Intravenous rutin in rat exacerbates isoprenaline-induced cardiotoxicity likely due to intracellular oxidative stress

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Objectives: Rutin, quercetin-3-*O*-rutinoside, a natural flavonol glycoside, has shown various *in vitro* benefits with potential use treating human diseases, especially cardiovascular system disorders. Antioxidant properties are assumed to underlie the majority of these benefits. Yet rutin pro-oxidant properties have been reported as well. Our research group has recently shown aggravating effects on isoprenaline (ISO)-induced cardiotoxicity in Wistar:Han rats after 24 hours.

Methods: This study was designed to examine in more detail the reasons for the negative effects of rutin (11.5 and 46 mg/kg, i.v.) after administration of ISO (100 mg/kg, s.c.) in rats within 2 hours of continuous experiment and in the H9c2 cardiomyoblast-derived cell line.

Results: Like our previous findings, rutin did not (11.5 or 46 mg/kg, i.v.) reduce the ISO-induced mortality within 2 hours although the lower dose significantly reduced cardiac troponin T (cTnT) and partly improved the histological findings. In contrast, the higher dose increased the mortality in comparison with solvent (1.26% w/v sodium bicarbonate). This was not caused by any specific haemodynamic disturbances. It appears to be associated with oxidative stress as rutin enhanced intracellular reactive oxygen species formation *in vitro* and had the tendency to increase it *in vivo*.

Conclusions: Rutin, likely due to its pro-oxidative effects, can exacerbate catecholamine cardiotoxicity depending on the dose used.

Keywords: Catecholamine, Isoprenaline, Flavonoid, Rutin, Wistar rat, H9c2 cell line, Reactive oxygen species

Introduction

Flavonoids, which are ubiquitous plant polyphenolic secondary metabolites, are an integral part of the human diet.[1,2] Recent epidemiological studies have evaluated the relationship between flavonoid intake and benefits in case of various pathologies including those of the cardiovascular system.[2–4] The proposed benefits are probably caused by combination of reactive oxygen species (ROS) scavenging activity, iron/copper chelation, inhibition of free radical-forming enzymes, decrease in expression of inflammatory signalling molecules, and other factors like direct vasodilatory and antiplatelet potential.[5–11] However, flavonoids can have pro-oxidative effects as well. These could be based on their transient metal-reducing potential and consequent increase in formation of the hydroxyl radical *via* Fenton reaction.[12–14]

The endogenous catecholamines are hormones and neurotransmitters in the sympathetic and central nervous systems. However, excessive concentrations can trigger acute myocardial infarction (AMI).[15,16] Interestingly, a cardiotoxic dose of the synthetic non-selective β-agonist isoprenaline (isoproterenol, ISO) induces a pathological state which resembles the acute phase of AMI as shown by marked release in

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cardiac troponin T (cTnT), calcium overload, ST segment (J-point) elevation, R wave amplitude decrease, and histopathological disturbances.[17–23] Although the pathogenesis of ISO-induced cardiotoxicity is multifactorial, overstimulation of β-adrenoceptors, auto-oxidation of catecholamines followed by formation of ROS, energy metabolism dysfunction, microthrombi formation, and membrane permeability alterations play substantial roles.[16–18,21,24,25]

Stanely Mainzen Prince and coworkers[26–29] reported that oral pre-treatment with rutin (40 or 80 mg/kg/day, 42 days, p.o.) had broad preventive effects on various aspects of ISO-induced cardiotoxicity (150 mg/kg/day, 2 days, s.c.) in rats. Similarly, administration of quercetin (10 mg/kg/day, 7 days, p.o.) appeared to be protective against cardiotoxic doses of ISO as well (100 mg/kg/day, respectively 85 mg/kg/day, s.c., 2 days).[30] The beneficial effects of the flavonols were particularly associated with their antioxidant, lipid-lowering, mitochondrial structure and function improving, and membrane stabilizing properties. However, Stanely Mainzen Prince and coworkers did not show data on rat survival, haemodynamics, arrhythmias after administration of cardiotoxic doses of ISO, and pharmacokinetics of the flavonoids.

On the other hand, our research group found that a single i.v. dose of rutin in rats either had no effect (11.5 mg/kg) or aggravated (46 mg/kg) ISO-induced cardiotoxicity (100 mg/kg, s.c.) after 24 hours.[31] Since such effects are in clear contrast to the foregoing studies, we reanalysed the effects of quercetin on ISO-induced cardiotoxicity. We used the same experimental design as Prince and Sathya[30] and we evaluated survival, haemodynamic, and ECG variables as well. Surprisingly, we found that oral administration of quercetin did not ameliorate ISO-induced cardiotoxicity.[32]

We decided to return to the issue of the i.v. administered rutin and to analyse the reason for higher mortality rate in rats receiving rutin as a premedication. This study was aimed at detailed comparison of the early pathophysiological effects of administered rutin (11.5 and 46 mg/kg, i.v.) on ISO-induced cardiotoxicity (100 mg/kg, s.c.) to explain the mechanisms of its direct negative effects.

Material and methods

Chemicals

Rutin hydrate, sodium bicarbonate, ISO hydrochloride, and urethane were purchased from Sigma-Aldrich Inc. (USA). Water for injection was purchased from Braun (Germany). Ultrapure water was produced by Milli-Q RG (Merck Millipore, USA).

In vivo studies using Wistar:Han rats

Wistar:Han male rats (MediTox, Czech Republic) were kept in an air-conditioned room at constant temperature and humidity. They had free access to a standard pellet diet for rodents (Velaz, Czech Republic) and tap water. After 2 weeks of acclimatization, the rats were deprived of food for 12 hours before the experiments.

The study was approved by the Ethics Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové (Study No. MŠMT 2437/2012-30), and it 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).

Haemodynamic study

Rats weighing on average 392 g were randomly divided into nine groups:

1. control groups received i.v. either sodium bicarbonate dissolved in water for injection (1.26% w/v, the solvent) or rutin dissolved in a solution of sodium bicarbonate as a slow bolus: (a) sodium bicarbonate – 2.3 ml/kg (SB2.3, six rats), (b) sodium bicarbonate – 4.6 ml/kg (SB4.6, six rats), (c) rutin – 11.5 mg/kg (2.3 ml/kg, Ru11.5, six rats), and (d) rutin – 46 mg/kg (4.6 ml/kg, Ru46, six rats). The higher dose of rutin was an equimolar dose to the clinical dose of the standard iron chelator – deferoxamine (50 mg/kg) and was chosen to enable comparison with other compounds.
2. ISO groups received i.v. either a solution of sodium bicarbonate, a solution of rutin, as described above, or saline (S, 4.6 ml/kg), and a freshly prepared solution of ISO dissolved in water for injection 5 minutes later (100 mg/kg, s.c.): (a) SB2.3+ISO (nine rats), (b) SB4.6+ISO (seven rats), (c) Ru11.5+ISO (seven rats), (d) Ru46+ISO (seven rats), and (e) S4.6+ISO (nine rats).

All rats were anaesthetized with urethane (1.2 g/kg, i.p.) before surgery and recording procedures were as described in our previous studies.[33,34] Briefly, a pressure transducer MLT0380/D (AdInstruments, Australia) was connected via a polyethylene catheter 0.5/1.0 mm (filled with heparinized saline 10 IU/ml) to the left common iliac artery. A high-fidelity pressure–volume micromanometer catheter (Millar PV-catheter SPR-838 2F, 4E, 9 mm; Millar Instruments Inc., USA) was inserted into the left ventricle via the right common carotid artery. The pressure transducer and Millar PV-catheter, together with s.c. electrodes for the ECG standard limb lead II (MLA1215, AdInstruments), were connected to the PowerLab apparatus equipped with software LabChart 7 (AdInstruments). After 15 minutes of equilibration, the drug formulation/solvent was administered as a slow i.v. bolus via the saphenous vein (400 µl/minute; 'Genie' Kent syringe pump, Kent Scientific Corporation, USA). ISO was administrated s.c. 5 minutes later if indicated. Haemodynamic and ECG

variables were then recorded continuously for 2 hours. The results were calculated as the percent change vs. baseline level (at time 0 minute, before ISO administration). The temperature was maintained at 36.5 ± 0.5°C (TCAT-2LV Controller, Physiterm Instruments Inc., USA). All calibrations were performed as described.[33]

At the end of the experiment, blood samples were collected from the abdominal aorta into a heparinized tube (BD Vacutainer, BD, USA) and surviving rats were sacrificed using 1 M aqueous solution of potassium chloride (i.v., Sigma-Aldrich Inc., USA). Afterwards, the heart was excised and weighed. The wet ventricles weight was expressed as an index, i.e. weight of ventricles in grams divided by body weight in grams, and expressed in per thousand (‰). The apical part of the heart was separated for further histological analysis and the base part was frozen at −20°C for further biochemical analysis as follows.

Biochemical analysis

cTnT, vitamin E, and vitamin C were measured in serum. cTnT was determined using electrochemiluminescence immunoassay which uses two monoclonal antibodies specifically targeted against cTnT (Elecsys 2010, Roche, Czech Republic). After deproteinization, vitamin E was analysed fluorimetrically using the HPLC system LC-10A (Shimadzu, Japan). Vitamin C was determined using capillary electrophoresis followed by UV detection (System P/ACE 5100, Beckman Coulter Inc.).

Frozen samples of the base part of the heart were dried, weighed, and digested by microwave digestion using nitric acid and hydrogen peroxide (Milestone MLS 1200 MEGA, Milestone Corporation, Italy). Calcium was determined photometrically using flame photometry (Efox 5053, Eppendorf, Germany). Copper was analysed using graphite furnace atomic absorption spectrometry (Unicam, Solaar 959, UK) and zinc was determined using flame atomic absorption spectrometry (Solaar 959, Unicam, UK).

Histological analysis

The excised heart ventricles were rapidly fixed in cold 10% buffered neutral formalin solution for at least 24 hours. After fixation, a heart tissue block was processed using the conventional paraffin-embedding technique. Paraffin sections 5–7 μm were stained with haematoxylin–eosin and Goldner's green trichrome.

The heart tissue was fixed by immersion in 3% glutaraldehyde, postfixed in 1% OsO_4 (both in phosphate buffer at pH 7.2–7.4), dehydrated in acetone and propylene oxide, and embedded in the resin mixture Durcupan ACM and Epon 812. Semi-thin sections about 1 μm were cut on the ultramicrotome LKB and stained with toluidine blue.

Evaluation of pathological changes included focal cardiomyocyte damage or small multifocal degeneration with mild degree of inflammatory process, extensive myofibrillar degeneration and/or diffuse inflammatory process, and necrosis with diffuse inflammatory process. For the topographic localization of the heart tissue we used a semi-quantitative scale as follows: (−) absence of pathological changes, (+) mild, (++) moderate, and (+++) marked changes. Photo documentation and image digitizing were performed using the light microscope, OLYMPUS AX-70 (Olympus Ltd, Japan), using a digital camera, Pixelink PL-A642 (Vitana Corp., Canada) and an image analysis software, NIS – ELEMENTS AR 3.2 (Laboratory Imaging, Czech Republic).

Glutathione in whole blood

Rats weighing on average 380 g were randomly divided into eight groups of four rats each and the same experimental design as described above, i.e. control groups: (a) SB2.3, (b) SB4.6, (c) Ru11.5, and (d) Ru46; and ISO groups: (a) SB2.3+ISO, (b) SB4.6+ISO, (c) Ru11.5+ISO, and (d) Ru46+ISO.

The rats were anaesthetized using urethane and the left common iliac artery was cannulated for blood withdrawal. After 15 minutes of equilibration, the drug formulation/solvent was administered as a slow i.v. bolus via the saphenous vein using the same infusion rate as described above. ISO was administrated s.c. 5 minutes later if indicated. Whole blood (150 μl) was collected into a heparinized tube (BD Vacutainer, BD, USA) at the predefined time intervals following the administration of ISO (0, 1, 5, 15, 30, 60, and 120 minutes). Immediately after the sampling, 1-methyl-2-vinylpyridinium trifluoromethanesulphonate (Oxis Research, USA) was added to the blood samples for analysis of oxidized glutathione (GSSG) to prevent spontaneous glutathione oxidation. Afterwards, the samples of oxidized and total glutathione (GSH_t) were stored at −80°C until analysis.

GSSG, ratio of reduced and oxidized glutathione (GSH/GSSG ratio), and GSH_t were determined colorimetrically using the BIOXYTECH GSH/GSSG-412 kit according to the instructions of the manufacturer (Item No. 21040, Oxis Research, USA) and a spectrophotometer Helios Gamma equipped with VisionLite Software 2.2 (ThermoFisher Scientific Inc., USA). The results were calculated as percent of change vs. baseline level (at time 0 minute, before ISO administration).

8-Isoprostane in rat plasma

Rats weighing on average 350 g were randomly divided into four groups of four rats involving only

the higher dose regimes and the same order as above: SB4.6, Ru46, SB4.6+ISO, and Ru46+ISO.

The administration of the drug formulation/solvent and whole blood withdrawal followed the protocol described above. Whole blood (150 µl) was collected at the predefined time intervals following the administration of ISO (0, 5, 15, 30, 45, 60, 90, 120, 150, 180, and 240 minutes). Immediately after the sampling, blood was centrifuged (1733 × g, 10 minutes, a centrifuge MPW-52, MPW Med. Instruments, Poland) in the presence of 0.005% *tert*-butylhydroxytoluene and plasma was stored at −80°C until analysis.

The free fraction of 8-isoprostane was determined in plasma using the 8-isoprostane EIA kit according to the instructions of the manufacturer (Item No. 516351, Cayman Chemical Company, USA) and a spectrophotometer Synergy HT Multi-Detection Microplate Reader (BioTec Instruments Inc., USA). The results were calculated as percent of change vs. baseline level (at time 0 minute, before ISO administration).

Pharmacokinetic study

A rat weighing 525 g was anaesthetized with urethane and cannulated for blood withdrawals in the same way as described above. Afterwards, rutin dissolved under the same conditions as above was administered at a dose of 46 mg/kg as a slow i.v. bolus via the saphenous vein using the same infusion rate as in the pharmacodynamic study. Whole blood (150 µl) was sampled at predefined time intervals following the administration of the drug formulation (0, 1, 5, 15, 30, 45, 60, 120, 180, and 240 minutes). Immediately after the sampling, whole blood was centrifuged (2500 × g, 10 minutes; a centrifuge MPW-52, MPW Med. Instruments, Poland) and plasma was stored at −80°C until analysis.

Rutin concentrations in plasma were assessed using the UHPLC–MS/MS method. This was a system consisting of Acquity UPLC (Waters Corporation, Czech Republic) and Quattro Micro triple quadrupole mass spectrometer (Waters Corporation, Czech Republic). The separation was performed on a BEH Shield RP C18 (2.1 × 100 mm, 1.7 µm) using gradient elution with methanol and 0.1% formic acid. All injected solutions were stored in the autosampler at 4°C. The partial loop with needle overfill mode was set up to inject 5 µl. The analytical column was kept at 40°C by a column oven.

The MS conditions were finely tuned in positive polarity ESI mode as follows: capillary voltage: +3200 V, ion source temperature: 130°C, extractor: 3.0 V, RF lens: 0.5 V. The desolvation gas was nitrogen at a flow 800 l/hour and at 450°C. Nitrogen was also used as a cone gas (100 l/hour) and argon as a collision gas. Analyses were performed in selected reaction monitoring mode using the precursor ion $[M + H]^+$ and the corresponding product ion (610.9 > 303.3). The cone voltage was 20 V, collision energy 25 eV, and the dwell time was 0.2 seconds. MassLynx MS Software 4.1 (Waters Corporation, Czech Republic) was used for MS control and data acquisition. QuanLynx Software (Waters Corporation, Czech Republic) was used for data processing and peak integration. The sample pre-treatment of plasma samples consisted in fast and simple protein precipitation. Plasma (50 µl) was precipitated with 100 µl of acetonitrile. After 10 minutes the samples were centrifuged for 10 minutes. The supernatant was then diluted 200× with a mixture of acetonitrile/water (2:1), filtered through a PTFE membrane with 0.22 µm pores and injected into a UHPLC system.

Pharmacokinetic parameters were calculated by Kinetica 5.1 (Thermo Fisher Scientific Inc., USA) and PKSolver both using the non-compartmental analysis of plasma after the i.v. bolus input and the linear trapezoidal method.[35]

In vitro studies using H9c2 cell line

The H9c2 cell line derived from embryonic BD1X rat heart tissue was obtained from the American Type Culture Collection (ATCC, USA).[36] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Switzerland) supplemented with 10% heat-inactivated foetal bovine serum (Lonza, Switzerland), 1% penicillin/streptomycin solution (Lonza, Switzerland), and 10 mM HEPES buffer (pH 7.4; Sigma-Aldrich, USA). Cell cultivation was held in 75 cm^2 tissue culture flasks from Techno Plastic Products AG (TPP, Switzerland) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured twice a week when they reached approximately 90% confluence.

For particular experiments, cells were seeded into appropriate microplates or Petri dishes (TPP, Switzerland) at given cellular density. Twenty-four hours prior to all cellular experiments, the medium was changed for serum-free cell-culture medium (pyruvate-free DMEM, Sigma-Aldrich, USA) supplemented with 1% penicillin/streptomycin solution (Lonza, Switzerland) and 10 mM HEPES buffer (pH 7.4; Sigma-Aldrich, USA). Serum deprivation was used to stop cellular proliferation to mimic the situation in post-mitotic cardiomyocytes.[37] Pyruvate was omitted because it is an antioxidant and could interfere with ROS-related toxicity.

Glutathione in H9c2 cell line

H9c2 cells seeded in Petri dishes (Ø150 mm) at a density of 3 000 000 cells/dish were pre-incubated with rutin (1–1000 µM) and ISO (0.1–1000 µM) was

added 5 minutes later. After 2-hour co-incubation, cells were washed twice with ice-cold PBS, harvested by scraper, and centrifuged. Supernatants were discarded, pellets of cells were resuspended in 175 µl of PBS, and aliquots were sampled into microtubes, i.e. (a) 100 µl was mixed with 10 µl of 1-methyl-2-vinylpyridinium trifluoromethanesulphonate (Oxis Research, USA) preventing the spontaneous oxidation of glutathione for GSSG assessment; (b) 50 µl was used for GSH_t assessment; and (c) 25 µl for protein assessment. Each step was kept on ice. The samples were stored at −80°C until analysis. GSSG, GSH/GSSG ratio, and GSH_t were determined in the same way as in whole blood.

2′,7′-Dichlorodihydrofluorescein diacetate assay

To assess intracellular ROS formation, measurement of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, USA) fluorescence intensity was used. This originally non-fluorescent reagent diffuses passively through the cellular membranes, its acetate groups are metabolized by intracellular esterases and then oxidized by ROS formed inside the cell (particularly by hydroxyl radicals) to green-fluorescent 2′,7′-dichlorofluorescein (DCF; $\lambda_{ex} = 485$ nm, $\lambda_{em} = 525$ nm). The fluorescence intensity is proportional to the intracellular concentration of hydroxyl radical leading to its quantitative measurement.

H9c2 cells seeded in a 96-well plate at a density of 10 000 cells/well were washed twice with ADS buffer (116 mM NaCl, 5.3 mM KCl, 1.2 mM $MgSO_4$, 1.13 mM NaH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$, 20 mM HEPES; pH 7.4) and loaded with 10 µM H_2DCF-DA in ADS buffer. After 60-minute incubation, the buffer was discarded, the cells were washed twice with ADS buffer, and pre-incubated with rutin for 5 minutes. ISO was then added (both in ADS buffer). All solutions were pre-warmed to 37°C. Fluorescence intensity was measured at time 0 and 2 hours of co-incubation at 37°C using a microplate spectrophotometer Tecan Infinite 200 M (Tecan Austria, Austria). Hydrogen peroxide and *tert*-butyl hydroperoxide (*t*BHP) (10–250 µM) were used as positive controls. Intracellular ROS formation after 2 hours was expressed as the percentage of the untreated control (100%).

Statistical analysis

Data are expressed as means ± SEM. Significant outliers were excluded using Grubb's test. Differences were considered significant at $P < 0.05$, unless indicated otherwise. Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, USA).

Survival was analysed using Kaplan–Meier survival curves and the log-rank test. cTnT, vitamin E and vitamin C, elements in myocardium, wet ventricle weight index, and glutathione and intracellular ROS formation in the H9c2 cell line were evaluated using one-way ANOVA followed by Tukey's multiple comparison test. The normality of the data was tested by D'Agostino–Pearson test. Time-dependent variables (haemodynamics, glutathione in whole blood, and 8-isoprostane in rat plasma) were analysed using two-way ANOVA followed by Tukey's multiple comparison test.

Results

In an earlier publication we found that a single dose of rutin (46 mg/kg, i.v.) aggravated ISO-induced cardiotoxicity and reduced survival within 24 hours of administration.[31] In the present study, we firstly analysed the effects of rutin and sodium bicarbonate (the solvent) on ISO-induced mortality. No control rats receiving either the solvent or rutin died in the haemodynamic study. The mortality rate for the ISO groups receiving either sodium bicarbonate at the lower dose or the lower dose of rutin was similar while the higher dose of rutin resulted in a higher mortality rate than the corresponding solvent group. Moreover, there was a clear tendency of the higher dose of the solvent to prevent mortality, but this was not significant in comparison with the lower dose of the solvent (Supplementary material Fig. A.1). Moreover, we did a *post hoc* analysis of the mortality rates (Fig. 1). Even though the *in vivo* studies (haemodynamic study, glutathione in whole blood, and 8-isoprostane in plasma) followed different protocols and the frequent blood withdrawals clearly affected the outcome, the results for 2-hour mortality rate were similar, i.e. the *post hoc* analysis (Fig. 1) and Kaplan–Meier survival curves of the individual *in vivo* studies (Supplementary material Figs. A.1–A.4). Only one rat receiving rutin (without ISO) at a dose of 46 mg/kg died during the 8-isoprostane study after 115 minutes. In general, the frequent blood withdrawals during this 4-hour study were associated with the higher mortality rates (Supplementary material Fig. A.4).

As the solvent, 1.26% sodium bicarbonate, could have affected the results due to its slight alkalinity, we did an additional experiment, in which saline was given in the same volume as the solvent (4.6 ml/kg). There were no significant differences between groups (Supplementary material Fig. A.2).

Based on our previous 24-hour study, in which rutin increased cardiac output dose-dependently after 24 hours,[31] and published papers which suggest that flavonoids can decrease blood pressure,[38] we assessed haemodynamic variables as these effects could have

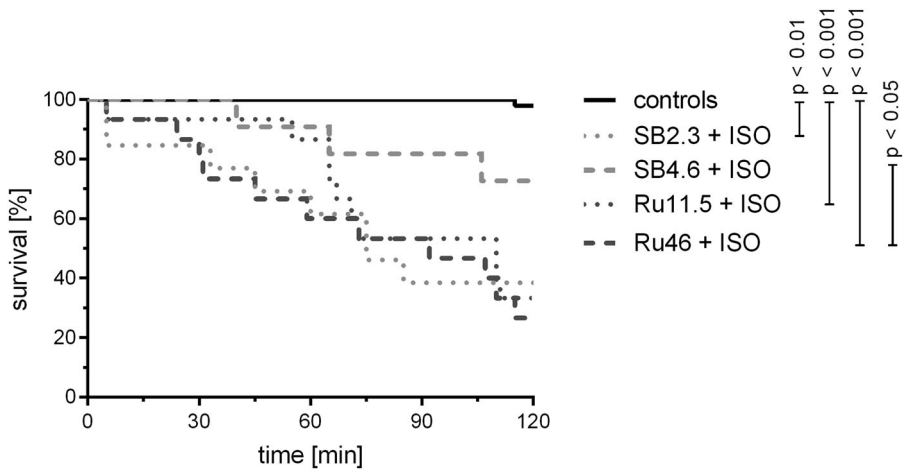

Figure 1 Kaplan–Meier survival curve of all *in vivo* studies involving the administration of the solvent (sodium bicarbonate, SB, 2.3 or 4.6 ml/kg, i.v.) or rutin (Ru, 11.5 or 46 mg/kg, i.v.), i.e. controls, and their combination with ISO (100 mg/kg, s.c.). The statistical analysis was performed using the log-rank test.

a negative influence on the early stages of ISO-induced cardiotoxicity. Neither the solvent or rutin themselves nor their combination with ISO significantly modified the haemodynamic variables, in comparison with the corresponding group, during or at the end of the 2-hour continuous recording (Supplementary material Figs. A.5 and A.6). Even if short-term differences were observed for several haemodynamic variables, such fluctuations were apparently caused by increase in intravascular volume rather than a pharmacological action of rutin itself. Analysing ECG variables, ISO-induced mortality was caused by different types of ventricular dysrhythmias including *torsades de pointes* and AV blocks. There were no specific dysrhythmias in individual groups suggesting rather their coincidence. Moreover, no positive or negative effect of rutin on their frequencies was observed.

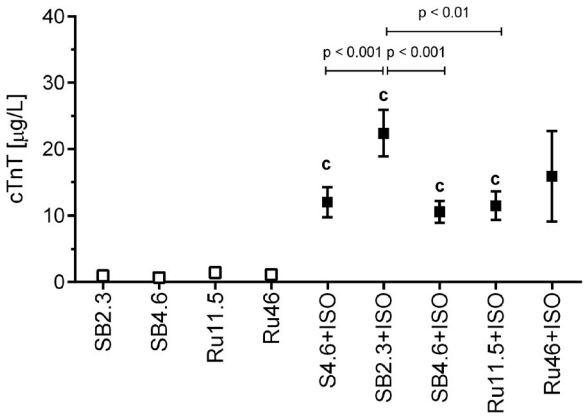

Figure 2 Serum concentration of cTnT after 2-hour haemodynamic study involving the administration of the solvent (sodium bicarbonate, SB, 2.3 or 4.6 ml/kg, i.v.) or rutin (Ru, 11.5 or 46 mg/kg, i.v.), i.e. controls, and their combination with ISO (100 mg/kg, s.c.) including the comparison with saline (S, 4.6 ml/kg, i.v.). Data are expressed as means ± SEM. The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance: c vs. the corresponding control group at $P < 0.001$. Because of high mortality in the group Ru46+ISO, its data were not included in the statistical analysis.

In agreement with previously published data, the administration of ISO (100 mg/kg, s.c.) induced a marked release of cTnT into blood. The lower dose of rutin reduced it significantly in comparison with the corresponding ISO group, while there was a dose-dependent tendency of higher dose of rutin to increase cTnT release. However, it was not feasible to assess the hypothesis statistically because of the low survival in the Ru46+ISO group. Interestingly, in agreement with the mortality rate, the higher dose of sodium bicarbonate had a preventive effect, in comparison with the lower dose. Like the mortality, the administration of saline instead of sodium bicarbonate was associated with the same cTnT release. Rats that received the solvent or rutin without ISO had only a negligible cTnT release (Fig. 2). No significant differences were found between serum concentrations of vitamin E and vitamin C (Supplementary material Fig. A.7). Similarly, there were no disturbances in myocardial levels of calcium, copper, or zinc (data not shown).

The co-administration of sodium bicarbonate and ISO showed massive diffuse inflammatory infiltrations in all parts of the ventricle using haematoxylin–eosin and Goldner's trichrome green stainings after 2-hour haemodynamic study. Indeed, the presence of large quantities of phagocytes, mast cells together with significant interstitial oedema and foci of necrotic cardiomyocytes were observed, confirming significant alterations of the heart tissue (Fig. 3C). Although rutin partly ameliorated some of ISO-induced histopathological changes, local capillary hyperaemia, mild interstitial oedema with dilation or swelling of cardiomyocytes, and slight inflammatory cell infiltration were still found in the endocardium and partially

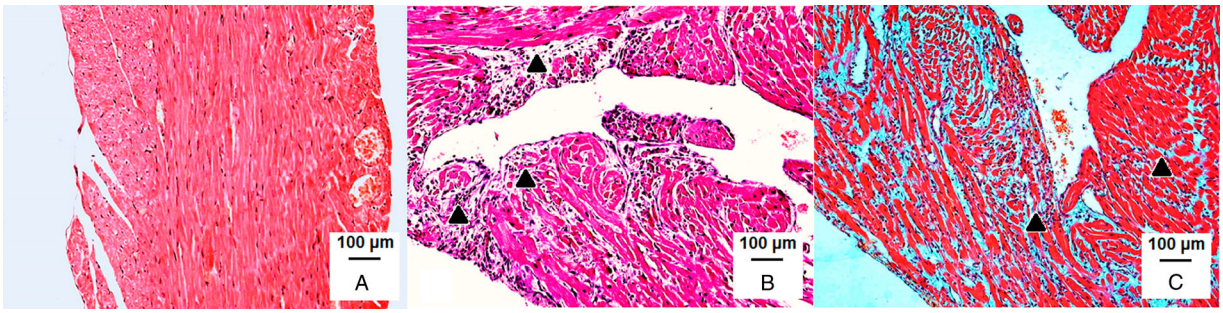

Figure 3 Overview of histological findings in endocardium and myocardium after 2-hour haemodynamic study. Staining: haematoxylin–eosin and Goldner's trichrome green. Representatives of control groups (A; rutin 11.5 mg/kg, i.v.) and ISO groups (100 mg/kg, s.c.) involving a premedication with rutin (B; 11.5 mg/kg, i.v.) or sodium bicarbonate (C; the solvent, 2.3 ml/kg, i.v.). In contrast to normal structural architecture in the control groups (A), marked alterations were found after the administration of ISO (B). Although rutin partly ameliorated histopathological changes, local capillary hyperaemia, mild interstitial oedema with dilation or swelling of cardiomyocytes, and slight inflammatory cell infiltration of phagocytes (▲) were still observed (C). There were no marked differences between the lower dose and the higher dose of rutin, resp. the solvent (not shown).

in the pericardium (Fig. 3B, Table 1). The structural architecture of all parts of the ventricle was normal in the control groups with the exception of those for rutin in which a slight local capillary hyperaemia was found (Fig. 3A). It is worth mentioning that no marked differences were observed between the lower dose and the higher dose of rutin and the solvent groups, at all. Moreover, these findings were confirmed at the cellular level using toluidine blue staining (Supplementary material Fig. A.8). The analysis of wet ventricle weight index showed no significant changes (Supplementary material Fig. A.9).

As haemodynamic study and ECG were unable to explain the reason for the higher mortality, we focused on evaluating oxidative stress. Again, there were no significant changes in GSSG, GSH/GSSG ratio, and GSH_t levels in whole blood after 2 hours. Interestingly, the higher dose of rutin itself increased the level of GSSG, however, there were no significant differences between the control and ISO groups during the experiment (Supplementary material Fig. A.10). For this reason, we used a simpler model – the rat cardiomyoblast-derived cell line, H9c2, in order to further examine the influence of both rutin and ISO on glutathione levels. Here, as expected, ISO tested over the broad concentration range of 0.1–1000 µM led to slight increase in GSSG levels (Fig. 4A). Rutin not only failed to prevent the ISO-

Table 1 Semi-quantitative analysis of histopathological changes in epicardium, myocardium, and endocardium of the left ventricle after 2-hour haemodynamic study

Pathological changes/experimental groups		SB2.3	SB4.6	Ru11.5	Ru46	SB2.3+ISO	SB4.6+ISO	Ru11.5+ISO	Ru46+ISO
Local capillary hyperaemia	Endocardium	–	–	+	+	+++	+++	++	++
	Myocardium	–	–	+	+	+++	+++	+	+
	Epicardium	–	–	+	+	+++	+++	++	++
Interstitial oedema with dilation	Endocardium	–	–	–	–	+++	+++	++	++
	Myocardium	–	–	–	–	+++	+++	+	+
	Epicardium	–	–	–	–	+++	+++	++	++
Swelling of the binder fibres	Endocardium	–	–	–	–	+++	+++	++	++
	Myocardium	–	–	–	–	+++	+++	+	+
	Epicardium	–	–	–	–	+++	+++	++	++
Necrotic changes of cardiomyocytes	Endocardium	–	–	–	–	++	++	+	+
	Myocardium	–	–	–	–	++	++	+/–	+/–
	Epicardium	–	–	–	–	++	++	+	+
Myofibrils fragmentation	Endocardium	–	–	–	–	++	++	+	+
	Myocardium	–	–	–	–	++	++	+	+
	Epicardium	–	–	–	–	++	++	+	+
Fragmentation of muscle trabeculae	Endocardium	–	–	–	–	+	+	–	–
	Myocardium	–	–	–	–	+	+	–	–
	Epicardium	–	–	–	–	+	+	–	–
Presence of macrophages/mast cells	Endocardium	–	–	–	–	+++	+++	++	++
	Myocardium	–	–	–	–	+++	+++	++	++
	Epicardium	–	–	–	–	+++	+++	++	++

Evaluation of pathological changes was expressed with a semi-quantitative scale as follows: (–) absence of pathological changes and (+) mild, (++) moderate, and (+++) marked changes. The study involved administration of the solvent (sodium bicarbonate, SB, 2.3 or 4.6 ml/kg, i.v.) or rutin (Ru, 11.5 or 46 mg/kg, i.v.), i.e. controls, and their combination with ISO (100 mg/kg, s.c.).

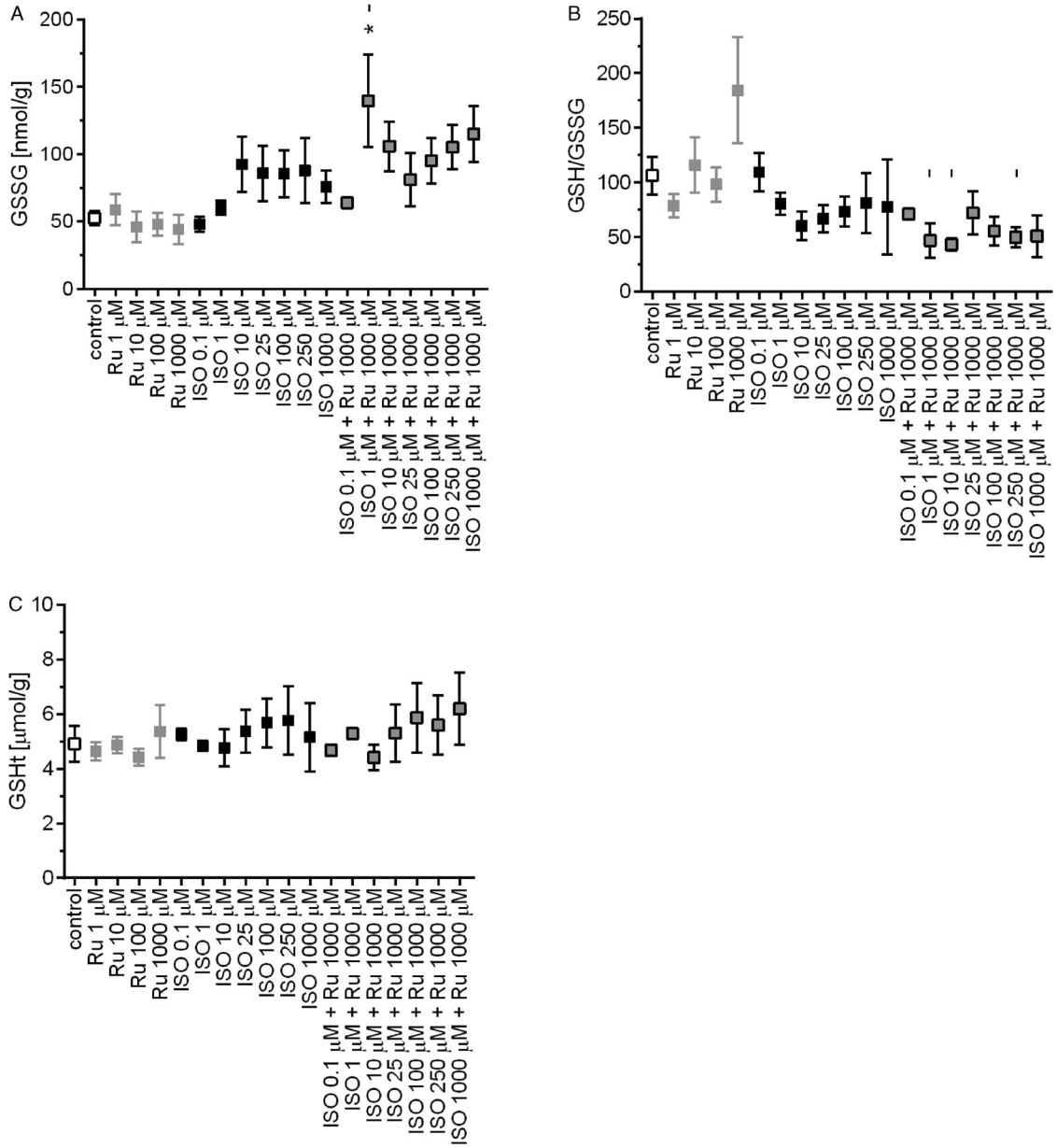

Figure 4 GSSG (A), the GSH/GSSG ratio (B), and GSH_t (C) in the H9c2 cell line exposed to rutin (Ru, 1–1000 μM), ISO (0.1–1000 μM), or their combination, i.e. ISO (0.1–1000 μM) and Ru (1000 μM). Data are expressed as means ± SEM of at least two independent experiments, which were performed in duplicates. The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance: *$P < 0.05$ vs. control; – $P < 0.05$ vs. Ru 1000 μM.

induced increase in GSSG, it increased it. However, this was significant only at the lowest concentration of ISO (Fig. 4A). Similarly, a slight increase in glutathione oxidation was revealed through decrease in the GSH/GSSG ratio (Fig. 4B). Although rutin at the highest concentration tended to increase GSH_t levels, there were no significant changes (Fig. 4C). This may also be the reason for the lower GSH/GSSG ratio (Fig. 4B).

Owing to the low/even negligible changes in plasma glutathione and the H9c2 cell line, we used a more selective biomarker of free radical-initiated peroxidation, 8-isoprostane. In this case, the free fraction level in plasma increased significantly in the SB4.6+ISO group compared with solvent ($P < 0.001$). The two-way ANOVA showed an overall significant difference between groups sets ($P < 0.001$) but the *post hoc* Tukey's multiple comparison showed no significant differences at various time intervals between groups, likely due to the high variability in the Ru46+ISO group (Fig. 5).

Due to the minor changes in oxidative stress found in plasma, intracellular ROS formation was determined using the H_2DCF-DA assay. As seen in Fig. 6, none of the concentrations of ISO induced ROS production inside H9c2 cells after 2-hour incubation in comparison with the control group. Interestingly, a dose-dependent decrease was observed. However, co-incubation with rutin had the opposite effect. The most significant induction was seen with

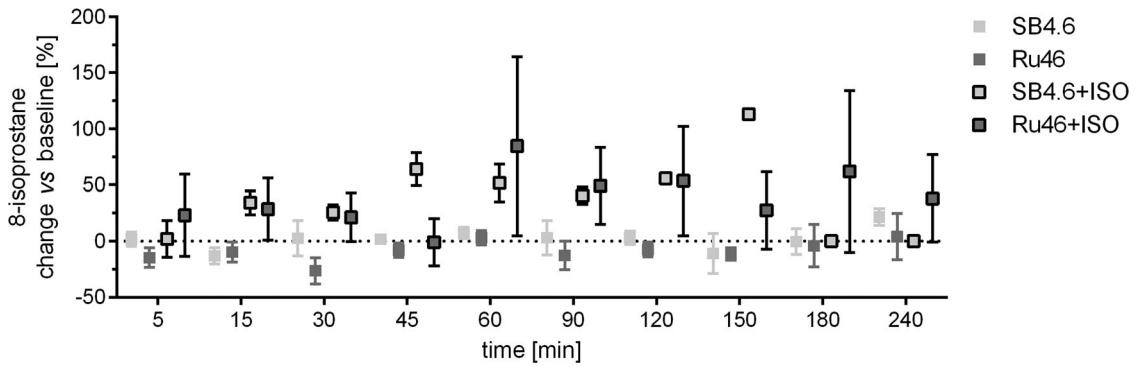

Figure 5 Changes of the free fraction of 8-isoprostane in plasma following the administration of the solvent (sodium bicarbonate, SB, 4.6 ml/kg, i.v.) or rutin (Ru, 46 mg/kg, i.v.), i.e. controls, and their combination with ISO (100 mg/kg, s.c.). The changes were calculated vs. baseline level (at time 0 minute, before ISO administration). Data are expressed as means ± SEM. Each sample was tested at least in duplicate. Two-way ANOVA showed significant differences between means of data sets ($P < 0.001$).

the combination of 1000 μM rutin and 10 μM ISO. As predicted the positive controls, *t*BHP, and hydrogen peroxide dose-dependently resulted in intracellular ROS formation (Supplementary material Fig. A.11).

To confirm the presence of rutin in rat plasma, whole blood was withdrawn from one rat for analysis of the pharmacokinetic profile after the slow i.v. bolus (Supplementary material Fig. A.12 and Supplementary material Tab. A.1). Calculations were carried out using two independent programmes (Kinetica 5.1 and PKSolver). The minor difference between the area under the curve at the last analysed time-point (AUC_{0-t}) and that extrapolated to infinity ($AUC_{0-\infty}$) confirmed sufficient duration of the study and thus accuracy of analysed pharmacokinetic parameters.[39]

Discussion

Rutin, quercetin-3-*O*-rutinoside, a natural flavonol glycoside, has shown various beneficial pharmacological properties which could be useful in the treatment of many human diseases, particularly those of the cardiovascular system.[40] These benefits have been mostly attributed to its antioxidant effects involving the combination of scavenging activities towards various ROS (e.g. superoxide, hypochlorite, and peroxynitrate), iron/copper chelation properties, and inhibition of

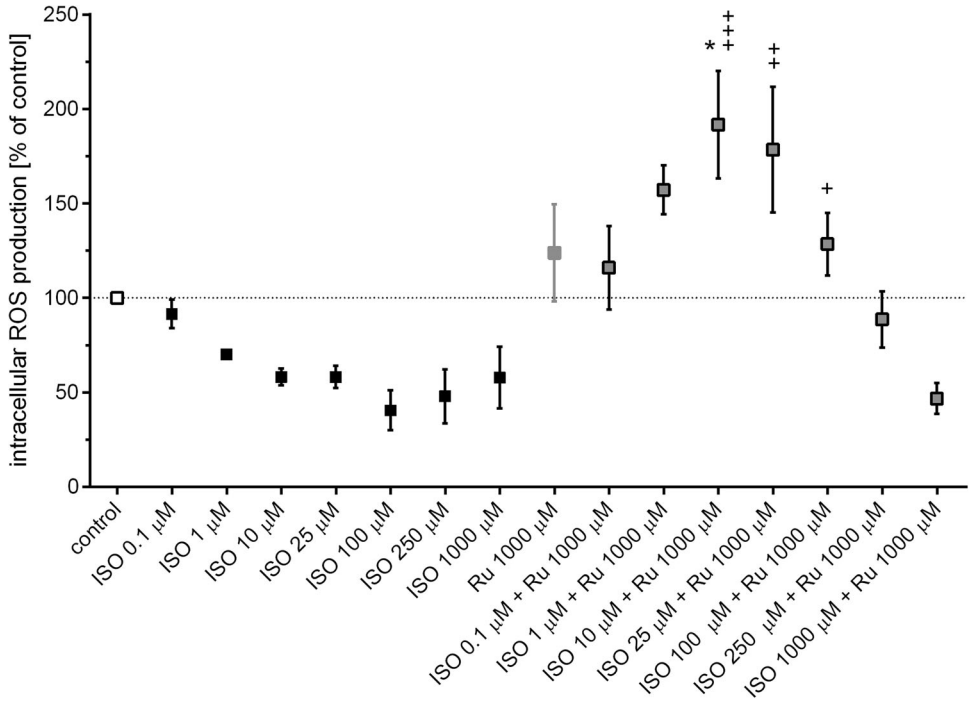

Figure 6 Intracellular ROS formation in H9c2 cardiomyoblasts determined using the H_2DCF-DA assay after 2-hour treatment with ISO (0.1–1000 μM), rutin (Ru, 0.1–1000 μM), or their combination, i.e. ISO (0.1–1000 μM) and Ru (1000 μM). Data are expressed as means ± SEM; $n = 3$. The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance: *$P < 0.05$ vs. control; + $P < 0.05$, ++ $P < 0.01$, and +++ $P < 0.001$ vs. the corresponding ISO group without rutin.

free radical-forming enzymes (e.g. xanthine oxidase) and anti-inflammatory effects.[6,8,11,41–44] It may improve endothelial function by augmenting NO production and inhibiting human platelet aggregation, as well.[45,46] However, rutin can also have pro-oxidative effects, which could be based on its metal-reducing potential and increased formation of the hydroxyl radical via Fenton chemistry.[12] This may also depend on other factors such as concentrations used, presence of other reductants, and/or the atmospheric oxygen access.[47]

Stanely Mainzen Prince and coworkers reported a broad spectrum of prophylactic effects of rutin (40 or 80 mg/kg/day, p.o., 42 days) in the case of various aspects of ISO-induced cardiotoxicity (150 mg/kg/day, s.c., 2 days), in the same rat strain as used in this study. The above authors suggested the beneficial effects of rutin were associated with free radical scavenging activity, lipid-lowering effects, both mitochondrial structure and function improvement, and membrane stabilizing properties in particular.[26–29] It is not easy to reconcile such results with the findings concerning the current study and our published study over 24 hours.

Firstly, Stanely Mainzen Prince and coworkers administered rutin orally for 42 days. We used a single i.v. dose. This is an important factor as rutin is not absorbed in the intestine. A number of clinical and experimental studies have reported that rutin is cleaved in the caecum via bacterial microflora and further metabolized by both bacterial and human enzymes to form various metabolites such as quercetin and its glucuronide and sulphate, tamarixetin, isorhamnetin, and various phenolic acids, resulting to no absorption of the parent rutin itself.[31,48–52] Moreover, the relatively short elimination half-life of i.v. rutin (Supplementary material Fig. A.10 and Supplementary material Tab. A.1) suggests that the positive effects of oral rutin on the ISO-induced oxidative stress were caused by its metabolites, including quercetin conjugates and small phenolic acids, and/or some adaptation mechanism(s).[53–55] Therefore, if rutin had positive effects, these benefits probably were not caused by rutin *per se*.

Secondly, no mortality data were provided by Stanely Mainzen Prince and coworkers, surprisingly, only positive results. Moreover, the results were linearly related to dose. It is very uncommon that 42-day oral treatment results in a high linear dose/effect relationship. To test this, we performed a similar analysis to the one we reported recently.[56] In short, mean control values were considered 100% (untreated rats), the mean ISO values were considered 0% (the pathological state), and mean effects of rutin were expressed as percent inhibition due to ISO effect. Surprisingly, a linear relationship describing inhibition of ISO-induced cardiotoxicity was found in most of the cases. Indeed, the linear regression coefficients in 5/34 cases were equal to 1.000 (15%), in 15/34 (44%) cases higher than 0.990, and in 25/34 (74%) cases higher than 0.975, respectively. Such high linearity for so many measured variables in the studies of Stanely Mainzen Prince and coworkers is unlikely. It is also well known that the bioavailability and pharmacokinetics of oral rutin are non-linear.[49,50,57]

In contrast, we found that a single i.v. dose of rutin either did not influence (11.5 mg/kg) or aggravate (46 mg/kg) ISO-induced cardiotoxicity (100 mg/kg, s.c.) in Wistar rats after 24 hours.[31] In agreement, the higher dose of rutin resulted in increased mortality rate from 30 to 52%, while the lower dose had no effect on mortality. Moreover, such outcomes are in agreement with cTnT concentrations; myocardial calcium levels, and *in vitro* experiments, in which co-incubation with higher concentrations of rutin was found to significantly increase ISO-induced toxicity in the H9c2 cell line.[31]

Similar findings were confirmed in this study. Indeed, rutin was not able to protect myocardium against ISO-induced cardiotoxicity within 2 hours of continuous experiment. Although the mortality rate was higher in general, likely due to invasive approach and/or frequent blood withdrawals, the outcome was similar (Fig. 1). Here, we discovered three interesting facts: (1) the lower dose of rutin did not affect the mortality rate but it decreased cTnT release after 2 hours, (2) the greater volume of administered solution itself was protective regardless whether it was saline or 1.26% sodium bicarbonate, (Fig. 1), and (3) both doses of rutin had some positive effect on histological myocardial changes after 2 hours due to ISO regardless of the high mortality rate and different effect on cTnT release. The results extended our previous findings demonstrating that i.v. rutin was unable to provide sufficient protection against ISO-induced cardiotoxicity. pH is markedly reduced during ischaemia[58] and we speculated that slightly alkaline solvent might afford some degree of protection but this was not the case as saline was similarly protective. One reason could be that ISO results in massive vasodilation due to β_2-adrenergic hyperstimulation together with myocardial hyperstimulation through β_1-receptors causing an acute state of circulatory shock[21] and increase in circulating blood by administration of a higher dose of fluid can partly reverse it.

The reason why both doses of rutin had partial protective effects from the histological findings after 2 hours without any effect on histology after 24 hours[31] or mortality is not clear. Since there was a marked difference between acute (2 hours) changes in myocardial histology which were mainly of an

inflammatory character and subacute (24 hours) changes which included myocardial necrosis, we suggest the acute positive effects may be based on the anti-inflammatory effects of flavonoids.[5,59]

Apropos oxidative stress variables in blood, rutin itself dose-dependently increased the level of GSH_t after 24 hours, however, ISO abolished this. The effect appears to correspond with our outcomes, in which both GSH_t and the GSH/GSSG ratio tended to increase at the higher concentration of rutin in the H9c2 cardiomyoblast-derived cell line (Fig. 4B and C). No significant differences in thiobarbituric acid reactive substances or vitamin E have been found after 24 hours.[31] The latter corresponds to this study, in which no significant changes in blood variables were observed 2 hours after the ISO treatment. This was valid for the continuous analysis of GSSG in blood as well. Even though there were only minor differences in oxidative stress-related parameters between groups, the mortality rates were markedly different. These data clearly suggest that antioxidant markers and markers of oxidative stress in blood might not be the valid markers of ISO-induced cardiotoxicity described.[60,61]

Interestingly, the inability of ISO to induce detectable formation of ROS was in accordance with our previous studies.[62,63] This phenomenon has also been described by Costa *et al.*,[64] where no changes were shown in lipid peroxidation, protein carbonylation, or activity of various antioxidant enzymes after up to 3 hours of incubation of isolated adult rat cardiomyocytes with 500 μM adrenaline. Moreover, direct antioxidant activity of catecholamines under pro-oxidant conditions has been described. Adrenaline elicited intracellular survival pathways in isolated rat cardiomyocytes resembling those described for the phenomenon of ischaemic preconditioning.[65] This may explain the above mentioned changes in glutathione in our 2- and 24-hour *in vivo* studies as well.

Although we found no convincing data in whole blood, our results clearly showed that rutin aggravated intracellular oxidative stress under *in vitro* conditions (Fig. 6) and was not able to reverse oxidative stress caused by ISO (Fig. 4). However, the clinical impact of these effects is unknown because the mortality rates for SB2.3/4.6+ISO and Ru11.5+ISO were not different, and there was a decrease in cTnT levels in Ru11.5+ISO (Figs. 1 and 2). Similarly, in contrast to the cell experiments, analysis of the free fraction of 8-isoprostane in rat plasma, a specific marker of free radical-initiated peroxidation of arachidonic acid,[66] revealed only differences between ISO and sodium bicarbonate (Fig. 5). Conclusively, although Stanely Mainzen Prince and coworkers reported positive effects of oral rutin on various oxidative stress markers, this does not mean that rutin is able to revert negative haemodynamic effects and clinical outcome (mortality) of ISO administration. In line with our previous findings that refute any positive effect of oral quercetin on ISO-induced cardiotoxicity,[32] claiming rutin is an oral protective substance against ISO-induced cardiac injury will need detailed study showing haemodynamic, ECG, and mortality rates as well. However, from our quercetin findings, we suspect such outcomes are unlikely, mainly due to the complex mechanisms of ISO-induced cardiotoxicity.

Conclusion

In contrast to studies reporting a wide spectrum of preventive effects of continuous oral administration of rutin against ISO-induced cardiotoxicity, the results described here of the direct effects of a single i.v. dose of rutin within 2 hours confirmed our previous findings of no direct protection of rutin. Rutin did not reduce the mortality rate and it increased intracellular ROS formation *in vitro* and had the tendency to increase it *in vivo.* The higher dose of sodium bicarbonate (4.6 ml/kg, 1.26% w/v solution) was found to protect rat myocardium possibly due to assuaging the state of shock accompanying ISO-induced cardiotoxicity. This is supported by significant decrease in cTnT level and greater survival rate.

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Disclaimer statements

Contributors None.

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Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval The study was approved by the Ethics Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové (Study No. MŠMT 2437/2012-30), and it 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).

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