UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
Katedra farmakologie a toxikologie

Výzkum látek s potenciálem chelatovat železo a jejich případné využití v terapii akutního infarktu myokardu

Screening of iron-chelating substances and their potential use in the therapy of acute myocardial infarction

DISERTAČNÍ PRÁCE

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Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně pod vedením svého školitele prof. MUDr. Radomíra Hrdiny, CSc. a konzultanta PharmDr. Přemysla Mladěnky, Ph.D. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

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Mgr. Tomáš Filipský

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ABSTRAKT

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Železo je esenciálním elementem všech živých organismů. Za fyziologických podmínek je úroveň volného/nevázaného železa v organismu velmi nízká. V případě některých patologických stavů, je ovšem tato homeostáza železa narušena a může dojít k poškození orgánů v důsledku zvýšené tvorby reaktivních forem kyslíku.

Ischemická choroba srdeční je celosvětově nejčastější příčinou morbidity a mortality. Její nejzávažnější formou je akutní infarkt myokardu (AIM). Již během časné ischémie se do krevního řečiště uvolňují katalyticky aktivní elementy, především zmíněné železo, ale také měď. Po obnovení krevního průtoku (reperfuzi) se mohou účastnit produkce biologicky nejnebezpečnějšího oxidantu – hydroxylového radikálu tzv. železem/mědí katalyzovanou Fentonovou reakcí. Tudíž se zde nabízí potencionálně aplikovatelný farmakoterapeutický přístup k léčbě tohoto onemocnění – terapie látkami chelatujících železo/měď.

Cílem této disertační práce byl výzkum látek chelatujících železo a jejich vliv na isoprenalinový model AIM včetně charakterizace některých dříve nezkoumaných aspektů tohoto modelu.

V *in vitro* části studie byla k analýze účinků testovaných látek při (pato)fyziologicky relevantních pH použita: 1) spektrofotometrická ferrozinová metoda k detekci účinnosti chelatace železa a 2) HPLC analýza k zjištění anti/pro-oxidační aktivity s použitím kyseliny salicylové jako indikátoru tvorby hydroxylového radikálu. Kromě toho byl vyvinut nový, finančně nenáročný, ale přesný analytický přístup pro

zjištění stechiometrie komplexu chelátor:železo za využití UV–Vis spektrofotometrie. *In vivo* část studie byla zaměřena na zhodnocení vlivu dexrazoxanu (20,4 mg/kg, i.v.) na isoprenalinový model AIM (100 mg/kg, s.c.) u Wistar:Han potkanů. Kromě toho byly také popsány časné patologické změny a vzájemné vztahy mezi biochemickými markery a funkčními parametry srdeční dys/funkce po aplikaci isoprenalinu. V těchto *in vivo* studiích byla použita termodiluční metoda nebo invazivní měření tlaku a objemu v levé srdeční komoře.

Pro chelatační účinky flavonoidů se jako nejvýhodnější pozice jevila 6,7-dihydroxylová skupina. Baikalein, obsahující tuto strukturu, měl srovnatelnou schopnost chelatovat železo jako referenční chelátor železa – deferoxamin. Nicméně jeho vliv na inhibici Fentonovy reakce byl menší. 3-hydroxy-4-keto substituce s dvojnou vazbou v poloze 2 a pyrokatecholový kruh B (např. kvercetin) byly rovněž spojeny s významnými chelatačními účinky. Na druhou stranu vliv těchto flavonoidů na Fentonovu reakci byl spíše minimální. V některých případech byl pozorován dokonce nežádoucí pro-oxidační účinek.

Ačkoli mezi dlouho známé podskupiny syntetických chelátorů železa patří 1-fenyl-3-methyl-4-acyl-pyrazol-5-ony, poznatky týkající se jejich biologické aktivity jsou spíše limitované. Některé sloučeniny prokázaly vyšší účinnost při pH 4,5 než v klinické praxi používaný deferoxamin. Za zmínku stojí prototypová sloučenina H_2QpyQ, tj. 2,6-bis[4(1-fenyl-3-methylpyrazol-5-on)karbonyl]pyridin, jejíž schopnost chelatovat železo rostla s klesajícím pH. Podle našich znalostí je to vůbec první sloučenina, která má takovéto vlastnosti. Kromě toho většina acylpyrazolonů patřila mezi velmi účinné inhibitory Fentonovy reakce, jež lze srovnávat s deferoxaminem.

Jednou z důležitých vlastností chelátorů je i stechiometrie vytvořeného komplexu ve vztahu k pH. Jelikož je u některých látek, např. flavonoidů, stechiometrie komplexů stále nejasná, byl k jejímu určení vyvinut nový analytický přístup za využití UV–Vis spektrofotometrie. Hlavní výhodou oproti standardní Jobově metodě se jeví jeho schopnost určit stechiometrii a případně i kinetiku tvorby komplexu i u slabých chelátorů železa.

Studie zaměřená na analýzu časných patologických změn po aplikaci kardiotoxické dávky isoprenalinu prokázala, že z patogenetického hlediska diastolická dysfunkce předchází systolické dysfunkci a že k její samotné indukci je stimulace pouze

β_2-adrenergních receptorů nedostatečná. Kromě toho sérové koncentrace srdečního troponinu (cTnT) významně korelovaly se závažností poškození myokardu u potkanů (např. s přetížením myokardu vápníkem – pozitivní korelace a tepovým objemem – negativní korelace). Na druhou stranu korelace cTnT s markery oxidačního stresu byly spíše nevýrazné (glutathion a vitamin C) nebo nulové (vitamin E a TBARS – reaktivní formy thiobarbiturové kyseliny, z angl. thiobarbituric acid reactive substances). Vztah mezi cTnT a dalšími parametry byl exponenciální s výjimkou koncentrace vápníku v srdeční tkáni, kde byl nalezen vztah, který popisuje mocninná funkce.

V 24hodinovém experimentu podání dexrazoxanu vedlo k částečnému poklesu mortality, snížení koncentrace vápníku v srdeční tkáni, ke zlepšení histopatologického nálezu a hemodynamických parametrů. Kontinuální 2hodinový experiment prokázal, že dexrazoxan není schopen ovlivnit isoprenalinem navozené atrioventrikulární bloky a jeho vliv na hemodynamické parametry byl zde spíše minimální. Navíc *in vitro* experimenty naznačily, že chelatační vlastnosti dexrazoxanu nehrají významnou roli v kardioprotektivním mechanismu.

Závěrem lze shrnout, že série *in vitro* experimentů může u chelátorů železa alespoň částečně predikovat pozitivní nebo i negativní vliv na isoprenalinový model AIM. Některé aspekty bude ale nutné ověřit v dalších zejména *in vivo* experimentech.

ABSTRACT

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Title of doctoral thesis:	Screening of iron-chelating substances and their potential use in the therapy of acute myocardial infarction

Iron is an essential element virtually for all living organisms. The concentration of free/unbound iron is very low at physiological conditions. However, in several pathological states, its homeostasis is impaired which may lead to an organ damage due to the increased production of reactive oxygen species.

Coronary heart disease is the main cause of morbidity and mortality worldwide. Its most serious form is acute myocardial infarction (AMI). During the early ischaemia, catalytically active elements, particularly iron and copper, are released into the blood circulation. After restoration of blood flow (reperfusion), these elements may participate in the production of biologically the most potent oxidant – hydroxyl radical *via* iron/copper catalysed Fenton reaction. Therefore, a therapy based on administration of iron/copper-chelating agents could be a potential pharmacotherapeutic approach in the treatment of this disease.

The main aim of this doctoral thesis was a screening of iron-chelating substances and their influence on isoprenaline model of AMI including characterisation of several previously unknown aspects of this model.

In vitro study analysed effects of the tested substances at (patho)physiologically relevant pH conditions by: 1) the spectrophotometric ferrozine methodology detecting iron-chelating properties, and 2) the HPLC analysis determining anti/pro-oxidative activities by the use of salicylic acid as the indicator of the formation of hydroxyl radical. Moreover, a new inexpensive but precise analytical approach for a

determination of a stoichiometry of the complex chelator:iron using UV–Vis spectrophotometry was evolved. *In vivo* part of the study was aimed at an evaluation of effects of dexrazoxane (20.4 mg/kg, i.v.) on isoprenaline model of AMI (100 mg/kg, s.c.) in Wistar:Han rats. Furthermore, early pathological changes and relationships among various biochemical and functional parameters of cardiac dys/function were described after the administration of isoprenaline. In these *in vivo* studies, the thermodilution method or the invasive measurement of pressure and volume in the left heart ventricle were used.

In flavonoids, the 6,7-dihydroxy structure was the most effective substitution for iron-chelation. Baicalein, in which this group is incorporated, possessed a similar ability to chelate iron as a reference iron chelator deferoxamine. However, its influence on the inhibition of Fenton reaction was lower. The 3-hydroxy-4-keto conformation together with 2,3-double bond and the catecholic B ring (*e.g.* quercetin) were associated with a substantial iron-chelating properties as well. On the other hand, the influence of these structures on Fenton reaction was rather minimal, and in several cases, even undesirable pro-oxidative effect was observed.

Although synthetic iron chelators from the group of 1-phenyl-3-methyl-4-acyl-pyrazol-5-ones have been known for many years, data on their biological activity are rather limited. Some of the tested substances were even more potent iron chelators at pH 4.5 than the clinically used standard – deferoxamine. Of particular interest is a prototype compound H_2QpyQ, *i.e.* 2,6-bis[4(1-phenyl-3-methylpyrazol-5-one)carbonyl]pyridine, which iron-chelating affinity increased when pH was decreasing. To our knowledge, it is the first compound having such properties. Moreover, most of the tested acylpyrazolones were powerful inhibitors of Fenton chemistry as deferoxamine.

One of the most important features of iron chelators is the stoichiometry of a formed complex in relation to pH. Moreover, in certain substances, *e.g.* flavonoids, the stoichiometry of the complex has been still unknown, therefore, a new analytical approach using UV–Vis spectrophotometry was evolved. The major benefit of this approach, compared to the standard Job's method, seems to be its capability to reveal the stoichiometry and the kinetic aspects of formation of the complex in chelators with moderate affinity to iron, as well.

The study focused on the analysis of early pathological changes after administration of the cardiotoxic dose of isoprenaline showed that diastolic dysfunction preceded systolic dysfunction and β_2-adrenoreceptor stimulation alone was not sufficient for its induction. Moreover, serum concentration of cardiac troponin T (cTnT) correlated strongly with the degree of myocardial injury in rats (*e.g.* calcium overload – positive correlation, stroke volume – negative correlation). On the other hand, correlations between cTnT and oxidative stress parameters were weak (for glutathione and vitamin C) or were not found (for serum vitamin E and TBARS – thiobarbituric acid reactive substances levels). Relationships between cTnT and other parameters were exponential with the exception of myocardial calcium, where a power function was found.

In a 24-hour experiment, the administration of dexrazoxane resulted in the partial decrease in mortality, reduction of myocardial calcium overload and improvement in histological impairment and peripheral haemodynamic disturbances. Continuous 2-hour experiments showed that dexrazoxane did not influence isoprenaline-induced atrioventricular blocks and had little effect on the measured haemodynamic parameters. Complementary *in vitro* experiments suggested that iron-chelating properties of dexrazoxane apparently did not play the major role in the cardioprotective mechanism.

It can be concluded that *in vitro* analysis of iron chelators may at least partially predict their positive or even negative influence on isoprenaline model of AMI. On the other hand, some aspects should be confirmed by additional *in vivo* experiments.

OBSAH

1. SEZNAM ZKRATEK

Apo-Tf	apo-transferin
AIM	akutní infarkt myokardu
CABG	akutní aortokoronární bypass, z angl. coronary artery bypass graft
CK-MB	kreatinkináza – izoforma MB
cTnI	srdeční troponin I
cTnT	srdeční troponin T
Dcytb	duodenální cytochrom b
DFO	deferoxamin
DMT-1	transportér pro dvojmocné kovy, z angl. divalent metal trasporter-1
EDTA	ethylendiaminotetraoctová kyselina
H_2QpyQ	2,6-bis[4(1-fenyl-3-methylpyrazol-5-on)karbonyl]pyridin
HCP-1	hem přenašečový protein 1, z angl. heme carrier protein-1
PCI	primární perkutánní koronární intervence, z angl.
PCIH	2-pyridylkarboxaldehyd isonikotinoyl hydrazon
PIH	pyridoxal isonikotinoyl hydrazon
SIH	salicylaldehyd isonikotinoyl hydrazon
TBARS	reaktivní formy thiobarbiturové kyseliny, z angl. thiobarbituric acid reactive substances
Tf	transferin
TfR1	transferin-receptor-1

2. ÚVOD

Ischemická choroba srdeční je celosvětově nejčastější příčinou morbidity a mortality a rovněž představuje závažný socio-ekonomický problém. Její nejzávažnější formou je akutní infarkt myokardu (AIM). Vývoj nových léčiv na základě detailní analýzy patofyziologických mechanismů vedoucích k AIM je tedy velmi žádoucí.

Jelikož časná fáze AIM je spojena s masivním uvolněním katecholaminů a volných redoxně aktivních přechodných kovů (železa a mědi), které mohou vstupovat do Fentonovy reakce, tzn. katalyzovat tvorbu vysoce reaktivního hydroxylového radikálu, a přispívat tedy k dalšímu poškození nejen srdeční tkáně, nabízí se zde hned několik potencionálně aplikovatelných přístupů k terapii tohoto závažného onemocnění.

V základním kardiologickém výzkumu je s oblibou po desítky let využíván katecholaminový model AIM. Ačkoli přesný patofyziologický mechanismus katecholaminové kardiotoxicity nebyl dodnes uspokojivě vysvětlen, lze patologické změny následující po aplikaci kardiotoxické dávky syntetického katecholaminu isoprenalinu v mnohých aspektech považovat za velmi podobné časným změnám, ke kterým dochází u AIM u lidí.

V průběhu mého čtyřletého doktorského studia ve studijním oboru Farmakologie a toxikologie (Katedra farmakologie a toxikologie, Farmaceutická fakulta v Hradci Králové, Univerzita Karlova v Praze) byly provedeny *in vitro* a *in vivo* analýzy a studie se zaměřením na výzkum látek chelatujících železo a jejich vlivu na isoprenalinový model AIM včetně charakterizace některých dříve nezkoumaných aspektů tohoto modelu:

1) *in vitro* analýza schopností flavonoidů chelatovat železo, jejich redoxních vlastností a vlivu na železem katalyzovanou Fentonovu reakci, tj. anti- a pro-oxidační účinky flavonoidů;
2) *in vitro* analýza schopností nových syntetických chelátorů, zejména acylpyrazolonů, chelatovat železo;
3) vývoj nového *in vitro* analytického přístupu ke stanovení stechiometrie komplexu chelátor:železo pomocí UV–Vis spektrofotometrie;

4) *in vivo* studie popisující časné hemodynamické změny po podání kardiotoxické dávky isoprenalinu;
5) *in vivo* studie analyzující vztah mezi různými biomarkery srdeční funkce/dysfunkce a markery oxidačního stresu;
6) *in vivo* studie hodnotící účinky chelátoru železa dexrazoxanu na isoprenalinový model AIM.

3. TEORETICKÁ ČÁST

3. 1. Železo

Nedlouho po „velkém třesku“ se železo stalo nejen významnou součástí vesmíru, hvězd a planet, ale i esenciální složkou pro vznik života na Zemi (Ilbert a Bonnefoy 2013; Sheftel et al. 2012). V zemské kůře je železo 4. nejrozšířenějším prvkem a 2. nejrozšířenějším kovem ihned po hliníku (≈ 5 %). Rovněž se všeobecně předpokládá, že zemské jádro je složené především ze železa (Ilbert a Bonnefoy 2013).

Železo (Fe, lat. *Ferrum*) je 26. prvkem periodické soustavy prvků a jeho oxidační čísla se mohou pohybovat v rozmezí –II ($Na_2[Fe(CO)_4]$) až +VI (K_2FeO_4) (Bleackley a Macgillivray 2011). Avšak za běžných podmínek v biologický systémech je železo přítomno pouze ve dvou oxidačních stavech: Fe(II) a Fe(III) (Arredondo a Núñez 2005). Reaktivita a rozpustnost tohoto elementu výrazně závisí na fyzikálně-chemických vlastnostech prostředí (teplota, pH, povaha komplexotvorného ligandu, atd.) (Ilbert a Bonnefoy 2013). Zatímco při absenci molekulárního kyslíku je Fe(II) stabilní, v jeho přítomnosti má tendenci velmi rychle oxidovat za vzniku Fe(III) (Arredondo a Núñez 2005). Při neutrálním pH probíhá tato reakce velmi rychle, ale při nízkém pH velmi pomalu s výjimkou biologických systémů. Kromě toho je Fe(II) při neutrálním pH velmi dobře rozpustné ve vodě (≈ 10^{-1} mol/l) na rozdíl od Fe(III), které precipituje ve formě oxyhydroxidů železa s výrazně nižší rozpustností (≈ 10^{-17} mol/l), (Ilbert a Bonnefoy 2013; Valko et al. 2005).

Jelikož závislost života na železe předcházela okysličení pozemské atmosféry, živé organismy si byly nuceny vyvinout nejen účinné obranné mechanismy proti potenciálně nebezpečným reakcím mezi železem a kyslíkem, ale i prostředky, které zvýší dostupnost železa z vnějšího prostředí, např. siderofory – produkty mikroorganismů vytvářející ve vnějším prostředí komplexy s Fe(III), které jsou následně absorbovány za účasti specifických receptorů (Arredondo a Núñez 2005; Ilbert a Bonnefoy 2013; Sheftel et al. 2012).

3. 1. 1. Železo v lidském organismu

Jednou ze základních biologických vlastností železa je jeho schopnost zajišťovat transport elektronů v redoxních reakcích probíhajících v buňce (Ilbert a Bonnefoy 2013; Papanikolaou a Pantopoulos 2005; Sheftel et al. 2012). Nezastupitelné jsou jeho role při transportu kyslíku, syntéze ATP a buněčné proliferaci a diferenciaci. Rovněž je důležitou složkou imunitního systému (Kohgo et al. 2008; Mladěnka et al. 2005).

V lidském těle se nachází přibližně 3-5 g železa (70kg muž má ≈ 3,5 g železa, tj. 50 mg/kg) (Bleackley a Macgillivray 2011; Ilbert a Bonnefoy 2013; Muñoz 2009). Ženy v premenopauze mají však železa méně než muži (Muñoz 2009). Z celkového množství železa se 65 % nachází v hemoglobinu, 10 % je obsaženo v myoglobinu a 25 % připadá na zásobní formy železa – ferritin a hemosiderin (Muñoz 2009; Valko et al. 2005). Zbývající železo je součástí dalších metaloproteinů – enzymů (≈ 1 %; např. cytochromy P-450, cytochromoxidázy, peroxidázy), transportních proteinů (≈ 0,1 %; např. transferin) (Mladěnka et al. 2005; Muñoz 2009).

Obdobně jako ostatní biogenní prvky je železo absorbováno z přijaté stravy. Lidská strava denně obsahuje 15-20 mg železa, avšak za fyziologických podmínek je tělo schopno přijmout pouze 1-2 mg/den; výjimečně až 6 mg/den (Mladěnka et al. 2005; Muñoz 2009). Průměrná denní absorpce železa je v rovnováze s jeho průměrnými denními ztrátami, ke kterým dochází především prostřednictvím exfoliace buněk gastrointestinální sliznice a krvácením (u žen menstruací) (Muñoz 2009). Jelikož lidský organismus postrádá aktivní mechanismus exkrece železa, procesy jeho absorpce a distribuce musí být tedy úzkostlivě řízeny řadou proteinů a peptidů (De Freitas a Meneghini 2001; Fuqua et al. 2012; Ganz a Nemeth 2012; Kohgo et al. 2008; Mladěnka et al. 2005).

Z potravního hlediska lze železo obecně klasifikovat jako hemové a nehemové (Sharp 2010). K jeho uvolnění ze stravy napomáhají kromě kyselého prostředí žaludku a proximální části duodena i proteolytické enzymy (Fuqua et al. 2012; Sharp 2010). Hemová forma železa (hem) je výrazněji zastoupena v potravinách živočišného původu, kde je součástí hemoproteinů hemoglobinu a myoglobinu (Fuqua et al. 2012). Samotný hem je omezeně rozpustný, ale s ostatními komponenty stravy vytváří rozpustnější komplexy (Fuqua et al. 2012). Bylo prokázáno, že k absorpci hemu dochází

mechanismem endocytózy zprostředkované receptorem, ale vysokoafinitní receptor nebyl dosud identifikován (Fuqua et al. 2012; West a Oates 2008). Nedávno byl popsán enterocytární hemový transportér HCP-1 (hem přenašečový protein 1, z angl. heme carrier protein-1/proton coupled folate transporter), nicméně tento protein má velmi malou afinitu k hemu a jeho role spočívá spíše v absorpci folátů (Fuqua et al. 2012; Shayeghi et al. 2005; West a Oates 2008). Po vlastní absorpci hemu se v enterocytu působením hemoxygenázy uvolňují volné Fe(III) (Fuqua et al. 2012; West a Oates 2008). Detailnější mechanismus absorpce hemové formy železa není znám, podrobněji je však popsána absorpce nehemového železa (Kohgo et al. 2008; Mladěnka et al. 2005; Sharp a Srai 2007; West a Oates 2008).

Nehemové železo je přítomno jak v živočišné, tak v rostlinné stravě, kde je součástí nejrůznějších proteinů, např. ferritinu, resp. vakoul rostlinných buněk (Fuqua et al. 2012; Sharp 2010). Nicméně velká část nehemového železa se nachází ve formě železitých iontů, jejichž rozpustnost a biodostupnost je značně omezená (Fuqua et al. 2012; Ilbert a Bonnefoy 2013; Sharp 2010; Valko et al. 2005). Během procesu trávení pravděpodobně rovněž dochází v určité míře i ke vzniku různých stabilních nebo přechodných komplexů, které mohou absorpci železa jak napomáhat, tak ji inhibovat, např. komplexy s kyselinou askorbovou a aminokyselinami cysteinem nebo histidinem, resp. třískovinami (Sharp a Srai 2007). Na druhou stranu přesný mechanismus absorpce železa, které je součástí zásobního proteinu ferritinu, není podobně jako u hemu dosud znám (Fuqua et al. 2012).

Absorpce železa je zprostředkována kartáčovým lemem duodena a minoritně i proximální části jejuna (Fuqua et al. 2012; Mladěnka et al. 2005; Muñoz 2009). Ionty Fe(III) jsou před vlastní absorpcí redukovány na Fe(II) prostřednictvím Dcytb (duodenální cytochrom b, z angl. duodenal cytochrome b), který se nachází na apikální membráně enterocytů (McKie 2008; Mladěnka et al. 2005). Kromě této reduktázy může hrát významnou roli i další reduktáza Steap2 (six transmembrane epithelial antigen of the prostate 2) (McKie 2008; Ohgami et al. 2006). Po redukci jsou železnaté ionty aktivně transportovány na téže membráně přítomným nespecifickým transportérem DMT-1 (transportér pro dvojmocné kovy, z angl. divalent metal trasporter-1) (Espinoza et al. 2011; Mims a Prchal 2005; Mladěnka et al. 2005). Kromě Fe(II) se tento transportér podílí také na přenosu dalších dvojmocných iontů - Zn(II), Mn(II), Co(II),

Cd(II), Cu(II), Ni(II) a Pb(II) s výjimkou Ca(II) a Mg(II) (Gunshin et al. 1997). V enterocytu se železo může stát součástí zásobního ferritinu nebo „volné“ nízkomolekulární formy železa anebo je uvolněno do krevní cirkulace (Fuqua et al. 2012; Mladěnka et al. 2005).

Transport železa z enterocytu do krevní cirkulace je zprostředkován transmembránovým proteinem – ferroportin-1 (Anderson a Vulpe 2009). Jelikož ferroportin-1 s nejvyšší pravděpodobností přenáší železo ve formě Fe(II) a transferin váže pouze Fe(III), je na bazolaterální membráně důležitá přítomnost specifického analogu ceruloplazminu s ferroxidázovou aktivitou tzv. hephaestinu (Anderson a Vulpe 2009; Fuqua et al. 2012; Vulpe et al. 1999).

Uvolnění železa z enterocytu do krevní cirkulace je systémově regulováno hepcidinem (Ganz a Nemeth 2012; Peslova et al. 2009; Tandara a Salamunic 2012). Hepcidin, peptid primárně produkovaný hepatocyty, se váže na bazolaterální membráně enterocytů přítomný exportér železa ferroportin-1, což má za následek internalizaci a degradaci tohoto transportéru a výrazný pokles uvolňování železa z enterocytu do krevní cirkulace (Nemeth et al. 2004). Kromě toho absorpce železa je regulována enterocytární koncentrací železa a hypoxií (Fuqua et al. 2012). Významnou roli v lokální regulaci má systém regulačních proteinů – iron responsive element/iron regulatory protein system, který ovlivňuje expresi genů metabolismu železa (Wang a Pantopoulos 2011).

Za fyziologických podmínek úroveň nevázaného železa v krevní cirkulaci je velmi nízká – často nepřekročí hranici 1 μmol/l nebo je nedetekovatelná. Většina železa je totiž vázaná na transportní protein transferin (Tf) (Anderson 1999; Mladěnka et al. 2005; Nemeth et al. 2004). Apo-transferin (Apo-Tf) má dvě vysokoafinitní vazebná místa pro Fe(III). Po navázání železa se transferin (Tf-Fe_2) váže na specifický membránový transferin-receptor-1 (TfR1). Po vazbě dochází k endocytóze vzniklého komplexu. Kyselé prostředí endozómu vede k postupnému uvolnění Fe(III), apo-Tf a TfR1. Fe(III) je zredukováno na Fe(II) a prostřednictvím intracelulárního DMT-1 přeneseno do cytoplasmy buňky, kde se po oxidaci stává součástí zásobní formy železa, tj. ferritinu, nebo vstupuje do různorodých buněčných procesů anebo se stane součástí málo popsané frakce volného/nevázaného železa. Význam kompartmentu nevázaného

železa stoupá u onemocnění spojených s přetížením organismu železem (Mladěnka et al. 2005).

3. 1. 2. Přetížení organismu železem

Onemocnění spojená s přetížením organismu železem jsou závažná onemocnění vedoucí k progresivnímu a v některých případech k nevratnému poškození orgánů již před objevením klinických projevů (Fernandes 2012; Fleming a Ponka 2012; Inati et al. 2010). Nicméně rozvoj neodvratitelných následků orgánového poškození lze zpomalit nebo dokonce zastavit (Fleming a Ponka 2012). Klinické manifestace mohou zahrnovat srdeční selhání, diabetes mellitus, hepatomegalii, cirhózu jater, hypogonadismus, impotenci, pigmentaci kůže a hepatocelulární karcinom (Cassinerio et al. 2012; Flaten et al. 2012; Murphy a Oudit 2010; Siah et al. 2006). Některá onemocnění jsou v lidské populaci celkem běžná, některá jsou však vzácnější.

Mezi známá genetická onemocnění spojená s nadbytkem železa patří obzvláště hereditární/primární hemochromatóza. Toto autosomálně recesivní onemocnění je způsobeno mutací HFE genu, který je lokalizován na 6. chromosomu, což má za následek zvýšené vstřebávání železa buňkami střevní sliznice (Crownover a Covey 2013). Základem léčby tohoto onemocnění je flebotomie. Nejdříve probíhají pravidelné týdenní odběry krve (450-500 ml) do dosažení požadované koncentrace sérového ferritinu (≈ 50 μg/l), následně stačí jeden odběr za 3 měsíce. V případě nutnosti lze aplikovat kombinovanou terapii s deferoxaminem (Crownover a Covey 2013; Flaten et al. 2012).

Velice významnou příčinou morbidity a mortality je přetížení organismu železem vznikající na podkladě pravidelných krevních transfuzí při terapii talasémií (Kontoghiorghes et al. 2000). Talasémie je dědičné krevní onemocnění, při němž je narušena tvorba proteinové složky hemoglobinu. α-talasemie, která je neslučitelná se životem, je dána poruchou tvorby α-řetězců a β-talasemie je dána poruchou syntézy β-řetězců. Forma minor je heterozygotní s jedním genem normálním a obvykle nevyžaduje žádnou terapii. Forma major je homozygotní nebo heterozygotní se dvěma různými mutacemi (Nienhuis a Nathan 2012). Léčba β-talasémie major je založena na

podání chelátorů – deferoxaminu, deferipronu anebo deferasiroxu (Flaten et al. 2012; Neufeld 2010).

3. 2. Akutní infarkt myokardu

Kardiovaskulární onemocnění řadíme mezi civilizační choroby. Obzvláště ischemická choroba srdeční je nejčastější příčinou morbidity a mortality v tzv. západní civilizaci a představuje rovněž závažný socio-ekonomický problém (Celermajer et al. 2012; Laslett et al. 2012; Negi a Anand 2010). Podle údajů WHO z roku 2008 byly kardiovaskulární onemocnění celosvětově příčinou ≈ 30 % úmrtí, resp. ≈ 13 % v případě ischemické choroby srdeční (WHO 2011a; WHO 2011b).

Nejzávažnější formou ischemické choroby srdeční je akutní infarkt myokardu (AIM). Podle patologické definice je pro AIM charakteristická akutní ložisková nekróza myokardiálních buněk v důsledku prolongované ischémie myokardu (Hlinomaz et al. 2011). Na základě klinické definice je AIM charakterizován jako typický vzestup s následným poklesem srdečních biomarkerů nekrózy s alespoň jednou hodnotou nad 99. percentilem horního limitu normy a současně musí být splněno alespoň jedno z následujících kritérií: a) klinické ischemické symptomy; b) známky AIM na EKG (elevace nebo deprese ST-úseků, změny T-vln, nová blokáda levého Tawarova raménka anebo vývoj patologického Q-kmitu); c) nově vzniklá regionální porucha kinetiky (dysfunkce, resp. hybnost, lévé komory – hypokineze, akineze nebo dyskineze), průkaz nové ztráty viabilního myokardu při zobrazovacích metodách (např. selektivní koronarografií) (Hlinomaz et al. 2011; Widimský a Špaček 2004).

Rizikové faktory AIM lze obecně rozdělit na neovlivnitelné (genetická predispozice, pohlaví, věk) a ovlivnitelné (porucha metabolismu lipidů, arteriální hypertenze, diabetes mellitus, abdominální typ obezity, kouření, nedostatečná pohybová aktivita a konzumace ovoce a zeleniny a rovněž psychosociální faktory). Na vzniku AIM se mohou rovněž podílet i další méně specifické vnější faktory (Dahlöf 2010; Kloner 2006; Nawrot et al. 2011; Yusuf et al. 2004)

3. 2. 1. Klinické projevy a diagnostika akutního infarktu myokardu

Základním subjektivním klinickým symptomem akutních koronárních syndromů včetně AIM je bolest na hrudi, která je způsobena podrážděním nervových zakončení v ischemických (nikoliv nekrotických) oblastech myokardu. Pro tuto anginózní bolest je charakteristický vznik v klidu nebo při malé námaze, větší intenzita, delší trvání a nedostatečná reakce na nitroglycerin. Nemocní často popisují kruté, svíravé bolesti za hrudní kostí (stenokardie) trvající 20 minut až 12 hodin, které vyzařují do horních končetin, krku, čelisti, zad nebo epigastria. Jindy popisují pouze neurčitý tlak na přední straně hrudníku. Stenokardie bývají někdy doprovázené úzkostí, pocením, dušností nauzeou, zvracením, slabostí nebo palpitacemi. AIM může proběhnout i asymptomaticky nebo s minimálními příznaky (10-30 % infarktů) (Hlinomaz et al. 2011; Widimský a Špaček 2004).

Jak bylo zmíněno v definici AIM, mají laboratorní nálezy v diagnostice zcela nezastupitelnou roli. Nejdůležitějšími ukazateli poškození myokardu jsou srdeční troponiny – srdeční troponin T (cTnT) nebo srdeční troponin I (cTnI). Myokardiální izoforma kreatinkinázy (CK-MB) a myoglobin mají dnes spíše pomocný význam (Brancaccio et al. 2010; Hlinomaz et al. 2011; Widimský a Špaček 2004). Srdeční troponiny jsou pro poškození myokardu vysoce specifické a za fyziologických podmínek jsou téměř pod limitem detekce (Adamcová et al. 2007; Daubert a Jeremias 2010; Hochholzer et al. 2010).

Zásadním a naprosto nepostradatelným vyšetřením je elektrokardiografie. Pro rozpoznání akutní ischémie je rozhodující zhodnocení úseků ST. Přetrvání elevací úseků ST déle než 20 min je typické pro AIM s elevacemi úseků ST (tzv. STEMI: ST-Elevation Myocardial Infarction), který vyžaduje rychlou reperfuzní léčbu. Na druhé straně nepřítomnost elevací nebo depresí úseků ST (tzv. NSTEMI: Non-ST-Elevation Myocardial Infarction) na EKG diagnózu AIM nevylučuje (Hlinomaz et al. 2011; Widimský a Špaček 2004).

Nepostradatelnými metodami používanými k diagnostice AIM a jeho komplikací jsou rovněž zobrazovací metody - echokardiografie nebo selektivní koronarografie (Hlinomaz et al. 2011; Widimský a Špaček 2004).

3. 2. 2. Terapie akutního infarktu myokardu

V léčebné péči o nemocné lze rozlišit přednemocniční, nemocniční a následnou ambulantní fázi (Hlinomaz et al. 2011). Základem péče o nemocného je velmi rychlá reakce na rozvíjející se akutní koronární syndrom. V případě náhle vzniklých anginózních bolestí neustupujících do 5 min po sublinguálním podání nitroglycerinu by mělo následovat podání kyseliny acetylsalicylové (400-500 mg) a okamžité zavolání zdravotnické záchranné služby. Lékař prvního kontaktu při podezření na AIM ihned podá kyselinu acetylsalicylovou (160-325 mg p.o; i.v.), vhodné je i rutinní podáni klopidogrelu (300-600 mg p.o.). Důležité je odstranění nebo alespoň zmírnění anginózních bolestí pomocí nitroglycerinu (p.o.; i.v. při hypertenzi a srdečním selhávání) a pomocí opioidních analgetik (morfin 2-5 mg i.v.; fentanyl 1-2 mg i.v.). U nemocných s tepovou frekvencí > 60 tepů/min, kteří nemají projevy srdečního selhání a nemají kontraindikace, je vhodné podání betablokátorů (metoprolol 5-15 mg i.v.). Při bradykardii (< 45 tepů/min) je podáván atropin (0,5-3,0 mg i.v.). V průběhu transportu do nemocnice pacienti rovněž inhalují kyslík. Akutní srdeční selhání je v přednemocniční fázi léčeno diuretiky (furosemid i.v.). Při hypertenzi jsou dále podávány betablokátory, nitráty (i.v.) a kaptopril (25 mg p.o.), při hypotenzi vazokonstriktory (noradrenalin, dopamin, dobutamin, i.v.) a v indikovaném případě je nutný dostatečný i.v. přísun tekutin. V případě nutnosti je zapotřebí zahájit kardiopulmonální resuscitaci (Hlinomaz et al. 2011; Widimský a Špaček 2004).

Na základě anamnézy a zjištěných elevací úseků ST alespoň ve dvou svodech změřených na 12-ti svodovém EKG je nejracionálnější terapií tohoto AIM reperfuzní léčba – primární perkutánní koronární intervence (PCI). V tomto případě kromě podání kyseliny acetylsalicylové a klopidogrelu musí ještě předcházet podání heparinu (70-100 U/kg i.v.). Vhodné je i podání inhibitorů GP IIb/IIIa destičkových receptorů (abciximab i.v., eptifibatid i.v.). V tomto případě se podává méně heparinu (max. 70U/kg i.v.). U velmi malé skupiny nemocných je indikován akutní aortokoronární bypass (CABG, z angl. coronary artery bypass graft) (Hlinomaz et al. 2011; Widimský a Špaček 2004).

Trombolytická léčba je vzhledem ke své dostupnosti a ceně stále ve světě nejčastější používanou reperfuzní strategií. V České republice se vzhledem k všeobecné dostupnosti PCI používá pouze minimálně v případě nedostupnosti léčby první volby a

absencí kontraindikací. Dříve nejvíce rozšířena streptokináza byla nahrazena rekombinovanými tkáňovými aktivátory plazminogenu – alteplázou a tenekteplázou (Hlinomaz et al. 2011).

V prvních 3 hodinách jsou PCI a trombolýza z hlediska mortality podobně účinné, mezi 3. a 12. hodinou AIM s elevacemi úseků ST je ale už PCI jasně výhodnější (Hlinomaz et al. 2011).

V případě AIM bez elevací úseků ST (NSTEMI) je ≈ 60 % nemocných ošetřeno PCI, ≈ 20 % CABG a zbytek je léčen medikamentózně (Hlinomaz et al. 2011).

3. 2. 3. Ischemicko-reperfuzní poškození

Nezbytné obnovení krevního zásobení po déle trvající ischemii myokardu provedené výše zmíněnými postupy je ale často spojeno s dalším poškozením kardiomyocytů, tzv. ischemicko-reperfuzním poškozením (Hausenloy a Yellon 2013). Podstatou tohoto poškození je již samotné okysličení dříve ischemické tkáně s následným nárůstem v produkci volných radikálů (Ambrosio et al. 1991; Ambrosio et al. 1987).

Již během časné ischémie se do krevního řečiště uvolňují katalyticky aktivní elementy, především železo a měď, které se mohou po obnovení krevního průtoku účastnit produkce biologicky nejnebezpečnějšího oxidantu – hydroxylového radikálu železem/mědí katalyzovanou Fentonovou reakcí (reakce 1) (Berenshtein et al. 2002; Halliwell a Gutteridge 1992; Chevion et al. 1993).

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

Reakce 1. Železem/mědí katalyzovaná Fentonova reakce

3. 2. 4. Model akutního infarktu myokardu

V experimentálním výzkumu AIM a v následném *in vivo* screeningu nových terapeutických možností jeho léčby se již od roku 1959 používá jako induktor modelového poškození srdeční tkáně syntetický katecholamin isoprenalin (Chappel et al. 1959; Rona et al. 1959a; Rona et al. 1959b). Časné patologické změny následující po aplikaci kardiotoxické dávky isoprenalinu lze v mnohých aspektech považovat za velmi podobné časným změnám, ke kterým dochází u AIM u lidí (mj. uvolnění endogenních katecholaminů, elevace ST-segmentu na EKG, uvolnění srdečního troponinu T, nekróza srdeční tkáně a přetížení vápníkem) (Díaz-Muñoz et al. 2006; Filipský et al. 2012b; Chagoya de Sánchez et al. 1997; Kloner 2006; Rona 1985; Vliegenthart et al. 2002; York et al. 2007).

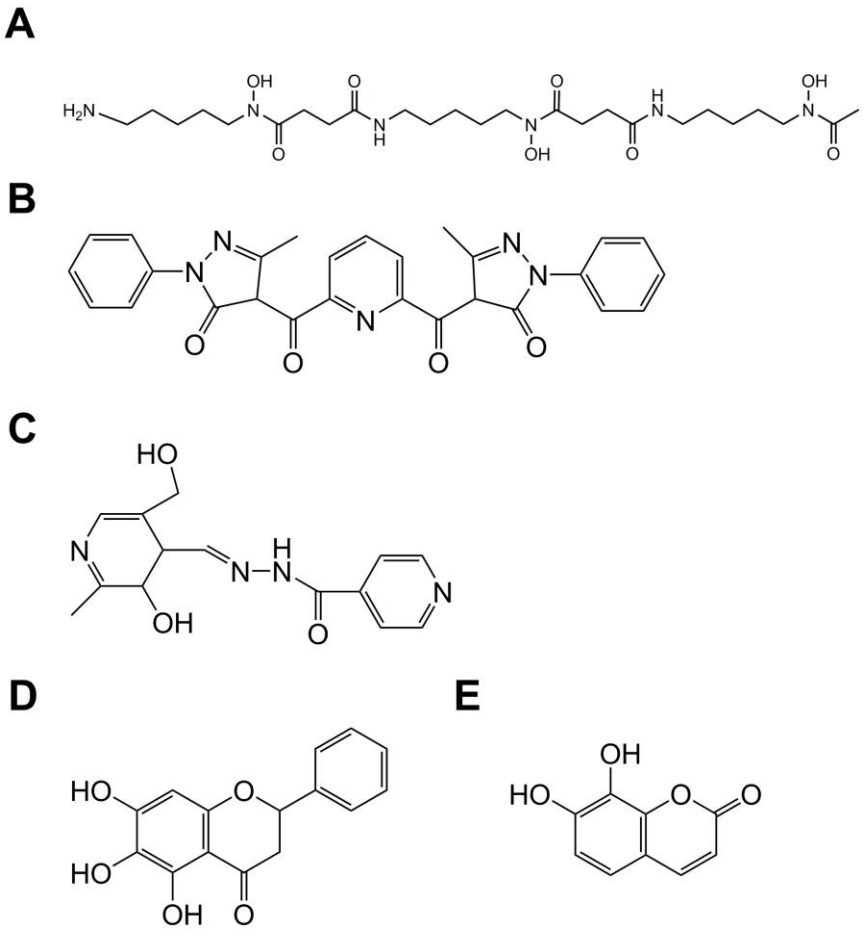
Přestože se problematikou tohoto modelu zabývalo velké množství studií, definitivní, pravděpodobně multifaktoriální, patofyziologický mechanismus nebyl dosud spolehlivě vysvětlen (Filipský et al. 2012b; Mladěnka et al. 2009a; Rona 1985; Zhang et al. 2008).

K hlavním patofyziologickým mechanismům isoprenalinového modelu patří nadměrná stimulace β-adrenergních receptorů a následná dysbalance v energetickém metabolismu myokardu. K poškození srdeční tkáně přispívá tvorba volných radikálů a oxidačních produktů isoprenalinu, agregace krevních destiček s tvorbou mikrotrombů a změny v permeabilitě buněčné membrány (Bindoli et al. 1992; Díaz-Muñoz et al. 2006; Kloner 2006; Mladěnka et al. 2009a; Remião et al. 2001; Schömig 1990; Todd et al. 1980; Zhang et al. 2008). Rovněž dochází ke zvýšení koncentrace vápníku v srdeční tkáni a s tím související kontraktilní dysfunkci (Gross et al. 1999; Chatelain a Kapanci 1984; Korff et al. 2006)

Kromě isoprenalinového modelu AIM existují i další modely, které simulují AIM, resp. ischemicko-reperfuzní poškození, např. často používaný standardní nefarmakologický model AIM ligatury koronárních arterií (Bloom a Davis 1972; Hasenfuss 1998; Zhang et al. 2011)

3. 3. Chelátory železa

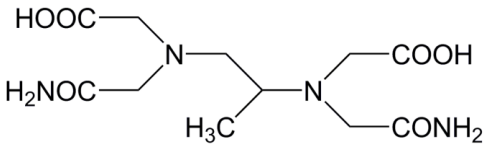
Chelátory železa představují rozsáhlou skupinu přírodních a syntetických látek s variabilní chemickou strukturou, jejichž společnou vlastností je schopnost vázat atomy železa (obr. 1) (Filipský et al. 2012a; Kalinowski a Richardson 2005; Mladěnka et al. 2011; Mladěnka et al. 2010a; Pierre et al. 2003; Sharpe et al. 2011; Zhou et al. 2012).



Obr. 1. Příklady chemických struktur chelátorů železa:
(**A**) deferoxamin, (**B**) aroylhydrazon PIH, (**C**) H_2QpyQ, (**D**) baikalein, (**E**) daphnetin.

V klinické praxi se však můžeme setkat pouze s několika zástupci této farmakoterapeutické skupiny – deferoxaminem, deferipronem, deferasiroxem a

dexrazoxanem, tzn. chelátory účinnými, relativně bezpečnými, cenově dostupnými a s výjimkou deferoxaminu i přijatelnou compliance pacientů (Bernhardt 2007; Cvetkovič a Scott 2005; Kwiatkowski 2011). Tato léčiva jsou indikována samostatně nebo v kombinacích ve velmi řídkých případech akutní intoxikace železem, ke které dochází především u dětí, nebo mnohem častěji u chronického přetížení organismu železem. Chronické přetížení organismu železem nastává kromě ojedinělých případů hereditární hemochromatózy zejména po podávání častých krevních transfuzí zejména u pacientů s talasémií (Flaten et al. 2012; Chang a Rangan 2011; Kwiatkowski 2011; Neufeld 2010). Dexrazoxan je používán u pacientů s nádorovými onemocnění jako prevence kardiotoxických účinků antracyklinových cytostatik (Cvetkovič a Scott 2005). Nicméně mechanismus účinku vysvětlovaný schopností jeho metabolického produktu ADR-925 (obr. 2) vázat železo a minimalizovat tvorbu hydroxylového radikálu je nyní důkladně přehodnocován (Šimůnek et al. 2009).

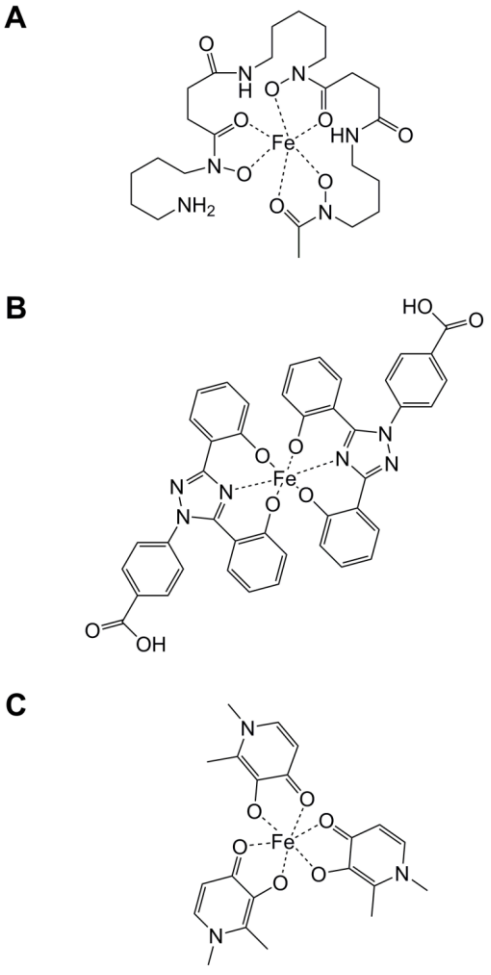


Obr. 2. ADR-925

Kromě farmakoterapie zmíněných onemocnění schválenými léčivy jsou látky chelatující železo experimentálně testovány i v celé řadě dalších indikací. V současné době je základní výzkum látek s chelatačním potenciálem zaměřen nejen na nádorová, ale i na neurodegenerativní onemocnění, např. Alzheimerovu chorobu, Parkinsonovu chorobu a Friedreichovu ataxii, a akutní infarkt myokardu, resp. ischemicko-reperfuzní poškození srdeční tkáně (Hašková et al. 2011a; Hider et al. 2011; Jomova et al. 2010; Kalinowski a Richardson 2005; Li et al. 2011; Mladěnka et al. 2009b; Zatloukalová et al. 2012). Některé chelátory železa prokázaly také antibiotický účinek (Tam et al. 2003).

3. 3. 1. Fyzikálně-chemické a farmakologické vlastnosti chelátorů železa

Koordinační číslo železa je 6, což odpovídá počtu koordinačně-kovalentních vazeb mezi centrálním atomem železa a jednovaznými ligandy s výsledným oktaedrickým uspořádáním koordinační sféry komplexu. V závislosti na počtu donorových atomů v molekule ligandu lze chelátory železa rozdělit na šestivazné (např. deferoxamin), třívazné (např. deferasirox) a dvojvazné (např. deferipron) (obr. 3) (Dubey et al. 2007; Tam et al. 2003; Zhou et al. 2012).



Obr. 3. Příklady komplexů chelátorů železa:
(**A**) deferoxamin (1:1), (**B**) deferasirox (2:1), (**C**) deferipron (3:1).

Z farmakoterapeutického hlediska jsou zásadními faktory, které mohou ovlivnit klinickou aplikaci samotných chelátorů, kromě lipofility, molekulové hmotnosti a potencionální toxicity, především selektivita chelátoru, struktura komplexu a jeho případná redoxní aktivita a v neposlední řadě také faktory vnějšího prostředí (např. pH) (Kalinowski a Richardson 2005; Zhou et al. 2012).

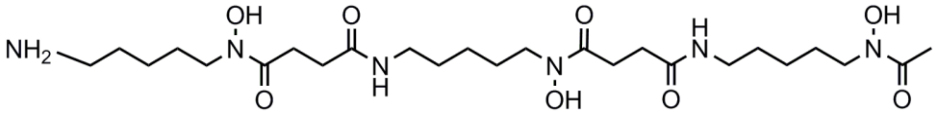
Ideální chelátor železa by měl být vysoce selektivní obzvláště pro Fe(III), aby byla minimalizována chelatace ostatních esenciálních elementů a nedošlo k nežádoucímu rozvoji jejich deficience (Zhou et al. 2012). Bohužel mnohé ligandy obsahující především karboxylovou skupinu a dusík vykazují i nezanedbatelnou afinitu především k dvojmocným biogenním prvkům, např. Zn(II) (Hershko 2005; Zhou et al. 2012). Výjimkou se zdá být za biologických podmínek měď, která je v organismu velice pevně vázána na proteiny (např. ceruloplasmin) (Rae et al. 1999).

Struktura komplexu by měla zabránit účasti vázaného železa na nežádoucí tvorbě volných radikálů, a proto by atom železa měl být chelatován tak, aby byl minimalizovat jeho přímý kontakt s kyslíkem nebo peroxidem vodíku. (Zhou et al. 2012). Kromě toho chelátory s nižší afinitou k železu/mědi, resp. komplexy s nižší stechiometrií, jsou náchylné k potenciaci produkce vysoce toxického hydroxylového radikálu železem/mědí katalyzovanou Fentonovou reakcí (Mira et al. 2002; Zhou et al. 2012). Klasickým příkladem takových látek jsou např. flavonoidy nebo kumariny (Macáková et al. 2012; Mira et al. 2002; Mladěnka et al. 2011; Mladěnka et al. 2010a). Kromě dříve uvedeného jevu, který stojí za nárůstem produkce hydroxylového radikálu, stojí za zmínku i související usnadnění nežádoucí redoxní reakce železa/mědi takovými chelátory, které mají schopnost vázat jak Fe(II), tak Fe(III), resp. Cu(I) a Cu(II) (Mira et al. 2002; Zhou et al. 2012). Na druhou stranu v některých indikacích je vyšší toxicita komplexu žádoucí, např. při terapii nádorových onemocnění (Kalinowski a Richardson 2005).

Důležitým faktorem, který rovněž ovlivňuje samotný účinek chelátorů železa je již zmíněné pH (Filipský et al. 2012a; Mira et al. 2002; Mladěnka et al. 2011; Mladěnka et al. 2010a). Jelikož v průběhu rozvoje různých onemocnění dochází i k zásadnímu narušení fyziologických hodnot pH, je tedy nadmíru žádoucí sledovat chelatační účinky i za různých patofyziologických pH podmínek (Ambrosio et al. 1987; Mladěnka et al. 2005; Parolini et al. 2009).

3. 3. 2. Deferoxamin

Deferoxamin (DFO; desferrioxamin B, derivát kyseliny hydroxámové, obr. 4) je šestivazebný chelátor železa, který byl identifikován a izolován z houby *Streptomyces pilosus* v 60. letech 20. století (Bickel et al. 1960). Jeho přirozenou funkcí je působit jako siderofor, tj. látka, která umožňuje mikroorganismům získávat železo z vnějšího prostředí (Sharpe et al. 2011). Úplná syntéza DFO byla popsána v roce 1988 (Bergeron a Pegram 1988).



Obr. 4. Deferoxamin.

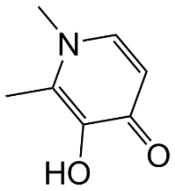
Již v roce 1968 byl DFO registrován jako první léčivá látka určená k léčbě akutních i chronických onemocnění spojených s přetížením organismu železem (Desferal® – deferoxamin mesylát, Ciba-Geigy – Novartis) (Bernhardt 2007; Sharpe et al. 2011). Přestože jsou k terapeutickému použití schváleny další chelátory železa, deferoxamin i nadále zůstává zlatým standardem v klinické praxi (Sharpe et al. 2011).

DFO, který je poměrně velkou a vysoce hydrofilní molekulou, chelatuje především extracelulárně (Flaten et al. 2012). Nicméně facilitovaný transportní systém napomáhá jeho průniku i do hepatocytů, ve kterých váže hepatocelulární železo a brání jeho potenciální redistribuci a rozvoji komplikací (Hershko 2005; Kwiatkowski 2011; Zhou et al. 2012). Jelikož se po perorálním podání DFO z gastrointestinálního traktu téměř nevstřebává a po i.v. podání má vysokou clearance, musí být tedy DFO aplikován kontinuální subkutánní nebo intravenózní infuzí. K dosažení terapeutických koncentrací je podáván 8-12 hodin 5-7 dní v týdnu v dávce 25-60 mg/kg, což je spojené s nižší compliance pacientů (Flaten et al. 2012; Kwiatkowski 2011; Zhou et al. 2012). Jedna molekula DFO váže s vysokou afinitou a selektivitou jeden atom železa, především Fe (III) (Hershko 2005; Kalinowski a Richardson 2005). Vzniklý komplex je kineticky

stabilní s relativně nízkou lipofilitou (Zhou et al. 2012). Exkrece probíhá nejen močí, ale i žlučí (eliminační poločas 5-10 min) (Hershko 2005; Zhou et al. 2012). Možnými nežádoucími účinky, obzvláště u dětí, jsou poruchy sluchu, poruchy zraku, lokální a alergické reakce, infekce, syndrom dechové tísně, neurologické poruchy a průjem (Flaten et al. 2012; Kwiatkowski 2011). V případě rychle infuze se může vyskytnout hypotenze (Flaten et al. 2012).

3. 3. 3. Deferipron

Deferipron (L1; 1,2-dimethyl-3-hydroxypyrid-4-on, obr. 5) je dvojvazný chelátor syntetizovaný již v roce 1981 (Kontoghiorghes et al. 1987; Piga et al. 2010). Deferipron je prvním perorálně účinným chelátorem železa, který byl schválen k léčbě chronického přetížení organismu železem způsobeným krevními transfuzemi (Ferriprox® – deferipron, ApoPharma – Apotex, registrováno EMA v roce 1999, resp. FDA v roce 2011) (Flaten et al. 2012; Piga et al. 2010). Deferipron vytváří s železem komplexy v molárním poměru 3:1 a pravděpodobně působí, na rozdíl od DFO, především intracelulárně (Flaten et al. 2012).



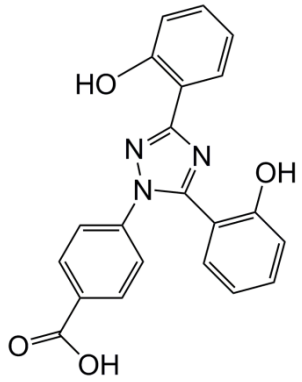
Obr. 5. Deferipron.

Po perorálním podání je deferipron velmi rychle absorbován v gastrointestinálním traktu (Flaten et al. 2012). V játrech probíhá jeho metabolizace glukoronidací a následně je samotné léčivo, resp. komplex deferipronu a železa, eliminován téměř kompletně ledvinami (eliminační poločas – 47-134 min) (Flaten et al. 2012; Piga et al. 2010). Jelikož deferipron má malou lipofilní molekulou, snadno

proniká do buněk, obzvláště kardiomyocytů, ve kterých chelatuje železo a brání rozvoji závažných srdečních komplikací (Kwiatkowski 2011; Piga et al. 2010). Deferipron je běžně podáván ve formě tablet nebo roztoku určeného k perorálnímu podání ve třech denních dávkách (75-100 mg/kg/d) (Kwiatkowski 2011). Nežádoucí účinky zahrnují gastrointestinální diskomfort, zvýšení hladiny jaterních enzymů, únavu, bolest hlavy, bolesti kloubů a načervenalé/hnědé zabarvení moči; vzácná je neutropenie/ agranulocytóza (Kwiatkowski 2011; Piga et al. 2010).

3. 3. 3. Deferasirox

Deferasirox (ICL670, 4-[3,5-*bis*(2-hydroxyfenyl)-1,2,4-triazol-1-yl]-benzoová kyselina, obr. 6) je vysoce selektivní třívazný chelátor železa, který byl získán na základě racionální syntézy a screeningu více než 700 látek (Hershko 2005; Stumpf 2007). Deferasirox je perorálně účinný chelátor, jehož indikací je terapie chronického přetížení organismu železem způsobeného krevními transfuzemi s cílem předcházet rozvoji závažných komplikací (Exjade®, Novartis, registrováno FDA v roce 2005, resp. EMA v roce 2012) (Kwiatkowski 2011; Pathare et al. 2009; Stumpf 2007). Deferasirox vytváří komplexy v chelatačním poměru 2:1 (Dubey et al. 2007; Stumpf 2007).

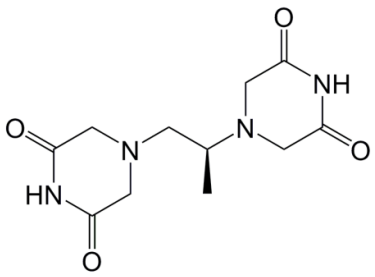


Obr. 6. Deferasirox.

Deferasirox je dostupný ve formě tablet určených k přípravě perorální suspenze (Kwiatkowski 2011). Biologická dostupnost je přibližně 70 % a na jejím zvýšení se podílí strava (Stumpf 2007). Vazba na plazmatické bílkoviny je vysoká (99 %, primárně albumin) (Stumpf 2007). Deferasirox, resp. komplex, je metabolizován primárně glukuronidizací a poté je eliminován především žlučí (Stumpf 2007). Eliminační poločas (11-19 hod) umožnuje jeho podání v jedné denní dávce (20-40 mg/kg) (Hershko 2005; Kwiatkowski 2011). Nežádoucími účinky jsou především zvýšená hladina kreatininu, vyrážka, bolest hlavy, gastrointestinální diskomfort, zvýšené hladiny jaterních enzymů a jaterní a renální insuficience (Kwiatkowski 2011; Stumpf 2007).

3. 3. 4. Dexrazoxan

Dexrazoxan (ICRF-187, 4-[(2*S*)-2-(3,5-dioxopiperazin-1-yl)propyl]piperazin-2,6-dion, cyklický derivát EDTA, obr. 7) je jedinou schválenou látkou s kardioprotektivními účinky vůči nežádoucí kardiotoxicitě antracyklinových antibiotik, které jsou používané v terapii mnoha nádorových onemocnění (Cardioxane®, Novartis; Zinecard®, Pfizer) (Cvetkovič a Scott 2005).



Obr. 7. Dexrazoxan.

Na základě klinických studií je přistupováno k intravenóznímu podání dexrazoxanu za účelem prevence pozdních kardiovaskulárních komplikací, především srdečního selhání, v případě, kdy kumulativní dávka antracyklinového antibiotika

překročí definovanou hranici (u doxorubicinu >300 mg/m^2, u epirubicinu >540 mg/m^2. Dávkový poměr dexrazoxanu a doxorubicinu/epirubicinu je 10:1 (Cvetkovič a Scott 2005; Hasinoff a Herman 2007; Testore et al 2008).

Mechanismus účinku dexrazoxanu na základě všeobecně akceptované hypotézy primárně spočívá ve schopnosti jeho otevřeného analogu, resp. hydrolytického produktu, ADR-925 (obr. 2) chelatovat železo (Cvetkovič a Scott 2005). Nicméně tento mechanismus účinku založený na chelataci a tedy inhibici tvorby volných radikálu, obzvláště hydroxylového radikálu, je důkladně přehodnocován. Na mechanismu účinků dexrazoxanu se rovněž podílí inhibice topoizomerázy II_β (Jordan et al 2009; Hasinoff a Herman 2007; Šimůnek et al. 2009).

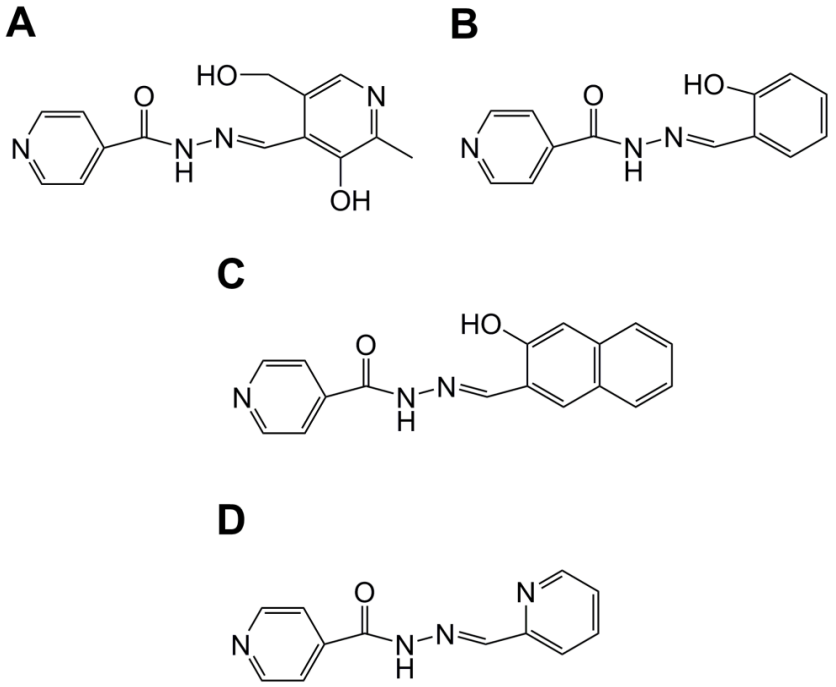
Po intravenózním podání je velmi rychle distribuován do tkání. Vazba na plazmatické bílkoviny je velmi nízká (< 2 %). Na ADR-925 je enzymaticky hydrolyzován nejen ve tkáních, tj. játrech, ledvinách a srdci, ale i v erytrocytech a leukocytech. Eliminace probíhá především močí v nezměněné formě (eliminační poločas 2-2,7 hod). Nejčastějšími nežádoucí účinky jsou gastrointestinální diskomfort (např. nauzea, zvracení, stomatitidy) a hematologické reakce zahrnující anémii a leukopenii (Cvetkovič a Scott 2005).

3. 3. 5. Aroylhydrazony

Experimentálně hojně testovanými látkami jsou i nejrůznější analogy třívazných chelátorů ze skupiny aroylhydrazonů (obr. 9) (Kalinowski a Richardson 2005; Whitnall a Richardson 2006). Na vzniku komplexních vazeb se v těchto molekulách podílí karbonylový a fenolový kyslík a dusík iminové skupiny. Díky tomu se tvoří komplexy se stechiometrií chelátor:železo 2:1 (Kalinowski a Richardson 2005).

Tyto chelátory mají vysokou schopnost chelatovat železo s možnou klinickou indikací nejen v terapii přetížení organismu železem, ale i v léčbě neurodegenerativních onemocnění (Kalinowski a Richardson 2005; Whitnall a Richardson 2006). Látky této skupiny mají rovněž nezanedbatelné antiproliferační účinky potencionálně využitelné v terapii nádorových onemocnění a protektivní účinky vůči katecholaminové

kardiotoxicitě (Hašková et al. 2011b; Kalinowski a Richardson 2005; Macková et al. 2012; Mladěnka et al. 2009b).

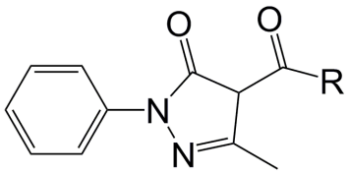


Obr. 9. Zástupci aroylhydrazonů.
(**A**) PIH (pyridoxal isonikotinoyl hydrazon),
(**B**) SIH (salicylaldehyd isonikotinoyl hydrazon),
(**C**) 311
(**D**) PCIH (2-pyridylkarboxaldehyd isonikotinoyl hydrazon).

3. 3. 6. Acylpyrazolony

Acylpyrazolony (deriváty 1-fenyl-3-methyl-4-acylpyrazol-5-onu; obr. 8) patří mezi syntetické chelátory, které obsahující z pohledu koordinační chemie esenciální β-diketo skupinu (Marchetti et al. 2005; Pettinari et al. 2002). Přestože je jejich schopnost chelatace známa již několik desetiletí, aplikace těchto látek zůstává limitována především na analytickou a technologickou oblast (Marchetti et al. 2005). Jelikož jejich zajímavou vlastností je schopnost chelatace železa i při nízkém pH, které lze

zaznamenat při nádorových onemocněních nebo AIM, mohou i tyto látky být předmětem rozsáhlejšího preklinického výzkumu (Ambrosio et al. 1987; Filipský et al. 2012a; Parolini et al. 2009).

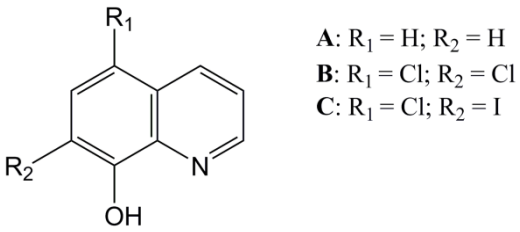


Obr. 8. Základní chemická struktura 1-fenyl-3-methyl-4-acylpyrazol-5-onů.

3. 3. 7. Hydroxychinoliny

Dvojvazné chelátory obsahující strukturu 8-hydroxychinolinu (obr. 10) mají silnou schopnost vázat železo v chelatačním poměru 3:1 (Pierre et al. 2003). Nicméně jejich velkou nevýhodou je nízká selektivita (Pierre et al. 2003).

Tyto syntetické lipofilní látky mají kromě antibiotických účinků (v klinické praxi kloroxin) značný potenciál i v léčbě nejrůznějších chorob spojených s narušením homeostázy železa, např. neurodegenerativních a nádorových onemocnění (Budimir et al. 2011; Oliveri et al. 2012; Whitnall a Richardson 2006).



Obr. 10. Zástupci hydroxychinolinů.
(A) 8-hydroxychinolin, (**B**) kloroxin, (**C**) kliochinol.

3. 3. 8. Flavonoidy

Flavonoidy jsou hojně se vyskytující polyfenolické rostlinné sekundární metabolity, jejichž přítomnost v potravinách je všeobecné považována za prospěšnou pro lidské zdraví (Bravo 1998; Bubols et al. 2013; Landete 2012). Kromě širokého spektra pozitivních vlastností (protizánětlivé, antidiabetické, hepato/gastro-protektivní, antibiotické účinky, atd.) stojí za zmínku především jejich účinky na kardiovaskulární systém, které jsou pravděpodobně zprostředkovány vychytáváním volných radikálů, schopností chelatovat železo, interakcemi s enzymy (např. inhibice xantinoxidázy) a případně přímým vasodilatačním účinkem (Arora et al. 1998; Bravo 1998; Mladěnka et al. 2010b; van Acker et al. 1998). Nicméně byly popsány rovněž i jejich negativní účinky, např. pro-oxidační vlastnosti (Macáková et al. 2012; Mladěnka et al. 2009c).

3. 4. Reference

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4. RECENZOVANÉ ODBORNÉ ČLÁNKY PUBLIKOVANÉ V ČASOPISECH S IMPAKT FAKTOREM A SOUVISEJÍCÍ S TÉMATEM DISERTAČNÍ PRÁCE A JEJICH KOMENTÁŘ

4. 1. *In vitro* analysis of iron chelating activity of flavonoids

4. 2. Iron reduction potentiates hydroxyl radical formation only in flavonols

4. 3. *In vitro* characteristics of 1-phenyl-3-methyl-4-acylpyrazol-5-ones iron chelators

4. 4. Mathematical calculations of iron complex stoichiometry by direct UV–Vis spectrophotometry

4. 5. Acute initial haemodynamic changes in a rat isoprenaline model of cardiotoxicity

4. 6. Common biomarkers of oxidative stress do not reflect cardiovascular dys/function in rats

4. 7. Dexrazoxane provided moderate protection in a catecholamine model of severe cardiotoxicity

4. 1. In vitro analysis of iron chelating activity of flavonoids

MLADĚNKA, Přemysl, MACÁKOVÁ, Kateřina, FILIPSKÝ, Tomáš, ZATLOUKALOVÁ, Libuše, JAHODÁŘ, Luděk, BOVICELLI, Paolo, SILVESTRI, Ilaria P., HRDINA, Radomír, SASO, Luciano. In vitro analysis of iron chelating activity of flavonoids. *Journal of Inorganic Biochemistry*. 2011, **105**(5), 693-701. ISSN 0162-0134.

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Flavonoidy jsou hojně se vyskytující rostlinné sekundární metabolity, u kterých bylo mimo jiné prokázáno, že mohou příznivě ovlivnit lidské zdraví. Některé z jejich pozitivních účinků jsou alespoň částečně spojeny se schopností chelatovat železo.

V této *in vitro* studii, jsme se zaměřili na zhodnocení železo-chelatačních vlastností a stability vytvořených komplexů u 26 zástupců flavonoidů při 4 patofyziologicky relevantních pH (4,5; 5,5; 6,8; 7,5). Jejich komplexotvorné vlastnosti byly porovnány se standardním chelátorem železa – deferoxaminem. Ke stanovení chelatace byla použita naší pracovní skupinou vyvinutá spektrofotometrická ferrozinová metoda.

In vitro experimenty navázaly na dřívější studie a potvrdily, že pro vytvoření komplexu s železem existuje několik potenciálních komplexotvorných míst. Nejvýhodnější pozicí se ukázala v literatuře relativně málo zmiňovaná 6,7-dihydroxylová skupina. Baikalein, obsahující tuto strukturu, měl srovnatelnou schopnost chelatovat železo jako deferoxamin při všech testovaných pH. Kromě toho stechiometrie vytvořených komplexu baikaleinu s železem byla totožná se stechiometrií platnou pro komplex železa s deferoxaminem, tj. 1:1. 3-hydroxy-4-keto substituce s dvojnou vazbou v poloze 2 a pyrokatecholový kruh B byly rovněž spojeny s významnou chelatační schopností. Nicméně pyrokatecholový kruh B chelatoval železo výrazně méně při kyselém pH. Kvercetin a myricetin, u kterých lze nalézt všechny výše zmíněné 3 strukturní znaky, chelatovaly železo podobně jako baikalein, resp. deferoxamin, při neutrálním pH, ale byly jednoznačně méně účinné při nižším pH. Na druhou stranu z literatury známá 5-hydroxy-4-keto substituce představuje jen slabé a nestabilní

chelatační místo. Isolované hydroxyl, methoxyl, keto nebo *ortho* methoxy-hydroxy substituce neměly na schopnost chelatovat železo významný vliv.

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In vitro analysis of iron chelating activity of flavonoids

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ABSTRACT

Flavonoids have been demonstrated to possess miscellaneous health benefits which are, at least partly, associated with iron chelation. In this in vitro study, 26 flavonoids from different subclasses were analyzed for their iron chelating activity and stability of the formed complexes in four patho/physiologically relevant pH conditions (4.5, 5.5, 6.8, and 7.5) and compared with clinically used iron chelator deferoxamine. The study demonstrated that the most effective iron binding site of flavonoids represents 6,7-dihydroxy structure. This site is incorporated in baicalein structure which formed, similarly to deferoxamine, the complexes with iron in the stoichiometry 1:1 and was not inferior in all tested pH to deferoxamine. The 3-hydroxy-4-keto conformation together with 2,3-double bond and the catecholic B ring were associated with a substantial iron chelation although the latter did not play an essential role at more acidic conditions. In agreement, quercetin and myricetin possessing all three structural requirements were similarly active to baicalein or deferoxamine at the neutral conditions, but were clearly less active in lower pH. The 5-hydroxy-4-keto site was less efficient and the complexes of iron in this site were not stable at the acidic conditions. Isolated keto, hydroxyl, methoxyl groups or an *ortho* methoxy–hydroxy groups were not associated with iron chelation at all.

1. Introduction

The direct scavenging potential of flavonoids has been traditionally attributed to their positive effects on human health. The current research has been gradually changing this dogma by emphasizing their transition metals chelation properties and direct interaction with some enzymes and blood/vascular cells, which may have important roles in their influence on human being [1,2]. The metal chelation can be responsible, at least partly, both for the documented antioxidant capacity of flavonoids and for some other activities (e.g., inhibition of lipooxygenases by chelation/reduction of iron in their active site) [3]. The former includes inhibition of both iron based Fenton reaction with prevention of the production of the hydroxyl radical and lipid peroxidation initiated by trace levels of copper/iron although, particularly, the contribution of direct scavenging effect and metal chelation is very difficult to assess [4]. Notwithstanding such difficulties, iron chelation represents an important part of biological activity of flavonoids.

Although iron chelation activities of flavonoids (Fig. 1A) had been reported by many researchers, some biologically relevant information and comparison among flavonoids and to a clinically used iron chelator, e.g. deferoxamine (Fig. 1B), is missing [5–11]. The published studies uniformly reported the three most common iron binding sites of flavonoids: 1) catecholic ring B, 2) 3-hydroxy-4-keto and 3) 5-hydroxy-4-keto conformation [6,8]. Most published papers analyzed easily commercially available natural flavonoids and, therefore, less is known about the effects of other suggested potentially chelating agents with substitutions, which are less usual in natural flavonoids, e.g. an *ortho*-dihydroxy group in ring A was also shown to be a potent chelation site [12].

In addition, the influence of different pH conditions was mostly neglected in the previous studies, as most papers used neutral pH and some pH 5.5. In particular, different pH conditions may have pathophysiological importance: 1) pathological acidosis has been detected in inflammatory conditions including acute myocardial infarction and tumors [13,14], and 2) the physiological differences in pH in various body compartments (e.g. stomach and lysosomes) play also unambiguously roles. In this study, four different pHs starting from neutral conditions over a range of 3 pH units to acidic conditions were selected in the pathophysiological relation to the iron homeostasis. The most acidic pH used (4.5) is documented in lysosomes, which are organelles important in iron traffic [15,16].

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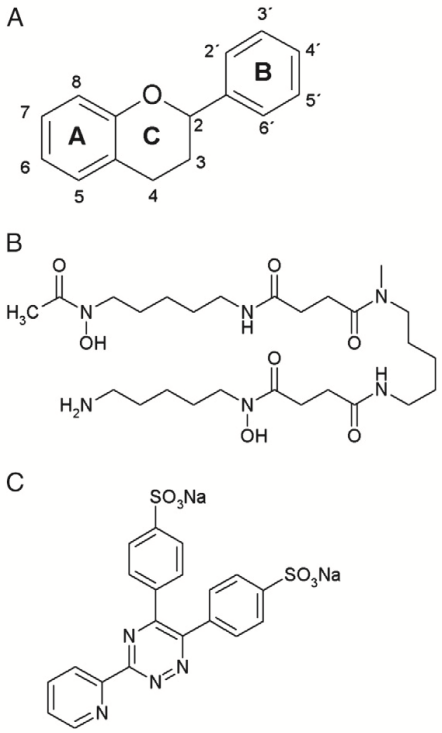


Fig. 1. Chemical structures. A: General structure of flavonoids, B: Deferoxamine, and C: Ferrozine.

Other two acidic pHs (5.5 and 6.8) were chosen to mimic severe or moderate ischaemia, where iron is known to participate in tissue damage [14].

This study was therefore aimed at the detailed analysis of iron chelation by flavonoids at different patho/physiologically relevant pH conditions and at comparison of their effects with a standard iron chelator deferoxamine. Moreover, contributions of the various chemical groups, both iron binding and non-iron binding elements, were assessed by comparing 26 flavonoids in all tested conditions.

2. Materials and methods

2.1. Reagents

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine, Fig. 1C), ferrous sulfate heptahydrate, ferric chloride hexahydrate, hydroxylamine, dimethyl sulfoxide (DMSO), sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), HEPES sodium salt and all flavonoids (Fig. 2), with exception of negletein and mosloflavone, were purchased from Sigma-Aldrich (Germany), deferoxamine from Novartis (Switzerland). Negletein and mosloflavone were synthesized through a convergent synthesis starting from chrysin as previously reported [17].

2.2. Iron chelation assessment

Ferrozine is a specific reagent which forms a magenta colored complex (absorption maximum at 562 nm) with ferrous ions [18]. The methodology was adjusted also for the measurement of total iron (ferrous + ferric) chelation in our previous study [19]. Shortly, various concentrations of flavonoid DMSO solutions were mixed for a period of 2 min with ferrous or ferric iron (final concentration 50μM) in 15 mM acetate (pH 4.5 and 5.5) or HEPES (pH 6.8 and 7.5) buffers. In case of ferrous ions, ferrozine (50μL, 5 mM aqueous solution) was added to the mixture immediately. For the assessment of total iron at pH 4.5, hydroxylamine aqueous solution (50μL, 10 mM) was added firstly in order to reduce remaining ferric ions into the ferrous ones, which were measured after the addition of ferrozine. Hydroxylamine was added to the mixtures for pH 7.5, as well by virtue of inhibition of ferrous oxidation at this pH.

All experiments were performed in 96-well microplates. Each concentration of the sample was measured at least twice with the addition of ferrozine and twice without ferrozine (blank). Absorbance was measured immediately after addition of ferrozine and 5 min later by the spectrophotometer Anthos reader 2010 (Anthos Labtec Instruments, Salzburg, Austria). If not indicated otherwise, results were calculated from measurements after 5 min. Concentration of ferrous ions corresponds linearly to the absorbance [18] and was checked in each experiment. For comparison of iron-chelating activity, deferoxamine was used as a standard iron chelator.

2.3. Statistical analysis

The amount of remaining iron was calculated from the difference of absorbance between the tested sample (with ferrozine) and its corresponding blank (without ferrozine) divided by the difference of the control sample (the known amount of iron without the tested substance) and its control blank.

The normalized dose-dependent curves with 95% confidence intervals were constructed by GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, California, U.S.A) for each condition (ferrous/total iron, pH). Each curve of an efficient substance was composed from at least five points (first point 0% chelation, last point 100% chelation). The efficiencies of iron chelation at the concentration ratio 10:1 and 1:1 (substance:iron) were calculated from the curve equation:

$$y = 100^{*}(1 + 10\ \exp((\log EC50 - x)^{*}k))$$

y the iron chelation efficiency in %;
x the common logarithm of concentration ratio substance: iron; and
k the slope of the curve.

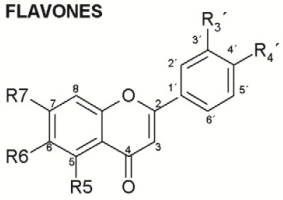
Ferrozine must be considered as an iron chelator, therefore some degree of competition between ferrozine and the tested substance should be awaited. Therefore, the measurement was performed at two time points, immediately after addition of ferrozine (0 min) and 5 min later. At time 0, ferrozine probably reacts with free non-chelated iron or with iron loosely bound to a tested substance. Within the 5 min interval, a competition for chelated iron occurs. Ferrozine can therefore remove iron from the complex formed with a tested substance if the complex is not stable. But it does not remove iron from the stable complexes with known powerful iron chelators, e.g. deferoxamine [19]. Therefore, the stability (s) can be calculated as follows:

$$s = y_5 / y_0,$$

where y_5 is the iron chelation efficiency at 5 min and y_0 at time 0.

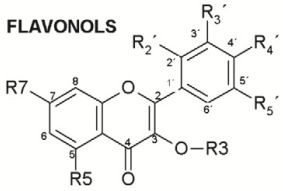
Data are expressed as means ± SD. Differences were considered significant at $p \leq 0.05$, unless stated otherwise. Differences in iron chelation at ratios 10:1 and 1:1 between a flavonoid and deferoxamine

FLAVONES



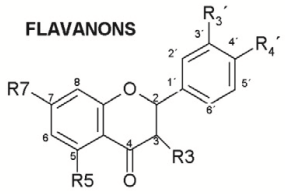
flavone	R_5	R_6	R_7	R_3'	R_4'
flavone	H	H	H	H	H
5-hydroxyflavone	OH	H	H	H	H
7-hydroxyflavone	H	H	OH	H	H
chrysin	OH	H	OH	H	H
apigenin	OH	H	OH	H	OH
luteolin	OH	H	OH	OH	OH
negletein	OH	OH	OCH_3	H	H
baicalein	OH	OH	OH	H	H
mosloflavone	OH	OCH_3	OCH_3	H	H
diosmin	OH	H	O-Glc-Rha	OH	OCH_3

FLAVONOLS



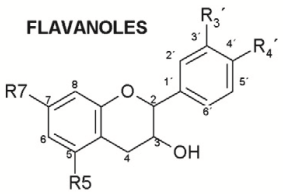
flavonol	R_3	R_5	R_7	R_2'	R_3'	R_4'	R_5'
3-hydroxyflavone	H	H	H	H	H	H	H
kaempferol	H	OH	OH	H	H	OH	H
quercetin	H	OH	OH	H	OH	OH	H
morin	H	OH	OH	OH	H	OH	H
myricetin	H	OH	OH	H	OH	OH	OH
rutin	-Glc-Rha	OH	OH	H	OH	OH	H
troxerutin	-Glc-Rha	OH	O-C_2H_4-OH	H	O-C_2H_4-OH	O-C_2H_4-OH	H

FLAVANONS



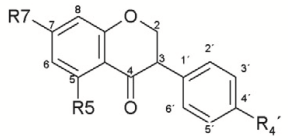
flavanon	R_3	R_5	R_7	R_3'	R_4'
naringenin	H	OH	OH	H	OH
naringin	H	OH	O-Glc-Rha	H	OH
hesperetin	H	OH	OH	OH	OCH_3
hesperidin	H	OH	O-Glc-Rha	OH	OCH_3
taxifolin	OH (conf. S)	OH	OH	OH	OH

FLAVANOLES



flavanol	R_5	R_7	R_3'	R_4'	conformation
epicatechin	OH	OH	OH	OH	2R, 3R
catechin	OH	OH	OH	OH	2R, 3S

ISOFLAVONOIDS



isoflavonoid	R_5	R_7	R_4'
daidzein	H	OH	OH
genistein	OH	OH	OH

Fig. 2. Chemical structures of all flavonoids tested in this study. Glc: glucose; and Rha: rhamnose.

and among flavonoids (functional group analysis) were carried out by comparing 95% confidence intervals. The differences in iron complexes stability were performed by one-way ANOVA test followed by Bonferroni's Multiple Comparison Test.

3. Results

All flavonoids, except flavone, 7-hydroxyflavone and daidzein, were able to chelate iron at different pH conditions in various degrees (Fig. 3).

At neutral or close to neutral conditions (pH 6.8 and 7.5), many of the tested flavonoids were potent iron chelators (Fig. 3A and B). Notwithstanding different iron chelating properties, all flavonoids formed stable complexes with iron in those conditions (Fig. 4A). Some flavones, flavonols and even flavanones, in particular those with the adjacent hydroxyl groups in ring A or ring B and/or free 3-hydroxyl group, were able to reach the efficacy of standard iron chelator deferoxamine for chelation of ferrous iron. The tested flavanones without catecholic B ring were clearly the less efficient group of flavonoids with only moderate effects at pH 7.5.

In more acidic pH, ferrous chelation was less expressed with few exceptions in flavonoids as compared to deferoxamine. The tested flavanones were almost without any effect. At pH 5.5, only baicalein with free 5,6,7- hydroxyl groups and flavonols with 3-hydroxy-4-keto-5-hydroxy conformation and with at least two free hydroxyl groups in ring B in an *ortho* position reached the efficacy of deferoxamine

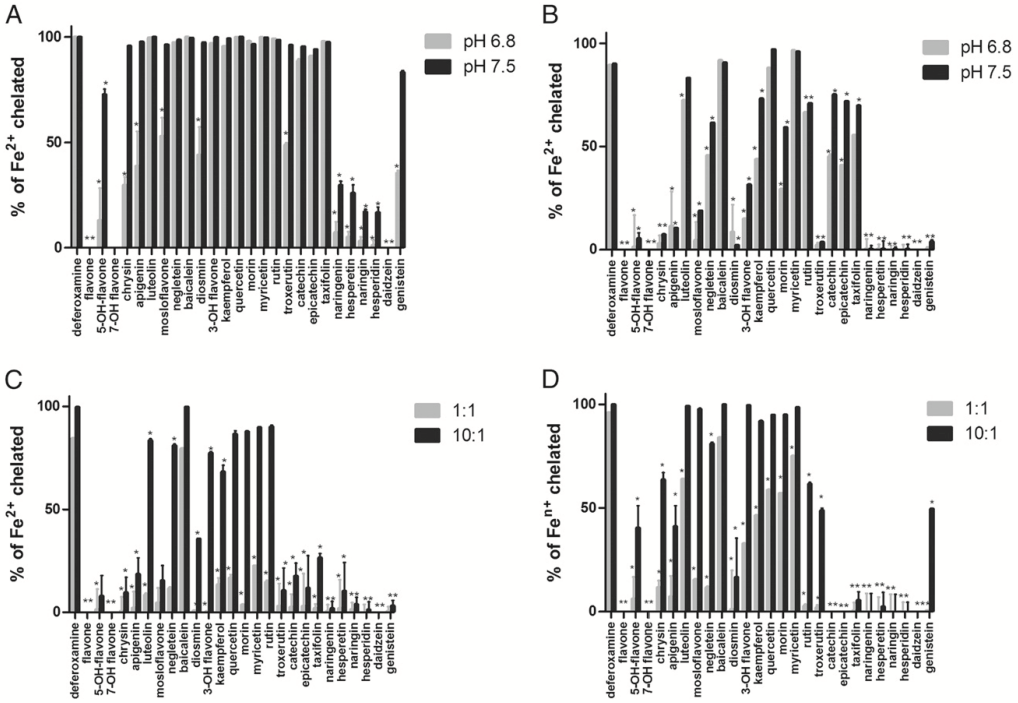


Fig. 3. Iron chelation by flavonoids. Ferrous chelation at pH 6.8 and 7.5 (A):, the concentration ratio 10:1 (B):, the ratio 1:1 flavonoid:iron, respectively, at pH 5.5 (C), and total iron chelation at pH 4.5 (D). The efficiency of iron chelation was compared to deferoxamine: * $p<0.05$.

(Fig. 3C). Baicalein was similarly active to deferoxamine even in the ratio 1:1 (Fig. S1 in Supplementary material) in contrast to the above mentioned flavonols, which achieved the efficacy of deferoxamine only in the ratio 10:1. Again here, the complexes of flavonoids with ferrous ions were stable at pH 5.5 (data not shown).

At pH 4.5, flavonoids did not chelate nearly any ferrous iron in the ratio 1:1, with exception of baicalein (18±2%). In the ratio 10:1, baicalein chelated 70±2% of iron, morin chelated 35±2% of iron, while other flavonoids did not chelate more than 25% of iron (data not shown). The efficiency of baicalein was not significantly different from standard iron chelator deferoxamine (75±1%). Both deferoxamine and baicalein complexes were stable (101±2% and 98±1%, respectively).

The total iron chelation at pH 4.5 represented a clear difference to ferrous chelation. Baicalein and flavonols with at least free 3-hydroxyl group and some flavones reached or had almost the same potency as deferoxamine. In contrast to other more neutral pH, the catecholic B ring without 3-hydroxy-4-keto or 5-hydroxy-4-keto conformation and 2,3 double bond had no effect on iron chelation. Moreover, some flavonoids, particularly those with 5-hydroxy-4-keto conformation without 3-hydroxyl group and/or the catecholic B ring, did not form stable iron complexes (Fig. 4B).

To characterize the contribution of various functional groups in detail, the iron chelating effect of close relative flavonoids were compared:

1) The hydroxyl groups in ring A (Fig. 5, and Fig. S2 in Supplementary material). Increasing the number of free hydroxyl groups in ring A improved the potency: baicalein with the free 5,6,7-hydroxyl groups was more potent than negletein with the free 5,6-hydroxyl groups. The latter was more potent than 5-hydroxyflavone.
2) The hydroxyl groups in ring B (Fig. 6, Fig. S3 and S4 in Supplementary material). In disharmony to the ring A, there was no difference between catechol (two hydroxyl groups in an *ortho* possition) and pyrogallol (three adjacent hydroxyl groups) in ring B. Similarly, one hydroxyl group or two hydroxyl groups in a *meta* position did not represent any advantage when compared to flavonoids with no hydroxyl group in ring B. Generally, there were no differences between flavonols morin (two hydroxyl groups in meta positions 2′ and 4′ in ring B) and kaempferol (one hydroxyl group in position 4′ in ring B only) with the exception for ferrous chelation at pH 4.5. Moreover, catechol or pyrogallol in ring B was associated with higher chelation only at neutral or close to neutral conditions (pH 6.8 and 7.5). At more acidic conditions, there were no differences among flavonoids with one, two or three hydroxyl groups in ring B at the tested concentrations.
3) The 3-hydroxyl group (Fig. 7, Fig. S5 and S6 in Supplementary material). The free 3-hydroxyl group in flavonoids with the 4-keto group generally favored the increase in iron chelation, but the presence of a powerful iron chelation site (the catecholic ring B) at the neutral conditions may hide the contribution of this hydroxyl group. Similarly, the 3-hydroxyl group appeared to be associated with high affinity for ferric ions and, therefore, ferric iron chelation was markedly enhanced at the acidic conditions by its presence.
4) The 5-hydroxyl group (Fig. 8A and Fig. S7 in Supplementary material). The free 5-hydroxyl group in flavonoids with the 4-keto group may enhance the iron chelating effect of flavonoids, particularly at the neutral or close to neutral conditions (pH 6.8–7.5). Its contribution for iron chelating activity was generally lower than that of the free 3-hydroxyl group, e.g., see 3-hydroxyflavone vs. kaempferol and 5-hydroxyflavone vs. kaempferol.

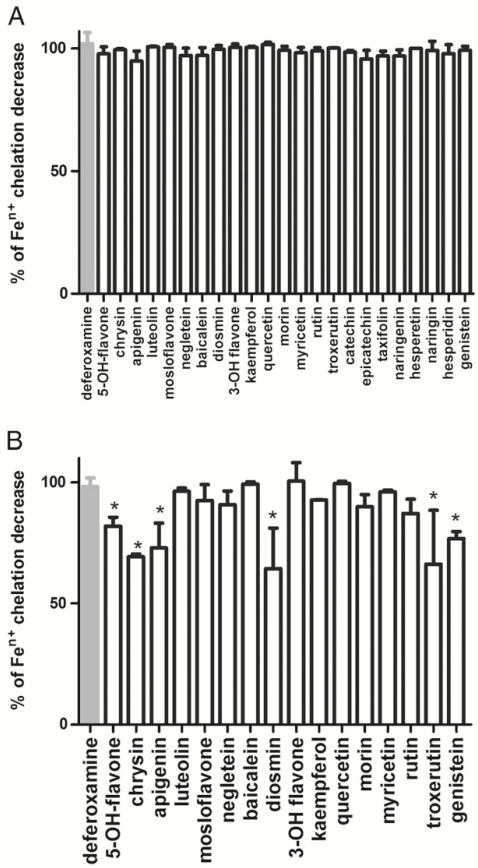


Fig. 4. The stability of iron-flavonoid complexes at pH 6.8 for ferrous iron (A) and at pH 4.5 for total iron (B). Data are shown for at least moderate iron chelators (i.e., at minimum 20% of iron chelation at ratio 10:1, flavonoid:iron). The data compare the change in iron chelation efficacy at time 0 and at time 5 min. Similar results as for pH 6.8 were also found for pH7.5 and 5.5 (not shown in the graph). Statistical significance: * $p<0.01$ vs. deferoxamine.

5) The 2,3-double bond (Fig. 8B and Fig. S8 in Supplementary material). This double bond appeared to increase the iron chelation effect as can be demonstrated by comparison of quercetin with taxifolin.
6) A keto group in position 4 (Fig. 8C and Fig. S8 in Supplementary material). The keto group alone apparently had low effect on iron chelation, but it is an important component of the 3-hydroxyl-4-keto or 5-hydroxyl-4-keto chelation sites and, therefore, its removal was associated with attenuation of iron chelation (demonstrated for taxifolin vs. catechin at the acidic conditions, when the catecholic B ring did not play a substantial role).
7) The position of ring B (Fig. 8D and Fig. S9 in Supplementary material) did not seem to have any effect as isoflavonoid genistein and flavon apigenin with the same functional groups had the same efficacy.
8) Additional groups. Stereoisomery in flavanols concerning the position of the 3-hydroxyl group was not important for iron chelation. Carbohydrate alone did not have any contribution for chelation, rather when bound to a known chelation site, it may decrease the chelation potency (e.g., see rutin vs. quercetin in Fig. 7B). In flavanones, there were no differences between aglycones hesperetin and naringenin and their corresponding glycosides hesperidin and naringin in the tested concentrations.

4. Discussion

Deferoxamine is an iron chelator used for the treatment of iron overload for many decades [20,21]. Its high affinity for ferric iron renders it a suitable substance for the comparison of iron chelating activity. Moreover, it chelates also ferrous iron with high affinity under aerobic conditions (see Fig. 3), probably because the formation of more stable ferric-deferoxamine complex is facilitated by the presence of oxygen [22,23]. Deferoxamine is a hexadentate iron chelator, it forms complexes with ferric iron in the stoichiometric ratio 1:1 and this ratio was also found with the use of ferrozine methodology [19,24]. The potential of flavonoids to chelate iron has been well documented, but here, for the first time, it has been shown that some flavonoids are similarly active as deferoxamine (Fig. 3). These flavonoids include those with the catecholic B ring with the 3-hydroxyl-4-keto conformation and the 2,3-double bond and flavonoids with a 6,7-dihydroxy conformation in ring A. The former flavonoids including myricetin and quercetin reached the activity of deferoxamine only at or near to the physiological pH, while the latter represented by baicalein demonstrated the same activity as deferoxamine at all tested pH. The fact that baicalein is more active iron chelator than other common flavonoids was assumed recently by other authors [5,12].

The data of this study show that 6,7-dihydroxy conformation in ring A is the most efficient iron chelating site followed by the 3-hydroxy-4-keto site with the 2,3-double bond and the catecholic B ring. The 5-hydroxy-4-keto site is another possible iron binding site, but this study showed and other researchers confirmed that its importance is lower when compared to the previously mentioned sites [7]. In addition, the complex formed at this site is less stable and iron can be easily released at more acidic conditions (see Fig. 4B). Generally, different dissociation of protons of hydroxyl groups seems to explain the differences in iron chelation among flavonoids and their different iron chelating behavior in various pH, too: The dissociation at the acidic conditions is favored more in the 7-hydroxyl group than in the 4′-hydroxyl group and is less probable in the 5-hydroxyl group [25]. Indeed, baicalein with the 6,7-dihydroxyl groups is very efficient even at the acidic condition. The catecholic B ring is efficient at the neutral conditions, but has only low activity at the acidic conditions. The 5-hydroxy-4-keto site has only moderate activity at all pH conditions. In harmony with other studies, iron is not steadily bound by flavonoids with an isolated keto group, isolated hydroxyl groups, methoxyl groups or hydroxyl group with adjacent methoxyl group [11,26]. A certain exception is morin, which likely forms an additional chelation site between 2′-hydroxyl group of ring B and 3-hydroxyl group of ring C, probably because of easy ionization of 2′-hydroxyl group [25]. But this chelation site seems to be weak, and relevant only in the absence of more potent chelation sites. Carbohydrate moieties did not increase iron binding and, therefore, it may be concluded that they have no iron chelating effects.

The comparison of structural differences and iron chelating activity of the tested flavonoids confirmed the previous findings. Increasing the number of hydroxyl groups in ring B did not linearly increase the chelating activity (Fig. 6). This means that one hydroxyl group or none hydroxyl group has no chelation activity, while there is no difference between the catecholic and pyrogallol B ring suggesting that two hydroxyl groups are sufficient for iron chelation and additional adjacent hydroxyl group did not provide any further advantage concerning iron chelation. Apparently, contradictory results were found in flavones ring A where increasing the number of hydroxyl groups improved the iron chelation potential. This is not because of three hydroxyl groups have higher iron chelating potential than two hydroxyl groups but likely due to the different chelating sites. Proton dissociation and the subsequent iron chelation activity likely follows this pattern: the 6,7-dihydroxy>5,6-dihydroxy>5-hydroxyl-4-keto site. This pattern can be supported by our recent

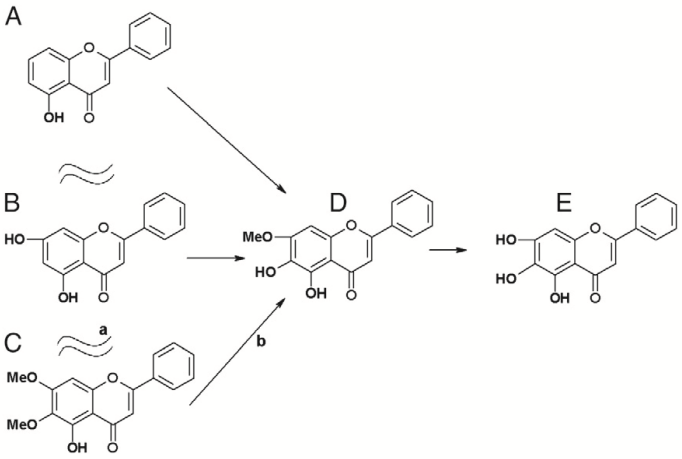


Fig. 5. The effect of the number of hydroxyl groups in ring A. A: 5-hydroxyflavone, B: chrysin, C: mosloflavone; D: negletein, and E: baicalein. The direction of arrows shows more potent compound ($p<0.05$). ≈ No difference between compounds. [a] The total iron chelation at pH 4.5 was an exception, where mosloflavone was significantly more efficient chelator. [b] At pH 4.5, mosloflavone was similarly efficient to negletein in chelation of the total iron (ferric + ferrous ions).

findings, where methylation in basic media of baicalein occurs firstly on hydroxyl group on C-7, then on hydroxyl group on C-6 and finally on hydroxyl group on C-5. In analogy, demethylation in acidic media occurs in reverse order [17]. Therefore, baicalein with 6,7-dihydroxy-chelation site has higher potency than negletein with 5,6-dihydroxy groups and both agents are more potent than 5-hydroxyflavone.

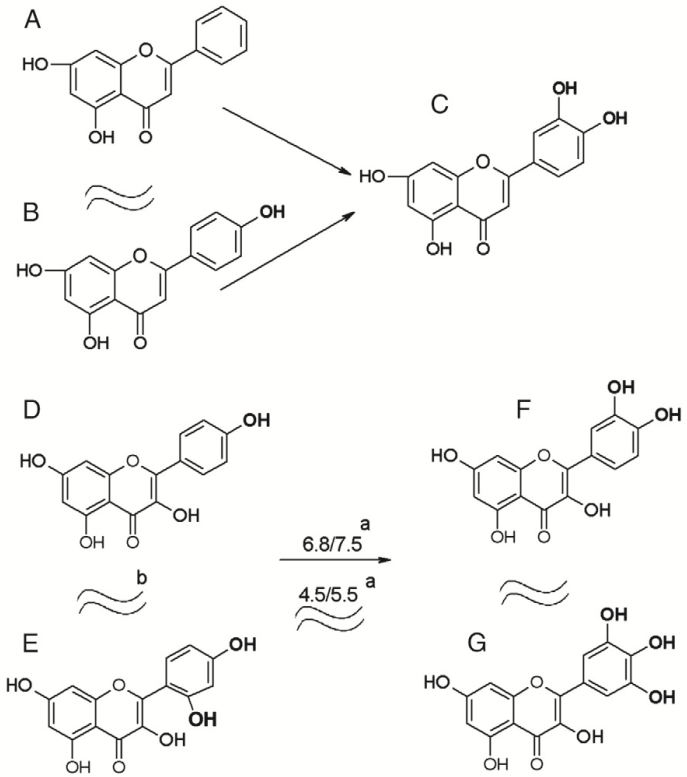


Fig. 6. The effect of the number of hydroxyl groups in ring B. A: chrysin, B: apigenin, C: luteolin, D: kaempferol, E: morin, F: quercetin, and G:myricetin. The direction of arrows shows more potent compound ($p<0.05$). ≈ No difference between compounds. [a] Significance was found at pH 6.8 and 7.5, but no difference was found at pH 5.5 for ferrous chelation and pH 4.5 (total iron chelation). [b] The only exception is ferrous chelation at pH 4.5.

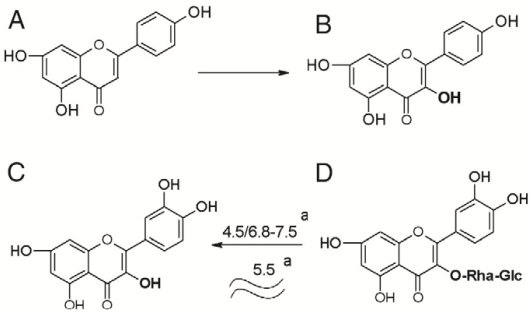


Fig. 7. The effect of the 3-hydroxyl group. A: apigenin, B: kaempferol, C: quercetin, and D: rutin. The direction of arrows shows more potent compound ($p<0.05$). ≈ No difference between compounds. [a] Significance was found at pH 4.5 for total iron chelation and at pH 6.8 and 7.5 for ferrous chelation, but no difference was found at pH 5.5 for ferrous chelation.

Indeed, NMR-spectra implicated that oxygens in the positions 6 and 7 of baicalein represent the iron binding sites [12].

The published results showed equivocal conclusions and, hence, the stoichiometry of flavonoid:iron complexes could be generally estimated with difficulties. This may be, at least partly, caused by the different pH conditions, solvents and methodological approach, e.g., non-buffered spectrophotometric experiments in methanol suggested rutin:Fe^{2+} complex to be 2:3, while spectrophotometry showed 2:1 complex at pH 7.4. Similarly, spectrophotometric approach observed complex of quercetin with ferrous iron with stoichiometry 1:2, while electrospray ionization mass spectrometry ($pH \leq 5.5$) documented a mixture of 1:1 and 2:1 complexes [8,10,27]. The methodology used in this paper with ferrozine approach is not primarily suitable for the establishment of stoichiometry because of its competitive manner, i.e., competition between ferrozine and flavonoids for ferrous iron. Some conclusions could be drawn in condition where the complexes were apparently very stable and thus ferrozine could not remove iron from the complex. The simplest way for the estimation of stoichiometry is a measurement of iron chelation in molar ratio flavonoid:iron 1:1. At neutral pH, baicalein, quercetin and myricetin chelated approximately 100% iron. This suggests complexes with 1:1 or eventually even higher stoichiometry, while morin, negletein, catechin, epicatechin and taxifolin chelated around 50% of iron indicating complex 2:1 flavonoid:iron, respectively. 3-hydroxyflavone chelated about 33% of iron at the same ratio implicating 3:1 complex with iron. From the equimolar ratio at pH 4.5 some stoichiometry for ferric ions chelation can be estimated, as well. Again, baicalein has the ratio 1:1, quercetin, kaempferol,

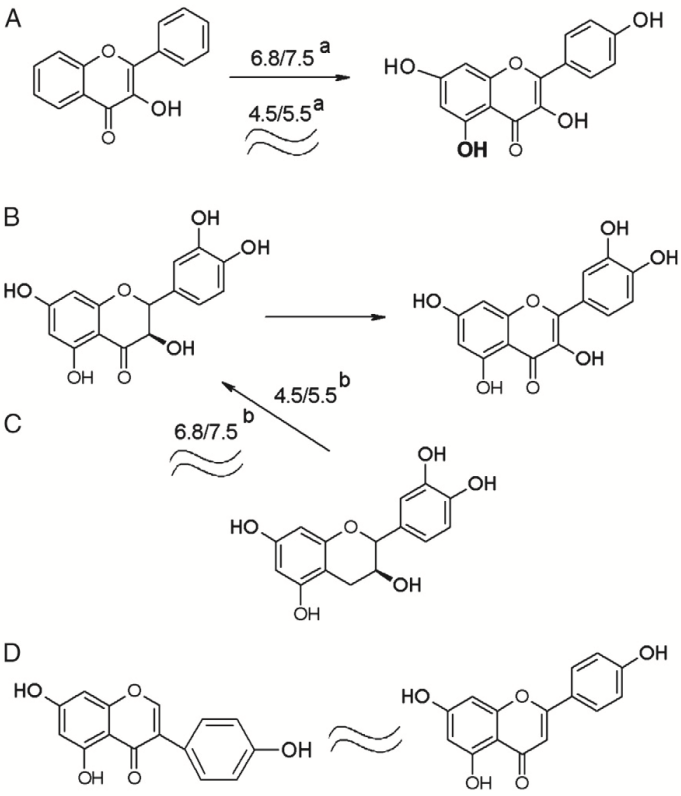


Fig. 8. The effects of other principal functional groups: the 5-hydroxyl group (A, 3-hydroxyflavone vs. kaempferol), the 2–3 double bond (B, taxifolin vs. quercetin), the 4-ketogroup (C, taxifolin vs. catechin) and position of B-ring (D, apigenin vs. genistein). The direction of arrows shows more potent compound ($p<0.05$). ≈ No difference between compounds. [a] Significance was found for ferrous chelation at pH 6.8 and 7.5, no differences were found at lower pH for ferrous chelation and at pH 4.5 for total iron chelation. [b] Significance was found for ferrous chelation at pH 5.5 and total iron chelation at pH 4.5 but no differences were found at pH 6.8 and 7.5 for ferrous chelation.

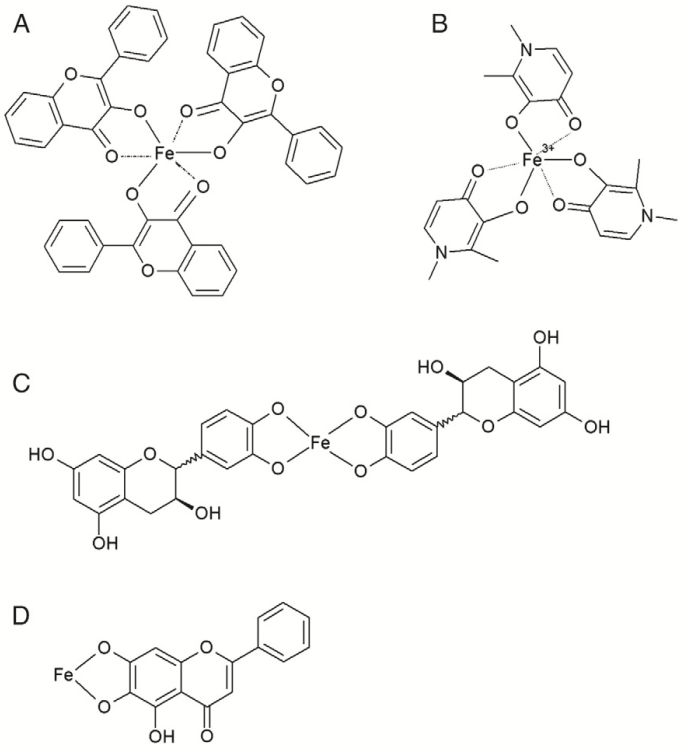


Fig. 9. The proposed iron binding with flavonoids and deferiprone. A: The 3:1 complex of 3-hydroxyflavone with iron. B: The 3:1 complex of ferric iron with deferiprone. C: Iron binding with catechin (2:1) and D: Iron binding with baicalein (1:1).

myricetin, morin 2:1 and 3-hydroxyflavone 3:1. These results imply that:

1) Three 3-hydroxy-4-keto groups form a complex with one iron atom both at the neutral and acidic conditions (Fig. 9A). Note that similarly to 3-hydroxyflavone of which chelating site is formed by keto-hydroxy component, another clinically used iron chelator deferiprone with keto-hydroxyl site forms the complex with the same stoichiometry (Fig. 9B) [28].
2) Two adjacent hydroxyl groups are necessary for binding of one iron atom with exception of baicalein (Fig. 9C and D). The catecholic B ring has low, if any, impact on chelation at the acidic conditions, while at the neutral conditions 2 catecholic B rings bind one atom of iron.
3) Myricetin and quercetin apparently use both 3-hydroxy-4-keto- (5-hydroxy) and the catecholic B ring binding sites, at the neutral conditions which lead finally to 1:1 (or higher) stoichiometry.
4) The precise stoichiometry of 3-hydroxy-4-keto-5-hydroxy conformation cannot be assessed with certainty, but it seems to be 2:1.

Two important issues have to be discussed. Firstly, this study did not analyze ferric chelation at higher pH due to difficulties associated with ferric reduction. Generally, ferric iron has limited solubility at neutral pH, it is firmly bound in the organism to transport and storage molecules and, therefore, it plays probably a minor role in hydroxyl radical formation or direct lipid peroxidation. But for the completion of iron chelating data, a spectrophotometric study is being performed in our laboratory to resolve this limitation. The second issue is the possibility of iron reduction by flavonoids, which was demonstrated in other studies. Ferric iron can be chelated by polyphenols, but simultaneously it can be reduced, which may facilitate its catalytic role in reactive species production [9,19,29,30]. Therefore, the study analyzing iron reducing potential of flavonoids should be performed in the future.

5. Conclusions

This study showed that flavonoids with the 6,7-dihydroxy iron chelation site (e.g., baicalein) are similarly potent iron chelators as clinically used deferoxamine. Flavonols (containing the 3-hydroxyl group, the 4-ketogroup and the 2,3-double bond) with the catecholic B ring could be similarly active to deferoxamine, but only at the neutral pH or its proximity. Flavonoids with 5-hydroxyl-4-keto chelation site are less potent iron chelators.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2011.02.003.

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Supplementary data

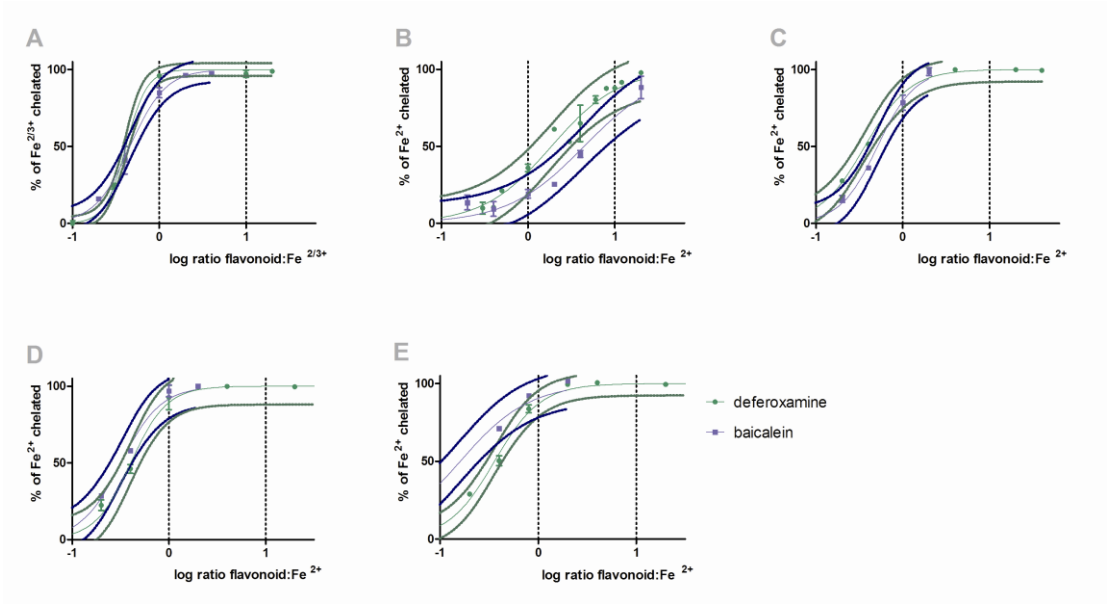

Fig. S1. The comparison of the iron chelating activity of baicalein and deferoxamine.
Total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 4.5 (**B**), pH 5.5 (**C**), pH 6.8 (**D**) and pH 7.5 (**E**) were compared by use of 95 % confidence intervals. In all cases, the iron chelating activity of baicalein was not inferior to that of deferoxamine.

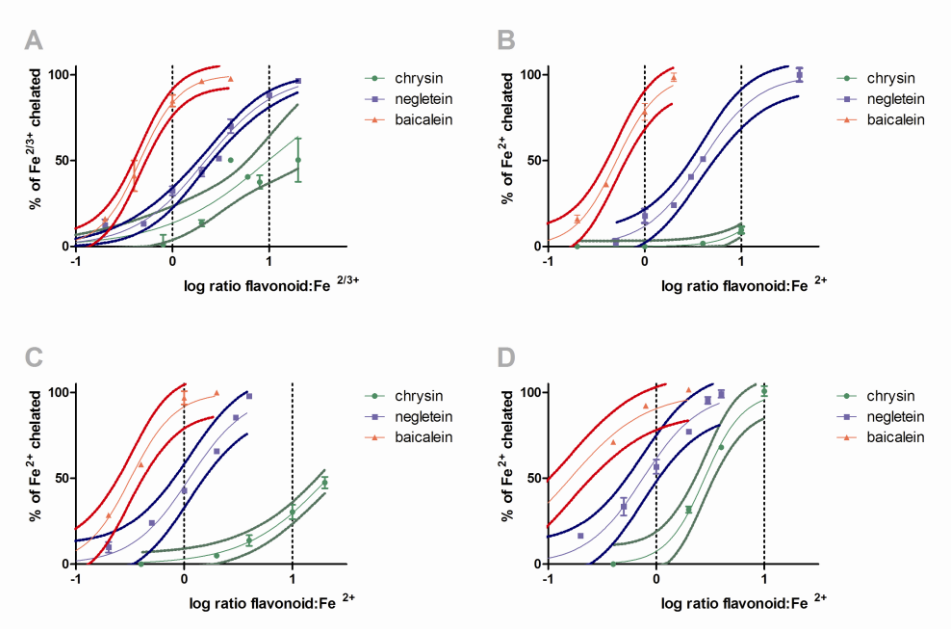

Fig. S2. The comparison of the effect of increasing number of ring A hydroxyl groups substitutions.
Chrysin (one hydroxyl group), negletein (two hydroxyl groups) and baicalein (three hydroxyl groups) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals. In all cases, baicalein was more potent than negletein and negletein was more potent than chrysin.

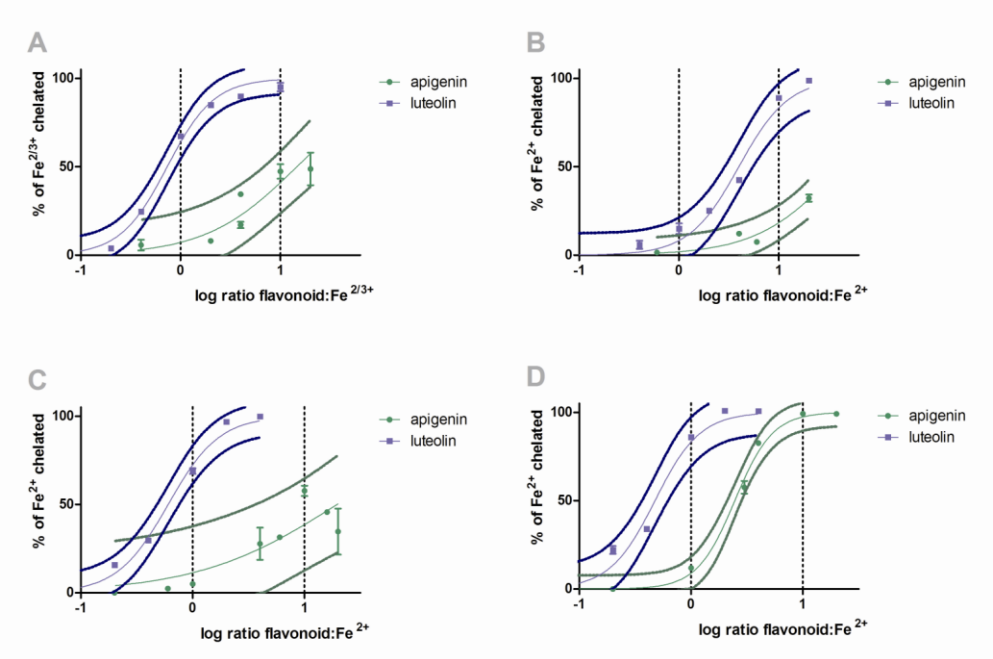

Fig. S3. The comparison of the effect of ring B hydroxyl groups substitutions in flavons.

Apigenin (one hydroxyl group) and luteolin (two hydroxyl group in an *ortho* position) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals. In all cases, luteolin was more potent than apigenin.

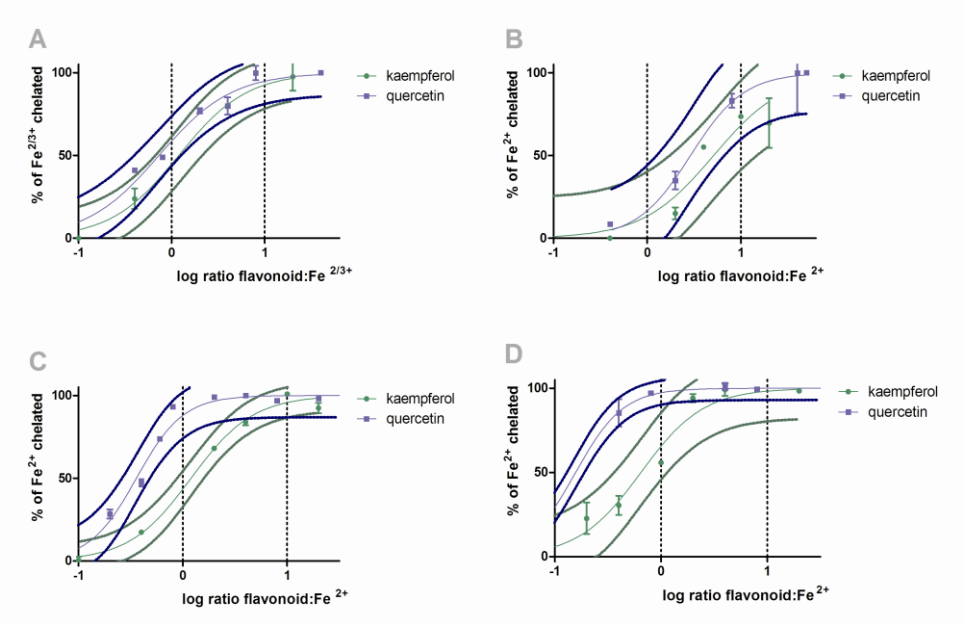

Fig. S4. The comparison of the effect of ring B hydroxyl groups substitutions in flavonols.

Kaempferol (one hydroxyl group) and quercetin (two hydroxyl group in an *ortho* position) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals.

Both flavonoids were similarly potent at lower pH (total iron chelation at pH 4.5 and ferrous chelation at pH 5.5), while at pH 6.8 and 7.5, quercetin was more potent iron chelator in ratio 1:1 (log ratio = 0; x = 0), flavonoid:iron, respectively.

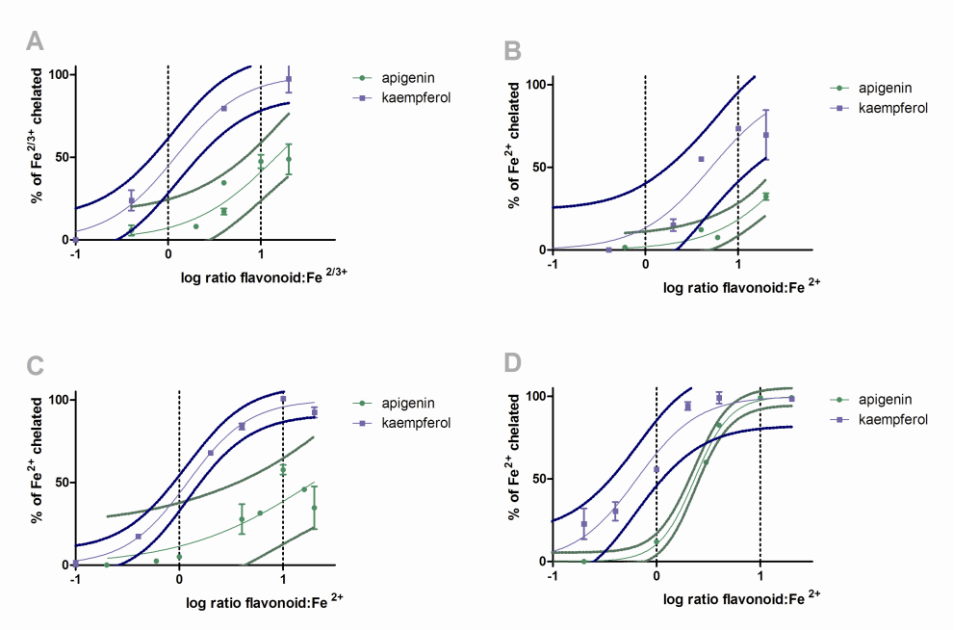

Fig. S5. The influence of the 3-hydroxyl group.

Two corresponding flavonoids differing in the 3-hydroxylgroup (kaempferol with and apigenin without the group) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals.In all cases, kaempferol was more potent than apigenin.

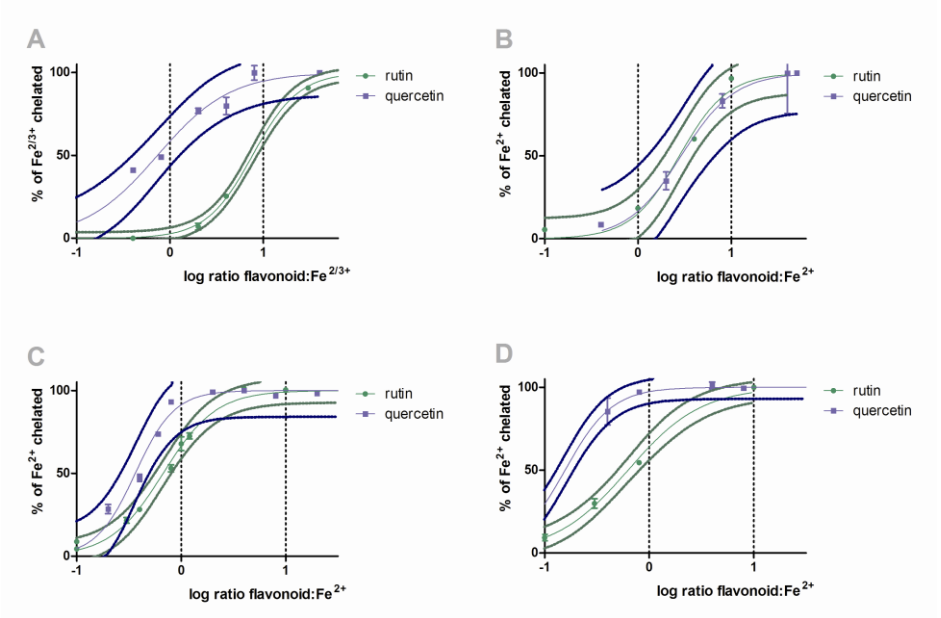

Fig. S6. The influence of the 3-hydroxyl group substitution by a sugar moiety.

Two relative flavonoids differing in 3-hydroxylgroup derivation by a sugar (rutin and its aglycone quercetin) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals.

Quercetin was much more potent total iron chelator at pH 4.5, more potent ferrous chelator at pH 7.5 and slightly more potent at pH 6.8. At pH 5.5, there was no difference in ferrous chelation between these flavonoids.

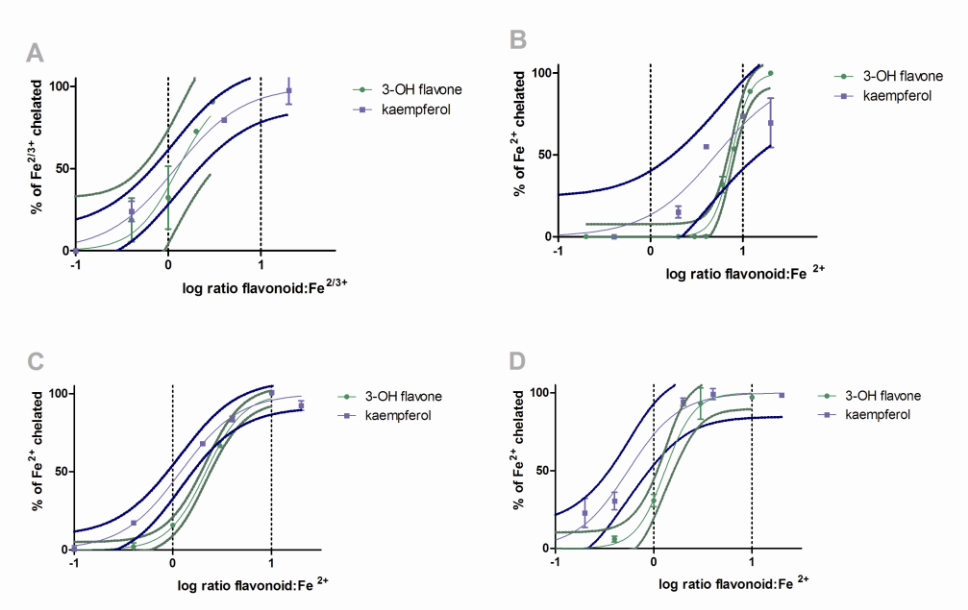

Fig. S7. The influence of the 5-hydroxyl group.

Two relative flavonoids differing in 5-hydroxylgroup (3-hydroxyflavone *vs.* kaempferol) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals. The 5-hydroxyl substitution improved ferrous chelation very slightly at pH 6.8 and pH 7.5, but there were no differences at pH 5.5 for ferrous chelation and at pH 4.5 for total iron chelation.

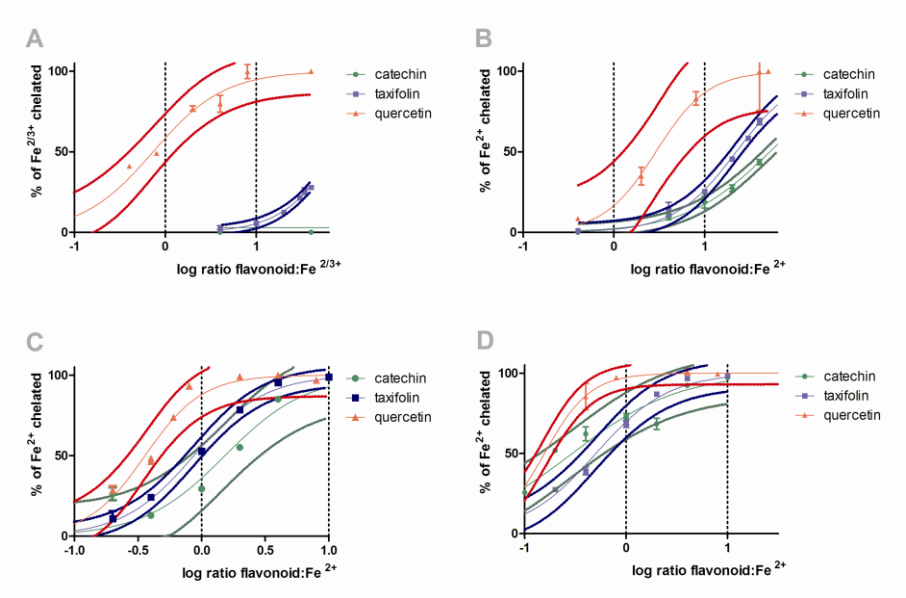

Fig. S8. The influence of the 4-keto group and the 2,3-double bound.Three relative flavonoids differing in the 4-keto and the 2-3-double bound (quercetin having both, taxifolin having only the 4-ketogroup and catechin without any) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals.

Quercetin was the most potent iron chelator in all cases, while taxifolin was more potent than catechin only at higher ratios flavonoid:iron than 10:1 (log ratio = 1; x=1) and in more acidic conditions (total iron chelation at pH 4.5 and ferrous chelation at pH 5.5).

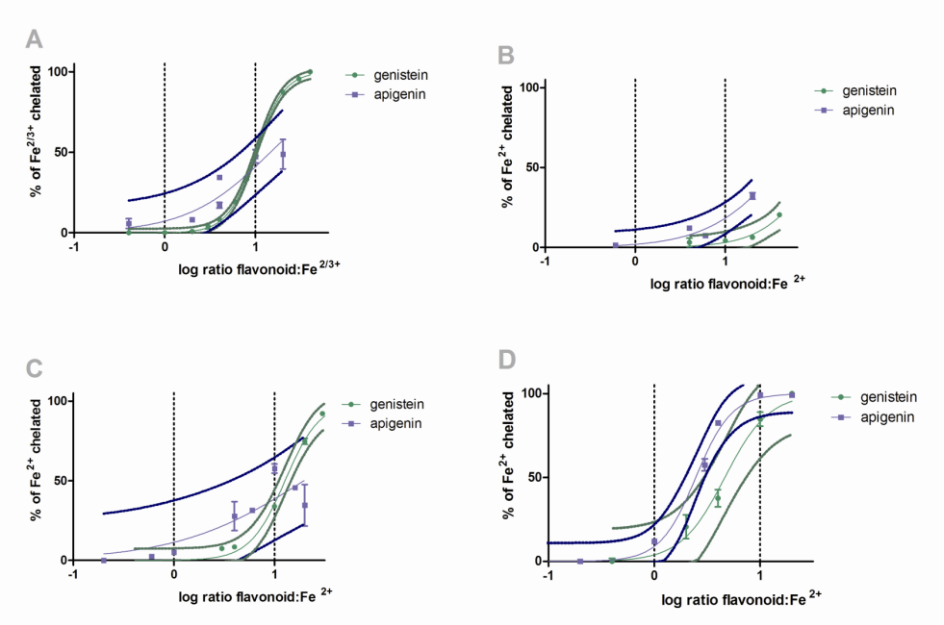

Fig. S9. The influence of the position of ring B.

Two corresponding flavonoids differing in the position of ring B (isoflavone genistein *vs.* flavones apigenin) were compared for their total iron chelating activity at pH 4.5 (**A**) and ferrous chelating activity at pH 5.5 (**B**), pH 6.8 (**C**) and pH 7.5 (**D**) by use of 95 % confidence intervals. There were no significant differences in all cases within the ratio 1:1 and 10:1, flavonoid:iron, respectively.

4. 2. Iron reduction potentiates hydroxyl radical formation only in flavonols

MACÁKOVÁ, Kateřina, MLADĚNKA, Přemysl, FILIPSKÝ, Tomáš, ŘÍHA, Michal, JAHODÁŘ, Luděk, TREJTNAR, František, BOVICELLI, Paolo, SILVESTRI, Ilaria P., HRDINA, Radomír, SASO, Luciano. Iron reduction potentiates hydroxyl radical formation only in flavonols. *Food Chemistry*. 2012, **135**(4), 2584-2592. ISSN 0308-8146.

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Podstatnou součástí lidské stravy jsou rovněž flavonoidy, které jsou považovány za zdraví prospěšné látky. Nicméně, někteří zástupci této skupiny mohou mít z důvodu svých redoxních vlastností i nežádoucí pro-oxidační účinky.

Tato studie byla zaměřena na zhodnocení anti/pro-oxidačních vlastností u 26 flavonoidů při patofyziologicky relevantních pH, tj. schopnosti redukovat železité ionty, a vlivu na inhibici/potenciaci produkce hydroxylového radikálu. Ke stanovení byla použita spektrofotometrická ferrozinová metoda, resp. HPLC analýza s použitím kyseliny salicylové jako indikátoru tvorby hydroxylového radikálu.

Statisticky významné redoxní vlastnosti byly zaznamenány v kyselém prostředí především u flavonolů a flavanolů s pyrokatecholovým kruhem. Za zmínku stojí především pro-oxidační vliv kvercetinu a kaempferolu na železem katalyzovanou Fentonovu reakci. Na jednu stranu některé flavonoly prokázaly při velmi nízkých koncentracích pozitivní antioxidační účinky, ale na druhou stranu při podrobné analýze koncentrační závislosti byly jejich antioxidační účinky při vyšších testovaných koncentracích již minimální nebo dokonce měly vlastnosti pro-oxidační. Zatímco flavonoly morin a rutin měly jednoznačně koncentračně závislé pro-oxidační účinky, 7-hydroxyflavon a hesperetin patřily mezi jediné flavonoidy, které s rostoucí koncentrací snižovaly tvorbu hydroxylového radikálu.

Na závěr lze konstatovat, že nadměrná konzumace flavonoidů může v některých případech vést i k manifestaci jejich nežádoucích pro-oxidačních účinků.

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Iron reduction potentiates hydroxyl radical formation only in flavonols

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ABSTRACT

Flavonoids, substantial components of the human diet, are generally considered to be beneficial. However, they may possess possible pro-oxidative effects, which could be based on their reducing potential. The aims of this study were to evaluate the ability of 26 flavonoids to reduce ferric ions at relevant pH conditions and to find a possible relationship with potentiation of hydroxyl radical production. A substantial ferric ions reduction was achieved under acidic conditions, particularly by flavonols and flavanols with the catecholic ring B. Apparently corresponding bell-shaped curves displaying the pro-oxidant effect of flavonols quercetin and kaempferol on iron-based Fenton reaction were documented. Several flavonoids were efficient antioxidants at very low concentrations but rather inefficient or pro-oxidative at higher concentrations. Flavonols, morin and rutin were progressively pro-oxidant, while 7-hydroxyflavone and hesperetin were the only flavonoids with dose-dependent inhibition of hydroxyl radical production. Conclusively, administration of flavonoids may lead to unpredictable consequences with few exceptions.

1. Introduction

Flavonoids represent substantial components of human diet and although GIT metabolism and absorption largely limits their bioavailability, the daily food intake can cause significant increase in their human plasma levels (Scalbert & Williamson, 2000). Flavonoids have been largely promoted for their broad potential health benefits in the past and thus markedly consumed by patients in the form of herbal medicines or dietary supplements. However, no definite and unequivocal evidence concerning their clinical effects has been reported so far (Chahar, Sharma, Dobhal, & Joshi, 2011; Galati & O'Brien, 2004; Mladenka, Zatloukalova, Filipsky, & Hrdina, 2010b). Their uncritically assessed positive effects were mostly attributed to their reactive oxygen species (ROS) scavenging activity, which was confirmed by many different antioxidant assays. On the other hand, substantial data have been published about their pro-oxidative properties too (Galati & O'Brien, 2004; Prochazkova, Bousova, & Wilhelmova, 2011; Sakihama, Cohen, Grace, & Yamasaki, 2002). Probably due to different approaches, it is still not clear which flavonoids and conditions can be associated with pro-oxidative properties. Moreover, the mechanism(s) of pro-oxidation action are still a topic of discussion. The involvement of redox-active transient metals, namely copper and iron, or enzymes in the generation of ROS by flavonoids has been suggested (Cao, Sofic, & Prior, 1997; Galati & O'Brien, 2004; Prochazkova et al., 2011; Sakihama et al., 2002). Interestingly, antioxidant effects are sometimes measured by ferric ions reduction at acidic pH (originally designed as "ferric reduction ability of plasma", today usually named "ferric reducing antioxidant power" or simply FRAP). There are very good correlations between this assay and redox potential and sufficient correlations with other antioxidant assays (Firuzi, Lacanna, Petrucci, Marrosu, & Saso, 2005; Zhang et al., 2011). Thus, although this assay clearly reflects the reducing potential of the tested compounds and the majority of antioxidants expresses a certain degree of activity in this assay (Pulido, Bravo, & Saura-Calixto, 2000), one very important factor is neglected – possible risk of potentiation of Fenton chemistry. During the Fenton reaction, ferrous ion catalyses production of hydroxyl radical, while being simultaneously oxidised to ferric ion. Therefore, ferrous ions recovery by a reductant may intensify Fenton reaction (Aruoma, Murcia, Butler, & Halliwell, 1993), hence rendering FRAP assay pathophysiologically less relevant. Similar pathways of reactions were documented for copper as well (Sakihama et al., 2002). It need not be

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emphasised that the reducing properties of ascorbic acid are commonly used for the potentiation of the Fenton reaction (Aruoma et al., 1993; Laughton, Halliwell, Evans, & Hoult, 1989).

The first aim of this study was to evaluate ferric ions reducing properties of flavonoids at selected (patho)physiologically relevant pHs. The second aim was to find the relationship between ferric ions reducing properties of flavonoids and potentiation of the Fenton reaction. The choice of the tested pHs was based on the fate of iron and flavonoids in the human organism and relevant pathological states. For example, low pH facilitates iron absorption in the proximity of the gastro-duodenal junction, lysosomes are important organelles for iron kinetics and ischaemia markedly disturbs physiological pH. Similarly, lower pH was documented in tumours (Ambrosio, Zweier, Jacobus, Weisfeldt, & Flaherty, 1987; Donovan & Andrews, 2004; Kurz, Terman, Gustafsson, & Brunk, 2008; Parolini et al., 2009).

2. Materials and methods

2.1. Reagents

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt (ferrozine), ferrous sulphate heptahydrate, ferric chloride hexahydrate, hydroxylamine, dimethyl sulphoxide (DMSO), sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), HEPES sodium salt, salicylic acid, 1,4-dioxane, 2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids and all tested flavonoids (Supplementary data, Fig. S1), with exception of negletein and mosloflavone, were purchased from Sigma–Aldrich (Steinheim, Germany). Negletein and mosloflavone were synthesised through a convergent synthesis starting from chrysin, as was previously reported (Righi et al., 2010). Deferoxamine was purchased from Novartis (Basle, Switzerland) and methanol for HPLC was from JT Baker (Phillipsburg, NJ).

2.2. Assessment of ferric ions reduction potential

The degree of iron reduction was established by use of ferrozine methodology as previously reported by us (Mladenka et al., 2010a). Ferrozine forms a magenta-coloured complex with ferrous ions and this reaction is specific to these ions because ferric ions do not react with ferrozine (Stookey, 1970). Hence, the assessment of ferric ions reduction potential can be easily established in experiments, in which ferric ions were mixed with flavonoids together, and afterwards, the amount of reduced ferrous ions was evidenced by ferrozine.

Briefly, various 15 mM buffers (acetate buffers for pH 4.5 and 5.5 and HEPES buffers for pH 6.8 and 7.5) were firstly mixed with different concentrations of the tested compound dissolved in DMSO. Freshly prepared aqueous solution of ferric ions (50 μl, 250 μM) was added and mixture was stirred for 2 min. Afterwards, aqueous solution of ferrozine (50 μl, 5 mM) was added and absorbance was measured at 562 nm 5 min later by Anthos Reader 2010 (Anthos Labtec Instruments, Salzburg, Austria). The assay for non-buffered conditions was identical with the exception that no buffer was added into the mixture. In all sets of experiments, hydroxylamine (50 μl, 10 mM) was used as positive control (100% iron reduction) (Mladenka et al., 2010a).

2.3. Measurement of inhibition of iron mediated production of hydroxyl radicals

As generally known, ferrous ions react with hydrogen peroxide to produce hydroxyl radical (the Fenton reaction) (Halliwell & Gutteridge, 1999). The formed radical can be trapped by salicylic acid and its ensuing products (2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids) can be detected by HPLC (Nappi & Vass, 1998).

Briefly, ferrous ions were mixed with the tested compounds dissolved in methanol in different concentration ratios for 2 min. Salicylic acid and hydrogen peroxide (concentration of the both substances was 7 mM) were added subsequently, and afterwards, the mixture was analysed by HPLC (Philips PU 4100 pump, Philips, UK), Eclipse Plus C18 column (4.6 × 100 mm, 3.5 μm, Agilent, Santa Clara, CA), with UV–vis detector (Ecom LCD 2083; Ecom, Prague, Czech Republic), using 40% methanol and 0.085% aqueous solution of phosphoric acid as mobile phase. The only exception was taxifolin, because the peak of taxifolin interferes with dihydroxybenzoic acids at the abovementioned settings, the procedure of analysis was modified: mobile phase consisted of 0.1% aqueous solution of formic acid and methanol, gradient elution was used by increasing the methanol content from 5% to 15% within 5 min and afterwards to 40% within 10 min. All experiments were checked by addition of 'internal standard', i.e., known amounts of 2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids.

2.4. Statistical analysis

Results are expressed as mean ± SD. The differences among the tested substances were compared by use of one-way ANOVA test followed by Dunnett's multiple comparison test. Correlations were analysed by Pearson's test. All statistical analysis was performed by GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA).

3. Results

Firstly, ferric ions reductive properties of flavonoids were established at four (patho)physiologically relevant pHs and under non-buffered conditions as well. None of the tested flavonoids was able to reduce ferric ions at pH 7.5 or pH 6.8. At pH 5.5 only flavanols catechin, epicatechin and partly taxifolin were efficient reducing agents. At pH 4.5 and at non-buffered conditions the number of reducing flavonoids increased markedly. Only flavone, all tested monohydroxyflavones, flavones without the adjacent hydroxyl groups in ring A and the catecholic group in ring B (chrysin, apigenin, mosloflavone, diosmin), isoflavones (daidzein and genistein) and troxerutin were not able to significantly reduce ferric ions at any tested conditions. Comparing pH 4.5 and non-buffered conditions, the results were similar but not equal (full curves of the efficient flavonoids are depicted in Supplementary data, Figs. S2 and S3). Baicalein, which was a very potent reducing agent at non-buffered conditions but a relatively weak reducing agent at pH 4.5, and partly rutin, which demonstrated the opposite behaviour, were exceptions. The results summarising the maximal ferric ions reducing potential of flavonoids are shown in Fig. 1. The statistical analysis confirmed that the most potent reducing agents were epicatechin and catechin at all tested conditions.

The detailed statistical analysis among flavonols and flavanols (Fig. 2A) and flavones (Fig. 2B) emphasised that there was no direct relationship between the number of hydroxyl groups in ring A and/or ring B, and reducing activity. More likely, the localisation of the hydroxyl groups had the major role. The most efficient structural features included the 3-hydroxy group with catecholic ring B and 5,6-dihydroxy substitution. The role of the 2,3-double bond was variable depending on conditions. Its presence increased the reducing potential at pH 4.5 and decreased it at pH 5.5 and had no influence at non-buffered conditions. Contrarily, the presence of the 4-keto group diminished the ferric ions reducing potential of flavonoids under all conditions. The 3-hydroxyl group alone was not associated with reduction but its presence enhanced the reducing potential of reducing group(s). Interestingly, its

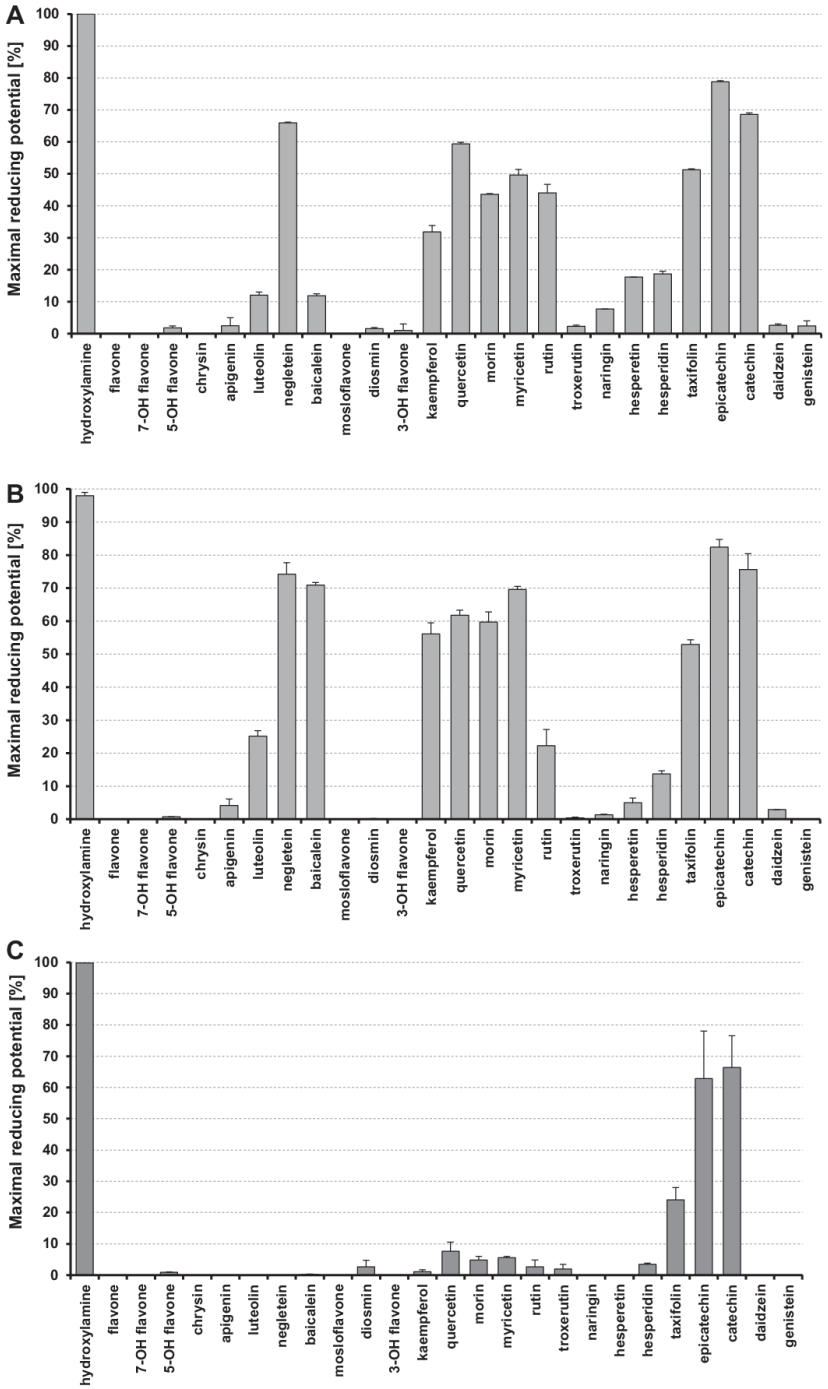

Fig. 1. Summary of the maximal ferric reducing potential of the tested flavonoids. (A) pH 4.5, (B) non-buffered conditions and (C) pH 5.5. The bars show the maximal percentage of the reduced ferric ions. The statistical comparison among the relevant flavonoids is shown in Fig. 2 for better clarity. Naringenin is not shown because the colour of its complex with iron interfered with ferrozine assay.

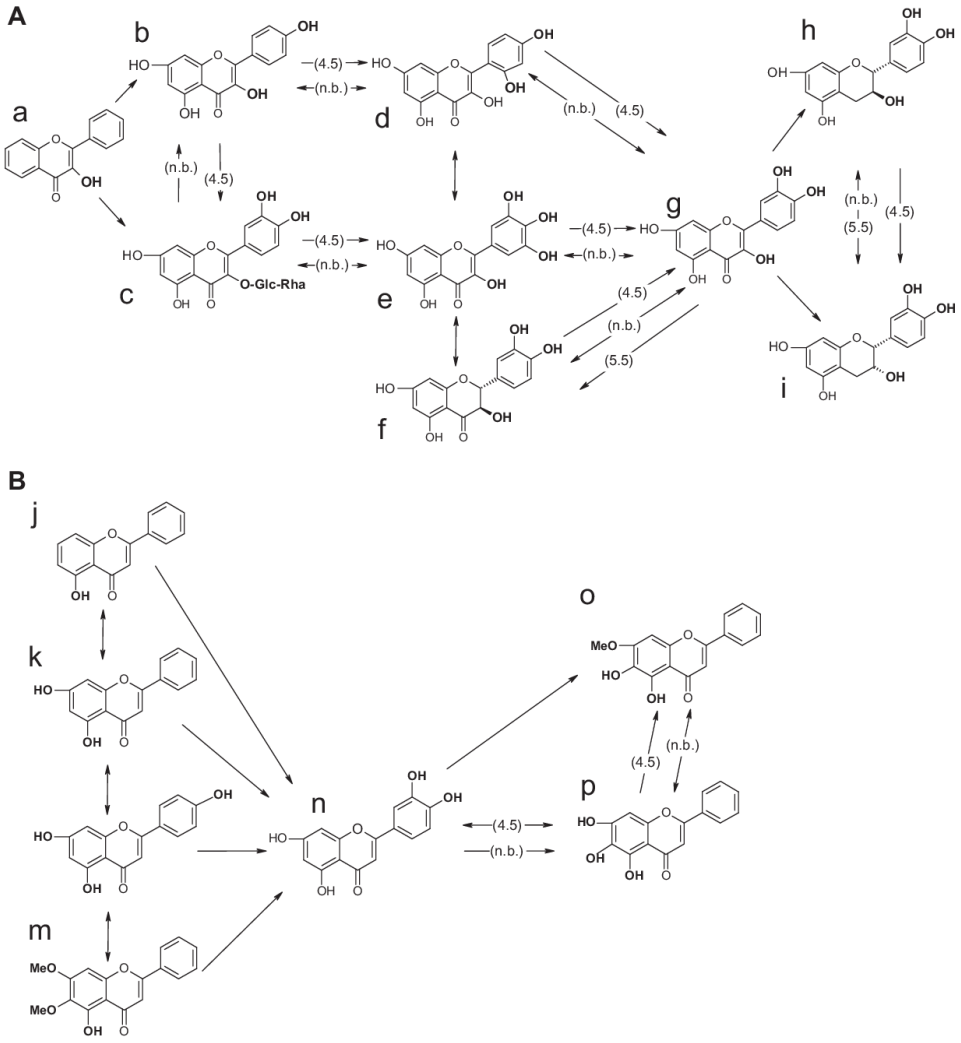

Fig. 2. Differences in the maximal ferric ions reducing potential of flavonols and flavanols (A) and flavones (B). a: 3-hydroxyflavone, b: kaempferol, c: rutin, d: morin, e: myricetin, f: taxifolin, g: quercetin, h: catechin, i: epicatechin, j: 5-hydroxyflavone, k: chrysin, l: apigenin, m: mosloflavone, n: luteolin, o: negletein and p: baicalein. Direction of arrows shows more powerful ferric ions reducing agent (at $p < 0.05$), double-head arrow means no significant difference. Flavonoids on the left side (a, j–m) did not produce any significant reduction potential. Because most of the tested flavonoids did not significantly reduce iron at pH 5.5, comparison at this pH is shown only among taxifolin, quercetin, catechin and epicatechin. n.b. – non-buffered conditions; 4.5 – at pH 4.5 and 5.5 – at pH 5.5.

configuration may possess some influence, since epicatechin was more efficient at pH 4.5 than catechin. The presence of three hydroxyl groups in ring B did not improve the reducing potential, rather a decrease was observed at pH 4.5. The 2′-hydroxyl group increased the reducing potential under acidic conditions but not under non-buffered conditions.

Similar to flavonols, the catecholic B ring was associated with enhanced ferric ions reduction in flavones. But interestingly, in contrast to flavonols, the monohydroxylated ring B did not evoke any significant reduction, supporting the role of the 3-hydroxyl group. The presence of the 5,6-dihydroxy group was linked with a significantly higher ferric ions reducing potential than the catecholic B ring in flavones. Again, the three adjacent hydroxyl groups in ring A did not improve the ferric ions reducing potential. Moreover, a marked decrease was observed under acidic conditions.

The curves illustrating the ferric ions reducing potential of different molar ratios of the tested flavonoid:Fe^{3+} (log scale) followed in most cases the bell-shaped character. This is because their ferric ions reducing potential rose with increasing concentration of the flavonoids up to a peak and thereafter dropped in a similar manner with further increase in the concentration (examples are shown in Fig. 3 and all curves of reducing flavonoids in Supplementary data, Figs. S2 and S3). At pH 4.5 the maximum reduction potential was achieved at a molar ratio of 1:1 (flavonoid:iron). Exceptions were flavones and flavonols with the catecholic or pyrogallol ring B (quercetin, myricetin and luteolin) where the maximum was

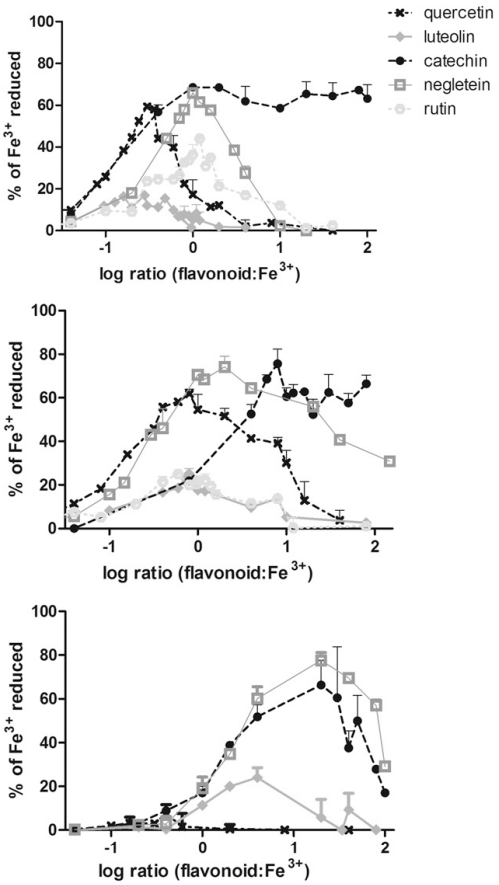

Fig. 3. Representative examples of the curves showing the relationship between log molar concentration ratio (flavonoid:Fe^{3+}) and reducing potential at pH 4.5 (A), at non-buffered conditions (B) and at pH 5.5 (C).

shifted to the left, i.e., between the molar ratio 1:10 and 1:1 of flavonoid:iron (Fig. 3A). Under non-buffered conditions, the maximum reduction potential was mostly seen at the molar ratio 1:1 (Fig. 3B). At pH 5.5 the curves of efficient reducing agents, namely flavanols and taxifolin, had the maximal reducing peak at higher concentration ratios (Fig. 3C).

Because of the considerable reducing potential of the tested flavonoids, we performed additional experiments to assess whether these properties correspond to the intensification of the iron-catalysed Fenton reaction.

There were five types of responses:

- *Progressive antioxidant effect:* the antioxidant effect increased with the concentration of flavonoid. This was a typical behaviour for iron-chelator deferoxamine as a standard substance, but on the other hand, it was rather exceptional in flavonoids. The only cases were 7-hydroxyflavone and hesperetin (Fig. 4A).
- *Low antioxidant:* the antioxidant effects were seen only at very low ratios of flavonoid to iron, but the curve slowly reverted, usually in the proximity of ratio 1:1. Such examples are 3-hydroxyflavone, taxifolin or isoflavonoid daidzein (Fig. 4A, Supplementary data, Figs. S4 and S5).

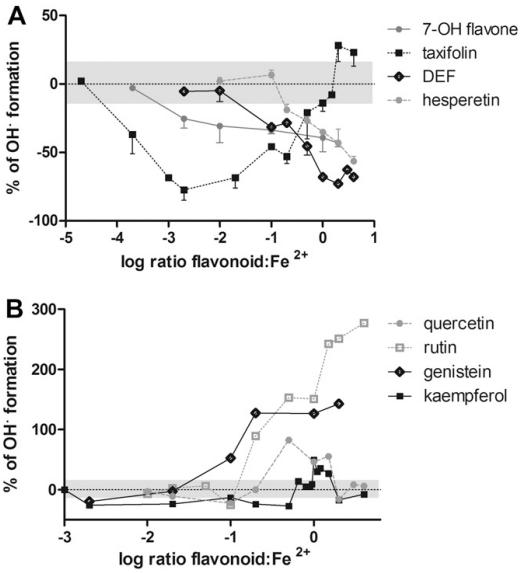

Fig. 4. Prototypical curves of flavonoids and deferoxamine (DEF) showing the influence on the iron-mediated Fenton reaction. (A) Antioxidant effects, (B) pro-oxidant behaviour. In Fig. 4B, SDs are not shown for increased clarity. Grey area indicates the error of the method.

- *Bell-shaped curves:* low concentrations had low or no antioxidant effects while comparable concentrations of iron and flavonoids (i.e. in the proximity of concentration ratio 1:1) were pro-oxidant and further increases in the concentration of flavonoid led to a decrease in pro-oxidation effect or to an antioxidant effect. This behaviour was seen mainly in flavonols; typical examples were kaempferol and quercetin (Fig. 4B). Interestingly, flavone chrysin seemed to follow the same pattern (Supplementary data, Fig. S4C).
- *Progressive pro-oxidant effect:* flavonoids morin, rutin, naringin and genistein fit into this class (Fig. 4B, Supplementary data, Figs. S4 and S5).
- No or negligible effect at all tested concentration ratios – troxerutin and flavanols catechin and epicatechin (Supplementary data, Fig. S5).

While the behaviour of various flavonoids was clearly different from the standard iron chelator deferoxamine, we compared their effects on the Fenton reaction at three different ratios (Fig. 5):

(a) at the concentration ratio 1:10 (compound:iron); under these conditions deferoxamine was only partly efficient since it cannot chelate all iron at this ratio (Mladenka et al., 2010a),
(b) at the concentration ratio 1:1 where deferoxamine should chelate all iron, since it forms complexes with 1:1 stoichiometry. Interestingly, several flavonoids can form complexes with iron at the same stoichiometry (Mladenka et al., 2011),
(c) at the concentration ratio 2:1 since many flavonoids chelate iron at this ratio under acidic conditions (Mladenka et al., 2011). From Fig. 4A it is clear, that in the case of deferoxamine, there was no significant difference between ratios 1:1 and 2:1. This is likely because the excess of deferoxamine was not associated with improved Fenton reaction inhibition.

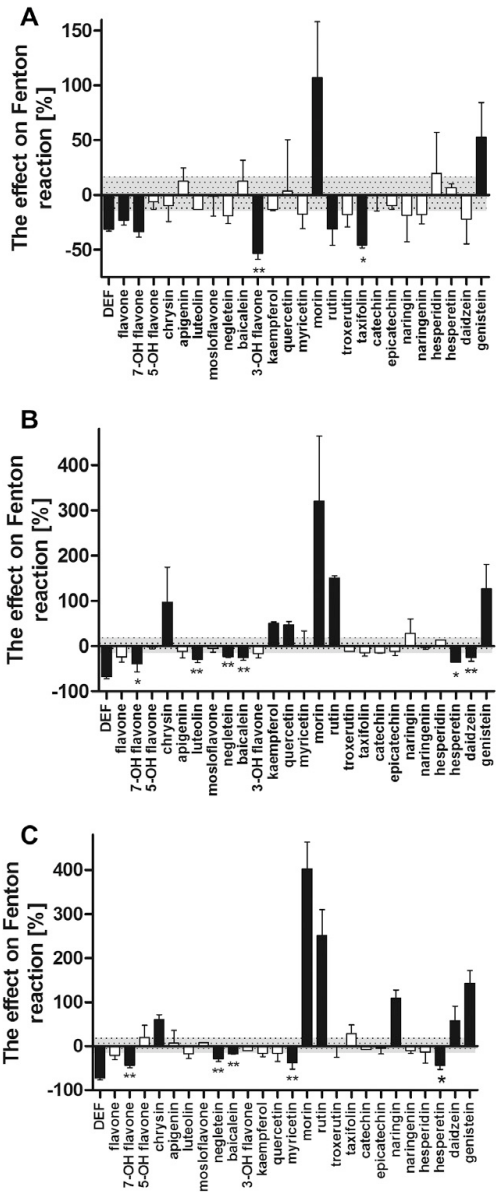

Fig. 5. Effects of flavonoids and deferoxamine (DEF) on the Fenton reaction at the concentration ratios (flavonoid or DEF:iron) 1:10 (A), 1:1 (B) and 2:1 (C), respectively. White bars mean that flavonoid was neither pro-oxidant nor antioxidant at a given ratio (error of the method is shown as grey dotted area). The statistical significance is shown for antioxidant activity vs. deferoxamine: $^{*}p < 0.01$, $^{**}p < 0.001$.

Summarising the described data concerning the Fenton reaction it appeared that monohydroxylated flavonoids, as well as simple flavone, were similarly efficient inhibitors of the Fenton reaction at low ratios; in particular, 3-hydroxyflavone was more efficient than deferoxamine in the ratio 1:10 (compound:iron). Taxifolin was an apparent exception. It was the most efficient inhibitor of the Fenton reaction even at very low concentration ratios (Fig. 4A, 1:1000 – 1:100; taxifolin:iron). But its inhibitory potential dropped with increasing concentrations, although at concentration ratio 1:10 remained more potent than deferoxamine. At the concentration ratio 1:1 and 2:1 (compound:iron), none of the tested flavonoids were more potent than deferoxamine. However, 7-hydroxyflavone, negletein, baicalein and hesperetin were efficient at both mentioned ratios. Contrarily, some flavonoids, in particular morin and genistein, were apparently pro-oxidant at all three selected ratios.

Comparing the data from ferric ions reduction and the Fenton reaction assays, it was apparent that the direct relationship between reduction of ferric ions and potentiation of the Fenton reaction was not confirmed for the majority of flavonoids. Real exceptions were flavonols (Fig. 6 and Supplementary data, Table 1). Flavonols with bell-shaped curves (kaempferol and quercetin) correlated or tended to have correlations between ferric ions reducing properties and intensifying effects on the Fenton reaction. Progressively pro-oxidant flavonols (morin and rutin) showed such correlation at concentrations up the concentration ratio flavonol:iron 1:1 (axis *x*, log = 0 in Fig. 6), i.e. up to their maximal reduction peaks.

4. Discussion

In the past, the antioxidant effects of flavonoids were almost exclusively linked to their hydrogen-donating effects and considered to be identical with their reducing properties. To date, many studies have confirmed and compared the reducing potential of different flavonoids. Because the majority of these studies used the FRAP assay, which is principally based on measuring the amount of reduced ferric ions in their marked excess, the reducing potential of flavonoids appears to be linear (Firuzi et al., 2005). The relevance of this assay in relation to iron is rather low since plasma or cellular concentrations of free iron are negligible at physiological conditions. Even a marked release of free iron after ischaemia (during reperfusion) can hardly be considered as a multiple excess of free iron (Berenshtein et al., 2002). Moreover, in the substantial excess of iron, the FRAP assay is not able to relevantly consider iron-chelating properties of flavonoids, in contrast to the ferrozine methodology analysing both the low and high concentration ratios of the tested compound to iron. Thus we suppose that the bell-shaped curves measured in this study are more relevant for flavonoid-iron interactions. Recently, we have reported that chelation of ferrous ions at pH 4.5 is low with exception of baicalein. On the other hand, some flavonoids, especially those with the free 3-hydroxyl group, are able to chelate ferric ions at the concentration ratio 2:1 (Mladenka et al., 2011). On the contrary, complexes at the concentration ratio 1:1 seem to be unstable at this pH, as can be deduced from the maximal ferric ions reduction (Fig. 5A, Supplementary data, Fig. S2). The importance for the different ratios can be supported by the study of Mira et al. (2002) who measured the reducing potential by the ferrozine methodology at pH 5.5 only at the concentration ratio 1:4 (flavonoid:iron). Hence, the outcome of that study is not identical with our data.

In spite of differences in the reducing curves, the FRAP values appear to correspond with the maximum percentage of reduced ferric ions at both non-buffered condition and pH 4.5. In this assay, flavone or other flavonoids with only one isolated hydroxyl group, either in position 3 or in other positions, did not exhibit any significant reduction potential. Similarly, isolated hydroxyl groups in ring A in flavones (5,7-dihydroxy group in chrysin and methoxy–hydroxy substitution in mosloflavone) or aliphatic hydroxyl groups in troxerutin did not reduce ferric ions, as well. FRAP assays con-

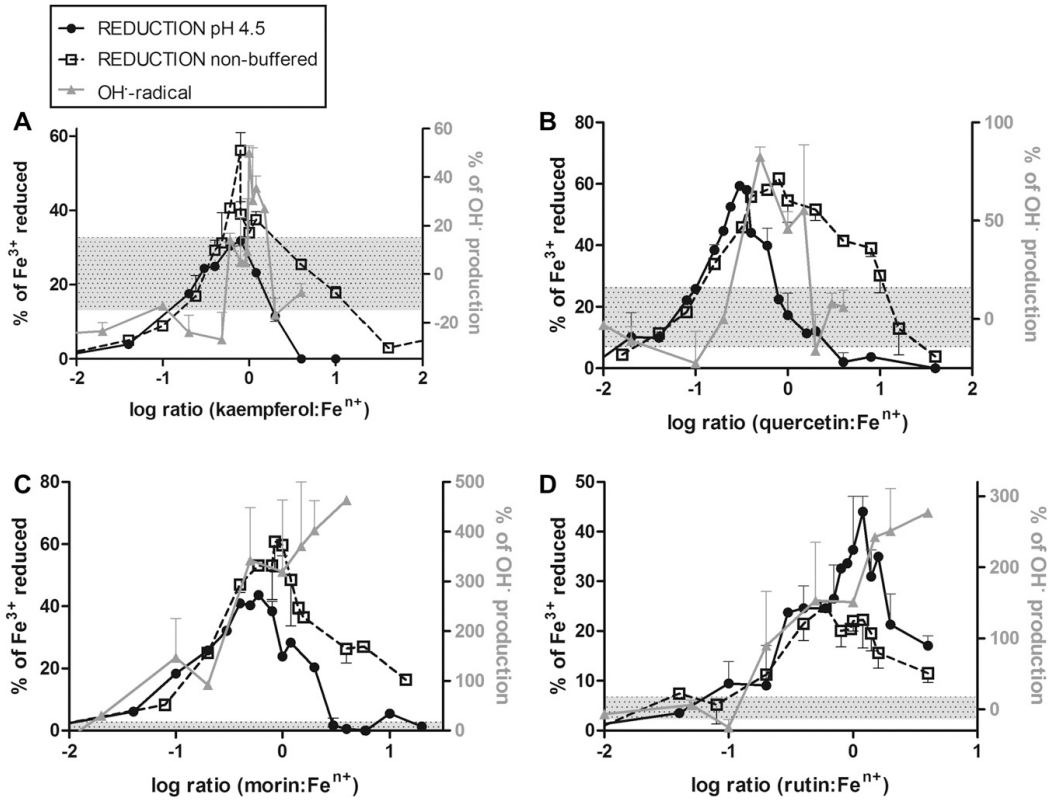

Fig. 6. Relationship between ferric ions reduction and effect on the Fenton reaction in flavonols. (A) kaempferol, (B) quercetin, (C) morin and (D) rutin. Left axis *y* and black curves represent the percentage of ferric ions reduced, right axis *y* and grey line show the influence of the compound on the Fenton reaction. Dotted grey area represents the error of the Fenton reaction assay.

firm that 5-hydroxyflavone and 7-hydroxyflavone together with chrysin do not reduce iron at all but some moderate iron reduction is observed with 3-hydroxyflavone (Firuzi et al., 2005). The difference may consist in the lower pH used in the FRAP methodology. An isolated hydroxyl group in ring B did not reduce ferric ions (apigenin and the tested isoflavonoids), but the presence of 3-hydroxyl group converted non-reducing apigenin into reducing kaempferol. These results are also in accordance with the FRAP results, in which a negligible or no activity is found for apigenin, a very low activity is exerted by isoflavonoids, daidzein and genistein, and a marked activity is observed in the case of kaempferol (Firuzi et al., 2005; Zhang et al., 2011). Flavonoids with the catecholic ring B, in particular flavonols or flavanols with the free 3-hydroxyl group, were very potent reducing agents. In comparison to the catecholic ring, the pyrogallol ring B or A did not increase the reduction potential. Again, those results agree with FRAP assay (Firuzi et al., 2005; Zhang et al., 2011). Similar to pH 4.5 in our assay, myricetin with pyrogallol ring B is less potent than quercetin with catecholic ring B (Firuzi et al., 2005; Zhang et al., 2011). In addition, the methoxy–hydroxy substitution of ring B is on one hand less potent than catecholic ring B, but on the other hand has comparable activity with the pyrogallol ring B (Zhang et al., 2011). The FRAP assay shows that the influence of the 5-hydroxyl group is rather neutral, since fisetin, a quercetin congener without the mentioned hydroxyl group, is only slightly less active than quercetin (Firuzi et al., 2005). Both catecholic rings A and B showed similar reduction potentials. Interestingly, in flavanones, the presence of methoxy–hydroxy substitution of ring B carried some limited reduction potential, which was higher in comparison to one isolated hydroxyl group in ring B. Similar finding shows FRAP (Firuzi et al., 2005; Zhang et al., 2011). The absence of both the 2,3-double bond and 4-keto group improved the reduction potential (quercetin vs. catechin and epicatechin) in our reduction assays but not in the FRAP assay, in which quercetin is by far the most potent reducing agent (Firuzi et al., 2005; Pulido et al., 2000; Zhang et al., 2011). The difference could be probably explained by different pH conditions used in the assays, since pH has been shown to influence the redox potential of polyphenols (Hagerman et al., 1998). It should be also mentioned that endogenous antioxidants are less potent than flavonoids in FRAP assay: (1) uric acid shows only limited reducing potential comparable to that of flavanones with methoxy–hydroxy ring B and (2) ascorbic acid is roughly three times less active than quercetin (Firuzi et al., 2005; Pulido et al., 2000).

One marked exception is baicalein, which was a similarly active reducing agent at non-buffered conditions to its analogue negletein with blocked 7-hydroxyl group by a methyl group. Contrarily, at acidic pH, baicalein was a slightly active reducing agent while negletein remained very active. This difference could be explained by the fact that baicalein is a very active iron chelator, even in comparison to the standard iron chelator deferoxamine under acidic conditions (Mladenka et al., 2011). But its chelating potential under non-buffered conditions is much lower (unpublished data).

Whereas the reduction activity of the tested flavonoids is in sufficient agreement with published studies, on the other hand, effects on the Fenton reaction or, in general, on metal-based oxidation are very different to the published papers, particularly in relation to the used methodology and transient metals (Cao et al., 1997; Laughton et al., 1989; Sugihara, Arakawa, Ohnishi, & Furuno, 1999). Chiefly, studies reported potentiation of the Fenton reaction at neutral pH by use of EDTA–ferric ions. However, this may be pathophysiologically less relevant because pure ferric salt or ferric-ADP or ferric citrate do not intensify the Fenton reaction in the presence of flavonoid, in contrast to EDTA–ferric ions (Laughton et al., 1989). The reason may lie in the solubility. Ferric ions have very low solubility at neutral pH, but EDTA improves it. Indeed, in this study, no reduction of ferric ions by flavonoids was observed at neutral or slightly acidic pH. We aimed to compare the ferric ions reduction potential of the tested flavonoids at relevant pHs with potentiation of the Fenton reaction. This hypothesis was confirmed only in the cases of flavonols, namely quercetin and kaempferol. Both flavonols had similar ferric ions reducing potential curves and the Fenton reaction curves depicting the changes in production of hydroxyl radical (Fig. 6). This behaviour could be explained by the fact that except for their reducing potentials, they are active iron chelators even under acidic conditions (Mladenka et al., 2011). Therefore, at lower concentration they are reducing iron, while at higher one, they are able to firmly chelate iron. In contrast, morin and rutin showed similar ferric ions reduction curves and, at the same time, they had pro-oxidant properties. Rutin, in contrast to all previously mentioned flavonols, does not contain the free 3-hydroxyl group and is less potent iron chelator at acidic conditions. Morin contains the 2′-hydroxyl group which lies in the proximity of the 3-hydroxyl group. Hence, it seems that the presence of the free 3-hydroxyl group is very important in this issue. Although not all outcomes from the study of Sugihara et al. (1999) are in agreement with our study, morin was similarly to this study clearly pro-oxidant in ferrous ions-based (without hydrogen peroxide) lipid peroxidation. Myricetin was an exception since it has not been pro-oxidant at any concentration ratio implying that the presence of the pyrogallol ring B can scavenge hydroxyl radical or inhibit its formation, notwithstanding its powerful ferric ions reducing properties, which were the base of pro-oxidant behaviour in other experiments (Laughton et al., 1989). The manner of action of flavonoids described by the bell-shaped curve was published in few studies in the past; e.g., ferric ions/EDTA/hydrogen peroxide-based assay showed the bell-shaped behaviour of quercetin, with the maximum pro-oxidation peak in the proximity of the concentration ratio 1:1 (quercetin:iron). The pro-oxidation effect with rather a plateau in the case of morin and a dose dependent pro-oxidation effect of naringenin is observed (Yen, Duh, Tsai, & Huang, 2003). The bell-shaped DNA degradation in bleomycin–ferric ions assay is well documented in the case of quercetin and myricetin (Laughton et al., 1989). Interestingly, concerning the copper, flavonoids have been shown to rather inhibit copper-based oxidation, in contrast to ferrous-based oxidation, although reverse bell-shaped curves have been also observed (Cao et al., 1997; Sugihara et al., 1999).

Generally, behaviour of flavonoids towards the Fenton reaction was very different in comparison to the standard iron chelator deferoxamine. Deferoxamine progressively blocked the Fenton reaction in apparent relation to its iron-chelating properties. A dose-dependent inhibition of the Fenton reaction was observed also in the case of 7-hydroxyflavone. There are two important differences: (1) 7-hydroxyflavone is not able to chelate iron (Mladenka et al., 2011) and (2) 7-hydroxyflavone decreased hydroxyl radical production in much lower concentrations, e.g., 1:1000 flavonoid:iron, respectively. Therefore in accordance to the literature, the probable explanation is that 7-hydroxyflavone and other flavonoids are direct scavengers of hydroxyl radical (Bochoráková, Paulová, Slanina, Musil, & Táborská, 2003). In fact, many flavonoids were active inhibitors of the Fenton reaction in very low ratios while pro-oxidant or ineffective in the proximity of ratio 1:1. This likely reflects the fact that the amount of the formed complex in very low ratios did not markedly influence the chemistry of the Fenton reaction and the decisive factor is their scavenging potential. Contrarily, at higher ratios, the complexes of iron with flavonoids are capable of redox cycling and this may balance or even prevail over their scavenging potential. Of interest is the influence of the 7-hydroxyl group since 7-hydroxyflavone and hesperetin were the only mentioned dose-dependent antioxidants. Any modification of these structures, e.g. by a saccharide moiety in the case of hesperidin or addition of the 5-hydroxyl group in chrysin absolutely abolished the inhibitory effect on hydroxyl radical production. The effect of 5-hydroxyl group is of particular interest since hesperetin contains also the 5-hydroxyl group like chrysin but the behaviour is completely opposite. The likely explanation is the different stereochemistry of the molecule. Nearly planar flavones with 5-hydroxyl-4-keto group and 2,3-double bond are able to form unstable complexes with iron even at low pH, in contrast to non-planar flavanones with 5-hydroxyl-4-keto group but without 2,3-double bond, which do not chelate almost any iron in that condition (Mladenka et al., 2011). In addition, the concomitant substitution of ring B markedly modified the activity as naringenin was inactive at most concentrations and its glycoside with blocked 7-hydroxyl group was even pro-oxidant. Although pro-oxidant effects of chrysin were documented by others too, it should be mentioned that hesperetin was shown to be pro-oxidative in a ferric ions/EDTA/hydrogen peroxide based assay (Sugihara et al., 1999; Yen et al., 2003). We suppose that non-physiological addition of EDTA influences the assay since, as was mentioned, it improves solubility of ferric ions at used neutral pH. In general, slight modification of flavonoid structure was associated with very different behaviour, e.g., both flavone apigenin and its corresponding isoflavone genistein did not reduce ferric ions but genistein was apparently pro-oxidant in comparison to neutral behaviour of apigenin. A low effect of apigenin on iron-based pro-oxidation has been documented by others, as well (Sugihara et al., 1999).

Previously, we have shown that baicalein exerted similar potent iron-chelating properties to deferoxamine (Mladenka et al., 2011), and therefore we have suggested it as a promising iron chelator. However, in this study, its inhibition of hydroxyl radical formation was rather moderate, probably reflecting its ferric ions reducing properties. Of interest is that very active reducing agents negletein, catechin and epicatechin did not promote the Fenton reaction. Similarly, another study documented that the influence of catechin on hydroxyl radical formation was rather low (Hagerman et al., 1998). However, depending on the methodological approach, the effect of catechin may be very different, e.g., any effect in the presence of ferric ions and hydrogen peroxide, dose-dependent antioxidant effect in the presence of Fe^{3+}/H_2O_2/ascorbic acid, and pro-oxidant in the presence of Fe^{3+}/EDTA (Chobot, Huber, Trettenhahn, & Hadacek, 2009). In contrast to flavanols, taxifolin with the 4-keto group was a very efficient reductant and its $OH^{\cdot}$-scavenging potential was very high and significant even at ratios 1:1000 (taxifolin:iron). The marked potency of taxifolin and its close congeners in comparison to other flavonoids was also documented towards the copper-based Fenton reaction (Cao et al., 1997; Sugihara et al., 1999). However it seemed to be lost or even reversed at higher taxifolin:iron concentration ratios (Fig. 4A).

Although many flavonoids can behave as pro-oxidant elements, they can be still useful in specific pathological or pharmacological conditions:

(1) Iron absorption is achieved in the proximity of the gastroduodenal junction, where the pH is lower than in the intestine (Donovan & Andrews, 2004). Thus, it is worth considering the results from pH 4.5 or pH 5.5, in which ferric ions were reduced into ferrous, and hence, the improvement in iron absorption may take place. In particular, flavanols catechin and epicatechin seem to be suitable since their reducing potential was preserved at pH 5.5 and their influence on the Fenton reaction was neutral.
(2) As mentioned above, ischaemia with subsequent reperfusion leads to the release of free iron which is redox active and its participation in the Fenton reaction has been observed (Berenshtein et al., 2002). The previous idea that flavonoids may decrease the Fenton reaction consequences does not seem to be absolutely correct since many flavonoids were able to potentiate the chemistry of the Fenton reaction in this study. The 7-hydroxyflavone and hesperetin are the only promising candidates for this pathological state.
(3) The situation in cancer is contrary. The idea that the anticancer activity of flavonoids may be, at least partly, associated with increased ROS production has been suggested (Galati & O'Brien, 2004). The fact that morin and rutin efficiently induced cell apoptosis (Romero, Paez, Ferruelo, Lujan, & Berenguer, 2002) supports outcomes from this study and suggests that pro-oxidant flavonoids may act by this mechanism.
(4) Iron overload conditions – flavonoids are mostly potent iron chelators (Mladenka et al., 2011) and they did not appear to reduce ferric ions at physiological pH.

Interestingly quercetin was shown to extend the life span of nematode *Caenorhabditis elegans* in low doses but to shorten it in higher concentration (Pietsch et al., 2011). This hormetic effect was explained by influence on ROS production. It would be thus of interest to test other flavonoids with different influence on Fenton chemistry using the same model.

Conclusively, a systemic administration of flavonoids with the exceptions of 7-hydroxyflavone and hesperitin appears to have unpredictable impact, because low levels may have antioxidant and higher ones pro-oxidant effects. This may, at least, partly explain the controversy among various studies analysing the effects of flavonoids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.06.107.

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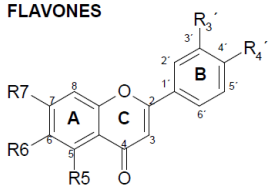
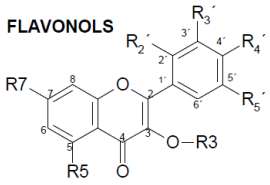
Supplementary data

FLAVONES



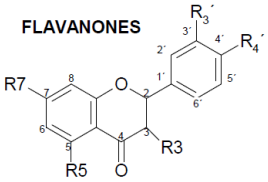
flavones	R_5	R_6	R_7	R_3'	R_4'
flavone	H	H	H	H	H
5-hydroxyflavone	OH	H	H	H	H
7-hydroxyflavone	H	H	OH	H	H
chrysin	OH	H	OH	H	H
apigenin	OH	H	OH	H	OH
luteolin	OH	H	OH	OH	OH
negletein	OH	OH	OCH_3	H	H
baicalein	OH	OH	OH	H	H
mosloflavone	OH	OCH_3	OCH_3	H	H
diosmin	OH	H	O-Glc-Rha	OH	OCH_3

FLAVONOLS



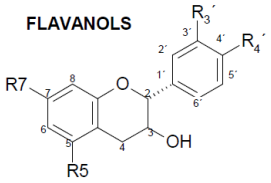
flavonols	R_3	R_5	R_7	R_2'	R_3'	R_4'	R_5'
3-hydroxyflavone	H	H	H	H	H	H	H
kaempferol	H	OH	OH	H	H	OH	H
quercetin	H	OH	OH	H	OH	OH	H
morin	H	OH	OH	OH	H	OH	H
myricetin	H	OH	OH	H	OH	OH	OH
rutin	-Glc-Rha	OH	OH	H	OH	OH	H
troxerutin	-Glc-Rha	OH	O-C_2H_4-OH	H	O-C_2H_4-OH	O-C_2H_4-OH	H

FLAVANONES



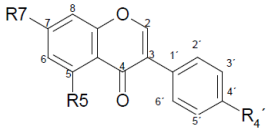
flavanones	R_3	R_5	R_7	R_3'	R_4'	configuration
naringenin	H	OH	OH	H	OH	2RS
naringin	H	OH	O-Glc-Rha	H	OH	2RS
hesperetin	H	OH	OH	OH	OCH_3	2S
hesperidin	H	OH	O-Glc-Rha	OH	OCH_3	2S
taxifolin	OH	OH	OH	OH	OH	2R, 3R

FLAVANOLS



flavanols	R_5	R_7	R_3'	R_4'	configuration
(-)-epicatechin	OH	OH	OH	OH	2R, 3R
(+)-catechin	OH	OH	OH	OH	2R, 3S

ISOFLAVONOIDS



isoflavonoids	R_5	R_7	R_4'
daidzein	H	OH	OH
genistein	OH	OH	OH

Fig. S1. Chemical structures of the tested flavonoids.

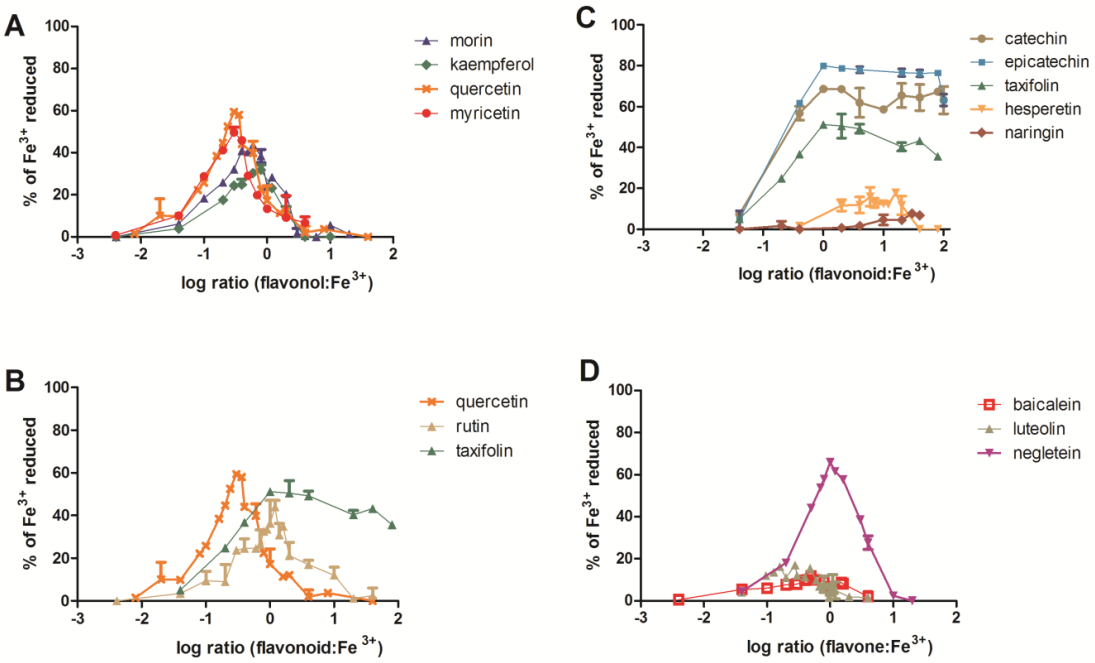


Fig. S2. Iron reduction at pH 4.5. Figure shows active reducing flavonoids (maximum reduction >5%). **A**: flavonols, **B**: flavanols and taxifolin, **C**: effect of 3-OH group and 2,3-double bond, **D**: flavones. Data represents mean ± SD.

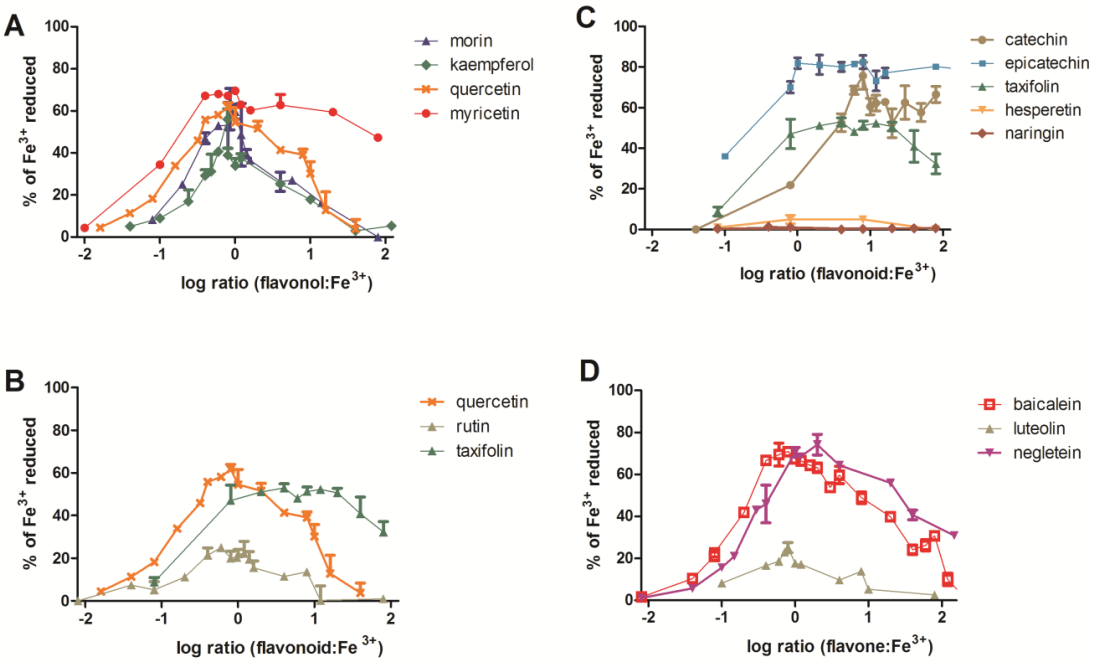


Fig. S3. Iron reduction in non-buffered conditions. Figure shows active reducing flavonoids (maximum reduction >5 %). **A**: flavonols, **B**: flavanols and taxifolin, **C**: effect of 3-OH group and 2,3-double bond, **D**: flavones. Data represents mean ± SD.

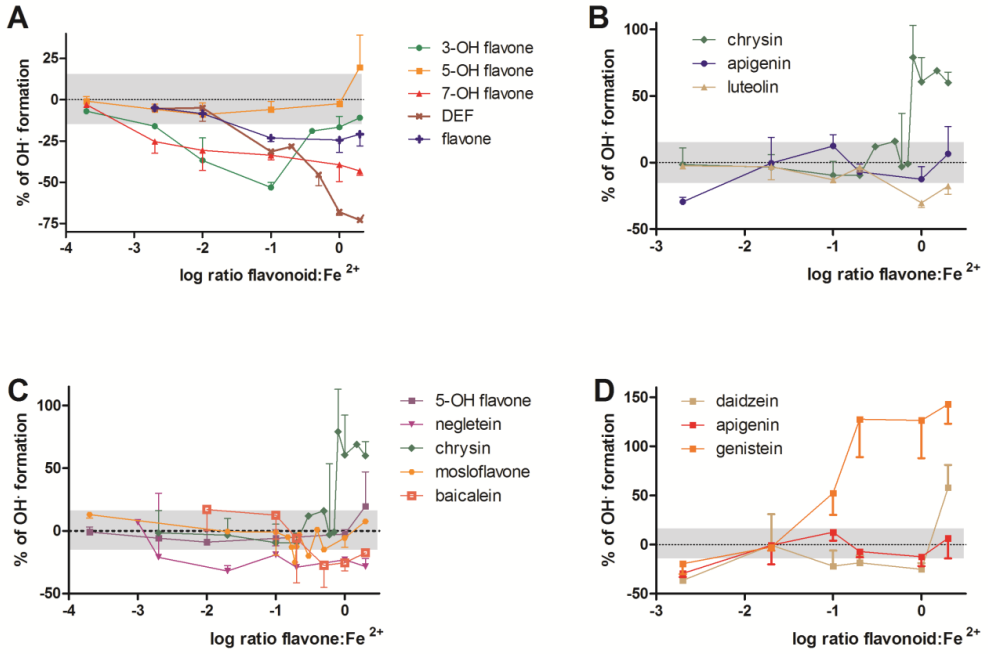


Fig. S4. Effect of flavonoids on Fenton chemistry I. **A**: monohydroxyflavones, flavones and deferoxamine, **B**: influence of hydroxyl groups in ring B in flavones, **C**: influence of A-ring substitution in flavones. **D**: isoflavonoids and apigenin. Data represents mean ± SD.

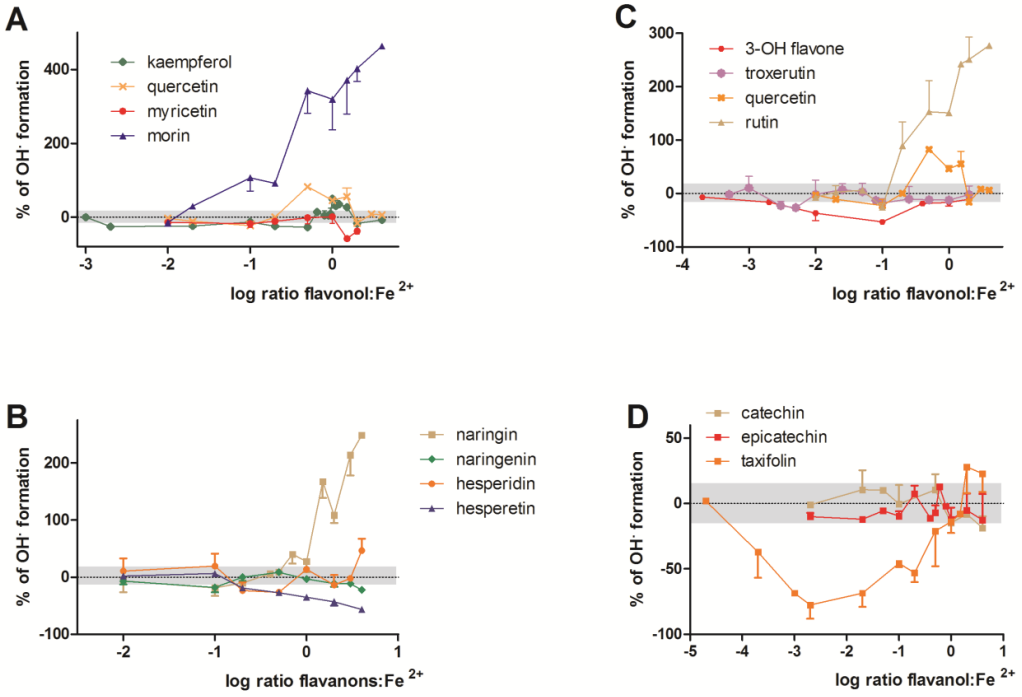


Fig. S5. Effect of flavonoids on Fenton chemistry II. **A**: influence of hydroxyl groups in ring B in flavonols, **B**: influence of the 3-hydroxyl group and 2,3-double bond, **C**: flavanones. **D**: flavanols and taxifolin. Data represents mean ± SD.

Table 1. Correlations between ferric ions reducing potential of flavonols at pH 4.5 or in non-buffered conditions and effect on the iron-catalysed Fenton reaction.

flavonol	**kaempferol**	**quercetin**	**morin**	**rutin**
concentration ratio range	whole tested range	whole tested range	up to 1:1 morin:Fe	up to 1:1 rutin:Fe
pH 4.5	0.66 (p=0.05)	0.31 (p=0.41)	0.82 (p=0.09)	0.87 (p=0.01)
non-buffered conditions	0.46 (p=0.26)	0.64 (p=0.36)	0.91 (p=0.03)	0.84 (p=0.04)

Table summarizes Pearson correlation coefficients as well as their significance. The corresponding curves are shown in the main document *(Fig. 6)*.

4. 3. In vitro characteristics of 1-phenyl-3-methyl-4-acylpyrazol-5-ones iron chelators

FILIPSKÝ, Tomáš, MLADĚNKA, Přemysl, MACÁKOVÁ, Kateřina, HRDINA, Radomír, SASO, Luciano, MARCHETTI, Fabio, PETTINARI, Claudio. In vitro characteristics of 1-phenyl-3-methyl-4-acylpyrazol-5-ones iron chelators. *Biochimie*. 2012, **94**(1), 125-131. ISSN 0300-9084.

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Chelátory železa představují skupinu poměrně strukturně odlišných látek, jejichž společnou vlastností je schopnost vázat železo. V dnešní době tato skupina sloučenin zaznamenává velmi rychlý vývoj nejen díky aplikacím v nových experimentálních indikacích ale i díky rozličným požadavkům na účinnost a bezpečnost léčiv.

Ačkoli mezi dlouho známé podskupiny syntetických chelátorů železa patří 1-fenyl-3-methyl-4-acyl-pyrazol-5-ony, poznatky týkající se jejich biologické aktivity jsou spíše limitované. V této studii jsme se tedy zaměřili na analýzu chelatačních vlastností u vybraných zástupců acylpyrazolonů. Kromě toho byl popsán i jejich vliv na železem katalyzovanou Fentonovu reakci. Ke stanovení účinků byla použita spektrofotometrická ferrozinová metoda, resp. HPLC analýza s použitím kyseliny salicylové jako indikátoru produkce hydroxylového radikálu.

Všichni testovaní zástupci chelatovali železité ionty bez výjimky, ale jejich schopnost vázat ionty železnaté byla silně závislá na acylové substituci. Přestože chelatační vlastnosti vůči železnatým iontům při různých patofyziologicky relevantních pH nebyly výrazněji odlišné, některé sloučeniny prokázaly vyšší účinnost při pH 4,5 než v klinické praxi používaný deferoxamin. Za zmínku stojí prototypová sloučenina H_2QpyQ, tj. 2,6-bis[4(1-fenyl-3-methylpyrazol-5-on)karbonyl]pyridin, jejíž schopnost chelatovat železo rostla s klesajícím pH. Navzdory zmíněným rozdílům v chelatačních vlastnostech většina testovaných acylpyrazolonů patřila mezi velmi účinné inhibitory Fentonovy reakce, jenž lze srovnávat i s deferoxaminem.

Na závěr lze konstatovat, že acylpyrazolony patří mezi účinné látky chelatující železo a do budoucna si větší pozornost rozhodně zaslouží specifický chelátor H_2QpyQ především díky svým chelatačním vlastnostem při nízkém pH.

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Research paper

In vitro characteristics of 1-phenyl-3-methyl-4-acylpyrazol-5-ones iron chelators

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ABSTRACT

Iron chelators represent a group of structurally different compounds sharing the ability of iron binding. The group has been evolving in recent years mainly due to novel experimental indications associated with variable requirements for iron chelators. A group of synthetic 1-phenyl-3-methyl-4-acyl-pyrazol-5-ones has been known for many years but data on their potential biological activity are rather limited.

In this study, we analysed a series of these compounds for their iron-chelating properties as well as for their effects on iron based Fenton chemistry. For the former ferrozine spectrophotometric method and for the latter HPLC method with salicylic acid were used.

All of the tested compounds were very efficient ferric chelators but their ferrous-chelating effects differed according to the acyl substitution. Notwithstanding various ferrous chelation activities, the individual Fe^{2+}-affinities were not significantly different through pathophysiologically relevant pH conditions and some of the tested substances were more potent ferrous chelators at pH 4.5 than clinically used standard deferoxamine. Of particular interest is H_2QpyQ /2,6-bis[4(1-phenyl-3-methylpyrazol-5-one)carbonyl]pyridine/ which iron-chelating affinity increased when pH was decreasing. In spite of ferrous chelation differences, most of the tested acylpyrazolones were similarly active powerful inhibitors of Fenton chemistry as deferoxamine.

Conclusively, acylpyrazolones are efficient iron chelators and H_2QpyQ may represent a prototype of novel specific chelators designated particularly for chelation at acidic conditions.

1. Introduction

Iron chelators include a large spectrum of both natural and synthetic compounds with very different chemical structures [1,2]. Most of tested chelators chelates iron by use of two oxygens and these chelators possess very high affinity for ferric ions in physiological conditions [2,3]. Oxygens for iron binding may originate from various functional groups, which are generally catecholic, aromatic or hydroxamate keto-hydroxy or even carboxyl-hydroxy conformations [1,3]. The keto–keto conformation found in 4-acylpyrazol-5-ones is rather a rare type of iron-chelating site but it has been recently investigated for iron-chelating properties. On the other side, it should be noted that keto–keto compounds evidence enol tautomery [4–6].

Clinical approved indications for iron chelators are limited at the present. Deferoxamine (DEF), deferiprone and deferasirox are used for the treatment of iron excess in overtransfused patients or less commonly in hereditary iron overload diseases and infrequently in acute iron intoxications. Dexrazoxane is used for the prevention of anthracycline cardioxicity but the influence of iron chelation in this indication has been largely discussed [7]. The experimentally investigated use is much larger, it involves the treatment of acute myocardial infarction, neurodegenerative diseases, infection and tumours [8–11]. For some of the mentioned pathological states, specific properties of chelators are needed, e.g. in tumours and acute myocardial infarction, a lower pH condition is playing a significant role, therefore iron chelators have to retain their iron-chelating activity at acidic pH. Recently we showed that DEF is less active ferrous chelator in acidic conditions than in neutral pH [12]. In addition, in tumours iron bound to a chelator may promote the

Abbreviations: DEF, deferoxamine; H_2QpyQ, 2,6-bis[4(1-phenyl-3-methylpyrazol-5-one)carbonyl]pyridine.

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formation of reactive oxygen species through redox cycling in order to destroy malignant cells while this effect is an absolutely excluding criterion for most of other indications [10,13,14].

Acylpyrazolones have been known for more than one century and their interactions with various metals have been well documented, but their use is presently rather analytical and only a few derivatives were tested for their possible effects on human being [6,15,16]. Because it has been suggested that their iron-chelating activity is preserved at lower pH, these drugs seem to be interesting targets for a preclinical *in vitro* analysis. Therefore the aim of this study was 1) to test iron-chelating activity of various substitutions of the basic 1-phenyl-3-methyl-4-acyl-pyrazol-5-one structure at different pathophysilogically important pH conditions, 2) to test whether these substances may enhance redox activity (potentiate hydroxyl radical production by Fenton chemistry), 3) to compare their *in vitro* iron based properties with the standard iron chelator deferoxamine.

2. Materials and methods

2.1. Reagents

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), ferrous sulfate heptahydrate, ferric chloride hexahydrate, hydroxylamine, dimethyl sulfoxide (DMSO), sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), HEPES sodium salt, salicylic acid, 1,4-dioxane, 2,3-dihydrobenzoic and 2,5-dihydrobenzoic acids were purchased from Sigma–Aldrich (Germany). Methanol for HPLC was from J Baker (USA).

Monoacylpyrazolones (Fig.1A) were prepared by following the general method below described for 4-benzoyl-3-methyl-1-phenylpyrazol-5-one (HQ^{Ph}): 3-Methyl-1-phenylpyrazol-5-one (15.0 g, 0.088 mol) was placed in a flask equipped with a stirrer separating funnel and a reflux condenser. Dry 1,4-dioxane (80 ml was added by warming and to the clear solution calcium hydroxide (12.0 g, 0.162 mol) and then benzoyl chloride (13.0 g, 0.090 mol) was added, the latter dropwise for 10 min. The mixture was heated to reflux for 4 h and then poured into 2 mol dm^3 HCl (300 ml) to decompose the calcium complex. A light brown precipitate immediately formed, which was separated by filtration from the solution and dried under reduced pressure at 50 °C. Recrystallisation was performed by treating the solid with hot methanol: slow cooling of the solution afforded a yellow crystalline powder. Its analytical and spectroscopic data agree with those reported in literature [17].

Bis(acyl)pyrazolones (Fig.1B) were synthesized according to the following method for 2,6-bis[4(1-phenyl-3-methylpyrazol-5-one carbonyl]pyridine (H_2QPyQ): To the solution of 15 g (0.088 mol) of 3-methyl-1-phenylpyrazole-5-one in hot dioxane, 12 g (0.162 mol) of $Ca(OH)_2$ were added, and the resulted mixture was refluxed for 30 min. Then to the suspension formed 8.77 g (0.043 mol) of 2,6-pyridinedicarbonyl dichloride was added dropwise followed by refluxing of reaction mixture for 24 h. The precipitate formed was treated by 350 ml of 2 N HCl and then filtered off and, stirred for an hour in concentrated HCl and then recrystallized from methanol in 88% yield. Its analytical and spectroscopic data agree with those reported in literature [18].

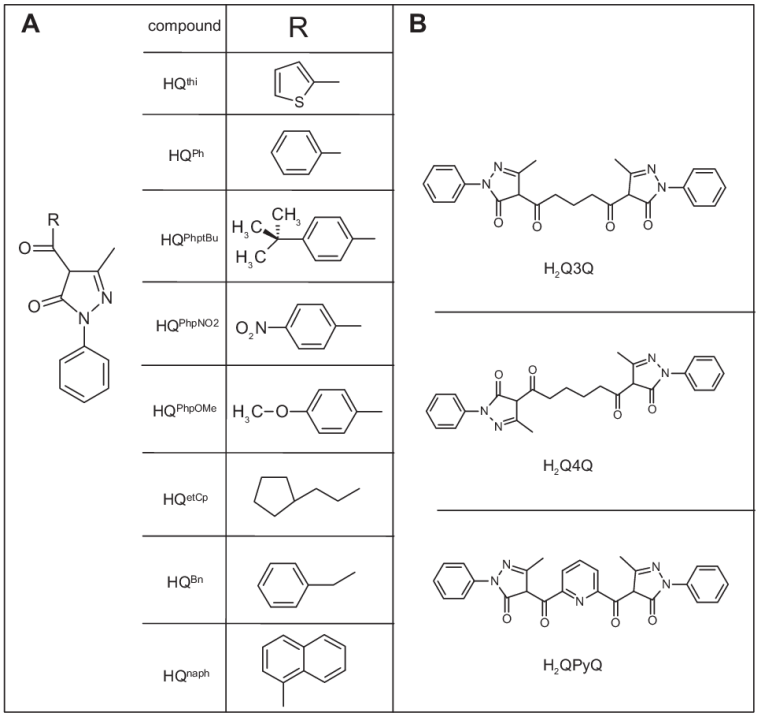

Fig. 1. Structures of pyrazolones tested in this study. A: simple pyrazolones, B: compounds with 2 pyrazolone cores.

2.2. Methods

2.2.1. Assessment of iron chelation

The degree of iron chelation was established by use of ferrozine methodology as previously reported by us [12,19]. Ferrozine forms a magenta coloured complex with ferrous ions and this reaction is specific to these ions because ferric ions do not react with ferrozine [20]. The methodology was extended for the testing of ferric ions or more precisely of total iron chelation in acidic conditions by addition of a suitable reductant, e.g. hydroxylamine. Shortly, in various 15 mM buffers (acetate for pH 4.5 and 5.5 and HEPES for pH 6.8 and 7.5), different concentrations of the tested compound dissolved in DMSO (final concentrations ranging from 10 μM to 10 mM) were mixed with ferrous/ferric ions (final concentration 50 μM) for 2 min. At pH 7.5, hydroxylamine (final concentration 2 mM) was added before ferrous ions in order to ascertain that iron will remain in ferrous form. Similarly, in the case of total iron chelation, hydroxylamine was added owing to the reduction of non-chelated ferric iron and mixed for additional 1 min. Then ferrozine water solution (final concentration 1 mM) was added and absorbance was measured at 562 nm immediately and 5 min later on Anthos Reader 2010 (Anthos Labtec Instruments, Austria). Results were calculated from measurements after 5 min, the difference 5 min vs. immediate measurement was used for the assessment of the complex stability. Concentration of ferrous ions corresponds linearly to the absorbance and was checked in each experiment.

The amount of remaining ferrous ions was calculated from the difference of absorbance between the tested sample (with ferrozine) and its corresponding blank (without ferrozine) divided by the difference of the control sample (the known amount of iron without the tested substance) and its control blank. The normalized dose-dependent curves with 95% confidence intervals were constructed by GraphPad Prism version 5.0 for Windows, GraphPad Software (USA) for each condition (ferrous/ferric iron, pH).

2.2.2. Assessment of ferric reduction

The methodology was very similar to ferric chelation, i.e. various concentrations of tested compounds were mixed with ferric ions (final concentration 50 μM) in different buffers for 2 min. Ferrozine was added thereafter and the colour formation corresponded to the amount of reduced ferric, i.e. ferrous, ions. Hydroxylamine was used only as positive control (100% reduction). Calculation of reduced iron was the same as in the assessment of iron chelation.

2.2.3. Measurement of inhibition of iron based production of hydroxyl radicals

As generally known, ferrous ions with hydrogen peroxide form hydroxyl radicals (Fenton reaction) [21]. Formed radicals may be trapped by use of salicylic acid and ensuing products (2,3-dihydrobenzoic and 2,5-dihydrobenzoic acids) can be detected by HPLC [22]. Shortly, ferrous ions were mixed with the tested compounds in different concentration ratios for 2 min (final concentrations of the tested compounds ranged from 30 μM to 3 mM). Salicylic acid and subsequently hydrogen peroxide (concentration of both substances was 7 mM) were added and the mixture was analysed by HPLC (pump Philips PU 4100, Philips, UK; Eclipse Plus C18, 4,6 × 100 mm, 3.5 μm, Agilent, USA with UV–VIS detector ECOM LCD 2083, Ecom, Czech Republic) with 40% methanol and 0.085% of water solution of phosphoric acid as mobile phase. All experiments were controlled by addition of "internal standard", i.e. the known amount of 2,3-dihydrobenzoic and 2,5-dihydrobenzoic acids.

2.3. Statistical analysis

Results are expressed as mean ± SD. The differences in ferrous chelation were compared by 95% confidence intervals of chelation curves and differences in inhibition of Fenton chemistry by one-way ANOVA test followed Dunnett's multiple comparison test (GraphPad Prism version 5.0 for Windows, GraphPad Software, USA).

3. Results

All of the tested compounds chelated ferrous and ferric iron although with different affinity. There were marked differences in ferrous iron chelation among the tested pyrazolones but interestingly, with exception of H_2QpyQ, there were not significant differences in ferrous chelation in individual pyrazolones through different pH conditions: Fig. 2A shows chelation curves for HQ^{etCp}, an example of majority of tested chelators with stable ferrous chelation potency through various pathophysiologically important pH. H_2QpyQ is an exception in the context of this group because its ferrous chelation potency decreases with increasing pH (Fig.2B). Summarized differences in ferrous chelation are depicted in Fig. 3.

All tested substances were very active ferric chelators, there were only minor differences among tested substances related to the stoichiometry of formed complexes at acidic conditions. All simple pyrazolones (e.g. pyrazolones with only one pyrazolone ring) formed apparently the complexes with ferric iron with the stoichiometry 3:1 (Fig.2C, represented by $HQ^{etCp} - x = 0 \rightarrow$ log ratio HQ^{etCp}:$Fe^{3+} = 0 \rightarrow$ ratio 1:1 $\rightarrow y =$ approximatelly 33%, i.e. in the ratio 1:1 about 33% of iron was chelated – hence ratio 3:1), while compounds with two pyrazolone cores chelated ferric iron in the ratio 3:2 (H_2Q3Q and H_2Q4Q, in Fig.2C $x = 0 \rightarrow y = 75\%$) or 2:1 ($H_2$QPyQ, in Fig.2C $x = 0 \rightarrow y = 50\%$). Additional experiments confirmed that similarly to ferrous chelation, ferric chelation activity of H_2QPyQ was more pronounced in acidic conditions than in neutral conditions (Supplementary data).

Because of marked differences in ferrous chelation, statistical comparison of the tested chelators was performed by use of 95% confidential intervals (example is shown in Fig. 4 and summary in Fig. 5). The less efficient ferrous chelators were thienyl and *p*-nitrophenyl derivates. In contrast, the most effective chelators were doubled structures (H_2Q3Q and H_2Q4Q). When comparing the substitutions of the basic core, the ferrous chelation affinity increased from thienyl through phenyl to naphthyl substitution. Benzyl derivative had the same efficacy as phenyl. Additional substitutions on phenyl ring markedly influenced the affinity ranging from *p*-nitrophenyl (the lowest) through *p*-methoxyphenyl to *p*-*tert*-butylphenyl. The latter compound was the most active ferrous chelator from the tested simple pyrazolones. 2-cyclopropylethyl substitution was also very effective and was comparable to that of naphthyl. In contrast to ferric iron chelation, there was a slight difference between 3-C and 4-C bridged pyrazolones. The former appeared to be more efficient. As mentioned above, the activity of H_2QpyQ was influenced by pH. At pH 4.5 its activity was between those of naphthyl and phenyl, at pH 5.5 it dropped to that of phenyl and at neutral or slightly acidic conditions it was even lower, comparable roughly to those of thienyl. Deferoxamine is a very effective ferrous chelator at pH 5.5–7.5 where its activity was even higher than that of all pyrazolones tested here, but at pH 4.5 its activity was comparable with the activity of H_2QpyQ at this pH, thus about 1 order lower than the most active substances tested in this study (Fig. 3).

Complexes of ferric/ferrous iron with the tested substances were stable again with exception of H_2QpyQ, which loses concentration-dependently iron from its complexes in competition with ferrozine.

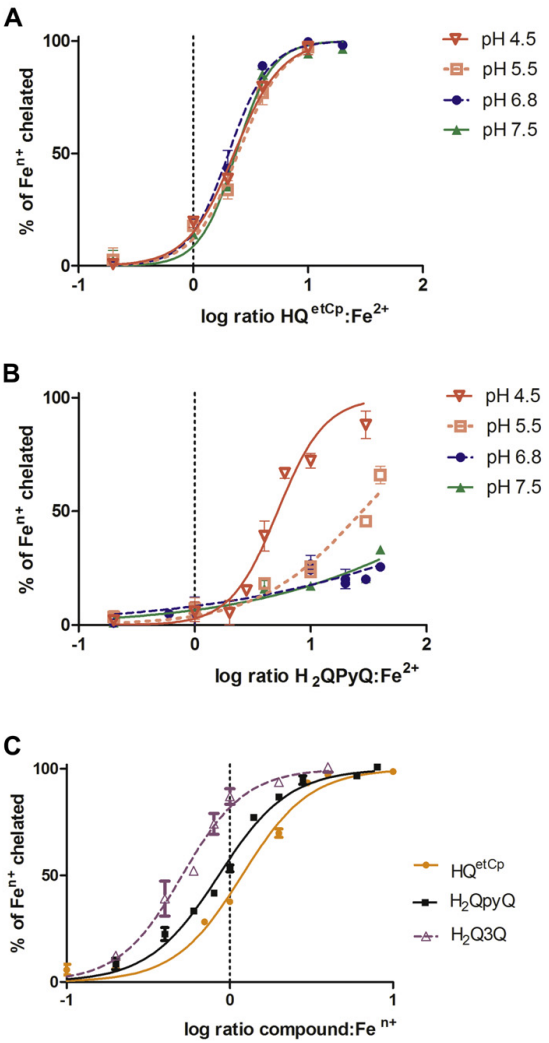

Fig. 2. Iron-chelating curves of the selected pyrazolones. A: ferrous chelation by HQ^{etCp} at different pH conditions. B: ferrous chelation by H_2QpyQ at different pH conditions. C: total iron chelation at pH 4.5. This figure shows the dependence of percentage of chelated iron on the concentration ratio of the tested compound to iron in the logarithmic plot.

None of the tested chelators significantly reduced ferric iron into ferrous one at any of the tested pH conditions (data not shown).

Additional experiments were performed in order to confirm, whether ferrous chelation affinity of these compounds corresponds with inhibition of Fenton chemistry (Fig. 6). Deferoxamine, as well as the majority of the tested pyrazolones, were able to markedly inhibit Fenton chemistry and their effects were almost complete at ratio 2:1 chelator:ferrous iron, respectively. In general, the inhibition of hydroxyl radical formation was dose dependent and majority of pyrazolones showed almost identical behaviour (an example is shown in Fig. 6B). The only exceptions were H_2QPyQ and interestingly *p*-methoxyphenylderivative. H_2Q4Q was not tested at

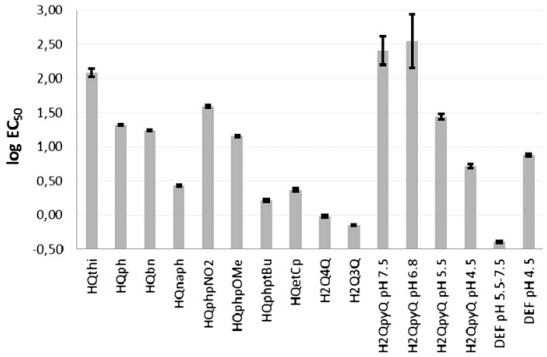

Fig. 3. Orientation comparison of the tested compounds for ferrous chelation by log EC_{50}. Log EC_{50} means common logarithm of concentration ratio of compound:iron at which 50% of iron is chelated. With exception of H_2QpyQ and DEF all compounds have their log EC_{50} determined from iron chelation curves at pH 7.5.

this ratio because of limited solubility, but repeated experiment at ratio 1:10 showed that the substance was the only pro-oxidative compound in this study (Fig. 7). In comparison to DEF, only 2-cyclopropylderivative was more active than deferoxamine.

4. Discussion

The administrations of iron chelators represent the only useful tool for the treatment of iron overload, but on the other hand, excessive iron removal by iron chelators may be disadvantageous [3,23]. Therefore specific iron chelators, e.g. redox or pH sensitive, are desired [24]. In this study, we documented pH dependent iron-chelating properties of H_2QPyQ. This compound was shown to increase its ferrous-chelating properties when pH is decreasing (Fig. 2B), while the previously tested substances with iron-chelating properties are generally more potent ferrous chelators at neutral pH and their ferrous-chelating properties decreases with pH [3,12,19]. H_2QPyQ may therefore represent a novel type of selective iron chelators, which possess only low iron chelation activity under physiological conditions and its iron-chelating activity is activated upon drop in pH. In theory, such pH dependent chelation behaviour may be very useful in the treatment of acute myocardial infarction, when iron is released and pH is dropped.

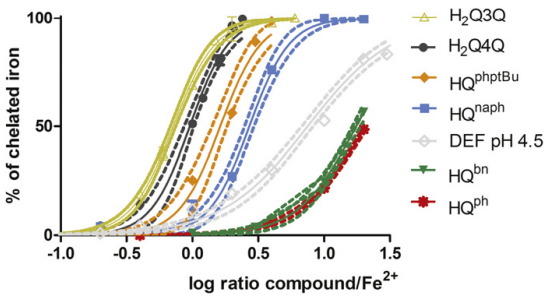

Fig. 4. Comparison of ferrous chelation by selected chelators by 95% confidence intervals. From the left to the right ferrous chelation activity decreases in the following order: H_2Q3Q > H_2Q4Q > *tert*-butylphenyl derivative > naphthyl derivative > benzyl or phenyl derivatives, which had the same ferrous chelation effects. Data represent iron chelation at pH 7.5, for comparison, DEF at pH 4.5 is shown.

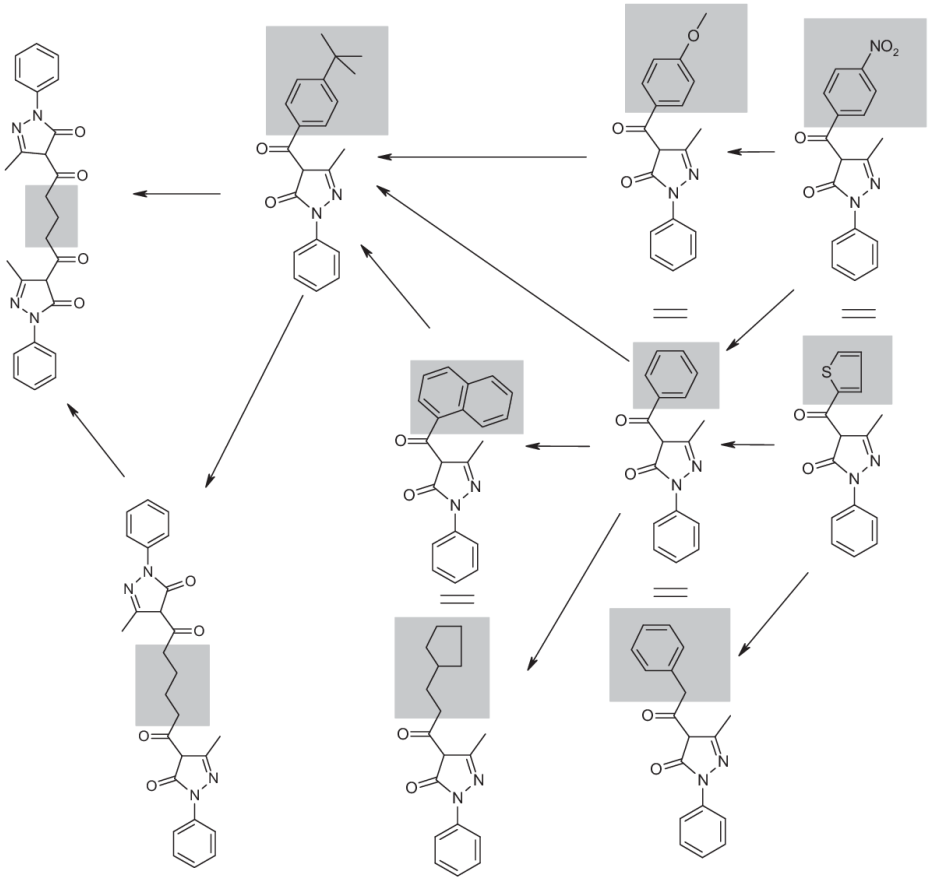

Fig. 5. Summary of ferrous chelation potency. The direction of an arrow means a significantly more potent chelator. H_2QpyQ and DEF are not shown because their ferrous chelation potency alters with pH condition.

Upon recovery of neutral pH, the drug can only slightly chelate iron. On the other hand, H_2QPyQ is rather an early prototype of such drug, because its iron-chelating properties are relatively low, in particular, it chelated ferrous iron with the same potency as DEF at pH 4.5, but much less at pH 5.5, which is likely more relevant in ischaemic tissue where drop of the pH below 6 was observed [13]. In addition, its inhibition of Fenton chemistry was lower than that of DEF and other active pyrazolones (Fig. 7). The chemical reason why H_2QPyQ is more active at acidic conditions is not clear. It may be speculated that under neutral conditions ferrous ions are complexed by nitrogen from pyridine and two adjacent oxygens from keto groups, e.g. the stoichiometry of the complex is 2:1, H_2QPyQ:Fe^{2+}, respectively. But this 2:1 ferrous complex is likely not stable, in contrast to 2:1 ferric complex (Fig. 2). In acidic conditions, ferrous ions cannot be chelated by nitrogen from pyridine group, because this nitrogen is hydrogenated and therefore the chelation site seems to be only two oxygens from keto groups. In additions, if nitrogen is hydrogenated, the enol form formation is facilitated and the complex may be more stable.

The simple acylpyrazolones are considered as bidentate chelators, i.e. they can form complexes with stoichiometry 3:1 chelator:iron, respectively. This was well documented with ferric ions by others and also in this study [6,25]. The stoichiometry of doubled structures apart from the mentioned H_2QPyQ is more complicated. From our results it appears that H_2Q3Q and H_2Q4Q are forming complexes 3:2, chelator:iron respectively. Such stoichiometry was formerly reported for H_2Q4Q [26]. Notwithstanding, information concerning ferric chelation was known, the relevant comparable data on ferrous chelation have not been published as yet. In this study we showed that ferrous chelation affinity differs markedly among individual acylpyrazolones (Figs. 3–5). The differences among ferrous chelation potency are probably primarily explained by different acidity of enol-hydrogens. In particular, *p*-nitrophenyl derivative was among the less efficient ferrous chelator. This compound has likely the most acidic enol-hydrogen because of negative mesomeric and inductive effects of the nitro group. Positive mesomeric and less expressed negative inductive effect of p-methoxyphenylderivative range this compound between p-nitrophenyl and p-*tert*-butylphenyl derivatives. The latter has the less acidic hydrogen due to positive inductive effect of *tert*-butyl group and was the most potent ferrous chelator from the tested simple pyrazolones. Interestingly, despite these differences, all simple acylpyrazolones as well as H_2Q3Q and H_2Q4Q chelated ferrous ions with the same affinity through different pathophysiologically relevant pH conditions. The stability of these complexes

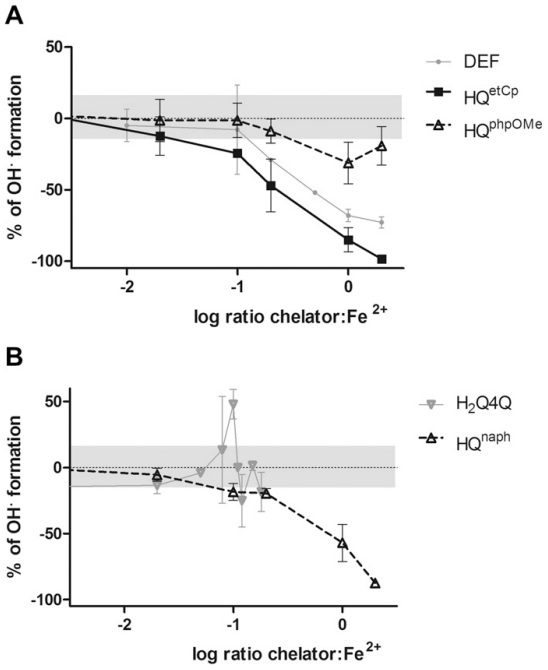

Fig. 6. The effects of the selected iron chelators on Fenton chemistry. A: comparison of active chelators and DEF. B: comparison of active chelator HQ^{naph} and pro-oxidant chelator H_2Q4Q. The graph shows the percentage of hydroxyl radical formation (positive values means potentiation, negative inhibition). The grey area is the error of the method.

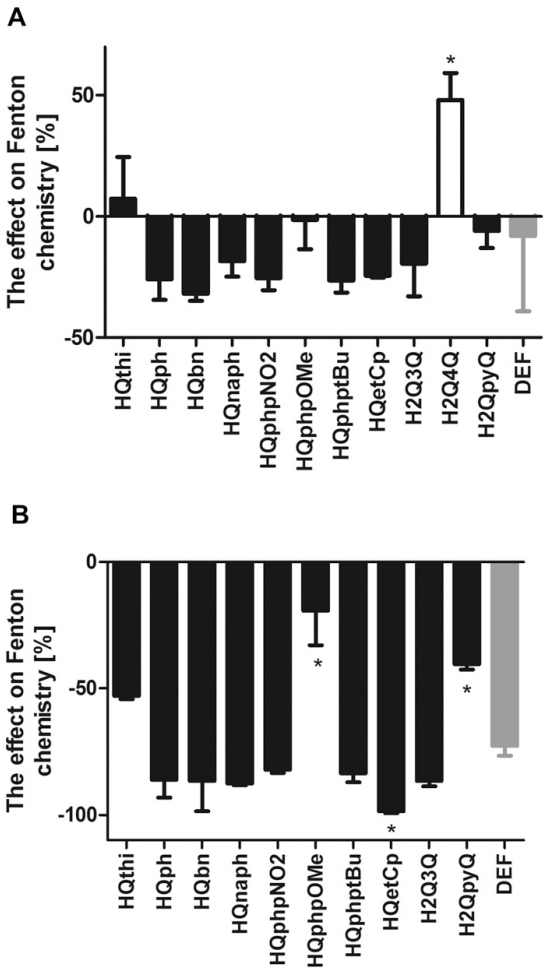

Fig. 7. The effects of the tested chelators on Fenton chemistry in the ratio 1:10 (A) and 2:1 (B) chelator:iron, respectively. Statistical significance: $*p < 0.05$ vs. DEF.

over the large range of pHs was previously demonstrated with other chelators of diketone type [5]. As mentioned above, this is an obvious contrast to DEF which ferrous-chelating properties decrease with pH ([12] and Fig. 3). This may represent an advantage because their ferrous-chelating properties are not influenced by some pathological processes, e.g. acidosis.

A particular disadvantage of some chelators represents the possible iron redox cycling with consequent potentiation of the formation of reactive oxygen species. In principle, the risk may be expressed mainly 1) in non-selective chelators, e.g. those which bind both Fe^{2+} as well Fe^{3+}, 2) in chelators, which are potent antioxidants and may reduce ferric ions into ferrous ions, e.g. polyphenols but as well diketonic curcumin, 3) in chelators, where the chelator cannot absolutely imprison the iron (e.g. small chelators as EDTA or steric hindrance) [3,4,12,27,28]. Therefore, we tested all compounds for their iron reducing properties and their influence on hydroxyl radical formation. None of the tested compound reduced ferric ions and pro-oxidant properties were observed only in the case of H_2Q4Q but in a very narrow range (Figs. 6 and 7). The apparent contrast of partly pro-oxidant H_2Q4Q to antioxidant H_2Q3Q may lie in steric hindrance. According to chelation results from this study, H_2Q4Q seems to form complexes 2:1 with ferrous ions, where iron may be accessible for small molecules like hydrogen peroxide, while H_2Q3Q complexes with 3:2 stoichiometry probably tightly imprison Fe^{2+} rendering it inaccessible. It is of interest that most of the tested chelators inhibited strongly Fenton chemistry in the face of clearly different ferrous-chelating affinity. This finding necessitates additional experiments.

5. Conclusions

This study showed that 1-phenyl-3-methyl-4-acyl-pyrazol-5-ones compounds represent interesting group of iron chelators with high affinity for ferric ions and different affinity for ferrous ions. None of the tested acylpyrazolones was able to reduce ferric ions and most of them were powerful inhibitors of Fenton chemistry. Because of their stable ferrous-chelating properties which are influenced by miscellaneous substitutions, they could be beneficial in different pathologies. In addition, H2QpyQ is a prototype of a specific iron chelator, which iron-chelating properties are increasing while pH is decreasing.

Acknowledgements

The authors wish to thank Mrs. Irena Rejlová for her excellent technical assistance. This work presentation was supported by

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biochi.2011.09.024.

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Supplementary data

Methods

Absorption spectra of H_2QpyQ and H_2QpyQ-Fe^{3+} were measured in various pathophysiologically relevant pH conditions (pH 4.5-7.5, 15 mM acetate or HEPES buffers) in the wavelength range from 220 to 600 nm by use of UV–Vis spectrophotometer Helios Gamma (Thermo Fisher Scientific, USA) equipped with software VisionLite 2.2 (Thermo Fisher Scientific, USA).

Shortly, H_2QpyQ was dissolved in methanol and measured alone or with an excess of ferric ions. The final concentration of ferric ions was 250 μM or 500 μM while the concentration of H_2QpyQ ranged from 20 μM to 60 μM in order to obtain various ratios of ferric ions excess and reasonable absorbance values. Because of instability of ferric chloride solutions in neutral or slightly acidic conditions, experiments were re-performed with ferric tartrate (Sigma-Aldrich, USA). The concentration of free ferric ions was checked by use of ferrozine upon reduction with hydroxylamine before each experiment.

Results

H_2QpyQ UV–VIS spectra showed two absorption maxima at 235 ± 2 nm and 275 ± 5 nm depending on pH. Upon addition of ferric ions to H_2QpyQ, bathochromic shifts of both H_2QpyQ maxima to 247 ± 3 nm and 309 ± 0 nm (pH 4.5) or 287 ± 9 nm (pH 5.5) were observed in acidic conditions suggesting a complex formation (Fig.S1 and S2). At pH 6.8 less expressed shifts were observed (Fig.S3) while at pH 7.5 no clear peak shifts were found (Fig. S4). These results implies that H_2QpyQ Fe^{3+}-chelating activity is better in acidic than in neutral conditions.

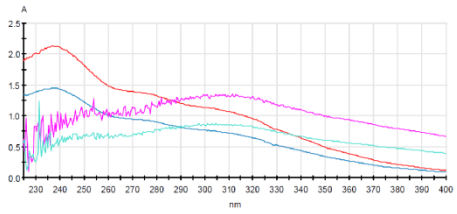

Figure S1. Spectrum of H_2QpyQ with/without ferric ions at pH 4.5. Red line and dark blue line represent H_2QpyQ in concentrations 60 and 40 μM, respectively. Purple line is H_2QpyQ and Fe^{3+} in the ratio 1:6 and light blue H_2QpyQ and Fe^{3+} in the ratio 1:10.

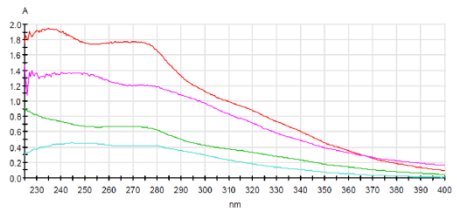

Figure S3. Spectrum of H_2QpyQ with/without ferric ions at pH 6.8. Red line and green line represent H_2QpyQ in concentrations 60 and 20 μM, respectively. Purple line is H_2QpyQ and Fe^{3+} in the ratio 1:6 and light blue H_2QpyQ and Fe^{3+} in the ratio 1:10.

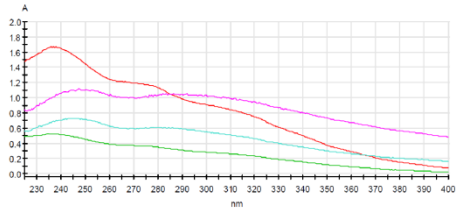

Figure S2. Spectrum of H_2QpyQ with/without ferric ions at pH 5.5. Red line and green line represent H_2QpyQ in concentrations 60 and 20 μM, respectively. Purple line is H_2QpyQ and Fe^{3+} in the ratio 1:6 and light blue H_2QpyQ and Fe^{3+} in the ratio 1:10.

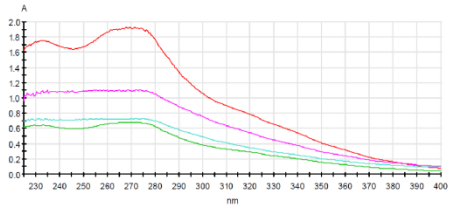

Figure S4. Spectrum of H_2QpyQ with/without ferric ions at pH 7.5. Red line and green line represent H_2QpyQ in concentrations 60 and 20 μM, respectively. Purple line is H_2QpyQ and Fe^{3+} in the ratio 1:6 and light blue H_2QpyQ and Fe^{3+} in the ratio 1:10.

4. 4. Mathematical calculations of iron complex stoichiometry by direct UV–Vis spectrophotometry

FILIPSKÝ, Tomáš, ŘÍHA, Michal, HRDINA, Radomír, VÁVROVÁ, Kateřina, MLADĚNKA, Přemysl. Mathematical calculations of iron complex stoichiometry by direct UV–Vis spectrophotometry. *Bioorganic Chemistry*. 2013, http://dx.doi.org/10.1016/j.bioorg.2013.06.002. ISSN 0045-2068.

(IF 2011 – 1.211)

Látky schopné vázat železo jsou v současné době hojně experimentálně testovány v různých patologických stavech. Jelikož jsou jejich potencionální indikace různorodé, tak i požadavky na jejich fyzikálně-chemické a farmakoterapeutické vlastnosti se liší. Jedním z důležitých faktorů je i stechiometrie vytvořeného komplexu ve vztahu k pH. Kromě toho u některých látek, např. flavonoidů, je stechiometrie komplexů stále nejasná.

Cílem této studie bylo vyvinout nový, rychlý a precizní analytický přístup pro zjištění stechiometrie komplexu za využití UV–Vis spektrofotometrie. Jednotlivé popsané postupy byly validovány na 10 různých látkách při různých patofyziologicky relevantních pH a porovnány s již známou standardní Jobovou metodou.

V případě výrazného rozdílu mezi absorpčním maximem sloučeniny a odpovídajícím absorpčním maximem komplexu bylo dostatečným postupem k určení stechiometrie změření samotné absorbance komplexu při vlnové délce absorpčního maxima komplexu. Nicméně ve většině případů byl rozdíl mezi absorpčními maximy nedostatečný, a proto byly pro výpočet stechiometrie použity matematicky náročnější doplňující metody. S využitím těchto metod byla stechiometrie popsána u všech testovaných látek. Hlavní výhodou těchto postupů oproti standardní Jobově metodě se jeví jejich schopnost určit stechiometrii a případně i kinetiku tvorby komplexu i u slabých chelátorů železa.

Na závěr lze konstatovat, že s využitím nových metod lze nejen lépe vysvětlit mnohá protichůdná zjištění týkající se stechiometrie komplexů, ale i samotné mechanismy chelatace.

Mathematical calculations of iron complex stoichiometry by direct UV–Vis spectrophotometry

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ABSTRACT

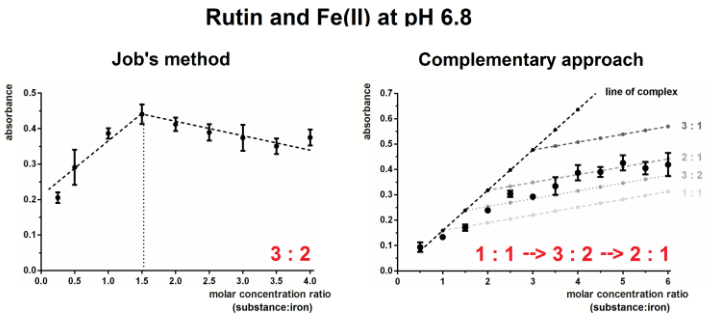
The effects of iron-chelating agents on miscellaneous pathologies are currently largely tested. Due to various indications, different properties for chelators are required. A stoichiometry of the complex in relation to pH is one of the crucial factors. Moreover, the published data on the stoichiometry, especially concerning flavonoids, are equivocal.

In this study, a new complementary approach was employed for the determination of stoichiometry in 10 iron-chelating agents, including clinically used drugs, by UV–Vis spectrophotometry at relevant pH conditions and compared with the standard Job's method.

This study showed that the simple approach based on absorbance at the wavelength of complex absorption maximum was sufficient when the difference between absorption maximum of substance and complex was high. However, in majority of substances this difference was much lower (9-73 nm). The novel complementary approach was able to determine the stoichiometry in all tested cases. The major benefit of this method compared to the standard Job's approach seems to be its capability to reveal a reaction stoichiometry in chelators with moderate affinity to iron.

In conclusion, using this complementary method may explain several previous contradictory data and lead to a better understanding of the underlying mechanisms of chelator's action.

GRAPHICAL ABSTRACT



KEYWORDS

Iron; Chelator; Complex; Job's method; Stoichiometry; Spectrophotometry

HIGHLIGHTS

- UV–Vis spectrophotometric characterization of iron complex stoichiometry
- Evaluation of iron complex stoichiometry by the standard Job's method
- Development of a new complementary approach and corresponding calculations
- The complementary approach may be able to reveal even a reaction stoichiometry

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1. INTRODUCTION

In the past decades, there has been a rapid development of novel iron-chelating agents. Many of them have shown a promising potential in the therapy of iron overload and non-iron overload pathologies [1, 2]. Despite of the enormous interest of investigators, to date, a limited number of iron chelators is clinically used. Deferoxamine, deferasirox or deferiprone are indicated in patients, who require a long-term treatment with blood transfusions in haematological disorders [3, 4]. Moreover, in epidemiologically rare cases, deferoxamine can be clinically used for the management of acute iron intoxication [5]. Dexrazoxane is an approved cardioprotective agent that effectively protects against anthracycline-induced cardiotoxicity although the involvement of its iron-chelating product has recently been questioned [6, 7].

Apart from the above mentioned clinically used iron chelators, there is a wide spectrum of structurally different substances of the both natural and synthetic origin sharing the ability for chelation of Fe(II) and/or Fe(III) [8]. Beyond iron overload conditions, iron-chelating agents are experimentally tested in the prevention/treatment of acute myocardial infarction, neurodegenerative diseases and cancer [1, 4, 7, 9-11].

Thus, in relation to the mentioned approved or examined indications, different properties for iron chelators are required. Activity of iron chelator may be affected by many factors, *e.g.* pH. Differences in pH due to both physiological (*e.g.* in upper part of the intestine, where the absorption of iron occurs) and pathological (*e.g.* ischaemic myocardial tissue and cancer) aspects may significantly influence chelation of iron [12-14].

Moreover, low stability of the complex may allow or even potentiate the reaction of iron with hydrogen peroxide (Fenton chemistry) with the known generation of the most harmful biological oxidant, hydroxyl radical [15]. In order to prevent this reaction, the complex chelator-iron has to be very stable. Complexes with a lower stoichiometry, where all coordination sites are not fully occupied, are more prone to the production of hydroxyl radical, but on the other hand, such complexes may be useful in the therapy of cancer [1, 15]. Hence the knowledge of the stoichiometry of the complex may be also of potential clinical significance.

To date, the characterizations of Fe(II)/Fe(III) complexes at different pH conditions are rather scarce. This may be likely associated with difficulties in a methodological approach, *e.g.* apparently contradictory findings have been published concerning the stoichiometry of the complexes in flavonoids [16, 17]. Therefore, the aim of this study was to develop a simple, precise and rapid UV–Vis spectrophotometric approach usable at different (patho)physiologically relevant pH. Novel mathematical calculations of the stoichiometry, and a standard method of continuous variation, also known as the Job's method, were employed and their advantages and disadvantages disclosed.

2. MATERIALS AND METHODS

2.1. Reagents

Deferoxamine was purchased from Novartis (Switzerland). Deferasirox was isolated from Exjade tablets (Novartis, Switzerland) by extraction with hot ethanol and then precipitation by water. NMR (Varian Mercury-Vx BB 300 instrument, operating at 300 MHz for 1H, 75 MHz for 13C, Palo Alto, CA, USA) and MS spectra (Agilent 500 Ion Trap LC/MS, Santa Clara, CA, USA) of the product were in accordance with literature and elemental analysis (Fisons EA 1110, Milano, Italy) revealed its sufficient purity (calculated: C 67.56, H 4.05, N 11.25; found: C 67.46, H 4.14, N 11.29) [18]. Deferiprone was a kind gift from ApoPharma Inc. (Apotex Inc., Canada), ethylenediaminetetraacetic acid disodium salt (EDTA), 8-hydroxyquinoline, chloroxine, quercetin and rutin were purchased from Sigma–Aldrich Inc. (USA). Pyridoxal isonicotinoyl hydrazone (PIH) and salicylaldehyde isonicotinoyl hydrazone (SIH) were synthesized as was described previously [19]. The tested iron-chelating agents are depicted in Fig. 1.

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazinedisulphonic acid sodium salt (ferrozine), ferrous sulphate heptahydrate, ferric chloride hexahydrate, ferric tartrate, hydroxylamine hydrochloride, sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), HEPES sodium salt were purchased from Sigma–Aldrich Inc. (USA) and methanol from J.T. Baker (Avantor Performance Materials, Inc., USA). Ultrapure water was used throughout this study.

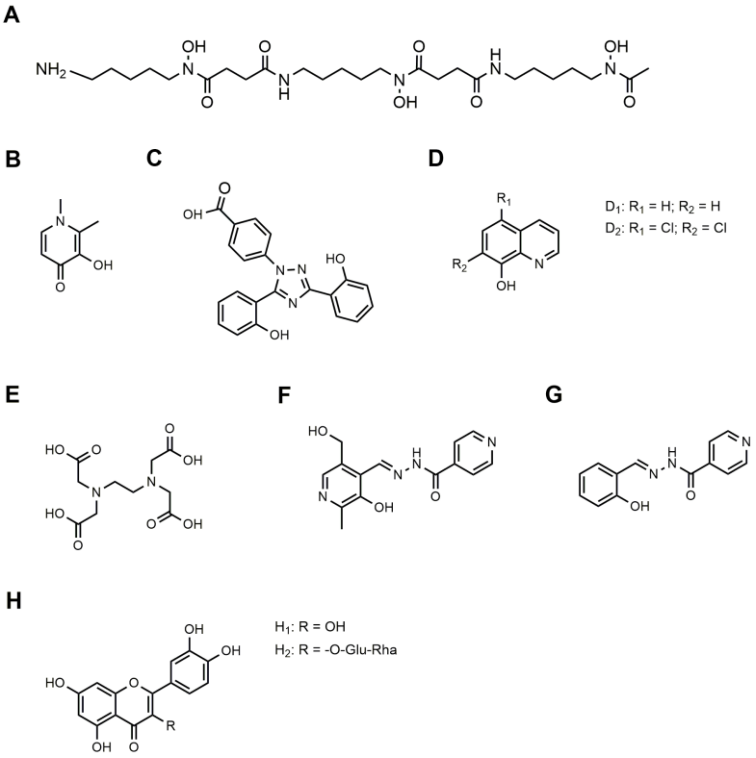

Fig. 1: Iron-chelating agents tested in this study.
Deferoxamine (**A**), deferiprone (**B**), deferasirox (**C**), 8-hydroxyquinoline (**D_1**), chloroxine (**D_2**), EDTA (**E**), PIH (**F**), SIH (**G**), quercetin (**H_1**) and rutin (**H_2**).

2.2. Assessment of iron concentration in stock solutions

Before each experiment, a concentration of iron in stock solutions was routinely checked by a spectrophotometric reagent – ferrozine. Ferrozine specifically reacts with Fe(II) and gives a stable magenta coloured complex with a single absorption maximum at 562 nm [20]. Therefore, an aqueous solution of ferrozine (final concentration 1.67 mM) was used for a direct determination of Fe(II) concentration, which linearly corresponds to the absorbance of the formed complex with ferrozine.

Moreover, the above-mentioned approach was slightly modified for an assessment of Fe(III) by an addition of a reducing agent – hydroxylamine (final concentration 3.33 mM). Afterwards, Fe(III) was reduced into Fe(II) and its concentration was subsequently determined by ferrozine.

2.3. Iron and pH conditions

The assessment of stoichiometry was performed at four (patho)physiologically relevant pH values (4.5, 5.5, 6.8 and 7.5). For the both lower pHs, 15 mM acetate buffers were used, while 15 mM HEPES buffer was used for pH 6.8. Because oxidation of Fe(II) significantly raises in the course of time at pH 7.5, hydroxylamine was added in the final concentration of 5 mM to the HEPES buffer in order to prevent Fe(II) oxidation [21]. For the determination of Fe(III) chelation at pH 7.5, HEPES buffer without hydroxylamine was used.

Two kinds of ferric solutions, *i.e.* ferric chloride hexahydrate and ferric tartrate, were tested because of low solubility of Fe(III) at higher pHs. In this study, ferric chloride hexahydrate was used at pHs 4.5 and 5.5 and ferric tartrate at pHs 6.8 and 7.5.

2.4. Ultraviolet–visible spectrophotometry

All experiments were performed in semi-micro polystyrene or ultraviolet-transparent cuvettes (BrandTech Scientific Inc., The United Kingdom) and absorbance was measured by the use of spectrophotometer Helios Gamma equipped with VisionLite Software 2.2 (ThermoFisher Scientific Inc., USA.).

2.4.1. Assessment of absorption maxima of iron-chelating agent and its complex

Firstly, absorption spectra ranging from 220 to 800 nm with wavelength(s) of absorption maximum(a) of a tested substance (λ_{Smax}) were determined at all pH conditions. Molar absorption coefficients of the substance (ε_S) were calculated according to the Lambert–Beer law. Similarly, a determination of the wavelength(s) of absorption maximum(a) of the complex (λ_{Cmax}) and the corresponding molar absorption coefficients (ε_C) were accomplished by the use of iron excess at different concentration ratios ranging from 1:6 to 1:50 (substance:iron). The blank was composed from a buffer and a solvent of the substance (methanol or water) at the ratio 2:1 in the case of Fe(II). Because the absorbance of Fe(III) disturbed the measurement, the assessment of ferric complexes was slightly modified by an inclusion of ferric aqueous solution into the blank.

2.4.2. Job's method

The Job's method, also known as the method of continuous variation, is a simple analytical approach which is used to the determination of stoichiometry of two interacting components. In this method, the total molar concentration of two reactants is kept constant while their molar concentration ratios are continuously varied throughout the series of samples (Fig. 2A) [22].

Briefly, an aqueous solution of Fe(II) or Fe(III) was mixed for 3 min with a methanolic/aqueous solution of a tested substance at different molar concentration ratios ranging from 1:3 to 6:1 (substance:iron) at all tested pHs and afterwards absorption spectra were immediately measured. The blank was composed from a buffer and a solvent at the ratio 2:1, respectively.

2.4.3. Complementary methods based on mathematical calculations of the stoichiometry

In addition to the standard Job's approach, complementary mathematical calculations were employed. Compared to the Job's method, the total molar concentration of the tested substance was continuously varied, while the molar concentration of Fe(II) or Fe(III) was kept constant throughout the series of samples (Fig. 2B).

The preparation of different molar concentration ratios ranging from 1:3 to 6:1 (substance:iron) was identical to the above described protocol.

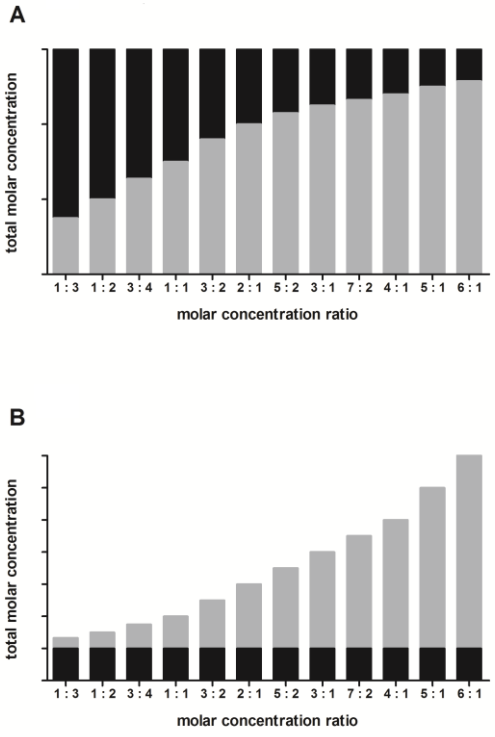

Fig. 2: A schematic depiction of the Job's method (**A**) and the complementary method (**B**).
The gray columns correspond to the molar concentration of a substance and the black columns correspond to the molar concentration of iron. In the Job's method, the molar concentration ratios of the substance to iron are continuously changing while the total molar concentration is kept constant. In the complementary method, there was a constant molar concentration of iron while a molar concentration of the substance continuously varied. The ratios signify substance to iron.

a) Absorbance at absorption maximum of the complex (Method I)

A determination of the stoichiometry according to the Method I was based on a simple evaluation of absorbance of a series of samples at the wavelength of absorption maximum of complex λ_{Cmax}.

b) Symmetry of the absorption maximum of the complex (Method II)

This method was based on a simple assumption that in the theory the absorption maximum of a complex is symmetric, if there are no interfering proximal absorption maxima (Fig. 3). Thus, at similar distance (d) from λ_{Cmax} to the left (λ_{sym1}) or to the right (λ_{sym2}), the absorbance of the complex $A_{C_{\lambda sym1}}$ was proposed to be the same as the absorbance of the complex $A_{C_{\lambda sym2}}$ (eqs. 1-3).

$$\lambda_{sym1} = \lambda_{Cmax} - d \qquad \text{(eq. 1)}$$

$$\lambda_{sym2} = \lambda_{Cmax} + d \qquad \text{(eq. 2)}$$

$$A_{C_{\lambda sym1}} = A_{C_{\lambda sym2}} \qquad \text{(eq. 3)}$$

According to the known additive character of absorbance, a measured absorbance was additively composed from the absorbance of the formed complex and the non-reacted substance at any wavelength. Therefore in the theory, if the absorbance of the complex $A_{C_{\lambda sym2}}$ was sufficient (>0.1) and the absorbance of the substance $A_{S_{\lambda sym2}}$ was zero, the measured absorbance $A_{\lambda sym2}$ should be equal to the absorbance of the complex $A_{C_{\lambda sym2}}$ (eqs. 4 and 5).

$$A_{\lambda_{sym2}} = A_{S_{\lambda sym2}} + A_{C_{\lambda sym2}} \qquad \text{(eq. 4)}$$

$$A_{\lambda_{sym2}} = A_{C_{\lambda sym2}} \qquad \text{(eq. 5)}$$

Thus, the measured absorbance $A_{\lambda sym1}$ was directly used for the assessment of the molar concentration of the non-reacted substance (eqs. 6 and 7):

$$A_{\lambda_{sym1}} = A_{S_{\lambda sym1}} + A_{C_{\lambda sym1}} \qquad \text{(eq. 6)}$$

and because of eqs. 3 and 5:

$$A_{\lambda_{sym1}} = A_{S_{\lambda sym1}} + A_{\lambda_{sym2}} \qquad \text{(eq. 7)}$$

Considering the Lambert–Beer law (eq. 8), the molar concentration of the non-reacted substance (c_S) was calculated as follows (eq. 9):

$$A_{S_{\lambda sym1}} = c_S \times \varepsilon_{S_{\lambda sym1}} \times \ell \qquad \text{(eq. 8)}$$

in which ℓ was the known width of cuvette and $\varepsilon_{S_{\lambda sym1}}$ was the molar absorption coefficient of the substance at λ_{sym1},

$$c_S = \frac{A_{\lambda_{sym1}} - A_{\lambda_{sym2}}}{\varepsilon_{S_{\lambda sym1}} \times \ell} \qquad \text{(eq. 9)}$$

Afterwards, the chelation ratio (X) was calculated according to the eq. 10, in which c_{S_0} was the initial molar concentration of a substance and c_{Fe} was the final molar concentration of iron in the sample.

$$X = \frac{c_{S_0} - c_S}{c_{Fe}} \qquad \text{(eq. 10)}$$

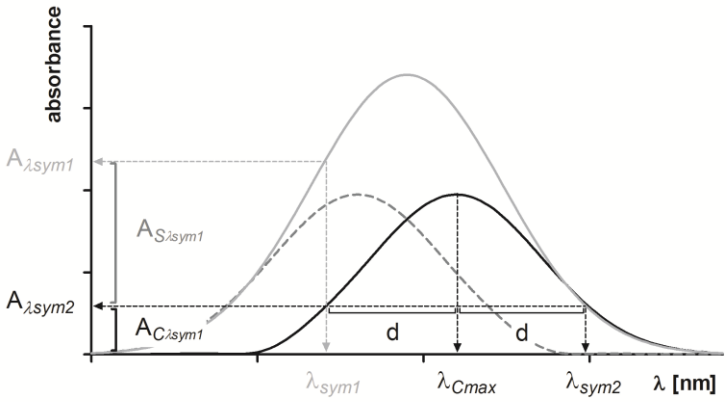

Fig. 3: The method of the symmetry of absorption maximum of the complex.
The measured absorption spectrum (grey curve) is the sum of the absorption spectrum of the non-reacted substance (dark grey dashed curve) and the absorption spectrum of the formed complex (black curve).

c) Calculation using the absorption maximum of the substance (Method III)

A calculation of the stoichiometry using the absorption maximum of the substance was based on the determination of the molar concentration of the non-reacted substance (c_S) likewise in the previous methodology. However, the absorbance at the wavelength of absorption maximum of substance ($A_{\lambda_{Smax}}$) was used (see Supplementary data Fig. S1A). Similarly, $A_{\lambda_{Smax}}$ was the sum of the absorbance of the non-reacted substance and the formed complex (analogously to the eq. 6). Hence, considering the Lambert–Beer law, the molar concentration of the non-reacted substance (c_S) was calculated as follows (eqs. 11-13):

$$A_{\lambda_{Smax}} = c_S \times \varepsilon_{S_{\lambda Smax}} \times \ell + c_C \times \varepsilon_{C_{\lambda Smax}} \times \ell \qquad \text{(eq. 11)}$$

in which $\varepsilon_{S_{\lambda Smax}}$ and $\varepsilon_{C_{\lambda Smax}}$ were the molar absorption coefficients of the substance and the formed complex, respectively, at the wavelength of absorption maximum of the substance λ_{Smax}. The unknown molar concentration of the complex (c_C) was substituted by conversion to the molar concentration equivalents of the substance:

$$c_C + c_S = c_{S_0} \qquad \text{(eq. 12)}$$

And hence the concentration of the non-reacted substance (c_S) was:

$$c_S = \frac{\frac{A_{\lambda Smax}}{\ell} - \varepsilon_{C_{Smax}} \times c_{S_0}}{\varepsilon_{S_{\lambda Smax}} - \varepsilon_{C_{\lambda Smax}}} \qquad \text{(eq. 13)}$$

Afterwards, the calculation of the stoichiometry was accomplished according the eq. 10.

d) Calculation using the absorption maximum of the complex (Method IV)

The calculation of the stoichiometry using the absorption maximum of the complex was analogous to the Method III with one exception that the absorbance was measured at the wavelength of absorption maximum of the complex (λ_{Cmax}) (see Supplementary data Fig. S1B).

e) Theoretical determination of absorbance of the complex at the wavelength of its absorption maximum (Method V)

This method was based on a construction of theoretical lines mimicking absorbance of the most probable stoichiometries. Basically, because the molar concentration of iron was stable throughout the complementary approach (Fig. 2B), absorbance was firstly raising dependently on the formation of complex as long as all added substance reacted with iron and formed the complex (eqs. 14 and 15):

$$A_{\lambda Cmax} = A_{C_{\lambda Cmax}} \qquad \text{(eq. 14)}$$

$$A_{C_{\lambda Cmax}} = c_{S_0} \times \varepsilon_{C_{\lambda Cmax}} \times \ell \qquad \text{(eq. 15)}$$

In a certain point, at which all iron was exhausted, the absorbance $A_{\lambda Cmax}$ raised only dependently on the absorbance of the further added (non-reacted) substance $A_{S_{\lambda Cmax}}$ (eqs. 16 and 17):

$$A_{\lambda Cmax} = A_{C_{\lambda Cmax}} + A_{S_{\lambda Cmax}} \qquad \text{(eq. 16)}$$

$$A_{\lambda Cmax} = c_{eq} \times \varepsilon_{C_{\lambda Cmax}} \times \ell + (c_{S_0} - c_{eq}) \times \varepsilon_{S_{\lambda Cmax}} \times \ell \qquad \text{(eq. 17)}$$

The point of the molar concentration equilibrium (c_{eq}) was at $c_{S_0} = c_{Fe}$ for stoichiometry 1:1, $c_{S_0} = 2 \times c_{Fe}$ for stoichiometry 2:1, etc. The comparison of the measured absorbance with these theoretical lines was able to reveal the searched stoichiometry or even the reaction stoichiometry at different molar concentration ratios (Fig. 4). At the wavelength of absorption maximum of the substance, the identical approach was used.

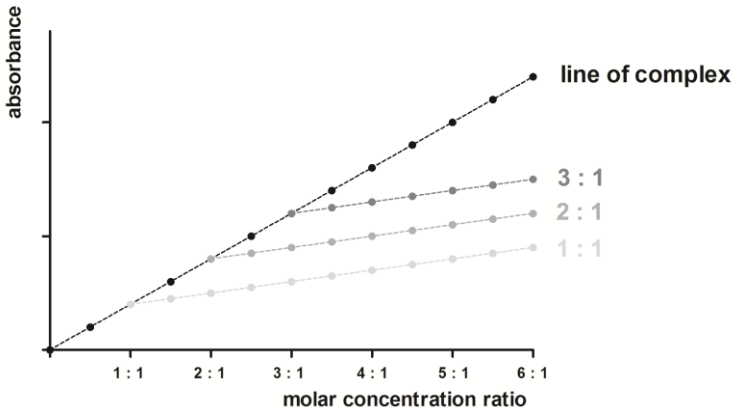

Fig. 4: Theoretical determination of absorbance (Method V).
The black line corresponds to the absorbance of the formed complex at the excess of iron. The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron.

f) Theoretical determination of the sum of absorbance of the non-reacted substance and complex at absorption maximum of substance (Method VI)

This calculation was similar to the previous methodology based on the construction of theoretical lines mimicking the absorbance of the most probable stoichiometries.

The principle was the same, *i.e.* firstly, the absorbance depended on the formation of complex up to the point, in which the whole iron was consumed for the formation, and thereafter the absorbance was dependent only on the added (non-reacted) substance (eqs. 14-16). But in the contrast to the Method V, it presumed that diverse complexes with different molar absorption coefficients were formed in the excess of iron. Therefore, the absorbance was not rising linearly up to the concentration equilibrium. Thus, the lines depending only on the non-reacted substance were constructed directly from the measured absorbance at the most probable chelation ratios (1:1, 2:1, 3:1, *etc.*).

The identical approach was used at the wavelength of the absorption maximum of the complex.

2.5. Data analysis

The majority of experiments, in particular those that determined unclear stoichiometry, were performed at least in duplicates with two different stock solutions. On the other hand, some experiments, which gave unequivocal outcomes, were performed as a single measurement after the concentration of both reagents was calibrated.

Data are expressed as means ± SD. In appropriate cases, a single measurement is depicted for better lucidity in figures.

3. RESULTS AND DISCUSSION

First, the absorption spectra of all 10 analysed substances and their iron complexes were measured and compared. The tested substances varied markedly in the positions of their absorption maxima and in the arithmetic differences between the absorption maxima of the pure substance and its corresponding complex. In almost all tested cases at pH ≥ 5.5, there were no apparent differences in absorption maxima between Fe(II) or Fe(III) complexes. This suggests that only one type of iron-substance complex was formed and thus the data were summarized. Since ferrous ions may be oxidized in the complex with strong iron chelators under physiological pH, ferric complexes were likely formed [23].

Quercetin was an exception because there was a marked difference between the absorption maximum of the complex at different pH conditions (Tab. 1), which is in agreement with the previously published data [24].

substance	λ_{Smax} [nm]	λ_{Cmax} [nm]	λ shift [nm]
8-hydroxyquinoline	240 ± 0 307 ± 1	250 ± 0 355 ± 0 456 ± 0 575 ± 0	10-268
chloroxine	247 ± 0 278 ± 1 316 ± 2	261 ± 1	14
deferasirox	246 ± 0 293 ± 1	309 ± 2	14
deferiprone	280 ± 0	227 ± 1 289 ± 3	9
deferoxamine	225 ± 1	430 ± 0	205
EDTA	×	256 ± 0	×
PIH	218 ± 2 296 ± 1 342 ± 1	233 ± 3 310 ± 2 367 ± 2 463 ± 2	14-123
quercetin	370 ± 0	434 ± 2[a] 443 ± 3[b]	64-73[a,b]
rutin	356 ± 2	401 ± 5	45
SIH	218 ± 5 288 ± 0 330 ± 0	231 ± 7 303 ± 0 350 ± 0	13-20

Tab. 1: Summarized wavelengths of absorption maxima of the tested substances and their complexes with iron.

λ shift means the difference between absorption maximum(a) of the tested substance and the complex with iron. Quercetin: [a]at pH 4.5-6.8; [b]at pH 7.5. × - no absorption maximum of EDTA was found.

At pH 4.5 the iron-chelating activity differed among the tested substances. 8-hydroxyquinoline, SIH, PIH, quercetin and rutin had low affinity for Fe(II) but not for Fe(III) at this pH.

In three iron-chelating agents, there were marked shifts of the absorption maximum(a) of the pure substance and the formed complex (deferoxamine and 8-hydroxyquinoline) or the pure substance (EDTA) did not absorb in the measured range of wavelengths. In these cases, the analytical approach was not complicated. The Job's method or Method I was sufficient for the assessment and no additional approaches were necessary (see summarized data in Tab. 2). Examples are shown for deferoxamine (Fig. 5 and see Supplementary data Fig. S2), EDTA (see Supplementary data Fig. S3) and 8-hydroxyquinoline (see Supplementary data Fig. S4). The EDTA-iron complex's absorption maximum was localized at the low wavelength (256 nm), at which Fe(III) is known to exert some absorbance. But this fact did not interfere with the assessment in low concentrations of iron and EDTA. The resulting stoichiometries are in full accordance with available literary data, since both deferoxamine and EDTA are hexadentate iron chelators, and hence 1:1 ratios, as expected, were confirmed in this study as well [1, 15]. 8-Hydroxyquinoline formed 3:1 complexes which is in agreement with the bidentate nature of this iron-chelating agent [25].

In all other tested substances, all described methodological approaches were applied (see summarized data in Tab. 2).

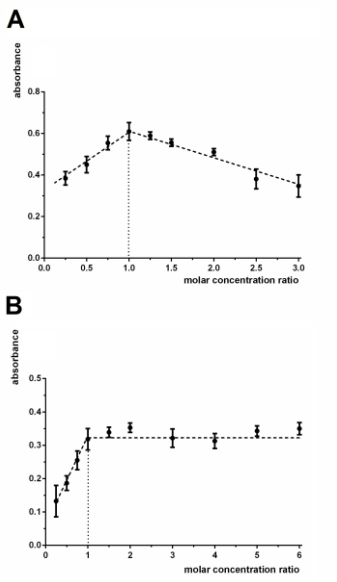

Fig. 5: Assessment of deferoxamine-Fe(II) complex.

Figure shows the standard Job's method (**A**) and simple method I (**B**) at pH 7.5. Absorbance was read at λ_{Cmax} (430 nm). The ratios signify substance to iron.

The total molar concentration of deferoxamine and iron was 0.5 mM for the Job's method. In the Method I, the final molar concentration of deferoxamine was from 0.025 to 0.6 mM while that of iron was constantly 0.1 mM. The assessment was performed in duplicates.

substance/method	Job's	I	II	III	IV	V	VI
8-hydroxyquinoline	✓	✓	-	-	-	-	-
chloroxine	✓	✓	✓	✓	✓	✓	✓
deferasirox	✓	×	×	×	×	✓	✓
deferiprone	✓	×	✓	×	×	×	✓
deferoxamine	✓	✓	-	-	-	-	-
EDTA	✓	✓	-	-	-	-	-
PIH	✓	✓	(✓)	×	×	✓	✓
quercetin	✓	✓	(✓)	(✓)	(✓)	✓	✓
rutin	✓	×	(✓)	(✓)	(✓)	✓	✓
SIH	✓	×	(✓)	×	×	✓	✓

Tab. 2: Summarized results of the described methodological approaches.

✓ successful method, (✓) partially efficient method, × unsuccessful method, - the analysis was not performed.

Partially efficient method means that the method was able to suggest the correct stoichiometric ratio but the measured points were not in the full agreement with the theoretical lines.

The Method I, based on the use of the wavelength of the absorption maximum of complex (λ_{Cmax}), was able to successfully determine the chelation stoichiometry in several other cases due to the character of the absorption spectra (chloroxine, quercetin and PIH), but not in other tested chelators (deferasirox, rutin and SIH; Supplementary data Figs. S5, S7 and S9).

The Method II was able to clearly identify the stoichiometry in chloroxine (Supplementary data Fig. S8E) and deferiprone. In several cases (Tab. 2), the method suggested apparently the correct stoichiometric ratio, but the resulting graph did not fit in the expected scheme. This was the case of quercetin or SIH, where the ratio 1:1 and 2:1, respectively, was suggested but the chelation lines were not identical with the theoretical lines (Fig. 6A and Supplementary data Fig. S7E). In the case of deferasirox, the Method II completely failed. The Methods III and IV were even less efficient and gave the clear result only in the case of chloroxine (Supplementary data Fig. S8).

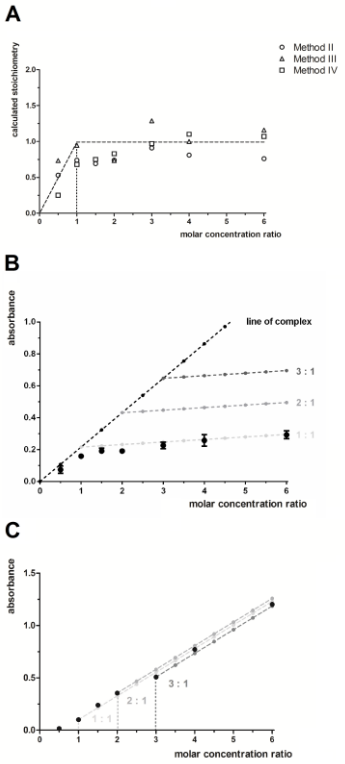

Fig. 6: Quercetin and Fe(III) at pH 7.5.

Complementary approach – the plots of Method II, III and IV (**A**), the plot of Method V (**B**) and the plot of Method VI (**C**), in which all lines are almost identical suggesting the formation of the complex at the stoichiometry 1:1. The final molar concentration of iron was 0.01 mM and the final molar concentrations of quercetin were 0.005-0.06 mM.

In Fig. 6B, the light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. In this figure, tsingle measurements are depicted for better lucidity. In this figure, the single measurements are depicted for better lucidity.

The reasons for the success/failure of the Methods II, III and IV likely consisted in three factors: (a) a small difference between wavelengths of absorption maxima of the substance and its complex with iron, (b) a presence of another absorption maximum in the proximity of analysed absorption maximum and (c) a low difference between molar absorption coefficients of the substance and its complex at analysed wavelength(s).

The stoichiometries of the both tested flavonoids, rutin and quercetin, were identified by all Methods II-VI suggesting that the wavelength difference of 45 nm between the tested substance and its complex was sufficient for these methodologies. In all other compounds (chloroxine, deferasirox, deferiprone, SIH and PIH), the difference was much lower ranging approximately from 9 to 25 nm. One exception was PIH, in which a distant absorption maximum at 463 nm (difference 121 nm) was observed as well. But at this wavelength, absorbance of the complex was very low (Supplementary data Tab. S1) which did not enable the precise

calculation. As mentioned previously, the methods III and IV failed with an exception of chloroxine in all mentioned substances suggesting that the difference between wavelengths (factor a) is the principal factor for the applicability of those methods.

Although the difference between wavelengths in the case of chloroxine was quite small, comparable to deferiprone, and smaller than PIH or SIH, these methods were applicable. The reason likely lay in the steepness of the absorption maxima, *i.e.* the differences between the molar absorption coefficients (factor c; Supplementary data Tab. S1). The second factor (b), the presence of a close absorption maximum, may substantially contribute to the failure as well. This was apparently true for deferasirox, PIH and SIH (Supplementary data Figs. S5A, S7A and S10A, respectively).

The Methods V and VI were the most efficient and were able to reveal the stoichiometry in all cases. The only exception was deferiprone in the case of Method V. The reason for failure of Methods III, IV and V in the case of deferiprone, in contrast to successful Methods II and VI, can be likely explained by the dependence of the former methods on the assessment of molar absorption coefficients of complex. The probable explanation was the very small difference between the wavelengths of pure deferiprone and its complex (9 nm), which blunted the correct calculation of the molar absorption coefficient of the complex. Indeed, there was almost no difference between the molar absorption coefficient of pure deferiprone and its complex at the λ_{Cmax} (Supplementary data Tab. S1).

Using the Job's method and the complementary mathematical calculations for the determination of chelation stoichiometry, it was disclosed that all tested substances chelated iron in various manners and the obtained ratios were consistent with the previously published data – deferasirox 2:1 (Fig. S5) [15, 26]; quercetin 1:1 (Fig. 6 and Supplementary data Fig. S6) [27]; SIH 2:1 (Supplementary data Fig. S7) [28-30]; chloroxine 3:1 (Supplementary data Fig. S8) [25]; rutin 1:1, 3:2 and 2:1 (Fig. 7 and Supplementary data Fig. S9) [16, 17]; PIH 2:1 (Fig. 8, Supplementary data Fig. S10) [28-30]; and deferiprone 3:1 (Supplementary data Fig. S11) [28].

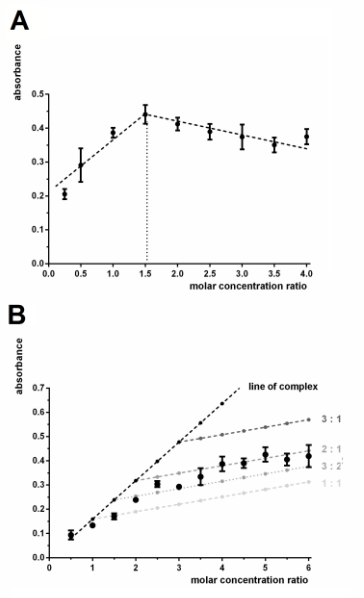

Fig. 7: Rutin and Fe(II) at pH 6.8.

Job's method – the Job's plot at λ_{Cmax} (404 nm) (**A**).The total molar concentration of rutin and iron was 0.05 mM.

Complementary approach – the plot of Method V (**B**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of rutin were 0.005-0.06 mM.

In Fig. 7B, the black line corresponds to the absorbance of the formed complex at the excess of iron. The light grey line mimics the absorbance at the stoichiometry 1:1, the dotted grey line at 3:2, the dashed grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. The assessment was performed with 4 new stock solutions.

Another feature of the theoretical methods, especially of the Method V, was demonstrated within the assessment of rutin's stoichiometry at pH 6.8. Comparing the proposed values for absorbance with the measured ones, it was found that rutin may chelate iron at various chelation ratios (1:1, 3:2 and 2:1, rutin to iron, respectively) depending on its concentration (Fig. 7B). On the other hand, the Job's method showed only one ratio 3:2 (Fig. 7A). Comparing these two different methodological approaches led to

a slight superiority of the complementary approach. Therefore, this finding may explain the diverse results in flavonoids from the different studies [16, 17].

This method is not able to determine the iron oxidation status in the complex. Notwithstanding this limitation may be of importance for chemical screening of novel specific ferrous chelators, this is of lower importance for a pharmacological study. The main question in the later is the efficacy of a tested substance to chelate ferrous or ferric iron while the oxidation status of the complex is of secondary importance.

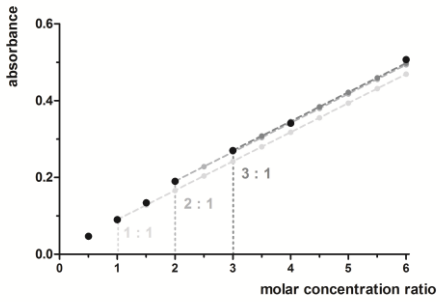

Fig. 8: PIH and Fe(III) at pH 7.5.

Complementary approach – the plot of Method V. The final molar concentration of iron was 0.01 mM and the final molar concentrations of PIH were 0.005-0.06 mM.

The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The line 3:1 is identical with the line 2:1 suggesting the latter complex. The ratios signify substance to iron. In this figure, the single measurement is depicted for better lucidity.

4. CONCLUSION

This study reported the novel calculations for the assessment of stoichiometry of chelators with iron. This approach can be useful in the confirming of the chelation stoichiometry and moreover, it may reveal the reaction stoichiometry in chelators with a moderate affinity to iron. Since the data on stoichiometry of the complexes of several substances were apparently different among miscellaneous studies, particularly in flavonoids, we suggest that for the correct stoichiometry calculation, the both Job's and the complementary approaches should be used.

5. APPENDIX

Supplementary data associated with this article can be found, in the online version, athttp://dx.doi.org/10.1016/j.bioorg. 2013.06.002.

6. ACKNOWLEDGEMENT

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Supplementary data

Calculations using absorption maximum of substance
and absorption maximum of complex
(Method III and Method IV)

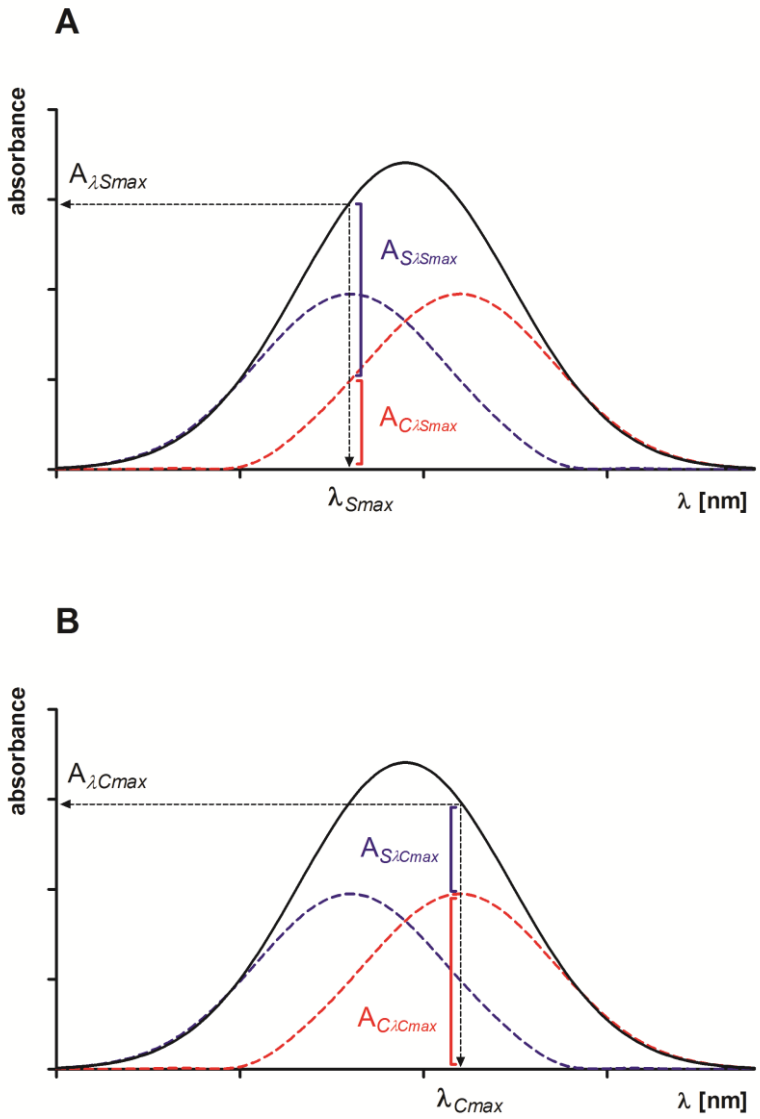

Fig. S1: Calculations using the absorption maximum of a substance (**A**) and the absorption maximum of the complex (**B**). The measured absorption spectrum (black curve) is the sum of the absorption spectrum of the non-reacted substance (blue dashed curve) and the absorption spectrum of the formed complex (red dashed curve).

Deferoxamine and Fe(II) at pH 7.5

Job's method and Complementary approach – Method I

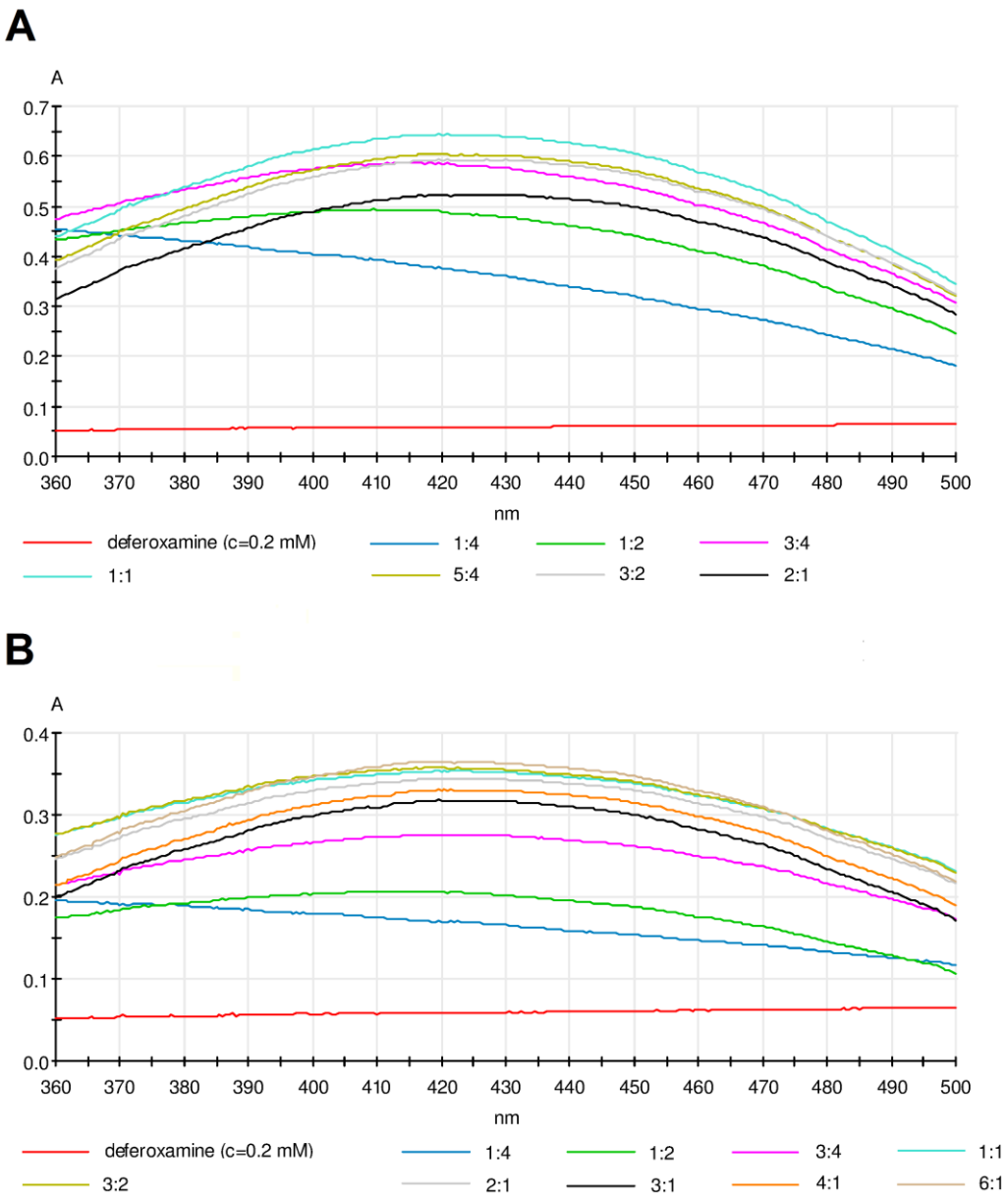

Fig. S2: Deferoxamine and Fe(II) at pH 7.5.

Job's method – the absorption spectra (**A**). The total molar concentration of deferoxamine and iron was 0.5 mM.

Complementary approach – the absorption spectra (**B**). The final concentration of iron was 0.1 mM and the final concentrations of deferoxamine were 0.025-0.6 mM.

The ratios signify substance to iron. In this figure, the absorption spectra of the single measurements are depicted.

EDTA and Fe(II) at pH 7.5

Job's method and Complementary approach – Method I

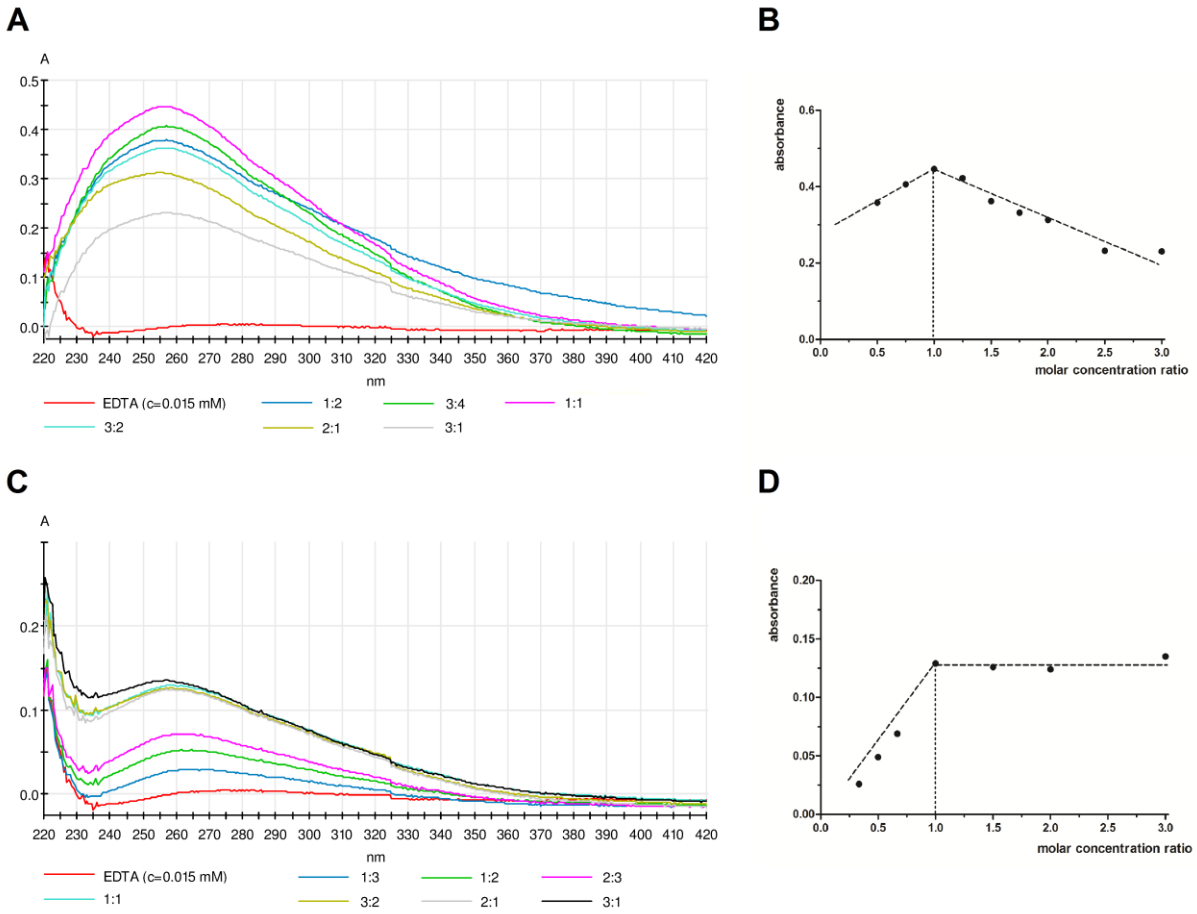

Fig. S3: EDTA and Fe(II) at pH 7.5.

Job's method – the absorption spectra (**A**) and the Job's plot at λ_{Cmax} (256 nm) (**B**). The total molar concentration of EDTA and iron was 0.1 mM.

Complementary approach – the absorption spectra (**C**) and the plot of Method I (**D**). The final molar concentration of iron was 0.015 mM and the final molar concentrations of EDTA were 0.005-0.045 mM.

The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

8-Hydroxyquinoline and Fe(III) at pH 7.5

Job's method and Complementary approach – Method I

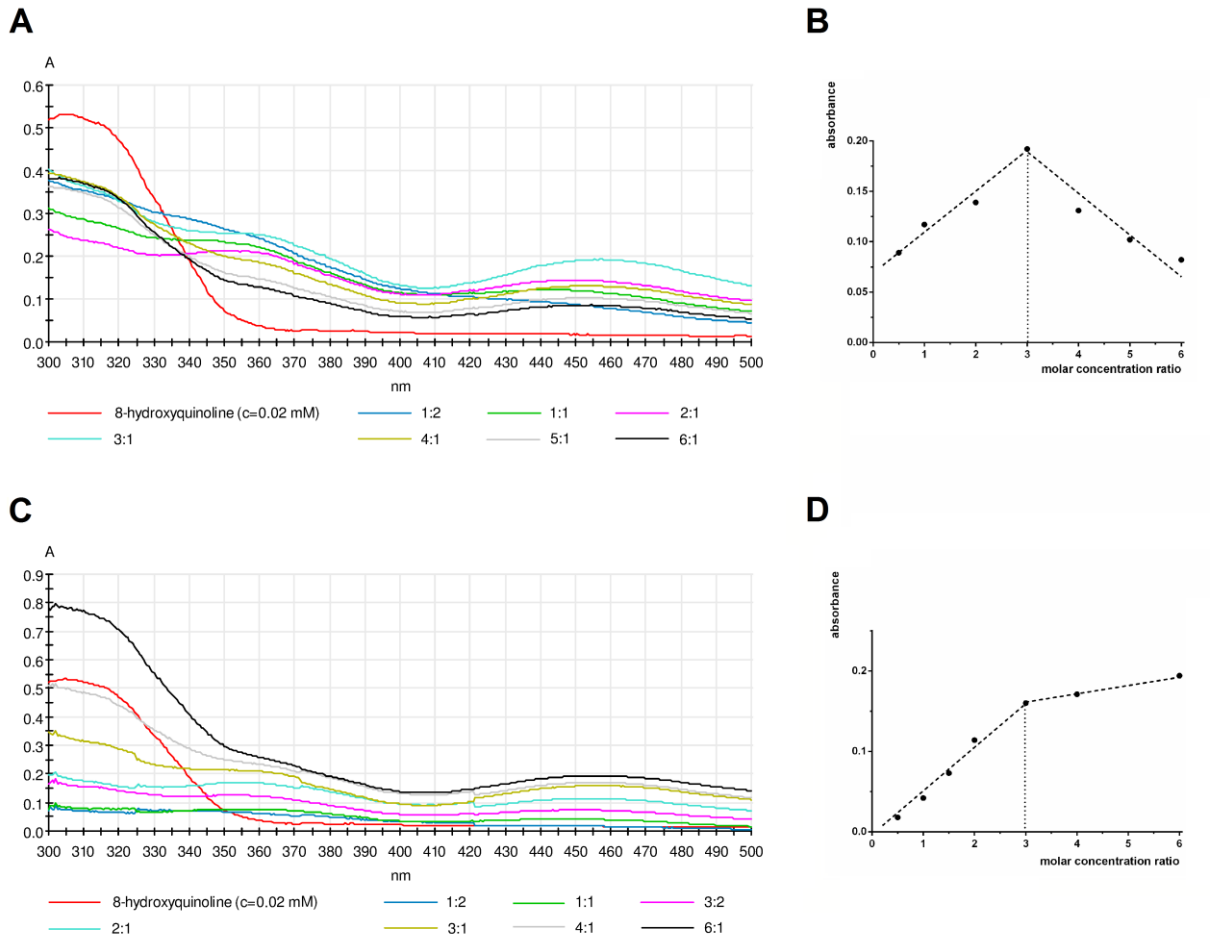

Fig. S4: 8-Hydroxyquinoline and Fe(III) at pH 7.5.

Job's method – the absorption spectra (**A**) and the Job's plot at λ_{Cmax} (460 nm) (**B**). The total molar concentration of 8-hydroxyquinoline and iron was 0.2 mM.

Complementary approach – the absorption spectra (**C**) and the plot of Method I (**D**). The final molar concentration of iron was 0.05 mM and the final molar concentrations of 8-hydroxyquinoline were 0.025-0.3 mM.

The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

Deferasirox and Fe(II) at pH 7.5

Absorption spectra, Job's method and Complementary approach – Method I

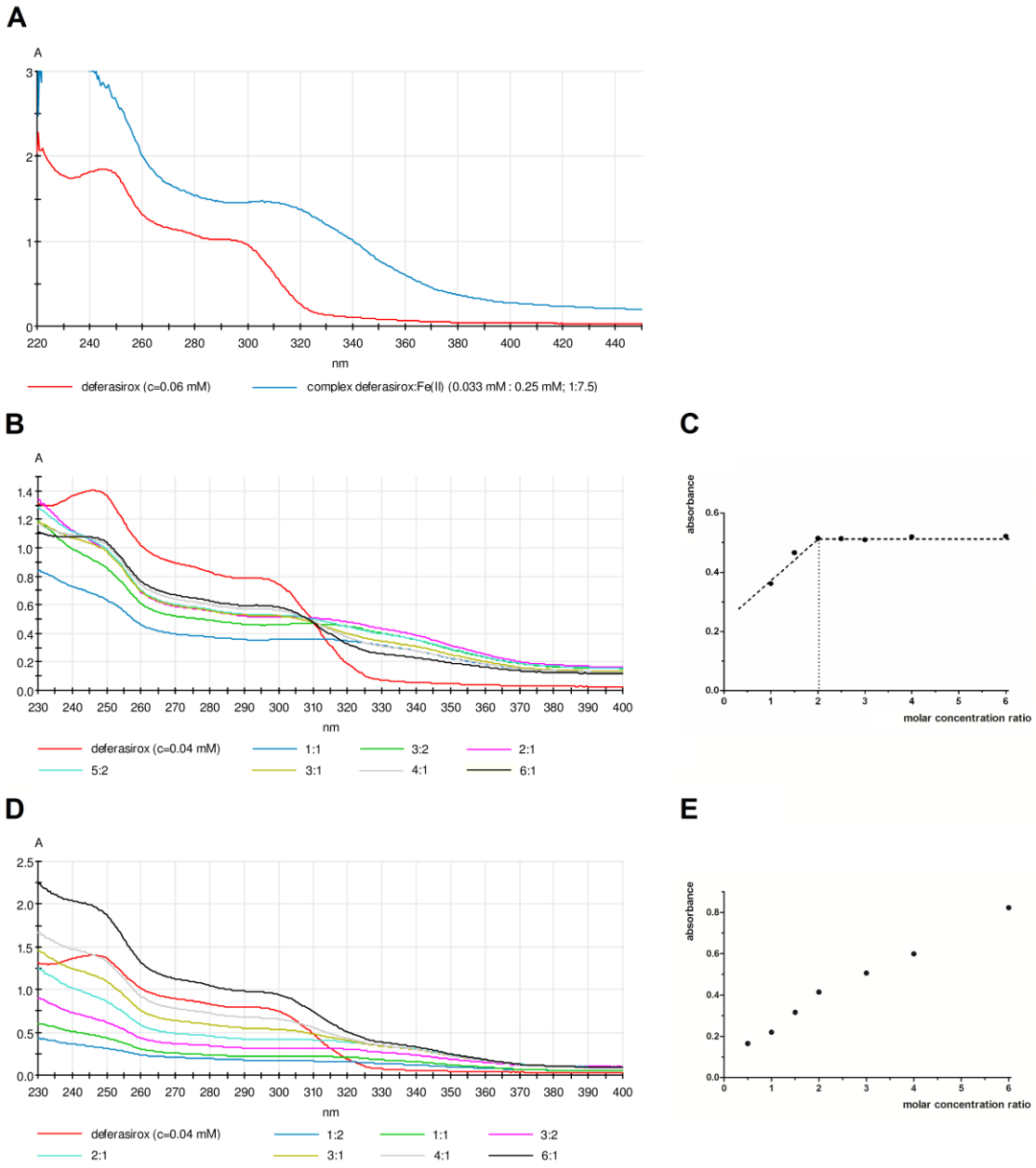

Fig. S5: Deferasirox and Fe(II) at pH 7.5.

Absorption spectra – deferasirox and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (309 nm) (**C**). The total molar concentration of deferasirox and iron was 0.04 mM.

Complementary approach at λ_{Cmax} – the absorption spectra (**D**) and the plot of Method I (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of deferasirox were 0.005-0.06 mM. This method was unsuccessful.

The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

Quercetin and Fe(III) at pH 7.5

Absorption spectra, Job's method and Complementary approach – Method I

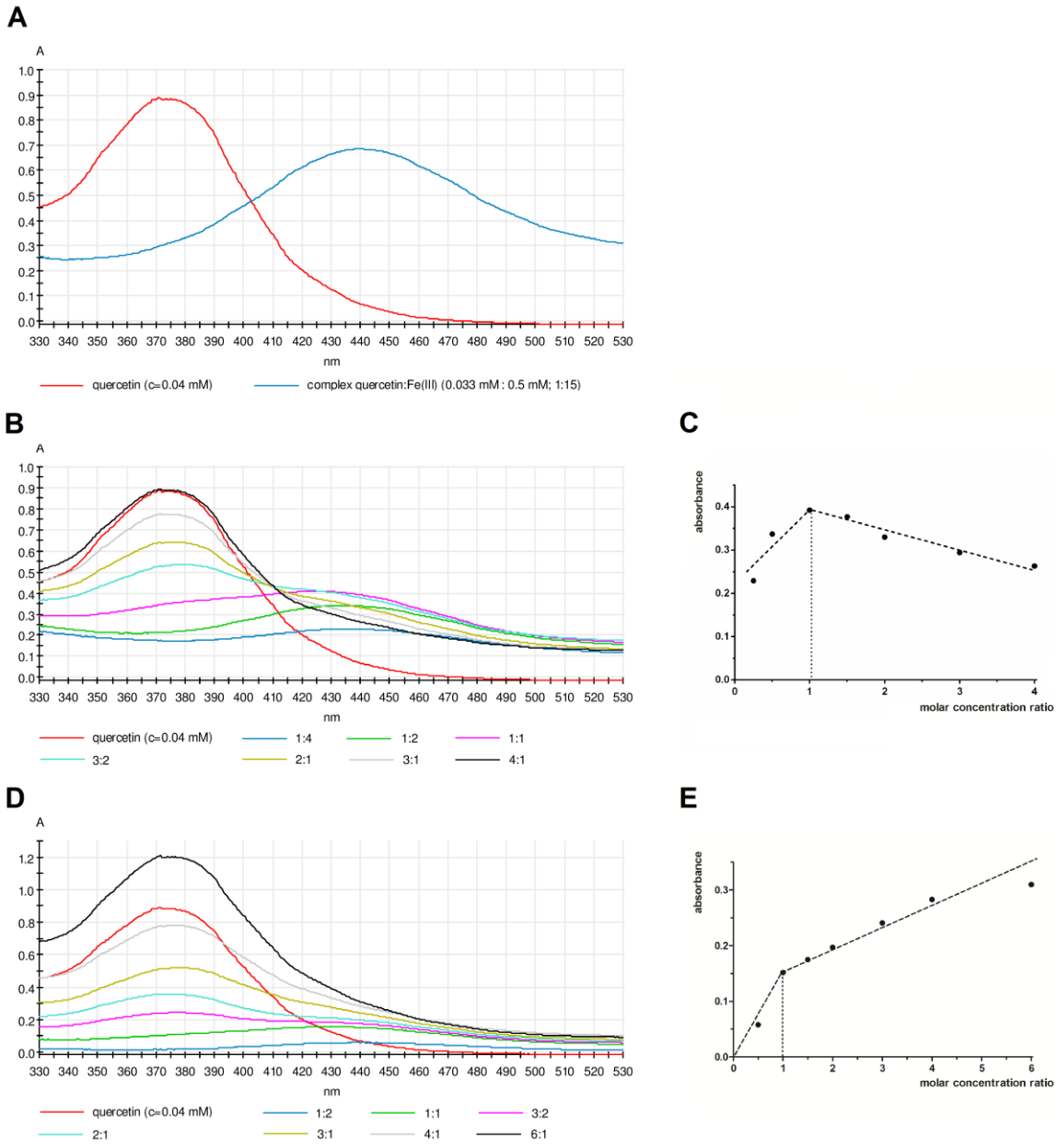

Fig. S6: Quercetin and Fe(III) at pH 7.5.

Absorption spectra – quercetin and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (443 nm) (**C**). The total molar concentration of quercetin and iron was 0.05 mM.

Complementary approach – the absorption spectra (**D**) and the plot of Method I (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of quercetin were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

SIH and Fe(II) at pH 7.5

Absorption spectra, Job's method and Complementary approach – Method II

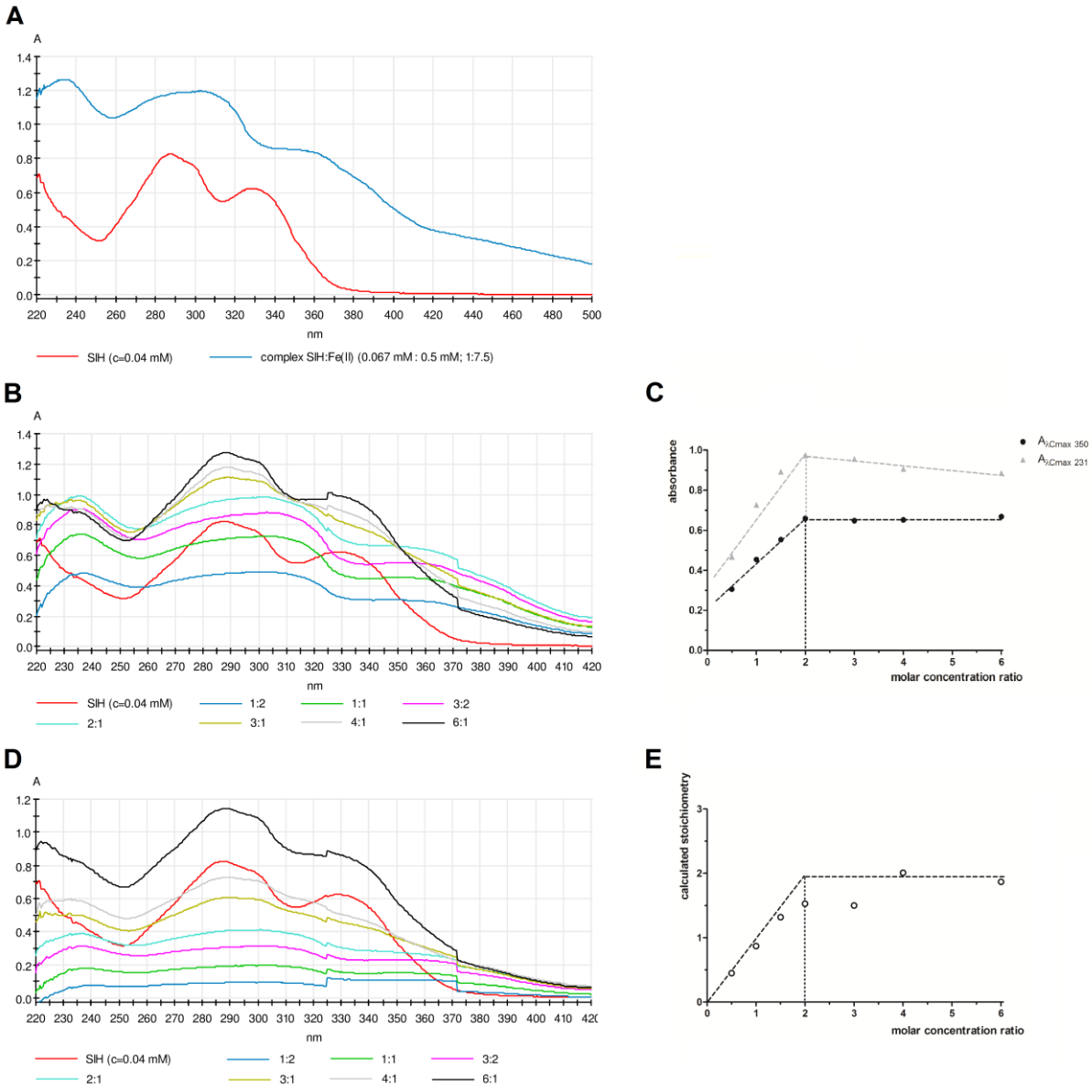

Fig. S7: SIH and Fe(II) at pH 7.5.

Absorption spectra – SIH and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plots at λ_{Cmax} (231 and 350 nm) (**C**). The total molar concentration of SIH and iron was 0.075 mM.

Complementary approach – the absorption spectra (**D**) and the plot of Method II (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of SIH were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

Chloroxine and Fe(II) at pH 5.5

Absorption spectra, Job's method and Complementary approach – Method II-IV

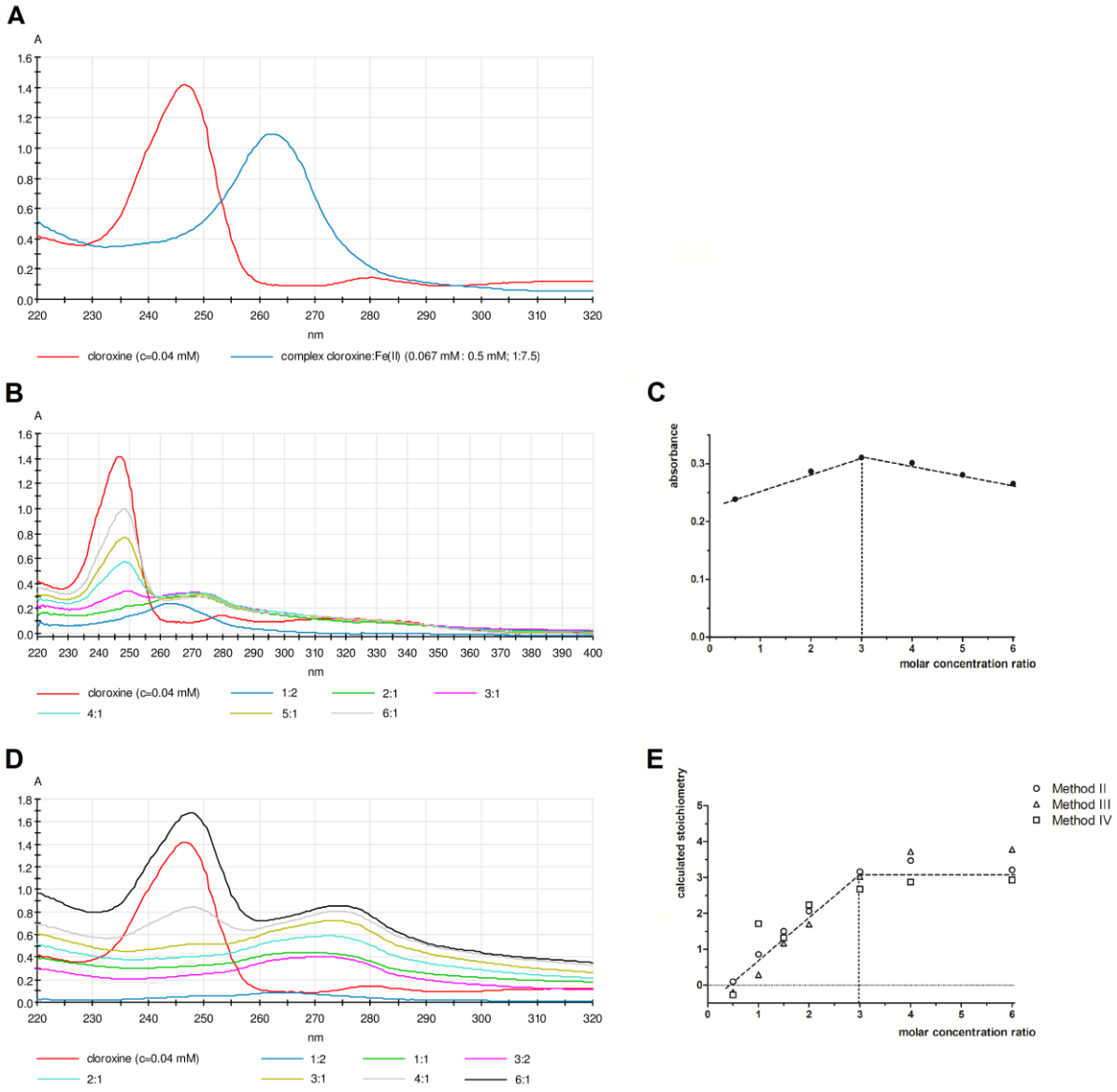

Fig. S8: Chloroxine and Fe(II) at pH 5.5.

Absorption spectra – chloroxine and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (262 nm) (**C**). The total molar concentration of chloroxine and iron was 0.05 mM.

Complementary approach – the absorption spectra (**D**) and the plots of Method II, III and IV (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of chloroxine were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

Rutin and Fe(II) at pH 6.8

Absorption spectra, Job's method and Complementary approach

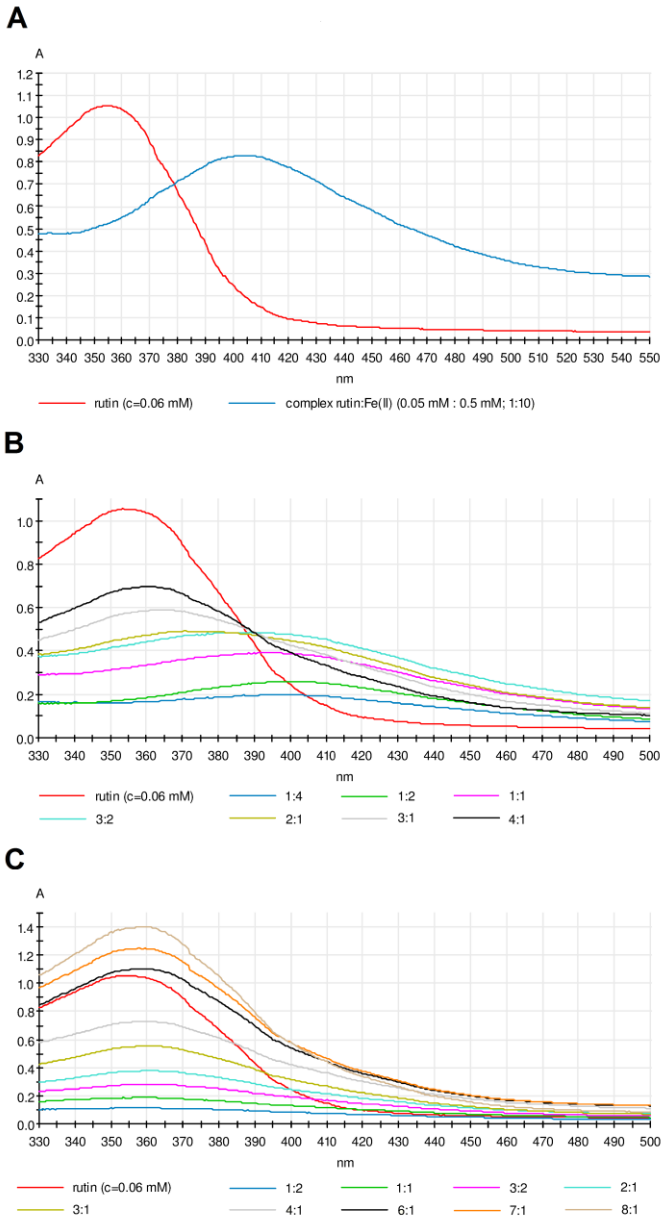

Fig. S9: Rutin and Fe(II) at pH 6.8.

Absorption spectra – rutin and its complex (**A**).

Job's method – the absorption spectra (**B**). The total molar concentration of rutin and iron was 0.05 mM.

Complementary approach – the absorption spectra (**C**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of rutin were 0.005-0.08 mM. Corresponding plots are shown in Fig. 7 in the main article.

The ratios signify substance to iron. In this figure, the absorption spectra of the single measurements are depicted.

PIH and Fe(III) at pH 7.5

Absorption spectra, Job's method and Complementary approach

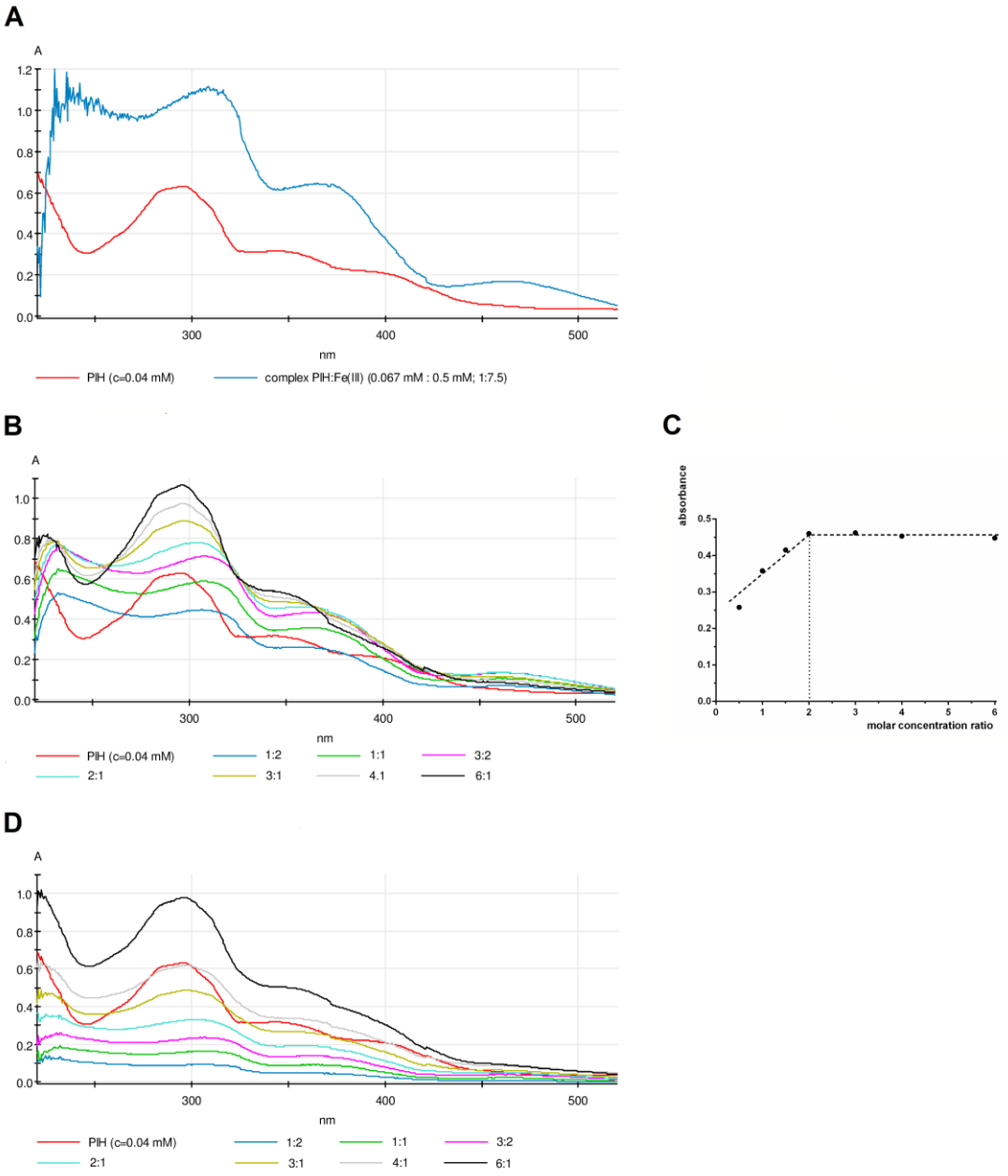

Fig. S10: PIH and Fe(III) at pH 7,5.

Absorption spectra – PIH and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (366 nm) (**C**). The total molar concentration of PIH and iron was 0.075 mM.

Complementary approach – the absorption spectra (**D**).The corresponding plot (Method VI) is shown in Fig. 8 in the main article. The final molar concentration of iron was 0.01 mM and the final molar concentrations of PIH were 0.005-0.06 mM.

The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

Deferiprone and Fe(III) at pH 7.5

Absorption spectra, Job's method and Complementary approach

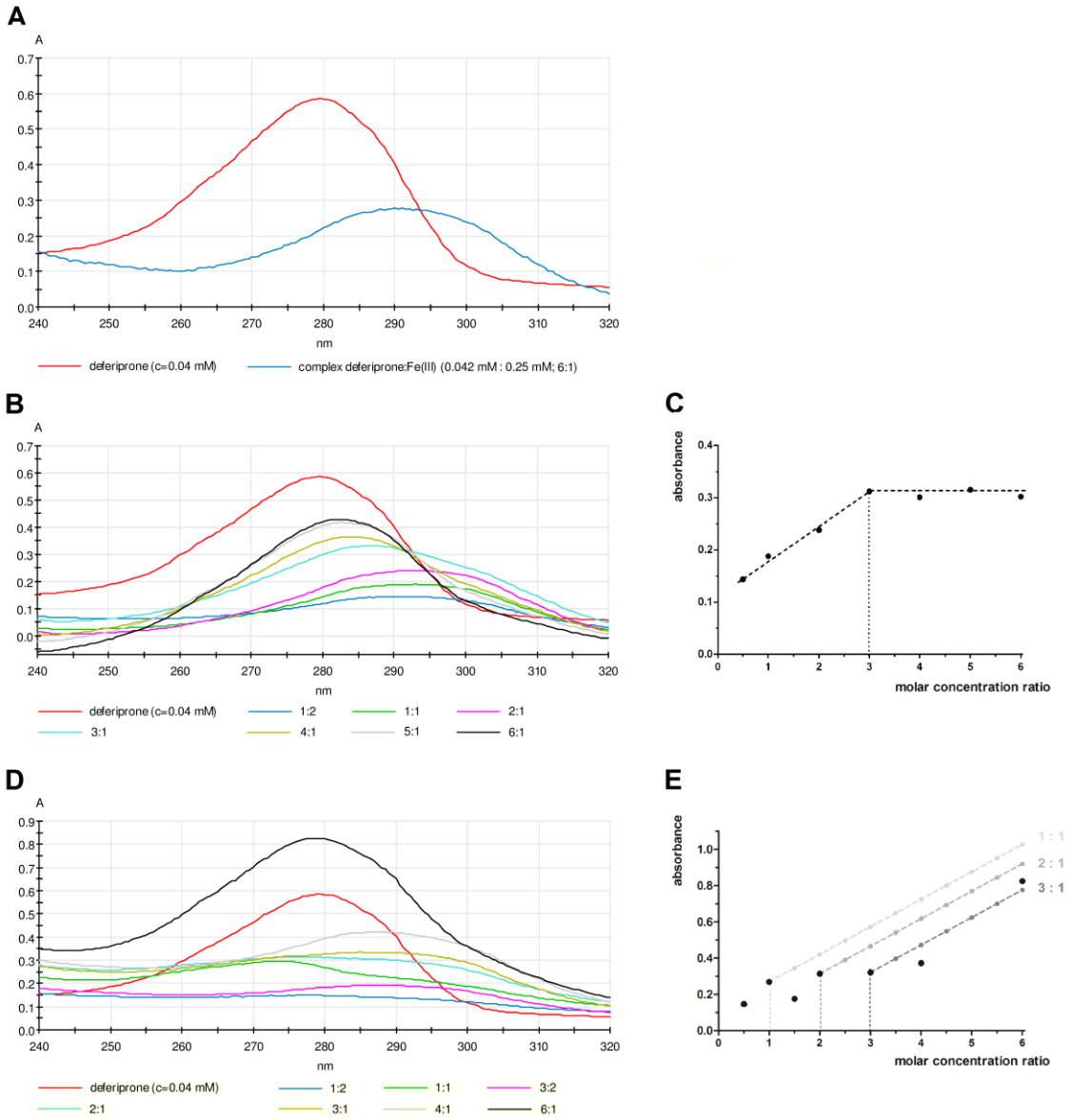

Fig. S11: Deferiprone and Fe(III) at pH 7.5.

Absorption spectra – deferiprone and its complex (**A**).

Job's method – the absorption spectra (**B**) and the Job's plot at λ_{Cmax} (305 nm) (**C**). The total molar concentration of deferiprone and iron was 0.05 mM.

Complementary approach – the absorption spectra (**D**) and the plot of Method VI (**E**). The final molar concentration of iron was 0.01 mM and the final molar concentrations of deferiprone were 0.005-0.06 mM.

The light grey line mimics the absorbance at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1. The ratios signify substance to iron. In this figure, the single measurements are depicted for better lucidity.

Tab. S1: Summarized molar absorption coefficients and corresponding differences at the analyzed wavelengths.

substance	ε_S [$mol^{-1}.cm^{-1}.l$]	ε_C [$mol^{-1}.cm^{-1}.l$]	$\lvert \varepsilon_S - \varepsilon_C \rvert$ [$mol^{-1}.cm^{-1}.l$]
chloroxine	$\varepsilon_{\lambda 247}$ = 34 803 ± 4 033 $\varepsilon_{\lambda 261}$ = 9 059 ± 1 557	$\varepsilon_{\lambda 247}$ = 14 337 ± 6 700 $\varepsilon_{\lambda 261}$ = 21 973 ± 6 502	λ_{247} = 20 465 λ_{261} = 12 914
deferasirox	$\varepsilon_{\lambda 246}$ = 30 815 ± 518 $\varepsilon_{\lambda 309}$ = 10 771 ± 875	$\varepsilon_{\lambda 246}$ = 35 258 ± 9 196 $\varepsilon_{\lambda 309}$ = 18 666 ± 4 412	λ_{246} = 4 443 λ_{309} = 7 895
deferiprone	$\varepsilon_{\lambda 280}$ = 14 730 ± 1 093 $\varepsilon_{\lambda 289}$ = 9 800 ± 2 012	$\varepsilon_{\lambda 280}$ = 8 490 ± 1 853 $\varepsilon_{\lambda 289}$ = 9 948 ± 2 204	λ_{280} = 6 240 λ_{289} = 148
PIH	$\varepsilon_{\lambda 218}$ = 16 007 ± 1353 $\varepsilon_{\lambda 233}$ = 11 133 ± 1 644 $\varepsilon_{\lambda 296}$ = 16 425 ± 1 486 $\varepsilon_{\lambda 310}$ = 13 423 ± 1 391 $\varepsilon_{\lambda 342}$ = 7 909 ± 487 $\varepsilon_{\lambda 367}$ = 5 569 ± 381 $\varepsilon_{\lambda 463}$ = 260 ± 179	$\varepsilon_{\lambda 218}$ = 13 630 ± 3 298 $\varepsilon_{\lambda 233}$ = 14 900 ± 4 630 $\varepsilon_{\lambda 296}$ = 14 162 ± 2 090 $\varepsilon_{\lambda 310}$ = 14 627 ± 2 751 $\varepsilon_{\lambda 342}$ = 9 686 ± 1 191 $\varepsilon_{\lambda 367}$ = 10 732 ± 1 501 $\varepsilon_{\lambda 463}$ = 3 084 ± 879	λ_{218} = 2 377 λ_{233} = 3 766 λ_{296} = 2 263 λ_{310} = 1 205 λ_{342} = 1 777 λ_{367} = 5 164 λ_{463} = 2 824
quercetin	$\varepsilon_{\lambda 370}$ = 23 838 ± 887 $\varepsilon_{\lambda 434/443}$ = 983 ± 192	$\varepsilon_{\lambda 370}$ = 8 247 ± 1 713 $\varepsilon_{\lambda 434/443}$ = 15 887 ± 3 346	λ_{370} = 15 591 $\lambda_{434/443}$ = 14 905
rutin	$\varepsilon_{\lambda 356}$ = 17 210 ± 1 346 $\varepsilon_{\lambda 401}$ = 2 319 ± 706	$\varepsilon_{\lambda 356}$ = 10 219 ± 1 342 $\varepsilon_{\lambda 401}$ = 16 674 ± 1 924	λ_{356} = 6 991 λ_{401} = 14 356
SIH	$\varepsilon_{\lambda 218}$ = 16 875 ± 3 577 $\varepsilon_{\lambda 231}$ = 9 200 ± 1 700 $\varepsilon_{\lambda 288}$ = 18 020 ± 1 147 $\varepsilon_{\lambda 303}$ = 14 473 ± 1 325 $\varepsilon_{\lambda 330}$ = 13 725 ± 804 $\varepsilon_{\lambda 350}$ = 6 844 ± 688	$\varepsilon_{\lambda 218}$ = 17 195 ± 4 412 $\varepsilon_{\lambda 231}$ = 20 325 ± 3 631 $\varepsilon_{\lambda 288}$ = 16 243 ± 1 927 $\varepsilon_{\lambda 303}$ = 16 850 ± 1 533 $\varepsilon_{\lambda 330}$ = 11 726 ± 1 153 $\varepsilon_{\lambda 350}$ = 11 279 ± 1 110	λ_{218} = 320 λ_{231} = 11 125 λ_{288} = 1 777 λ_{303} = 2 378 λ_{330} = 1 999 λ_{350} = 4 435

ε_S – molar absorption coefficient of substance, ε_S – molar absorption coefficient of complex,

$\lvert \varepsilon_S - \varepsilon_C \rvert$ – absolute difference between the coefficients

4. 5. Acute initial haemodynamic changes in a rat isoprenaline model of cardiotoxicity

FILIPSKÝ, Tomáš, ZATLOUKALOVÁ, Libuše, MLADĚNKA, Přemysl; HRDINA, Radomír. Acute initial hemodynamic changes in a rat isoprenaline model of cardiotoxicity. *Human & Experimental Toxicology*. 2012, **31**(8), 830-843. ISSN 0960-3271.

(IF 2011 – 1.772)

Ve výzkumu patofyziologie akutního infarktu myokardu se již od konce 50. let minulého století používá jako induktor modelového poškození srdeční tkáně syntetický katecholamin isoprenalin s neselektivní β-agonistickou aktivitou. Přestože se problematikou isoprenalinového modelu zabývalo velké množství studií, přesný mechanismus časné patogeneze po podání kardiotoxické dávky isoprenalinu není dosud spolehlivě vysvětlen.

V této studii jsme se zaměřili na analýzu časných hemodynamických změn po aplikaci kardiotoxické dávky isoprenalinu (100 mg/kg, s.c.) u Wistar:Han potkanů. Úvodní experimenty byly provedeny za použití termodiluční metody a u hlavních experimentů s dvouhodinovým záznamem hemodynamický parametrů byl použit Millar katétr. Doplňkové experimenty se salbutamolem byly zaměřeny na objasnění role β_2-adrenergních receptorů.

V průběhu 1. minuty po podání isoprenalinu došlo k razantnímu poklesu arteriálního krevního tlaku (−40 %), nárůstu tepové frekvence (+30 %) a poklesu tepového objemu (−30 %). Během dvou minut po aplikaci signifikantně kleslo dotížení (−40 %) a předtížení (−10 %). Rovněž byla významně ovlivněna diastola (tzn. zkracení intervalu fáze isovolumické relaxace o 50 % a plnící fáze o 40 %). Na druhé straně byl zaznamenán markantní, ale krátkodobý, nárůst kontraktility levé komory (+100 %). Je ovšem nutné zmínit, že v průběhu celého dvouhodinového pokusu se kontraktilita myokardu nesnížila oproti bazálním hodnotám, naopak zůstala spíše zvýšená. Na základě těchto časných hemodynamických změn a rovněž minimálních změnách v ejekční frakci lze předpokládat, že příčinou časné patogeneze je diastolická dysfunkce. Podání salbutamolu (116 mg/kg, s.c.) nevedlo k zásadním časným změnám

hemodynamických parametrů s výjimkou významného poklesu diastolického krevního tlaku a dotížení.

Tato studie tedy prokázala, že z patogenetického hlediska diastolická dysfunkce předchází systolické dysfunkci a že k její samotné indukci je stimulace pouze β_2-adrenergních receptorů nedostatečná.

Original Article

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Acute initial haemodynamic changes in a rat isoprenaline model of cardiotoxicity

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Abstract

The synthetic catecholamine isoprenaline (ISO) has been used as an inductor in the acute myocardial infarction model for more than a half century. Despite the fact that many articles were published on this topic, precise early haemodynamic pathology remains unknown. Acute haemodynamic changes were measured in rats; first, in preliminary experiments by the thermodilution method; and second, in main experiments continuously for 2 h using a Millar catheter. Animals received saline or ISO in the cardiotoxic dose (100 mg/kg, subcutaneously). Also, additional experiments were performed with salbutamol in order to evaluate the role of β_2-receptors. ISO caused a rapid, within 1 min, approximately 40% decrease in arterial blood pressures, 30% increase in the heart rate, and 30% decrease in the stroke volume. Within the first 2 min, the changes were followed by decreases in afterload (−40%), preload (−10%), diastolic relaxation (−50%), diastolic filling (−40%), and a marked, but short-term, increase in the left ventricle contractility (+100%). Ejection fraction did not significantly change, suggesting diastolic dysfunction. Salbutamol, with the exception of diastolic pressure and afterload, did not substantially influence other parameters. In conclusion, this study demonstrated that diastolic dysfunction precedes systolic dysfunction and β_2-receptor stimulation alone is not sufficient for an early induction of diastolic dysfunction.

Keywords

catecholamines, diastolic dysfunction, isoprenaline, myocardial injury, salbutamol

Introduction

Endogenous catecholamines, adrenaline and noradrenaline, are the essential components of the sympathetic nervous system, which are responsible for maintaining the cardiovascular homeostasis. However, catecholamines are cardiotoxic under certain circumstances and thus may contribute to additional cardiac impairments. The known examples are chronic heart failure, stress cardiomyopathy, arrhythmias, and acute myocardial infarction (AMI).[1–4] Infarctions may be triggered by catecholamines themselves, and it is the base for the experimental use of catecholamines as a model of AMI.[5,6] Interestingly, a synthetic catecholamine isoprenaline (ISO) with nonselective β-adrenergic agonistic activity has demonstrated a superior activity in mimicking the AMI in laboratory animals in comparison with that of endogenous catecholamines.[5] Although the haemodynamic effects of therapeutic doses of ISO are well known, the precise pathophysiology of the ISO model of AMI, in which doses of ISO are much larger, is not known. This model has been used for more than a half century notwithstanding. Additionally, the reason for ISO superiority in the induction of AMI over the endogenous catecholamines is also unknown.

Current knowledge emphasizes that both β-adrenergic receptor and catecholamines redox cycling (including the production of a row of their oxidation

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products and production of reactive oxygen species) are involved in the pathophysiology of catecholamine-induced cardiac impairment.[4,7,8] Although oxidative stress is important, the first event in the pathophysiology represents the overstimulation of both β-adrenergic receptors in the cardiovascular system. It leads to marked stimulation of the myocardium having positive inotropic, chronotropic, and dromotropic effects due to the activation β_1-receptors. Also, it causes a decrease in total peripheral resistance (TPR) as a consequence of the stimulation of β_2-receptors. In the therapeutic doses of ISO, the stimulation of β-receptors leads to an increase in cardiac output together with the reinforcement of myocardial contractility and improvement in the myocardial relaxation.[9,10] Interestingly, the consequences of the cardiotoxic doses are quite the opposite, that is they cause decreases in cardiac output and stroke volume. The decreases are associated with impaired systolic and diastolic functions.[11–13] Although lots of data have been published on the biochemical and histological findings regarding the cardiotoxic doses of ISO, the early haemodynamic processes in the cardiovascular system, which cause a drop in the stroke volume remain unclear. Therefore, this study was aimed at detailed analysis of haemodynamic changes, in particular contractility and diastolic function, after the administration of cardiotoxic doses of ISO. Additionally, we investigated the involvement of β_2-receptors in the cardiac injury.

Materials and methods

Animals

Totally 35 Wistar:Han male rats were obtained from Biotest s.r.o. (Czech Republic). The rats were housed in cages located in a special air-conditioned room with a periodic light–dark cycle for 2 weeks. During this period, the rats were provided with free access to tap water and standard pellet diet for rodents. After the acclimatization period, the healthy rats weighing approximately 370 g were used for the experiments described below.

The study was performed under the supervision of the Ethical Committee of 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).

Anaesthesia

After 12 h of fasting, the rats were anaesthetized with intraperitoneal injection containing aqueous solution of urethane (Sigma-Aldrich, USA) at a dose of 1.2 g/kg.

Haemodynamic study by thermodilution method

A polyethylene catheter (0.5/1.0 mm filled with heparinised saline 50 IU/ml) was inserted into the right internal jugular vein for injection of the indicator (saline). Its temperature was approximately 10°C. A thermocatheter (outer diameter 0.8 mm) was introduced through the common left carotid artery into the aortic arch. Another polyethylene catheter filled with heparinised saline was inserted into the left common iliac artery. The catheter was connected to the blood pressure transducer BPR-01. Both the transducer and thermocatheter were linked to the apparatus for the measurement of haemodynamic variables Cardiosys equipped with Cardiosys 1.1. software (Experimetria Ltd, Hungary) using a thermodilution transpulmonary method according to the Stewart-Hamilton principle.[14]

Haemodynamic study by pressure–volume recording using a micromanometer catheter

The left common iliac artery was connected to a pressure transducer MLT0380/D (AdInstruments, Australia) via a polyethylene catheter (0.5/1.0 mm filled with heparinised saline 50 IU/ml). A high-fidelity pressure–volume micromanometer catheter (Millar pressure–volume catheter SPR-838 2F, 4E, 9 mm, Millar Instruments Inc., USA) was inserted into the left heart ventricle through the right common carotid artery. Both pressure transducer and Millar pressure–volume catheter with the subcutaneous electrodes of the electrocardiography (ECG) standard limb lead II MLA1215 (AdInstruments, Australia) were connected to PowerLab with LabChart 7 software (AdInstruments, Australia).

Drug administration and measurement

The healthy Wistar:Han male rats were randomly divided into groups. All of them were of statistically similar average weight.

Thermodilution method

Following 15 min of equilibration, 10 rats received isoprenaline (Sigma-Aldrich, USA) in the dose equal to 100 mg/kg subcutaneously (sc). Cardiac output and

derived parameters were measured by the administration of 100 µl of cold saline[14] in the following time intervals—0 (baseline level), 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, and 240 min.

Pressure–volume recording using a micromanometer catheter

Following 15-min of equilibration, the calibration of parallel volume necessary for the estimation of real blood volume in the left ventricle was performed using 20 µl of 25% w/w natrium chloride solution with 900 IU/ml of heparin—the hypertonic saline calibration.[15] Five minutes after calibration, 15 rats received ISO (100 mg/kg sc—the same dose as in the previous setting) or salbutamol hemisulphate sc (5 rats, Sigma-Aldrich, USA) in the equimolar dose of ISO (116 mg/kg sc) or the solvent—control group (5 rats, 2 ml/kg of saline sc, Braun, Germany). Haemodynamic as well as ECG monitoring continued for 2 h after the administration of drug/drugs. After 2 h, an additional hypertonic saline calibration was performed. At the end of the experiment, a blood sample was collected from the abdominal aorta into the heparinised test tube (170 IU/10 ml) and conductance signal was calibrated using cuvettes of known volumes.[16]

Following the experiment, all surviving animals were killed painlessly using intravenous administration of 1 ml 1 M aqueous solution of potassium chloride (Sigma-Aldrich, USA).

Calculation of parameters

The Millar catheter records two electrical signals: the first one for pressure and the second one for conductance/volume. The pressure electrical output signal varies proportionally with changes in pressure; therefore, a simple internal calibration was performed in each experiment in order to obtain the precise intraventricular pressure values. However, the conductance signal is not directly transformable in the intraventricular blood volume. It depends on more factors (equation (1))[15]:

$$G(t) = \frac{\alpha}{\mathrm{L}^2} \times \sigma_\mathrm{b} \times \mathrm{V}(t) + \ G_\mathrm{p} \qquad (1)$$

where $G(t)$ is the measured conductivity, α is the dimensionless constant, σ_b is the specific blood conductivity, L is the distance between electrodes, V_t is the actual blood volume in the left ventricle and G_p is the so-called parallel conductance, which reflects the electrical field of the surrounding tissues (e.g. left ventricular wall, right ventricle, and lung tissue). Therefore, two calibrations are necessary in order to obtain the precise volume of blood in the left ventricle, the first one is the above mentioned hypertonic saline calibration (for the assessment of parallel conductance) and the second one is the cuvette calibration (for the assessment of σ_b). Details can be found in references[15] and [16]. Thus, the conductance signal without repeated calibrations can be very easily influenced by many factors. In particular, changes in blood viscosity and parallel tissue conductivity were relevant in our experiments. Therefore, the volume signal was calibrated at the beginning and end of each experiment, and stroke volume and related parameters data were reliable during the first 15 min of the experiment. Changes at the end of the experiment (2 h) were only approximate because it was not possible to carry out the cuvette calibration during this experiment.

TPR was calculated as mean arterial blood pressure divided by the cardiac output. Left ventricular $\mathrm{d}P/\mathrm{d}t_\mathrm{max}$ (maximal pressure rise in the isovolumic phase in the left ventricle) divided by left ventricular end-diastolic volume (LVEDV) was designated as $(\mathrm{d}P/\mathrm{d}t_\mathrm{max})/V$ and $\mathrm{d}P/\mathrm{d}t_\mathrm{max}$ divided by instantaneous pressure at this maximum as $(\mathrm{d}P/\mathrm{d}t_\mathrm{max})/p$. Other parameters had the common meaning. Tau (the time constant of left ventricular isovolumic pressure decay) was calculated by Weiss method.[17]

Data analysis

Data are expressed as means ± SEM. In the case of thermodilution method, differences versus baseline values (i.e. before drug administration) were compared by means of Student's paired T test. Differences between groups (pressure–volume recording) were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Outliers were excluded by Grubb's test. Statistical software GraphPad Prism 5 for Windows (GraphPad Software, USA) was used for all statistical analysis. Differences between groups were considered significant at $p \leq 0.05$, unless otherwise indicated.

Results

Thermodilution method

ISO as a nonselective β-receptor agonist is expected to cause vasodilation and acceleration in the heart

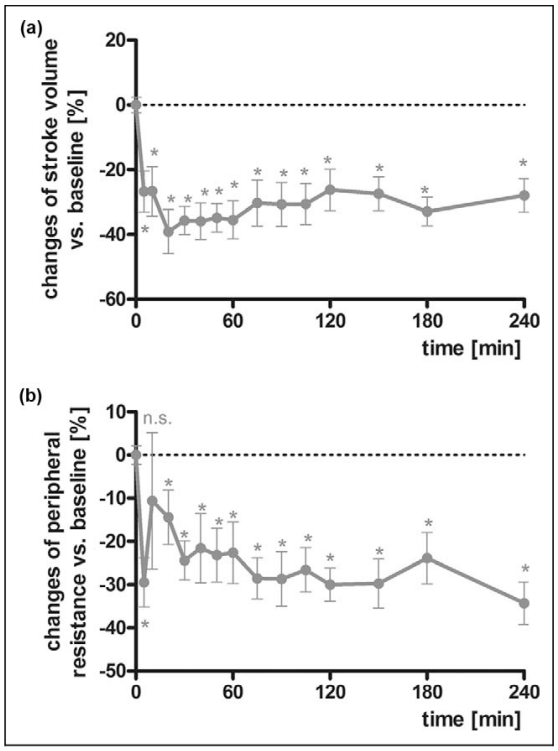

Figure 1. The stroke volume (a) and total peripheral resistance (b) after subcutaneous (sc) administration of 100 mg/kg isoprenaline (ISO) measured by the thermodilution method. Changes in the measured parameters and statistical significance were calculated versus baseline levels (at time 0, i.e. before ISO administration); *$p < 0.05$.

rate. Indeed, a rapid increase in the heart rate and a decrease in both systolic and diastolic blood pressures were documented after ISO administration.[18] We observed similar changes in the heart rate and blood pressure (for details see Supplementary Figure S1). These changes were accompanied by decreases in stroke volume and TPR (Figure 1). All mentioned variables changed at the first stroke volume measurement (5 min after ISO administration) and remained mostly at the same level during the whole experiment (4 h). Of the 10 animals, 4 died within the first 70 min and were not included in the analysis.

Pressure–volume recording

In order to characterize the ISO-induced cardiovascular effects in detail, we continuously analysed the ISO-treated animals using the Millar catheter. This approach enables continuous measurements in contrast to the thermodilution method, but (1) it is more invasive and therefore it enables only shorter experiments (in case of ISO up to 2 h); (2) it requires repeated calibration in pathologically changing myocardium for a precise measurement of intraventricular blood volume and derived parameters (for more details see 'Parameters calculation' and 'Discussion' sections).

Changes in the heart rate and blood pressures after ISO administration shown in the preceding section were confirmed. In addition, the changes were rapid and significant already 30 s after sc administration of ISO (Figure 2(a) and (b)). Diastolic and systolic blood pressures followed the same pattern. Stroke volume decreased as well, but the decrease started approximately 5–10 s after changes occurred in the blood pressure and the heart rate (see Supplementary Figure S2).

To investigate the cause of the fall in stroke volume, we analysed ejection fraction, parameters of loading conditions (preload and afterload), and systolic and diastolic functions. There was no significant change in ejection fraction (Figure 2(c)) between controls and ISO-treated group; ISO tended to increase ejection fraction, suggesting that the responsible factor may be an impaired diastolic function.

As loading conditions may influence the assessment of systolic and diastolic functions, we first determined the loading condition parameters. The extent of preload can be deduced from the left ventricular end-diastolic pressure (LVEDP).[19] ISO caused a rapid, but relatively mild (10%) and short-term, decrease in this parameter (Figure 3(a)). After 5 min, the differences were not significant. The assessment of afterload was accomplished by the assessment of TPR. A marked 40% drop in TPR was found after the administration of ISO (Figure 3(b)).

As loading conditions were apparently changing, the sensitivity of contractility (systolic) parameters were enhanced by minimizing the preload influences $((dP/dt_{max})/V)$ or by reducing afterload influences $((dP/dt_{max})/p)$.[20] Nevertheless, both the derived parameters showed qualitatively similar kinetics as the nonderived dP/dt_{max} (Figure 3(c) and Supplementary data Figure S3). It suggests that the loading conditions could not be considered as the main factor of contractility changes. Contractility increased approximately to 100% of baseline value at 25 s after ISO administration. Although this contractility peak was only transitive, dP/dt_{max} remained rather elevated for the rest of the experiment.

Diastole has two distinctive phases, the isovolumetric relaxation and the filling phase. The former

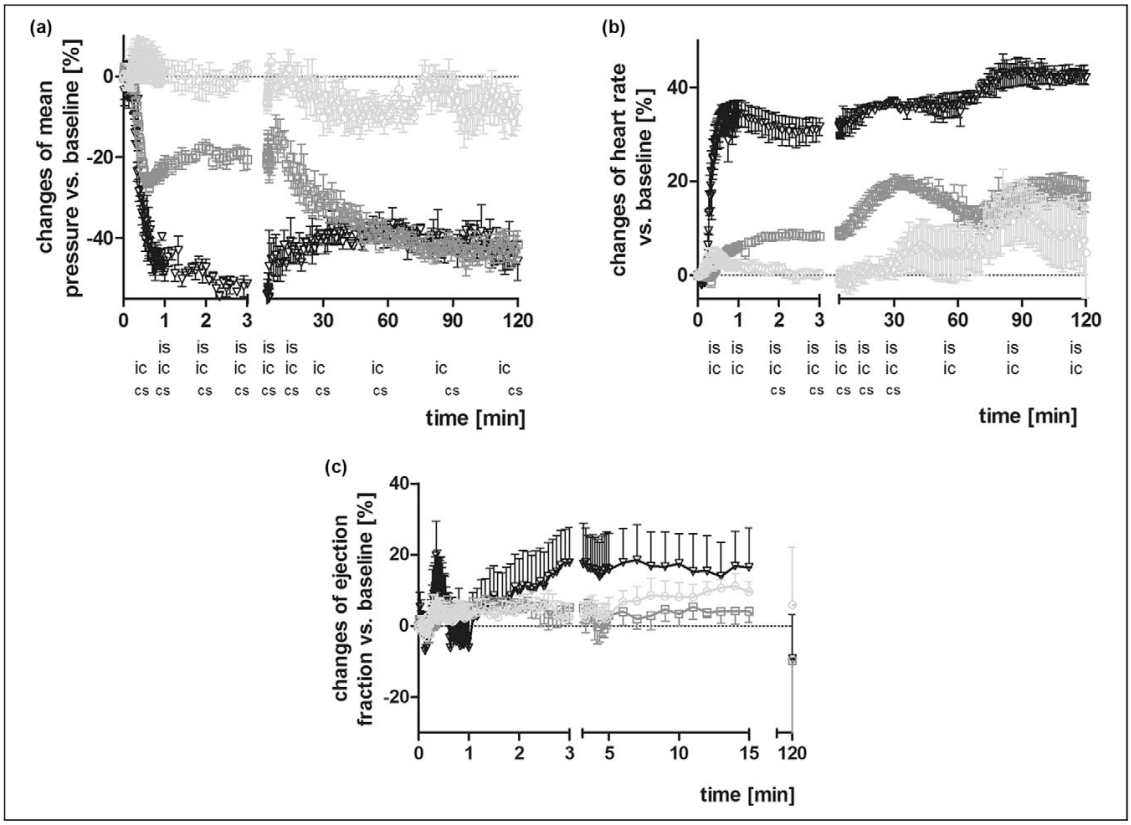

Figure 2. Basic haemodynamic parameters. The mean arterial blood pressure (a), the heart rate (b), and the ejection fraction (c) after the administration of isoprenaline (ISO; black), salbutamol (dark grey), and saline (grey). Statistical significance at $p < 0.05$: is: ISO versus salbutamol; ic: ISO versus control; cs: salbutamol versus control.

one can be characterized by peak negative dP/dt. As this parameter is also influenced by loading conditions, we verified these results by the assessment of tau which is less affected by these parameters.[17,21] Both parameters gave similar outcome; however, the tau interindividual and intraindividual variability in the ISO group was very high and some cardiac cycles could not be quantified by use of the tau (data not shown). Hence, we present the relaxation data as peak negative dP/dt (Figure 4(a)). Relaxation indicated marked 50% depression of the baseline value within the first minute of ISO administration. It remained there for the whole 2 h of the experiment. The filling phase can be characterized by maximal volume rise dV/dt_{max} (Figure 4(b)). Once again, this phase appeared to be depressed and it reached its maximal decrease (40%) within the first 2 min. After the initial decrease, marked variability in the parameter was observed.

During the course of the experiment, marked ECG modifications were recorded (e.g. ST-elevation/depression, T amplitude rise, and P-R interval prolongation) in ISO-treated animals. Of the 15 ISO-treated animals, 7 died. The deaths were caused by serious dysrhythmias that started by ventricular extrasystoles and in most cases were followed by atrioventricular block of various degrees, polymorphic ventricular tachycardia, and/or ventricular fibrillation, which led to death. No mortality was observed in the controls.

To reveal the role of individual β-adrenergic receptor subtypes in ISO-induced cardiotoxicity, we performed additional experiments with the selective β_2-agonist salbutamol. In contrast to ISO, salbutamol caused only a slight and very slow increase in the heart rate and no rapid change in the stroke volume (Figure 2(b), Supplementary data Figure S2). Blood pressures, as in the animals-treated with ISO, dropped very rapidly, but the decrease (around 20%) did not

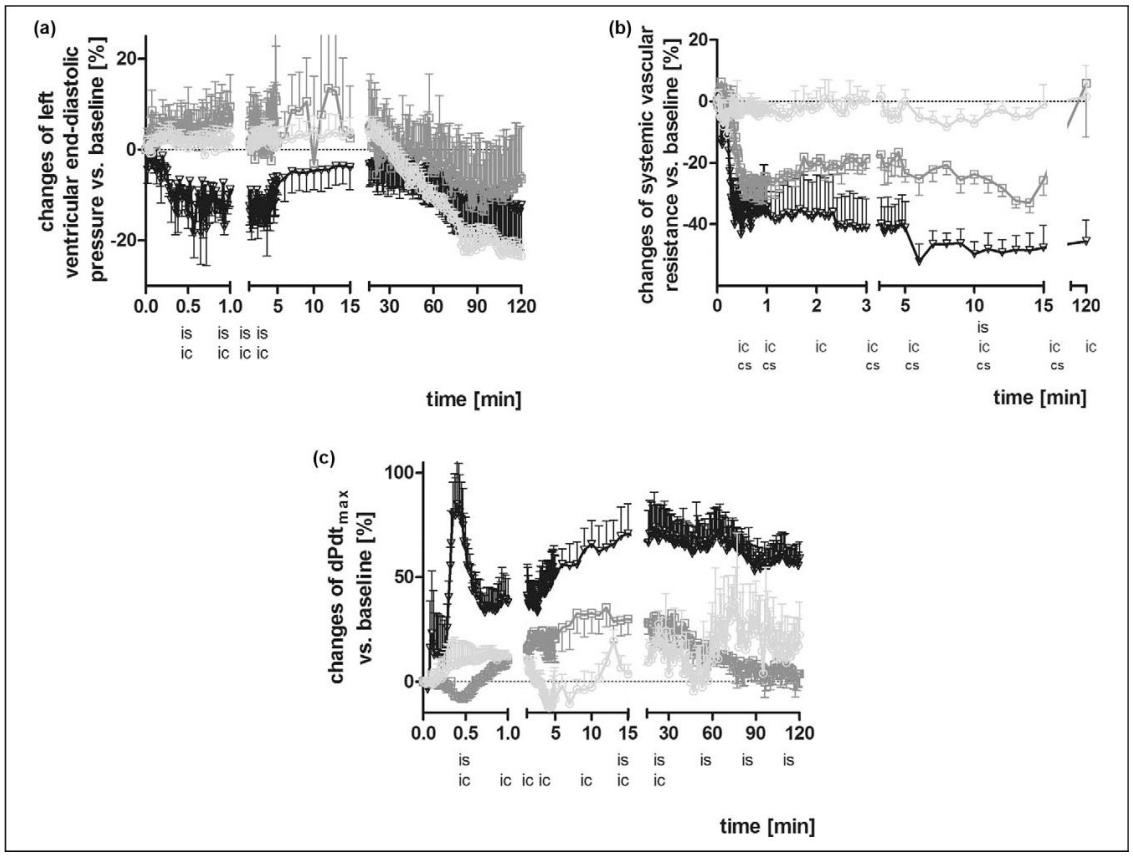

Figure 3. Parameters of preload, afterload and contractility. The left ventricular end-diastolic pressure (a), the total peripheral resistance (b), and the parameter dP/dt_{max} (c) after administration of isoprenaline (ISO; black), salbutamol (dark grey), and saline (grey). Statistical significance at $p < 0.05$: is: ISO versus salbutamol; ic: ISO versus control; cs: salbutamol versus control.

reach the ISO effect (more than 40%) within the first 30 min (Figure 2(a)). After 30 min, there were no significant changes between ISO and salbutamol (about a 40% decrease in both agents). Concordantly to the stroke volume data, salbutamol decreased more diastolic blood pressure than the systolic one (in case of ISO, both pressures dropped in similar manner, see Supplementary data Figure S2). Salbutamol, in contrast to ISO, apparently did not influence preload (Figure 3(a)), but there were mostly no significant differences between ISO and salbutamol in TPR (Figure 3(b)). These results collectively imply that TPR was decreased to a similar extent by both drugs due to the activation of β_2-adrenergic receptors.

Concerning the left intrinsic ventricular contractility and relaxation (Figures 3(c) and 4), the influence of salbutamol was rather low and, with exception of $(dP/dt_{max})/p$, there were no significant differences between salbutamol-treated group and the controls. On the other hand, differences between ISO and salbutamol were mostly significant. However, it should be mentioned that salbutamol caused short-term but significant decrease in peak negative dP/dt within the first minute after its administration. But this decrease was not associated with the depression in diastolic filling. In addition, salbutamol, in contrast to ISO, rather increased the diastolic filling rate (Figure 4(b)).

No rats died in the salbutamol-treated group during the 2-h experiment.

Discussion

ISO is a synthetic catecholamine with β_1- and β_2-agonistic activity. ISO is rarely used in pharmacotherapy,

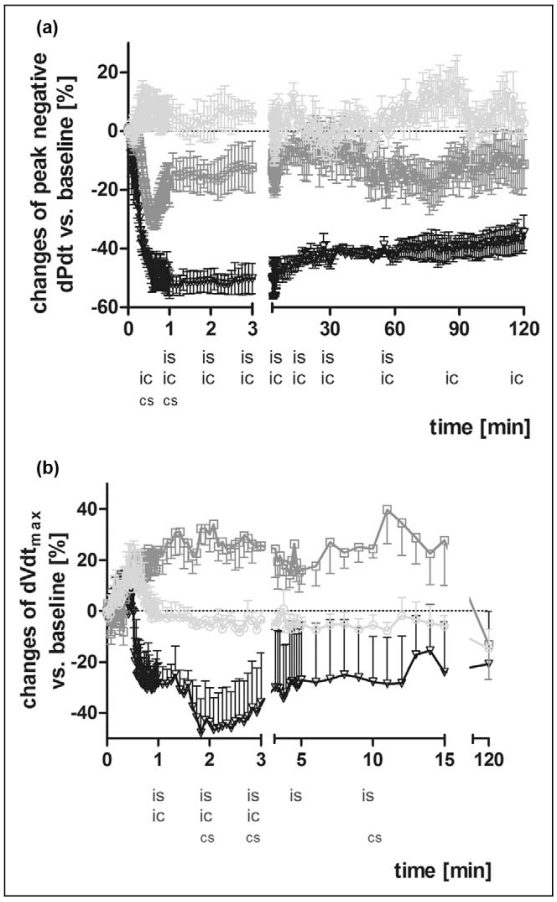

Figure 4. Parameters of left ventricular diastolic function. Peak negative d*P*/d*t* (a parameter of left ventricular isovolumetric relaxation) (a) and d*V*/dt_{max} (maximal volume rise in the left ventricular diastolic filling phase) (b) after administration of isoprenaline (ISO; black), salbutamol (dark grey), and saline (grey). Statistical significance at $p < 0.05$: is: ISO versus salbutamol; ic: ISO versus control; cs: salbutamol versus control.

at present. Due to its positive dromotropic effect, it could be administered in cases of atrioventricular blockade. Apart from this, its β_1 activity in the heart leads to positive chronotropic, ionotropic, and also bathmotropic effects. β_2 activity in the vascular system is primarily associated with vasodilation of the blood vessels in skeletal muscles. Therefore, the administration of low ISO doses mimics moderate doses of adrenaline, which stimulates primarily β-receptors and leads to increased cardiac output (e.g. increases in the heart rate and contractility) in line with a decrease in TPR.[10] However, it is in contrast to what we found in our study in which we used cardiotoxic doses of ISO. The volume of the blood ejected from the left ventricle is primarily dependent on systolic (contractility) and diastolic functions of the heart. Both phases are markedly dependent on loading conditions (preload and afterload). While the loading parameters can be reliably determined,[19,22–24] the precise determination of intrinsic contractility and relaxation has remained unanswered for many decades. Many approaches have been proposed, some of them are new and their validity should be verified or demands some specific processes (e.g. vena cava occlusion) or additional equipment (e.g. echocardiography).[25–31] In this study, we used a maximal pressure rise in the isovolumic phase (dP/dt_{max}) as a parameter of cardiac contractility. We are aware that dP/dt_{max} is dependent on preload and afterload. Therefore, we modified this parameter in order to attenuate the loading interferences: preload influence can be diminished by division of dP/dt_{max} by LVEDV ((dP/dt_{max})/V), and afterload by division with instantaneous left ventricular pressure at the dP/dt_{max} ((dP/dt_{max})/p).[20,22] A parameter of diastolic relaxation peak negative dP/dt also depends on the loading conditions.[17] An additional factor—tau, a time constant of left ventricular isovolumic pressure decay, is less influenced by the loading conditions; but in ischaemic conditions, like in our model, its assessment may be blunted.[17,27] Generally, it appears that profound reduction in peak negative dP/dt is associated with an increase in tau and other relaxation parameters, suggesting relaxation depression.[27] Thus, with respect to marked changes in peak negative dP/dt in this study, this parameter was considered to be a sufficient predictor of diastolic relaxation.

In this experiment, the administration of ISO led to rapid decreases in preload, afterload (Figure 3) and myocardial diastolic function within 30 s (Figure 4). On the contrary, the myocardial contractility increased temporarily to 100% of the resting value (Figure 3(c)). This increase was followed by its decrease, but contractility remained elevated for the whole course of the experiment. This initial increase in contractility is in accordance with the haemodynamic action of ISO on β_1-adrenergic receptors. This can be confirmed by the administration of selective β_2-agonist salbutamol, which caused similar rapid reduction in afterload (Figure 3(b)), however, not a sudden peak in contractility (Figure 3(c)). Therefore, the main cause of the transient contractility peak in ISO group was not the reduced afterload. Salbutamol

brought about only a moderate increase in contractility which was probably caused by the activation of the sympathetic nervous system or by the partial activation of β_1-adrenergic receptors or by stimulation of presynaptic β_2-adrenergic receptors with subsequent endogenous catecholamine release. The latter was documented for another β_2-agonist clenbuterol.[32] Tachycardia, which can also elevate dP/dt_{max},[22] should be considered to be an additional factor. Nevertheless, this fact might not play a significant role in this study because after initial increase in the heart rate, tachycardia, in contrast to dP/dt_{max} curve, remained relatively stable.

The decrease in afterload in the ISO group, confirmed in this study by decreased TPR (Figure 3(b)), may rather improve the stroke volume; therefore, it apparently does not play any role in the decreases in stroke volume. Moreover, as the ejection fraction did not change, it indicates that the decrease in the stroke volume was apparently associated with diastolic dysfunction or decreased preload. The cause of the decrease in preload is not known but generalized vasodilation or/and tachycardia could have played a role. When comparing ISO with salbutamol, it seems that tachycardia may be the main factor because both drugs decreased afterload in a similar manner. This can be explained by the fact that therapeutic doses of ISO caused an increase in preload in clinical studies.[10] The increase in preload was observed in patients with coronary heart disease after the enhancement in heart rate. On the other hand, healthy patients demonstrated a decrease in preload after the same increment of the heart rate, which was similar in this study.[33] Moreover, the relationship between diastolic function/dysfunction and LVEDP, the current marker of preload is not linear.[34] Therefore, it seems that the main factor was the impaired diastolic function. This conclusion may be supported by several findings: (1) tachycardia after sc administration of ISO shortened the diastole and deteriorated diastolic blood supply to the working myocardium. In addition, the same dose reduced the myocardial blood flow in previous studies; (2) a reduced diastolic pressure may be an additional factor in the deterioration of myocardial blood supply; (3) an increase in water content of myocardium after ISO administration, suggesting oedema was very rapid in previous studies; (4) ischaemia which is well described in the myocardium after higher doses of ISO causes relaxation disturbances; (5) the speediness of diastolic dysfunction documented in this study seems to be very rapid, different studies documented very rapid impairment in the myocardial homeostasis, for example some increase in lactate and inorganic phosphate even 30 s after ISO administration; (6) deterioration of the relaxation decreases the stroke volume and cardiac output, notwithstanding the increase in the heart rate.[17,27,34,35] The augment in the heart rate generally causes an opposite increase in dP/dt_{min}, with a decrease in tau. Therefore, it improves the relaxation both in animals and in healthy humans. Nevertheless, the response may differ in patients with coronary heart disease.[17,19,33] Similarly, normal doses of noradrenaline, dobutamine, and ISO or stimulation of the sympathetic nervous system improved ventricular relaxation.[9,17,19,21,36]

Some questions may arise from the fact that salbutamol produced a decrease in peak negative dP/dt too (Figure 4). A decrease in preload (although it is known that the decreased preload means lower dP/dt_{min})[17] can be excluded because the preload was normal in salbutamol group and normalized in ISO group, regardless of the fact that peak negative dP/dt remained depressed. The probable cause of peak negative dP/dt decrease in salbutamol group was the very rapid decrease in blood pressure, in particular diastolic blood pressure. It was likely related to lower myocardial perfusion and transient ischaemia associated with a transient diastolic dysfunction. Contrarily, in ISO-treated group, the peak negative dP/dt continued to drop within the first minute and remained depressed through the whole course of the experiment. It indicates persistently impaired diastolic function. In addition, salbutamol, in contrast to ISO, improved diastolic filling phase.

ISO cardiotoxicity is associated with both diastolic and systolic dysfunction in later phases,[12,13] and this study shows that diastolic dysfunction is very rapid and observable prior to systolic dysfunction. The finding that diastolic dysfunction precedes systolic one was documented in humans with coronary heart disease, arterial hypertension, left ventricular hypertrophy, and aging-related myocardial changes.[33,37] Diastolic relaxation is not a passive event, it requires energy, too. Therefore, the drop in ATP content may markedly deteriorate the relaxation as it happened probably in this case.[35,38] Calcium may play an important role in the relaxation and contraction effects. Low doses of ISO accelerate calcium reuptake by sarcoplasmic reticulum, leading first to the improvement in relaxation and in higher doses to an increase in the slow inward calcium current through voltage-gated calcium channels and increase in contractility. Very high doses

or prolonged activation led to intracellular 'calcium overload' with its known consequences.[9,11,38–40]

This study had some limitations which mostly arise from the used methods. In particular, the precise study of ventricular volumes is dependent on many variable factors.[15,16] Some of them are changing after ISO administration (e.g. blood viscosity due to an increased platelet aggregation and coagulation, oedema of the heart tissue).[5,35,41] Therefore, the ventricular volume changes and their derived parameters cannot be established with precision within the whole course of the 2-h experiment. We used 15 min, implying a relatively short period in which these changes are of minor importance. We did not establish the pharmacokinetics of the administered drugs, but the results indicate that both drugs were rapidly absorbed. Following our previous studies as well as the results of others, the dose of ISO was chosen so that it would be significantly cardiotoxic.[11,35,42]

Pharmacological doses of ISO are known to improve left ventricular contraction and relaxation together with the stroke volume and ejection fraction,[10] but the cardiotoxic dose is associated with marked systolic and diastolic dysfunction. In this study, we demonstrated that the cardiotoxic sc dose of 100 mg/kg of ISO produces rapid diastolic dysfunction and on the other hand improves the systolic function of the myocardium. The observed early diastolic dysfunction is primarily associated with the overstimulation of β_1-adrenergic receptors, and the stimulation of β_2-adrenergic receptors alone is not associated with the early myocardial diastolic dysfunction.

Acknowledgements

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Declaration of Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplementary data

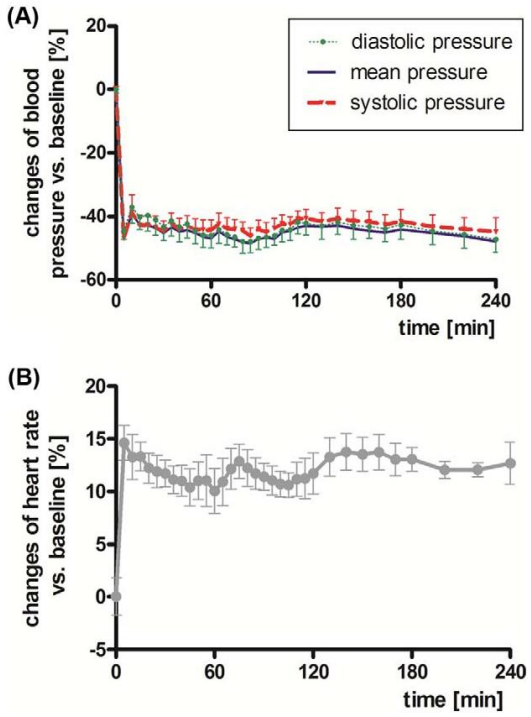

Figure S1. Arterial blood pressures (A) and the heart rate (B) after subcutaneous (sc) administration of 100 mg/kg isoprenaline (ISO) measured by the thermodilution method. Changes and statistical significance were calculated versus baseline levels before ISO administration. Changes in heart rate were significant at $p < 0.01$ from the first measurement at fifth minute and blood pressures were significant at $p < 0.001$ from the first measurement at the fifth minute.

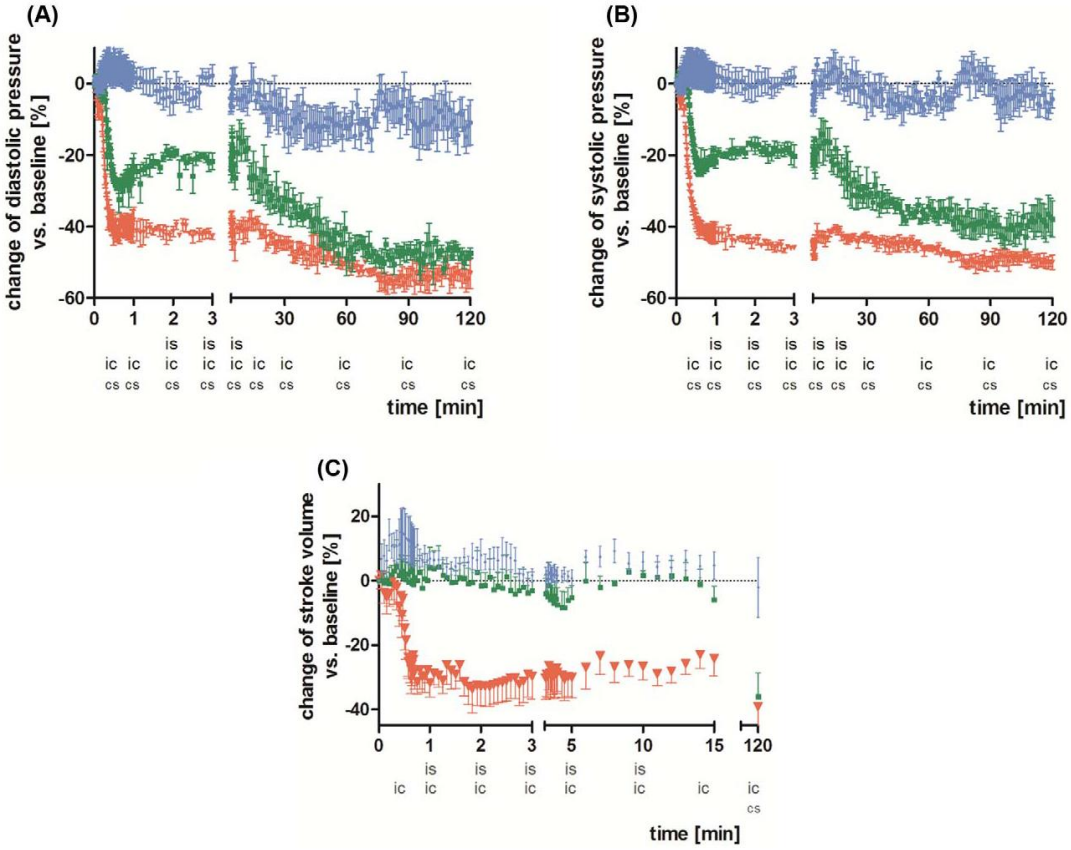

Figure S2. Arterial diastolic (A), systolic (B) blood pressures, (C) and stroke volume after the application of 100 mg/kg isoprenaline (ISO; red), salbutamol (green) and saline (blue)—the Millar catheter-based method. Statistical significance: is: ISO versus salbutamol at $p < 0.05$; ic: ISO versus control at $p < 0.05$; cs: salbutamol versus control at $p < 0.05$.

4. 6. Common biomarkers of oxidative stress do not reflect the cardiovascular dys/function

MLADĚNKA, Přemysl, ZATLOUKALOVÁ, Libuše, FILIPSKÝ, Tomáš, VÁVROVÁ, Jaroslava, HOLEČKOVÁ, Magdalena, PALICKA, Vladimir, HRDINA, Radomír. Common biomarkers of oxidative stress do not reflect the cardiovascular dys/function. *Biomedical Papers*. 2013, **157**, X. ISSN 1213-8118.

(IF 2011 – 0.702)

Časná diagnostika akutních koronárních syndromů, především akutního infarktu myokardu, i přes značný pokrok ve výzkumu biomarkerů poškození srdeční tkáně a oxidačního stresu zůstává oblastí zasluhující si pozornost.

Cílem této studie bylo popsat vzájemné vztahy mezi biochemickými markery a funkčními parametry srdeční dys/funkce u Wistar:Han potkanů rozdělených do dvou skupin (kontrolní a isoprenalinová skupina, celkem 145 potkanů). Podání kardiotoxické dávky isoprenalinu (100 mg/kg, s.c.) simulovalo patologický stav, který je v časné fázi v mnoha aspektech podobný akutnímu infarktu myokardu u lidí.

Sérové koncentrace srdečního troponinu (cTnT), esenciálního biomarkeru poškození srdeční tkáně u lidí, silně korelovaly se závažností poškození myokardu u potkanů (např. s přetížením myokardu vápníkem – pozitivní korelace a tepovým objemem – negativní korelace). Na druhou stranu korelace cTnT s markery oxidačního stresu byly spíše nevýrazné (glutathion a vitamin C) nebo nulové (vitamin E a TBARS - reaktivní formy thiobarbiturové kyseliny, z angl. thiobarbituric acid reactive substances). Vztah mezi cTnT a dalšími parametry byl exponenciální s výjimkou koncentrace vápníku v srdeční tkáni, kde byl nalezen vztah, který popisuje mocninná funkce.

Na základě provedené studie nelze běžné biomarkery oxidačního stresu použít k diagnostice srdeční dys/funkce po podání nekrogenní dávky isoprenalinu u potkanů.

Common biomarkers of oxidative stress do not reflect cardiovascular dys/function in rats

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Background. Predicting cardiovascular events remains challenging despite the range of known biomarkers.
Aim. To establish relationships between various biochemical and functional parameters of the cardiovascular system.
Method. The relationship between cardiovascular dys/function and various biomarkers was examined in 145 experimental rats half of which received isoprenaline 100 mg/kg s.c. to induce cardiac impairment.
Results. Serum concentration of cardiac troponin T (cTnT), a known marker of cardiac derangement, correlated strongly with degree of myocardial injury (e.g. calcium overload, stroke volume) but correlations between cTnT and oxidative stress parameters were weak (for glutathione and vitamin C) or not found (for serum vitamin E and plasma thiobarbituric acid reactive substances levels). Relationships between cTnT and other parameters were exponential with the exception of myocardial calcium, where a power function was found.
Conclusions. Commonly used biomarkers of oxidative stress cannot reliably predict cardiovascular dys/function in experimental rats.

Keywords: oxidative stress, cardiovascular disease, catecholamines, acute myocardial infarction

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INTRODUCTION

Cardiovascular diseases are a leading cause of mortality and morbidity worldwide, accounting for around 17 million of deaths each year. Although recent trends in health care have slightly reduced fatal cardiovascular events, the incidence cardiovascular disorders is not decreasing. Moreover, the high prevalence of these diseases is no longer the prerogative of developed nations. Risk factors for cardiovascular diseases are well known, but predicting a cardiovascular event remains challenging, despite the number of known biochemical markers[1-4]. Involvement of oxidative stress in some cardiovascular diseases is clearly suggested in most studies, although the relationship of oxidative stress markers and cardiovascular mortality is equivocal and clinical use of antioxidants is inconclusive[5,6].

Coronary heart disease accounts for more than 40% of cardiovascular deaths and its most serious form, acute myocardial infarction (AMI), is the principal cause of chronic heart failure[1]. Elevated catecholamines are known risk factors for a cardiovascular event and the synthetic catecholamine, isoprenaline has been used for about a half a century for inducing AMI and subsequent heart failure in small laboratory animals[7-10]. In our previous study, we found in a very small number of animals some correlations among various parameters in healthy and AMI-induced animals[10]. The study reported here was aimed at a more detailed analysis: 1) to confirm the association of the cardiovascular injury and its known biochemical markers; 2) to analyze the relationship of common biomarkers of oxidative stress and degree of cardiovascular dys/function. The animals were intentionally not stratified to mimic normal population with the presence or absence of cardiovascular disorders, as well as treated and untreated patients. Moreover, the use of animals in this study enabled analysis of myocardial elements, in particular calcium and this cannot be performed in humans.

METHODS

Animals and measurements

Our group recently analysed the effects of different drugs with iron chelating properties on the isoprenaline model of cardiac toxicity in rats. Most of the data have already been published as studies of the effects of iron chelating drugs on this model[10-14]. The present study was an analysis of the data from a total of 145 young male Wistar: Han rats (Biotest s.r.o. Konárovice, Czech Republic) from another viewpoint. All experiments conformed 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) and were performed

with the approval of the Ethics Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové.

The experimental procedure was the same in all animals. Detailed methodology can be found in our publications (e.g. ref. 10, 12, 13). In brief, all animals received i.v. various agents with iron chelating properties or solvent; 72 animals received 100 mg/kg isoprenaline (Sigma-Aldrich, USA) s.c. in a 5 min interval to induce myocardial impariment; 73 rats formed the controls. After 24 h, the anaesthesized animals (urethane 1.2 g/kg i.p., Sigma-Aldrich, USA) were connected to the instrument Cardiosys® (Experimentria Ltd, Hungary) with software Cardiosys 1.1. for the measurement of haemodynamic variables. At the end of the experiment, blood was withdrawn and the heart excised for analysis of antioxidants, enzymes and metal ions in the myocardium. Cardiac troponin T (cTnT), vitamin E, and vitamin C were measured in serum, thiobarbituric acid reactive substances (TBARS) in plasma, antioxidant enzymes SOD and GPx in erythrocytes and the total glutathione in the whole blood. Standard approaches were used for the analysis: SOD and GPx were determined by commercial kits (Randox, United Kingdom), cTnT by electroluminescence immunoassay (Roche Diagnostics, Germany), vitamin E by fluorimetric detection, the total blood glutathione and vitamin C by UV detection. Iron, copper, and selenium were determined using graphite furnace atomic absorption spectrometry. Zinc was determined using flame atomic absorption spectrometry and calcium was measured photometrically using flame photometry.

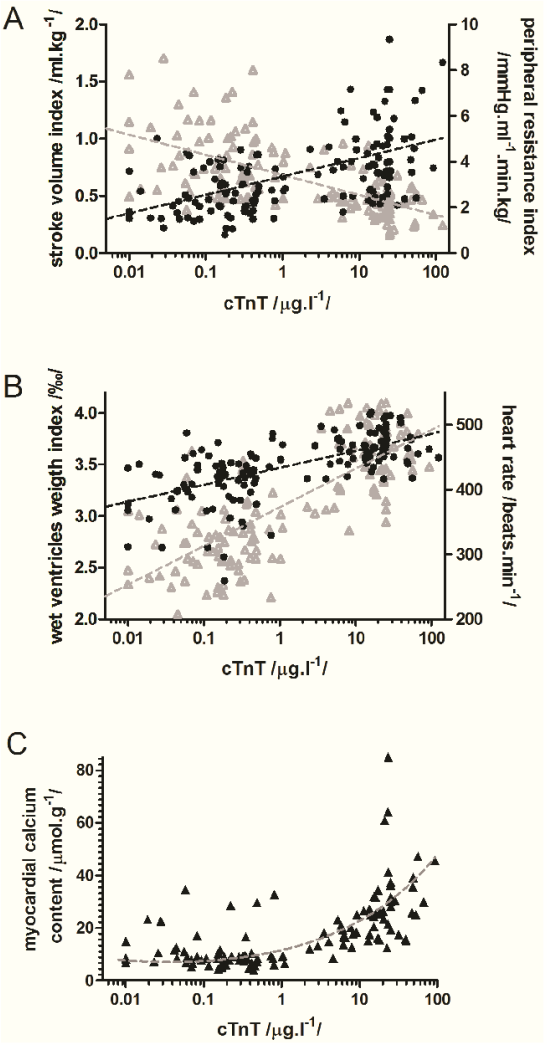

Fig. 1. Relationship of cardiac troponin T in serum with stroke volume, peripheral resistance index (A), wet ventricle weight index, heart rate (B) and myocardial calcium content (C).

Equations are as follows: the stroke volume index = -0.17 log (cTnT) + 0.68 /R^2=0.37/; the peripheral resistance index = 0.76 log (cTnT) + 3.38 /R^2=0.24/; the wet ventricle weight index = 0.36 log (cTnT) +3.09 /R^2=0.55/ ; the heart rate = 25 log (cTnT) + 434 /R^2=0.39/; the myocardial calcium content = 1.45 $cTnT^{0.25}$ + 10.25 $cTnT^{0.33}$ /R^2=0.53/.

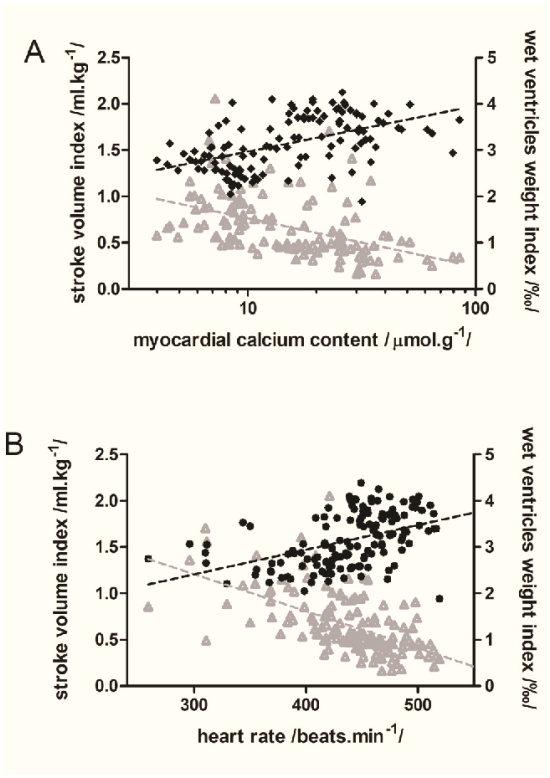

Fig. 2. Relationship of myocardial calcium content (A) and heart rate (B) with the wet ventricle weight and stroke volume index.

Equations are as follows: the stroke volume index = -0.52 log (Ca) + 1.29 /R^2=0.21/; the wet ventricle weight index = 0.99 log (Ca) +1.98 /R^2=0.27/; the stroke volume index = - 0.004 x heart rate + 2.404 /r^2=0.34/; the wet ventricle weight index = 0.005 x heart rate + 0.815 /r^2=0.20/.

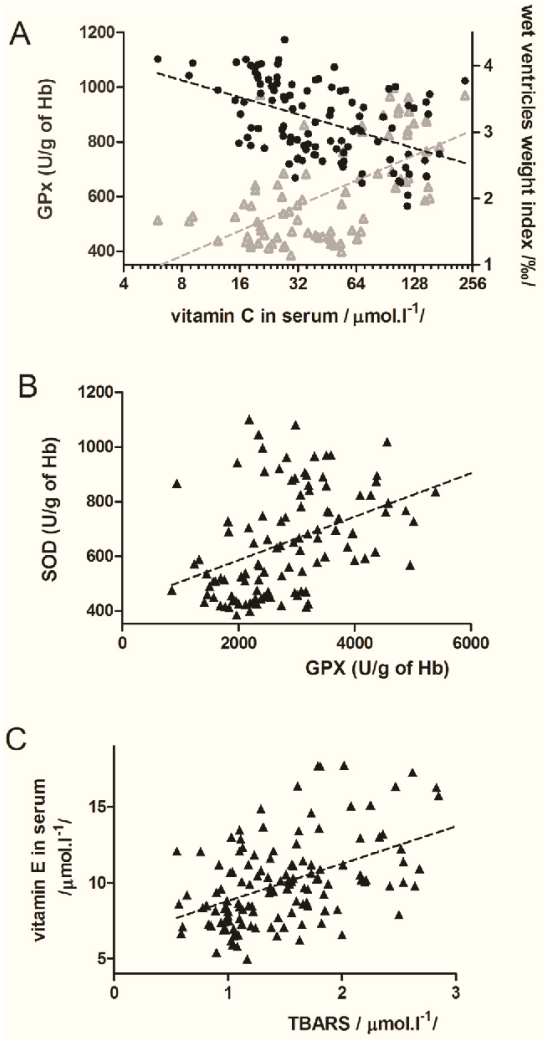

Fig. 3. Relationship of vitamin C serum concentration with the wet ventricle weight and GPx activity (A), of GPx and SOD activities (B) and between TBARS concentration and vitamin E serum level (C).
Equations are: GPx = 305.3 x log (vitamin C) + 106.9 /R^2=0.36/; wet ventricle weight index = -0,85 x log (vitamin C) + 4.55 /R^2=0.23/; SOD = 0.080 GPx +426.2 /r^2=0.19/; vitamin E = 2.46 x TBARS + 6.35 /r^2=0.23/

"Double product" was calculated as a product of the systolic blood pressure and heart rate and the total peripheral resistance was calculated as the mean arterial blood pressure divided by cardiac output. Stroke volume, wet ventricles weight and the total peripheral resistance are expressed as indices (variables divided by the body weight).

Statistical analysis

Each data set was firstly checked by D'Agostino-Person normality test for Gaussian distribution. Data sets with non-Gaussian distribution were analysed by the use of non-parametric Spearman's correlation test and those with Gaussian distribution with Pearson's test. The minimal level of the statistical significance was $P \leq 0.05$. Data with the correlation coefficient higher than 0.45 were analyzed by linear or non-linear regression. All data analyses were performed by the use of GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

The correlations were performed for all parameters with the exception of the derived parameters (e.g. double product and blood pressure). Significant correlation coefficients are shown in Table 1. The detailed relationship of the variables with correlation coefficients higher than 0.45 is depicted in Fig. 1-3.

There was a high correlation between levels of cTnT and total peripheral resistance, wet ventricle weight, myocardial calcium content, heart rate but only a weak correlation of cTnT with diastolic blood pressure and the "double product". Except for calcium concentration, the relationship was linear in the semilogarithmic plot. A more complicated relationship of the type of a power function was found between myocardial calcium level and serum cTnT. A negative strong association was found for cTnT and stroke volume, again with a linear character in the semilogarithmic plot. A weaker negative association with total blood glutathione and serum vitamin C concentrations was disclosed. No significant association was found for vitamin E, TBARS, SOD and other myocardial elements with cTnT.

The relationships of heart rate, myocardial calcium levels and wet ventricle weight with these parameters were very similar to those of cTnT (Table 1, Fig. 2 *vs.* Fig. 1).

The total peripheral resistance was likewise positively associated with cTnT, myocardial calcium, wet ventricle weight and heart rate and negatively with the total blood glutathione. But in addition to these parameters, a positive moderate correlation was found with TBARS.

Stroke volume index correlated positively with the total blood glutathione and weakly negatively with TBARS, GPx and myocardial zinc concentration in addition to the stronger correlations mentioned (cTnT, Ca, wet ventricle weight, heart rate). The total blood glutathione correlations were mostly less expressed, but the mentioned negative correlation with total peripheral resistance supports the previous results.

For serum antioxidants (vitamin C and vitamin E), the correlations were rather opposite. Vitamin C correlated very negatively with wet ventricle weight and moderately with cTnT. Relatively high positive correlations of vitamin C were found with myocardial zinc level and activities of erythrocytes antioxidant enzymes SOD and GPx. Of interest is that the relationship of vitamin C with other parameters was of an exponential character (Fig. 3A). In contrast, vitamin E did not correlate with cTnT, total

Table 1. Significant correlation coefficients among measured variables.

	diastolic blood pressure	systolic blood pressure	stroke volume	double product	peripheral resistance	heart rate	wet ventricles weight	Zn	Fe	Se	Cu	Ca	SOD	GP_x	TBARS	vitamin E	vitamin C	GSH
cTnT	0.24**	n.s.	-0.66***	0.36***	0.54***	0.67***	0.74***	n.s.	n.s.	n.s.	n.s.	0.70***	n.s.	-0.19*	n.s.	n.s.	-0.30**	-0.28***
GSH	n.s.	n.s.	0.43***	-0.17*	-0.35***	-0.29***	n.s.	-0.29**	0.20*	n.s.	0.24**	-0.30***	n.s.	-0.27**	n.s.	n.s.	-0.19*	
vitamin C	n.s.	n.s.	n.s.	n.s.	n.s.	-0.23*	-0.51***	0.41***	n.s.	-0.21*	n.s.	n.s.	0.43***	0.57***	n.s.	n.s.		
vitamin E	-0.19*	-0.20*	n.s.	n.s.	n.s.	n.s.	n.s.	-0.20*	n.s.	n.s.	-0.28**	n.s.	-0.30**	-0.39***	0.47***			
TBARS	n.s.	n.s.	-0.23**	n.s.	0.27**	n.s.	n.s.	n.s.	-0.32***	0.24**	-0.24**	n.s.	n.s.	n.s.				
GPx	n.s.	n.s.	-0.25**	n.s.	0.20*	n.s.	-0.39***	0.40***	-0.25**	n.s.	n.s.	n.s.	0.49***					
SOD	n.s.	n.s.	n.s.	n.s.	0.19*	n.s.	-0.26**	0.22*	-0.32***	n.s.	0.20*	n.s.						
Ca	n.s.	n.s.	-0.56***	0.22*	0.44***	0.44***	0.53***	n.s.	-0.26**	n.s.	n.s.							
Cu	0.30**	0.34**	n.s.	0.27**	n.s.	n.s.	n.s.	n.s.	0.20*	-0.24**								
Se	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.									
Fe	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.29**										
Zn	n.s.	n.s.	-0.20*	n.s.	0.19*	n.s.	n.s.											
wet ventricles weight	0.25**	n.s.	-0.42***	0.32***	0.41***	0.52***												
heart rate	0.45***	n.s.	-0.63***															
stroke volume	-0.17*	n.s.																

Zn, myocardial zinc content; Fe, myocardial iron content; Se, myocardial selenium content; Ca, myocardial calcium content; SOD, erytrocyte superoxide dismutase; GPx, erythrocyte glutathione peroxidase; GSH, total blood glutathione. Vitamin E and vitamin C were measured in the serum, TBARS in the plasma
Notes: n.s. – non-significant, * significant at $P<0.05$, ** $P<0.01$, *** $P<0.001$

blood glutathione, wet ventricle weight, myocardial calcium content, stroke volume, heart rate or total peripheral resistance at all. However, its negative correlation with the erythrocyte enzymes SOD and GPx activities, and a positive one with TBARS, are of note. Analogously to vitamin E, TBARS did not correlate with other variables or only slightly. For GPx, in addition to the mentioned correlations, a relatively strong correlation was found with myocardial zinc concentration. Myocardial selenium and iron did not correlate with almost any of the variables. Interestingly, there was an intermediately strong correlation of myocardial copper level with diastolic and systolic blood pressures.

In the analysis of relationship among haemodynamic parameters, an interesting correlation was found between heart rate and diastolic blood pressure, but not with systolic blood pressure.

DISCUSSION

Biomarkers of the cardiovascular injury

cTnT and cTnI are the most commonly used biomarkers of cardiac injury at the moment. They are used successfully in the diagnosis of AMI with better sensitivity over previously used biomarkers. Various studies have described excellent negative correlations of cTnT concentration with the left ventricular contractility impairment or cardiomyocyte viability and positive one with myocardial infarction size[15,16]. In our previous study, we established a correlation of the cTnT concentration with cardiac function parameters in a relatively small number of animals[10]. In the present study, the relation of the cTnT levels with the other haemodynamic variables was further confirmed, extended and mathematically evaluated. The dependence of cTnT concentration on most of measured parameters was of an exponential character, e.g. it could be transformed into a linear relationship in the semilogarithmic plot. Similarly, other authors reported the exponential relationship between cardiomyocyte viability and cTnT level[15]. A more complicated relationship (power function) was found between myocardial calcium content and cTnT level.

One prominent feature of the various cardiovascular diseases is the "calcium overload"[17,18]. Like cTnT, calcium myocardial levels correlated in a similar manner with the parameters of cardiovascular function. Moreover, in accordance with cTnT, these dependences were again of an exponential type. Our results show the extent of calcium overload can be estimated from cTnT concentration, stroke volume index and heart rate. Interestingly, heart rate correlated positively not only with calcium overload and cTnT, but as well with the diastolic blood pressure and negatively with the stroke volume index. Thus, elevated heart rate suggests impaired cardiovascular function. This has been well documented in humans, where increased heart rate is an important predictor of mortality[19]. High blood pressure is another risk factor of cardiovascular diseases[20] and thus some degree of relation to other parameters was expected. The diastolic blood pressure correlated positively with cTnT concentration and wet ventricle weight, but the systolic blood pressure did not correlate with cardiovascular function parameters at all. A limited correlation for systolic blood pressure was with myocardial copper content. Copper may affect the cardiovascular function, e.g. elevated serum copper levels were found in patients with AMI in relation to the severity of the disease[21]. A serum zinc disturbance was shown in AMI (ref.[21,22]) and we found an association between myocardial zinc level and the mean blood pressure in our previous study[10]. However, this association was not confirmed in the present study. Generally, myocardial zinc concentration fluctuation was relatively low in this study - 91% of data were in a narrow range (0.80-1.20 μmol/L). Studies on myocardial selenium levels in AMI are inconsistent[23-25], but at least within 30 days after AMI, selenium concentration seems to be stable[25] and, indeed, only few very weak correlations were found in the present study. In contrast, iron homeostasis perturbance accompanies AMI, but the serum iron concentration did not correlate with the biochemical markers of cardiac injury in accordance with our study[26,27].

The wet ventricle weight index reflects indirectly the pathological changes in the myocardium, which may include infiltration of leucocytes with inflammatory response and fibrosis, as well. In the present study, owing to the relatively shorter time after cardiovascular insult (i.e. 24 h after isoprenaline administration), the former was rather present[10]. But still, this factor correlated strongly with the cardiovascular injury.

Interrelationship among parameters of oxidative stress and the cardiovascular dys/function

A very recent review[6] of oxidative stress biomarkers and prediction of cardiovascular diseases showed equivocal results. The authors suggested that the currently used markers probably do not precisely reflect the oxidative stress status and/or the different methodological approaches used may be the cause of the variable conclusions. They found, e.g. that the relationship between oxidized LDL and the risk of cardiovascular events may be positive or negative in different sub-populations of patients. Other studies in patients suffering from AMI (with/without reperfusion) or stable/unstable angina pectoris are inconsistent, as well[25,28-33]. The present study findings are in the harmony with this review. The prediction of cardiovascular dysfunction from the parameters of oxidative stress was of very limited significance, although between some antioxidants and the parameters of oxidative stress significant correlations were found. The only partial exception seems to be the level of total blood glutathione, which correlated weakly positively with stroke volume and negatively with heart rate and myocardial calcium content. The level of oxidized or reduced glutathione may correlate more strongly but this was not measured in this series of experiments and remains to be established in our further study. The complexity of the antioxidant system and an unknown relationship to the cardiovascular function could be demonstrated in the case of serum vitamin C concentration. Vitamin C levels correlated strongly neg-

atively with the wet ventricle weight and less importantly with cTnT concentration. However no correlation was found with calcium levels, which, as mentioned above, shows strong correlations with both previously cited parameters. In addition, an obviously contradictory finding is the weak negative correlation of vitamin C with total blood glutathione. Interestingly, serum vitamin C concentrations, myocardial zinc levels and erythrocyte antioxidant enzymes SOD and GPx activities mutually correlated positively, but again, no or only very approximate relation to cardiovascular dys/function was detected. Associations of vitamin E levels are even more confounding. The published studies suggested the central role of vitamin E in the antioxidant system and the necessity to cooperate with other antioxidants, in particular with vitamin C. But in the contrast to vitamin C, vitamin E did not correlate in this study with any measured parameter of the cardiovascular dysfunction (with the exception of the weak negative correlation with blood pressure). TBARS has been used as an indicator of malondialdehyde, a stable product of lipid peroxidation, and therefore a marker of oxidative stress. However, malondialdehyde is the only one of several substances that react with thiobarbituric acid and malondialdehyde measured by HPLC did not correlate with TBARS (ref.[6,30]). Other studies found no correlation of TBARS with the biochemical parameters of cardiac injury or plasma iron, but positive correlation with C-reactive protein was found[27,29,32]. The highly significant positive correlation of vitamin E with TBARS (Fig. 3C) is therefore of unknown meaning and in contrast another study showed in extreme exercisers, a more specific marker of lipid peroxidation F_2-isoprostane correlated negatively with vitamin E level[34,35].

There are two probable explanations for the failure of the markers of oxidative stress to predict the cardiovascular dys/function: 1) the currently used biomarkers do not accurately reflect oxidative stress; 2) the oxidative stress status does not correspond to the cardiovascular dys/function. The first reason seems not to be very likely because the cardiovascular diseases in general, as well as the used model of cardiotoxicity, are considered to be associated with oxidative stress[5,36-38] and even if TBARS may be not an accurate indicator of oxidative stress, vitamins E and C or antioxidant enzymes should at least partly react on the oxidative stress status. Therefore, we are of that opinion, that even if oxidative stress is an accompanying factor of the cardiovascular derangement, the commonly used oxidative stress biomarkers cannot be used for the assessment of cardiovascular dysfunction.

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CONFLICT OF INTEREST

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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4. 7. Dexrazoxane provided moderate protection in a catecholamine model of severe cardiotoxicity

ZATLOUKALOVÁ, Libuše, FILIPSKÝ, Tomáš, MLADĚNKA, Přemysl, Semecký, Vladimír, MACÁKOVÁ, Kateřina; HOLEČKOVÁ, Magdalena, VÁVROVÁ, Jaroslava; PALICKA, Vladimir, HRDINA, Radomír. Dexrazoxane provided moderate protection in a catecholamine model of severe cardiotoxicity. *Canadian Journal of Physiology and Pharmacology*. 2012, **90**(4), 473-484. ISSN 0008-4212.

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V současné době převládá hypotéza, že příznivé účinky dexrazoxanu vůči antracyklinové kardiotoxicitě jsou převážně spojeny s jeho schopností chelatovat železo. Nicméně tento mechanismu účinku je důkladně přehodnocován. Kromě účasti na patogenezi antracyklinové kardiotoxicity se železo výraznou měrou podílí i na patogenezi kardiotoxicity katecholaminové.

V této studii jsme se tedy zaměřili na zhodnocení vlivu intravenózně podaného dexrazoxanu na isoprenalinový model akutního infarktu myokardu u Wistar:Han potkanů. Za účelem vysvětlení mechanismu/ů kardioprotekce dexrazoxanu byly provedeny 2 typy *in vivo* experimentů: a) 24hodinový; b) 2hodinový. Potkanům byl nejdříve podán dexrazoxan (20,4 mg/kg, i.v.) a za 5 minut následovalo podání kardiotoxické dávky isoprenalinu (100 mg/kg, s.c.). Součástí těchto experimentů byla analýza hemodynamických parametrů, EKG a také biochemická a histopatologická vyšetření. Rovněž byly provedeny dodatečné *in vitro* experimenty analyzující schopnosti jednotlivých léčiv a jejich kombinace chelatovat/redukovat železo a ovlivnit železem katalyzovanou Fentonovu reakci.

V 24hodinovém *in vivo* experimentu podání dexrazoxanu vedlo k částečnému poklesu mortality, snížení koncentrace vápníku v srdeční tkáni a ke zlepšení histopatologického nálezu a hemodynamických parametrů. Kontinuální 2hodinový experiment prokázal, že dexrazoxan není schopen ovlivnit isoprenalinem navozené atrioventrikulární bloky a jeho vliv na hemodynamické parametry byl zde spíše minimální.

Protektivní účinky dexrazoxanu vůči isoprenalinové karditoxicitě jsou na základě této studie pravděpodobně zprostředkovány inhibicí pozdních srdečních změn a nižší incidencí ventrikulárních fibrilací v důsledku snížení koncentrace vápníku v srdeční tkáni. *In vitro* experimenty naznačily, že chelatační vlastnosti dexrazoxanu nehrají významnou roli v kardioprotektivním mechanismu.

Dexrazoxane provided moderate protection in a catecholamine model of severe cardiotoxicity

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Abstract: Positive effects of dexrazoxane (DEX) in anthracycline cardiotoxicity have been mostly assumed to be associated with its iron-chelating properties. However, this explanation has been recently questioned. Iron plays also an important role in the catecholamine cardiotoxicity. Hence in this study, the influence of DEX on a catecholamine model of acute myocardial infarction (100 mg/kg of isoprenaline by subcutaneous injection) was assessed: (*i*) the effects of an intravenous dose of 20.4 mg/kg were analyzed after 24 h, (*ii*) the effects were monitored continuously during the first two hours after drug(s) administration to examine the mechanism(s) of cardioprotection. Additional in vitro experiments on iron chelation/reduction and influence on the Fenton chemistry were performed both with isoprenaline/DEX separately and in their combination. DEX partly decreased the mortality, reduced myocardial calcium overload, histological impairment, and peripheral haemodynamic disturbances 24 h after isoprenaline administration. Continuous 2 h experiments showed that DEX did not influence isoprenaline induced atrioventricular blocks and had little effect on the measured haemodynamic parameters. Its protective effects are probably mediated by inhibition of late myocardial impairment and ventricular fibrillation likely due to inhibition of myocardial calcium overload. Complementary in vitro experiments suggested that iron chelation properties of DEX apparently did not play the major role.

Key words: dexrazoxane, catecholamine, iron, acute myocardial infarction, cardiotoxicity, isoprenaline, myocardium.

Résumé : Les effets positifs du dexrazoxane (DEX) sur la cardiotoxicité des anthracyclines ont surtout été attribués à ses propriétés de chélateur de fer. Cette explication a cependant été remise en question récemment. Le fer joue aussi un rôle dans la cardiotoxicité des catécholamines. C'est ainsi que dans cette étude, l'influence du DEX a été évaluée dans un modèle d'infarctus aigu du myocarde induit par les cathécolamines (100 mg/kg d'isoprénaline sous-cutané) : (*i*) les effets d'une dose intraveineuse de 20,4 mg/kg de DEX ont été analysés après 24 h et (*ii*) les effets ont été suivis de manière continue au cours des deux premières heures suivant l'administration du ou des médicaments afin d'examiner les mécanismes responsables de cardioprotection. Des expériences additionnelles in vitro de chélation/réduction du fer et de l'influence sur la chimie de Fenton ont été réalisées avec l'isoprénaline et le DEX utilisés séparément ou conjointement. Le DEX diminuait partiellement la mortalité, réduisait la surcharge calcique de myocarde, les anomalies histologiques et les perturbations hémodynamiques périphériques 24 h après l'administration d'isoprénaline. Les expériences réalisées en continu pendant les deux premières heures ont montré que le DEX n'avait pas d'influence sur le blocage atrioventriculaire induit par l'isoprénaline et avait peu d'effet sur les paramètres hémodynamiques mesurés. Ses effets protecteurs sont probablement dus à l'inhibition des dysfonctions tardives du myocarde et de la fibrillation ventriculaire, vraisemblablement à cause d'une inhibition de la surcharge calcique du myocarde. Les expériences complémentaires réalisées in vitro ont suggéré que les propriétés du DEX dans la chélation du fer ne jouaient apparemment pas le rôle principal.

Mots-clés : dexrazoxane, catécholamine, fer, infarctus aigu du myocarde, cardiotoxicité, isoprénaline, myocarde.

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Abbreviations: Abbreviations: AMI, acute myocardial infarction; AV, atrioventricular; DEX, dexrazoxane; ISO, isoprenaline; ROS, reactive oxygen species; PCTH, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone.

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Introduction

Dexrazoxane (DEX) is the only approved drug for the prevention of anthracycline cardiotoxicity, and few studies reported its positive effects on cardiac impairment caused by ischaemia-reperfusion or chronic catecholamine administration (Cvetković and Scott 2005; Flandina et al. 1990; Hrdina et al. 2000; Popelová et al. 2009; Ramu et al. 2006). However, its protective effect has been traditionally attributed to the inhibition of reactive oxygen species (ROS) formation due to chelation of iron ions, but recent findings questioned this (Popelová et al. 2008; Simůnek et al. 2009).

Administration of synthetic catecholamine isoprenaline (ISO) leads to a pathological state, similar in many aspects to acute myocardial infarction (AMI) in humans. Although the pathophysiology is very complicated and not fully understood, the involvement of overstimulation of β-adrenergic receptors, catecholamine redox cycling, and iron has been well documented both in vivo as well as in vitro experiments (Hašková et al. 2011*b*; Rona 1985). Recently, we reported that iron chelator 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH) provided marked protection on the mentioned model (Mladenka et al. 2009*b*). Therefore, if the traditional hypothesis is correct, DEX may also be protective in this model. Moreover, DEX appears to have some advantages in comparison with other drugs with iron-chelating properties: (*i*) a recent study suggested its non-iron based direct ROS scavenging properties (Galetta et al. 2009); (*ii*) DEX enters easily into the intracellular compartments (Dawson 1975); (*iii*) in comparison with other iron-chelating agents, DEX is a prodrug, which can be directly activated by iron (Buss and Hasinoff 1995; Hasinoff 1998; Hasinoff and Aoyama 1999*b*). In particular, this latter fact may be advantageous and can be likely responsible for its low toxicity. Other iron chelators may actually withdraw iron from the physiologically important biochemical pathways, and hence they have narrower therapeutic range (Cohen et al. 2004; Liu and Hider 2002).

The only study examining the effect of DEX on ISO cardiotoxicity was by Flandina et al. (1990). In that study, ISO was administered in a dose of 20 mg/kg once a week for 5 weeks, and the protective effects were noted. However, it should be emphasized that such experimental design corresponds rather with a model of chronic heart failure (Carll et al. 2011). On the contrary, this study aims at the analysis of acute effect of DEX in the ISO-model of AMI. This enables the comparison with other iron chelators, which we previously tested on the same model (Mladenka et al. 2009*b*, 2009*c*, 2009*d*). Additional aims were: (*i*) to analyse acute haemodynamic effects of DEX within the period of 2 h after its intravenous (i.v.) administration and to check whether they may be associated with cardioprotection; (*ii*) to verify the involvement of iron chelation in the cardioprotective effects.

Material and methods

Animals

Wistar:Han male rats obtained from Biotest s.r.o. (Czech Republic) were used after 2 weeks of acclimatization. The animals were maintained in an air-conditioned room, and free access to a standard pellet diet for rodents and tap water was allowed. Animals fasted for 12 h 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).

24 h experiments

Animals weighing approximately 350 g were randomly divided into the following groups:

1. Control group I (7 animals): 2 mL/kg of saline subcutaneous injection (s.c.) (Braun, Germany)
2. ISO group I (10 animals): ISO 100 mg/kg s.c. (Sigma-Aldrich, Germany, dissolved in saline)
3. DEX group I (7 animals): 20.4 mg/kg of DEX i.v. (Cardioxane®, Chiron, UK)
4. DEX- ISO group I (8 animals): rats received DEX 5 min before ISO in the same dose as described above

Twenty-four hours after drug administration, rats were anaesthetized with urethane (1.2 g/kg intraperitoneal injection (i.p.), Sigma-Aldrich, Germany). A polyethylene catheter (0.5/1.0 mm filled with heparinised saline 50 IU/mL) 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) was inserted into the left iliac artery, which was connected to the blood pressure transducer BPR-01 of the apparatus for measurement of haemodynamic variables (Cardiosys, Experimentria Ltd, Hungary) using the software package Cardiosys version 1.1. For the measurement of cardiac output, a thermodilution transpulmonary method was used according to the Stewart-Hamilton method (Spiller and Webb-Peploe 1985).

The measurements of haemodynamic parameters were carried out following a 15 min equilibration period after the surgical procedure. Functional variables (cardiac output, stroke volume, blood pressure, heart rate) were averaged from four recordings performed in 5 min intervals. Results are expressed as indices (measured variable divided by the body weight) except for the blood pressure, heart rate, and the "double product" (systolic blood pressure multiplied by heart rate). The last 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.

After haemodynamic measurement, a blood sample (approximately 5 mL) was collected from the abdominal aorta into the heparinised test tube (170 IU). The animal was then sacrificed by i.v. potassium chloride (1 mmol/L, Sigma-Aldrich, Germany) overdose. Heart ventricles were excised, weighted (for the assessment of wet ventricles weight), and frozen at –20 °C for further analysis of the selected elements content. The wet ventricles weight is expressed as an index, i. e., weight of ventricles in grams divided by the weight of the body in kilograms, thus expressed in per thousand (‰).

Histological analysis

After the autopsy, the apical parts of the hearts were fixed in a Bouin solution and processed for light microscopy. Tissues were dehydrated in increasing concentrations of ethanol,

embedded in paraffin, and sectioned into 5–8 μm slices. The tissue sections were stained with haematoxylin and eosin. 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). Semiquantitative histological analysis was performed from 20 serial sections, by the assessment of the relative occurrence of observed histological impairment related to area of tissue uniformly by 200× magnification.

Biochemical analysis of the blood

Cardiac troponin T (cTnT) and vitamin E were measured in serum and total glutathione in the whole blood. cTnT was determined by electrochemoluminescence immunoassay (Elecsys 2010, Roche Diagnostics), which employs two monoclonal antibodies specifically directed against cTnT. Capillary electrophoresis was used for separation of glutathione, which was measured by UV detection (System P/ACE 5100, Beckman) at 200 nm. After deproteinisation, 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, weighted, 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, UK), zinc was determined using flame atomic absorption spectrometry (Unicam, Solaar 959, UK), and calcium was measured photometrically using flame photometry (Eppendorf, Efox 5053, Germany). Results are expressed as $\mu mol \cdot g^{-1}$ (iron, copper, zinc, calcium) or $nmol \cdot g^{-1}$ (selenium) of dry tissue.

2 h experiments

Rats weighing approximately 370 g were anaesthetized using i.p. urethane (the same dose as above) before administration of drugs. A pressure transducer MLT0380/D (AdInstruments, Australia) was connected by a polyethylene catheter 0.5/1.0 mm (filled with heparinised saline 10 IU/mL) with the common left iliac artery. Millar PV-catheter SPR-838 (2F, 4E, 9 mm; Millar Instruments, Inc., USA) was inserted in the left cardiac ventricle through the right carotid artery. Both pressure transducer and Millar PV-catheter, together with subcutaneous electrodes for the ECG standard limb lead II (MLA1215, AdInstruments, Australia), were connected to the PowerLab apparatus equipped with software LabChart version 6 (AdInstruments, Australia). Drug (s) were administered to rats after a 15 min equilibration period following the surgical procedures:

- Control group IIa (3 animals), same as control group I (i.e., s.c. saline); the control group IIb (3 animals): rats received 1 mL/kg of saline i.v.
- DEX group II (5 animals), ISO group II (9 animals), and DEX-ISO group II (5 animals) received drugs as mentioned above

Animals were monitored for 2 h. The animal temperature was maintained during the entire experiment at 36.5 °C ± 0.5 °C (TCAT-2LV Controller, Physiterm Instruments Inc., USA). The experiment was terminated in the same way as in the 24 h experiments.

In vitro experiments

Ferrous chelation was measured by ferrozine, as previously described by us (Mladenka et al. 2010). Various concentrations of water solution of DEX and (or) ISO were mixed with water ferrous solutions in different buffers (pH 4.5–7.5) before the addition of water solution of ferrozine. Absorbance was measured at wavelength 562 nm using the spectrophotometer Anthos reader 2010 (Anthos Labtec Instruments, Austria). Ferric ions reduction was assessed in a similar setting. Ferric ions were first mixed with DEX and (or) ISO in the buffers. Thereafter, ferrozine was added to measure the concentration of ferrous ions, i.e., the concentration of reduced iron (Mladenka et al. 2010).

Ferrous ions form with hydrogen peroxide hydroxyl radicals (Fenton reaction), which may be trapped by use of salicylic acid, and ensuing products (2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids) can be detected by HPLC. The effects of ISO and DEX on the Fenton reaction were measured in the previous reports (Filipský et al. 2011). Ferrous ions were mixed with the tested compounds at different concentration ratios. Methanolic solution of salicylic acid, and subsequently water solutions of hydrogen peroxide (concentration of both substances was 7 mmol/L) were added. The mixtures were analysed by HPLC (pump Philips PU 4100, Philips, UK; Eclipse Plus C18, 4.6 × 100 mm, 3.5 μm, Agilent, USA with UV–vis detector ECOM LCD 2083, Ecom, Czech Republic) with 40% methanol and 0.085% of a water solution of phosphoric acid as mobile phase. All experiments were controlled by addition of internal standards.

Except for methanol for HPLC (JT Baker, USA), all other chemicals necessary for in vitro study were purchased from Sigma-Aldrich (Germany).

Data analysis

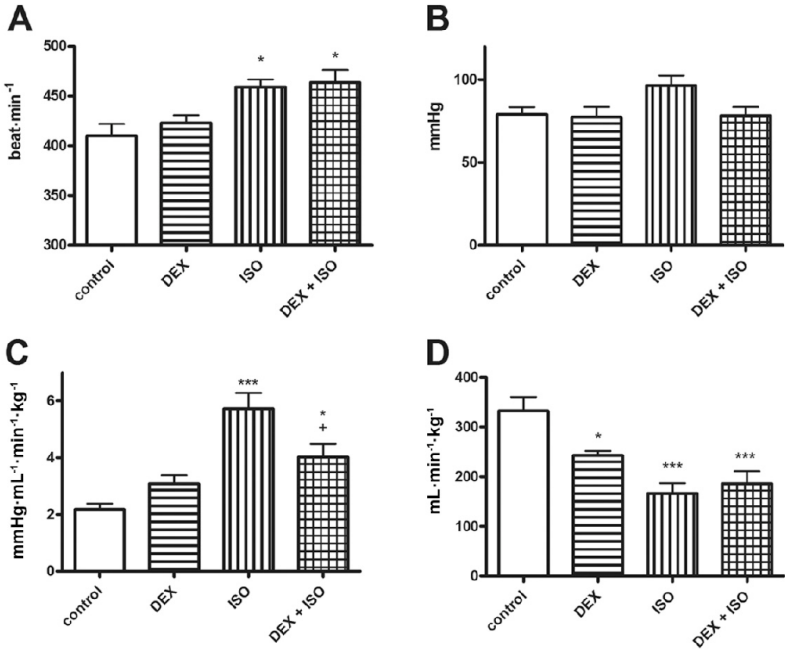
Data are expressed as means ± SEM. Groups were compared by one-way ANOVA test, followed by Dunnett multiple comparison test (24 h experiments) or by two-way ANOVA test, followed by Bonferroni post-tests (continuously monitored experiments) using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences between groups were considered significant at $p \leq 0.05$, unless indicated otherwise.

Results

Administration of 100 mg/kg of ISO s.c. to rats resulted in 30% mortality (3 rats of 10 died in variable time intervals within 24 h). Intravenous premedication of rats with 20.4 mg/kg of DEX reduced the mortality to 12.5% (1 of 8 rats died within 1 h after ISO administration). None of animals died in both the control and DEX groups.

Cardiotoxic effects of ISO were associated with a decrease in cardiac output and increases in peripheral resistance and heart rate after 24 h (Fig. 1). Diastolic blood pressure tended to be increased in the ISO group, while systolic blood pressure was not changed 24 h after administration of ISO, compared with the controls. DEX did not influence haemodynamic parameters of healthy animals with the exception of

Fig. 1. Haemodynamic parameters after administration of ISO, DEX, or their combination. Control animals received saline only. (A) heart rate, (B) diastolic blood pressure, (C) peripheral resistance index, (D) cardiac index. Statistical significance: *, $p < 0.05$ vs. control, **, $p < 0.01$ vs. control, ***, $p < 0.001$ vs. control, +, $p < 0.05$ vs. ISO.



a significant decrease in cardiac output (Fig. 1D). Administration of DEX before ISO partially ameliorated peripheral resistance without affecting elevated heart rate and decreased cardiac output.

Principal biochemical and morphological parameters, associated with cardiac impairment, were influenced by administered drugs in agreement with haemodynamic results (Fig. 2), i.e., ISO caused elevations in cTnT, myocardial calcium content, and cardiac wet ventricle weight. These variables were affected by DEX only partly in the case of myocardial calcium content. There were no significant differences in total glutathione in the blood, vitamin E in the plasma and in myocardial iron, zinc, selenium, and copper concentrations among groups (data not shown). Partial amelioration of ISO-cardiotoxicity after DEX administration was supported by histological analysis (Fig. 3 and Table 1)

To characterize the mechanism(s) of the partial protective effect of DEX and the mechanism associated with the decreased cardiac output in DEX group in more detail, additional experiments were performed. Acute effects of ISO, DEX, and their combination were monitored continuously during 2 h after administration of the above-mentioned drugs. Because cardiovascular parameters can be acutely influenced by the volume of the administered solutions, additional groups of animals received saline i.v. in adequate volume. There were no significant changes between i.v. and s.c. saline groups in any measured parameters. For better clarity, data of both control groups are presented together (the controls).

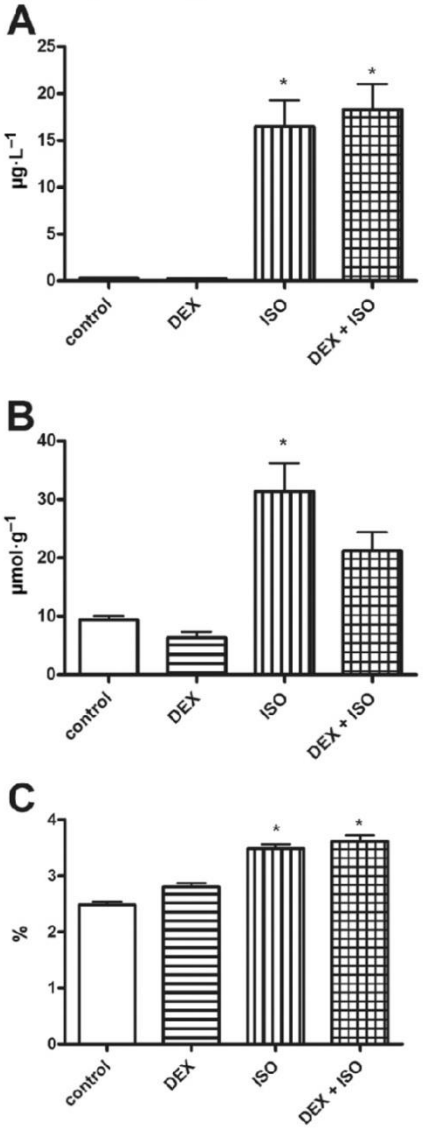
Similarly to the acute experiments, ISO caused 33% mortality (3 of 9 animals died) within 100 min. Two animals died of atrioventricular (AV) block and one of ventricular fibrillation. DEX decreased the mortality (1 of 5 rats died of AV block after 75 min), while none of animals died after administration of saline or DEX alone.

Within the first minute, the s.c. administration of ISO caused a marked increase in heart rate and a decrease in blood pressures. These effects were accompanied by a decrease in the parameter of ventricular relaxation dP/dt_{min} (Fig. 4). Contrarily, the parameter of ventricular contractility, dP/dt_{max}, rather increased and did not fall below the control levels in both ISO groups during the entire experiment (data not shown). Although there was a tendency for DEX to attenuate acute haemodynamic effects of ISO, no significant differences were found, with the exception of a short time interval in the case of mean blood pressure (Figs. 4A–4B).

DEX alone has only little effect on healthy animals with the exception of myocardial contractility, which was reduced at the end of the experiment (Fig. 5).

While DEX protective effects were not associated with haemodynamic improvement, they may be linked with the prevention of fatal dysrhythmias. ISO caused rapid elevation/depression in ST junction, T wave amplitude, and P-R interval (Fig. 6). There were no significant changes in P wave duration or amplitude between controls and ISO. The same was true for QRS complex duration. The QT interval cannot be measured precisely in rats (Beinfield and Lehr 1968), and hence it was not included in the ECG data analysis. DEX was not able to reduce prolongation of the P-R interval and

Fig. 2. Principal biochemical and morphological parameters related to ISO cardiotoxicity and the effects of DEX. (A) cardiac troponin T in serum, (B) myocardial calcium content, (C) wet ventricles weight index. Statistical significance: *, $p < 0.001$ vs. control.



S-T segment elevation, suggesting that it had not any influence on AV block and ischaemia (Figs. 6A and 6C). Analysing the ECG of all animals together, only an insignificant tendency to reduce T-wave amplitude was documented (Fig. 6). However, when examining individual animals, some protection was suggested in all but one rat (Fig. 7E vs. Figs. 7C, 7D, and 7F).

Fig. 3. Histological findings after administration of ISO, DEX, and their combination. (A) Control animals did not differ from the animals administered DEX only (not shown). (B) In contrast to healthy or DEX animals, marked alteration in the heart tissue was seen after administration of ISO. In particular, marked inflammation with leucocytes infiltration and cardiomyocytes degeneration was present. (C) Similar changes, although less expressed, were found after administration of DEX+ISO. Haematoxylin-eosin, 200× magnification.

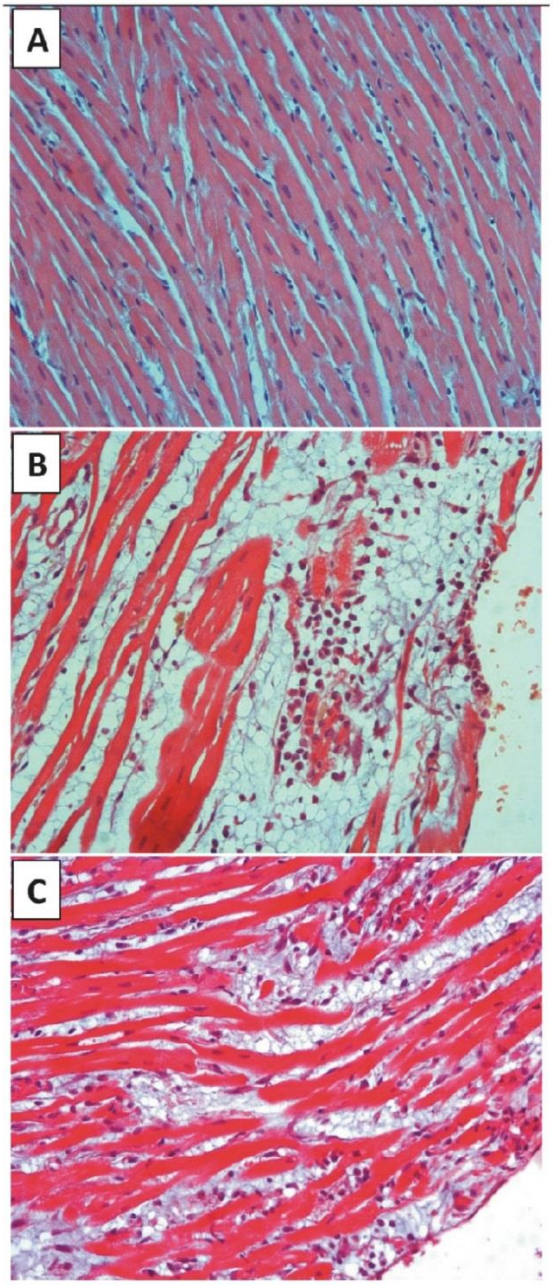


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Table 1. Semiquantitative analysis of histopathological changes in the left ventricle 24 h after administration of drugs.

Finding / drugs	Control / DEX			ISO			ISO + DEX		
	ENDO	MYO	EPI	ENDO	MYO	EPI	ENDO	MYO	EPI
Capilary congestion		+		++	+++	++	+	++	+
Interstitial dilatation with exsudation				+++	++	+	++	+	+
Swelling of connective fibres				++	++	+	++	+	+
Necrotic changes of the cardiomyocytes					+++			++	
Fragmentation of myofibrils				++	+++	+	+	++	+
Fragmentation of myocardial muscles					++			+	
Polymorphonuclear infiltration				+++	+++	++	++	++	+
Macrophages / mast cells				+	+				
Fibroblasts									

Note: The number of crosses corresponds to the severity of impairment, i.e., the percentage of the occurrence of the observed findings related to the tissue section area: + <10%, ++ 10%–30%, +++ >30%. All the assessments were performed by 200× magnification. Abbreviations: ENDO, endocardium; MYO, myocardium; EPI, epicardium.

Inflammation and the lactate elevation with subsequent acidosis play important roles in the ISO cardiotoxicity (Blasig et al. 1985; Rona 1985). Thus, additional in vitro experiments were performed taking in the account different pH conditions (Fig. 8). DEX is considered to be a chelating prodrug, but it has been suggested that it could be directly transformed by free ferrous ions to an efficient iron chelator (Buss and Hasinoff 1995). Indeed, some time-dependent ferrous chelation was confirmed in this study, but only at neutral or close to neutral pH; acidic conditions blocked this transformation (Figs. 8A and 8B). It is known that catecholic compounds (e.g., ISO) can chelate iron as well. We therefore carried out supplemental experiments to mimic drug(s) administration from the in vivo part of this study. DEX and ISO were administered alone or together in equimolar concentrations in 5 min intervals, and their influence on iron chelation was analysed. ISO was a much better iron chelator in this setting, but interestingly at pH 5.5 DEX blocked the iron-chelating properties of ISO, in particular in the ratio 40:1 drugs/iron, respectively (Figs. 8C and 8D). Because catecholic-ring containing compounds may also reduce iron (Mladenka et al. 2010) and as a result may be associated with the risk of pro-oxidative properties, iron-reducing analysis of ISO/DEX was undertaken. DEX did not reduce iron at all, but ISO showed marked iron reduction at pH 5.5, which was not influenced by co-administration of DEX (Fig. 8E). No reduction by ISO was found at pH 6.8 or higher (data not shown). We therefore performed experiments to confirm that these iron-reducing effects are associated with pro-oxidation, i.e., potentiation of the Fenton chemistry. Indeed, ISO mildly enhanced production of hydroxyl radical by the Fenton chemistry. Although DEX was able to block ISO-enhancing effects on the Fenton chemistry, it was not able to block the Fenton reaction itself (Fig. 8F). Conclusively, it seems that although iron-chelating properties of DEX may have some impact on ISO-mediated toxicity in vitro, it appears that iron chelation is not the main mechanism of DEX- protective effects against ISO cardiotoxicity in vivo.

Discussion

Iron is an essential element for many important life processes. But even transient iron homeostasis derangement may have significant pathophysiological consequences in clinical practice. This is probably due to catalytic role of iron in the production of the most potent biological oxidant, hydroxyl radical. Therefore, physiologically, iron homeostasis is meticulously regulated in the organism (Mladenka et al. 2005). Pathologically, a marked increase in free redox-active intracellular iron with subsequent release of this loosely bound iron into the circulation was observed in a model of AMI (Berenshtein et al. 2002). On the other hand, excessive iron removal stops cell growth and may be thus disadvantageous except in the treatment of tumours (Kalinowski and Richardson 2005). The narrow therapeutic range between excessive iron chelation and chelation of free "harmful" iron represent a significant therapeutic problem. DEX is a pro-drug, which is slowly transformed into its active iron-chelating metabolite (s) spontaneously at neutral conditions or more rapidly by enzymes (Hasinoff and Aoyama, 1999*a*, 1999*b*). Moreover, it was shown that this transformation may be markedly accelerated by iron, in particular by ferrous ions (Buss and Hasinoff 1995). We confirmed this transformation at neutral or close to neutral conditions (Fig. 8B). In theory, DEX seems to be an ideal iron-chelating agent possessing high chelation efficacy in "iron overload" states, but simultaneously it does not excessively remove iron at physiological conditions.

Experimental administration of large doses of catecholamines, in particular those of the synthetic β-agonist ISO, mimics in some aspects AMI in humans (Mladenka et al. 2009*a*; Rona 1985). This model may be pathophysiologically relevant. Among others, it has been proven that endogenous catecholamines are markedly released during AMI, which may contribute to further deterioration of the disorder (Kloner 2006; Lameris et al. 2000). The precise mechanisms of catecholamine myocardial injury have not been established yet. The overstimulation of β-adrenoceptors is the first step in the pathogenesis of catecholamine cardiotoxicity, but the involvement of iron (and (or) copper) and ROS with redox cycling of catecholamines has been suggested. In fact, iron chelators have been shown to improve catecholamine cardiotoxicity to various extents. Similarly, blockade of adrenergic receptors did not influence positively all aspects of the injury (Bloom and Davis 1972; Hašková et al. 2011*b*; Mladenka et al. 2009*b*, 2009*c*; Neri et al. 2007; Persoon-Rothert et al. 1989; Remião et al. 2002). In the light of this data, it is not surprising that iron chelators cannot influence all pathogenetic steps of catecholamine injury (Mladenka et al. 2009*b*,

Fig. 4. Acute haemodynamic changes after administration of ISO, saline (controls), DEX or combination DEX+ISO. A: mean blood pressure B: the parameter of left ventricular relaxation – negative peak of dP/dt - dP/dt_{min}. Results are expressed as percentil difference vs. baseline values (before administration of drugs). Statistical significance: *, p <0.05 vs. control, **, p <0.01 vs. control, ***, p <0.001 vs. control; +, p <0.05 vs. ISO. For better clarity, SEM and statistical significance vs. control are shown only in 1, 3, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. The controls and DEX were not significantly different at any value and any time interval and their errors are depicted only upwards. Significant differences between ISO and DEX+ISO at the level of p <0.05 were found only in case of the mean blood pressure, but in a relatively short time interval (from 38 to 58 min).

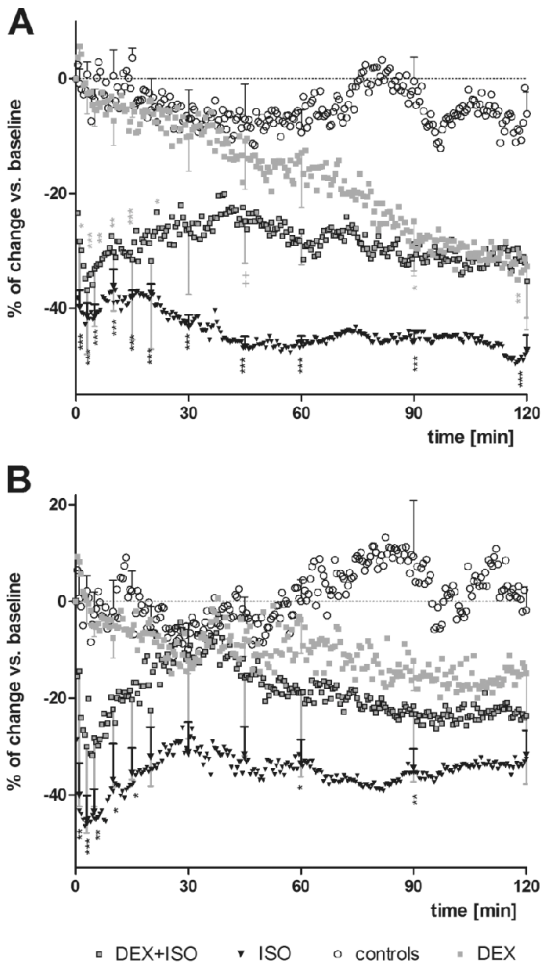
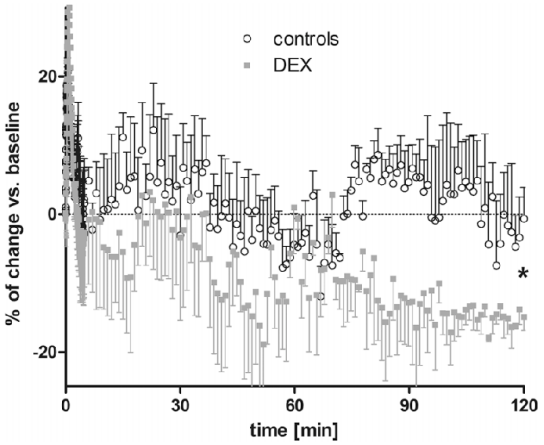


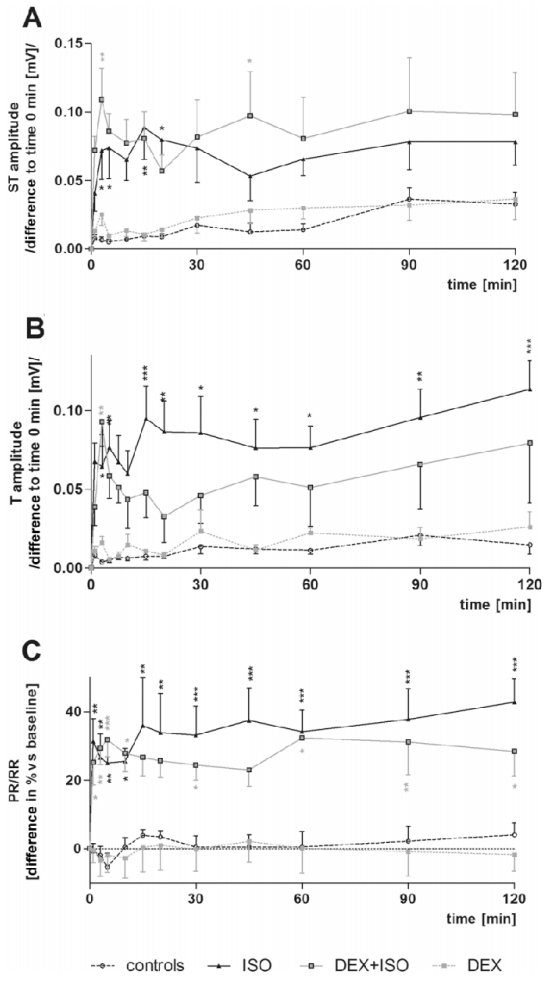
Fig. 5. Myocardial contractility. The plot depicts dP/dt_{max}, a parameter of myocardial contractility, after administration of saline or DEX in a dose of 20.4 mg per kg. *, significant difference at p < 0.05 at the end of the experiment (120 min).



2009*c*). Generally, the mortality from ISO injury seems to be caused within the first two hours by arrhythmias, or later by myocardial structure impairment (Chagoya de Sánchez et al. 1997; Mladenka et al. 2009*a*). In our recent study, iron chelator PCTH, in the equimolar dose to that of DEX used in this study, absolutely inhibited the ISO-caused mortality (Mladenka et al. 2009*b*), so it had to inhibit both early arrhythmias and late myocardial impairment. It this study, DEX did not inhibit all arrhythmias (2 animals of 13 died within first 2 h), but apparently it inhibited, at least partly, the myocardial impairment (none of DEX treated animals died from late ISO-associated myocardial impairment consequences).

ISO-induced arrhythmias were fatal because of AV blocks or ventricular fibrillation. AV blocks seems to be a typical feature seen after high doses of catecholamines. Contrarily, ventricular fibrillation dominates in other models of AMI (e.g., coronary artery occlusion) (Barta et al. 2008). Indeed, prolongation of the P-R interval observed in this study (Fig. 6C) is a predisposition to AV blocks. DEX was not able to prevent P-R interval prolongation, and hence it had no or little effect on AV blocks, but it seems that it blocked ventricular fibrillation. This finding is not novel, because DEX has been shown to reduce risk of dysrhythmias in anthracyclines cardiotoxicity (Galetta et al. 2005) and the effects of DEX on both calcium overload and iron chelation/ROS scavenging are known to diminish the incidence of ventricular fibrillation (Bernier et al. 1986; Clements-Jewery et al. 2002; Singal et al. 1982). Based on in vitro data of this study and the fact that PCTH absolutely inhibited mortality and therefore abolished all fatal arrhythmias (Mladenka et al. 2009*b*), it seems that iron chelation is not the principal mechanism of positive effect of DEX on ISO cardiotoxicity. On the other hand, the question concerning DEX-scavenging activity of ROS has not been solved yet. Both recent and former studies demonstrated that DEX had in vitro and in vivo significant non-iron based direct ROS scavenging activity, and such effects may also contribute to the attenuation of dysrhythmias occurrence (Galetta et al. 2009; Hüsken et al. 1995). In contrast, some in vitro studies reported participation of DEX on iron-based hydroxyl radi-

Fig. 6. Abnormalities in ECG parameters after application of ISO, saline (controls), DEX, or combination DEX+ISO. (A) ST junction (segment) amplitude, (B) T wave amplitude, (C) relative P-R interval (measured as P-R interval divided by the length of the cycle). Results are expressed as absolute arithmetic difference vs. baseline (time 0, i.e., before administration of drugs) in case of S-T junction and T-wave amplitudes and percentual difference vs. baseline in case of P-R interval. Statistical significance: *, $p < 0.05$ vs. control, **, $p < 0.01$ vs. control, ***, $p < 0.001$ vs. control. There were no significant differences between the controls and DEX, or DEX+ISO and ISO alone in any of the measured ECG parameters.

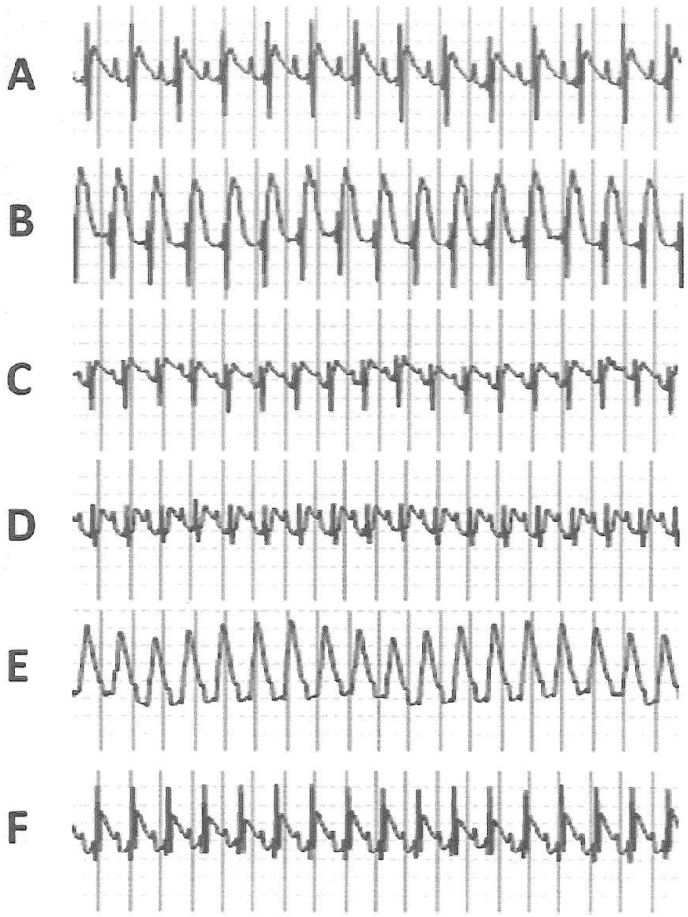


cal formation (Diop et al. 2000; Thomas et al. 1993). In this and another recent study, however, it was shown that DEX in the presence of iron had no pro-oxidative nor antioxidant effect (Hašková et al. 2011*a*). This supports the assumption that iron chelation is not the main effect of DEX. The fact that DEX blocked the mild pro-oxidative effect of ISO on the Fenton chemistry is not sufficient to explain DEX effects, because DEX has no influence on the Fenton chemistry itself, in contrast to other iron chelators (Filipský et al. 2011).

Apart from ferrous and ferric ions, DEX may chelate with fairly similar affinity copper, calcium, manganese, and zinc (Diop et al. 2000; Huang et al. 1982). This chelating non-selectivity may be, in principle, both positive and negative. A chronic study observed increased zinc excretion (Von Hoff et al. 1981). However, this study did not document any derangement in myocardial copper, zinc, and selenium concentrations, suggesting that the single injection cannot disrupt the homeostasis of these metals. Rather, chelation of calcium seems to be important in the DEX protective effect, because DEX partly inhibited myocardial calcium overload (Fig. 2B). This may contribute not only to the inhibition of ventricular fibrillation, which may be triggered by calcium overload and subsequent delayed after-depolarisation, but also to inhibition of late myocardial impairment. This suggestion is in harmony with histological analysis, which confirmed the partly protective effect of DEX attributable to the inhibition of calcium overload. In addition, it has been known that inhibition of calcium overload cannot absolutely reverse cardiac impairment (Bloom and Davis 1972). And here again, the different mechanism of DEX activity is supported by the fact that PCTH was not able to significantly modify calcium overload and had no impact on histological findings (Mladenka et al. 2009*b*). Mild calcium chelation is the probable cause of the observed decreased cardiac output in 24 h experiments (Fig. 1D), which was likely caused by a decreased contractility observed 2 h after DEX administration (Fig. 5). Interestingly, the described changes in myocardial contractility do not seem to be clinically important, since in the majority of experiments, DEX ameliorated a decreased contractility caused by administration of anthracyclines (Cvetković and Scott 2005; Popelová et al. 2009).

An earlier report with chronic ISO administration (5 times 20 mg/kg each weak s.c.) documented the protective effect of chronic DEX pre-treatment (5 times 100 mg/kg i.p., 30 min before ISO) on the ISO-induced injury (Flandina et al. 1990). The results of this study cannot be easily compared, since the study was rather a model of chronic heart failure. No mortality was observed in ISO group during the experiment, but the documented protective effect on myocardial histology in that study seems to confirm results from our study. A significant level of protection was observed with premedication of DEX before onset of ischaemia in in vitro or ex vivo models of AMI (Hasinoff 2002; Persoon-Rothert et al. 1989; Ramu et al. 2006). However, such type of studies neglected the substantial contribution of the immune cells in the pathogenesis of myocardial injury (Jordan et al. 1999). Activated immune cells potentiate inflammation, which can decrease DEX efficacy because of acidic environment of the inflamed tissue. Additional factors responsible for the failure of DEX to more efficiently inhibit the acute ISO cardiac injury probably lies in the insufficient biotransformation to its iron-chelating metabolite(s). Administration of DEX 5 min before ISO may be insufficient for the formation of efficient intracellular concentration(s) of active metabolite(s), which represent(s) a clear difference to the repeated administration of DEX in chronic studies. Administration of DEX in sufficient time before administration of ISO may be associated with more effective protection by DEX, but it is necessary to

Fig. 7. ECG standard limb lead II records. (A) Two second ECG tracings of a control rat (the same ECG tracing was found in animals administered with DEX or rats before administration of drug/s/); (B) typical tracing 120 min after ISO administration (note marked S-T junction elevation), and (C–F) all survived combination group rats.



treat AMI as soon as possible in clinical practice. Therefore this possibility was clinically irrelevant and was not checked in this study. An additional factor contributing to a low protective effect of DEX against ISO cardiotoxicity may be a low dose of DEX. Iron released during ischaemia is generally low, and the amount of excreted iron did not rise with an increase in the dose of DEX in clinical studies (Berenshtein et al. 2002; Tetef et al. 2001). Thus, the above-mentioned factor plays only minor, if any, role in this study.

Conclusions

This study showed that DEX had some cardioprotective effects in the ISO model of AMI that we used. In particular, DEX partly decreased the mortality, late myocardial impairment consequences, calcium overload, peripheral haemodynamic disturbances, and possibly the incidence of ventricular fibrillation. At the same time, it had no effect on the occurrence of AV blocks produced by ISO administration. The effect of DEX documented in this study is in an apparent contrast to the previously reported positive effects of other lipophilic iron chelator PCTH using the same model of myocardial injury. In addition, although DEX blocked in vitro ISO pro-oxidative effect in the Fenton chemistry, it was not able to attenuate this iron-catalysed process. Therefore, the original idea that iron chelation is the main mechanism of cardioprotection in this model, was not confirmed, rather the influence on calcium homeostasis may play a more important role in the mechanism of DEX cardioprotection.

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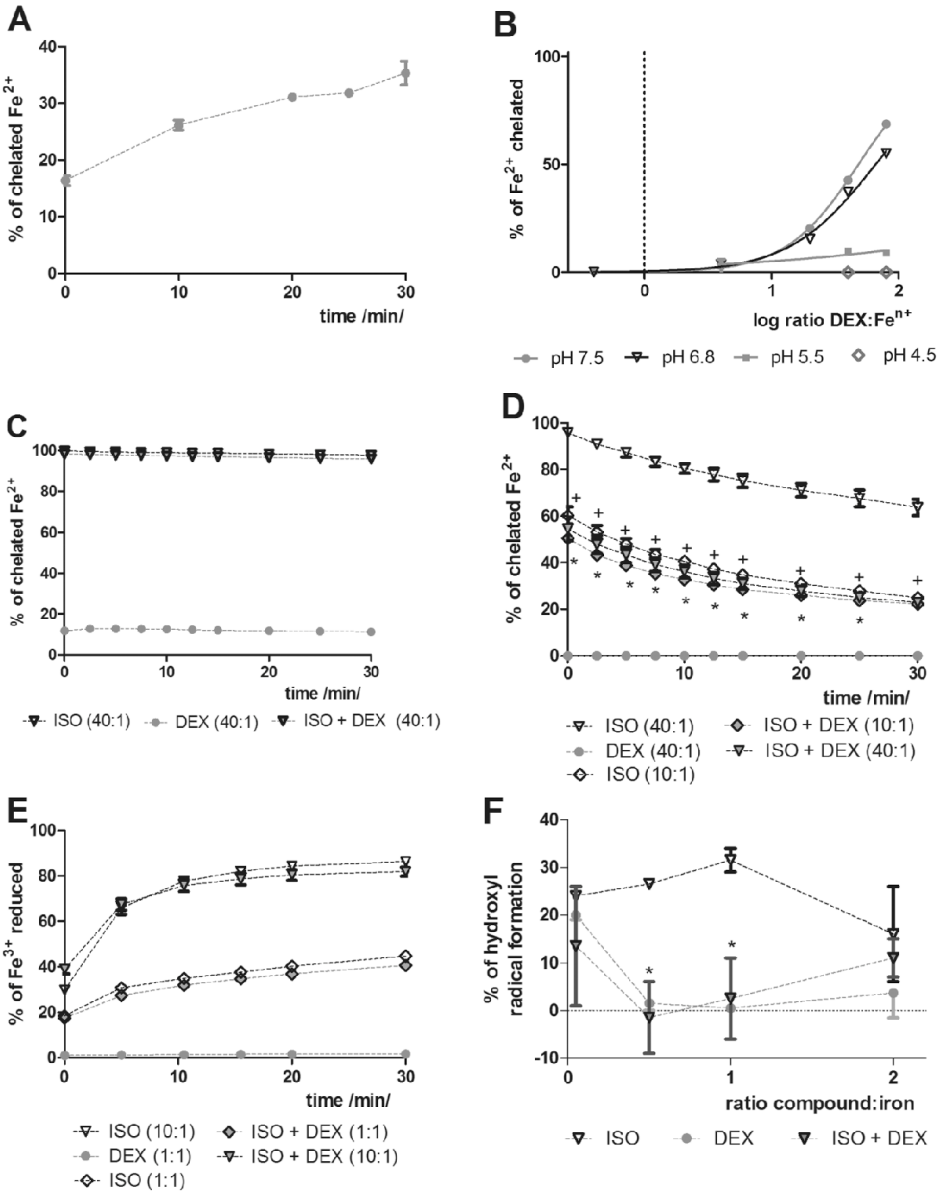
Fig. 8. In vitro experiments. (A) Time-dependent increase in iron chelation by DEX (pH 6.8, ratio DEX/Fe^{2+} 40:1). (B) Chelation of ferrous ions by DEX at various pH (30 min incubation, DEX/Fe^{2+}). (C–D) Chelating effect of DEX, ISO, and combination of both at (C) pH 6.8 and (D) pH 5.5. DEX and (or) ISO were incubated with ferrous ions in 40 or 10 times excess for 5 min and measured in various time intervals after ferrozine administration. At pH 6.8, identical curves were observed for ISO 40:1 and 10:1 (not shown). Statistical significance at $p < 0.05$: + ISO vs. ISO+DEX at ratio 40:1, * ISO vs. ISO+DEX in ratio 10:1. (E) Ferric iron reduction at pH 5.5 (ISO, DEX, or combination were incubated with ferric ions for 5 min and measured in various time intervals after ferrozine administration). (F) Effect of ISO, DEX, and combination ISO+DEX on the Fenton chemistry. Data are expressed as mean ± SD. Statistical significance at $p < 0.05$: *, ISO vs. ISO +DEX.



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5. PODÍL KANDIDÁTA NA JEDNOTLIVÝCH PUBLIKACÍCH

Disertační práce je souhrnem 7 odborných publikací (4. 1. – 4. 7.). Kandidát je prvním autorem publikací 4. 3., 4. 4. a 4. 5. a spoluautorem publikací 4. 1., 4. 2., 4. 6. a 4. 7.

Ve studii 4. 1. se autor podílel zejména na *in vitro* stanovení chelatačních vlastností flavonoidů pomocí ferrozinové metody, zpracování a analýze dat a přípravě vlastního rukopisu.

Ve studii 4. 2. se kandidát podílel především na *in vitro* stanovení redukčních vlastností flavonoidů pomocí ferrozinové metody a přípravě vlastního rukopisu ve stejné šíři jako u předešlé studie.

Ve studii 4. 3. se jako první autor publikace podílel rozhodující mírou na *in vitro* stanovení chelatačních vlastností acylpyrazolonů pomocí ferrozinové metody, zpracování a analýze dat a sepsaní vlastního rukopisu.

Ve studii 4. 4. jako první autor publikace koordinoval všechny *in vitro* experimenty a podílel se rozhodující mírou na všech stanoveních stechiometrie komplexu za využití UV–Vis spektrofotometrie, zpracování a analýze dat a sepsaní vlastního rukopisu.

Ve studii 4. 5. jako první autor publikace koordinoval a prováděl *in vivo* experimenty analyzující časné hemodynamické změny a EKG po podání kardiotoxické dávky isoprenalinu a odběry vzorků a rovněž se podílel na zpracování a analýze dat a sepsaní vlastního rukopisu.

Ve studii 4. 6. analyzující biomarkery oxidačního stresu a srdeční dys/funkce se kandidát podílel na analýze dat a přípravě vlastního rukopisu.

Ve studii 4. 7. hodnotící vliv dexrazoxanu na isoprenalinový model akutního infarktu myokardu se autor podílel stejnou měrou jako první autorka na *in vivo* experimentech, odběrech vzorků, zpracování a analýze dat, přípravě vlastního rukopisu a náročném recenzním řízení.

6. ZÁVĚR

Železo je esenciálním elementem všech živých organismů. Nezastupitelné jsou jeho role při transportu kyslíku, syntéze ATP a buněčné proliferaci a diferenciaci. Jelikož lidský organismus postrádá aktivní mechanismus exkrece železa, procesy jeho absorpce a distribuce musí být bedlivě řízeny celou řadou proteinů a peptidů. Za fyziologických podmínek je úroveň volného/nevázaného železa v organismu velmi nízká.

V případě přetížení organismu železem mohou vzplanout závažná onemocnění vedoucí k poškození orgánů. Toto narušení homeostázy železa je spojeno se signifikantním nárůstem koncentrace volného/nevázaného železa, které se může podílet na tvorbě reaktivních forem kyslíku. K příkladům narušení této homeostázy patří akutní infarkt myokardu (AIM). Základem péče o nemocného je zde velmi rychlá reakce na rozvíjející se akutní koronární syndrom. Nejracionálnější terapií AIM je reperfuzní léčba – primární perkutánní koronární intervence (PCI). Obnovení krevního průtoku po déle trvající ischemii myokardu je ale často spojeno s dalším poškozením kardiomycytů, tzv. ischemicko-reperfuzním poškozením. Již během časné ischémie se do krevního řečiště uvolňují katalyticky aktivní elementy, především železo a měď, které se mohou po obnovení krevního průtoku účastnit produkce vysoce toxického hydroxylového radikálu železem/mědí katalyzovanou Fentonovou reakcí. Tudíž se zde nabízí potencionálně aplikovatelný farmakoterapeutický přístup k léčbě tohoto závažného onemocnění – terapie látkami chelatujících železo/měď.

Chelátory železa představují rozsáhlou skupinu přírodních a syntetických látek s variabilní chemickou strukturou, jejichž společnou vlastností je schopnost vázat atomy železa. V klinické praxi se však můžeme setkat pouze s několika zástupci této farmakoterapeutické skupiny – deferoxaminem, deferipronem, deferasiroxem a dexrazoxanem, tzn. chelátory účinnými, relativně bezpečnými, cenově dostupnými a s výjimkou deferoxaminu i přijatelnou compliance pacientů.

V této práci byly detailně analyzovány účinky flavonoidů a acylpyrazolonů *in vitro*, tj. železo-chelatační vlastnosti a vliv na železem katalyzovanou Fentonovu reakci. U flavonoidů se pro jejich chelatační účinky jako nejvýhodnější substituce jevila 6,7-

dihydroxylová skupina. Baikalein, obsahující tuto strukturu, měl srovnatelnou schopnost chelatovat železo jako deferoxamin při všech testovaných pH. 3-hydroxy-4-keto substituce s dvojnou vazbou v poloze 2 a pyrokatecholový kruh B byly rovněž spojeny s významnými chelatačními účinky. Nicméně pyrokatecholový kruh B chelatoval železo výrazně méně při kyselém pH. Kvercetin a myricetin, u kterých lze nalézt všechny výše zmíněné 3 strukturní znaky, chelatovaly železo podobně jako baikalein, resp. deferoxamin, jen při neutrálním pH, ale byly jednoznačně méně účinné při nižším pH. Na druhou stranu z literatury známá 5-hydroxy-4-keto substituce představuje jen slabé a nestabilní chelatační místo. Isolované hydroxyl, methoxyl, keto nebo *ortho* methoxy-hydroxy substituce neměly na schopnost chelatovat železo významný vliv. Nicméně někteří zástupci této skupiny mohou mít z důvodu svých redoxních vlastností i nežádoucí pro-oxidační účinky. V důkladné analýze byl přímý vztah redukce – prooxidace nalezen jen u některých flavonoidů. Obecně byly účinky na železem katalyzovanou Fentonovu reakci velmi různorodé. Některé flavonoly prokázaly při velmi nízkých koncentracích pozitivní antioxidační účinky, ale na druhou stranu při podrobné analýze koncentrační závislosti byly jejich antioxidační účinky při vyšších testovaných koncentracích již minimální nebo dokonce měly vlastnosti pro-oxidační. Zatímco flavonoly morin a rutin měly jednoznačně koncentračně závislé pro-oxidační účinky, 7-hydroxyflavon a hesperetin patřily mezi jediné flavonoidy, které s koncentrační závislostí snižovaly tvorbu hydroxylového radikálu. Je nutné připomenout, že poslední dvě zmíněné látky mají minimální chelatační potenciál.

Ačkoli mezi dlouho známé podskupiny syntetických chelátorů železa patří 1-fenyl-3-methyl-4-acyl-pyrazol-5-ony, poznatky týkající se jejich biologické aktivity jsou spíše limitované. Všichni testovaní zástupci chelatovali železité ionty bez výjimky, ale jejich schopnost vázat ionty železnaté byla silně závislá na acylové substituci. Přestože chelatační vlastnosti vůči železnatým iontům při různých patofyziologicky relevantních pH nebyly výrazněji odlišné, některé sloučeniny prokázaly vyšší účinnost při pH 4,5 než v klinické praxi používaný deferoxamin. Za zmínku stojí H_2QpyQ, tj. 2,6-bis[4(1-fenyl-3-methylpyrazol-5-on)karbonyl]pyridin, jehož schopnost chelatovat železo rostla s klesajícím pH. Navzdory zmíněným rozdílům v chelatačních vlastnostech patřily tyto látky mezi velmi účinné inhibitory Fentonovy reakce, jenž lze zde srovnávat i s deferoxaminem.

Jednou z důležitých vlastností chelátorů je i stechiometrie vytvořeného komplexu ve vztahu k pH. Jelikož je u některých látek, např. flavonoidů, stechiometrie komplexů stále nejasná, byl vyvinut nový analytický přístup pro zjištění stechiometrie komplexu za využití UV–Vis spektrofotometrie a porovnán s již známou Jobovou metodou. V případě výrazného rozdílu mezi absorpčním maximem sloučeniny a odpovídajícím absorpčním maximem komplexu bylo dostatečným postupem k určení stechiometrie změření samotné absorbance komplexu při vlnové délce absorpčního maxima komplexu. Nicméně ve většině případu byl rozdíl mezi absorpčními maximy nedostatečný, a proto byly pro výpočet stechiometrie použity matematicky náročnější doplňující metody. S využitím těchto metod byla stechiometrie popsána u všech testovaných látek. Hlavní výhodou těchto postupů oproti standardní Jobově metodě se jeví jejich schopnost určit stechiometrii a případně i kinetiku tvorby komplexu i u slabých chelátorů železa.

Studie zaměřená na analýzu časných hemodynamických změn po aplikaci kardiotoxické dávky isoprenalinu odhalila pokles arteriálního krevního tlaku, tepového objemu, dotížení a předtížení a nárůst tepové frekvence velmi rychle po s.c. podání isoprenalinu. Rovněž byla významně ovlivněna diastola (tzn. zkrácení intervalu fáze isovolumické relaxace a plnící fáze). Na druhou stranu byl zaznamenán markantní, ale krátkodobý, nárůst kontraktility levé komory. Je ovšem nutné zmínit, že v průběhu pokusu se kontraktilita myokardu nesnížila oproti bazálním hodnotám, naopak zůstala spíše zvýšená. Na základě popsaných změn lze předpokládat, že příčinou časné patogeneze je diastolická dysfunkce. Podání salbutamolu nevedlo k zásadním časným změnám hemodynamických parametrů s výjimkou významného poklesu diastolického krevního tlaku a dotížení.

Sérové koncentrace srdečního troponinu (cTnT), esenciálního biomarkeru poškození srdeční tkáně u lidí, silně korelovaly se závažností poškození myokardu u potkanů (např. s přetížením myokardu vápníkem – pozitivní korelace a tepovým objemem – negativní korelace). Na druhou stranu korelace cTnT s markery oxidačního stresu byly spíše nevýrazné (glutathion a vitamin C) nebo nulové (vitamin E a TBARS - reaktivní formy thiobarbiturové kyseliny, z angl. thiobarbituric acid reactive substances). Vztah mezi cTnT a dalšími parametry byl exponenciální s výjimkou

koncentrace vápníku v srdeční tkáni, kde byl nalezen vztah, který popisuje mocninná funkce.

V 24hodinovém *in vivo* experimentu i.v. podání dexrazoxanu vedlo k částečnému poklesu mortality, snížení koncentrace vápníku v srdeční tkáni, ke zlepšení histopatologického nálezu a hemodynamických parametrů. Kontinuální 2hodinový *in vivo* experiment prokázal, že dexrazoxan není schopen ovlivnit isoprenalinem navozené atrioventrikulární bloky a jeho vliv na hemodynamické parametry byl zde spíše minimální. Navíc *in vitro* experimenty naznačily, že chelatační vlastnosti dexrazoxanu nehrají významnou roli v kardioprotektivním mechanismu.

Na základě provedených studií lze na závěr konstatovat, že schopnost chelatovat železo a anti/pro-oxidačních vlastnosti flavonoidů a syntetických acylpyrazolonů závisí nejen na jejich chemické struktuře, ale i na pH vnějšího prostředí. K objasnění mnoha protichůdných zjištění týkající se stechiometrie komplexů, ale i samotných mechanismů chelatace, lze použít nový matematický model pro výpočet stechiometrie. *In vivo* studie prokázala, že z patogenetického hlediska diastolická dysfunkce předchází systolické dysfunkci a že k její samotné indukci je stimulace pouze β_2-adrenergních receptorů nedostatečná. Kromě toho běžné biomarkery oxidačního stresu nelze použít k diagnostice srdeční dys/funkce u potkanů. Protektivní účinky dexrazoxanu vůči isoprenalinové karditoxicitě byly pravděpodobně zprostředkovány inhibicí pozdních srdečních změn a nižší incidencí ventrikulárních fibrilací v důsledku snížení koncentrace vápníku v srdeční tkáni. Chelatační vlastnosti dexrazoxanu nehrály významnější roli v jeho kardioprotektivním mechanismu.

Závěrem lze shrnout, že série *in vitro* experimentů může u chelátorů železa alespoň částečně predikovat pozitivní nebo i negativní vliv na isoprenalinový model AIM u potkanů. Některé aspekty bude ale nutné ověřit v dalších zejména *in vivo* experimentech.

7. SEZNAM ODBORNÝCH PUBLIKACÍ

7. 1. Recenzované publikace v odborných časopisech s IF

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(IF 2010 – 3.317)

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7. 2. Přednášky na konferencích

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Hradec Králové, 15.–17. září 2010

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