawa K, Osawa Y, Yoshida K, and Shimizu M. Biochemical changes of myocardial necrosis induced by isoproterenol and protective effects of beta-blocker and anti-inflammatory drugs. *Chem Pharm Bull (Tokyo)* 1974; **22**: 669-675.

105 van Acker FA, Hulshof JW, Haenen GR, Menge WM, van der Vijgh WJ, and Bast A. New synthetic flavonoids as potent protectors against doxorubicin-induced cardiotoxicity. *Free Radic Biol Med* 2001; **31**: 31-37.

106 Varadarajan SG, An J, Novalija E, Smart SC, and Stowe DF. Changes in [Na(+)](i), compartmental [Ca(2+)], and NADH with dysfunction after global ischemia in intact hearts. *Am J Physiol Heart Circ Physiol* 2001; **280**: H280-293.

107 Vetterlein F and Schmidt G. Effects of isoprenaline on functional capillary density in the subendocardial and subepicardial layer of the rat myocardium. *Basic Res Cardiol* 1980; **75**: 526-536.

108 von Kanel R, Mills PJ, Ziegler MG, and Dimsdale JE. Effect of beta2-adrenergic receptor functioning and increased norepinephrine on the hypercoagulable state with mental stress. *Am Heart J* 2002; **144**: 68-72.

109 Voogd A, Sluiter W, van Eijk HG, and Koster JF. Low molecular weight iron and the oxygen paradox in isolated rat hearts. *J Clin Invest* 1992; **90**: 2050-2055.

110 Weinberg ED. The therapeutic potential of lactoferrin. *Expert Opin Investig Drugs* 2003; **12**: 841-851.

111 Weinberg ED. Therapeutic potential of iron chelators in diseases associated with iron mismanagement. *J Pharm Pharmacol* 2006; **58**: 575-584.

112 Wexler BC and McMurtry JP. Allopurinol amelioration of the pathophysiology of acute myocardial infarction in rats. *Atherosclerosis* 1981; **39**: 71-87.

113 WHO. Nomenclature and criteria for diagnosis of ischemic heart disease. Report of the Joint International Society and Federation of Cardiology/World Health Organization task force on standardization of clinical nomenclature. *Circulation* 1979; **59**: 607-609.

114 Widimský P and Špaček R. Infarkt myokardu. In *Kardiologie* (Aschermann M, Ed.) Galén Praha 2004; 688-712

115 Wilcox L, Borradaile N, and Huff M. Antiatherogenic Properties of Naringenin, a Citrus Flavonoid. *Cardiovasc Drug Rev* 1999; **17**: 160–178.

116 Winsor T, Mills B, Winbury MM, Howe BB, and Berger HJ. Intramyocardial diversion of coronary blood flow: effects of isoproterenol-induced subendocardial ischemia. *Microvasc Res* 1975; **9**: 261-278.

117 Zhang Y, Li H, Zhao Y, and Gao Z. Dietary supplementation of baicalin and quercetin attenuates iron overload induced mouse liver injury. *Eur J Pharmacol* 2006; **535**: 263-269.

118 Zheng JS, Christie A, De Young MB, Levy MN, and Scarpa A. Synergism between cAMP and ATP in signal transduction in cardiac myocytes. *Am J Physiol* 1992; **262**: C128-135.

III. ARTICLES RELATED TO DOCTORAL THESIS

1. The fate of iron in the organism and its regulatory pathways

Review article

Mladěnka P, Hrdina R, Hübl M, Šimůnek T. The fate of iron in the organism and its regulatory pathways. Acta Medica (Hradec Kralove) 2005; 48(3):127-35

Summary

Iron is an essential element involved in many life-necessary processes. Interestingly, in mammals there is no active excretion mechanism for iron. Therefore iron kinetics has to be meticulously regulated. The most important step for regulation of iron kinetics is absorption. There are several proteins known to participate in iron absorption and its regulation, but present knowledge is still not sufficient for complete understanding of the entire process. Surprisingly, the iron regulation at the molecular level is better described. This article discusses also iron delivery to the cells and iron fate inside the cells.

Iron is an essential element for virtually all living cells. Many life-important processes, among others oxygen transport, ATP production and DNA-synthesis, could not exist without iron. Cellular iron deficiency stops the cell growth and ultimatelly leads to the cell death. The most important property of iron, for which iron is a necessary component of many enzymes, is its ability to donate and receive an electron, i.e. to convert between its ferrous (Fe^{2+}) and ferric form (Fe^{3+}). However this useful feature can be dangerous under some conditions, because iron is also known to generate free radicals.

The body of an adult man consists normally of 35 to 45 mg of iron per kilogram. The largest amount of iron is stored in circulating erythrocytes (1,8 g), parenchymatic cells of the liver (1 g), reticuloendothelial macrophages (0,6 g), bone marrow (0,3 g) and muscles (0,3 g) (6).

Absorption

Absorption takes place in absorptive villi of the small intestine, near of the gastro-duodenal junction (22). At this place, in the proximity of stomach the pH is still low and it assists in the decomposition of dietary iron. Iron is presented in food either locked in heme or as ferric ions bound to some molecule. The process of absorption of heme iron is little known while more information is available about non-heme iron. Firstly, ferric ions have to be converted to ferrous ions. This transformation is realized on the apical (luminal) membrane (71) by means of a heme-based ferric reductase - duodenal cytochrome b (57). Expression of cytochrome b is stimulated by hypoxia, iron deficiency (71) and hypotransferrinaemia and depressed by iron overload (57).

Normally 1 to 2 mg of iron is absorbed daily, the absorption can rarely increase above 6 mg (22).

After the conversion, ferrous ions are transported through apical membrane by divalent metal transporter 1 (DMT-1 or previosly named divalent cationt transporter, DCT-1; natural resistance associated macrophage protein 2, Nramp2) localized on the same membrane. Except Fe^{2+}, DMT-1 has an unusually broad substrate range including also Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}, but interestingly it does not transport calcium and magnesium (34). Xu et al. discovered that a simple mutation can dramatically increase calcium permeability indicating similarity between DMT-1 and calcium channels (86). DMT-1 mediated transport is active, proton-coupled and depends on the cell membrane potential. This 561 amino acids protein with 12 transmembrane segments is ubiquitously expressed, most notably just in the proximal duodenum (34). DMT-1 acts also as intracellular transporter *(see the paragraph "Transport and endocytosis of iron into the cells" and Figure 2).* Importance of DMT-1 was manifested by Fleming et al. who found severe defects in intestinal iron absorption and erythroid iron utilization in microcytic, hypochromic anaemic homozygous mk/mk mice which carry missense (glycine/arginine) mutation in DMT-1 (28). DMT1 is upregulated by dietary iron deficiency (34).

The enterocyte-entered iron can become a part of poorly defined intracellular labile iron pool, be incorporated into ferritin or be released into blood. The mechanism of release into the circulation has not been fully elucidated. Present knowledge indicates that a transmembrane protein named ferroportin1 (named also metal transport protein 1, MTP1) is responsible for this process (1, 50). Overexpression of ferroportin1 in tissue cultures caused intracellular iron depletion (1). Significance of ferroportin1 is documented in zebrafish with mutations in ferroportin1 gene, such animals were unable to synthetize this transporter and developed

hypochromic anemia (23). Ferroportin1 has 10 transmembrane domains and acts probably also as ferric reductase (a NADP/adenine specific site was identified). This may be due to assumption that ferric ions need to be firstly reduced to ferrous ions and than can be transported by ferroportin1 from the cell into the circulation. But a clear evidence that ferroportin1 transports ferrous or/and ferric ions has not yet been published (50). Ferroportin1 is expressed in many cells, but not in plasma membrane of macrophages (1), in jejunum and ileum (23). The function of ferroportin1 in iron metabolism could be different in miscellaneous cells (*see also Regulation of iron metabolism at the molecular level).* Ferroportin1 was also identified at the basal surface of placental syncytiotrophoblasts, which indicates its possible role in delivery of iron from the mother to the fetus (23*).*

Osaki et al. found that ferrous ions exported from the cell require before loading onto transferrin their conversion to ferric ions (66). For some decades it has been known that without copper, iron remains in enterocytes and its movement into the circulation is seriously impaired (52). These two facts together led finally to suggestion that ceruloplasmin, which has ferroxidase activity, can be involved in basolateral iron transport (66). Studying sex-linked anaemic (sla) mice, a ceruloplasmin analogue was discovered and it was named after a Greek god of metalworking, haephastin (81). Haephastin is a multicopper oxidase, which in contrast to soluble ceruloplasmin, has a transmembrane domain and therefore is a membrane protein (24). At the present, haephastin is considered to be involved in intestinal iron transport (20,24) while ceruloplasmin in iron transport in some other tissues, e.g. in the liver (24,35).

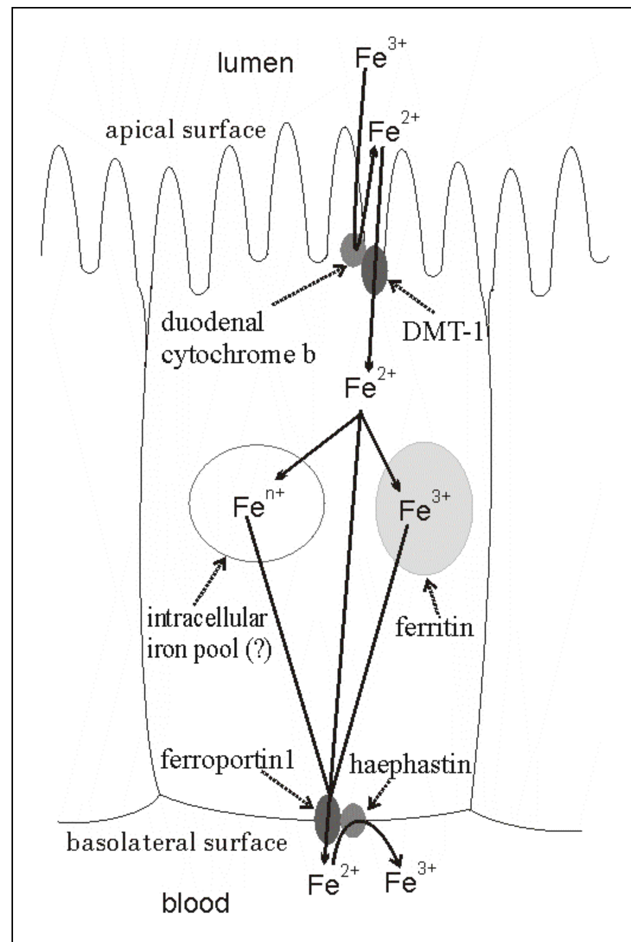


Fig. 1 Schematic illustration showing iron absorption from the lumen of small intestine and its transit through enterocyte. Iron is presented in food mainly in the form of ferric ions, which are converted by the use of duodenal cytochrome b to ferrous ions. Fe^{2+}-ions are transported through the enterocyte membrane by divalent metal transporter-1 (DMT-1). The fate of iron in the enterocyte can be: 1) incorporation into ferritin, 2) becoming part of intracellular labile iron pool or 3) direct transit to the blood. Transport through the enterocyte/blood membrane occurs via ferroportin-1. For loading of iron onto transferrin, its conversion to ferric ions, probably by haephastin, is needed.

Transport and endocytosis of iron into the cells

Transferrin (Tf) is the main iron carrier in human, approximately 3 mg of iron is bound to this protein. Apo-Tf possesses two high affinity ferric binding sites, while it has very low affinity for ferrous ions. Normally only about 30% of Tf is saturated by iron (6). Plasma level of non-transferrin bound iron is extremely low and it normally does not exceed 1 μmol/l and is often under detection limits (4). Non-Tf bound iron represents iron bound to other proteins, like ferritin, and also non-protein iron, probably mainly attached to citrate or possibly the citrate-acetate complex (33). Importance of non-Tf bound iron is accentuated in iron-overload disorders.

Iron-loaded transferrin (Tf-Fe_2) is cleared from the blood by specific binding to its cell membrane receptors, transferrin-receptor 1 (TfR1) or cubilin receptor (48). Transferrin-receptor 2 (TfR2) does not seem to play a role in iron uptake; its role is discussed later. The binding of Tf to TfR1 has been described elsewhere (6, 87). TfR1 exists as a membrane homodimer that binds one diferric Tf per monomer (72). Attachment of Tf to TfR1 results in the endocytosis of the whole complex. The endosome is acidified by ATP-dependent proton pump, iron, apo-Tf and TfR1 are subsequently released from the complex. Apo-Tf and TfR1 return to the cell surface for re-utilization. Ferric ions are reduced to ferrous ions because only Fe^{2+} can be transported through the endocyte-membrane into cytoplasm by the use of DMT-1. There is, however, an important question, whether the reduction of iron occurs before or after being released from Tf. It was shown that Tf-Fe_2 loses ferric ions under acid conditions (30), but also NADH diferric reductase activity on the cell membrane of hepatocytes was documented (77). Ferric ions could be reduced by that reductase to ferrous ions, which, as mentioned above, have low affinity to Tf and can be easily released from Tf. It was pointed out that TfR1 is needed for NADH differic

reductase activity and it is well possible that this reductase enters into endosome together with Tf-TfR1 complex (77). Some authors disclaimed existence of a specific NADH differic reductase (10, 78). Nowadays no clear evidence of such enzyme has been published, but there is also no evidence that differic transferrin reduction does not occur, it may be mediated by some non-specific NADH-reductase.

Recently, another transferrin membrane receptor was discovered - transferrin receptor 2 (TfR2) (44). TfR2 is composed of 2 protein transcripts: α and β. Transcript α manifests 45% identity and 66% similarity to TfR1 extracellular domain. TfR2 expression was found to be limited mainly to the liver, its elevated levels are presented in erythroid precursors while other tissues displayed low expression (44). Surprisingly no TfR2 was detected in mature red blood cells (17). It is suggested that the function of TfR2 is distinct from that of TfR1. There are some indices supporting this assumption: TfR2 affinity to Tf-Fe_2 is 30 times lower as compared to TfR1 and cell expression of TfR2 in cell culture corresponded with cell cycle, rather than with iron levels (43). Mice lacking TfR1 die before birth disclosing importance of TfR1 for erythropoesis and neurological development (55) and insufficiency of TfR2 to substitute the function of TfR1. TfR2 acts probably as an iron regulator, its involvement in iron metabolism is described in detail in the paragraph „*Regulation of iron absorption*."

Interestingly, mammals without Tf can live as it ensues from rare cases of atransferrinemia (12). They manifest hypochromic anemia and have increased iron absorption, but surprisingly their total plasma iron concentration is decreased rather than increased (4) and development of tissues except for the red blood cells is normal (22). Non-Tf iron must therefore be available for tissues apart from the red blood cells precursors that have absolute need for Tf bound iron.

Wright and colleagues performed many experiments on rat liver and described a high efficient, carrier-mediated and saturable mechanism of non-Tf transport (85). But the responsible carrier has not been identified as yet.

Recently multifunctional membrane receptor cubilin was demonstrated to play quantitatively important role in iron supply for the renal proximal tubules. Cubilin mediates the endocytosis of Tf-Fe_2 (48) and therefore can be classified as the third TfR.

At this place, it has to be mentioned a glycoprotein named lactoferrin. This protein reveals a high degree of homology at amino acid sequence level (60) and also the three dimensional conformation level with transferrin (2). Even if the structure of both proteins is very similar, they differ significantly in their localization and function. While transferrin does not allow the existence of free iron in the circulation, lactoferrin may perform the same function on mucosal surfaces. Lactoferrin was also documented to have antimicrobial, anti-inflammatory and antitumoral properties (15,53,79).

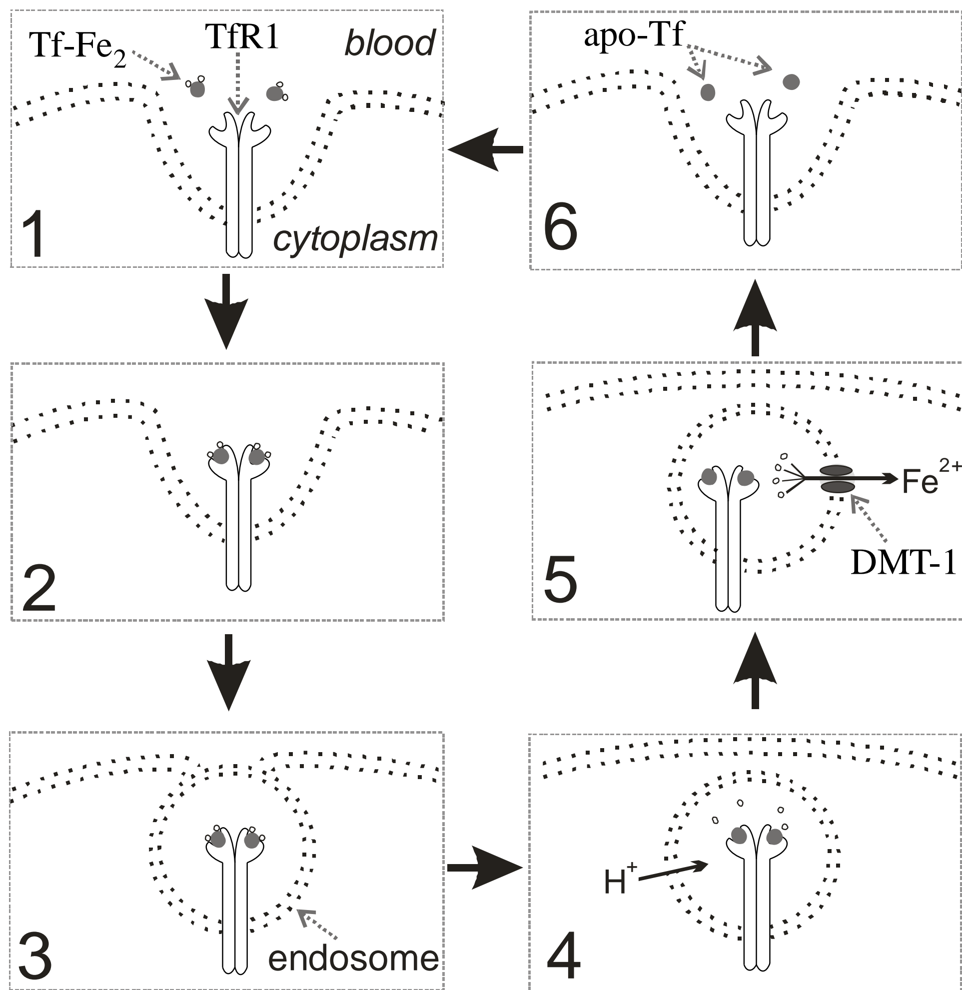


Fig. 2 The schematic view of iron uptake via TfR1-mediated endocytosis in erythroid precursors, as modified according to Andrews, 1999 (6). **1.** Iron-loaded transferrin (Tf-Fe_2) in the blood and TfR1 receptor on the cell surface. **2.** Two Tf-Fe_2 bind to one TfR1. **3.** Internalization of complex Fe-Tf-TfR1. **4.** The endosome is acidified by ATP-dependent proton pump and iron (ferric/ferrous?) is released from the complex. **5.** Ferrous ions are transported through endosome membrane by use of divalent metal transporter 1 (DMT-1). 6. The recyclation of TfR1 and release of apo-Tf into the blood.

Iron inside cells

After the DMT-1-mediated transport the intracellular fate of iron is somewhat unclear. It can be stored inside ferritin, moved to mitochondria, where it can be used for synthesis of heme or Fe-S clusters, or can become a part of mysterious chelatable (labile) iron pool.

Ferritin is a 24-subunits containing protein shell with an inner core where iron is stored as ferrihydrite. The cavity can store up to 4500 atoms of iron, but it usually accommodates about 2000 atoms. Apo-ferritin is heterogenous about 441 kDa large protein composed from variable numbers of 21 kDa heavy (H) or 19 kDa light (L) subunits. Ferritin composition is different in various tissues. Iron passes in form of its ferrous ion through one of the 6 pores of internal cavity of apo-ferritin being oxidized to ferric ion by ferroxidase activity of H-subunit. Inside the ferritin structure, iron becomes a part of a growing crystal of ferrihydrite $(FeOOH)_x$ (40). If iron is needed, it can be easily released from ferritin, but the exact mechanism of iron "escape" and re-reduction to its ferrous form has not yet been published. Normally only isolated particles of ferritin can be seen inside the cells with the exception of the hemopoietic bone marrow and reticuloentdothelial system cells, where more frequent particles can be found (40). Small amount of ferritin normally occurs in the blood and it is usually proportional to the quantity of total body iron store (41).

The second cellular iron store compound is hemosiderin. Hemosiderin is a heterogenous and rather insoluble particle, which contains except iron also proteins, carbohydrates and lipids. It is considered to be a degraded form of ferritin and its localization in siderosomes (40), iron containing lysosomes, suggests its possible danger for the cells. Heavily iron-loaded siderosomes

were shown to be less stable probably due to the iron-induced lipid peroxidation of the lysosomal membrane (69).

While there is quite a good knowledge of iron storage compounds, there is little familiarity concerning iron transport into mitochondria. A protein named frataxin could play some role. Its mutation leads to neurodegenerative disorder described as Friedreich's ataxia. Experiments on yeast with homologous gene revealed an accumulation of iron in the mitochondrion (70). Accumulation of iron inside mitochondria was observed also in patients suffering from Friedreich's ataxia in some tissues (14). Therefore it has been thought that frataxin could play some role in the iron transport from and/or into mitochondria. Defects in Fe-S protein assembly have been recently observed in experiments on yeast implying possible involvement of frataxin in Fe-S cluster maturation (61).

Excretion and iron recirculation

Humans have no specific excretion mechanism for iron. Iron is eliminated only by exfoliation of enterocytes or by menstruation bleeding. The eliminated amount usually corresponds with the absorbed amount, which is normally 1 to 2 mg/day.

As mentioned above, most iron is localized in the red blood cells. At the end of their life, the red blood cells are phagocyted by specialized population of reticuloendothelial macrophages. Degradation of hemoglobin liberates iron which is then returned to the circulation by binding to transferrin. Transferrin then distributes the iron through the body and delivers it to iron-requiring cells, preferentially again for hemoglobin synthesis. When iron is needed for hemoglobin synthesis, most cells are capable to release it into the circulation.

Iron-containing proteins

As mentioned above, due to its ability of accept and donate electron iron forms the essential part of many enzymes. Iron is a common component of metalloproteins, where it can be directly ligated to the protein through Fe-S bound or be firmly closed in the heme structure. Heme proteins, including hemoglobin, myoglobin and many enzymes (cytochromes P-450, cytochromeoxidases, peroxidases) are known longer than the Fe-S proteins. Discovery of iron-sulfur clusters, where iron is bound to the protein through sulfur, opened in the 1960s new insight into iron metabolism and function (46). It was pointed out that Fe-S clusters form enzymatic sites of dehydratases, e.g. bacterial enzymes and human aconitases (8), and also the human enzyme succinate dehydrogenase (76).

Enzyme aconitase (aconitase hydratase) converts citrate to isocitrate. There are two different aconitases in mammals, 83 kDa large mitochondrial (m-acon) and 98 kDa cytosolic (c-acon). Both aconitases are encoded by nuclear DNA but their genes are located on different chromosomes. Both are very similar 4-domains proteins with 30% sequence identity and containing [4Fe-4S] cluster necessary for their enzymatic activity (73). Interestingly, acon is an unusual case of Fe-S proteins, because only three irons are ligated directly to cysteines of protein while the fourth (marked as Fe_a, *see also Fig.4*) is attached to an inorganic sulfur of cluster and a hydroxyl group (8). Indispensability of Fe_a for enzymatic activity of acon ensues from the fact that it represents the binding site for carboxyl and hydroxyl groups of citrate (9). After translation m-acon is directed to mitochondria, where it executes an important step of Krebs cycle. Conversely, the enzymatic role of c-acon is obscure, it may regulate the non-protein bound iron (65), the non-enzymatic role of c-acon is discussed in the next paragraph.

Succinate dehydrogenase is another Krebs cycle enzyme. It comprises 3 different types of Fe-S clusters, [4Fe-4S], [3Fe-3S] and [2Fe-2S] (76), representing the only known enzyme containing all known types of Fe-S clusters.

Regulation of iron metabolism at the molecular level

There is quite a good knowledge concerning the posttranscriptional regulation of iron metabolism. Iron regulatory elements (IREs) were identified in the untranslated regions of mRNA encoding miscellaneous proteins involved in iron kinetics and energy metabolism. IREs are about 30-nucleotides long stem-loop, or more precisely stem-bulge-stem-loop (*see Fig. 3*), structures that present specific binding sites for cytoplasmic iron regulatory proteins (IRPs) IRP1 and IRP2.

Under conditions of iron excess, IRPs do not possess affinity to bind IREs, on the contrary, when iron is scarce, IRPs bind to IREs. It is generally accepted that if IRE lies in the 5´end of mRNA, IRE-IRP binding inhibits mRNA translation. If it lies in the 3´end, the IRE-IRP binding protects mRNA against degradation and synthesis of protein is enhanced (47). This rule can be applied in many proteins associated with iron and energy metabolism containing IREs. IREs were found in 5´end of mRNA of H- and L- ferritin chains (7), m-acon (75) or erythroid 5-aminolevulic acid synthase (e-alas) (13,21,59), a key protein in erythroid heme synthesis. Binding of IRPs to IREs of these mRNAs stops protein synstesis by inhibiting the stable association of mRNA with the small ribosomal subunit (31). Increased levels of iron therefore raise synthesis of ferritin as well as heme/hemoglobin and ATP production (49,75). At the 3´end of TfR1 mRNA five types of IREs, named A-E, were found (19). Between the IRE C and IRE D lies an instable site, which can be easily recognized by nucleases and the mRNA degradation

consequently starts. If IRP binds to IRE, this instable site is not accesible for endonucleotic clip, mRNA is stabilized and TfR1 synthesis enabled (39). In TfR2 mRNAs, no known IRP was detected, suggesting another type of regulation (44).

Discovery of ferroportin1 regulation represents only one known exception at this time. Ferroportin1 mRNA contains IRE as well in its 5´untranslated region (1,58). But its regulation is somewhat mysterious. In duodenal epithelial cells of iron-deprived mice an augmented expression of ferroportin1 was documented while in iron-replete it diminished. The reverse situation prevails in Kupffer cells of the liver: iron-deprived showed less expression while iron-repletion augmented expression (1).

On the basis of phylogenetic comparison, IREs are considered as strong conserved mRNA structures (80). Henderson et al. examined an optimal sequence and structure of IRE. They confirmed that loop and bulge seem to be the most necessary part of IRE. The loop was formerly considered to have 6 unpaired bases –CAGUGX (X – any nucleotide). Henderson et al. outlined paring between pyrimidine and purine bases at positions 1 and 5, respectively. In known IREs cytosine (pos. 1) and guanidine (pos. 5) are localized at these positions. Also combination of uracil (pos.1) and adenine (pos. 5) is sufficient to maintain the function of IRP1, but such combination has not yet been discovered in natural mRNAs (37). In harmony with this finding, positions 1 and 5 are much less accessible to chemical and nuclease attack than other loop positions (11). NMR spectroscopy confirmed the existence of hydrogen bond between positions 1 and 5 (49). It should be emphasized, that IRP2 requires conserved cytosine-guanidine pair (45). Three "free" nucleotides (AGU) at loop positions 2, 3 and 4 and unpaired cytosine bulge seems to be specific binding sites for IRP contact. Any substitution at these positions largely decreases

the IRP-IRE binding in most cases (37). On the contrary, nucleotides in stems can vary if the base-pairing remains retained. Disrupting base-pairing of the upper stem prevents or broadly decreases IRE-IRP binding (11, 54).

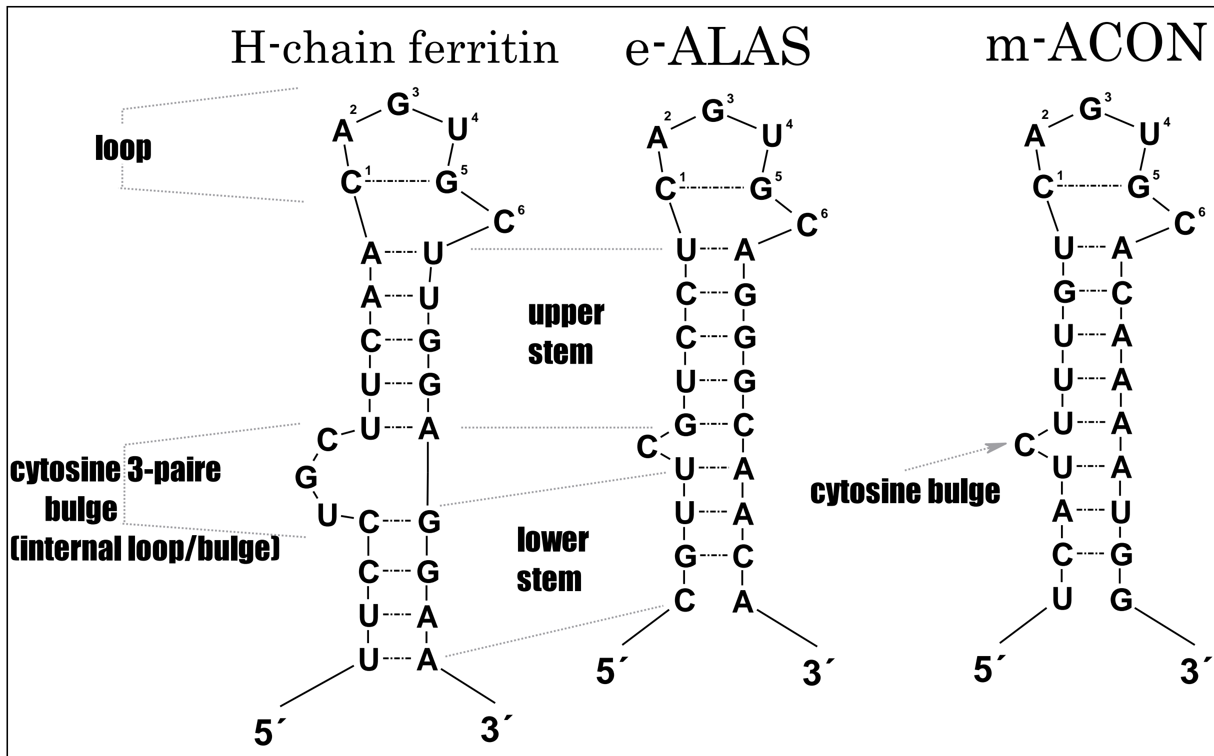


Fig. 3 Structures of some IREs. The primary structures of H-chain ferritin according to Henderson et al., 1994 (37), of e-alas and m-acon according to Ke et al., 1998 (45).

Iron regulatory proteins IRP1 and IRP2 have high sequence identity (57% identical and 79% similar) except for a 73 amino acid insertion in the case of IRP2 (73). IRP1 (formerly termed as iron regulatory factor IRF or iron responsive element binding protein IRE-BP) is 98 kDa bifunctional protein of 889 amino acids containing iron-sulfur cluster. When intracellular

iron is abundant, protein possess [4Fe-4S] cluster and act as c-acon, while the condition of low intracellular iron level occurs, the cluster somewhat decomposes and acts as IRP1. The disintegration of cluster involves more profound changes than a simple lose of Fe_a atom (*see previous section and Fig. 4*) (16). It should be mentioned, that m-acon lacks the IRE-binding activity (46). IRP2 (formerly assigned as IRP_B) is a 105 kDa protein (38) containing 963 amino acids (73). It is not composed of iron-sulfur cluster and it lacks aconitase activity (35). Like IRP1 also IRP2 binds to IREs in the state of iron deficiency. When iron is in excess, IRP2 is enzymatically degraded (45). Though IRP1 and IRP2 are very similar, they bind various IREs with different affinities: IRP1 affinity to all known IREs was detected to be analogous, but IRP2 binds 10-times more IREs with 3-nucleotide bulge than IREs with single cytosine bulge (45).

The regulation of various proteins involved in iron kinetics and energy metabolism depends on the strengh of IRP-IRE binding ensuing from IRE sequence. Supression of ferritin synthesis in iron-deplete condition is more efficient than those of m-acon (75), e-alas (59) or TfR1 (45). Ferritin mRNA therefore seems to be the most sensitive target for IRPs. The easiest explanation can ensue from the C-bulge structure. Cytosin 3-base bulge (sometimes described as internal loop/bulge) opposing one nucleotide presented in ferririn mRNA appeared to be 3-fold more effective than as single unpaired cytosine (37) presented in TfR, e-alas and m-acon mRNAs. Discovery of IRP2 may play the crucial role because of its higher affinity to ferritin IRE containing 3-base bulge than to other IREs with single cytosine, as described above. The distribution of IRPs in the organism is different. IRP2 prevails in the brain while IRP1 is more profoundly expressed in other tissues (73). High concentration of IRP2 was found out also in the

intestine in experiments with rodents (37). These findings, however, cannot sufficiently explain IRP2 contribution to iron protein regulation.

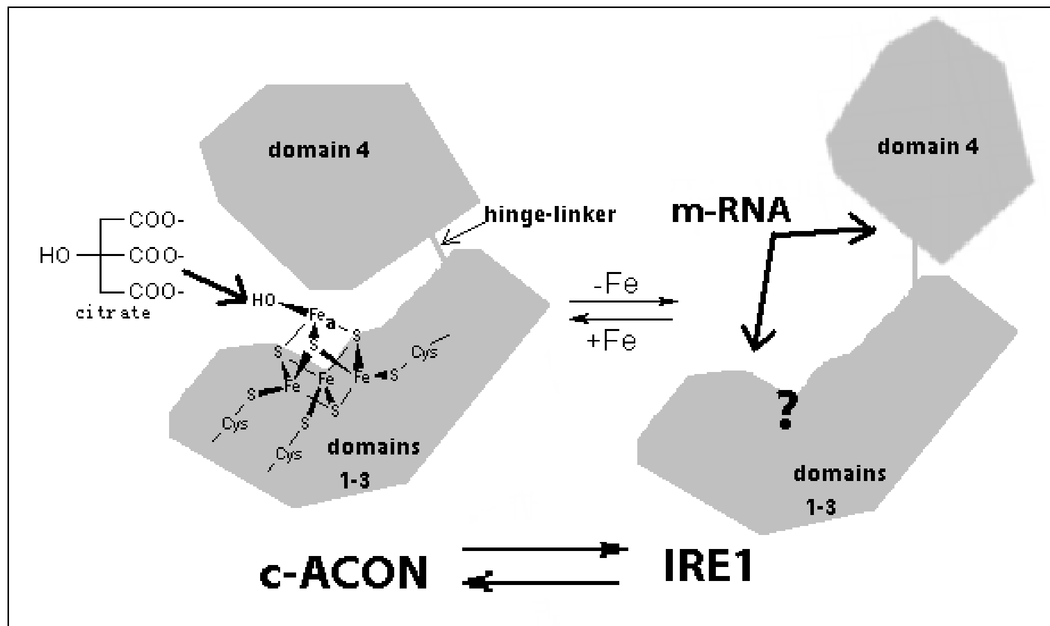


Fig. 4. The double function of iron responsible protein 1 / cytosolic aconitase. The structure of the cluster and protein according to Beinert and Kennedy, 1989 (9) and Klausner and Rouault, 1993 (46), respectively. In iron-replete status, the protein acts as c-acon, in iron deplete status, iron is released from the cluster, the cluster somewhat decomposes and the whole protein acts as IRP1. The double-function protein IRP1/c-acon is 4-domains protein with a cleft between domains 1-3 and 4 that is connected by a flexible hinge linker.

Phosphorylation of IRPs may explain some questions. Eisenstein et al. found two specific sites (around Ser 138 and Ser 711) of IREs which can be phosporylated. It is of interest that IRP1

contains both specific sites, but IRP2 only one with the important change of serine to alanine (27). Both sites were proven to be highly conserved in vertabrates (25, 27). It was documented that phosphorylation of Ser-138 site of IRP1 causes loss of c-acon activity by an impairment of Fe-S cluster (16,27). Previously, the enhanced IRE-IRS binding of phosphorylated IRPs was found (25). Interestingly, on Ser-138 phosphorylated IRP1 appears to be stabilized by iron deprivation but in situations of iron abundance it undergoes degradation similar to dissociation of IRP2 (27). Therefore phosphorylation can represent a regulatory mechanism with involvement of either iron or some other signals.

At least, it can be stated that the affinities of IRPs to IREs and distribution of IRPs are not accidental and reflect the accuracy of regulation. For the cell it is important to have some level of m-acon, because of its exigency in ATP synthesis. On the other hand, huge ferritin synthesis could create a deficiency in accessibility of iron for iron-protein synthesis (75). In the future, discovery of new proteins regulated by IRE sequences is awaited. At present their research is difficult due to variation of IRE-sequence not enabling fast analysis.

Iron was found to be the responsible element for regulation of some other proteins but not via the IRP-IRE system. In Drosophila IRE was found in the mRNA of succinate dehydrogenase (47), in human mRNA of the homologous enzyme such IRE is not comprised, but succinate dehydrogenase is still regulated by iron status. Iron supplementation was found to increase activity of succinate dehydrogenase as well as isocitrate dehydrogenase (64). This finding in the concordance with the described regulation of m-acon accentuates enhanced NADH and ATP production in the case of iron abundance (64).

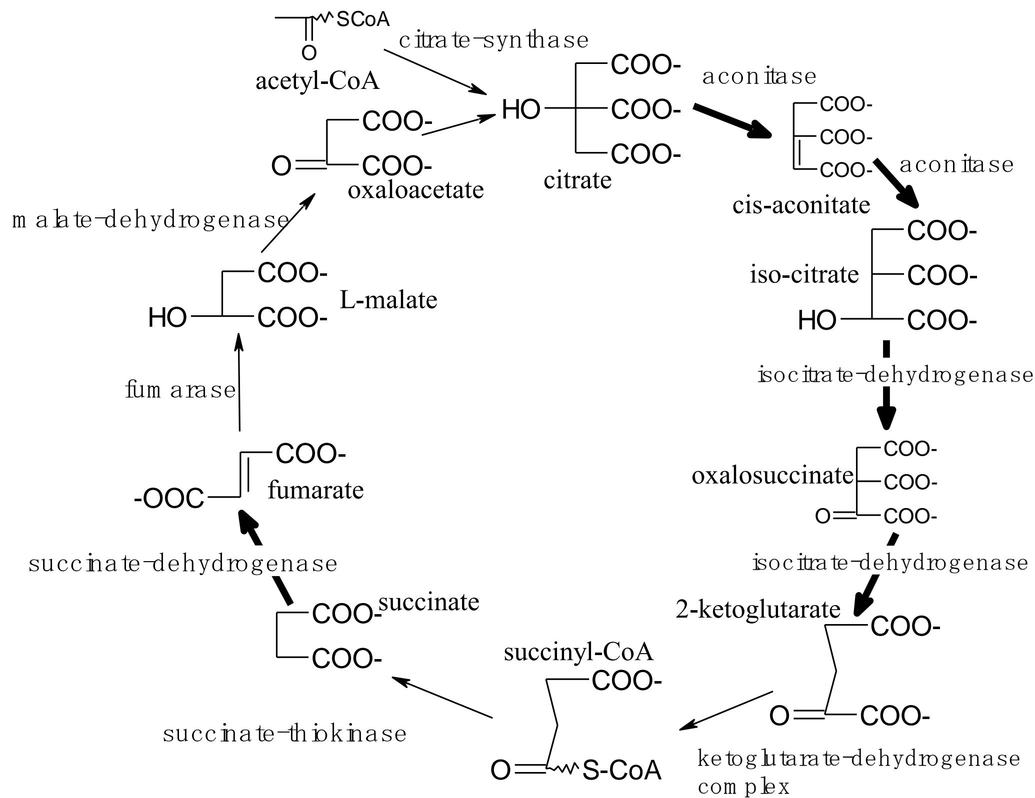


Fig. 5. Krebs cycle with iron regulated enzymes. Thick arrows show three enzymes which are known to be regulated by iron status.

Regulation of iron absorption

As generally accepted, the excess of iron is toxic for the cells. Because mammals lack a regulatory pathway for an active iron excretion, iron levels have to be tightly regulated by iron absorption.

For many years the process of regulation of iron absorption was so foggy, that terms like “stores regulator”, “erythropoetic regulator” and “mucosal block” (absorption regulator) were used to express three proposed ways for that regulation. The present knowledge is still not

sufficient, but it is known that at least two of these vague regulators may represent protein called hepcidin.

Hepcidin is a 25-amino acid peptide hormone, which contains 8 cysteine-residues, all connected by 4 intramolecular disulfide bounds. It is produced by the liver as a 84-amino acid long precursor. The name reflects both its site of origin and its significant antimicrobial properties (68). The group of Nicolas demonstrated dependence of iron status on the hepcidin levels: Transgenic animals overexpressing hepcidin have decreased levels of body iron, on the contrary mice lacking hepcidin manifested progressive iron accumulation though surprisingly dramatic decrease of iron stores in reticuloendothelial cells. Experimentally induced anemia decreases hepcidin mRNA levels in mice (62,63).

Patients with large hepatic adenomas have severe iron refractory anemia. Adenoma produces inappropriately high levels of hepcidin, when adenoma was resected or the liver was transplanted, anemia resolved spontaneously (84).

Decreased iron content in the diet evoked augmented formation of duodenal cytochrome b, DMT-1 and ferroportin1 (hephaestin did not changed) and depression in hepcidin synthesis in the liver (5). In conclusion, hepcidin has been proposed as a negative regulator of iron absorption and reticuloentothelial macrophage iron release.

Important role in the regulation of iron absorption plays also HFE-protein. It is a 343 amino acids integral membrane protein which reveals a tight homology to a major histocompatibility complex class I-like protein. Its significance in iron metabolism has been acknowledged for many years because of its defect in hereditary hemochromatosis, the most common autosomal recessive disorder known in human. Patients suffering from this disease show increased iron

absorption and develop iron overload. HFE-protein is localized in association with TfR1 and β_2-microglobulin in duodenum, predominantly in the crypt enterocytes and in the placenta (83). Lebron et al. showed that if HFE-protein is bound to TfR1, it reduces affinity of receptor for iron-loaded transferrin (51). However this presumption is questioned by others as the reported nanomolar changes in affinity do not seem to influence receptor binding properties (83). It was found out that one type of mutation in HFE (C282Y) inhibited HFE-protein interaction with β_2-microglobulin (26), leading to accelerated degradation of mutated HFE-protein (82). On the one hand, mice lacking β_2-microglobulin were shown to have the same manifestations like in hereditary hemochromatosis, i.e. impaired iron absorption regulation associated with increased iron absorption and iron overload (74), seemingly proposing important role of β_2-microglobulin in iron absorption control. But on the other hand, the second most common mutation of HFE gene (H63D), which was demonstrated to aggravate iron overload status (56), did not inhibit the interaction of β_2-microglobulin with HFE-protein (26). Mice with decreased levels of TfR1 seemed to have reduced iron absorption (55).

Present knowledge proposes that the level of serum transferrin-bound iron informs crypt enterocytes via HFE-protein and TfR1 about the total body iron status. If the body iron stores are low, crypt cells are targeted to differentiate in enterocytes with programmed elevated absorption of iron (3). This seems to be likely, because the response of iron demand lasts 2 or 3 days and the same period is necessary for crypt cells to migrate and differentiate in villus enterocytes. The programming of enterocytes involves the raised synthesis of DMT-1. This is well documented in hereditary hemochromatosis, where the augmented expression of DMT-1 was discovered (34, 88). Precise mechanism how HFE is involved in iron metabolism is not known nowadays.

Protein	The role in iron kinetics and its regulation
ceruloplasmin	oxidation of cell-released iron before binding to transferrin
cubilin	transport of iron in the renal proximal tubular cells
DMT-1	iron transport from the small intestine lumen into enterocytes and from endosome into cytoplasm
ferritin	iron storage
ferroportin1	iron transport from cell into the blood
haephastin	oxidation of cell-released iron before loading on transferrin
hemojuvelin	regulation of iron kinetics, modulator of hepcidin expression?
hemosiderin	iron storage (detoxication)
hepcidin	negative regulator of iron absorption and reticuloentothelial macrophage iron release ?
HFE-protein	regulation of iron kinetics ?
IRP1	regulation of iron kinetics
IRP2	regulation of iron kinetics
lactoferrin	iron chelation on mucosal surfaces and the involvement in the body defence against pathogens
TfR1	iron transport from the blood into cells
TfR2	regulation of iron kinetics?
transferrin	transport of iron in the circulation

Tab. 1 The overview of proteins related to iron kinetics and its regulation.

From the recent studies it ensues that TfR2 plays an important role in the iron regulation. Mice with mutation of TfR2 manifested hemochromatosis (29). Humans with mutated TfR2 suffer also from hemochromatosis, a disorder very similar to the hereditary hemochromatosis caused by HFE mutation (18). The link between the HFE and TfR2 is supported by finding that TfR2 and HFE-protein co-localize in the crypt duodenal cells (32).

Recently, a new mutation in HFE2 gene responsible for protein named hemojuvelin was discovered. The mutation in HFE2 causes juvenile hemochromatosis, a disorder

indistinguishable from hepcidin-deficiency. Hemojuvelin localization is restricted to similar tissues as that of hepcidin. Analysis of hemojuvelin reveals its possible function as membrane-bound receptor or secreted polypeptide hormone. Deleterious mutation of hemojuvelin reduces hepcidin levels despite iron overload (in such condition hepcidin expression is normally induced). It is thought that hemojuvelin acts as modulator of hepcidin expression (67) but further studies are needed for verification.

Conclusion

This review attempted to shed light on the fate of iron in the organism. Because the topic is large, it was necessary to omit willfully some important tasks. Anemias and diseases with iron overload were mentioned only for explication of physiological ways of iron metabolism. The fate of iron in the brain was reviewed successfully by Zecca et al. (87).

Abbreviations

DMT-1, divalent metal transporter; IRP1, iron regulatory protein 1; IRP2, iron regulatory protein 2; Tf, transferrin; Tf-Fe_2, iron-loaded transferrin; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2; c-acon, cytosolic aconitase; m-acon; mitochondrial aconitase; Ser, serin.

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References

1. Abboud S, Haile DJ. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Biol Chem. 2000 Jun 30;275(26):19906-12.

2. Abdallah FB, El Hage Chahine JM. Transferrins: iron release from lactoferrin.J Mol Biol. 2000 Oct 20;303(2):255-66.
3. Anderson GJ. Control of iron absorption. J Gastroenterol Hepatol. 1996 Nov;11(11):1030-2.
4. Anderson GJ. Non-transferrin-bound iron and cellular toxicity. J Gastroenterol Hepatol. 1999 Feb;14(2):105-8.
5. Anderson GJ, Frazer DM, Wilkins SJ et al. Relationship between intestinal iron-transporter expression, hepatic hepcidin levels and the control of iron absorption. Biochem Soc Trans. 2002 Aug;30(4):724-6.
6. Andrews NC. Disorders of iron metabolism. N Engl J Med. 1999 Dec 23;341(26):1986-95. Review. No abstract available. Erratum in: N Engl J Med 2000 Feb 3;342(5):364.
7. Aziz N, Munro HN. Iron regulates ferritin mRNA translation through a segment of its 5' untranslated region. Proc Natl Acad Sci U S A. 1987 Dec;84(23):8478-82.
8. Beinert H. Iron-sulphur clusters: agents of electron transfer and storage, and direct participants in enzymic reactions. Tenth Keilin memorial lecture. Biochem Soc Trans. 1986 Jun;14(3):527-33.
9. Beinert H, Kennedy MC. 19th Sir Hans Krebs lecture. Engineering of protein bound iron-sulfur clusters. A tool for the study of protein and cluster chemistry and mechanism of iron-sulfur enzymes. Eur J Biochem. 1989 Dec 8;186(1-2):5-15.
10. Berczi A, Sizensky JA, Crane FL, Faulk WP. Diferric transferrin reduction by K562 cells. A critical study. Biochim Biophys Acta. 1991 Apr 9;1073(3):562-70.
11. Bettany AJ, Eisenstein RS, Munro HN. Mutagenesis of the iron-regulatory element further defines a role for RNA secondary structure in the regulation of ferritin and transferrin receptor expression. J Biol Chem. 1992 Aug 15;267(23):16531-7.
12. Beutler E, Gelbart T, Lee P, Trevino R, Fernandez MA, Fairbanks VF. Molecular characterization of a case of atransferrinemia. Blood. 2000 Dec 15;96(13):4071-4.
13. Bhasker CR, Burgiel G, Neupert B, Emery-Goodman A, Kuhn LC, May BK. The putative iron-responsive element in the human erythroid 5-aminolevulinate synthase mRNA mediates translational control. J Biol Chem. 1993 Jun 15;268(17):12699-705.
14. Bradley JL, Blake JC, Chamberlain S, Thomas PK, Cooper JM, Schapira AH. Clinical, biochemical and molecular genetic correlations in Friedreich's ataxia. Hum Mol Genet. 2000 Jan 22;9(2):275-82.
15. Britigan BE, Serody JS, Cohen MS. The role of lactoferrin as an anti-inflammatory molecule. Adv Exp Med Biol. 1994;357:143-56.
16. Brown NM, Kennedy MC, Antholine WE, Eisenstein RS, Walden WE. Detection of a [3Fe-4S] cluster intermediate of cytosolic aconitase in yeast expressing iron regulatory protein 1. Insights into the mechanism of Fe-S cluster cycling. J Biol Chem. 2002 Mar 1;277(9):7246-54.
17. Calzolari A, Deaglio S, Sposi NM et al. Transferrin receptor 2 protein is not expressed in normal erythroid cells. Biochem J. 2004 Aug 1;381(Pt 3):629-34.
18. Camaschella C, Roetto A, Cali A et al. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. Nat Genet. 2000 May;25(1):14-5.
19. Casey JL, Hentze MW, Koeller DM et al. Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. Science. 1988 May 13;240(4854):924-8.
20. Chen H, Attieh ZK, Su T et al. Hephaestin is a ferroxidase that maintains partial activity in sex-linked anemia mice. Blood. 2004 May 15;103(10):3933-9.
21. Dandekar T, Stripecke R, Gray NK et al. Identification of a novel iron-responsive element in murine and human erythroid delta-aminolevulinic acid synthase mRNA. EMBO J. 1991 Jul;10(7):1903-9.
22. Donovan A, Andrews NC. The molecular regulation of iron metabolism. Hematol J. 2004;5(5):373-80.
23. Donovan A, Brownlie A, Zhou Y et al. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature. 2000 Feb 17;403(6771):776-81.
24. Eisenstein RS. Discovery of the ceruloplasmin homologue hephaestin: new insight into the copper/iron connection. Nutr Rev. 2000 Jan;58(1):22-6.

25. Eisenstein RS, Tuazon PT, Schalinske KL, Anderson SA, Traugh JA. Iron-responsive element-binding protein. Phosphorylation by protein kinase C. J Biol Chem. 1993 Dec 25;268(36):27363-70.
26. Feder JN, Tsuchihashi Z, Irrinki A, Lee VK, Mapa FA, Morikang E, Prass CE, Starnes SM, Wolff RK, Parkkila S, Sly WS, Schatzman RC. The hemochromatosis founder mutation in HLA-H disrupts beta2-microglobulin interaction and cell surface expression. J Biol Chem. 1997 May 30;272(22):14025-8.
27. Fillebeen C, Chahine D, Caltagirone A, Segal P, Pantopoulos K. A phosphomimetic mutation at Ser-138 renders iron regulatory protein 1 sensitive to iron-dependent degradation. Mol Cell Biol. 2003 Oct;23(19):6973-81.
28. Fleming MD, Trenor CC 3rd, Su MA et al. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat Genet. 1997 Aug;16(4):383-6.
29. Fleming RE, Ahmann JR, Migas MC et al. Targeted mutagenesis of the murine transferrin receptor-2 gene produces hemochromatosis. Proc Natl Acad Sci U S A. 2002 Aug 6;99(16):10653-8.
30. Gaber BP, Aisen P. Is divalent iron bound to transferrin? Biochim Biophys Acta. 1970 Nov 17;221(2):228-33.
31. Gray NK, Hentze MW. Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and e-alas mRNAs. EMBO J. 1994 Aug 15;13(16):3882-91.
32. Griffiths WJ, Cox TM. Co-localization of the mammalian hemochromatosis gene product (HFE) and a newly identified transferrin receptor (TfR2) in intestinal tissue and cells. J Histochem Cytochem. 2003 May;51(5):613-24.
33. Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A, Sadler PJ. Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. J Biol Chem. 1989 Mar 15;264(8):4417-22.
34. Gunshin H, Mackenzie B, Berger UV et al. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature. 1997 Jul 31;388(6641):482-8.
35. Guo B, Yu Y, Leibold EA. Iron regulates cytoplasmic levels of a novel iron-responsive element-binding protein without aconitase activity. J Biol Chem. 1994 Sep 30;269(39):24252-60.
36. Harris ZL, Durley AP, Man TK, Gitlin JD. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci U S A. 1999 Sep 14;96(19):10812-7.
37. Henderson BR, Menotti E, Bonnard C, Kuhn LC. Optimal sequence and structure of iron-responsive elements. Selection of RNA stem-loops with high affinity for iron regulatory factor. J Biol Chem. 1994 Jul 1;269(26):17481-9.
38. Henderson BR, Seiser C, Kuhn LC. Characterization of a second RNA-binding protein in rodents with specificity for iron-responsive elements. J Biol Chem. 1993 Dec 25;268(36):27327-34.
39. Hentze MW, Kuhn LC. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci U S A. 1996 Aug 6;93(16):8175-82
40. Iancu TC. Ferritin and hemosiderin in pathological tissues. Electron Microsc Rev. 1992;5(2):209-29.
41. Jacobs A, Worwood M. Ferritin in serum. Clinical and biochemical implications. N Engl J Med. 1975 May 1;292(18):951-6.
42. Juang HH. Modulation of iron on mitochondrial aconitase expression in human prostatic carcinoma cells. Mol Cell Biochem. 2004 Oct;265(1-2):185-94.
43. Kawabata H, Germain RS, Vuong PT, Nakamaki T, Said JW, Koeffler HP. Transferrin receptor 2-alpha supports cell growth both in iron-chelated cultured cells and in vivo. J Biol Chem. 2000 Jun 2;275(22):16618-25.
44. Kawabata H, Yang R, Hirama T et al. Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. J Biol Chem. 1999 Jul 23;274(30):20826-32.

45. Ke Y, Wu J, Leibold EA, Walden WE, Theil EC. Loops and bulge/loops in iron-responsive element isoforms influence iron regulatory protein binding. Fine-tuning of mRNA regulation? J Biol Chem. 1998 Sep 11;273(37):23637-40.
46. Klausner RD, Rouault TA. A double life: cytosolic aconitase as a regulatory RNA binding protein. Mol Biol Cell. 1993 Jan;4(1):1-5.
47. Kohler SA, Henderson BR, Kuhn LC. Succinate dehydrogenase b mRNA of Drosophila melanogaster has a functional iron-responsive element in its 5'-untranslated region. J Biol Chem. 1995 Dec 22;270(51):30781-6.
48. Kozyraki R, Fyfe J, Verroust PJ et al. Megalin-dependent cubilin-mediated endocytosis is a major pathway for the apical uptake of transferrin in polarized epithelia. Proc Natl Acad Sci U S A. 2001 Oct 23;98(22):12491-6.
49. Laing LG, Hall KB. A model of the iron responsive element RNA hairpin loop structure determined from NMR and thermodynamic data. Biochemistry. 1996 Oct 22;35(42):13586-96.
50. Le NT, Richardson DR. Ferroportin1: a new iron export molecule? Int J Biochem Cell Biol. 2002 Feb;34(2):103-8.
51. Lebron JA, Bennett MJ, Vaughn DE et al. Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. Cell. 1998 Apr 3;93(1):111-23.
52. Lee GR, Nacht S, Lukens JN, Cartwright GE. Iron metabolism in copper-deficient swine. J Clin Invest. 1968 Sep;47(9):2058-69.
53. Legrand D, Elass E, Pierce A, Mazurier J. Lactoferrin and host defence: an overview of its immuno-modulating and anti-inflammatory properties. Biometals. 2004 Jun;17(3):225-9.
54. Leibold EA, Laudano A, Yu Y. Structural requirements of iron-responsive elements for binding of the protein involved in both transferrin receptor and ferritin mRNA post-transcriptional regulation. Nucleic Acids Res. 1990 Apr 11;18(7):1819-24.
55. Levy JE, Jin O, Fujiwara Y, Kuo F, Andrews NC. Transferrin receptor is necessary for development of erythrocytes and the nervous system. Nat Genet. 1999 Apr;21(4):396-9.
56. Martins R, Picanco I, Fonseca A et al. The role of HFE mutations on iron metabolism in beta-thalassemia carriers. J Hum Genet. 2004 Nov 5 J Hum Genet. 2004;49(12):651-5
57. McKie AT, Latunde-Dada GO, Miret S et al. Molecular evidence for the role of a ferric reductase in iron transport. Biochem Soc Trans. 2002 Aug;30(4):722-4.
58. McKie AT, Marciani P, Rolfs A et al. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell. 2000 Feb;5(2):299-309.
59. Melefors O, Goossen B, Johansson HE, Stripecke R, Gray NK, Hentze MW. Translational control of 5-aminolevulinate synthase mRNA by iron-responsive elements in erythroid cells. J Biol Chem. 1993 Mar 15;268(8):5974-8.
60. Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J, Jolles P. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur J Biochem. 1984 Dec 17;145(3):659-76
61. Muhlenhoff U, Richhardt N, Ristow M, Kispal G, Lill R. The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins. Hum Mol Genet. 2002 Aug 15;11(17):2025-36.
62. Nicolas G, Bennoun M, Porteu A et al. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4596-601.
63. Nicolas G, Chauvet C, Viatte L et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest. 2002 Oct;110(7):1037-44
64. Oexle H, Gnaiger E, Weiss G. Iron-dependent changes in cellular energy metabolism: influence on citric acid cycle and oxidative phosphorylation. Biochim Biophys Acta. 1999 Nov 10;1413(3):99-107.
65. O'Halloran TV. Transition metals in control of gene expression. Science. 1993 Aug 6;261(5122):715-25.

66. Osaki S, Johnson DA, Frieden E. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J Biol Chem. 1966 Jun 25;241(12):2746-51.

67. Papanikolaou G, Samuels ME, Ludwig EH et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet. 2004 Jan;36(1):77-82.

68. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem. 2001 Mar 16;276(11):7806-10.

69. Peters TJ, Seymour CA. Acid hydrolase activities and lysosomal integrity in liver biopsies from patients with iron overload. Clin Sci Mol Med. 1976 Jan;50(1):75-8.

70. Radisky DC, Babcock MC, Kaplan J. The yeast frataxin homologue mediates mitochondrial iron efflux. Evidence for a mitochondrial iron cycle. J Biol Chem. 1999 Feb 19;274(8):4497-9.

71. Raja KB, Simpson RJ, Peters TJ. Investigation of a role for reduction in ferric iron uptake by mouse duodenum. Biochim Biophys Acta. 1992 Jun 10;1135(2):141-6.

72. Richardson DR. Mysteries of the transferrin-transferrin receptor 1 interaction uncovered. Cell. 2004 Feb 20;116(4):483-5.

73. Samaniego F, Chin J, Iwai K, Rouault TA, Klausner RD. Molecular characterization of a second iron-responsive element binding protein, iron regulatory protein 2. Structure, function, and post-translational regulation. J Biol Chem. 1994 Dec 9;269(49):30904-10.

74. Santos M, Schilham MW, Rademakers LH, Marx JJ, de Sousa M, Clevers H. Defective iron homeostasis in beta 2-microglobulin knockout mice recapitulates hereditary hemochromatosis in man. J Exp Med. 1996 Nov 1;184(5):1975-85.

75. Schalinske KL, Chen OS, Eisenstein RS. Iron differentially stimulates translation of mitochondrial aconitase and ferritin mRNAs in mammalian cells. Implications for iron regulatory proteins as regulators of mitochondrial citrate utilization. J Biol Chem. 1998 Feb 6;273(6):3740-6.

76. Singer TP, Johnson MK. The prosthetic groups of succinate dehydrogenase: 30 years from discovery to identification. FEBS Lett. 1985 Oct 14;190(2):189-98.

77. Sun IL, Navas P, Crane FL, Morre DJ, Low H. NADH diferric transferrin reductase in liver plasma membrane. J Biol Chem. 1987 Nov 25;262(33):15915-21.

78. Thorstensen K, Aisen P. Release of iron from diferric transferrin in the presence of rat liver plasma membranes: no evidence of a plasma membrane diferric transferrin reductase. Biochim Biophys Acta. 1990 Apr 9;1052(1):29-35

79. Valenti P, Berlutti F, Conte MP, Longhi C, Seganti L. Lactoferrin functions: current status and perspectives. J Clin Gastroenterol. 2004 Jul;38(6 Suppl):S127-9

80. von Darl M, Harrison PM, Bottke W. cDNA cloning and deduced amino acid sequence of two ferritins: soma ferritin and yolk ferritin, from the snail Lymnaea stagnalis L. Eur J Biochem. 1994 Jun 1;222(2):353-66.

81. Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat Genet. 1999 Feb;21(2):195-9.

82. Waheed A, Parkkila S, Zhou XY et al. Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with beta2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. Proc Natl Acad Sci U S A. 1997 Nov 11;94(23):12384-9.

83. Waheed A, Parkkila S, Saarnio J et al. Association of HFE protein with transferrin receptor in crypt enterocytes of human duodenum. Proc Natl Acad Sci U S A. 1999 Feb 16;96(4):1579-84.

84. Weinstein DA, Roy CN, Fleming MD, Loda MF, Wolfsdorf JI, Andrews NC. Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. Blood. 2002 Nov 15;100(10):3776-81.

85. Wright TL, Lake JR. Mechanisms of transport of nontransferrin-bound iron in basolateral and canalicular rat liver plasma membrane vesicles. Hepatology. 1990 Sep;12(3 Pt 1):498-504.

86. Xu H, Jin J, DeFelice LJ, Andrews NC, Clapham DE. A spontaneous, recurrent mutation in divalent metal transporter-1 exposes a calcium entry pathway. PLoS Biol. 2004 Mar;2(3):E50.
87. Zecca L, Youdim MB, Riederer P, Connor JR, Crichton RR. Iron, brain ageing and neurodegenerative disorders. Nat Rev Neurosci. 2004 Nov;5(11):863-73.
88. Zoller H, Pietrangelo A, Vogel W, Weiss G. Duodenal metal-transporter (DMT-1, NRAMP-2) expression in patients with hereditary haemochromatosis. Lancet. 1999 Jun 19;353(9170):2120-3.

2. The role of reactive oxygen and nitrogen species in cellular iron metabolism

Review article

Mladěnka P, Hrdina R, Hübl M, Šimůnek T. The role of reactive oxygen and nitrogen species in cellular iron metabolism. Free Radic Res. 2006 Mar;40(3):263-72.

Abstract:

The catalytic role of iron in the Haber-Weiss chemistry, which results in propagation of damaging reactive oxygen species (ROS), is well established. In this review we attempt to summarize the recent evidence showing the reverse: that reactive oxygen and nitrogen species can significantly affect iron metabolism. Their interaction with iron-regulatory proteins (IRPs) seems to be one of the essential mechanisms of influencing iron homeostasis. Iron depletion is known to provoke normal iron uptake via IRPs, superoxide and hydrogen peroxide are supposed to cause unnecessary iron uptake by similar mechanism. Furthermore, ROS are able to release iron from iron-containing molecules. On the contrary, nitric oxide appears to be involved in cellular defense against the iron-mediated ROS generation probably mainly by inducing iron removal from cells. In addition, NO may attenuate the effect of superoxide by mutual reaction, although the reaction product – peroxynitrite - is capable to produce highly reactive hydroxyl radicals.

Introduction

Iron is the most abundant transition metal in the living organisms and virtually all living cells need it for crucial metabolic pathways. Indeed, oxygen transport, ATP production or DNA-synthesis - all these basic processes require enzymes with iron as a cofactor. On the other hand, free or loosely bound iron is well known to generate free radicals that are responsible for various damages [1]. Therefore iron has to be firmly incorporated in proteins and its homeostasis must be meticulously controlled.

Cellular iron homeostasis is managed mainly by expression of transferrin receptor 1 (TfR1) and ferritin. The first is responsible for uptake of iron into the cell, while the latter for intracellular iron sequestration and cellular storage [2,3,4]. Both proteins are regulated by iron regulatory proteins.

Iron regulatory proteins

Expression of many proteins related to iron kinetics and energy metabolism is regulated post-transcriptionally by cytoplasmic proteins called iron regulatory proteins (IRPs). There are two IRPs (IRP1 and IRP2) and both are able to bind to the specific sequences in the untranslated regions of mRNA known as iron responsive elements (IREs) [2,3,4].

Iron entering the cell seems to become firstly a part of poorly defined intracellular labile iron pool. Such pool seems to sense cellular iron stores. Under conditions of iron excess (high iron pool), IRPs do not possess affinity to IREs, on the contrary, when iron is scarce, IRPs bind to IREs. If IRE is localized at the 5´end of mRNA, e.g. in H- and L- ferritin chains, the binding of an IRP to an IRE stops protein synthesis [5]. Conversely, association of an IRP with IREs at

the 3´end of TfR1-mRNA protects mRNA against degradation and synthesis of TfR1 is enhanced [6]. As a result, under the condition of iron lack (low iron pool), synthesis of TfR1 is augmented and that of ferritin stopped. When iron is abundant, the synthesis of ferritin and some other proteins involved in energy metabolism is increased, while TfR1 abated.

IRP1 is a bifunctional protein (*see Figure 1*) which can act as an iron regulatory protein (*described above*) or as a cytosolic aconitase (c-acon). In the state of iron repletion, this protein contains one specific $[4Fe\text{-}4S]^{2+}$ cluster with only three irons ligated directly to cysteines while the fourth (marked as Fe_a) is attached to an inorganic sulfur of the cluster [7,8]. This fourth iron is necessary for enzymatic activity. Such protein is c-acon and cannot bind IREs. When iron is scarce, this fourth iron atom is released, probably provoking the cluster decomposition, and such cluster-free protein obtains the IRE-binding activity and acts as IRP1 [9,10].

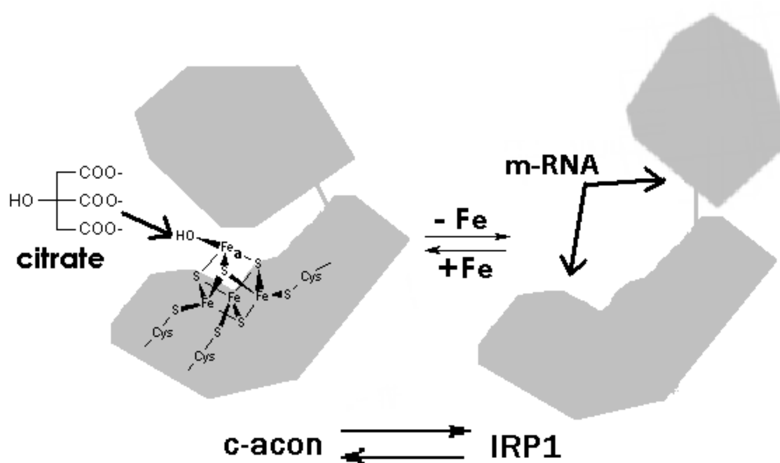


Fig. 1: The dual function of cytosolic aconitase / iron regulatory protein 1.

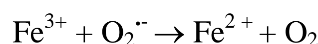
The structure of the cluster and protein according to Beinert and Kennedy [7] and Klausner and Rouault [8], respectively. In iron-repleted status, the protein acts as c-acon, in iron depleted status,

iron is released from the cluster, the cluster further decomposes, loses other 3 atoms of iron and such cluster-free protein acts as IRP1.

IRP2 does not contain the iron-sulfur cluster and lacks the aconitase activity. Like IRP1, IRP2 also binds to IREs in the state of iron deficiency [11]. When iron is in excess, IRP2 undergoes enzymatic degradation [12]. Mitochondrial aconitase (m-acon), an enzyme similar to c-acon, contains the Fe-S cluster as well, but it does not act as an IRP.

Free radicals

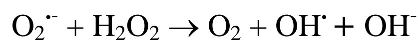
As mentioned above, under certain conditions, iron may facilitate formation of free radicals dangerous for the cells. The most potent oxidizing agent in biological systems is hydroxyl radical ($OH^{\bullet}$), which is generated by Haber-Weiss chemistry [1,13]: superoxide ($O_2^{\bullet-}$) converts ferric ions to ferrous ions and these react with hydrogen peroxide to produce hydroxyl radicals:



$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

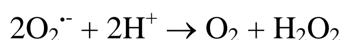
The latter reaction is known as Fenton reaction after the Fenton reagent containing hydrogen peroxide and ferrous salt. The whole process can be summarized in so-called Haber-Weiss reaction: Superoxide reacts with hydrogen peroxide in the presence of iron to produce molecular oxygen, hydroxyl radical and hydroxide anion:



$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-}$$

For the hydroxyl radical production two conditions have to be fulfilled: the presence of free iron and some reactive oxygen species (ROS). NADPH-oxidase can generate superoxide, its production is generally associated with inflammation caused by neutrophils and macrophages. Many tissues contain xanthine dehydrogenase, which can be easily converted to xanthine oxidase, an enzyme also known to generate superoxide and hydrogen peroxide [13,14]. It should be noted that superoxide is produced also in the respiratory chain of mitochondria, although compartmentization seems to confine superoxide to this organelle [15,16] even though superoxide crossing through the outer mitochondrial membrane by use of a voltage-dependent channel was recently proposed [17].

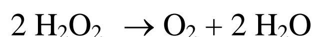
Superoxide is unstable and it is decomposed either spontaneously or much faster by superoxide dismutases (SODs) into hydrogen peroxide and molecular oxygen:



$$2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

There are two SODs in the cell: SOD-1, known as Cu,Zn-SOD, it is localized in the cytosol and SOD-2, marked as Mn-SOD, protecting the mitochondrial department.

Hydrogen peroxide is then converted by catalase or glutathione peroxidase into water and molecular oxygen:



$$2\,H_2O_2 \rightarrow O_2 + 2\,H_2O$$

It is thought that superoxide and hydrogen peroxide are present in the organism physiologically, which is supported by fact that SODs are ubiquitously and abundantly expressed [18]. Therefore iron - the second member of the Haber-Weiss chemistry - must be meticulously regulated in order to avoid cellular damages. In man free iron is scarce under physiological conditions. Nearly all iron is sequestered by proteins: in plasma is it bound to transferrin, in

various cells it is locked in the structure of ferritin, in the red blood cells iron is firmly incorporated to hemoglobin, in muscles to myoglobin. “Free” iron means iron with at least one iron coordination site open or occupied by a readily dissociable ligand. All formerly mentioned transport and storage proteins tightly complex all six coordination sites of iron and therefore such iron cannot produce hydroxyl radical. In contrast, iron bound to ADP, ATP or citrate remains “free”, because these molecules are not able to ligand all of its 6 coordination sites [19].

An imbalance in a cellular redox state, where the ROS production overwhelms anti-oxidant capacity, results in the state termed oxidative stress and recent evidence suggests that oxidative stress is a common denominator in many pathologies [1]. The prevention of cellular damages caused by elevated ROS production can be efficiently achieved with iron-chelating agents and this was demonstrated by numerous papers, including those of our group [20,21,22,23].

Superoxide and hydrogen peroxide

In the last decade it has become obvious that superoxide and hydrogen peroxide may be involved in iron metabolism disturbances. Extracellular hydrogen peroxide stimulates within 60 minutes IRP1 binding to IREs together with the decrease in c-acon activity, while the withdrawal of stimulus after 15 minutes does not change the induction of IRP [24,25,26,27]. On the contrary, IRP2 is not significantly affected by hydrogen peroxide [27]. Interestingly, the increased IRE-IRP1 binding is not observed with hydrogen peroxide and cytosolic fractions [25,28,29] or intracellulary produced hydrogen peroxide [26], even though the c-acon activity was inhibited in all cases. Blockade of the respiratory chain evokes production of superoxide and interestingly within 2 hours it activates the IRP1-binding. The latter effect appears to be mediated via

hydrogen peroxide formed from superoxide, as the activation of IRP1 corresponds with emergence of intracellular H_2O_2 [26].

Hydrogen peroxide possibly reacts directly with the [4Fe-4S] cluster of c-acon, releases one iron atom (Fe_a) and subsequently inhibits the c-acon activity but it does not convert c-acon into IRP1 [28,30]. In fact, one Fe atom release from c-acon was observed in yeast with extracellulary added hydrogen peroxide, which is a diffusible molecule and therefore easily penetrates the membranes [9]. The hydrogen peroxide diffusion can be observed in mammals as well, but activation of IRP1 with extracellular hydrogen peroxide occurs also when no detectable increase in intracellular hydrogen peroxide was measured [26]. The effect of extracellular hydrogen peroxide in mammal is therefore thought not to be related to direct interaction with c-acon/IRP1, but rather via some non-soluble, probably membrane-associated protein with further conduction of the signal inside the cell. The IRP1-activation was proposed to be based on phosphorylation, as its induction by hydrogen peroxide could be inhibited by okadaic acid, which acts as an inhibitor of type I/IIa protein phosphatases [27].

The rapid activation of IRP1 by a short stimulus of extracellular hydrogen peroxide can clarify some ROS-induced damages, especially in the ischemia/reperfusion injury, when ROS, formed by xanthine oxidase, can activate iron uptake inside the cells and contribute to the Haber-Weiss chemistry with its deleterious consequences [13].

The first study examining the results obtained with cell culture experiments in a more complex system was performed by Mueller *et al.* [31], and indeed, the authors, using the H_2O_2-generating system in perfused rat liver, were able to show the expected increase in IRP1-IRE binding.

Escherichia coli missing cytosolic SOD reveals 8-fold increased levels of free iron as compared to the control bacteria, which clearly demonstrates the role of superoxide radical in iron release. Released iron was shown to be mainly in ferrous state. Surprisingly, most iron did not originate from ferritin but from four cytosolic bacterial enzymes containing Fe-S cluster [32]. Indeed, Fe-S cluster containing aconitase of the same bacteria is reversibly inactivated by superoxide [33]. Cu, Zn-SOD deficient mice manifest reduced enzymatic activity of c-acon, reduced IRE-IRP1 binding - probably due to a decrease in IRP1 synthesis - but no change in IRP2 expression in the liver and noteworthy normal iron metabolism as demonstrated by unchanged levels of ferritin and TfR1 [34]. Experiments in Drosophila with silencing and genetic mutation of the cytosolic SOD also showed abated activity of c-acon, but, in contrast, the IRP1-IRE binding was strongly activated [16]. In Drosophila, some additional type of regulation can be expected, which is supported by the fact, that Drosophila does not possess the vertebrate highly conserved site (Ser 138) of IRP1 for phosphorylation [35]. Similarly, defect in SOD-2 results in the decrease of m-acon function [16]. Superoxide produced within the mitochondria was further shown to slightly increase the IRP1 binding and to decrease the c-acon activity [15,24]. Extracellulary produced superoxide has no effect on iron and energy metabolism [15,24] as should be expected because superoxide is not a diffusible molecule. Increased IRP1 binding and decreased c-acon activity, caused by mitochondrial superoxide, therefore most likely reflects its conversion into hydrogen peroxide.

Treatment of rat liver lysates with xanthine oxidase, which produces superoxide a hydrogen peroxide [14,36], manifested decreased IRP-IRE binding but surprisingly it did not affect c-acon

[37]. Macrophage cytosolic extracts with added xanthine oxidase showed highly reduced aconitase activity extract but did not exhibit significant effect on IRP1-IRE binding [28].

Similarly to hydrogen peroxide, superoxide also directly reacts with Fe-S clusters of various enzymes, releases iron and impairs their enzymatic activity. But in contrast to hydrogen peroxide, the cluster decomposition of c-acon seems to be more profound as documented by Flint et al. [38], who showed that bacterial enzymes containg the [4Fe-4S] cluster released at least three iron atoms when treated with superoxide. Interestingly, such cluster disintegration appears not to stimulate the IRP1-IRE binding. The degradation of Fe-S cluster is therefore probably not sufficient for conversion of c-acon into IRP1, or it is also possible that superoxide can oxidize some free sulfhydryl groups [18] and prevent the IRP-IRE binding. Finally, it should be emphasized, that mammals are probably better equipped with defense mechanisms against ROS, as they do not seem to develop free iron overload. In bacteria, iron, released from Fe-S clusters by superoxide, accelerates DNA damages caused by superoxide or by other ROS [32]. Whether the impairment of iron clusters caused by superoxide (and further plausible consequences, like DNA damages seen in bacteria) is minor in mammal cells, requires further examination. There are certain discrepancies among the different studies, which can be often explained by different methodical approach and this issue is further discussed in the chapter concerning the NO.

Additionally, ROS also appear to affect other iron containing molecules. Richardson and Ponka [39] examined cellular iron uptake from transferrin after exposure of cell cultures to ferric ammonium citrate. They found elevated uptake of iron which was not mediated by TfR1. Further investigation documented involvement of superoxide and/or hydrogen peroxide and possibly also hydroxyl radical in release of iron from transferrin and increased transport of iron into the cell

[39]. In fact, it is well known that superoxide can release iron from ferritin [40,41,42]. Withdrawal of iron from iron store and transport molecules can represent an important step in ROS propagation.

Nitric oxide

Nitric oxide (NO) is a free radical with very complex biological function. It is synthesized from L-arginine by three different NO-synthases (NOS). NO has high affinity to metals and many biological effects of NO can be attributed to its chemical interaction with iron: For example activation of guanylyl cyclase appears to be mediated by nitrosylation of heme iron [43] and Fe-S clusters are decomposed after interaction with NO [44].

Many authors have shown that NO reduces c-acon activity and consequently it increases the IRP1-IRE binding [27,28,45,46,47,48,49]. This IRP1-IRE binding is activated slowly and needs hours (3-12 hours - variably in different studies, which probably depends on means of NO production and its concentration used) for full effect [24,27,46,50]. The activation seems to be analogous to induction caused by iron depletion and requires the presence of NO during the whole activation period [24,27]. The modulatory effect of NO on IRP1 seems to be stronger than that of iron repletion, as NO has been shown to activate the IRP1-IRE binding also after hemin treatment, which typically diminishes the IRP-IRE binding [48]. NO is a diffusible molecule which easily crosses biological membranes. Published data suggest that NO attacks directly the Fe-S cluster of c-acon. An iron-nitrosyl complex is formed, where NO firstly coordinates the crucial Fe_a atom of the cluster, as indicated by rapid inactivation of c-acon activity [44]. This triggers further allosteric changes of the protein and finally slowly leads to the total decomposition of the cluster and transformation of protein into IRP1 [24,27,44,46,48,51].

Unfortunately, at this point the relative harmony between different studies concerning the interaction of NO and iron metabolism ends and the findings are becoming discrepant (*see Table 1*). In our opinion, the likely explanation of such diverse results could involve many aspects of experimental procedures. Following aspects appear to play a role:

- The type of the cell culture used. First, the liver iron metabolism differs in some aspects from other tissues (*for details see review*)[4]. Furthermore, there are some cell cultures (e.g. leukemia L1210 cells and F6 fibroblasts) that do not produce NO and these are suggested to have altered management of iron metabolism as well [46,48].
- The ratio of IRP2/IRP1. IRP2 binds with higher affinity to ferritin H-chain IRE in comparison to other IREs [12] and it seems to have therefore principal role in ferritin levels management. The high IRP2 content is present in macrophages and brain, while in most of other tissues it is lower [4,27,45,52].
- Experimental method of NO production and its concentration. To date, numerous different experimental approaches of NO production have been used. The most common is a stimulation of cytokine-inducible NOS (iNOS) by cytokines and lipopolysacharide. However, both agents are known to trigger multiple cellular responses and the results obtained by such treatment may be blunted by not easily eliminable confounders. The combination of LPS/IFNγ/TNF-α is documented to decrease total protein synthesis and may elicit some discrepancies among studies [53]. Other means are the iNOS gene transfection, NO gas, or the use of NO-releasing agents – e.g. the most frequently used SNAP (S-nitroso-N-acetyl-D,L-penicilamine) and others (*Table I*). It should be mentioned that there is some controversy with

SNAP as penicilamine may chelate iron, even though it was manifested that SNAP has only low iron chelating properties [54].

- Duration of NO exposure. As mentioned above, IRP1 activation by NO is a slow process.
- The amount of NO produced. The most commonly used method for evaluation of NO levels is the measurement of nitrite. But it seems that nitrite levels may not be a reliable indication of biological active NO as peroxynitrite may significantly augment the nitrite levels [55]
- Redox state of the cell (i.e. the "intracellular redox background"). Under physiological conditions, NO can be interconverted to its redox form - nitrosonium (NO^+) [56]. Nitrosonium is conjectured to nitrate proteins and its effects are implied to be similar to peroxynitrite (*see the next section*). Nitrosonium does not activate or can even abate the IRP1-IRE binding, but it dramatically decreases the IRP-2 binding activity [27,50]. Furthermore, nitrosonium has been shown to decrease the TfR mRNA levels and consequently the iron uptake by cells [55,57].
- Ambient oxygen conditions. Hypoxia is known to regulate IRP1 and IRP2 binding in opposite manner. IRP1-IRE binding appears to be diminished after hypoxia, while that of IRP2 elevated [58].

The first unresolved question is the interaction of NO with the ability of IRP2 to bind IRE. There are papers showing both an increase [27,48,53,59], a decrease [45,50,52,60,61] as well as unchanged binding [50,53]. Some light into this discrepancy gave the papers showing that production of NO by a combination of LPS and IFNγ decreased the binding activity, while SNAP did not [50], and

that IRP2 decrease is associated with LPS and IFNγ independently of NO [60]. Beyond the IRP-IRE interaction, NO induces the release of iron differently from various cells [50, 62, 63]. The iron efflux requires glutathione, and it is probably carried out by an energy-dependent membrane transport mechanism [62]. Stimulated iron release from cells diminishes the intracellular iron pool, but such condition of iron depletion also stimulates the IRPs-IREs binding. This fact can also explain the increased IRP2-IRE binding by NO-treatment in the study by Pantopoulos and Hentze [48], where the cells were transfected with iNOS gene. Such cells produce continually NO and they could have been chronically iron depleted, which resulted in IRP2 stabilization and accumulation. A conclusion cannot be made at this moment, but it appears that NO alone does not change directly the IRP2 affinity for IRE.

Authors	**Cell culture**	**NO product.**	**Time [h]**	**IRP2**	**TfR1 mRNA**	**TfR1**	**Fe uptake**	**FT mRNAs**	**FT or FT-H**	**FT-Fe**
Bouton et al., 1998[60]	RAW 264.7	LPS+IFNg	1-24	↓						
Cairo et al., 2002[45]	J774A.1*	SIN-1+SOD	1	↓						
Kim and Ponka, 1999[50]	RAW 264.7	LPS+IFNγ	10	↓	↓				↑	
		SNAP	10	**0**	↑				↓	
Kim and Ponka, 2000[61]	RAW 264.7	LPS+IFNγ	10	↓	↓		↓	**0**	↑	
Mulero and Brock, 1999 [64]	J774	LPS+IFNγ	16/24			↓	↓			↓
Oria et al., 1995 [54]	K562	SNAP	24		↑	↑		↑	**0**	↑
Pantopoulos and Hentze, 1995 [48]	B6.NOS	NOS	-	↑	↑			↑	↓**x**↑	
	RAW 264.7, J774.A1	LPS+IFNγ	12		↓					
Phillips et al., 1996[53]	FT02B	LPS+IFNγ†	4-24	↑	↓			**0**	↓	
		SNAP	4-24	**0**	↑					
Recalcati et al., 1998[52]	J774A.1	LPS+IFNγ	4 / 24	↓					↑	
		SNAP	24	↓					↑	
Richardson et al.,1995[57]	K562	SNAP	18		↑	↑				
Wang et al., 2005[59]	B6,H1299	SNAP	8	↑						

Table I. Papers concerning more aspects of NO interaction with iron metabolism and/or IRP2. Notes. IRP2: activation of IRP2; FT: ferritin; FT-H: ferritin H-chain; FT-Fe: iron ferritin content; J774A.1, J774, RAW 264.7 are mouse macrophage cell lines; K562 is an erythroleukemic cell line; B6 is a mouse fibroblast cell line; B6.NOS: mouse B6 fibroblasts transfected with NOS; FT02B is rat hepatoma cell line; H1299 is human lung cancer cell line; *precisely lysate of J774A.1; † in combination with tumor necrosis factor α (TNF-α); SIN-1: 3-morpholinosydnonimine; SOD: superoxide dismutase; IFNγ, interferon γ; NOS: NO-synthase; LPS: lipopolysacharide; SNAP: S-nitroso-N-acetyl-D,L-penicilamine; ↓: a decrease; ↑: an increase; 0: no change; ↓x↑: cannot be clearly determined.

It can be even speculated that activation of the IRP-IRE binding in state of iron starvation may be mediated via NO, but such mechanism is very unlikely, even though a regulatory loop between iron metabolism and the NO is known (*see Figure 2 and the last paragraph of this section*).

The increased IRPs-IRE binding stabilizes TfR1 mRNA and should therefore lead to augmented iron uptake. Studies concerning levels of TfR1 again appear to be divergent, but when the experiments using stimulation by LPS+IFNγ [48,50,53,61] are separated from other means of NO production [48,50,53,54,57], again a possible explanation arises: LPS with IFNγ may overcome the effect of NO and thus decrease the levels of TfR1 by a NO-independent manner. In agreement with this proposition, the increase in TfR1 density has been documented after NO treatment [54,57]. The only study reporting a decrease in TfR1 density[64] proved that this decrease was NO-independent and may be linked with LPS+IFNγ. Nevertheless, an expected raise in iron uptake from iron loaded transferrin (Tf-Fe_2) has not been observed and surprisingly the contrary

process has been discovered in various cell cultures [55,61,63,64]. The explanation of this unexpected process is hard to make at the moment. Watts and Richardson [63] proposed that reduced Fe uptake results, at least partly, from a decrease in levels of ATP, which is required for Tf-Fe_2-TfR1-mediated endocytosis. The reduced ATP levels may be caused by inhibition of some enzymes (mainly m-acon) of respiratory chain by NO. NO did not decrease the Tf binding to TfR1 [63], but some other NO interaction with iron uptake process cannot be precluded.

NO appears to elevate ferritin m-RNA levels probably by some unknown pretranslational mechanism [48,54], although in LPS+IFNγ treated cells no change in ferritin m-RNAs was reported [53,61]. Furthermore, both an increase [48,50,52,61] and a decrease in ferritin (total or H-chain) synthesis [48,49,50,53] have been described. But again, when the experiments using LPS+IFNγ are separated, NO seems to elevate ferritin m-RNA levels, but on the other hand (in the agreement with IRP-IRE theory) to decrease ferritin synthesis. It seems that LPS+IFNγ abate ferritin m-RNAs levels but paradoxically increase ferritin levels in non-hepatic cells. This statement is in harmony with known findings that ferritin synthesis is augmented in inflammation [65]. In the hepatic cells, however, an alternative iron metabolism control is expected.

In various cell cultures, the incorporation of iron from Tf-Fe_2 into ferritin was reduced differently after the stimulation with NO [62,63,64], although Oria et al. [54] reported the opposite effect in K562 cells.

For many years it was believed that NO induces iron release from ferritin [41]. Recently, however, Watts and Richardson [62] did not observe such phenomenon in cell lysates. Today, this discrepancy can be explained, as the former group used an agent afterwards shown to release

NO^+ [41], while the latter a NO-releasing agent [62]. Today, NO seems to intercept iron before it reaches ferritin [62] and it can be suggested that NO does not directly interact with iron stores within ferritin but it can by some indirect mechanism mobilize iron from ferritin. Whether such mechanism simply involves an adaptation to low cellular iron levels after increased iron efflux deserves further investigation.

NO may be a biological messenger used by cells to prevent intracellular damages caused by ROS. As tumours have been shown to contain more TfR1 receptors and generally they have more pronounced iron demands [66], by elevating iron release from the cell and inhibition of respiratory chain, NO can inhibit ROS-damages and suppress cell proliferation. This is proposed to represent the defense mechanism of activated macrophages against tumour cells and pathogens.

It was demonstrated that mRNA levels of iNOS are profoundly increased in iron deficiency and reduced in the opposite condition [67]. In agreement, ferrous ions were shown to abate the iNOS-stimulated synthesis of NO and iron chelator o-phenantroline augmented NO-synthesis in cell cultures. Interestingly, the same group also found that iNOS-mediated NO-production is not influenced by iron in rat astrocytoma C6 cell line, supporting the assumption that NO-signaling may be controlled differently among various cells [68].

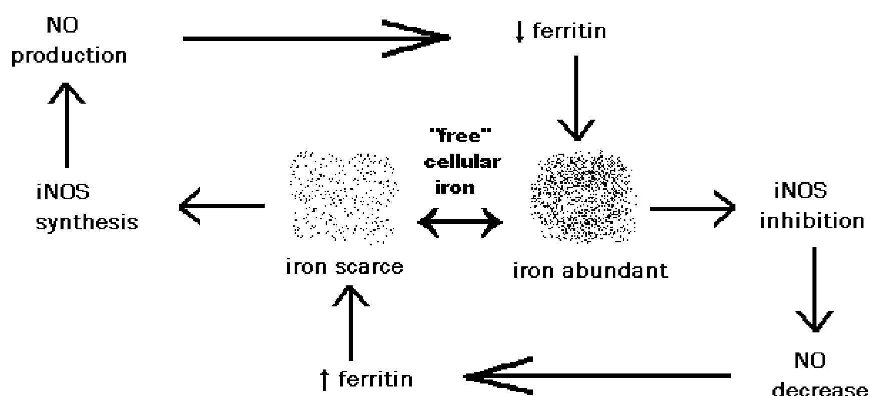


Fig. 2: Probable feedback regulation between iron and NO according to Weiss et al. [67] based on their own research. When the level of intracellular iron (probably reflected by labile iron pool) needed for metabolic processes is low, the iNOS induction is provoked. This is followed by an augmented NO production, decrease in ferritin synthesis and an increase in free iron pool. In the reversed situation, when intracellular iron is abundant, iNOS is not stimulated and the resulting low NO production results to an increase in ferritin synthesis and ultimately to abated intracellular iron.

Peroxynitrite

A new interesting question arises with the possible involvement of peroxynitrite in iron metabolism. Peroxynitrite ($ONOO^-$) is the reaction product of superoxide and nitric oxide [69] and it is considered to be a strong oxidant and a major cytotoxic agent produced during inflammation, sepsis, and ischemia-reperfusion. Peroxynitrite was documented to nitrate tyrosine residues of proteins [70] and in fact nitrosylation of c-acon/IRP1 was demonstrated in vitro [44,47]. In addition, peroxynitrite decreases c-acon activity in vitro more rapidly than NO and it has been shown to slightly enhance the IRP1-binding [28,44,47] or not change [27] or slightly decrease it [57]. When slight stimulation of IRP1-IRE was observed, this activation with NO and superoxide was far less potent, when compared to a situation when macrophages were stimulated to produce NO

only [47]. When peroxynitrite was produced extracellulary, c-acon was slightly inhibited and some IRP1 activation was present. NO, together with superoxide (produced within mitochondria), evoked pronounced inhibition of both aconitases and, paradoxically, it significantly stimulated the IRP1-IRE binding [24].

It is suggested that peroxynitrite is a hardly diffusible molecule and the likely explanation of some increase in IRP1-IRE binding is due to the readily diffusible NO. Such proposal is supported by the fact that peroxynitrite did not stimulate IRP1 binding in macrophage cell extract, but if SOD was added, stimulation of IRP1 binding occurred [28]. The paper of Cairo et al.[45] seemingly contradicts those findings. In this study, a lower concentration of peroxynitrite-producing agent was used and in this case a significant IRP1 activation was seen. But when a higher concentration was employed, no increase in IRP1 activity was observed in concordance to previous data [45]. Similar results were reported with recombinant IRP1 [46]. Nitration and/or oxidation seem to take place in a situation when peroxynitrite is produced in sufficient quantity. It may be possible that low concentration of peroxynitrite cannot modify IRP1 more than NO alone, i.e. it removes only Fe-S cluster of c-acon and transforms it into IRP1. The decrease in c-acon activity but no significant increase in IRP1 binding caused by higher concentrations of peroxynitrite can be explained by spheric hindrance of nitro group, oxidation of free sulfhydryl groups and/or by incomplete decomposition of the Fe-S cluster of c-acon/IRP1. Chemical modification evoked by higher concentration of peroxinitrite on c-acon/IRP1 can be confirmed by the lack of recovery of c-acon activity after treatment with ferrous sulfate and cysteine, which have been proven to be able to reconstruct the c-acon cluster and its enzymatic function [45]. Activation of IRP1 binding with mitochondrial production of peroxynitrite may depend not only

on NO, the potential role of superoxide and/or hydrogen peroxide cannot be omitted. In addition, peroxinitrite is known to decrease IRP-2 binding activity even in presence of an iron chelator [45].

Like superoxide and NO^+, peroxynitrite was also demonstrated to mobilize iron from ferritin *in vitro*, but surprisingly, its effect was less pronounced than that of either superoxide or NO^+ [41]. In addition, peroxynitrite can also interact with iron uptake from transferrin; a decrease in TfR mRNA and in Fe-uptake was observed in various cell cultures [55, 57].

It may be implied that, in macrophages during inflammatory processes (when superoxide is generated), the nitration of c-acon can on the one hand protect cells against the well-known consequences of iron excess [47] but on the other hand it cannot be omitted that peroxynitrite decomposes ($t_{1/2}$=0,5s) into hydroxyl and $NO_2^{\cdot}$ radicals [51]. These radicals may reduce cell viability and some effects of peroxynitrite can therefore be ascribed to its toxicity [57].

Conclusion

Correct iron metabolism is essential for maintenance of cellular homeostasis and both iron deficiency and iron overload are responsible for a number of even life-threatening pathologies. Reactive oxygen and nitrogen species are also abundant in cells under both physiological and pathological conditions and better understanding of the role of RONS in cellular iron trafficking is therefore of crucial importance.

This review aimed to show that ROS cause damage not only via the Haber-Weiss chemistry, but they can themselves affect the control of iron metabolism, provoke iron loading into the cells, mobilize iron from proteins, probably most easily from Fe-S clusters and therefore further aggravate the vicious circle of Fe/ROS-induced cellular damage.

On the contrary, nitric oxide appears to be involved in cellular protection against ROS. NO apparently protects cells by inhibition of exceeding iron uptake into the cells and by removing of iron from the cells.

Peroxynitrite also affects the cellular iron metabolism, but its impact seems to be weaker than that of other reactive species. On the one hand, it can be suggested that NO can protect cells against superoxide by reaction with it, but on the other hand, it has to be underlined that the reaction product - peroxynitrite - can be decomposed into highly toxic hydroxyl radical.

Abbreviations

c-acon, cytosolic aconitase; NOS, NO-synthase; IFNγ, interferon γ; iNOS, cytokine-inducible NOS; IRE, iron responsible element; IRP1, IRP2, iron regulatory proteins 1 and 2; LPS, lipopolysacharide; m-acon, mitochondrial aconitase; Ser, serin; SNAP, S-nitroso-N-acetyl-D,L-penicilamine; SOD, superoxide dismutase; ROS, reactive oxygen species; $Tf\text{-}Fe_2$, iron loaded transferrin; TfR1, transferrin receptor 1; TNF-α, tumor necrosis factor α.

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References

[1] Halliwell B, Gutteridge JM. Free radicals in biology and medicine. 3rd ed. New York: Oxford University Press; 1999

[2] Andrews NC. Disorders of iron metabolism. N Engl J Med 1999;341:1986-95

[3] Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. Cell 2004;117:285-97

[4] Mladenka P, Hrdina R, Hubl M, Simůnek T. The fate of iron in the organism and its regulatory pathways. Acta Medica (Hradec Kralove) 2005; in press.
[5] Aziz N, Munro HN. Iron regulates ferritin mRNA translation through a segment of its 5' untranslated region. Proc Natl Acad Sci U S A 1987;84:8478-82.
[6] Hentze MW, Kuhn LC. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci U S A 1996;93:8175-82
[7] Beinert H, Kennedy MC. 19th Sir Hans Krebs lecture. Engineering of protein bound iron-sulfur clusters. A tool for the study of protein and cluster chemistry and mechanism of iron-sulfur enzymes. Eur J Biochem 1989;186:5-15.
[8] Klausner RD, Rouault TA. A double life: cytosolic aconitase as a regulatory RNA binding protein. Mol Biol Cell 1993;4:1-5
[9] Brown NM, Kennedy MC, Antholine WE, Eisenstein RS, Walden WE. Detection of a [3Fe-4S] cluster intermediate of cytosolic aconitase in yeast expressing iron regulatory protein 1. Insights into the mechanism of Fe-S cluster cycling. J Biol Chem 2002;277:7246-54.
[10] Haile DJ, Rouault TA, Harford JB, Kennedy MC, Blondin GA, Beinert H, Klausner RD. Cellular regulation of the iron-responsive element binding protein: disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding. Proc Natl Acad Sci U S A 1992;89:11735-9.
[11] Guo B, Yu Y, Leibold EA. Iron regulates cytoplasmic levels of a novel iron-responsive element-binding protein without aconitase activity. J Biol Chem 1994;269:24252-60.
[12] Ke Y, Wu J, Leibold EA, Walden WE, Theil EC. Loops and bulge/loops in iron-responsive element isoforms influence iron regulatory protein binding. Fine-tuning of mRNA regulation? J Biol Chem 1998;273:23637-40.
[13] McCord JM. Iron, free radicals, and oxidative injury. Semin Hematol 1998;35:5-12.
[14] Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. J Physiol 2004;555:589-606
[15] Gardner PR, Raineri I, Epstein LB, White CW. Superoxide radical and iron modulate aconitase activity in mammalian cells. J Biol Chem 1995;270:13399-405.
[16] Missirlis F, Hu J, Kirby K, Hilliker AJ, Rouault TA, Phillips JP. Compartment-specific protection of iron-sulfur proteins by superoxide dismutase. J Biol Chem 2003;278:47365-9.
[17] Han D, Antunes F, Canali R, Rettori D, Cadenas E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J Biol Chem 2003;278:5557-63.
[18] Liochev SI, Fridovich I. Superoxide and iron: partners in crime. IUBMB Life 1999;48:157-61.
[19] Graf E, Mahoney JR, Bryant RG, Eaton JW. Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. J Biol Chem 1984;259:3620-4
[20] Hrdina R, Gersl V, Klimtova I, Simunek T, Mazurova Y, Machackova J, Adamcova M. Effect of sodium 2,3-dimercaptopropane-1-sulphonate (DMPS) on chronic daunorubicin toxicity in rabbits: comparison with dexrazoxane. Acta Medica (Hradec Kralove) 2002;45:99-105.
[21] Omar R, Nomikos I, Piccorelli G, Savino J, Agarwal N. Prevention of postischaemic lipid peroxidation and liver cell injury by iron chelation. Gut 1989;30:510-4.
[22] Simunek T., Boer C., Bouwman R. A., Vlasblom R., Versteilen A.M.G., Sterba M., Gersl V., Hrdina R., Ponka P., de Lange J. J., Paulus W.J., Musters R.J.P. SIH - a novel lipophilic iron chelator - protects H9c2 cardiomyoblasts from oxidative stress-induced mitochondrial injury and cell death. J Mol Cell Cardiol 2005;39:345-54.
[23] Simunek T, Klimtova I, Kaplanova J, Sterba M, Mazurova Y, Adamcova M, Hrdina R, Gersl V, Ponka P. Study of daunorubicin cardiotoxicity prevention with pyridoxal isonicotinoyl hydrazone in rabbits. Pharmacol Res 2005;51:223-31.
[24] Castro LA, Robalinho RL, Cayota A, Meneghini R, Radi R. Nitric oxide and peroxynitrite-dependent aconitase inactivation and iron-regulatory protein-1 activation in mammalian fibroblasts. Arch Biochem Biophys 1998;359:215-24.
[25] Martins EA, Robalinho RL, Meneghini R. Oxidative stress induces activation of a cytosolic protein responsible for control of iron uptake. Arch Biochem Biophys 1995;316:128-34.

[26] Pantopoulos K, Mueller S, Atzberger A, Ansorge W, Stremmel W, Hentze MW. Differences in the regulation of iron regulatory protein-1 (IRP-1) by extra- and intracellular oxidative stress. J Biol Chem 1997;272:9802-8.
[27] Pantopoulos K, Weiss G, Hentze MW. Nitric oxide and oxidative stress (H2O2) control mammalian iron metabolism by different pathways. Mol Cell Biol 1996;16:3781-8
[28] Bouton C, Raveau M, Drapier JC. Modulation of iron regulatory protein functions. Further insights into the role of nitrogen- and oxygen-derived reactive species. J Biol Chem 1996;271:2300-6.
[29] Pantopoulos K, Hentze MW. Activation of iron regulatory protein-1 by oxidative stress in vitro. Proc Natl Acad Sci U S A 1998;95:10559-63.
[30] Brazzolotto X, Gaillard J, Pantopoulos K, Hentze MW, Moulis JM. Human cytoplasmic aconitase (Iron regulatory protein 1) is converted into its [3Fe-4S] form by hydrogen peroxide in vitro but is not activated for iron-responsive element binding. J Biol Chem 1999;274:21625-30.
[31] Mueller S, Pantopoulos K, Hubner CA, Stremmel W, Hentze MW. IRP1 activation by extracellular oxidative stress in the perfused rat liver. J Biol Chem 2001;276:23192-6.
[32] Keyer K, Imlay JA. Superoxide accelerates DNA damage by elevating free-iron levels. Proc Natl Acad Sci U S A 1996;93:13635-40
[33] Gardner PR, Fridovich I. Inactivation-reactivation of aconitase in Escherichia coli. A sensitive measure of superoxide radical. J Biol Chem 1992;267:8757-63.
[34] Starzynski RR, Lipinski P, Drapier JC, Diet A, Smuda E, Bartlomiejczyk T, Gralak MA, Kruszewski M. Down-regulation of iron regulatory protein 1 activities and expression in superoxide dismutase 1 knockout mice is not associated with alterations in iron metabolism. J Biol Chem 2005;280:4207-12.
[35] Fillebeen C, Chahine D, Caltagirone A, Segal P, Pantopoulos K. A phosphomimetic mutation at Ser-138 renders iron regulatory protein 1 sensitive to iron-dependent degradation. Mol Cell Biol 2003 ;23:6973-81.
[36] McCord JM, Fridovich I. The reduction of cytochrome c by milk xanthine oxidase. J Biol Chem 1968;243:5753-60.
[37] Cairo G, Castrusini E, Minotti G, Bernelli-Zazzera A. Superoxide and hydrogen peroxide-dependent inhibition of iron regulatory protein activity: a protective stratagem against oxidative injury. FASEB J 1996;10:1326-35
[38] Flint DH, Tuminello JF, Emptage MH. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J Biol Chem 1993;268:22369-76.
[39] Richardson DR, Ponka P. Identification of a mechanism of iron uptake by cells which is stimulated by hydroxyl radicals generated via the iron-catalysed Haber-Weiss reaction. Biochim Biophys Acta 1995;1269:105-14.
[40] Biemond P, Swaak AJ, van Eijk HG, Koster JF. Superoxide dependent iron release from ferritin in inflammatory diseases. Free Radic Biol Med 1988;4:185-98.
[41] Reif DW, Simmons RD. Nitric oxide mediates iron release from ferritin. Arch Biochem Biophys 1990;283:537-41.
[42] Thomas CE, Aust SD. Reductive release of iron from ferritin by cation free radicals of paraquat and other bipyridyls. J Biol Chem 1986;261:13064-70.
[43] Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol 1990;30:535-60.
[44] Soum E, Drapier JC. Nitric oxide and peroxynitrite promote complete disruption of the [4Fe-4S] cluster of recombinant human iron regulatory protein 1. J Biol Inorg Chem 2003;8:226-32.
[45] Cairo G, Ronchi R, Recalcati S, Campanella A, Minotti G. Nitric oxide and peroxynitrite activate the iron regulatory protein-1 of J774A.1 macrophages by direct disassembly of the Fe-S cluster of cytoplasmic aconitase. Biochemistry 2002;41:7435-42.
[46] Drapier JC, Hirling H, Wietzerbin J, Kaldy P, Kuhn LC. Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. EMBO J 1993;12:3643-9.
[47] Gonzalez D, Drapier JC, Bouton C. Endogenous nitration of iron regulatory protein-1 (IRP-1) in nitric oxide-producing murine macrophages: further insight into the mechanism of nitration in vivo and its impact on IRP-1 functions. J Biol Chem 2004;279:43345-51.

[48] Pantopoulos K, Hentze MW. Nitric oxide signaling to iron-regulatory protein: direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. Proc Natl Acad Sci U S A 1995;92:1267-71.

[49] Weiss G, Goossen B, Doppler W, Fuchs D, Pantopoulos K, Werner-Felmayer G, Wachter H, Hentze MW. Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. EMBO J 1993;12:3651-7.

[50] Kim S, Ponka P. Control of transferrin receptor expression via nitric oxide-mediated modulation of iron-regulatory protein 2. J Biol Chem 1999;274:33035-42.

[51] Henry Y, Lepoivre M, Drapier JC, Ducrocq C, Boucher JL, Guissani A. EPR characterization of molecular targets for NO in mammalian cells and organelles. FASEB J 1993;7:1124-34.

[52] Recalcati S, Taramelli D, Conte D, Cairo G. Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. Blood 1998;91:1059-66.

[53] Phillips JD, Kinikini DV, Yu Y, Guo B, Leibold EA. Differential regulation of IRP1 and IRP2 by nitric oxide in rat hepatoma cells. Blood 1996;87:2983-92.

[54] Oria R, Sanchez L, Houston T, Hentze MW, Liew FY, Brock JH. Effect of nitric oxide on expression of transferrin receptor and ferritin and on cellular iron metabolism in K562 human erythroleukemia cells. Blood 1995;85:2962-6.

[55] Richardson DR, Neumannova V, Ponka P. Nitrogen monoxide decreases iron uptake from transferrin but does not mobilise iron from prelabelled neoplastic cells. Biochim Biophys Acta 1995;1266:250-60

[56] Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. Science 1992;258:1898-902

[57] Richardson DR, Neumannova V, Nagy E, Ponka P. The effect of redox-related species of nitrogen monoxide on transferrin and iron uptake and cellular proliferation of erythroleukemia (K562) cells. Blood 1995;86:3211-9

[58] Hanson ES, Foot LM, Leibold EA. Hypoxia post-translationally activates iron-regulatory protein 2. J Biol Chem. 1999 Feb 19;274(8):5047-52.

[59] Wang J, Chen G, Pantopoulos K. Nitric oxide inhibits the degradation of IRP2. Mol Cell Biol 2005;25:1347-53.

[60] Bouton C, Oliveira L, Drapier JC. Converse modulation of IRP1 and IRP2 by immunological stimuli in murine RAW 264.7 macrophages. J Biol Chem 1998;273:9403-8.

[61] Kim S, Ponka P. Effects of interferon-gamma and lipopolysaccharide on macrophage iron metabolism are mediated by nitric oxide-induced degradation of iron regulatory protein 2. J Biol Chem 2000;275:6220-6.

[62] Watts RN, Richardson DR. The mechanism of nitrogen monoxide (NO)-mediated iron mobilization from cells. NO intercepts iron before incorporation into ferritin and indirectly mobilizes iron from ferritin in a glutathione-dependent manner. Eur J Biochem 2002;269:3383-92.

[63] Watts RN, Richardson DR. Differential effects on cellular iron metabolism of the physiologically relevant diatomic effector molecules, NO and CO, that bind iron. Biochim Biophys Acta 2004;1692:1-15

[64] Mulero V, Brock JH. Regulation of iron metabolism in murine J774 macrophages: role of nitric oxide-dependent and -independent pathways following activation with gamma interferon and lipopolysaccharide. Blood 1999;94:2383-9.

[65] Konijn AM, Hershko C. Ferritin synthesis in inflammation. I. Pathogenesis of impaired iron release. Br J Haematol 1977;37:7-16.

[66] Kwok JC, Richardson DR. The iron metabolism of neoplastic cells: alterations that facilitate proliferation? Crit Rev Oncol Hematol 2002;42:65-78.

[67] Weiss G, Werner-Felmayer G, Werner ER, Grunewald K, Wachter H, Hentze MW. Iron regulates nitric oxide synthase activity by controlling nuclear transcription. J Exp Med 1994;180:969-76.

[68] Harhaji L, Vuckovic O, Miljkovic D, Stosic-Grujicic S, Trajkovic V. Iron down-regulates macrophage anti-tumour activity by blocking nitric oxide production. Clin Exp Immunol 2004;137:109-16.

[69] Huie RE, Padmaja S. The reaction of no with superoxide. Free Radic Res Commun 1993;18:195-9.

[70] Ischiropoulos H, Zhu L, Chen J, Tsai M, Martin JC, Smith CD, Beckman JS. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Arch Biochem Biophys 1992;298:431-7.

3. The effects of lactoferrin in a rat model of catecholamine cardiotoxicity

Mladěnka P, Semecký V, Bobrovová Z, Nachtigal P, Vávrová J, Holečková M, Palicka V, Mazurová Y & Hrdina R. The effects of lactoferrin in a rat model of catecholamine cardiotoxicity, Biometals 2008, in press

Abstract:

Lactoferrin is recently under intense investigation because of its proposed several pharmacologically positive effects. Based on its iron-binding properties and its physiological presence in the human body, it may have a significant impact on pathological conditions associated with iron-catalysed reactive oxygen species (ROS). Its effect on a catecholamine model of myocardial injury, which shares several pathophysiological features with acute myocardial infarction in humans, was examined.

Male Wistar rats were randomly divided into 4 groups according to the received medication: control (saline), isoprenaline (ISO, 100 $mg.kg^{-1}$ s.c.), bovine lactoferrin (La, 50 $mg.kg^{-1}$ i.v.) or a combination of La + ISO in the above-mentioned doses. After 24 hours, haemodynamic functional parameters were measured, a sample of blood was withdrawn and the heart was removed for analysis of various parameters.

Lactoferrin premedication reduced some impairment caused by ISO (e.g., a stroke volume decrease, an increase in peripheral resistance and calcium overload). These positive effects were likely to have been mediated by the positive inotropic effect of lactoferrin and by inhibition of ROS formation due to chelation of free iron. The failure of lactoferrin to provide higher protection seems to be associated with the complexity of catecholamine cardiotoxicity and with its hydrophilic character.

Background:

Lactoferrin is an innate iron-binding glycoprotein with many proposed, potentially positive pharmacological activities. It shares a high degree of homology with transferrin (Abdallah and El Hage Chahine, 2000; Baker and Baker, 2005; Metz-Boutigue *et al.*, 1984). In the human body, transferrin acts as an iron-transporting protein and is found predominantly in the blood, whereas lactoferrin is localized in exocrine secretes (e.g., saliva, tears, milk, bronchial mucus) and in the secondary granules of neutrophils (Weinberg, 2003, 2006). Its role in man is only partially known. Lactoferrin inhibits the growth of many pathological microorganisms and its presence on mucosal surfaces represents the primary antimicrobial defence system of the organism. This antimicrobial property at least partially reflects its iron-binding capacity. Its affinity to iron is very high, about 260 times higher than that of transferrin (Baker *et al.*, 1994) and, in contrast to transferrin, is able to retain iron under more acidic conditions (Abdallah and El Hage Chahine, 2000).

The catecholamine model of myocardial injury possesses many pathophysiological similarities with acute myocardial infarction (AMI). The isoprenaline (ISO, synthetic catecholamine) model is therefore often used as a non-invasive model of AMI (Hasenfuss, 1998; Chagoya de Sanchez *et al.*, 1997; Rona, 1985). The only possibility for myocardial tissue recovery in AMI represents the reperfusion of ischaemic myocardium. But the whole process, described as myocardial ischaemia-reperfusion (I-R), is associated with tissue derangement due to burst of hydroxyl radical catalyzed by free iron (Fenton reaction). In fact, studies confirmed increased levels of free intracellular iron and its release from ischaemic cells (Berenshtein *et al.*, 2002; Coudray *et al.*, 1994). Unfortunately, studies examining the effects of some iron chelators

on I-R reported divergent results (Bolli *et al.*, 1990; Reddy *et al.*, 1991). Lactoferrin, which has been documented to inhibit the Fenton reaction (Gutteridge *et al.*, 1981), may have some advantage in comparison to other iron chelators, in particular its endogenous origin.

Based on the aforementioned iron participation in the pathogenesis of myocardial damage, this work hypothesizes the potentially positive effects of lactoferrin in a catecholamine model of myocardial injury.

***Methods*:**

Animals

Young Wistar male rats obtained from Biotest s.r.o. (Konárovice, Czech Republic), weighing approximately 350 g, were used after two weeks of acclimatisation. The animals were maintained in an air-conditioned room and were allowed free access to a standard pellet diet for rodents and tap water. The study was performed under the supervision of the Ethical Committee of the Charles University in Prague, Faculty of Pharmacy in Hradec Králové. All experiments were performed in concordance with the guiding principles of laboratory animal care and use.

Study design

Animals were randomly divided into 4 groups:

control group (C, 7 animals) - received saline 1 $ml.kg^{-1}$ s.c.

isoprenaline group (I, 13 animals) – received 100 $mg.kg^{-1}$ of isoprenaline (ISO; Sigma-Aldrich,USA) in the aqueous solution s.c.

lactoferrin (La, 7 animals) – bovine lactoferrin 50 $mg.kg^{-1}$ (DMV International, USA) was administered i.v.

*lactoferrin + ISO (*LaI, 11 animals) – rats received lactoferrin i.v. 5 minutes before application of ISO in the same doses as above.

Saline and/or drug were administered 24 hours before surgical procedure.

Experimental procedure

Animals were fasting for 12 hours before the experiment and were anaesthetized with urethane (1.2 $g.kg^{-1}$ i.p.; Sigma-Aldrich, USA). A PE catheter (0.5/1.0 mm filled with heparinized saline 50 $IU.ml^{-1}$) was inserted into the right jugular vein for the injection of cold saline. A thermocatheter (o.d. 0.8 mm) was introduced through the left carotid artery into the aortic arc. Another PE catheter (0.5/1.0 mm filled with heparinized saline 50 $IU.ml^{-1}$) was inserted into the left iliac artery, which was connected with the blood pressure transducer BPR-01 of the apparatus for measurement of haemodynamic variables Cardiosys® (Experimentria Ltd, Hungary) with software Cardiosys V 1.1. This device uses the thermodilution transpulmonary method according to the Stewart-Hamilton principle (Spiller and Webb-Peploe, 1985).

The first measurement was carried out following 15 minutes equilibration period after the surgical procedure. Functional variables were obtained in a total of four times in 5 minute intervals. Results are expressed as an index (variable divided by the body weight) except for blood pressure, heart rate and "double product" (mean blood pressure multiplied by heart rate). The last parameter is commonly used as an index of cardiac oxygen consumption (Lentner, 1990). Peripheral resistance was calculated as the mean arterial blood pressure divided by cardiac output, and cardiac power as a product of the mean blood pressure and cardiac output.

Following haemodynamic measurements, approximately 5 ml of blood was withdrawn from the abdominal aorta to the heparinized test tube (170 IU). The animal was then sacrificed by i.v. KCl overdose (1 mM), after which the heart ventricles were excised, weighed and frozen at -20°C for further analysis of ion content.

Histological analysis

After the autopsy, a routine histological examination of cardiac ventricular tissue was performed. Tissue blocks of the transversely sectioned left and right ventricles (the region under the atria with heart apex) were fixed by immersion in 4 % paraformaldehyde for 3 days. Furthermore, 10 consecutive longitudinal paraffin sections through the ventricles were cut (7 µm in thickness) and subsequently stained with haematoxylin-eosin.

Biochemical parameters

Cardiac troponin T (cTnT) and vitamin E were measured in serum, malondialdehyde (MDA) was measured in plasma, antioxidant enzymes were measured in erythrocytes and total glutathione (GSH) was measured in the whole blood. cTnT was determined by electrochemiluniscence immunoassay (Elecsys 2010, Roche Diagnostics), which employs two monoclonal antibodies specifically directed against cTnT. MDA was measured as a red complex with thiobarbituric acid at 485, 532 and 560 nm using Beckman DU 640 spectrophotometer (Beckman, Palo Alto, USA). Capillary electrophoresis was used for the separation of glutathione, which was then measured by UV detection (System P/ACE 5100, Beckman) at 200 nm. Glutathione peroxidase (GPx) was determined spectrophotometrically using a commercial kit

(Ransel, Randox, United Kingdom) according to the manufacturer's instruction as a decrease of absorbance at wavelength 340 nm (Cobas Mira, Roche, Switzerland). Superoxide dismutase (SOD) was determined spectrophotometrically at 505 nm using a commercial kit (Ransod, Randox, U.K.). After deproteinization, the analysis of vitamin E (α-tocopherol) with fluorimetric detection was performed in a HPLC system HP1050 (Hewlett Packard, Germany).

Microelements in the heart tissue

Frozen samples of myocardial tissue were dried, weighed and digested by microwave digestion using nitric acid and hydrogen peroxide (Milestone MLS 1200 MEGA, Italy). Iron, copper and selenium were determined using graphite furnace atomic absorption spectrometry (Unicam, Solaar 959, U.K.), zinc was determined using flame atomic absorption spectrometry (Unicam, Solaar 959, U.K.) and calcium was measured photometrically using flame photometry (Eppendorf, Efox 5053, Germany). Results are expressed as $\mu mol.g^{-1}$ (iron, copper, zinc, calcium) or $nmol.g^{-1}$ (selenium) of dry tissue.

Statistics

Data are expressed as means ± SEM. Groups were compared by one-way ANOVA followed by Tukey's Multiple Comparison Test by means of GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, California, USA). Differences between groups were considered to be significant when $P \leq 0.05$, unless indicated otherwise.

Results:

Final numbers of rats were 7 in control, lactoferrin (La) and lactoferrin+ISO (LaI) groups and 9 in the ISO group (because of high results variability). None of the control animals or animals from the lactoferrin group died. One rat (group LaI) died during surgery. There was comparable mortality in I group and combined group LaI (4 and 3 animals, respectively). Functional parameters are summarized in Fig. 1 and Tab. 1.

parameter/group	**control**	**isoprenaline (ISO)**	**lactoferrin (La)**	**La+ISO**
heart rate (beats.min^{-1})	418 ± 10	453 ± 7*	429 ± 9	470 ± 10$^{*\Delta}$
diastolic blood pressure (mmHg)	110 ± 6	121 ± 9	135 ± 7	127 ± 6
systolic blood pressure (mmHg)	92 ± 4	102 ± 7	112 ± 5	107 ± 6
mean blood pressure (mmHg)	82 ± 4	93 ± 7	101 ± 5	98 ± 6
stroke volume index (ml.kg^{-1})	0.83 ± 0.07	0.36 ± 0.06*	1.19 ± 0.12*	0.69 ± 0.09$^{+\Delta}$
double product (mmHg.beats.min^{-1})	38215 ± 1644	46070 ± 3652	46250 ± 3523	50319 ± 2443*
cardiac power index (mmHg.ml.kg^{-1}.min^{-1})	31511 ± 2947	15917 ± 2239*	52504 ± 3490*	34481 ± 4987$^{+\Delta}$

Table 1. Functional parameters measured 24 hours after drug(s) administration. Results are expressed as mean ± SEM. * p<0.05 vs. control, $^{+}$ p<0.05 vs. ISO, $^{\Delta}$ p<0.05 vs. La.

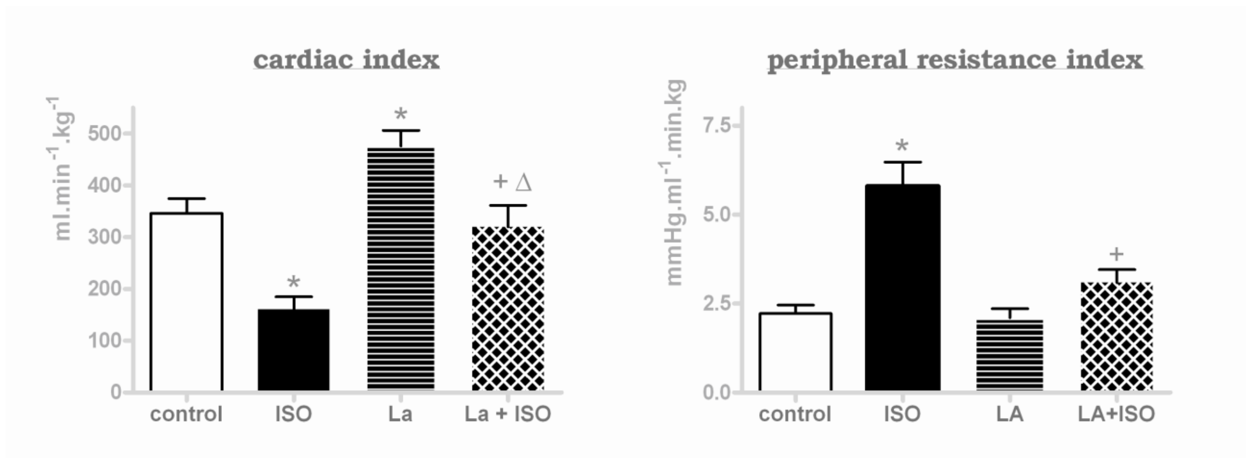

Figure 1. Stroke volume index and peripheral resistance index 24 hours after drug application.

* p<0.05 vs. control, $^{+}$ p<0.05 vs. ISO, $^{\Delta}$ p<0.05 vs. La.

Control and lactoferrin treated animals had negligible levels of serum cTnT and there was no statistical difference between myocardial calcium levels in these groups. Isoprenaline brought about a marked cTnT release and myocardial calcium overload. Lactoferrin premedication did not affect the release of cTnT, however, it decreased calcium overload caused by ISO (Fig. 2). Heart ventricle weight index was elevated in the ISO group when compared to the control or La groups, and contrarily, lactoferrin premedication rather worsened this increase (Fig.3).

There was no statistical significance in myocardial iron level (Tab.2). Similarly, no statistical significance was found in myocardial copper, selenium or zinc concentrations (Tab.2).

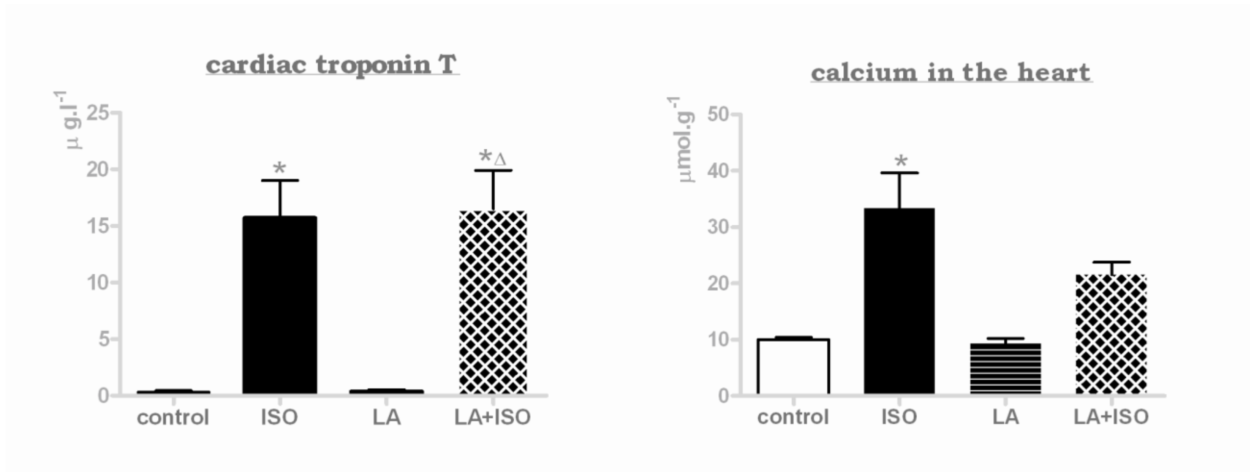

Figure 2. Cardiac troponin T in serum and calcium concentration in the myocardial tissue. * $p<0.01$ vs. control, $^{\Delta}$ $p<0.01$ vs. La.

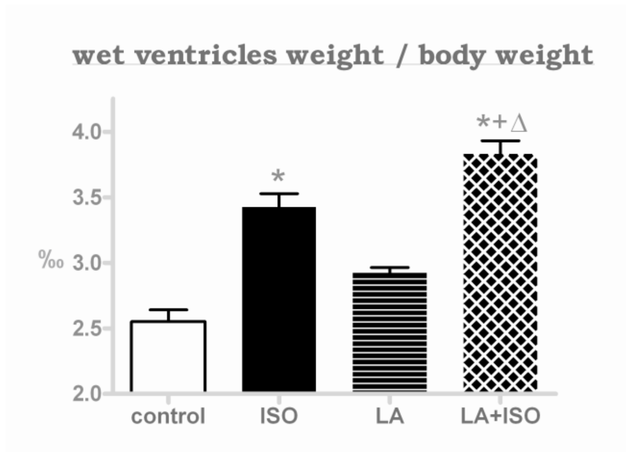

Figure 3. Wet ventricles weight index

* $p<0.001$ vs. control, $^{+}$ $p<0.05$ vs. ISO, $^{\Delta}$ $p<0.001$ vs. La.

Lactoferrin significantly elevated levels of total glutathione while significantly decreasing erythrocyte GPx and insignificantly SOD in healthy animals (Fig. 4, Tab.2). Moreover,

lactoferrin tended to decrease plasma thiobarbituric acid reactive substances (TBARS) but this decrease was not statistically significant (Tab.2). Other results concerning markers of ROS and antioxidants are shown in Tab. 2.

Parameter/group	Control	Isoprenaline (ISO)	Lactoferrin (La)	La +ISO
TBARS (μmol.l^{-1})	1.3 ± 0.1	1.6 ± 0.2	1.0 ± 0.1	1.4 ± 0.1
SOD (U.g^{-1} of Hb)	3092 ± 253	3288 ± 387	2461 ± 266	2138 ± 150^{*+}
Vitamin E (μmol.l^{-1})	9.0 ± 0.4	8.0 ± 0.5	8.2 ± 0.7	7.9 ± 0.7
Copper (μmol.g^{-1} of tissue)	0.58 ± 0.05	0.72 ± 0.03	0.76 ± 0.09	0.83 ± 0.07
Iron (μmol.g^{-1} of tissue)	3.8 ± 0.2	3.6 ± 0.2	4.2 ± 0.1	4.2 ± 0.2
Zinc (μmol.g^{-1} of tissue)	1.09 ± 0.01	1.09 ± 0.01	0.93 ± 0.08	1.13 ± 0.07
Selenium (nmol.g^{-1} of tissue)	7.8 ± 1.2	8.6 ± 1.0	11.3 ± 1.5	11.9 ± 1.5

Table 2. Biochemical parameters and myocardial element content 24 hours after drug administration. TBARS: thiobarbituric acid reactive substances in plasma; SOD: superoxide dismutase in erythrocytes; vitamin E in serum; copper, iron, selenium and zinc in the myocardial tissue. Results are expressed as mean ± SEM. * $p<0.05$ vs. control, $^{+}$ $p<0.05$ vs. ISO.

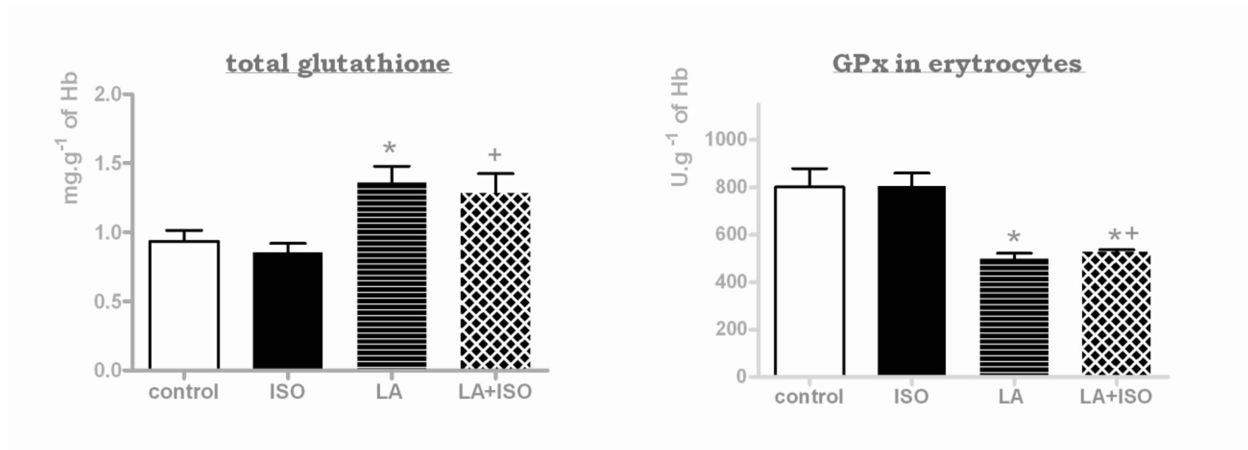

Figure 4. Total glutathione and glutathione peroxidase (GPx) in erythrocytes.

* $p<0.05$ vs. control, $^{+}$ $p<0.05$ vs. ISO.

The normal structure of myocardium was found in intact control animals (Fig.5A). Comparable findings were also visible after administration of lactoferrin (Fig.5B). In the isoprenaline group (Fig.5C), severe acute diffuse toxic damage with an inflammatory reaction was found in the whole myocardium (with a maximum in the subendocardial region, particularly in the heart apex). Myocytes with intensely eosinophilic cytoplasm prevailed, and scattered pyknotic nuclei were also seen. Mild inflammatory infiltrate and macrophages removing the debris of necrotic cells, as well as slight interstitial oedema, were also observed. The administration of lactoferrin (Fig.5D) did not affect morphological changes in myocardium induced by isoprenaline.

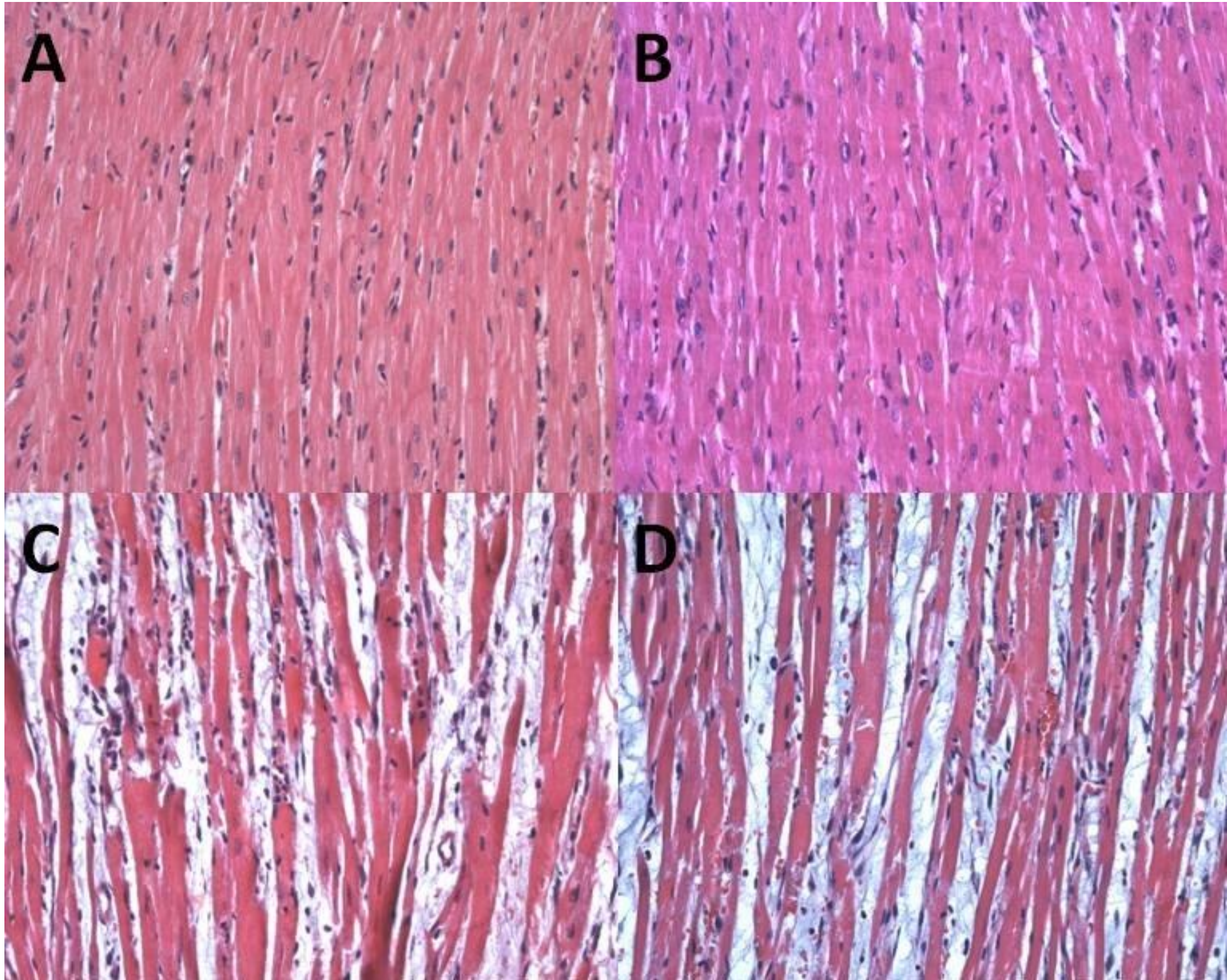

Figure 5. Histological examination of myocardium of all groups. In both control (**A**) and lactoferrin treated rats (**B**) unaffected myocardium was found: cardiomyocytes with centrally located oval-shaped nuclei and cytoplasm filled with cross-striated myofibrils. On the other hand, severe damage of cardiomyocytes resulted in their degeneration/necrosis, seen in both isoprenaline and isoprenaline + lactoferrin treated groups (**C**, **D**). Moreover inflammatory infiltrate and as interstitial oedema were present in this initial stage of myocardial damage.

Lactoferrin treatment did not affect morphological changes caused by the administration of isoprenaline. Haematoxylin–eosin. Direct magnification: 200x.

Discussion:

The administration of isoprenaline, a synthetic catecholamine with non-selective beta-agonist activity, leads to tachycardia and hypotension, which in sufficient doses, is associated with ischaemia followed by marked damage of the myocardium. This resembles, in some aspects, the acute myocardial infarction in man (Chagoya de Sanchez *et al.*, 1997; Rona, 1985). Ischaemia alters iron homeostasis and redox-active free (unbound or loosely bound) iron, which catalyses ROS-generation upon oxygen delivery restoration, appears in the circulation as well as intracellularly (Berenshtein *et al.*, 2002; Coudray *et al.*, 1994). An increase in the concentration of free iron in the myocardium may accompany certain medical procedures, and can be prevented by administration of apo-transferrin, a protein with similar structure to lactoferrin (Parkkinen *et al.*, 2006). Moreover, lactoferrin has a much higher affinity to iron compared with transferrin and was shown to inhibit iron-catalysed ROS formation and decrease ischaemia-reperfusion injury of corneal epithelial cells (Britigan *et al.*, 1994; Shimmura *et al.*, 1998). Some synthetic iron chelators have been well documented to alleviate the toxic effects of excess iron in various pathological conditions, where abundant iron may participate on ROS propagation (Kalinowski and Richardson, 2007; Kontoghiorghes, 2006; Sterba *et al.*, 2007). Similarly, this study documented partially protective effects of lactoferrin on catecholamine-mediated injury.

In contrast to transferrin, serum lactoferrin concentration is generally low. It rises significantly after exercise, sometimes reaching more than 5 $\mu g.ml^{-1}$, and in burnt patients may

even reach up to 40 $\mu g.ml^{-1}$ (Fielding *et al.*, 2000; Inoue *et al.*, 2004; Wolach *et al.*, 1984). In our study the serum lactoferrin levels were not measured; however, due to intravenous administration it can be assumed that its maximum concentration may reach about 1500 $\mu g.ml^{-1}$, which significantly exceeds physiological levels of endogenous lactoferrin. On the other hand, it is only about one half of normal human serum transferrin concentration (3150 $\mu g.ml^{-1}$) (Plomteux *et al.*, 1987). Lactoferrin has a very short initial plasma half-life of about 2 minutes and, therefore, only 15% of the administered dose remained in the plasma after 10 minutes. This first rapid disappearance corresponds to its distribution in the liver and partly in the spleen. Only about 1% of the administered dose remained in plasma after 10 hours (Bennett and Kokocinski, 1979; Karle *et al.*, 1979; Regoeczi *et al.*, 1985; Ward *et al.*, 1983). Based on these data, it may be concluded that high initial plasma levels of lactoferrin promptly dropped and were in physiologically relevant concentration ranges during the experiment.

There is some controversy concerning the role of lactoferrin in inflammation. On one hand, elevated levels of lactoferrin are found in patients with ischaemic stroke, and lactoferrin is known to promote leukocyte adhesion to the endothelial cells and to cause extravasation of plasma (Erga *et al.*, 2000; Kurose *et al.*, 1994; Oseas *et al.*, 1981; Santos-Silva *et al.*, 2002). On the other hand, lactoferrin release induced by leukocyte activation does not seem to amplify inflammation but, contrarily, to reduce the consequences of ROS generation during inflammation (Ward *et al.*, 1983; Weinberg, 2003). Especially, apo-lactoferrin released from neutrophils (iron-free lactoferrin) appears to have the most potent inhibitory effect on ROS-formation (Raghuveer *et al.*, 2002; Weinberg, 2003).

The dose used in this study (50 $mg.kg^{-1}$ i.v.) was not apparently pro-inflammatory in contrast to our preliminary experiments with higher doses (≥100 $mg.kg^{-1}$ i.v.). Moreover, lactoferrin had a tendency to decrease plasma TBARS and to increase total blood glutathione in comparison with healthy animals. There was an increase in wet ventricle weight in the lactoferrin group. However, such an increase was neither associated with obviously conspicuous morphological changes nor with an increase in serum cardiac troponin T concentration, thus suggesting its harmlessness. The only suspicious features are a significant decrease in erythrocyte GPx and a non-significant decrease in erytrocyte SOD. Erythrocyte enzymes are generally less susceptible to acute changes and indeed, ISO administration alone did not cause any change. Lactoferrin binds to various surface molecules and its interaction with some type of blood cells has been well documented (Britigan *et al.*, 1994; Brock, 2002). Therefore, it seems to be possible that these measurements may be blunted by lactoferrin binding to erythrocytes. This assumption must be verified with further examination. Generally, it may be inferred that pro-inflammatory effects of bovine lactoferrin in this study were negligible and of transient character.

Other observed effects of lactoferrin on healthy animals are not linked to its pro/anti-inflammatory properties, such as increased stroke volume and related cardiac indices. No change in the peripheral resistance and rather an increase in diastolic blood pressure, indicated that lactoferrin increased myocardial contractility (Fig. 1 and Tab. 1). This positive inotropic effect is very likely to be responsible for the partial amelioration of cardiovascular impairment caused by ISO. ROS has been well documented to impair physiological vascular function (Paffett and Walker, 2007). Lactoferrin binds extracellular free iron and subsequently inhibits ROS

formation. Therefore, the inhibition of increasing peripheral resistance, together with a tendency to decrease myocardial calcium overload, is very likely to be related to the iron chelation properties of lactoferrin. Insignificant differences in myocardial iron concentrations are not very surprising. Levels of released iron both during and after ischemic insult seem to be very small, albeit sufficient for ROS production. Therefore iron chelation in such conditions influences intracellular levels only marginally, in contrast to iron overloaded cells. Similarly, iron-loaded lactoferrin produces an increase in intracellular iron levels (Shimmura *et al.*, 1998; van Snick *et al.*, 1977).

Pathogenesis of catecholamine cardiotoxicity is multifactorial and not fully understood. It involves ROS-generation and adrenergic stimulation (Neri *et al.*, 2007; Rona, 1985). The first mechanism can be affected by iron chelation but the latter only partially, if at all. This may, at least in part, explain the failure of lactoferrin to decrease other catecholamine cardiovascular damage. The other important factor is the hydrophilic nature of lactoferrin and thus its limited intracellular penetration.

Conclusion

Our experiment did not show that the prophylactic administration of bovine lactoferrin (50 $mg.kg^{-1}$ i.v.), may reverse rat myocardial injury caused by s.c. administration of necrogenic dose of isoprenaline within a 24-hour period. Its partial protective effects are likely based on: 1) an unknown positive inotropic mechanism, which increases stroke volume index in healthy animals and therefore inhibits the drop in this parameter in catecholamine-treated animals; 2) extracellular iron chelation that inhibits an increase in peripheral resistance caused by isoprenaline insult. The failure of lactoferrin to provide a greater protection against

catecholamine cardiotoxic injury is likely to be associated mainly with its hydrophilic character and with the complexity of catecholamine cardiotoxicity. Further examination following this pilot study, which for the first time evaluated the direct effects of lactoferrin on cardiovascular function in healthy and isoprenaline-treated animals, is encouraged. One possible method may be the conjugation of lactoferrin with non-toxic polymers (Ward *et al.*, 1983), which may enable a smaller initial dose of lactoferrin and the prolongation of lactoferrin elimination half-time.

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References:

1. Abdallah FB and El Hage Chahine JM (2000) Transferrins: iron release from lactoferrin. J Mol Biol. 303: 255-66.
2. Baker EN, Anderson BF, Baker HM, Day CL, Haridas M, Norris GE, Rumball SV, Smith CA and Thomas DH (1994) Three-dimensional structure of lactoferrin in various functional states. Adv Exp Med Biol. 357: 1-12.
3. Baker EN and Baker HM (2005) Molecular structure, binding properties and dynamics of lactoferrin. Cell Mol Life Sci. 62: 2531-9.
4. Bennett RM and Kokocinski T (1979) Lactoferrin turnover in man. Clin Sci (Lond). 57: 453-60.
5. Berenshtein E, Vaisman B, Goldberg-Langerman C, Kitrossky N, Konijn AM and Chevion M (2002) Roles of ferritin and iron in ischemic preconditioning of the heart. Mol Cell Biochem. 234-235: 283-92.
6. Bolli R, Patel BS, Jeroudi MO, Li XY, Triana JF, Lai EK and McCay PB (1990) Iron-mediated radical reactions upon reperfusion contribute to myocardial "stunning". Am J Physiol. 259: H1901-11.
7. Britigan BE, Serody JS and Cohen MS (1994) The role of lactoferrin as an anti-inflammatory molecule. Adv Exp Med Biol. 357: 143-56.
8. Brock JH (2002) The physiology of lactoferrin. Biochem Cell Biol. 80: 1-6.
9. Coudray C, Pucheu S, Boucher F, Arnaud J, de Leiris J and Favier A (1994) Effect of ischemia/reperfusion sequence on cytosolic iron status and its release in the coronary effluent in isolated rat hearts. Biol Trace Elem Res. 41: 69-75.
10. Erga KS, Peen E, Tenstad O and Reed RK (2000) Lactoferrin and anti-lactoferrin antibodies: effects of ironloading of lactoferrin on albumin extravasation in different tissues in rats. Acta Physiol Scand. 170: 11-9.
11. Fielding RA, Violan MA, Svetkey L, Abad LW, Manfredi TJ, Cosmas A and Bean J (2000) Effects of prior exercise on eccentric exercise-induced neutrophilia and enzyme release. Med Sci Sports Exerc. 32: 359-64.
12. Gutteridge JM, Paterson SK, Segal AW and Halliwell B (1981) Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem J. 199: 259-61.

13. Hasenfuss G (1998) Animal models of human cardiovascular disease, heart failure and hypertrophy. Cardiovasc Res. 39: 60-76.
14. Chagoya de Sanchez V, Hernandez-Munoz R, Lopez-Barrera F, Yanez L, Vidrio S, Suarez J, Cota-Garza MD, Aranda-Fraustro A and Cruz D (1997) Sequential changes of energy metabolism and mitochondrial function in myocardial infarction induced by isoproterenol in rats: a long-term and integrative study. Can J Physiol Pharmacol. 75: 1300-11.
15. Inoue H, Sakai M, Kaida Y and Kaibara K (2004) Blood lactoferrin release induced by running exercise in normal volunteers: antibacterial activity. Clin Chim Acta. 341: 165-72.
16. Kalinowski DS and Richardson DR (2007) Future of toxicology-iron chelators and differing modes of action and toxicity: the changing face of iron chelation therapy. Chem Res Toxicol. 20: 715-20.
17. Karle H, Hansen NE, Malmquist J, Karle AK and Larsson I (1979) Turnover of human lactoferrin in the rabbit. Scand J Haematol. 23: 303-12.
18. Kontoghiorghes GJ (2006) New chelation therapies and emerging chelating drugs for the treatment of iron overload. Expert Opin Emerg Drugs. 11: 1-5.
19. Kurose I, Yamada T, Wolf R and Granger DN (1994) P-selectin-dependent leukocyte recruitment and intestinal mucosal injury induced by lactoferrin. J Leukoc Biol. 55: 771-7.
20. Lentner C (1990) Geigy Scientific Tables. Ciba-Geigy Limited: Basel.
21. Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J and Jolles P (1984) Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur J Biochem. 145: 659-76.
22. Neri M, Cerretani D, Fiaschi AI, Laghi PF, Lazzerini PE, Maffione AB, Micheli L, Bruni G, Nencini C, Giorgi G, D'Errico S, Fiore C, Pomara C, Riezzo I, Turillazzi E and Fineschi V (2007) Correlation between cardiac oxidative stress and myocardial pathology due to acute and chronic norepinephrine administration in rats. J Cell Mol Med. 11: 156-70.
23. Oseas R, Yang HH, Baehner RL and Boxer LA (1981) Lactoferrin: a promoter of polymorphonuclear leukocyte adhesiveness. Blood. 57: 939-45.
24. Paffett ML and Walker BR (2007) Vascular adaptations to hypoxia: molecular and cellular mechanisms regulating vascular tone. Essays Biochem. 43: 105-19.
25. Parkkinen J, Sahlstedt L, von Bonsdorff L, Salo H, Ebeling F and Ruutu T (2006) Effect of repeated apotransferrin administrations on serum iron parameters in patients undergoing myeloablative conditioning and allogeneic stem cell transplantation. Br J Haematol. 135: 228-34.
26. Plomteux G, Charlier C, Albert A, Farnier M, Pressac M, Vernet M, Paris M, Dellamonica C and Dezier JF (1987) [Reference values of serum transferrin in newborn infants, children and adults]. Ann Biol Clin (Paris). 45: 622-9.
27. Raghuveer TS, McGuire EM, Martin SM, Wagner BA, Rebouche CJ, Buettner GR and Widness JA (2002) Lactoferrin in the preterm infants' diet attenuates iron-induced oxidation products. Pediatr Res. 52: 964-72.
28. Reddy BR, Wynne J, Kloner RA and Przyklenk K (1991) Pretreatment with the iron chelator desferrioxamine fails to provide sustained protection against myocardial ischaemia-reperfusion injury. Cardiovasc Res. 25: 711-8.
29. Regoeczi E, Chindemi PA, Debanne MT and Prieels JP (1985) Lactoferrin catabolism in the rat liver. Am J Physiol. 248: G8-14.
30. Rona G (1985) Catecholamine cardiotoxicity. J Mol Cell Cardiol. 17: 291-306.
31. Santos-Silva A, Rebelo I, Castro E, Belo L, Catarino C, Monteiro I, Almeida MD and Quintanilha A (2002) Erythrocyte damage and leukocyte activation in ischemic stroke. Clin Chim Acta. 320: 29-35.
32. Shimmura S, Shimoyama M, Hojo M, Urayama K and Tsubota K (1998) Reoxygenation injury in a cultured corneal epithelial cell line protected by the uptake of lactoferrin. Invest Ophthalmol Vis Sci. 39: 1346-51.
33. Spiller P and Webb-Peploe MM (1985) Blood flow. Eur Heart J. 6 Suppl C: 11-8.
34. Sterba M, Popelova O, Simunek T, Mazurova Y, Potacova A, Adamcova M, Guncova I, Kaiserova H, Palicka V, Ponka P and Gersl V (2007) Iron chelation-afforded cardioprotection against chronic anthracycline cardiotoxicity: A study of salicylaldehyde isonicotinoyl hydrazone (SIH). Toxicology. 235: 150-66.
35. van Snick JL, Markowetz B and Masson PL (1977) The ingestion and digestion of human lactoferrin by mouse peritoneal macrophages and the transfer of its iron into ferritin. J Exp Med. 146: 817-27.

36. Ward PA, Till GO, Kunkel R and Beauchamp C (1983) Evidence for role of hydroxyl radical in complement and neutrophil-dependent tissue injury. J Clin Invest. 72: 789-801.
37. Weinberg ED (2003) The therapeutic potential of lactoferrin. Expert Opin Investig Drugs. 12: 841-51.
38. Weinberg ED (2006) Therapeutic potential of iron chelators in diseases associated with iron mismanagement. J Pharm Pharmacol. 58: 575-84.
39. Wolach B, Coates TD, Hugli TE, Baehner RL and Boxer LA (1984) Plasma lactoferrin reflects granulocyte activation via complement in burn patients. J Lab Clin Med. 103: 284-93.

4. The Novel Iron Chelator, PCTH, Reduces Catecholamine-Mediated Myocardial Toxicity

Mladěnka P, Kalinowski D, Hašková P, Bobrovová Z, Hrdina R, Šimůnek T, Nachtigal P, Semecký V, Vávrová J, Holečková M, Palicka V, Mazurová Y, Jansson P, & Richardson DR. The Novel Iron Chelator, PCTH, Reduces Catecholamine-Mediated Myocardial Toxicity. Chem Res Toxicol 2008, in press

Abstract:

Iron (Fe) chelators are used clinically for treatment of Fe overload disease. Iron also plays a role in the pathology of many other conditions and these potentially include the cardiotoxicity induced by catecholamines such as isoprenaline (ISO). The current study examined the potential of Fe chelators to prevent ISO cardiotoxicity. This was done as like other catecholamines, ISO contains the classical catechol moiety that binds Fe and may form redox-active and cytotoxic Fe complexes. Studies *in vitro* used the cardiomyocyte cell line, H9c2, which were treated with ISO in the presence or absence of the chelator, desferrioxamine (DFO), or the lipophilic ligand, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH). Both these chelators were not cardiotoxic and significantly reduced ISO cardiotoxicity *in vitro*. However, PCTH was far more effective than DFO, with the latter showing activity only at a high, clinically unachievable concentration. Further studies *in vitro* showed that interaction of ISO with Fe(II)/(III) did not increase cytotoxic radical generation, suggesting this mechanism was not involved. Further studies *in vivo* were initiated using rats pre-treated intravenously with DFO or PCTH before subcutaneous administration of ISO (100 mg/kg). DFO at a clinically used dose (50 mg/kg) failed to reduce catecholamine-cardiotoxicity, while PCTH at an equimolar dose totally prevented catecholamine-induced mortality and reduced cardiotoxicity. This study demonstrates PCTH reduced ISO-induced cardiotoxicity *in vitro* and *in vivo* demonstrating that Fe plays a role, in part, in the pathology observed.

Introduction

Iron (Fe) chelators are well known therapeutic agents administered to prevent complications associated with transfusional Fe overload in diseases such as β-thalassemia major (*1-6*). Iron chelators that are specifically designed for the treatment of Fe overload disease act by avidly binding Fe, preventing it from participating in deleterious Fenton chemistry that results in the generation of reactive oxygen species (ROS) and oxidative cell damage (*4, 7*).

Endogenous catecholamines such as epinephrine (Figure 1) and norepinephrine are known to bind Fe(III) through their catechol groups forming 3:1 ligand:Fe complexes (*8-10*) (Figure 1). Catecholamines are elevated in cardiovascular disease and may trigger acute myocardial infarction (AMI) (*11, 12*). In addition, sufficient doses of the synthetic catecholamine and non-selective β-adrenergic agonist, isoprenaline (ISO; Figure 1), evoke cardiac pathology which shares similarities to AMI (*13, 14*).

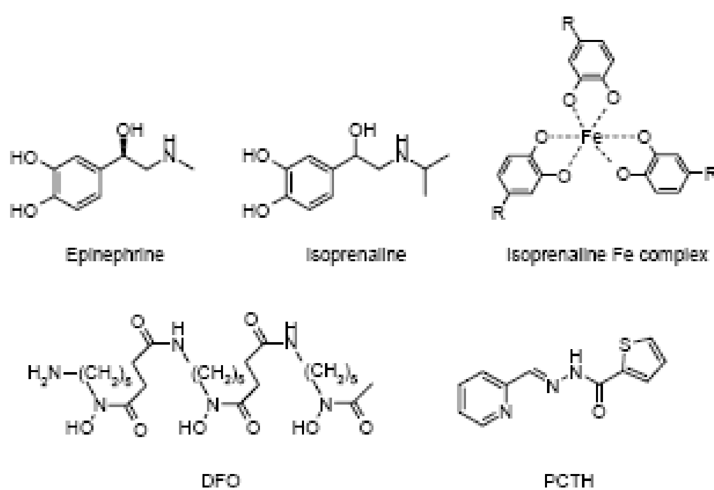


Figure 1. Chemical structures of epinephrine, isoprenaline (ISO), the 3:1 isoprenaline:Fe(III) complex, desferrioxamine (DFO) and 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH).

Catecholamines can produce cardiotoxicity through binding to adrenoceptors and also through oxidative pathways (*15*). However, the mechanisms involved in the latter process remain unclear. It is known that catecholamines undergo auto-oxidation and it has been suggested that the oxidative products of these agents are cardiotoxic (*16-18*). Previously, epinephrine was shown to increase the production of ROS in the presence of Fe and hydrogen peroxide (*19*), suggesting the ability of catecholamines to form redox-active Fe complexes (Figure 1). Considering this, it can be hypothesized that the generation of such complexes may participate in the myocardial tissue damage observed in ISO-induced cardiotoxicity and also AMI. The fact that anti-oxidants limit ISO-mediated cardiotoxicity suggests a role of free radicals in its pathogenesis (*20, 21*).

Based on the described pathogenesis of AMI-induced myocardial injury and the potential of catecholamines to form redox-active Fe complexes that could impart cytotoxicity, chelation therapy may be a useful intervention. A number of studies have investigated the role of the traditionally used chelator for Fe overload, namely, desferrioxamine (DFO; Figure 1), in models of AMI with conflicting results (*22-25*). It is well known that DFO has many limitations at binding intracellular Fe pools due to its high molecular weight, hydrophilicity and short plasma half-life (*7*). These problems result in poor Fe chelation efficacy and necessitates long subcutaneous (s.c.) infusion (12-24 h/day 5-6 days/week) (*7, 26*). Thus, the development of novel, lipophilic and orally-active Fe chelators is vital.

Iron chelators of the pyridoxal isonicotinoyl hydrazone (PIH) class have shown marked activity *in vitro* and *in vivo* (*27*). Their great advantage in comparison to DFO is their greater lipophilicity leading to oral bioavailability. However, the lack of patent protection of PIH has required the development of new ligands that maintain their most important features including lipophilicity (*7*). Such compounds include the 2-pyridylcarboxaldehyde isonicotinoyl

hydrazone (PCIH) series of chelators that show marked Fe chelation efficacy and are protected by national phase patents (*28*).

The PCIH analogs are a well characterized group of tridentate Fe chelators that form 2:1 ligand:metal complexes with Fe(II) (*29*). Of these ligands, the chelator, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH; Figure 1), has been shown to be a promising lead compound (*30, 31*). This chelator was previously observed to effectively mobilize intracellular Fe both *in vitro* in a number of cellular models (*29, 30, 32*) and demonstrated high efficacy and tolerability *in vivo* (*31*). In fact, PCTH was shown to be orally-effective in mice, having activity that was comparable to the orally-active chelators, PIH and deferiprone (L1) (*31*). Furthermore, the PCTH-Fe complex was found to be redox-inactive, acting in a protective manner to prevent Fenton chemistry and DNA damage (*29, 33*). In addition, PCTH was shown to be highly protective against H_2O_2-mediated cytotoxicity in a variety of cell types in culture (*34*). Collectively, these properties make PCTH an ideal candidate chelator for prevention of tissue impairment due to Fe-induced ROS generation.

The current study compared the *in vitro* and *in vivo* protective effects of the Fe chelators, DFO and PCTH, in catecholamine-induced cardiotoxicity. At a clinically-relevant dose, DFO could not reduce ISO-mediated cardiotoxicity and mortality in rats, while PCTH completely prevented catecholamine-induced mortality at an equimolar dose. In addition, PCTH significantly reduced signs of cardiotoxicity *in vitro* and *in vivo*, demonstrating this lipophilic Fe chelator has the potential to prevent ISO-mediated cardiotoxicity.

Experimental Procedures

Iron Chelators - Desferrioxamine (DFO) was from Novartis (Basel, Switzerland). 2-Pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH) was synthesized and characterized as previously described (*29, 30*).

***In Vitro* Cytotoxicity Experiments -** ***Cell Culture* -** The H9c2 cardiomyoblast line derived from embryonic rat heart tissue was from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco´s modified Eagle´s medium (DMEM, Lonza, Belgium) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 10 mM HEPES buffer (pH 7.4) in 75 cm^2 tissue culture flasks (Techno Plastic Products AG [TPP], Trasadingen, Switzerland). Incubations were performed at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were sub-cultured when they reached approximately 90% confluence.

For cytotoxicity experiments with neutral red (NR), cells were seeded in 96-well plates (TPP) at a density of 10,000 cells/well. For morphological assessment and nuclei staining (Hoechst 33342; Molecular Probes, Carlsbad, CA), cells were seeded at a density of 75,000 cells/well in 12-well plates (TPP). Then 24 h prior to experiments, the medium was changed to serum- and pyruvate-free DMEM (Sigma, St. Louis, MO). This was done to stop cellular proliferation to mimic the situation in post-mitotic cardiomyocytes. If the cells were kept in serum-containing medium, the observed effects (of ISO as well as Fe chelators) could be a mixture of not only cytotoxic, but also anti-proliferative activity. Pyruvate was removed from the medium since it is an anti-oxidant and may interfere with ROS-related toxicity. However, irrespective of serum or pyruvate deprivation, ISO toxicity was always observed. The H9c2 cells were subsequently incubated for 24 h at 37°C with or without ISO (250 µM; Sigma, St. Louis, MO) and Fe chelators (DFO or PCTH: 25-250 µM) in combination or alone. To dissolve PCTH, DMSO was used and an aliquot added to medium, leading to a final DMSO

concentration of ≤ 0.1% (v/v). At this concentration, DMSO had no effect on cellular viability.

Neutral Red Uptake Viability Assay - Cellular viability was determined using the cytotoxicity assay based on the ability of viable cells to incorporate NR. This is a well established assay showing sensitivity comparable or better than the MTT procedure (*35*). The weak cationic dye, NR, readily penetrates cell membranes by diffusion, accumulating intracellularly in lysosomes (*35*). After incubation with ISO and/or chelators, half the volume of medium from each well was removed and the same volume of medium with NR was added (final NR concentration: 40 µg/mL). After 3 h at 37°C, the supernatant was discarded and the cells fixed with 1% $CaCl_2$ in 0.5% formaldehyde for 15 min. The cells were then washed twice with PBS and solubilized with 1% acetic acid in 50% ethanol. The optical density of soluble NR was measured at 540 nm using a Tecan Infinite 200M plate reader (Tecan, Grodig, Austria). The viability of experimental groups was expressed as a percentage of the untreated control (100 %).

Apoptosis / Necrosis Estimations - Cellular death was determined using nuclei staining with Hoechst 33342 and propidium iodide (PI; Molecular Probes) that are well established and sensitive procedures to determine apoptosis and necrosis (*36*). Hoechst 33342 is a blue-fluorescent probe (λ_{ex}= 360 nm; λ_{em}= 460 nm) staining all nuclei. In apoptotic cells, chromatin condensation occurs and apoptotic cells can thus be identified as those with condensed and more intensely stained chromatin. The red (λ_{ex} = 560 nm; λ_{em}= 630 nm) DNA-binding dye, PI, is unable to cross the plasma membrane of living cells, but readily enters necrotic (or late-stage apoptotic) cells and stains their nuclei red. Cells were loaded with 10

μg/ml of Hoechst 33342 and 1 μg/mL of PI for 20 min at room temperature. Sample fields with approximately 300 cells were randomly selected and evaluated using an inverted epifluorescence microscope (Nikon Eclipse TS100, 10x Nikon air objective) with a digital camera (1300Q, VDS Vosskühler, Germany). Images were assessed with the software NIS-Elements AR 2.20 (Laboratory Imaging, Czech Republic). The cells were scored as "intact" (normal appearance of dark-blue Hoechst 33342-stained nucleus as well as absence of red PI-staining), "apoptotic" (condensed and/or fragmented nuclei but with no red PI staining - presumably apoptotic) and/or "PI+" (red PI-staining; necrotic or late-stage apoptotic). The number of intact, apoptotic and PI-positive cells were expressed as a percentage of the total number of nuclei counted.

Ascorbate Oxidation Assay to Assess Redox Activity of the ISO-Fe(III) Complex - To assess redox activity of the Fe complex of ISO, an established protocol was used to measure ascorbate oxidation (*37, 38*). In brief, ascorbic acid (0.1 mM) was prepared immediately prior to an experiment and incubated in the presence of Fe(III) (10 μM; added as $FeCl_3$), a 50-fold molar excess of citrate (500 μM) and the chelator (1-90 μM). Absorbance at 265 nm was measured after 10- and 40-min at room temperature and the decrease in intensity between these time points calculated. The results were expressed as Fe-binding equivalents (IBE). This was done due to the different coordination modes of the ligands to Fe, *i.e.*, DFO is hexadentate and forms 1:1 ligand:Fe complexes, while ISO is bidentate resulting in 3:1 complexes (Figure 1). A range of ligand:Fe IBE ratio's were used, namely, 0.1, 1, or 3. An IBE of 0.1 represents an excess of Fe to chelator, *i.e.*, 1 hexadentate chelator or 3 bidentate chelators in the presence of 10 Fe atoms. An IBE of 1 is equivalent to the complete filling of the coordination sphere, *i.e.*, Fe:DFO 1:1 or Fe:ISO 1:3. An IBE of 3 represents an excess of

chelator to Fe and is equal to either 3 hexadentate or 9 bidentate ligands in the presence of 1 Fe atom.

***H_2DCF Assay to Examine ISO-Induced ROS Generation* -** The generation of ROS by ISO was assessed using the widely implemented probe, 2',7'-dichlorodihydrofluorescein-diacetate (H_2DCF-DA; Sigma) (*39, 40*). This agent was hydrolysed to 2',7'-dichlorodihydrofluorescein (H_2DCF) *in vitro* and used for experiments. This reagent becomes highly fluorescent when oxidized by ROS (particularly hydroxyl radical and other highly oxidising species) to DCF (2′,7′-dichlorofluorescein) (*39, 40*). Fluorescence intensity is directly proportional to the level of ROS. Experiments were performed *in vitro* using a modification of a technique previously described (*41*). Briefly, cysteine (Cys; 100 μM; positive control) and ISO (100 μM) were tested for their ability to reduce ferric Fe ($FeCl_3$; 10 μM) to its ferrous form in a 25 mM HEPES buffer (pH 7.4). Then H_2O_2 (100 μM) was added to initiate the production of ROS. Fluorescence was measured using a plate reader (Victor 2, Wallac Oy, Turku, Finland) at λ_{ex} 485 nm and λ_{em} 530 nm at 5 min intervals.

***Preparation of ^{59}Fe-Transferrin* -** Transferrin (Tf; Sigma) was labeled with ^{59}Fe (Dupont NEN, MA, USA) to produce fully saturated diferric Tf (^{59}Fe–Tf), as previously described (*42, 43*).

***Effect of ISO, PCTH and DFO on ^{59}Fe Efflux from H9c2 Cells* -** Iron efflux experiments using established techniques (*44, 45*) were performed to examine the ability of ISO to mobilize ^{59}Fe from H9c2 cells compared to the well characterized chelators, DFO and PCTH (*30, 32*). The cells were initially prelabeled with ^{59}Fe-Tf (0.75 μM) for 30 h at 37°C

and washed four times with ice-cold PBS. The cells were then reincubated for 3 h at 37°C with DFO (25-250 μM) or PCTH (25-250 μM) in the presence or absence of ISO (250 μM). Subsequently, the overlying supernatant containing released ^{59}Fe was then separated from the cells using a pasteur pipette and placed in γ-counting tubes. The cells were removed from the plate in 1 mL of PBS using a plastic spatula and added to separate γ-counting tubes. Radioactivity was measured in both the cell pellet and supernatant using a γ-scintillation counter (Wallac Wizard 3, Turku, Finland).

***In Vivo* Experiment**

Animals - Young Wistar male rats (12 weeks-old, approximately 360 g) obtained from Biotest s.r.o. (Konárovice, Czech Republic) were used after two weeks of acclimatization. The animals were maintained in an air-conditioned room, allowed free access to a standard rodent pellet diet and water. Animals were fasted for 12 h prior to the experiment. The study protocol was approved by the Ethics Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové. This conforms to "The Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Study Design - Rats were randomly divided into 10 groups (Table 1) to achieve 7 animals in each group with the exception of those receiving isoprenaline (ISO), where the group consisted of 16 rats. Animals received vehicle (*i.e.*, either physiological saline [for DFO] or 20% propylene glycol [PG] in physiological saline for PCTH) or chelators dissolved in their corresponding vehicle *via* intravenous (i.v.) tail vein injection 5 min before administration of subcutaneous (s.c.) ISO in physiological saline. We have compared the

efficacy of DFO and PCTH *via* the i.v. route because unlike PCTH, DFO is not orally effective.

Measurement of Cardiac Function **-** Animals were anaesthetized with urethane (1.2 g/kg intraperitoneally [i.p.]; Sigma) 24 h after drug administration. A polyethylene catheter (0.5/1.0 mm filled with heparinized saline - 50 IU/mL) was inserted into the right jugular vein for injection of saline. A thermocatheter (o.d. 0.8 mm) was introduced through the left carotid artery into the aortic arch. Another catheter (0.5/1.0 mm filled with heparinized saline 50 IU/mL) was inserted into the left iliac artery. This was connected to the blood pressure transducer BPR-01 of the apparatus for measurement of hemodynamic variables using Cardiosys® (Experimentria Ltd, Hungary) with the software, Cardiosys V 1.1.

Cardiac function measurements were carried out following a 15 min equilibration period after the surgical procedure. Functional variables (stroke volume, blood pressure, heart rate) were averaged from four measurements performed at 5 min intervals. The mean blood pressure multiplied by heart rate (double product) is a parameter commonly used as an indirect indicator of cardiac oxygen consumption (*46*).

Following the hemodynamic measurements, the blood sample was collected from the abdominal aorta into heparinized (170 IU) test tubes. The animal was then sacrificed by i.v. KCl overdose (1 mM), heart ventricles were excised, weighed and frozen at -20°C for further analysis of selected metal content.

Stroke volume and wet ventricle weights were expressed as an index of body weight.

Histological Analyses - After autopsy, histological examination of cardiac ventricular tissue was performed. Tissue blocks of the transversely sectioned left and right ventricles (the region under the atria towards apex) were fixed by immersion for 3 days in a 4% formaldehyde solution that was freshly prepared from paraformaldehyde. Paraffin sections (7 μm in thickness) were then stained with hematoxylin-eosin.

Biochemical Analyses - Cardiac troponin T (cTnT) is a highly sensitive and specific biomarker of cardiotoxicity (*47*) and was measured in serum using an electro-chemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics). This assay employs two monoclonal antibodies specifically directed against cTnT with a detection limit of 25 μg/L (*47*).

For analysis of metal content, frozen samples of myocardial tissue were dried, weighed and dissolved by microwave digestion using nitric acid and hydrogen peroxide (Milestone MLS 1200 MEGA, Italy). Iron and selenium were determined using graphite furnace atomic absorption spectrometry (Unicam, Solaar 959, United Kingdom). Zinc was assessed using flame atomic absorption spectrometry (Unicam, Solaar 959, United Kingdom), while calcium was measured using flame photometry (Eppendorf, Efox 5053, Germany).

Statistics

Data are expressed as mean ± SEM. Two groups were compared by using Student's *t*-test. Differences between groups were considered to be significant at $p<0.05$.

Results

In Vitro Studies

DFO and PCTH rescue ISO-Induced Cytotoxicity in H9c2 Cells - While the ability of anti-oxidants to prevent ISO-induced cardiotoxicity has been assessed (*20, 21*), there have been no studies examining the role of Fe in this process by using specific Fe chelators. Previous studies examining the ability of DFO to prevent ischemia-reperfusion injury of the heart have demonstrated mixed results (*22-25*). This can be attributed, in part, to the poor membrane permeability of DFO that limits intracellular access (*48*). Considering this, we have compared the effect of DFO to the membrane-permeable ligand, PCTH (*30, 32*), in preventing ISO-mediated cardiotoxicity *in vitro* and *in vivo*.

Initial experiments examined the ability of DFO and PCTH (25–250 μM) to rescue the rat H9c2 cardiomyoblast cell line from the cardiotoxic effects mediated by ISO (250 μM) *in vitro* (Figure 2). After a 24 h exposure to ISO, pronounced changes in cellular morphology were evident in H9c2 cells compared to the relevant control. These observations included disruption of the cell monolayer, peripheral membrane blebbing and rounding up of cells with conspicuous nuclear shrinkage. Eventually, detachment of some cells from the substratum was observed (Figure 2). Exposure of cells for 24 h to 250 μM of either DFO or PCTH alone did not lead to significant alterations of cellular viability and induced no apparent change in cellular morphology. This confirms previous studies demonstrating that both DFO and PCTH were not markedly cytotoxic and are well tolerated *in vitro* and *in vivo* (*30, 31*). Interestingly, incubation of cells with either DFO or PCTH almost totally prevented the marked morphological alterations observed with ISO alone (Figure 2).

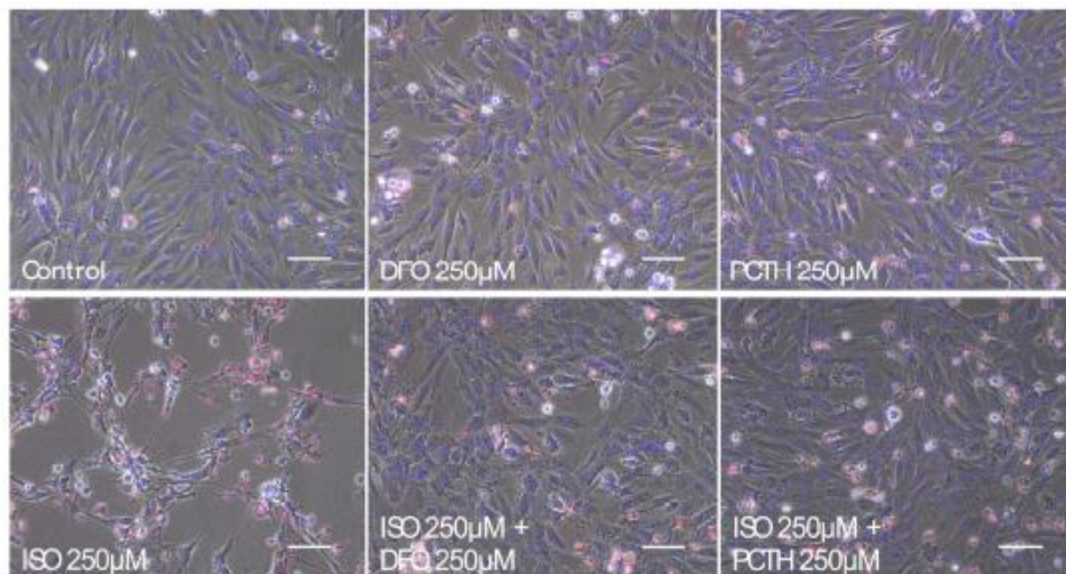


Figure 2. Cellular morphology and nuclear staining with Hoechst 33342 (blue) and propidium iodide (PI; red) after a 24 h incubation of H9c2 cardiomyoblasts with isoprenaline (ISO; 250 µM) alone or in a combination with the Fe chelators, DFO (250 µM) or PCTH (250 µM). Scale bars: 100 µm. Results are typical of 4 experiments.

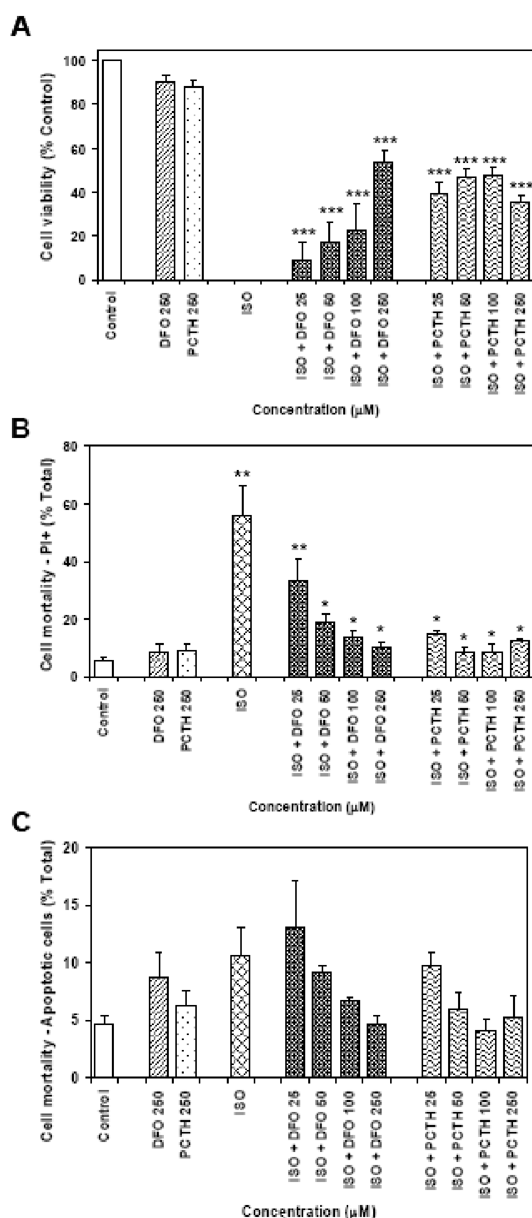


Figure 3. Increasing concentrations of the Fe chelators, PCTH and DFO (25-250 μM), rescue ISO (250 μM)-mediated cytotoxicity in the H9c2 cardiomyoblast cell line. (**A**) Cell viability, determined by neutral red uptake assay (expressed as a percentage of the untreated control group). **(B, C)** Cell mortality, determined by nuclear epifluorescence co-staining with PI and Hoechst 33342: **(B)** PI+ cells and **(C)** apoptotic cells (expressed as a percentage of the total

number of counted nuclei). Results are mean ± SEM (4 experiments). * versus ISO, $p<0.05$; ** versus control, $p<0.01$; *** versus ISO, $p<0.001$.

Consistent with the morphological assessment in Figure 2, vital imaging with NR revealed that DFO and PCTH alone did not significantly affect cellular viability or cell mortality (PI+ or apoptotic cells; Figure 3A-C). In contrast, a complete loss of cellular viability was observed after a 24 h incubation with ISO (250 μM; Figure 3A). Furthermore, in ISO-treated cells, PI-staining showed a marked and significant ($p<0.001$) increase (approximately 5-fold) of injured (necrotic and late stage-apoptotic) cells compared to the control (Figure 3B). Again, as demonstrated by morphological observations, co-incubation of cells with ISO (250 μM) and increasing concentrations of either DFO or PCTH (25–250 μM) resulted in a marked and significant ($p<0.001$) increase of cellular viability in comparison to ISO alone (Figure 3A). Similarly, combination of ISO with DFO or PCTH led to a pronounced decrease of PI+ cells in comparison to ISO alone (Figure 3B).

It is notable that co-incubation of DFO and ISO resulted in a marked increase in viability (Figure 3A) or decrease in mortality (Figure 3B) as a function of DFO concentration from 25-250 μM. Co-incubation of PCTH and ISO significantly ($p<0.001$) increased viability (Figure 3A) and significantly ($p<0.01$) decreased cell mortality (Figure 3B) relative to ISO alone, although no concentration dependence was noted. The concentration-dependence observed with DFO is consistent with its poor membrane permeability and the fact that high concentrations are needed to deplete cellular Fe pools (*49*). In contrast, PCTH is highly permeable, with previous studies demonstrating that concentrations of 25 μM or higher are effective at inducing maximum cellular Fe release (*30*). Thus, the use of 25-250 μM of PCTH in this study. It is likely that even at 25 μM, PCTH has sufficiently bound cellular Fe pools to

prevent the deleterious effects of Fe-mediated Fenton chemistry and that higher PCTH concentrations are no more effective.

Examining the effects of ISO on apoptosis, it is of interest that there were no significant differences in the number of apoptotic cells in any group, including a comparison of the control and ISO-treatment (Figure 3C). This may indicate that either the mode of cell death was predominantly by necrosis, or that apoptosis had occurred early in the 24 h incubation with ISO and that the cells had entered into secondary necrosis (*50*). It is now well established that the distinction between apoptosis and necrosis may not be clear and that a spectrum exists between these two extremes (*51*). Importantly, Fe chelation with DFO or PCTH in the presence of ISO has been shown to prevent cellular death (Figures 2, 3A, B), irrespective of whether it occurs *via* apoptosis or necrosis.

Effect of ISO on Ascorbate Oxidation

Considering the ability of both DFO and particularly PCTH at inhibiting ISO-induced cardiotoxicity *in vitro*, studies progressed to examine the redox activity of the ISO-Fe complex by assessing oxidation of the physiological substrate, ascorbate (Figure 4A). Assessment of this reaction has provided a useful indication of the redox activity of a variety of chelators in previous investigations (*37, 38, 52*). As positive controls, we implemented EDTA and di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) that increase this parameter (*37, 52*). In contrast, DFO and PCTH were used as negative controls as they do not induce ascorbate oxidation (*33*).

As shown previously (*33*), the internal controls, DFO and PCTH, prevented ascorbate oxidation. For instance, PCTH at all IBE's had little effect on ascorbate oxidation (87-95% of the control), while DFO decreased it to 35% and 25% at an IBE of 1 and 3, respectively

(Figure 4A). The positive controls, EDTA and Dp44mT, significantly ($p<0.001$) increased ascorbate oxidation, as expected (*37, 52*), particularly at IBEs of 1 and 3 (Figure 4A). On the other hand, ISO had little effect on ascorbate oxidation at IBEs of 0.1 and 1 and decreased ascorbate oxidation at an IBE of 3 to 70 ± 1% of the control. These studies indicate that the Fe complex of ISO does not readily act to induce ascorbate oxidation and can inhibit this reaction particularly at an IBE of 3 (Figure 4A).

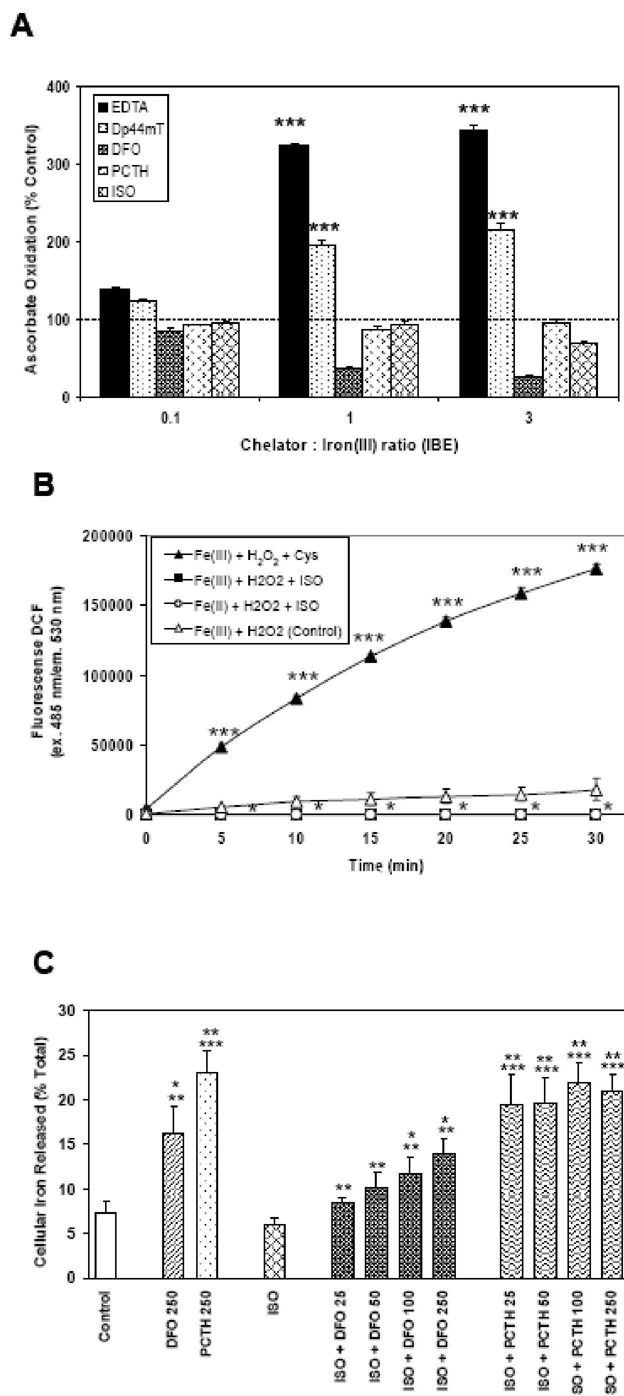


Figure 4. The lack of redox activity of the ISO-Fe complex in the absence of cells: **(A)** ascorbate oxidation, **(B)** DCF-DA oxidation studies, and **(C)** the ability of ISO to induce ^{59}Fe efflux from prelabelled H9c2 cells. **(A)** The Fe complex of ISO is not redox active in the absence of cells as demonstrated by the ascorbate oxidation assay. Chelators at iron-binding equivalent (IBE) ratios of 0.1, 1, and 3 were incubated in the presence of Fe^{III} (10 μM) and

ascorbate (100 μM). The UV absorbance at 265 nm was recorded after 10- and 40-min and the difference between the time points was calculated. The effects of ISO and PCTH were compared to the positive controls, DFO, EDTA and Dp44mT. **(B)** Production of reactive oxygen species was demonstrated by oxidation of the non-fluorescent probe H_2DCF to fluorescent DCF (5 *μ*M). To induce production of ROS, 100 *μ*M H_2O_2 was added to 25 mM HEPES buffer (pH 7.4) containing 10 *μ*M $FeCl_3$ and 100 *μ*M cysteine (-▲-) or 100 *μ*M isoprenaline (-●-). **(C)** H9c2 cells were incubated for 30 h at 37°C with ^{59}Fe-transferrin (^{59}Fe-Tf; [Tf] = 0.75 μM; [Fe] = 1.5 μM) to label cellular pools, washed and then reincubated for 3 h at 37°C with DFO (25-250 μM) or PCTH (25-250 μM) in the presence or absence of ISO (250 μM). The ^{59}Fe was then assessed in the cells and overlying medium using a γ-counter. Results are expressed as mean ± SEM of three experiments. * versus control, $p<0.05$; ** versus ISO, $p<0.01$; *** versus control, $p<0.001$.

Effect of ISO on H_2DCF Oxidation in the Absence of Cells

To further assess the effect of ISO on the redox activity of Fe, studies were initiated to examine the oxidation of the redox-sensitive probe, H_2DCF (*41*) (Figure 4B). As a positive control to induce H_2DCF oxidation, cysteine (Cys) was incubated in the presence of hydrogen peroxide and Fe(III). This significantly ($p<0.001$) increased DCF fluorescence at all time points (Figure 4B). In contrast, ISO significantly ($p<0.05$) decreased fluorescence after a 10 min incubation with Fe(II) or Fe(III) and H_2O_2. This suggested that the chelation of Fe by ISO acted to reduce ROS generation.

Effect of ISO on Inducing ^{59}Fe Efflux from Prelabeled H9c2 Cells

The finding that the interaction of ISO and Fe led to redox-inactive Fe complexes may indicate that at least part of the cardiotoxicity of this agent is due to its interaction with cellular Fe pools. To understand the interaction of ISO with cellular Fe, we examined the chelation efficacy of this catecholamine by assessing its ability to induce ^{59}Fe release from prelabeled H9c2 cells (Figure 4C). In these studies, ISO was compared to DFO and PCTH that have well characterized Fe chelation properties in cells (*30, 32*). These studies were done using standard procedures in our laboratory and provide data on the ability of these agents to permeate cell membranes and chelate intracellular Fe pools.

Prelabeled H9c2 cells incubated with control medium released very little ^{59}Fe (7% of total cell ^{59}Fe; Figure 4C), as found for other cell types (*29, 30, 45*). DFO (250 μM) alone significantly increased ^{59}Fe release (16% of total cell ^{59}Fe effluxed) relative to the control. PCTH (250 μM) was slightly more effective than DFO and significantly ($p<0.001$) increased ^{59}Fe release (to 23% of total cell ^{59}Fe) in comparison to control media, confirming its ability to penetrate cells (*29, 30, 32*). Despite the presence of the classical catechol Fe-binding moiety, ISO (250 μM) did not increase ^{59}Fe mobilization from cells in comparison to the control. As found in Figure 3A-C where a dose response was identified with DFO (25-250 μM), a concentration-dependent increase in ^{59}Fe efflux was observed when this ligand was combined with ISO (250 μM; Figure 4C). Consistent with previous results (Figure 3A-C), no dose response was observed with increasing concentrations of PCTH (25-250 μM) combined with ISO (250 μM), demonstrating optimal activity at 25 μM. Comparing activity of the ligands in the presence and absence of ISO, there was no significant alterations in ^{59}Fe release suggesting that ISO did not act to enhance mobilization of cellular ^{59}Fe pools during the 3 h reincubation. Similar observations were found after a 6 h reincubation (data not shown). It should be noted that longer incubations with ISO led to decreased cellular viability (Figure 2

and 3A) that could potentially alter cellular ^{59}Fe release. Hence, longer reincubation periods were not performed.

***In Vivo* Studies**

Mortality

Considering the protective effects of both DFO and PCTH in preventing ISO-mediated cardiotoxicity *in vitro* (Figures 2, 3A,B), subsequent studies examined the protective effects of Fe chelators against ISO-induced cardiotoxicity *in vivo*. Subcutaneous administration of 100 mg/kg of ISO in rats caused 31% mortality (5 of 16 animals) within 24 h (Figure 5A). Pre-treatment of rats with an i.v. injection of a clinically-relevant dose of DFO (50 mg/kg) or the lower dose of PCTH (10 mg/kg) 5 min prior to s.c. ISO administration, led to similar mortality (33% for both) as that observed after ISO alone (Figure 5A). However, pre-medication with a higher dose of PCTH (20 mg/kg – equimolar to the 50 mg/kg DFO dose), totally abolished ISO-associated mortality and this was in contrast with the mortality observed with ISO or ISO + PG groups (Figure 5A). In the control or solvent groups (*ie*., PG alone), no mortality occurred (Figure 5A).

Histopathology

Histological examination of the heart tissue was performed to assess the levels of cardiotoxicity in each treatment group (Figure 5B-E). Normal myocardial tissue was found in control-treated animals (Figure 5B) and comparable findings were also seen after administration of PCTH, DFO or PG (Figure 5C-E). In the ISO treatment group (Figure 5F), severe diffuse toxic damage with an inflammatory reaction was found in the whole myocardium (with maximum damage seen in the sub-endocardial region, particularly in the

heart apex). Cardiomyocytes with an intensely eosinophilic cytoplasm prevailed and degenerated or necrotic cells were also numerous. Slight focal interstitial edema, mild inflammatory infiltrate and the presence of macrophages were characteristically found (Figure 5F).

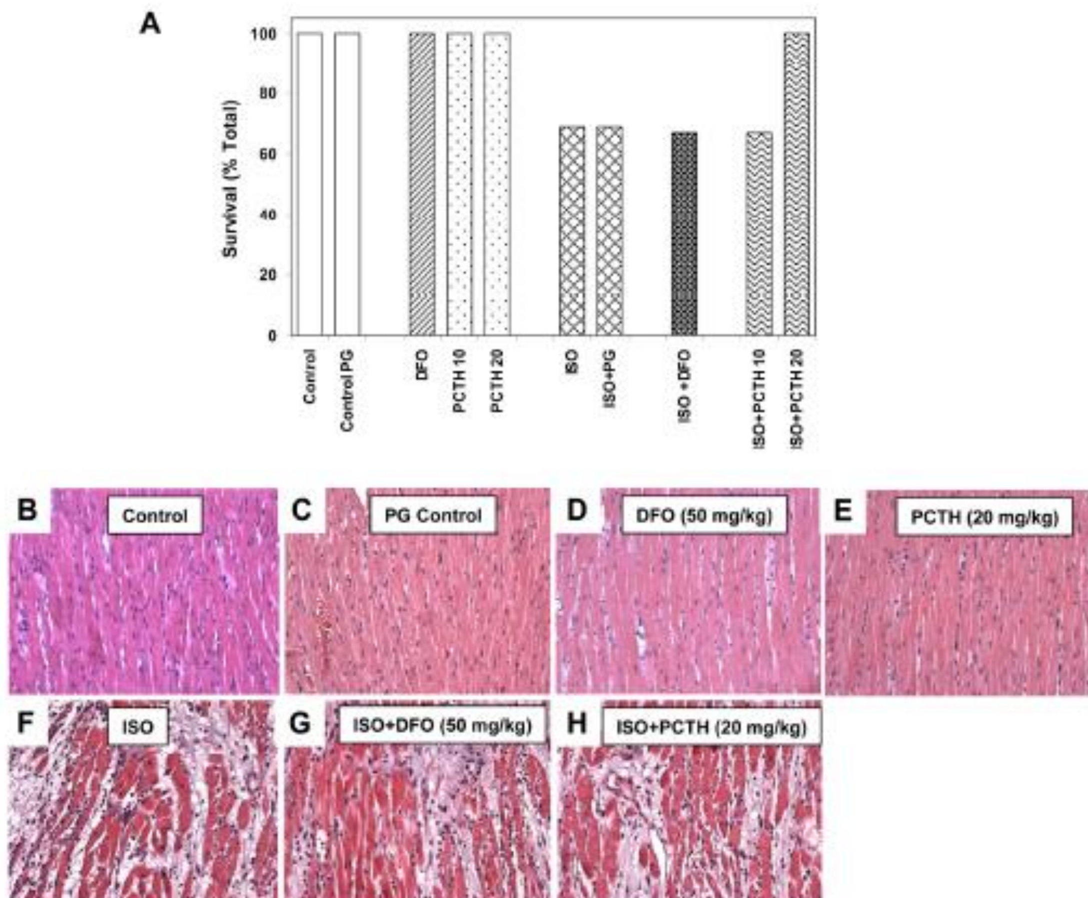


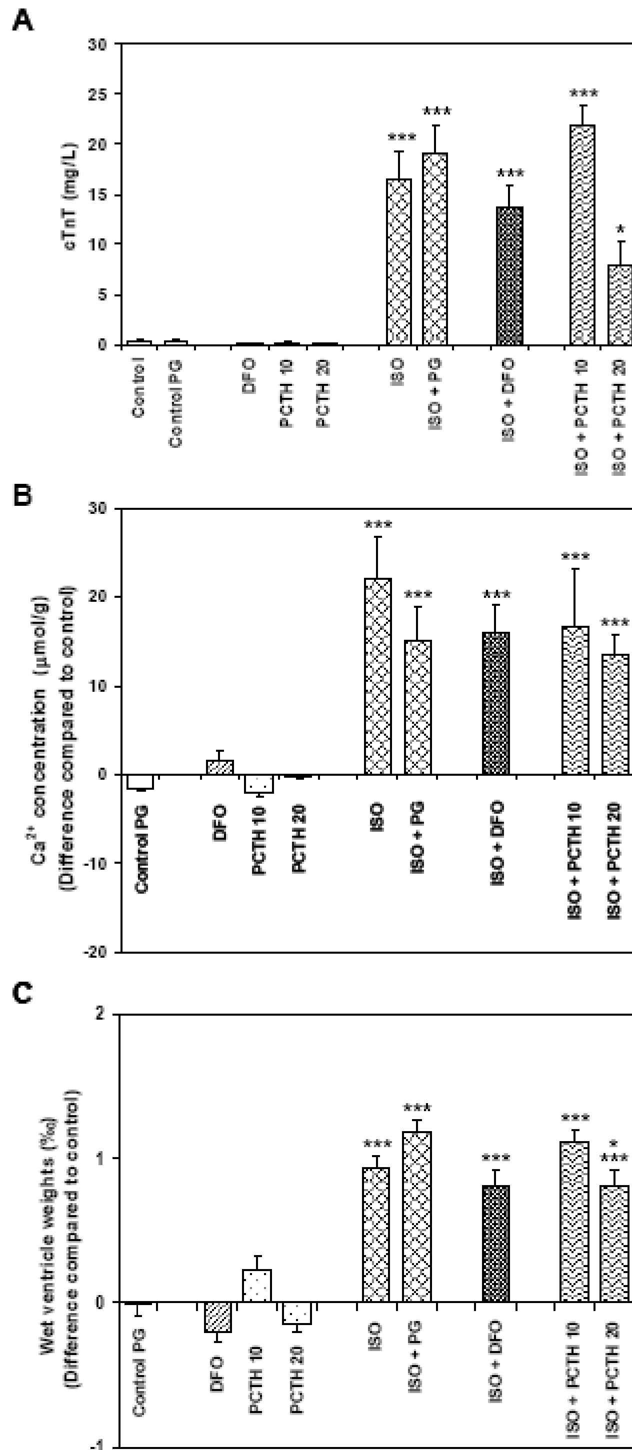
Figure 5. *In vivo* studies in which rats were treated with ISO with or without Fe chelator administration demonstrating: (**A**) survival rates for each treatment (expressed as a percentage of the total number of animals in each experimental group); **(B-H)** histology of the myocardium after treatment of rats with **(B)** control saline, **(C)** control propylene glycol (PG), **(D)** DFO (50 mg/kg), **(E)** PCTH (20 mg/kg), **(F)** ISO (100 mg/kg), **(G)** ISO (100 mg/kg) and

DFO (50 mg/kg) and **(H)** ISO (100 mg/kg) and PCTH (20 mg/kg). Results are mean ± SEM (n = 7-16 animals).

Although DFO was able to maintain cellular viability *in vitro* when co-incubated with ISO (Figure 2), this ligand (50 mg/kg) was unable to rescue the myocardium from the tissue damage mediated by ISO *in vivo* (Figure 5G). Interestingly, despite the decreased mortality of rats observed after treatment of ISO-treated rats with PCTH (20 mg/kg; Figure 5A), there was no effective rescue of the myocardium from the distinct morphological changes induced by ISO (Figure 5H). Together with the fact that even very high chelator concentrations *in vitro* did not totally prevent ISO-induced cytotoxicity (Figure 3A-C), these results suggest incomplete rescue of the effects of ISO by chelators *in vivo*. This could indicate that other factors insensitive to the effects of Fe chelation play significant roles in ISO-induced cardiotoxicity.

Markers of Myocardial Injury

Figure 6A shows serum levels of cTnT which represents released cTnT from myocardial cells and is a sensitive indice of cardiotoxicity *in vivo* (*47*). Controls and chelators did not produce any substantial release of cTnT, again demonstrating that the ligands alone were well tolerated at these doses. In contrast, ISO (100 mg/kg) or ISO + PG caused a significant ($p<0.001$) elevation in cTnT (ISO or ISO + PG; Figure 6A). Combination of ISO and DFO (50 mg/kg) did not decrease the cardiotoxicity observed with ISO (Figure 6A), while the 20 mg/kg dose of PCTH (but not the 10 mg/kg dose) significantly ($p<0.05$) decreased cTnT concentration (ISO + PCTH 20) in comparison to the relevant control (ISO + PG; Figure 6A).


A
cTnT (mg/L)
30
25
20
15
10
5
0
***
***
***
***
*
Control
Control PG
DFO
PCTH 10
PCTH 20
ISO
ISO + PG
ISO + DFO
ISO + PCTH 10
ISO + PCTH 20
B
Ca^{2+} concentration (µmol/g)
(Difference compared to control)
30
20
10
0
-10
-20
***
***
***
***
***
Control PG
DFO
PCTH 10
PCTH 20
ISO
ISO + PG
ISO + DFO
ISO + PCTH 10
ISO + PCTH 20
C
Wet ventricle weights (‰)
(Difference compared to control)
2
1
0
-1
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Control PG
DFO
PCTH 10
PCTH 20
ISO
ISO + PG
ISO + DFO
ISO + PCTH 10
ISO + PCTH 20

Figure 6. Examination of indices of cardiotoxicity *in vivo* after treatment with vehicles alone, ISO alone, chelators alone or chelators in the presence of ISO. The conditions were: control saline vehicle, PG vehicle, DFO (50 mg/kg), PCTH (10 or 20 mg/kg), ISO (100 mg/kg). (**A**) Serum cardiac troponin T (cTnT) levels 24 h after drug application; (**B**) myocardial calcium content (expressed as the difference compared to the saline control); (**C**) wet ventricle weight index (expressed as the difference in wet ventricle weight indices in comparison to the saline control). Results are mean ± SEM (n = 7-16 animals). * versus ISO + PG, $p<0.05$; *** versus control, $p<0.001$.

Myocardial calcium levels (Figure 6B) and the wet weight of the ventricle (Figure 6C) were also measured as markers of cardiotoxicity and found to be markedly and significantly ($p<0.001$) increased after ISO administration in comparison to the controls (Figure 6B, C). Neither DFO nor PCTH alone had any significant impact on calcium levels or ventricle weights in comparison to controls. Combination of ISO with either DFO or PCTH did not significantly affect myocardial calcium concentration relative to the controls, namely ISO alone or ISO + PG, respectively (Figure 6B). In ISO-treated rats, only the higher dose of PCTH (20 mg/kg) significantly decreased wet ventricle weight compared to the relative control (*i.e.*, ISO + PG).

Functional Parameters of the Heart

The data summarizing heart functional parameters are shown in Table 1. Administration of ISO significantly ($p<0.05$) decreased stroke volume index in comparison to the saline control. PG alone significantly decreased stroke volume index and this is the likely reason for the lack of difference between the PG + ISO and control PG groups. Heart rate was

significantly elevated in both ISO groups in comparison to their corresponding controls. Both Fe chelators alone did not affect stroke volume index as well as heart rate. Further, none of the chelator treatments were able to significantly attenuate the observed changes in heart rate or stroke volume index induced by ISO (Table 1). This demonstrated that Fe chelation did not affect the adrenergic activity of ISO. There were only minor differences between groups in regards to blood pressure with the exception of PG, which alone elevated systolic pressure in comparison to the saline control.

Metal Content of the Myocardium

There were no significant differences in the concentrations of Fe, selenium and zinc in the myocardium, except for copper (increased in control PG group) in all experimental groups (data not shown). It is not surprising that the low dose of PCTH used in the current study did not result in Fe-depletion since higher doses of the chelator, PCTH (100 mg/kg/*bd*), and longer durations of treatment are necessary for optimal Fe chelation and excretion (*31*).

Parameter / Group	control	control PG	DFO	PCTH 10	PCTH 20	ISO	PG + ISO	DFO + ISO	PCTH 10 + ISO	PCTH 20 + ISO
Stroke volume index /ml.min^{-1}.kg^{-1}/	0.81 ± 0.07	0.61 ± 0.05^{c}	0.77 ± 0.09	1.16 ± 0.15^{c}	0.69 ± 0.10	0.37 ± 0.05^{c}	0.49 ± 0.03	0.38 ± 0.03^{c}	0.51 ± 0.05	0.43 ± 0.03^{c}
Systolic pressure /mmHg/	108 ± 5	128 ± 7^{c}	119 ± 7	122 ± 9	132 ± 4	126 ± 7	132 ± 6	116 ± 3	109 ± 5^{i}	133 ± 7
Diastolic pressure /mmHg/	79 ± 4	89 ± 6	70 ± 2	79 ± 7	93 ± 6	95 ± 6	101 ± 3	87 ± 2	82 ± 5^{i}	104 ± 6
Heart rate /beats.min^{-1}/	410 ± 12	418 ± 18	406 ± 13	388 ± 22	412 ± 10	459 ± 7^{c}	466 ± 8^{c}	473 ± 13^{c}	465 ± 12^{c}	479 ± 15^{c}
Double product /mmHg.beats.min^{-1}/	36575±2172	42936±4052	36151±2417	36812±4720	43833±2506	48432±3218^{c}	51736±1625	45525±2052^{c}	42284±2648^{i}	54130±3429

Table 1. Functional parameters of the heart, heart rate and blood pressure. Results are mean ± SEM (*n* = 7-16 animals)

Abbreviations: PG, propylene glycol; PCTH 10 or 20, PCTH at 10 or 20 mg/kg

Statistical significance at $p<0.05$: c - versus relevant solvent group (control or control PG); i - versus relevant ISO group

Discussion

The synthetic catecholamine, ISO, has repeatedly been shown to evoke many similar pathogenic features as AMI (*13, 14, 53, 54*). Myocardial ischemia is associated with the progressive increase in endogenous catecholamine concentrations and has been observed in patients with AMI (*55, 56*). The harmful effects of catecholamines are hypothesized to be due to a number of mechanisms, including the induction of oxidative stress (*20, 21*). In the current study, we have examined the role of Fe in ISO-induced cardiotoxicity *in vitro* and *in vivo*. This was initiated due to evidence that catecholamines are well known to directly chelate Fe and could potentially form redox-active Fe complexes (*57, 58*). For the first time, we demonstrate that the lipophilic chelator PCTH rescues, in part, ISO-induced cardiotoxicity *in vitro* and *in vivo* illustrating a role for Fe in the pathology observed.

Both DFO and PCTH were able to impart significant cyto-protection against ISO toxicity using H9c2 cardiomyoblasts *in vitro*. However, DFO only approached the efficacy of PCTH in terms of protection at the highest tested concentration of 250 μM (Figure 3A). This is probably because of the lower membrane permeability and Fe chelation efficacy of this ligand (*48, 49*). Of significance, this DFO concentration greatly exceeds the highest plasma levels generally achieved in clinical settings, namely 10 μM (*59*). In contrast, the lipophilic and cell-permeable Fe chelator, PCTH (*29, 30, 32*), reduced ISO-mediated cardiotoxicity *in vitro* at only 25 μM (Figure 3A, B). Our *in vivo* rat model of ISO-mediated cardiotoxicity demonstrated that PCTH (20 mg/kg) completely prevented ISO-mediated mortality, while an equimolar dose of DFO did not (Figure 5A). These mortality results were supported by the significant reduction of serum

cTnT levels and wet ventricle weight comparing PCTH (20 mg/kg) treatment in comparison to the relevant ISO + PG control group (Figure 6A, C).

It is of interest that PCTH was able to significantly reduce ISO-induced cardiotoxicity *in vitro* and *in vivo*, but could not completely prevent these effects. It is notable that PCTH completely prevented ISO-mediated mortality in rats, but signs of cardiac damage were not (histology, calcium levels) or only partially (increased troponin release, wet ventricle weight) inhibited by PCTH pre-treatment. This finding was surprising, but it should be noted that the cardiotoxicity of catecholamines is not fully understood. In fact, it suggests that other mechanisms apart from those induced by the interaction of ISO with Fe also played important roles in ISO-induced cardiotoxicity.

Previous studies have demonstrated that cardiotoxicity of ISO can be mediated through two major mechanisms. First, β_1 and β_2 adrenoceptors mediating positive inotropic and chronotropic effects, results in ischemia due to myocardial hyper-activity, coronary hypo-perfusion and cytosolic Ca(II) overload (*60, 61*). Second, the generation of free radical species through mechanisms including the ability of catecholamines to undergo oxidative metabolism with the generation of ROS and the formation of toxic aminochromes (*18, 62-65*). Hence, chelation of Fe by DFO or PCTH would aid in the inhibition of some of the oxidative processes mediated by Fenton chemistry, but not the adrenoceptor-mediated mechanism. This explains the partial rescue of cardiotoxicity observed by these chelators. Indeed, as observed, the chelators had no effect on the positive inotropic and chronotropic effects of ISO (Table 1), suggesting that some of the cardiotoxicity observed *in vivo* is probably mediated through this mechanism. By

analogy, in a recent study, not all changes induced by catecholamines could be reverted by blockade of adrenergic receptors, suggesting the cytotoxic role of the oxidative processes (*63*).

With regards to the nature of Fe-mediated ISO-cardiotoxicity, it could be hypothesized that due to its structural similarity to epinephrine (Figure 1) and ability to bind Fe, ISO could form a cytotoxic redox-active Fe complex (*19*). Our studies examining both ascorbate oxidation and the DCF radical detection technique could not confirm increased redox activity of ISO-Fe(II) or Fe(III) complexes (Figure 4A, B). It is also known that ISO induces marked metabolic alterations in the heart which include inotropic and chronotropic effects, hypotension, metabolic acidosis, mitochondrial swelling and decreased indices of mitochondrial function including: oxygen consumption, respiratory quotient, ATP synthesis and membrane potential (*13, 66*). How these alterations precisely influence cardiomyocyte Fe trafficking are unknown.

Since the mitochondrion plays a key role in Fe metabolism (*67*), the marked alterations in this organelle could be significant in terms of Fe playing a cytotoxic role after ISO administration. It is also known that ISO induces ischaemia and this event has been associated with the release of “free” redox-active Fe (*68*). Again, this Fe release could be important to consider in terms of the chelator-mediated rescue from ISO-mediated cardiotoxicity. However, it is notable that ISO alone did not induce Fe release from cells nor was there any alteration in Fe mobilization mediated by DFO or PCTH in the presence of this catecholamine over 3-6 h (Figure 4C). This indicated that quantitative alterations in chelatable Fe pools did not occur in the presence of ISO, at least over the periods assessed.

Considering the facts above, the Fe-mediated toxicity of ISO could be due to the well described generation of redox-active ISO metabolites (*e.g.*, isoprenochrome, isoproterenol-*o*-quinone, leukoisoprenochrome *etc*) (*17, 18*). In the presence of Fe, this could lead to pronounced cytotoxicity *via* Fenton and Haber-Weiss chemistry, and hence, the partial rescue mediated by Fe chelators. In addition, transition metals have been shown to potentiate the conversion of ISO to isoprenochrome (*18*), leading to another potential mechanism by which chelators could inhibit ISO-mediated cardiotoxicity.

The effectiveness of particular Fe chelators to inhibit Fenton chemistry is linked with their chemical properties. The most important of these is the donor atoms involved in the coordination of Fe and the ability of the Fe complex to inhibit Fe-catalyzed ROS generation (*69*). Numerous Fe chelators (*e.g.,* EDTA) form redox-active Fe complexes that promote ROS formation, making them unsuitable as redox-protective agents (*33, 38*). In contrast, chelators with very low redox potentials, like DFO (-475mV), form very stable complexes with Fe(III) and are poor Fenton chemistry catalysts (*33, 38*). Analogously, the Fe complex of the lipophilic chelator, PCTH, is redox inactive, making it appropriate for Fe chelation therapy (*4, 29*).

Neither ISO nor the Fe chelators had any statistically significant effect on total Fe concentration in the myocardium. Previously, it has been well documented that administration of both DFO and PCTH led to elevated Fe excretion *in vivo* (*31, 70*). It is therefore likely that, due to the low dose and very limited length of treatment, the chelators in this study only resulted in binding Fe that was participating in the Fenton reaction, rather than inducing systemic Fe-depletion.

In summary, this investigation has shown that both DFO and PCTH were able to afford significant cytoprotection of cardiac cells against ISO toxicity *in vitro*. In contrast, *in vivo*, DFO at the highest clinically-relevant dose (equimolar to 20 mg/kg PCTH) showed no positive influence. On the other hand, the novel lipophilic Fe chelator, PCTH (20 mg/kg), was able to completely prevent ISO-mediated mortality *in vivo* and significantly attenuate some markers of cardiac injury caused by a necrogenic dose of ISO. This study demonstrates that further investigation of lipophilic Fe chelators such as PCTH is warranted for protection against catecholamine-induced cardiotoxicity.

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Abbreviations: AMI, acute myocardial infarction; cTnT, cardiac troponin T; DCF, 2',7'-dichlorofluorescein; DFO, desferrioxamine; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; H_2DCF, 2',7'-dichlorodihydrofluorescein; H_2DCF-DA 2',7'-dichlorodihydrofluorescein-diacetate; IBE, iron-binding equivalent; ISO, isoprenaline; NR, neutral red; PCIH, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone; PCTH, 2-

pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone; PG, propylene glycol; PIH, pyridoxal isonicotinoyl hydrazone; PI, propidium iodide; ROS, reactive oxygen species; Tf, transferrin.

References

(1) Davis, B. A., and Porter, J. B. (2000) Long-term outcome of continuous 24-hour deferoxamine infusion via indwelling intravenous catheters in high-risk beta-thalassemia. *Blood 95*, 1229-1236.

(2) Hershko, C., Link, G., and Konijn, A. M. (2002) Cardioprotective effect of iron chelators. *Adv. Exp. Med. Biol. 509*, 77-89.

(3) Hrdina, R., Gersl, V., Klimtova, I., Simunek, T., Machackova, J., and Adamcova, M. (2000) Anthracycline-induced cardiotoxicity. *Acta. Medica (Hradec Kralove) 43*, 75-82.

(4) Kalinowski, D. S., and Richardson, D. R. (2007) Future of toxicology--iron chelators and differing modes of action and toxicity: the changing face of iron chelation therapy. *Chem. Res. Toxicol. 20*, 715-720.

(5) Olivieri, N. F., and Brittenham, G. M. (1997) Iron-chelating therapy and the treatment of thalassemia. *Blood 89*, 739-761.

(6) Tam, T. F., Leung-Toung, R., Li, W., Wang, Y., Karimian, K., and Spino, M. (2003) Iron chelator research: past, present, and future. *Curr. Med. Chem. 10*, 983-995.

(7) Kalinowski, D. S., and Richardson, D.R. (2005) Evolution of iron chelators for the treatment of iron overload disease and cancer. *Pharmacol. Rev. 57*, 1-37.

(8) Jewett, S. L., Eggling, S., and Geller, L. (1997) Novel method to examine the formation of unstable 2:1 and 3:1 complexes of catecholamines and iron(III). *J. Inorg. Biochem. 66*, 165-173.

(9) Mentasti, E., Pelizzetti, E., and Baiocchi, C. (1976) Interactions of iron(III) with adrenaline, L-Dopa and other catechol derivatives. Electron-exchange kinetics and mechanism in acidic perchlorate media. *J. Inorg. Nucl. Chem. 38*, 2017-2021.

(10) Rajan, K. S., Davis, J. M., and Colburn, R. W. (1971) Metal chelates in the storage and transport of neurotransmitters: interactions of metal ions with biogenic amines. *J. Neurochem. 18*, 345-364.

(11) Kaye, D. M., Lefkovits, J., Jennings, G. L., Bergin, P., Broughton, A., and Esler, M. D. (1995) Adverse consequences of high sympathetic nervous activity in the failing human heart. *J. Am. Coll. Cardiol. 26*, 1257-1263.

(12) Kloner, R. A. (2006) Natural and unnatural triggers of myocardial infarction. *Prog. Cardiovasc. Dis. 48*, 285-300.

(13) Chagoya de Sanchez, V., Hernandez-Munoz, R., Lopez-Barrera, F., Yanez, L., Vidrio, S., Suarez, J., Cota-Garza, M. D., Aranda-Fraustro, A., and Cruz, D. (1997) Sequential changes of energy metabolism and mitochondrial function in myocardial infarction induced by isoproterenol in rats: a long-term and integrative study. *Can. J. Physiol. Pharmacol. 75*, 1300-1311.

(14) Rona, G. (1985) Catecholamine cardiotoxicity. *J. Mol. Cell. Cardiol. 17*, 291-306.

(15) Dhalla, K. S., Rupp, H., Beamish, R. E., and Dhalla, N. S. (1996) Mechanisms of alterations in cardiac membrane Ca2+ transport due to excess catecholamines. *Cardiovasc. Drugs Ther. 10 Suppl 1*, 231-238.

(16) Yates, J. C., and Dhalla, N. S. (1975) Induction of necrosis and failure in the isolated perfused rat heart with oxidized isoproterenol. *J. Mol. Cell. Cardiol. 7*, 807-816.

(17) Persoon-Rothert, M., van der Valk-Kokshoorn, E. J., Egas-Kenniphaas, J. M., Mauve, I., and van der Laarse, A. (1989) Isoproterenol-induced cytotoxicity in neonatal rat heart cell cultures is mediated by free radical formation. *J. Mol. Cell Cardiol. 21*, 1285-1291.

(18) Remiao, F., Carvalho, M., Carmo, H., Carvalho, F., and Bastos, M. L. (2002) Cu2+-induced isoproterenol oxidation into isoprenochrome in adult rat calcium-tolerant cardiomyocytes. *Chem. Res. Toxicol. 15*, 861-869.

(19) Nappi, A. J., and Vass, E. (1997) Comparative studies of enhanced iron-mediated production of hydroxyl radical by glutathione, cysteine, ascorbic acid, and selected catechols. *Biochim. Biophys. Acta 1336*, 295-302.

(20) Rajadurai, M., and Stanely Mainzen Prince, P. (2006) Preventive effect of naringin on lipid peroxides and antioxidants in isoproterenol-induced cardiotoxicity in Wistar rats: biochemical and histopathological evidences. *Toxicology 228*, 259-268.

(21) Rajadurai, M., and Stanely Mainzen Prince, P. (2007) Preventive effect of naringin on cardiac markers, electrocardiographic patterns and lysosomal hydrolases in normal and isoproterenol-induced myocardial infarction in Wistar rats. *Toxicology 230*, 178-188.

(22) Ambrosio, G., Zweier, J. L., Jacobus, W. E., Weisfeldt, M. L., and Flaherty, J. T. (1987) Improvement of postischemic myocardial function and metabolism induced by administration of deferoxamine at the time of reflow: the role of iron in the pathogenesis of reperfusion injury. *Circulation 76*, 906-915.

(23) Bolli, R., Patel, B. S., Jeroudi, M. O., Li, X. Y., Triana, J. F., Lai, E. K., and McCay, P. B. (1990) Iron-mediated radical reactions upon reperfusion contribute to myocardial "stunning". *Am. J. Physiol. 259*, H1901-1911.

(24) DeBoer, D. A., and Clark, R. E. (1992) Iron chelation in myocardial preservation after ischemia-reperfusion injury: the importance of pretreatment and toxicity. *Ann. Thorac. Surg. 53*, 412-418.

(25) Reddy, B. R., Wynne, J., Kloner, R. A., and Przyklenk, K. (1991) Pretreatment with the iron chelator desferrioxamine fails to provide sustained protection against myocardial ischaemia-reperfusion injury. *Cardiovasc. Res. 25*, 711-718.

(26) Hershko, C., Abrahamov, A., Konijn, A. M., Breuer, W., Cabantchik, I. Z., Pootrakul, P., and Link, G. (2003) Objectives and methods of iron chelation therapy. *Bioinorg. Chem. Appl.* 151-168.

(27) Richardson, D. R., and Ponka, P. (1998) Pyridoxal isonicotinoyl hydrazone and its analogues: Potential orally effective iron-chelating agents for the treatment of iron overload disease. *J. Lab. Clin. Med. 131*, 306-315.

(28) Richardson, D. R., Becker, E., and Bernhardt, P. V. (2000) Iron Chelators and Uses Thereof. Australian Patent Application 73992/00; European Patent Application 00962062.6; Japanese Patent Application 2001-521321; Indian Patent Application IN/PCT/2002/00286; Canadian Patent Application 2,383,721; New Zealand Patent Application 517660; United States Patent Application 10/069923; Indonesian Patent Application W-00200200762; Chinese Patent Application 00812399.3; National Phase of PCT/AU00/01050.

(29) Bernhardt, P. V., Chin, P., Sharpe, P. C., and Richardson, D. R. (2007) Hydrazone chelators for the treatment of iron overload disorders: iron coordination chemistry and biological activity. *Dalton Trans.* 3232-3244.

(30) Becker, E., and Richardson, D. R. (1999) Development of novel aroylhydrazone ligands for iron chelation therapy: 2-pyridylcarboxaldehyde isonicotinoyl hydrazone analogs. *J. Lab. Clin. Med. 134*, 510-521.

(31) Wong, C. S., Kwok, J. C., and Richardson, D. R. (2004) PCTH: a novel orally active chelator of the aroylhydrazone class that induces iron excretion from mice. *Biochim. Biophys. Acta 1739*, 70-80.

(32) Richardson, D. R., Mouralian, C., Ponka, P., and Becker, E. (2001) Development of potential iron chelators for the treatment of Friedreich's ataxia: Ligands that mobilize mitochondrial iron. *Biochim. Biophys. Acta 1536*, 133-140.

(33) Chaston, T. B., and Richardson, D. R. (2003) Redox chemistry and DNA interactions of the 2-pyridyl-carboxaldehyde isonicotinoyl hydrazone class of iron chelators: Implications for toxicity in the treatment of iron overload disease. *J. Biol. Inorg. Chem. 8*, 427-438.

(34) Lim, C. K., Kalinowski, D. S., and Richardson, D. R. (2008) Protection against hydrogen peroxide-mediated cytotoxicity by novel iron chelators in fibroblasts from control and Friedreich's ataxia patients. *Mol. Pharmacol. 74*, 225-235.

(35) Yancy, S. L., Shelden, E. A., Gilmont, R. R., and Welsh, M. J. (2005) Sodium arsenite exposure alters cell migration, focal adhesion localization and decreases tyrosine phosphorylation of focal adhesion kinase in H9C2 myoblasts. *Toxicol. Sci. 84*, 278-86.

(36) Simunek, T., Sterba, M., Popelova, O., Kaiserova, H., Adamcova, M., Hroch, M., Haskova, P., Ponka, P., and Gersl, V. (2008) Anthracycline toxicity to cardiomyocytes or cancer cells is differently affected by iron chelation with salicylaldehyde isonicotinoyl hydrazone. *Br. J. Pharmacol. Jun 9. [Epub ahead of print]*.

(37) Richardson, D. R., Sharpe, P. C., Lovejoy, D. B., Senaratne, D., Kalinowski, D. S., Islam, M., and Bernhardt, P. V. (2006) Dipyridyl thiosemicarbazone chelators with potent and selective anti-tumor activity form iron complexes with marked redox activity. *J. Med. Chem. 49*, 6510-6521.

(38) Chaston, T., Lovejoy, D., Watts, R. N., and Richardson, D. R. (2003) Examination of the anti-proliferative activity of iron chelators: Multiple cellular targets and the different mechanism of action of Triapine compared to Desferrioxamine and the potent PIH analogue 311. *Clin. Cancer Res. 9*, 402-414.

(39) Wang, M., Wei, A. Q., Yuan, J., Trickett, A., Knoops, B., and Murrell, G. A. C. (2002) Expression and regulation of peroxiredoxin 5 in human osteoarthritis. *FEBS Lett. 532*, 359-362.

(40) Yuan, J., Lovejoy, D. B., and Richardson, D. R. (2004) Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: in vitro and in vivo assessment. *Blood 104*, 1450-1458.

(41) Myhre, O., Andersen, J. M., Aarnes, H., and Fonnum, F. (2003) Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem. Pharmacol. 65*, 1575-1582.

(42) Richardson, D. R., and Baker, E. (1990) The uptake of iron and transferrin by the human malignant melanoma cell. *Biochim. Biophys. Acta 1053*, 1-12.

(43) Richardson, D. R., and Baker, E. (1992) Two mechanisms of iron uptake from transferrin by melanoma cells. The effect of desferrioxamine and ferric ammonium citrate. *J. Biol. Chem. 267*, 13972-13979.

(44) Richardson, D. R., Tran, E. H., and Ponka, P. (1995) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective anti-proliferative agents. *Blood 86*, 4295-4306.

(45) Baker, E., Richardson, D. R., Gross, S., and Ponka, P. (1992) Evaluation of the iron chelation potential of hydrazones of pyridoxal, salicylaldehyde and 2-hydroxy-1-naphthylaldehyde using the hepatocyte in culture. *Hepatology 15*, 492-501.

(46) Lentner, C. (1990) *Geigy Scientific Tables.*, Vol. 5, Ciba-Geigy Limited, Basel.

(47) Adamcova, M., Simunek, T., Kaiserova, H., Popelova, O., Sterba, M., Potacova, A., Vavrova, J., Malakova, J., and Gersl, V. (2007) In vitro and in vivo examination of cardiac troponins as biochemical markers of drug-induced cardiotoxicity. *Toxicology 237*, 218-228.

(48) Richardson, D. R., Ponka, P., and Baker, E. (1994) The effect of the iron(III) chelator, desferrioxamine, on iron and transferrin uptake by the human malignant melanoma cell. *Cancer Res. 54*, 685-689.

(49) Darnell, G., and Richardson, D. R. (1999) The potential of analogues of the pyridoxal isonicotinoyl hydrazone class as effective anti-proliferative agents III: The effect of the ligands on molecular targets involved in proliferation. *Blood 94*, 781-792.

(50) Goldspink, D. F., Burniston, J. G., Ellison, G. M., Clark, W. A., and Tan, L. B. (2004) Catecholamine-induced apoptosis and necrosis in cardiac and skeletal myocytes of the rat in vivo: the same or separate death pathways? *Exp. Physiol. 89*, 407-416.

(51) Clerk, A., Cole, S. M., Cullingford, T. E., Harrison, J. G., Jormakka, M., and Valks, D. M. (2003) Regulation of cardiac myocyte cell death. *Pharmacol. Ther. 97*, 223-261.

(52) Kalinowski, D. S., Yu, Y., Sharpe, P. S., Islam, M., Liao, Y-T., Lovejoy, D. B., Kumar, N., Bernhardt, P. V., and Richardson, D. R. (2007) Design, synthesis and characterization of novel iron chelators: Structure-activity relationships of the 2-benzoylpyridine thiosemicarbazone series and their 3-nitrobenzoyl analogs as potent anti-tumor agents. *J. Med. Chem. 50*, 3716-3729.

(53) Diaz-Munoz, M., Alvarez-Perez, M. A., Yanez, L., Vidrio, S., Martinez, L., Rosas, G., Yanez, M., Ramirez, S., and de Sanchez, V. C. (2006) Correlation between oxidative stress and alteration of intracellular calcium handling in isoproterenol-induced myocardial infarction. *Mol. Cell. Biochem. 289*, 125-136.

(54) Pinelli, A., Trivulzio, S., Tomasoni, L., Brenna, S., Bonacina, E., and Accinni, R. (2004) Isoproterenol-induced myocardial infarction in rabbits. Protection by propranolol or labetalol: a proposed non-invasive procedure. *Eur. J. Pharm. Sci. 23*, 277-285.
(55) Lameris, T. W., de Zeeuw, S., Alberts, G., Boomsma, F., Duncker, D. J., Verdouw, P. D., Veld, A. J., and van Den Meiracker, A. H. (2000) Time course and mechanism of myocardial catecholamine release during transient ischemia in vivo. *Circulation 101*, 2645-2650.
(56) Schomig, A. (1990) Catecholamines in myocardial ischemia. Systemic and cardiac release. *Circulation 82*, II13-22.
(57) Allen, D. R., Wallis, G. L., and McCay, P. B. (1994) Catechol adrenergic agents enhance hydroxyl radical generation in xanthine oxidase systems containing ferritin: implications for ischemia/reperfusion. *Arch. Biochem. Biophys. 315*, 235-243.
(58) Rajan, K. S., Davis, J. M., and Colburn, R. W. (1971) Metal chelates in the storage and transport of neurotransmitters: interactions of metal ions with biogenic amines. *J. Neurochem. 18*, 345-364.
(59) Summers, M. R., Jacobs, A., Tudway, D., Perera, P., and Ricketts, C. (1979) Studies in desferrioxamine and ferrioxamine metabolism in normal and iron-loaded subjects. *Br. J. Haematol. 42*, 547-555.
(60) Yeager, J. C., and Iams, S. G. (1981) The hemodynamics of isoproterenol-induced cardiac failure in the rat. *Circ. Shock. 8*, 151-163.
(61) Bloom, S., and Davis, D. L. (1972) Calcium as mediator of isoproterenol-induced myocardial necrosis. *Am. J. Pathol. 69*, 459-470.
(62) Behonick, G. S., Novak, M. J., Nealley, E. W., and Baskin, S. I. (2001) Toxicology update: the cardiotoxicity of the oxidative stress metabolites of catecholamines (aminochromes). *J. Appl. Toxicol. 21 Suppl 1*, S15-22.
(63) Neri, M., Cerretani, D., Fiaschi, A. I., Laghi, P. F., Lazzerini, P. E., Maffione, A. B., Micheli, L., Bruni, G., Nencini, C., Giorgi, G., D'Errico, S., Fiore, C., Pomara, C., Riezzo, I., Turillazzi, E., and Fineschi, V. (2007) Correlation between cardiac oxidative stress and myocardial pathology due to acute and chronic norepinephrine administration in rats. *J. Cell. Mol. Med. 11*, 156-170.
(64) Singal, P. K., Kapur, N., Dhillon, K. S., Beamish, R. E., and Dhalla, N. S. (1982) Role of free radicals in catecholamine-induced cardiomyopathy. *Can. J. Physiol. Pharmacol. 60*, 1390-1397.
(65) Singal, P. K., Beamish, R. E., and Dhalla, N. S. (1983) Potential oxidative pathways of catecholamines in the formation of lipid peroxides and genesis of heart disease. *Adv. Exp. Med. Biol. 161*, 391-401.
(66) Strubelt, O., and Siegers, C. P. (1975) Role of cardiovascular and ionic changes in pathogenesis and prevention of isoprenaline-induced cardiac necrosis. *Recent Adv. Stud. Cardiac Struct. Metab. 6*, 135-142.
(67) Napier, I., Ponka, P., and Richardson, D. R. (2005) Iron trafficking in the mitochondrion: novel pathways revealed by disease. *Blood 105*, 1867-1874.
(68) Berenshtein, E., Vaisman, B., Goldberg-Langerman, C., Kitrossky, N., Konijn, A. M., and Chevion, M. (2002) Roles of ferritin and iron in ischemic preconditioning of the heart. *Mol. Cell. Biochem. 234-235*, 283-292.
(69) Graf, E., Mahoney, J. R., Bryant, R. G., and Eaton, J. W. (1984) Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. *J. Biol. Chem. 259*, 3620-3624.
(70) Pippard, M. J., Callender, S. T., and Finch, C. A. (1982) Ferrioxamine excretion in iron-loaded man. *Blood 60*, 288-294.

5. Direct administration of rutin does not protect against catecholamine cardiotoxicity

Mladěnka P, Zatloukalová L, Šimůnek T, Bobrovová Z, Semecký V, Nachtigal P, Hašková P, Macková E, Vávrová J, Holečková M, Palicka V, Hrdina R. Direct administration of rutin does not protect against catecholamine cardiotoxicity. Toxicology 2008, in press

Abstract:

High levels of catecholamines are cardiotoxic and may trigger acute myocardial infarction (AMI). Similarly, the synthetic catecholamine isoprenaline (ISO) evokes a pathological state similar to AMI. During AMI there is a marked increase of free iron and copper which are crucial catalysts of reactive oxygen species formation. Rutin, a natural flavonoid glycoside possessing free radical scavenging and iron/copper chelating activity, may therefore be potentially useful in reduction of catecholamine cardiotoxicity as was previously demonstrated after its long-term peroral administration.

Male Wistar:Han rats received rutin (46 or 11.5 $mg.kg^{-1}$ i.v.) alone or with necrogenic dose of ISO (100 $mg.kg^{-1}$ s.c.). Haemodynamic parameters were measured 24 hours after drug application together with analysis of blood, myocardial content of elements and histological examination. Results were confirmed by cytotoxicity studies using cardiomyoblast cell line H9c2.

Rutin in a dose of 46 $mg.kg^{-1}$ aggravated ISO-cardiotoxicity while the dose of 11 $mg.kg^{-1}$ had no effect. These unexpected results were in agreement with *in vitro* experiments, where co-incubation with larger concentrations of rutin significantly augmented ISO cytotoxicity.

Our results, in contrast to previous studies in the literature, suggest that the reported positive effects of peroral administration of rutin were unlikely to have been mediated by rutin *per se* but probably by its metabolite(s) or by some other, at this moment, unknown adaptive mechanism(s), which merit further investigation.

Introduction:

Flavonoids, naturally occurring secondary plant metabolites, have been during the last decades under extensive investigation because of their claimed antioxidant, anti-inflammatory, antiallergic, antidiabetic, cardio-, hepato- and gastroprotective, antiviral and antineoplastic properties (Yao *et al.*, 2004). These proposed positive pharmacological activities have been mostly attributed to their free radical scavenging and metal chelating effects (van Acker *et al.*, 1998; Mira *et al.*, 2002; Firuzi *et al.*, 2005; Kaiserova *et al.*, 2007).

Increased endogenous catecholamine levels are associated with cardiac injury and may trigger acute myocardial infarction (AMI) (von Kanel *et al.*, 2002; Kloner, 2006). Indeed, synthetic catecholamine isoprenaline (isoprotenerol, ISO) is often used to evoke a pathological state in many aspects similar to AMI (Rona *et al.*, 1983; Diaz-Munoz *et al.*, 2006). The mechanism of catecholamine cardiotoxicity is not fully understood, it involves excessive stimulation of adrenergic receptors and oxidative stress enhanced by redox cycling of redundant catecholamines (Rona *et al.*, 1983; Behonick *et al.*, 2001; Remiao *et al.*, 2002). Even relatively small doses of ISO cause myocardial ischaemia (Winsor *et al.*, 1975) which is associated with marked increase in free iron and copper, which are subsequently released to the circulation and promote oxidative stress (Berenshtein *et al.*, 2002).

Rutin (quercetin-3-rutinoside), a natural flavonoid glycoside, being already clinically used for strengthening of capillaries (Wadworth and Faulds, 1992), possesses as other flavonoids iron/copper chelating and antioxidant activity and, based on the mentioned known pathological mechanism, appears to be a useful drug for reduction of catecholamine cardiotoxicity. Indeed, previous studies have suggested very promising effects of rutin on ISO-induced cardiac injury in

rats. Rutin, administered orally for 42 days, significantly improved both changes in the levels of lipids, lipoproteins and activity of Ca^{2+}-dependent Na^+/K^+ ATPase (Stanely Mainzen Prince and Karthick, 2007) as well as it reduced lipid peroxidation and increased myocardial antioxidative defence (Karthick and Stanely Mainzen Prince, 2006). The observed effects have been attributed to the antioxidant potential of rutin. However, no data have been shown on overall animal survival, cardiac function parameters, morphology changes in myocardium and the amount of rutin absorbed. Clinical or experimental studies have clearly documented that rutin is cleaved in the caecum and is not absorbed as the parent compound (Manach *et al.*, 1995; Choudhury *et al.*, 1999; Erlund *et al.*, 2000). It is therefore unknown whether the reported beneficial effect can be attributed directly to rutin or rather to its metabolites and/or to some other adaptation changes.

Hence, this study aimed to examine the potential direct protective effect of rutin, which may be based on its reactive oxygen species (ROS) scavenging properties and iron/copper chelating ability, to reduce myocardial impairment both in an *in vivo* rat catecholamine model of AMI and compare these results with *in vitro* cardiotoxicity experiments using rat cardiomyoblast cell line H9c2.

Methods:

Animals

Young Wistar:Han male rats obtained from Biotest s.r.o. (Konárovice, Czech Republic), weighing approximately 360 g, were used after two weeks of acclimatization. The animals were maintained in an air-conditioned room and were allowed free access to a standard pellet diet for rodents and tap water. Animals were fasting for 12 hours before the experiment. The study was

performed under the supervision of the Ethical Committee of the Charles University in Prague, Faculty of Pharmacy in Hradec Králové and it conforms to "The Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

IN VIVO study design

Animals were randomly divided into 6 groups:

- *control group* (C, 7 animals) received saline 1 $ml.kg^{-1}$ s.c.
- *isoprenaline group* (isoprotenerol, ISO, 10 animals) – received 100 $mg.kg^{-1}$ ISO (Sigma-Aldrich, USA) in the aqueous solution s.c.
- *rutin groups* – rats received either 11.5 mg kg^{-1} (Ru11, 7 animals) or 46 $mg.kg^{-1}$ of rutin (Sigma-Aldrich, USA) i.v. (Ru46, 7 animals).
- *combination groups* – rats received either 11.5 mg kg^{-1} (Ru11+ISO, 10 animals) or 46 $mg.kg^{-1}$ of rutin i.v. (Ru46+ISO, 17 animals) 5 minutes before application of ISO in the above mentioned dose.

Saline or drug(s) were administered 24 hours before the surgical procedures and measurements.

Cardiac function measurement

Animals were anaesthetized with urethane (1.2 $g.kg^{-1}$ i.p.; Sigma-Aldrich, USA). A polyethylene catheter (0.5/1.0 mm filled with heparinised saline 50 $IU.ml^{-1}$) was inserted into the right jugular vein for injection of cold saline (approximate temperature 10°C). A thermocatheter (o.d. 0.8 mm)

was introduced through the left carotid artery into the aortic arc. Another PE catheter (0.5/1.0 mm filled with heparinised saline 50 $IU.ml^{-1}$) was inserted into the left iliac artery, which was connected with the blood pressure transducer BPR-01 of the apparatus for measurement of haemodynamic variables Cardiosys® (Experimentria Ltd, Hungary) with software Cardiosys V 1.1. For the measurement of cardiac output, a thermodilution transpulmonary method was used according to the Stewart-Hamilton (Spiller and Webb-Peploe, 1985).

The measurements were carried out following 15 minute-lasting equilibration period after surgical procedure. Functional variables (cardiac output, stroke volume, blood pressure, heart rate) were averaged from four recordings performed in 5 minute intervals. Results are expressed as indices (measured variable divided by the body weight) except for the blood pressure, heart rate and "double product" (systolic blood pressure multiplied by heart rate). The latter parameter is commonly used as an indirect index of cardiac oxygen consumption. Total peripheral resistance was calculated as mean arterial blood pressure divided by cardiac output.

Following haemodynamic measurements the blood sample (approximately 5 ml) was collected from the abdominal aorta into the heparinised test tube (170 IU). Then the animal was sacrificed by i.v. KCl overdose (1 mM), heart ventricles were excised, weighed and frozen at -20°C for further analysis of selected elements content.

Histological analysis

After the autopsy, the apical parts of the hearts were fixed in Bouin´s solution and processed for light microscopy. Tissues were dehydrated in increasing concentrations of ethanol, paraffin embedded and sectioned to 5-8 µm. The tissue sections were stained with haematoxylin

and eosin, Masson´s trichrome and Weigert-van Gieson. Photo documentation and image digitizing were performed with the Olympus AX 70 light microscope, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Ottawa, Canada) with image analysis software NIS (Laboratory Imaging, Czech Republic).

Biochemical analysis of blood

Cardiac troponin T (cTnT) and vitamin E were measured in serum, malondialdehyde (MDA) in plasma and total glutathione in the whole blood. cTnT - highly sensitive and specific biomarker of myocardial injury (Adamcova *et al.*, 2007) - was determined by electrochemoluminescence immunoassay (Elecsys 2010, Roche Diagnostics), which employs two monoclonal antibodies specifically directed against cTnT. Malondialdehyde (MDA) was measured as a red complex with thiobarbituric acid (TBARS) at 485, 532 and 560 nm using Beckman DU 640 spectrophotometer (Beckman, Palo Alto, USA). Capillary electrophoresis was used for separation of glutathione, which was measured by UV detection (System P/ACE 5100, Beckman) at 200 nm. After deproteinization, analysis of vitamin E with fluorimetric detection was performed in an HPLC system HP1050 (Hewlett Packard, Germany).

Elements in the myocardium

Frozen samples of myocardial tissue were dried, weighed and digested by microwave digestion using nitric acid and hydrogen peroxide (Milestone MLS 1200 MEGA, Italy). Iron, copper and selenium were determined using graphite furnace atomic absorption spectrometry (Unicam, Solaar 959, U.K.), zinc was determined using flame atomic absorption spectrometry (Unicam, Solaar 959, U.K.) and calcium was measured photometrically using flame photometry

(Eppendorf, Efox 5053, Germany). Results are expressed as $\mu mol.g^{-1}$ (iron, copper, zinc, calcium) or $nmol.g^{-1}$ (selenium) of dry tissue.

***In vitro* cardiotoxicity assays**

The H9c2 cell line derived from the embryonic rat heart tissue was from the American Type Culture Collection (ATCC, U.S.A.). Cells were cultured in Dulbecco´s modified Eagle´s medium (DMEM, Lonza, Belgium) supplemented with 10% Foetal Bovine Serum, 1% Penicillin/Streptomycin and 10 mM HEPES buffer (Sigma) in 75 cm^2 tissue culture flasks (TPP, Switzerland) at 37°C in a humidified atmosphere of 5% CO_2. Cells were subcultured every 3-4 days once they reached 85-95% confluence. For cytotoxicity experiments with neutral red (NR), cells were seeded in 96-well plates (TPP) at a density of 10,000 cells per well. For morphology assessments, H9c2 cells were seeded at a density of 75,000 cells per well in 12-well plates (TPP). 24 hours prior experiments, medium was changed for serum- and pyruvate-free DMEM (Sigma, Germany). To dissolve rutin, 0.2% DMSO was present in the culture medium of all groups. At this concentration DMSO had no effect on cellular viability.

H9c2 cells were incubated for 24 hours with or without ISO (200 or 250 μM) and rutin (1 - 1000 μM) or iron chelator deferoxamine (Novartis Pharma, Switzerland, DFO - 250 μM). Changes in cellular morphology were documented using inverted microscope Nikon Eclipse TS100, 10x Nikon air objective, digital cooled camera (1300Q, VDS Vosskühler, Germany) and software NIS-Elements AR 2.20 (Laboratory Imaging, Czech Republic). Cellular viability was

determined using the cytotoxicity assay based on the ability of viable cells to incorporate neutral red (NR). NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulating intracellularly in lysosomes (Fotakis and Timbrell, 2006). For vital staining, half volume of medium from each well was removed and the same volume of medium with NR was added (final concentration 40 μg/ml). After incubation for 3 h at 37°C, the supernatant was discarded, cells were fixated with 1% $CaCl_2$ in 0.5% formaldehyde for 15 min, washed twice with PBS and solubilized with 1% acetic acid in 50% ethanol. The optical density of soluble neutral red was measured at 540 nm using Tecan Infinite 200M plate reader (Tecan, Austria). The viability of all experimental groups was expressed as percentage of untreated controls (100 %).

Data analysis

Data are expressed as means ± SEM. Groups were compared by one-way ANOVA followed by Tukey's Multiple Comparison Test by using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, California, U.S.A). Differences between groups were considered as significant at $P \leq 0.05$, unless indicated otherwise.

Results

***IN VIVO* experiments:**

Administration of 100 $mg.kg^{-1}$ of ISO s.c. caused 3 premature deaths (out of 10 experimental animals in this group) within 24 hours. Again, 3 animals from 10 died when animals were premedicated with 11.5 $mg.kg^{-1}$ of rutin before the administration of ISO,

however, the dose of 46 mg.kg^{-1} of rutin further increased the number of non-surviving animals to 53% (9 out of 17, in addition, 2 more animals died during anaesthesia). None of animals from control group or animals treated only with rutin (in both doses) died.

ISO in the above mentioned dose brought about a significant increase in myocardial calcium content and a marked cTnT release into circulation – see Fig. 1. Similarly to mortality, rutin premedication failed to improve both parameters. While the lower dose had no effect, the higher dose of rutin tended to rather aggravate the calcium overload and cTnT release. Rats who received saline or rutin alone, had only negligible cTnT release and normal calcium myocardial concentration, with exception of the higher dose of rutin, which itself insignificantly elevated myocardial calcium content.

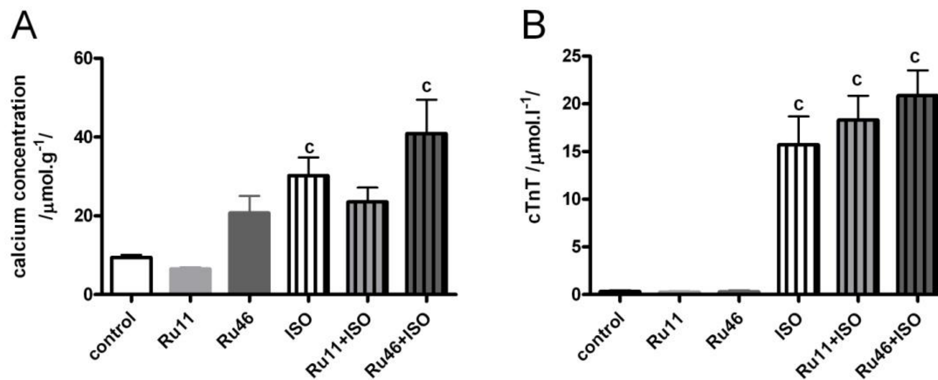

Figure 1. Myocardial calcium content (A) and serum cardiac troponin T concentrations (B) 24 hours after applications of rutin (Ru - 11.5 or 46 mg.kg^{-1} i.v.), isoprenaline (ISO - 100 mg.kg^{-1} s.c.) or their combinations to experimental rats. Statistical significance at $p < 0.05$: **c** vs. control group.

Rutin dose-dependently increased cardiac index (Fig. 2A) and showed a tendency to decrease peripheral resistance (Fig. 2B). Cardiac index drop caused by ISO was not reverted by either dose of rutin, although rutin in both doses significantly decreased the ISO-induced elevation in peripheral resistance. There were no significant differences among the groups in the values of blood pressure. Differences in heart rate reached statistical significance only in combination of the higher rutin dose with ISO. Other functional parameters as well as wet ventricles weight index are shown in Table 1.

Parameter / Group	**control**	**Ru11**	**Ru46**	**ISO**	**Ru11+ISO**	**Ru46+ISO**
Stroke volume index /ml.min.kg^{-1}/	0.81 ± 0.07	1.04 ± 0.19	1.09 ± 0.08	0.37 ± 0.05^{c}	0.58 ± 0.04	0.45 ± 0.08
Systolic blood pressure /mmHg/	108 ± 6	118 ± 5	135 ± 6	129 ± 8	117 ± 7	129 ± 9
Diastolic blood pressure /mmHg/	79 ± 3	90 ± 9	83 ± 4	96 ± 6	87 ± 5	98 ± 9
Heart rate /beats.min^{-1}/	408 ± 14	429 ± 25	413 ± 17	458 ± 8	464 ± 10	481 ± 11^{c}
Double product /mmHg.beat.min^{-1}/	36286 ± 2485	43028 ± 4698	41506 ± 3347	49437 ± 3533	44741 ± 2866	52107 ± 5119
Wet ventricles weight i. /‰/	2.4 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	3.5 ± 0.1^{c}	3.8 ± 0.2^{c}	3.6 ± 0.2^{c}

Table 1. Selected functional cardiac parameters and wet ventricle weight index 24 hours after applications of rutin (Ru - 11.5 or 46 mg.kg^{-1}), isoprenaline (ISO - 100 mg.kg^{-1}) or their combinations to experimental rats. Statistical significance at $p < 0.05$: c vs. control group. Wet ventricles weight index is expressed in ‰ (heart ventricles weight in mg divided by animal weight in grams).

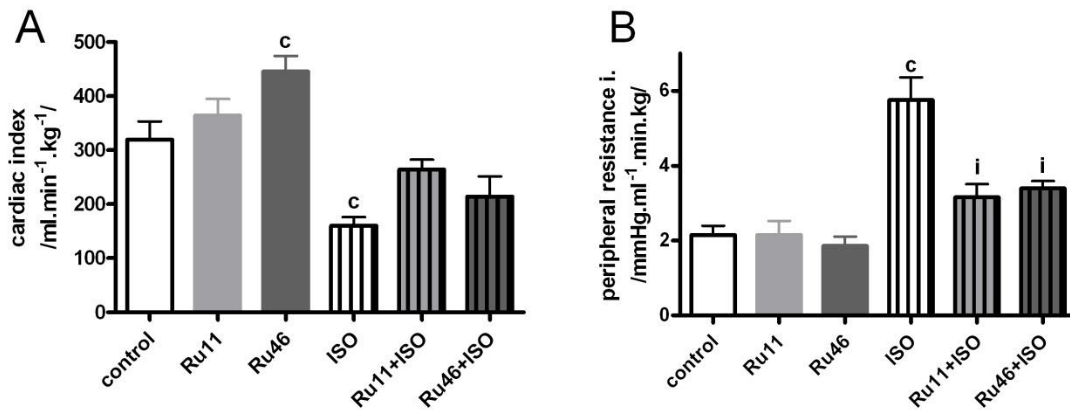

Figure 2. Cardiac index (A) and peripheral resistance index (B) 24 hours after applications of rutin (Ru - 11.5 or 46 mg.kg^{-1}), isoprenaline (ISO - 100 mg.kg^{-1}) or their combinations to experimental rats. Statistical significance at $p < 0.05$: **c** vs. control group, **i** vs. ISO group.

Administration of ISO had only small and insignificant influence on myocardial copper and zinc concentrations (Fig. 3). Interestingly, higher dose of rutin increased significantly copper levels in the myocardium while both doses of rutin decreased myocardial zinc content independently on ISO administration, although in combination with ISO the decrease was more pronounced. Difference among groups concerning myocardial iron and selenium content were not statistically significant (data not shown). Regarding parameters of oxidative stress in the blood, rutin dose-dependently increased levels of total glutathione (Fig. 4A). ISO treatment had only small effect on this parameter, although it nearly completely abolished the increase caused by rutin. ISO and combination groups had slightly higher levels of TBARS without statistical

significance (Fig. 4B). Serum vitamin E levels were comparable and without significant differences in all groups (data not shown).

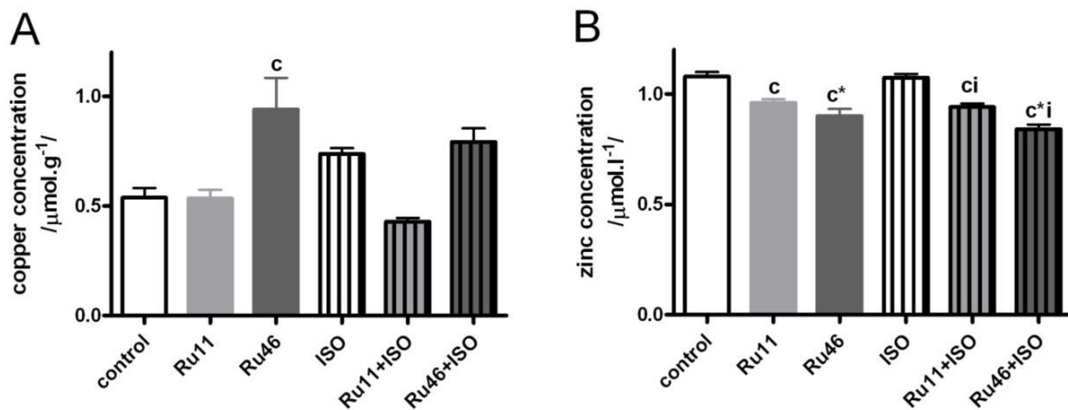

Figure 3. Myocardial copper content (A) and myocardial zinc content (B) 24 hours after applications of rutin (Ru - 11.5 or 46 $mg.kg^{-1}$), isoprenaline (ISO - 100 $mg.kg^{-1}$) or their combinations to experimental rats. Statistical significance: **c** vs. control group $p < 0.01$, **c*** vs. control at $p < 0.001$, **i** vs. ISO group at $p < 0.01$.

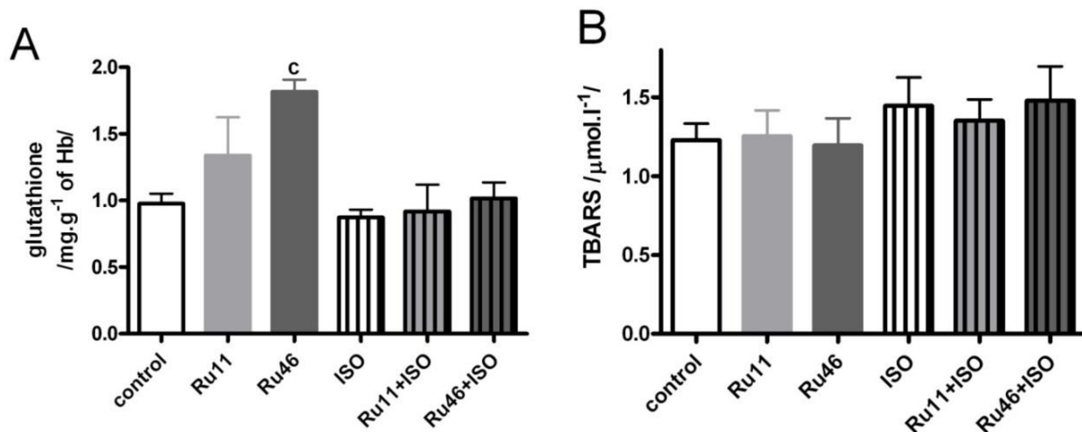

Figure 4. Blood levels of total glutathione (A) and plasma conjugated dienes - TBARS (B) 24 hours after applications of rutin (Ru - 11.5 or 46 $mg.kg^{-1}$), isoprenaline (ISO - 100 $mg.kg^{-1}$) or their combinations to experimental rats. Statistical significance: **c** vs. control group at $p < 0.01$.

The normal structure of myocardial tissue was found in both intact control animals and rutin treated rats by both doses (Fig. 5A). On the other hand, ISO treatment caused severe diffuse or focal damage of cardiomyocytes. Moreover, marked inflammatory infiltration located especially under endocardial epithelium (Fig. 5B) or in the thin fibrous tissue under epicardial epithelium was found, as well. In contrast, only rare infiltrate was presented in the area of central myocardium. Histopathological findings in the ISO group (Fig. 5B) and in combination groups ISO + rutin in both doses (Fig. 5C and 5D) were similar.

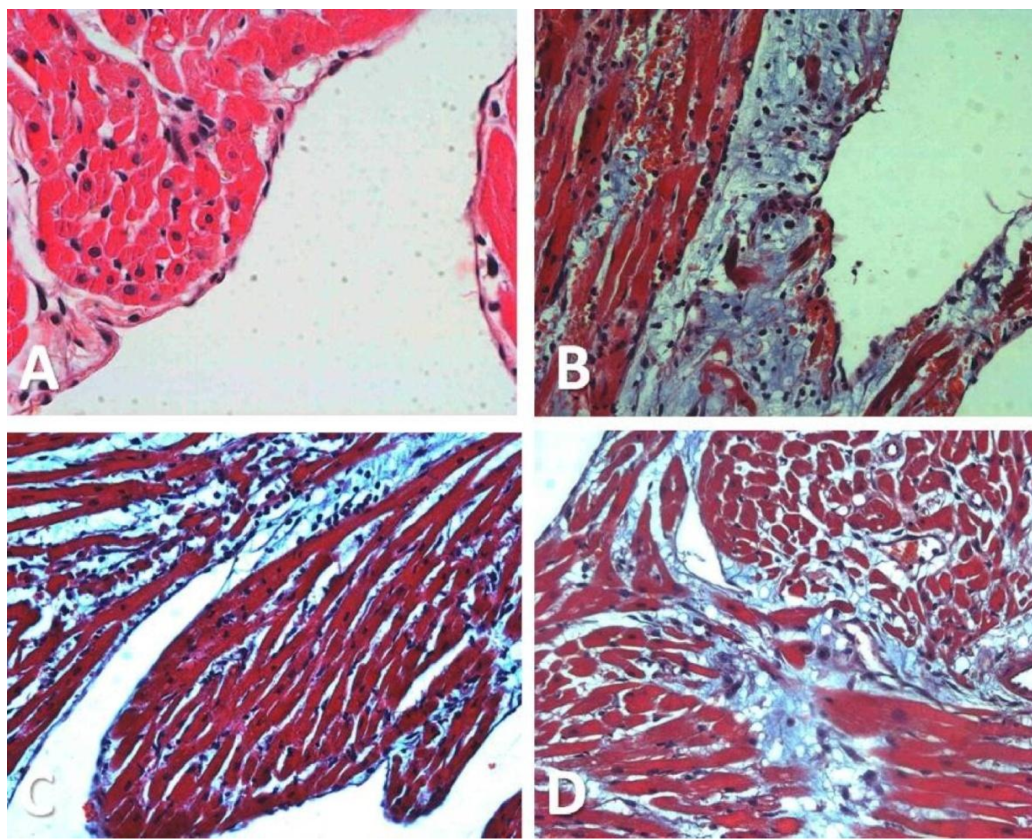

Figure 5. Effects of 24-hour incubation with 250 µM ISO and its combination with 30 µM rutin (Ru) or 250 µM deferoxamine (DFO) on cellular morphology of H9c2 cardiomyoblast cells. Scale bars: 100 µm.

***IN VITRO* experiments:**

In order to confirm the previous negative outcomes of *in vivo* experiments and to clarify the reason(s) for the failure of rutin to positively influence the ISO cardiotoxicity, the rutin effects on rat cardiomyoblast-derived cell line H9c2 were further studied with or without ISO.

As seen in Fig. 6A, 24-hour incubation with rutin was well tolerated by H9c2 cells. Significant decrease in cellular viability (to 74% of control values) was observed only with the highest, 1000 µM rutin concentration.

Exposure of cells to both 200 and 250 μM ISO resulted in pronounced changes of cellular morphology, including disruption of cellular monolayer, peripheral membrane blebbing and eventual rounding up of cells with conspicuous nuclear shrinkage. Eventually, detachment of some cells from the test plate bottom was observed (Fig. 7). The quality of changes was comparable with both used ISO concentrations, although they occurred earlier and were quicker with the higher (250 μM) dose. After 24 hours, vital imaging with neutral red revealed significant decrease of cellular viability which was partial (reduction to 46 % of control values) with 200 μM ISO and nearly complete at 250 μM ISO concentration (6 % of control).

Rutin, tested over broad concentration range of 1-1000 μM, failed to afford any protection against the ISO toxicity. Neither improvement of cellular morphology damage induced by ISO, nor increase in cellular viability were observed (Figs. 6, 7). While the lower (1-10 μM) rutin concentrations had negligible effects on ISO-induced toxicity, at higher concentrations (≥ 30 μM) rutin further decreased the viability of H9c2 cells, which was particularly obvious and statistically significant with the 200 μM ISO concentration (Fig. 6B). Of note, this significant aggravation of ISO toxicity was seen with 30-300 μM rutin concentrations, i.e. those at which rutin did not display any own significant toxicity (Fig. 6A). This suggests that potentiation, rather than simple addition of toxicities by ISO and rutin occurred.

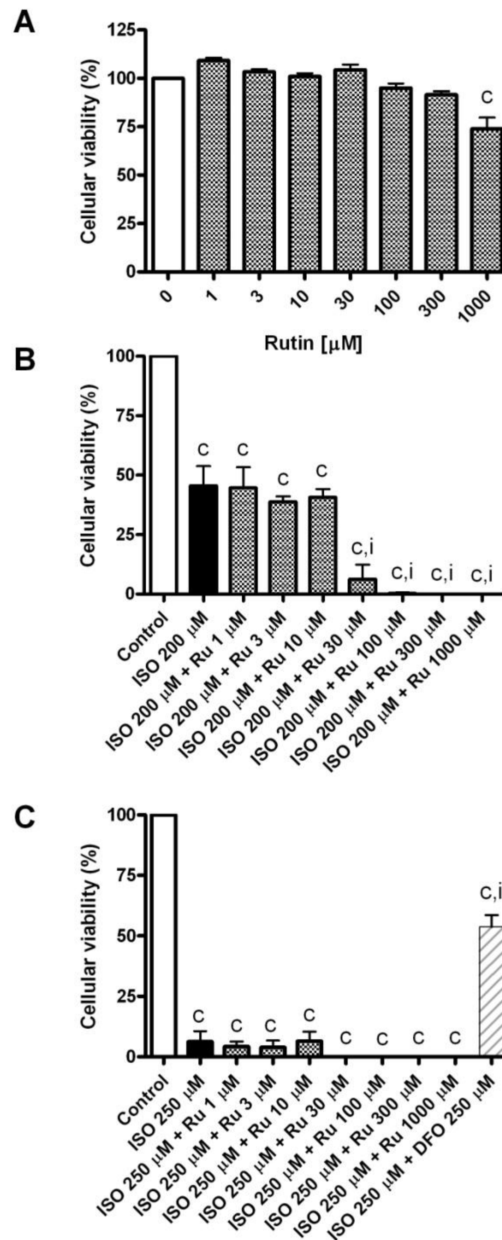

Figure 6. Cellular viability (percentage of untreated controls) of H9c2 cardiomyoblast cell line exposed to 1-1000 μM rutin (Ru) alone (panel A) or in combinations with 200 μM or 250 μM isoprenaline (ISO; panels B and C). Deferoxamine (DFO) was used as a positive cytoprotective

control. Statistical significance at $p<0.05$: c - against control cells, i - against ISO-treated group of cells. Means ± S.E.M. of at least 3 independent experiments.

In contrast, co-incubation of cells with 250 μM of ISO and iron chelator deferoxamine (DFO) resulted in a significant increase of cellular viability (Fig. 6C) as well as preservation of cellular morphology (Fig. 7).

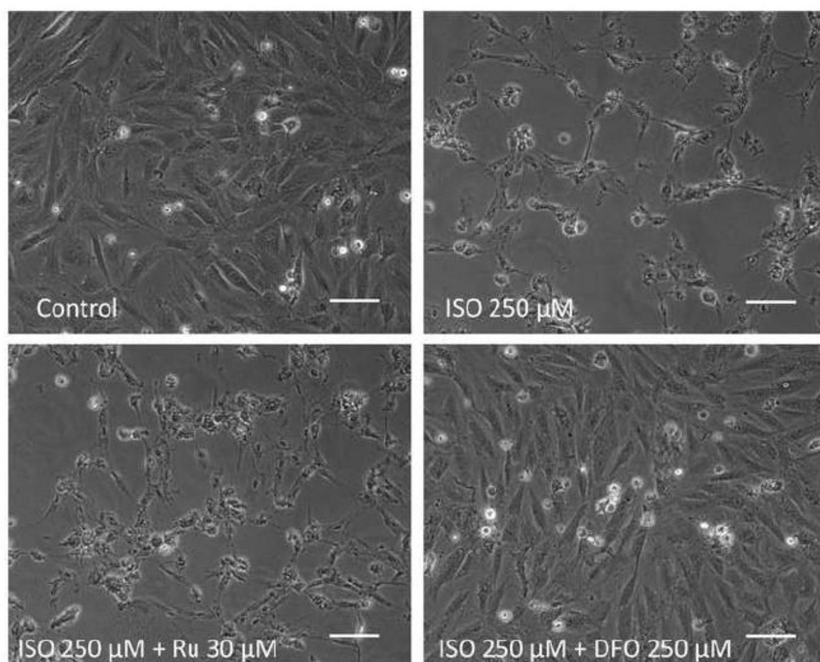

Figure 7. Effects of 24-hour incubation with 250 μM ISO and its combination with 30 μM rutin (Ru) or 250 μM deferoxamine (DFO) on cellular morphology of H9c2 cardiomyoblast cells. Scale bars: 100 μm.

Discussion

In the past, flavonoids have been uncritically promoted for their proposed positive pharmacological effects based on their antioxidant and/or metal-chelating properties. However, many rather negative results have emerged during the last years and our present data rather support these facts. The reasons for unequivocal failure of rutin to reduce the catecholamine cardiotoxicity appear to be complex and this requires discussing both the pathogenesis of catecholamine cardiotoxicity and pharmaco-toxicological properties of rutin.

ISO cardiotoxicity shares many similarities with AMI (Chagoya de Sanchez *et al.*, 1997; Diaz-Munoz *et al.*, 2006). ISO is a non-selective β-adrenoceptor agonist and its administration in large doses leads in the first phase to rapid hypotension and marked heart stimulation - acceleration in heart rate and increased contractility. This results in an insufficient myocardial oxygen supply, which is followed by myocardial ischaemia. Ischaemia is associated with an increased blood coagulation and ROS production based on a release of free iron and copper; this process is supposed to be similar to ischaemia during AMI (Pinelli *et al.*, 2004; Diaz-Munoz *et al.*, 2006). The final step is cardiomyocyte death by apoptosis or necrosis.

Based on these theoretical assumptions, flavonoids should possess good potential to be protective drugs due to their iron/copper binding and ROS scavenging properties. In fact, rutin was shown to scavenge ROS, namely superoxide, hypochlorite and peroxynitrate (Haenen *et al.*, 1997; Russo *et al.*, 2000; Moridani *et al.*, 2003; Firuzi *et al.*, 2004), to bind iron and copper (van Acker *et al.*, 1998), and to diminish oxidative injuries catalyzed by free iron (Afanas'ev *et al.*, 1989; Russo *et al.*, 2000; Mira *et al.*, 2002; Kaiserova *et al.*, 2007). Additionally, rutin was shown to inhibit xanthine oxidase, which is a principal ROS-generating enzyme and which

action is substantially augmented in ischaemic tissue as well as after ISO application (Russo *et al.*, 2000; Berry and Hare, 2004; Diaz-Munoz *et al.*, 2006). However, despite these reported beneficial properties, in our study rutin rather aggravated catecholamine myocardial injury both *in vitro* and *in vivo*.

There are at least three main underlying factors:

1. Hydrophilic character of rutin may represent a basis for distinction between its effects and those of its lipophilic aglycone - quercetin, which has been documented to penetrate the lipophilic barriers (Ferrali *et al.*, 1997). Rutin seems to be more active in inhibition of oxidative injury in hydrophilic ambient, while quercetin in lipophilic one (Afanas'ev *et al.*, 1989; Chen *et al.*, 1990). Moreover, lipophilicity correlates with protective antioxidant effects of polyphenols (Sestili *et al.*, 2002). Contrarily, highly hydrophilic iron chelator deferoxamine with documented low penetration inside the cells (Hershko *et al.*, 1978) was able to markedly reduce ISO toxicity in our *in vitro* experiments. Hence, differences in lipophilic/hydrophilic nature may have explained the failure of rutin to protect myocardium *in vivo*, but cannot explain the failure of rutin in the *in vitro* model and aggravation of catecholamine injury in both models.

2. Direct effects on cardiovascular system: Flavonoids have been shown to have positive inotropic and blood pressure-lowering effects (Chan *et al.*, 2000; van Acker *et al.*, 2001). In this study, rutin dose-dependently increased cardiac index and because the diastolic blood pressure was not substantially affected, this increase was probably evoked by increased cardiac inotropy. Direct effect on the blood vessels was not documented in this study in healthy animals, although it may play a role in animals with cardiovascular impairment as can be deduced from the inhibition of ISO-based peripheral resistance index elevation by both doses of rutin (Fig. 2B). In

the case of catecholamine injury, positive inotropic effect of rutin may have substantial impact, because it can lead to further augmentation in myocardial oxygen demand and more pronounced cardiac impairment.

3. There seems to be a narrow dose range between antioxidant and pro-oxidant properties of flavonoids. More factors (e.g. concentration, pH, presence of oxidative species) can have influence (Ratty and Das, 1988; Moran *et al.*, 1997; Galati and O'Brien, 2004; Boots *et al.*, 2007). There are several reports showing that some flavonoids caused drop in the cellular GSH levels and even leakage of lactate dehydrogenase (van Acker *et al.*, 2001). Such cellular derangement seems to be associated with severe damage of cell membrane, but these effects are unlikely in our study, where *in vivo* no detectable release of cTnT was observed after application of both doses of rutin. Moreover, rutin, in contrast to some other flavonoids, is a relatively safe drug as was documented by others and *in vitro* part of this study (Soares *et al.*, 2006). The rutin dose of 46 $mg.kg^{-1}$ was selected as equimolar to 50 $mg.kg^{-1}$ of iron chelator DFO, i.e. safe DFO dose in clinical practice, which was shown to protect myocardium in similar pathological models (Ambrosio *et al.*, 1987). This higher dose of rutin did not cause any apparent changes in myocardial histology, however, it evoked some derangement in myocardial elements (especially an insignificant increase in myocardial calcium content - Fig 1A - and significant increase in copper concentration – Fig 3A). Moreover, both doses of rutin decresed myocardial zinc content in a dose-dependent manner – Fig.3B. It may be hypothesised that these factors may further influence catecholamine cardiotoxicity, because, among others, copper and zinc are components of antioxidant enzymes and elevated calcium level suggests non-physiological myocardial changes. There is some consistency that flavonoids with catechol B-ring may produce ROS and

oxidize glutathione or form adduct with it (Galati and O'Brien, 2004). Whether this was the case, cannot be sufficiently explained at the moment, especially with respect to the observed dose-dependent increase in total glutathione (Fig. 4A).

The outcomes of the present study are in a sharp discrepancy with those of two studies of Karthick and Stanely Mainzen Prince (Karthick and Stanely Mainzen Prince, 2006; Stanely Mainzen Prince and Karthick, 2007). In their experiments, animals were pretreated for 42 days with rutin using intragastric tube and then ISO in a dose of 150 $mg.kg^{-1}$ s.c. was administered once daily for 2 days. These studies showed protective effect of p.o. rutin and authors concluded that rutin antioxidant activity was probably responsible for such protection. However, rutin is not absorbed following oral administration in humans or rats (Manach *et al.*, 1995; Choudhury *et al.*, 1999; Erlund *et al.*, 2000). Rather, it is cleaved by intestinal bacteria to numerous metabolites (e.g. quercetin, isorhamnetin, tamarixetin or various phenolic acids). Therefore, protective influence of p.o. rutin in those studies most likely has not been achieved with rutin itself, but rather by its active metabolites. On the contrary, rutin administered i.v. (as in our present study) is unlikely to undergo metabolization into quercetin (Choudhury *et al.*, 1999) and the same applies also for our *in vitro* experiments.

Even more important difference between our present and the previous studies is the fact, that while we have evaluated the direct effects of rutin on ISO-induced cardiotoxicity, Karthick and Stanely Mainzen Prince have injected ISO 24 and 48 hours after the last rutin administration, i.e. at the time when even its main metabolite quercetin should be in rats completely excreted into the bile and urine as the glucuronide and sulfate conjugates (Ueno *et al.*, 1983). Taking together with our present data, it is very likely that the original authors' assumption - that the

protective effects of rutin against ISO-cardiotoxicity in rats were due to the antioxidant potential of rutin – appears to be incorrect. Interestingly, recent evidence strongly suggests that some indirect action(s) of flavonoids are responsible for their beneficial effects (Halliwell *et al.*, 2005; Halliwell, 2007). As an example, the study of Lotito and Frei has suggested that the increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods is not caused by the flavonoids *per se*, but most likely due to the increased uric acid levels that result from competition in active renal secretion of flavonoids (Lotito and Frei, 2006).

Conclusions

In contrast to literature data reporting positive effects of 1.5-month daily oral administration of rutin on catecholamine cardiotoxicity, rutin administered i.v. acutely before ISO was not able to reduce the catecholamine cardiotoxicity; moreover, in its larger dose it further aggravated the ISO-induced cardiac damage. These *in vivo* data are in a good agreement with *in vitro* experiments.

The previously observed protection of catecholamine cardiotoxicity by peroral administration was therefore clearly have not been mediated by rutin *per se*. This conclusion is supported by the following facts: (i) i.v. rutin has no positive effects on catecholamine cardiotoxicity, (ii) rutin is cleaved to various metabolites by intestinal bacteria and is not absorbed as a parent compound, and (iii) the administration of rutin was stopped 24-48 hours before ISO, it is therefore unlikely, that sufficient levels of drug were in the circulation at the time of ISO-induced myocardial injury.

Hence, we support the emerging hypothesis that the beneficial effects of flavonoids are mediated by some indirect action(s). Further *in vitro* and *in vivo* studies are needed for better understanding of apparently highly complex action rutin (and/or its metabolites) in biological systems in order to fully exploit its protective potential while preventing its toxicity.

Acknowledgements

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REFERENCES:

Adamcova, M., Simunek, T., Kaiserova, H., Popelova, O., Sterba, M., Potacova, A., Vavrova, J., Malakova, J., and Gersl, V. (2007). In vitro and in vivo examination of cardiac troponins as biochemical markers of drug-induced cardiotoxicity. *Toxicology* **237**, 218-228.

Afanas'ev, I. B., Dorozhko, A. I., Brodskii, A. V., Kostyuk, V. A., and Potapovitch, A. I. (1989). Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* **38**, 1763-1769.

Ambrosio, G., Zweier, J. L., Jacobus, W. E., Weisfeldt, M. L., and Flaherty, J. T. (1987). Improvement of postischemic myocardial function and metabolism induced by administration of deferoxamine at the time of reflow: the role of iron in the pathogenesis of reperfusion injury. *Circulation* **76**, 906-915.

Behonick, G. S., Novak, M. J., Nealley, E. W., and Baskin, S. I. (2001). Toxicology update: the cardiotoxicity of the oxidative stress metabolites of catecholamines (aminochromes). *J Appl Toxicol* **21 Suppl 1**, S15-22.

Berenshtein, E., Vaisman, B., Goldberg-Langerman, C., Kitrossky, N., Konijn, A. M., and Chevion, M. (2002). Roles of ferritin and iron in ischemic preconditioning of the heart. *Mol Cell Biochem* **234-235**, 283-292.

Berry, C. E., and Hare, J. M. (2004). Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J Physiol* **555**, 589-606.

Boots, A. W., Li, H., Schins, R. P., Duffin, R., Heemskerk, J. W., Bast, A., and Haenen, G. R. (2007). The quercetin paradox. *Toxicol Appl Pharmacol* **222**, 89-96.

Diaz-Munoz, M., Alvarez-Perez, M. A., Yanez, L., Vidrio, S., Martinez, L., Rosas, G., Yanez, M., Ramirez, S., and de Sanchez, V. C. (2006). Correlation between oxidative stress and alteration of intracellular calcium handling in isoproterenol-induced myocardial infarction. *Mol Cell Biochem* **289**, 125-136.

Erlund, I., Kosonen, T., Alfthan, G., Maenpaa, J., Perttunen, K., Kenraali, J., Parantainen, J., and Aro, A. (2000). Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur J Clin Pharmacol* **56**, 545-553.

Ferrali, M., Signorini, C., Caciotti, B., Sugherini, L., Ciccoli, L., Giachetti, D., and Comporti, M. (1997). Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett* **416**, 123-129.

Firuzi, O., Lacanna, A., Petrucci, R., Marrosu, G., and Saso, L. (2005). Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. *Biochim Biophys Acta* **1721**, 174-184.

Firuzi, O., Mladenka, P., Petrucci, R., Marrosu, G., and Saso, L. (2004). Hypochlorite scavenging activity of flavonoids. *J Pharm Pharmacol* **56**, 801-807.

Fotakis, G., and Timbrell, J. A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* **160**, 171-177.

Galati, G., and O'Brien, P. J. (2004). Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic Biol Med* **37**, 287-303.

Haenen, G. R., Paquay, J. B., Korthouwer, R. E., and Bast, A. (1997). Peroxynitrite scavenging by flavonoids. *Biochem Biophys Res Commun* **236**, 591-593.

Halliwell, B. (2007). Dietary polyphenols: good, bad, or indifferent for your health? *Cardiovasc Res* **73**, 341-347.

Halliwell, B., Rafter, J., and Jenner, A. (2005). Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *Am J Clin Nutr* **81**, 268S-276S.

Hershko, C., Grady, R. W., and Cerami, A. (1978). Mechanism of iron chelation in the hypertransfused rat: definition of two alternative pathways of iron mobilization. *J Lab Clin Med* **92**, 144-151.

Chagoya de Sanchez, V., Hernandez-Munoz, R., Lopez-Barrera, F., Yanez, L., Vidrio, S., Suarez, J., Cota-Garza, M. D., Aranda-Fraustro, A., and Cruz, D. (1997). Sequential changes of energy metabolism and mitochondrial function in myocardial infarction induced by isoproterenol in rats: a long-term and integrative study. *Can J Physiol Pharmacol* **75**, 1300-1311.

Chan, E. C., Pannangpetch, P., and Woodman, O. L. (2000). Relaxation to flavones and flavonols in rat isolated thoracic aorta: mechanism of action and structure-activity relationships. *J Cardiovasc Pharmacol* **35**, 326-333.

Chen, Y. T., Zheng, R. L., Jia, Z. J., and Ju, Y. (1990). Flavonoids as superoxide scavengers and antioxidants. *Free Radic Biol Med* **9**, 19-21.

Choudhury, R., Srai, S. K., Debnam, E., and Rice-Evans, C. A. (1999). Urinary excretion of hydroxycinnamates and flavonoids after oral and intravenous administration. *Free Radic Biol Med* **27**, 278-286.

Kaiserova, H., Simunek, T., van der Vijgh, W. J., Bast, A., and Kvasnickova, E. (2007). Flavonoids as protectors against doxorubicin cardiotoxicity: role of iron chelation, antioxidant activity and inhibition of carbonyl reductase. *Biochim Biophys Acta* **1772**, 1065-1074.

Karthick, M., and Stanely Mainzen Prince, P. (2006). Preventive effect of rutin, a bioflavonoid, on lipid peroxides and antioxidants in isoproterenol-induced myocardial infarction in rats. *J Pharm Pharmacol* **58**, 701-707.

Kloner, R. A. (2006). Natural and unnatural triggers of myocardial infarction. *Prog Cardiovasc Dis* **48**, 285-300.

Lotito, S. B., and Frei, B. (2006). Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? *Free Radic Biol Med* **41**, 1727-1746.

Manach, C., Morand, C., Texier, O., Favier, M. L., Agullo, G., Demigne, C., Regerat, F., and Remesy, C. (1995). Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J Nutr* **125**, 1911-1922.

Mira, L., Fernandez, M. T., Santos, M., Rocha, R., Florencio, M. H., and Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radic Res* **36**, 1199-1208.

Moran, J. F., Klucas, R. V., Grayer, R. J., Abian, J., and Becana, M. (1997). Complexes of iron with phenolic compounds from soybean nodules and other legume tissues: prooxidant and antioxidant properties. *Free Radic Biol Med* **22**, 861-870.

Moridani, M. Y., Pourahmad, J., Bui, H., Siraki, A., and O'Brien, P. J. (2003). Dietary flavonoid iron complexes as cytoprotective superoxide radical scavengers. *Free Radic Biol Med* **34**, 243-253.

Pinelli, A., Trivulzio, S., Tomasoni, L., Bertolini, B., Brenna, S., Bonacina, E., and Vignati, S. (2004). Myocardial infarction non-invasively induced in rabbits by administering isoproterenol and vasopressin: protective effects exerted by verapamil. *Fundam Clin Pharmacol* **18**, 657-667.

Ratty, A. K., and Das, N. P. (1988). Effects of flavonoids on nonenzymatic lipid peroxidation: structure-activity relationship. *Biochem Med Metab Biol* **39**, 69-79.

Remiao, F., Carvalho, M., Carmo, H., Carvalho, F., and Bastos, M. L. (2002). Cu2+-induced isoproterenol oxidation into isoprenochrome in adult rat calcium-tolerant cardiomyocytes. *Chem Res Toxicol* **15**, 861-869.

Rona, G., Boutet, M., and Huttner, I. (1983). Reperfusion injury. A possible link between catecholamine-induced and ischemic myocardial alterations. *Adv Myocardiol* **4**, 427-439.

Russo, A., Acquaviva, R., Campisi, A., Sorrenti, V., Di Giacomo, C., Virgata, G., Barcellona, M. L., and Vanella, A. (2000). Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biol Toxicol* **16**, 91-98.

Sestili, P., Diamantini, G., Bedini, A., Cerioni, L., Tommasini, I., Tarzia, G., and Cantoni, O. (2002). Plant-derived phenolic compounds prevent the DNA single-strand breakage and cytotoxicity induced by tert-butylhydroperoxide via an iron-chelating mechanism. *Biochem J* **364**, 121-128.

Soares, V. C., Varanda, E. A., and Raddi, M. S. (2006). In vitro basal and metabolism-mediated cytotoxicity of flavonoids. *Food Chem Toxicol* **44**, 835-838.

Spiller, P., and Webb-Peploe, M. M. (1985). Blood flow. *Eur Heart J* **6 Suppl C**, 11-18.

Stanely Mainzen Prince, P., and Karthick, M. (2007). Preventive effect of rutin on lipids, lipoproteins, and ATPases in normal and isoproterenol-induced myocardial infarction in rats. *J Biochem Mol Toxicol* **21**, 1-6.

Ueno, I., Nakano, N., and Hirono, I. (1983). Metabolic fate of [14C] quercetin in the ACI rat. *Jpn J Exp Med* **53**, 41-50.

van Acker, F. A., Hulshof, J. W., Haenen, G. R., Menge, W. M., van der Vijgh, W. J., and Bast, A. (2001). New synthetic flavonoids as potent protectors against doxorubicin-induced cardiotoxicity. *Free Radic Biol Med* **31**, 31-37.

van Acker, S. A., van Balen, G. P., van den Berg, D. J., Bast, A., and van der Vijgh, W. J. (1998). Influence of iron chelation on the antioxidant activity of flavonoids. *Biochem Pharmacol* **56**, 935-943.

von Kanel, R., Mills, P. J., Ziegler, M. G., and Dimsdale, J. E. (2002). Effect of beta2-adrenergic receptor functioning and increased norepinephrine on the hypercoagulable state with mental stress. *Am Heart J* **144**, 68-72.

Wadworth, A. N., and Faulds, D. (1992). Hydroxyethylrutosides. A review of its pharmacology, and therapeutic efficacy in venous insufficiency and related disorders. *Drugs* **44**, 1013-1032.

Winsor, T., Mills, B., Winbury, M. M., Howe, B. B., and Berger, H. J. (1975). Intramyocardial diversion of coronary blood flow: effects of isoproterenol-induced subendocardial ischemia. *Microvasc Res* **9**, 261-278.

Yao, L. H., Jiang, Y. M., Shi, J., Tomas-Barberan, F. A., Datta, N., Singanusong, R., and Chen, S. S. (2004). Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* **59**, 113-122.

IV. SUMMARY

Background: Iron is an essential element necessary for many physiological processes involving oxygen transport, DNA-synthesis and ATP-formation. The fate of iron in the organism is tightly regulated especially at the absorption and distribution level probably mainly due to lack of specific active iron excretion mechanism. Any derangement of iron homeostatis may lead to appearance of free (unbound or loosely bound) iron, which can catalyse reactive oxygen species (ROS) production by Haber-Weiss chemistry.

Cardiovascular diseases, particularly coronary heart disease (CHD), remain notwithstanding recent scientific advances important therapeutic problem. The most serious form of CHD represents acute myocardial infarction (AMI). The pathophysiology of AMI involves in most cases initial ischaemic period caused by coronary blood flow derangement due to a thrombus formation. Ischaemia alters substantially tissue homeostasis with subsequent cytosolic free iron appearance. Reconstitution of coronary blood flow (reperfusion) represents the only way for myocardial tissue recovery although on the other hand, it is linked with a release of free redox-active iron in the circulation and formation of ROS both intracellularly as well extracellularly.

Iron chelators are a large group of drugs with very diverse structure. They are traditionally used as protective medication in conditions with suggested involvement of iron in ROS generation (e.g., thalassemic patients treated with blood transfusions and antracycline cardiotoxicity). Due to mentioned involvement of iron in the pathogenesis of AMI, drugs chelating iron may be useful in prevention of AMI-associated tissue impairment. The only drug

which has been tested extensively in occlusional models of AMI was deferoxamine. Regrettably, the published results have been contradictory.

Aim and methodology: This study was aimed to investigate the effects of drugs with iron chelating properties on catecholamine model of AMI. For this purpose, a synthetic catecholamine isoprenaline (ISO) with non-selective β-agonist activity was used in a dose of 100 $mg.kg^{-1}$ to evoke a pathological state in many aspects similar to AMI. Male Han:Wistar rats were pretreated i.v. with agents as follows: deferoxamine mesylate (50 $mg.kg^{-1}$), equivalent doses of 2-pyridylcarboxaldehyde-2-thiophenecarboxyl hydrazone (PCTH, 20.4 $mg.kg^{-1}$), rutin (46 $mg.kg^{-1}$) and lactoferrin in a dose of 50 $mg.kg^{-1}$ five minutes before application of ISO. Additional smaller doses of PCTH (10.2 $mg.kg^{-1}$) and rutin (11.5 $mg.kg^{-1}$) were administered to other rats for obtaining dose-reponse effects. 24 hours after drug application, animals were anaesthetized with urethane (1.2 $g.kg^{-1}$ i.p.). Cardiac function was assessed in terms of a thermodilution method using Cardiosys® apparatus (Experimetria Ltd.®, Hungary), blood was withdrawn for biochemical measurement and heart ventricles removed for weight, elements and histological analysis.

Results: Isoprenaline alone caused 30% mortality, a decrease in cardiac output associated with an increase in heart rate and peripheral resistance, marked elevation of serum cardiac troponin T (cTnT) concentration, an increase in wet ventricle weight, myocardial calcium overload and substantial impairment of cardiac tissue within 24 hours in comparison to the controls. There were no significant changes in plasma TBARS levels, serum vitamin C, total glutathione, erythrocyte antioxidant enzymes (SOD, GPx) and myocardial levels of zinc, selenium and iron.

Deferoxamine did not demonstrate any positive effect on ISO-impairment. PCTH in equimolar dose to that of deferoxamine hindered mortality, markedly reduced release of cTnT and partly but significantly reduced an increase in wet ventricles weight. Others parameters seemed not to be positively influenced by PCTH although some of its effect may be obscured due to used solvent – 20% water solution of propylene glycol, which alone demonstrated some myocardial derangement (e.g. a drop in cardiac index).

Both lactoferrin and rutin inhibited an increase in peripheral resistance caused by ISO. Notwithstanding these positive effects, rutin rather negatively influenced ISO-impairment. Namely, higher dose of rutin increased mortality to 53% and tended to intensify cTnT release, as well as calcium overload. Additionally, only higher dose of rutin elevated myocardial calcium levels in control animals too, and caused dose-dependent myocardial zinc homeostasis derangement. Except for the mentioned positive effect on peripheral resistence, lactoferrin restored cardiac index dropped by ISO and partly decreased calcium overload.

Discussion and conclusion: This study demonstrated for the first time that iron chelators can at least partly prevent myocardial injury caused by catecholamines and may probably protect myocardium from AMI consequences. The failure of complete inhibition of catecholamine myocardial injury seems to depend on the pathogenesis of ISO-cardiotoxicity. This is not fully understood but involves at least two main features – cardiac overstimulation by activation of ß-adrenoreceptors and ROS-generation due to released iron, as well due to catecholamines themselves. Lipophilic iron chelator PCTH may revert, at least partly, this injury probably because the fact that it chelates both intravascullar as well as intracellular free iron. Contrarily, hydrophilic, therapeutically standardly used chelator deferoxamine had no possitive effect on this

injury. Endogenous hydrophilic ferric chelator lactoferrin had modest influence on this injury, which were probably mediated by extracellular iron chelation (peripheral resistence, calcium overload) and/or by another unknown mechanism. By contrast, rutin appeared to aggravate this injury in the larger dose, which can be ascribed: i) to its proposed possible pro-oxidant properties; ii) to an increase in myocardial calcium level which may increase physiologically contractile force but under pathological condition may lead to acute heart failure or arrhythmias.

V. SOUHRN

ÚVOD: Železo je nezbytným prvkem pro celou řadu fyziologických procesů, mj. přenos kyslíku, syntézu DNA a tvorbu ATP. Osud železa v organismu je pečlivě regulován zejména na úrovni absorpce a distribuce pravděpodobně z důvodu neexistujícího specifického aktivního exkrečního mechanismu pro železo. Každé porušení homeostázy železa může vést k objevení se volného (nevázaného nebo slabě vázaného) železa, které je schopno katalyzovat tvorbu reaktivních forem kyslíku (ROS) prostřednictvím Haber-Weissova mechanismu.

Kardiovaskulární onemocnění, zejména pak ischemická choroba srdeční (ICHS), zůstávají přes recentní vědecké pokroky vážným medicinálním problémem. Nejzávažnější formou ICHS je akutní infarkt myokardu (AIM). Jeho patofyziologie zahrnuje ve většině případů iniciální ischemickou periodu v důsledku poruchy koronárního krevního proudu způsobeného trombózou nasedající obvykle na aterosklerotický postiženou koronární tepnu. Ischémie podstatně mění tkáňovou homeostázu s následným objevením se volného cytosolického železa. Obnova koronárního krevního průtoku (tzv. reperfúze) je jedinou možností k záchraně poškozeného myokardu, je ale na druhé straně spojena s uvolněním zmíněného volného železa do cirkulace a tvorbou ROS jak uvnitř tak vně buňky.

Chelátory železa představují rozsáhlou skupinu léčiv s rozmanitou strukturou. Tradičně se používají jako ochranné látky v patologických podmínkách spojených se zvýšenou tvorbou ROS v důsledku katalytického působení železa (např. u thalasemických pacientů léčených krevními transfúzemi a při prevenci antracyklinové kardiotoxicity). Vzhledem ke zmíněnému zapojení železa v patogenezi AIM, mohou být látky s želeto chelatační aktivitou užitečné v prevenci infarktového poškození myokardu. Jedinou látkou, která byla testována v této indikaci na

oklusivních modelech AIM byl deferoxamin. Publikované výsledky jsou bohužel značně nesourodé.

Cíl studie a metodologie: Tato studie směřovala ke zjištění účinků látek s železo chelatační aktivitou na katecholaminový model AIM. Pro tento účel byl využit syntetický katecholamin isoprenalin (ISO) s neselektivním β-agonistickým účinkem v dávce 100 $mg.kg^{-1}$ s.c., který vyvolává patologický stav v mnoha aspektech blízký AIM. Han:Wistar potkani byli premedikováni i.v. následujícími látkami: deferoxamin mesylát (50 $mg.kg^{-1}$), ekvivalentními dávkami 2-pyridylkarboxaldehyd-2-thiofenekarboxyl hydrazonu (PCTH, 20.4 $mg.kg^{-1}$), rutinu (46 $mg.kg^{-1}$) a laktoferinem v dávce 50 $mg.kg^{-1}$ vždy 5 minut před podáním ISO. Pro stanovení dávkové závislosti byly podány i nižší dávky PCTH (10.2 $mg.kg^{-1}$) a rutinu (11,5 $mg.kg^{-1}$). Za 24 hodin po aplikaci léčiv byla zvířata anestezována za pomocí uretanu (1,2 $g.kg^{-1}$ i.p.). Jejich srdeční funkce byla stanovena pomocí různých parametrů prostřednictví termodiluční metody za použití přístroje Cardiosys® (Experimetria Ltd.®, Maďarsko). Krev byla odebrána pro stanovení biochemických markerů a srdce vyjmuto pro zvážení srdečních komor, analýzu iontů a histopatologické vyšetření.

Výsledky: Podání ISO bylo spojeno s 30% mortalitou, snížením srdečního výdeje, tachykardií, zvýšením periferní cévní resistence, masivním uvolněním srdečního troponinu T (cTnT) do cirkulace, vzestupem vlhké hmotnosti komor, přesycením myokardu vápníkem a zjevnými patologickými abnormalitami v histologickém nálezu za 24 hodin v porovnání s kontrolními potkany. Naopak nebyly nalezeny statisticky významné změny v plasmatických hladinách malonyldialdehydu (měřeného jako TBARS), koncentracích vitamínu

C v plasmě, celkového glutationu v krvi, enzymatické aktivitě antioxidačních enzymů v červených krvinek (GPx a SOD) a koncentraci zinku, selénu a železa v srdeční tkáni.

Podání deferoxaminu nebylo spojeno s žádným ochranným vlivem na ISO-poškození. Naopak PCTH v ekvimolární dávce k deferoxaminu vedl k 100% přežití takto premedikovaných zvířat, významně snížil uvolnění cTnT a částečně redukoval vzestup vlhké hmotnosti komor po podání ISO. Ostatní parametry nebyly příznivě ovlivněny PCTH, je ale nutno zmínit, že některé jeho účinky mohly být zamaskovány při jeho podání v 20% vodném roztoku propylenglykolu, který sám vedl k určitému zhoršení myokardiální funkce (např. pokles srdečního výdeje).

Jak laktoferin tak rutin zablokoval vzestup periferní rezistence po podání ISO. Přes tento pozitivní účinek rutin spíše zhoršoval katecholaminové poškození myokardu. Vyšší dávka rutinu zvýšila mortalitu na 53% a současně prokázala tendenci k potenciaci uvolnění cTnT a zesílení přesycení myokardu vápníkem. Kromě toho, ze všech testovaných látek s železo chelatační aktivnou jen rutin ve vyšší dávce statisticky významně zvýšil myokardiální koncentrace vápníku i u kontrolních zvířat, kromě toho vedl k dávkově závislému poklesu hladiny zinku v myokardu. Mimo pozitivního účinku laktoferinu na periferní resistenci, laktoferin byl také schopen inhibovat pokles srdečního výdeje způsobený ISO a současně částečně omezit přesycení buněk vápníkem.

Diskuze a závěr: Tato studie je první svého druhu, které poukázala, že chelátory železa jsou schopny alespoň částečně snížit poškození způsobená katecholaminy a z tohoto důvodu i pravděpodobně ochránit myokard před následky AIM. Neúspěch těchto chelátorů železa úplně zabránit projevům katecholaminové kardiotoxicity závisí pravděpodobně na komplexnosti patofyziologie tohoto poškození, která není úplně pochopená. Je známo, že zahrnuje minimálně

dva hlavní mechanismy – nadměrnou stimulaci β-adrenoreceptorů a tvorbu ROS v důsledku jak uvolnění železa tak katecholaminů samotných. Lipofilní chelátor PCTH částečně inhiboval toto poškození asi v důsledku toho, že je schopen snížit následky zvýšených hladin železa nejen intravaskulárně ale také intracelulárně. Naopak hydrofilní, v terapii standardně používaný, chelátor deferoxamin neměl žádný vliv na dané poškození. Tělu vlastní hydrofilní chelátor železitých iontů laktoferin měl určitý pozitivní účinek na toto poškození, který byl pravděpodobně zprostředkován chelatací extracelulárního železa (periferní rezistence, snížení přesycení vápníkem) a/nebo jiným neznámým mechanismem. Rutin naproti tomu spíše zhoršuje ve vyšší dávce toto poškození. Důvody k tomu mohou být: i) jeho navržené pro-oxidativní vlastnosti; ii) vzestup hladin vápníku v myokardu, které mohou zvýšit fyziologicky inotropii, ale v případě patologických stavů vést naopak k akutnímu srdečnímu selhání nebo arytmiím.

VI. LIST OF PUBLISHED WORKS

1. Original papers in journals with peer-review

Firuzi O, **Mladěnka P**, Petrucci R, Marrosu G and Saso L. Hypochloride scavenging activity of flavonoids. J Pharm Pharmacol. 2004 Jun;56(6):801-7

Firuzi O, **Mladěnka P**, Riccieri V, Spadaro A, Petrucci R, Marrosu G, Saso L. Parameters of oxidative stress status in healthy subjects: their correlations and stability after sample collection. J Clin Lab Anal. 2006;20(4):139-48.

Mladěnka P, Semecký V, Bobrovová Z, Nachtigal P, Vávrová J, Holečková M, Palicka V, Mazurová Y & Hrdina R. The effects of lactoferrin in a rat model of catecholamine cardiotoxicity, Biometals 2008, in press

Mladěnka P, Zatloukalová L, Šimůnek T, Bobrovová Z, Semecký V, Nachtigal P, Hašková P, Macková E, Vávrová J, Holečková M, Palicka V, Hrdina R. Direct administration of rutin does not protect against catecholamine cardiotoxicity. Toxicology 2008, in press

Mladěnka P, Kalinowski D, Hašková P, Bobrovová Z, Hrdina R, Šimůnek T, Nachtigal P, Semecký V, Vávrová J, Holečková M, Palicka V, Mazurová Y, Jansson P, Richardson DR. The Novel Iron Chelator, PCTH, Reduces Catecholamine-Mediated Myocardial Toxicity. Chem Res Toxicol 2008, in press

2. Review articles

Mladěnka P, Hrdina R, Hübl M, Šimůnek T. The fate of iron in the organism and its regulatory pathways. Acta Medica (Hradec Kralove) 2005; 48(3):127-35

Mladěnka P, Hrdina R, Hübl M, Šimůnek T. The role of reactive oxygen and nitrogen species in cellular iron metabolism. Free Radic Res. 2006 Mar;40(3):263-72.

Hrdina R, **Mladěnka P**, Bobrovová Z a Hübl M. Farmakoterapie ichemické choroby srdeční. Praktické lékárenství 2007;2:62-5

3. Lectures on congresses

- **The 5th International Postgraduate Research Symposium on Pharmaceutics - Istanbul (Turkey), September 13- 15, 2007**
  - MLADĚNKA P, SEMECKÝ V, BOBROVOVÁ Z, NACHTIGAL P, ŠKRLE J, HRDINA R. Iron Chelators in Myocardial Ischemia-Reperfusion – Comparison of Endogenous Lactoferrin with Synthetic PCTH. Acta Pharmac Turcica, 2007, vol. 49 (suppl.), p. 11.

4. Posters on congresses – active presentation

- **55. Farmakologické Dny - Hradec Králové, August 31 – September 2, 2005**
  - HÜBL M, MLADĚNKA P, HRDINA R, PALIČKA V, VÁVROVÁ J, HOLEČKOVÁ M. EFFECT OF DEFEROXAMINE ON ISOPRENALINE MODEL OF ACUTE MYOCARDIAL INFARCTION IN RATS. Sborník abstraktů 55. Česko-slovenských farmakologických dnů. Hradec Králové, Lékařská fakulta Univerzity Karlovy 2005:13
- **56. Farmakologické Dny - Bratislava, September 6-8, 2006**
  - BOBROVOVÁ Z, HÜBL M, MLADĚNKA P, HRDINA R, PALIČKA V, VÁVROVÁ J, HOLEČKOVÁ M. Effect of PCTH on isoprenaline model of acute myocardial infarction in rats. Zborník prac 56. Farmakologické dni 2006 v Bratislavě 2006:84.
- **16th International Conference on Chelators (ICOC) - Limassol (Cyprus), October 25-31, 2006**
  - MLADĚNKA P, BOBROVOVÁ Z, HÜBL M, HRDINA R, NACHTIGAL P, VYKRUTOVÁ E, SEMECKÝ V. Effect of lactoferrin on a model of acute myocardial infarction in rats. Sborník abstrakt 16th ICOC. 2006: 76

- **29th world congress of the International Society for Heart Reasearch - Bologna (Italy), June 22-25, 2007**
  - MLADĚNKA P, HRDINA R, BOBROVOVÁ Z, HÜBL M, NACHTIGAL P, ŠKRLE J, SEMECKÝ V. Interrelationships of functional, biochemical and morphological variables in catecholamine cardiotoxicity. J Mol Cell Cardiol, 2007, vol. 42 (suppl. 1), p. 244.
- **The 5th International Postgraduate Research Symposium on Pharmaceutics - Istanbul (Turkey), September 13- 15, 2007**
  - HRDINA R, MLADĚNKA P, BOBROVOVÁ Z. Cardiac troponin T: A reliable biomarker of acute myocardial injury in rats. Acta Pharmac Turcica, 2007, vol. 49 (suppl.), p. 79.
- **28th Annual Meeting, European Section of the International Society for Heart Reasearch - Athens (Greece), May 28-31, 2008**
  - MLADĚNKA P., SEMECKÝ V., BOBROVOVÁ Z., FILIPOVÁ V., ZATLOUKALOVÁ L., HRDINA R. The influence of iron chelators on myocardial metal content after catecholamine cardiotoxic insult. J Moll Cell Cardiol, 2008, vol. 44:716-7.
- **EPHAR 2008 Congress - Manchester (Great Britain), July 12-17, 2008.**
  - MLADĚNKA P, ZATLOUKALOVÁ L, MACÁKOVÁ K., ŘEHÁKOVÁ Z., KUMAR PRASAD A, PARMAR PS, SASO L, HRDINA R. In vitro iron chelati-on activity of selected polyphenolic compounds. Fundam Clin Pharmacol, 2008; vol. 22 (suppl. 2), p. 58.
  - HRDINA R. MLADĚNKA P, ZATLOUKALOVÁ L, BOBROVOVÁ Z. Effects of iron chelators on acute isoprenaline cardiotoxicity. Fundam Clin Pharmacol, 2008; vol. 22 (suppl. 2), p.104-5

5. Posters on congresses – presentation by colleagues

- **57. Farmakologické Dny - Olomouc, Septembre 12- 14, 2007**
  - BOBROVOVÁ Z, HRDINA R, MLADENKA P, HUBL M, VAVROVA J, HOLECKOVA M, PALICKA V. Dose-dependence study of a novel iron chelator PCTH in a model of catecholamine cardiotoxicity. Biomed Pap, 2007, vol. 151 (suppl. 1), p. 13.
- **58. Farmakologické Dny - Praha, September 3-5, 2008**
  - ZATLOUKALOVÁ L, MLADĚNKA P, BOBROVOVÁ Z, VÁVROVÁ J, HOLEČKOVÁ M, PALIČKA V, HRDINA R. Isoprenaline Cardiotoxic Insult is Aggravated by Rutin. Prague Med Rep 2008; 109 (suppl.):S131-2
- **7th Joint Meeting of AFERP, ASP, GA, PSE & SIF - Athens (Greece), August 3-8,2008**
  - MACAKOVA M, MLADENKA P, REHAKOVA Z, ZATLOUKALOVA L HRDINA R, OPLETAL L, KARLICKOVA J. Ferrous and ferric chelation activity of selected natural flavonoids. Planta Med 2008; 74:946
- **20th IFCC Wordlab – Fortaleze (Brazil), September 28 – October 2, 2008**
  - HOLEČKOVÁ M, MLADĚNKA P, HRDINA R, PALIČKA V. Importance of myocardial element concentrations after catecholamine cardiotoxic injury. Clin Chem Lab Med 2008; 46(Special Suppl): S 530 – 531.