Cardioprotective Effect of Saffron Extracts against Acute Doxorubicin Toxicity in Isolated Rabbit Hearts Submitted to Ischemia-Reperfusion Injury

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Doxorubicin (DOX) is an anthracycline antibiotic routinely used as a chemotherapeutic agent for the treatment of solid tumours. However, DOX possesses an acute and cumulative cardiotoxicity due to free radical production. The present study was designed to investigate the possible protective effects of saffron (*Crocus sativus*) extracts against DOX-induced acute cardiotoxicity in isolated rabbit hearts submitted to 30 min global ischemia followed by 40 min reperfusion. DOX was delivered during reperfusion, without or with saffron given 5 min before ischemia or at reperfusion. Cardiodynamic, biochemical, and histopathological parameters were determined. In addition, to determine the expression of the AKT/mTOR/4EBP1 pathway, the levels of p38 MAPK and cardiac troponin T in heart homogenates were visualized by Western blotting. DOX administration during 40 min of reperfusion increased ischemic tissue damage, but did not act synergistically. Administration of saffron extracts during the first minutes of reperfusion significantly reduced oxidative myocardial damage, but was less effective when given before ischemia. Subsequent Western blot analysis revealed that saffron administration preserved cardiac troponin T proteins, inhibited the p38 MAPK pathway, and activated the AKT/mTOR/4EBP1 pathway in reperfusion- and DOX-treated rabbit hearts. In conclusion, saffron extracts, acting through antioxidant and antiapoptotic mechanisms, exhibited a protective effect against DOX-induced cardiotoxicity under ischemic condition.

Key words: Doxorubicin, Cardiotoxicity, Ischemia/Reperfusion, Saffron

Introduction

Doxorubicin (DOX) is a potent broad-spectrum chemotherapeutic agent belonging to the antitumour anthracycline antibiotic group of drugs. DOX is widely used in the treatment of many hematologic and solid tumour malignancies including breast, ovarian, and lung cancers, Hodgkin's disease, non-Hodgkin lymphoma, leukemia, and sarcomas. However, DOX causes dose-dependent cardiotoxicity mediated through various mechanisms, including free radical formation, membrane lipid peroxidation, and mitochondrial damage (Simůnek *et al*., 2009; Mordente *et al*., 2012; Tacar *et al*., 2013). Drug cardiotoxicity is a major concern to the pharmacological industry and it is one of the main reasons for non-approval, relabelling, warnings, and withdrawal of pharmaceutical compounds from the market.

Although issues related to DOX and cardiac toxicity have been well studied in normal conditions (Injac and Strukelj, 2008; Kalam and Marwick, 2013), the effects of this drug on the ischemic myocardium have not been investigated in detail. Cancers often occur in advanced age concomitantly with other diseases.

However, pre-existing heart disease is mostly considered as exclusion criteria in clinical studies testing the efficacy and potential adverse effects of antineoplastic drugs (Vera-Badillo *et al*., 2013). It is therefore necessary to investigate the off-target effects of anticancer or adjunct therapies in diseased conditions such as ischemia/reperfusion injury.

Myocardial ischemia (MI) is a deficit in blood perfusion; thus there is an imbalance between O_2 supply and demand, shifting the myocardium from aerobic to anaerobic metabolism (Rosano *et al*., 2008). The myocardium can tolerate brief periods of severe and even total MI without resultant cardiomyocyte death. Since MI results in the loss of contractile function and produces myocardial damage, it is therefore essential to restore the coronary flow to the ischemic myocardium (Rashed and Depre, 2006). However, reperfusion following a period of ischemia may cause injury to the myocardium. The production of excessive quantities of reactive oxygen species (ROS) is an important mechanism of reperfusion injury. O_2, when reintroduced into a previously ischemic myocardium, undergoes sequential reduction leading to the formation of ROS. Potent free radicals are produced within the first few minutes of reflow and play a crucial role in the development of reperfusion injury (Kutala *et al*., 2007).

To prevent free radical damage, the radicals should be converted to metabolically non-destructive molecules, or scavenged immediately after formation. The protective system preventing radical-mediated organ malfunction, disease processes, and aging is referred to as the antioxidative defensive system. Attention has been paid to the natural sources of antioxidants, such as phenolic and flavonoid compounds (Zhao and Zhao, 2010; Akhlaghi and Bandy, 2009). In this context, saffron is considered a spice possessing antioxidant activities attributed to two bioactive compounds, crocin and safranal (Karimi *et al*., 2010).

Despite extensive research, the mechanism of DOX-induced cardiotoxicity remains unknown, as are the mechanisms of the different chemicals acting as potential protectors (Bast *et al.*, 2007; Sahin *et al*., 2010). In terms of signaling pathways, it has been demonstrated that the cardiomyocytes in the myocardium are altered in response to anthracycline exposure. The activation of the PI3K/AKT/mTOR pathway represents the major mechanism of adaptive cardioprotection that is altered by anthracyclines. The delicate balance between pro- and antiapoptotic signaling that is based on the regulation of kinase pathways continues to pre-occupy researchers searching for anticancer molecules, however, an effective solution has not been found (Mordente *et al*., 2012).

Recent results from our laboratory indicated that a methanol/water extract from Lebanese saffron (*Crocus sativus*) is a powerful free radical scavenger *in vitro* (Makhlouf *et al*., 2011) as well as *ex vivo* in isolated heart perfused in normal condition and subjected to DOX or electrolysis as source of free radicals (Chahine *et al*., 2013). Thus, the aim of the present study was to test the possible acute cardioprotective effects of Lebanese saffron administered before ischemia, or during the first minutes of reperfusion, against free radical production under ischemic condition of an isolated rabbit heart in the presence or absence of DOX.

Materials and Methods

Saffron extraction

Dried stigmas of saffron (*Crocus sativus* L.) cultivated in the Bekaa valley, Lebanon – specimen identified and deposited at the Lebanese Ministry of Agriculture – were suspended at a concentration of 2 g/400 ml in a mixture of methanol and water (50:50, v/v) and magnetically stirred over 24 h at 4 °C in the dark. The solution was filtered, the solvent evaporated at 40 °C, the residue lyophilized, and the yield calculated. Safranal and crocin were determined by high-performance liquid chromatography (HPLC) and total polyphenols with Folin-Ciocalteu's reagent.

Animals

The study was performed with 72 male rabbits with body weights ranging between 1.5 and 2.5 kg and aged between 15 and 20 weeks. The animals were housed in a wide metallic cage under a 12-h light/12-h dark cycle. They were fed a standard granulated mixture with free access to water, and their weight was checked weekly. All experiments were performed at room temperature between 8 a.m. and 4 p.m. in accordance with international guidelines for the care and use of laboratory animals, and the protocol had been approved by the Research Ethics Committee of the Lebanese University, Beirut, Lebanon.

Isolated heart preparation

The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (90 mg/kg body weight). The hearts were dissected within 2 min

and perfused with Tyrode's solution and constant pressure (5.57 kPa) and 37 °C, and continuously gassed with a mixture of 95% O_2/5% CO_2 (v/v) according to the Langendorff technique (Skrzypiec-Spring *et al.*, 2007). Hearts were allowed to stabilize for 15 min, and those presenting any arrhythmias were discarded. The volume of the balloon inserted into the left ventricle was adjusted to maintain a left ventricular end diastolic pressure (LVEDP) of 1.3 kPa. All parameters were monitored throughout the experiment as previously described (Nemr *et al*., 2003; Chahine *et al.*, 2013).

Experimental design

Isolated hearts were randomly assigned to seven groups ($n = 10$ for each). Group 1: Control hearts perfused with standard Tyrode's solution for 70 min without any treatment. Groups 2, 3, and 4: After 20 min of stabilization, hearts were subjected to 30 min global ischemia followed by 40 min reperfusion, with or without saffron extracts (10 μg/ml) perfused during 5 min through the coronary circulation via the aortic cannula (in pre- or post-treatment before ischemia or at reperfusion). Groups 5, 6 and 7: DOX (5 μg/ml) was administered during 40 min of reperfusion, with or without saffron perfused before ischemia or at reperfusion according to the scheme in Fig. 1.

Biochemical study

Tissue

Hearts used for mechanical studies were freeze-clamped at the end of each protocol and conserved at −80 °C. Lipid peroxidation (LP) and superoxide dismutase (SOD) activities were determined by a spectrophotometric method using the thiobarbituric acid reactive substances (TBARS) assay kit (catalog no. 10009052) and SOD assay kit (catalog no. 706002), respectively, purchased from Cayman Chemical Company (Ann Arbor, MI, USA), according to the manufacturer's instructions.

Perfusate

The perfusate, eluted from the rabbit heart during reperfusion periods, was collected in an ice-cooled beaker for the spectrophotometric determination of the creatine kinase (CK) activity at 37 °C using a commercial assay kit from Abcam (Cambridge, MA, USA).

Histopathological study

Hearts were immersed in 10% formalin at the end of the experiment. They were fixed in paraffin, stained with hematoxylin and eosin, and thinly sliced using a microtome for visualization by light microscopy.

Western blot analysis

Heart tissue ($n = 4$ to 5 per group) was lysed in radioimmuno precipitation assay (RIPA) buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 100 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA). Tissue was homogenized by a PowerGene homogenizer (Fisher Scientific, Pittsburgh, PA, USA) and the homogenate centrifuged at $10{,}000 \times g$ for 15 min at 4 °C. The supernatant was collected, and proteins were quantified by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Fifty μg protein each from controls and treated groups were separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to Whatman nitrocellulose membranes (Fisher Scientific, Hampton, NH, USA). Membranes were probed with antibodies against total and phosphorylated AKT (Ser473), total and phosphorylated mTOR (Ser2448), total and phosphorylated P38 MAPK (Thr180/Tyr182) (Cell Signaling Technology, Danvers, MA, USA), total and phosphorylated 4EBP1 (Ser 65/Thr 70) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cardiac troponin T (Abcam), and β-actin (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analyses

The results are expressed as mean ± SD. Statistical analysis of variance (ANOVA) was followed by Bonferroni's multiple comparison post-hoc test. Results were considered statistically significant if $p < 0.05$.

Results

Cardiodynamics

The protocols used in this study on isolated rabbit hearts are summarized in Fig. 1. The effects of ischemia (I), reperfusion (R) or DOX and IR on heart function in the presence or absence of saffron extracts,

in pre-treatment or during the first minutes of reperfusion, are summarized in Table I and Fig. 1. In control hearts, cardiac parameters were stable throughout the entire perfusion period of 70 min. After 30 min of global ischemia, all parameters were affected, but recovered towards the initial values without reaching baseline values at 40 min. DOX administration (5 μg/ml) during reperfusion worsened the heart function, which recovered more slowly. Forty min following reperfusion, in hearts subjected to IR and DOX, the left ventricular pressure (LVP) was decreased to 56% and the left ventricular end diastolic pressure (LVEDP) increased to 200% compared to the respective controls. Saffron treatment prior to ischemia slightly ameliorated therecovery, while given at reperfusion it ameliorated the recovery of LVP and LVEDP, reaching 78% and 150%, respectively. Heart rate (HR) and coronary flow (CF) were also significantly improved. The HR decreased following IR and DOX treatment to nearly 66% and increased to 84% when saffron was administered at reperfusion. The CF also decreased after IR and DOX treatment to 77% and increased to 95.5% when saffron was given at reperfusion. Figure 2 shows typical tracings of the LVP in isolated rabbit hearts subjected to a period of global ischemia followed by reperfusion in the presence and absence of DOX (5 μg/ml) and saffron extract (SAF; 10 μg/ml). The used concentration of DOX was in accordance with the literature (Ramond *et al*., 2008; Gratia *et al*., 2012). For saffron, we used a concentration of 10 μg/ml for 5 min perfusion, as it was found in preliminary trials that higher concentrations have toxic effects on the heart contractility, while at lower concentrations the protective effect is not significant.

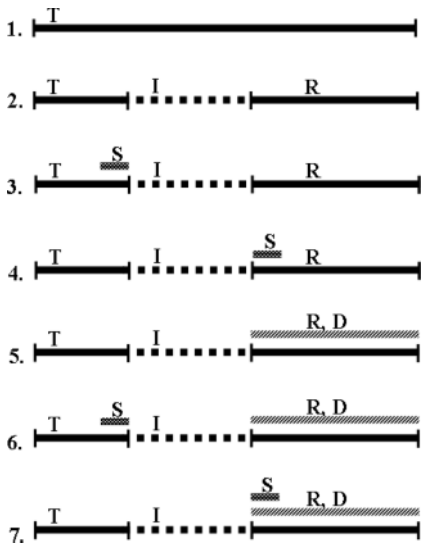

Fig. 1. Schematic representation of the protocol for isolated rabbit heart perfusion used to assess the effect of saffron (S) on ischemia (I)/reperfusion (R) and doxorubicin (D) cardiotoxicity.
Group 1: Control hearts perfused with standard Tyrode's solution (T) for 70 min without any treatment.
Groups 2, 3, and 4: After 20 min of stabilization, hearts were subjected to 30 min global ischemia followed by 40 min reperfusion, without or with saffron extracts (10 μg/ml) perfused during 5 min through the coronary circulation via the aortic cannula (in pre- or post-treatment before ischemia or at reperfusion).
Groups 5, 6, and 7: DOX (5 μg/ml) was administered during 40 min of reperfusion, without or with saffron (before ischemia or at reperfusion).

Arrhythmias

In the control group, there were practically no arrhythmias, very rare premature ventricular contrac-

Table I. Variation in the percentage of recovery of cardiodynamic parameters after 40 min of reperfusion in the different groups (controls: 100%). LVP, left ventricular pressure; LVEDP, left ventricular end diastolic pressure; HR, heart rate; CF, coronary flow; C, control; IR, ischemia reperfusion; S, saffron; D, doxorubicin; SIR, treatment with saffron before ischemia reperfusion; ISR, treatment with saffron at reperfusion; IRD, treatment with doxorubicin at reperfusion; SIRD, treatment with saffron before ischemia reperfusion and doxorubicin at reperfusion; ISRD, treatment with saffron and doxorubicin at reperfusion. $^{*}p < 0.05$ *vs.* C; $^{\#}p < 0.05$ *vs.* IRD.

Group	LVP	LVEDP	HR	CF
C	100	100	100	100
IR	$80.00 \pm 10.1^{*}$	$130 \pm 13.2^{*}$	$84.27 \pm 11.4^{*}$	$88.64 \pm 15.2^{*}$
SIR	$86.67 \pm 13.0^{*}$	$120 \pm 15.3^{*}$	$89.33 \pm 9.5^{*}$	95.45 ± 16.6
ISR	95.83 ± 15.3	110 ± 15.7	95.51 ± 10.2	100 ± 14.4
IRD	$55.83 \pm 11.8^{*}$	$200 \pm 14.6^{*}$	$65.73 \pm 12.7^{*}$	$77.27 \pm 13.9^{*}$
SIRD	$66.67 \pm 17.8^{*}$	$170 \pm 14.9^{*}$	$72.47 \pm 14.4^{*}$	$81.82 \pm 16.9^{*}$
ISRD	$78.33 \pm 14.6^{*\#}$	$150 \pm 13.9^{*\#}$	$84.27 \pm 11.5^{*\#}$	$95.45 \pm 15.4^{*\#}$

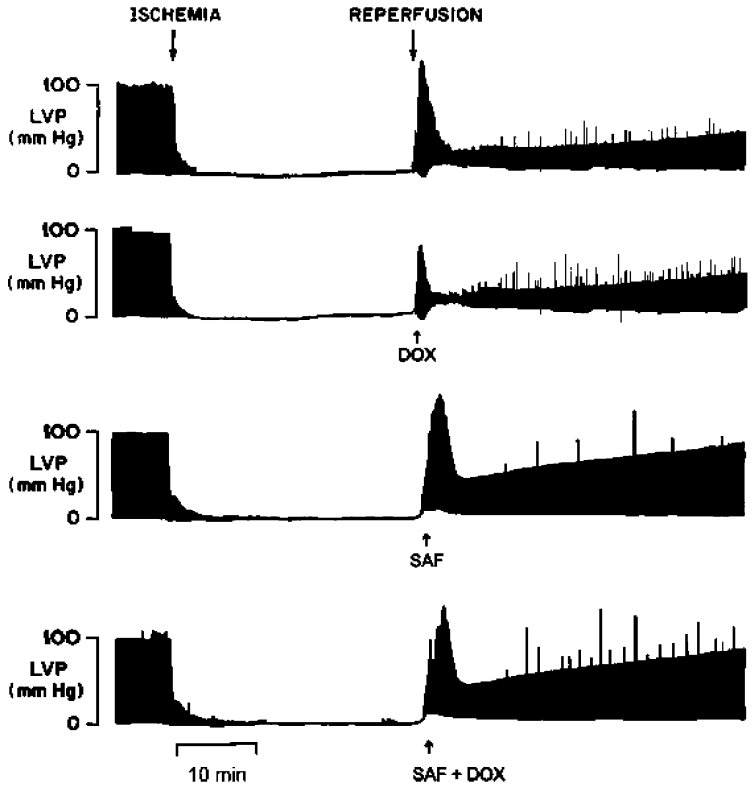

Fig. 2. Typical tracings of the left ventricular pressure (LVP) of isolated rabbit hearts subjected to a period of global ischemia followed by reperfusion in the presence and absence of doxorubicin (DOX; 5 μg/ml) and saffron extract (SAF; 10 μg/ml).

tions (PVC) were observed from time to time in some, but not all, rabbit hearts. During the first minutes of reperfusion, following 30 min of ischemia, a period of severe ventricular tachycardia (VT) appeared in all untreated hearts which was attenuated and replaced by frequent PVC. DOX, perfused during reperfusion, increased the occurrence of PVC. Hearts perfused with saffron (10 μg/ml) prior to ischemia still had the same pattern of PVC, however when administered at reperfusion, this treatment significantly reduced PVC generation. Saffron extracts exhibited potent antiarrhythmic effects against ischemia reperfusion alone and to a lesser extent against both reperfusion and DOX administration (Table II).

Biochemical parameters

As shown in Table III, hearts subjected to IR exhibited a significant increase in lipid peroxidation

Table II. Number of premature ventricular contractions (PVC) and percentages of ventricular tachycardia (VT) recorded throughout the reperfusion period in the various groups. C, control; IR, ischemia reperfusion; S, saffron; D, doxorubicin. Abbreviations of treatments as in Table I. $^{*}p < 0.05$ *vs.* IR; $^{\#}p < 0.05$ *vs.* IRD.

Electrophysiological parameter	C	IR	SIR	ISR	IRD	SIRD	ISRD
PVC (*n*)	5 ± 4	56 ± 11	40 ± 9	$28 \pm 7^{*}$	$92 \pm 16^{*}$	$80 \pm 11^{*}$	$47 \pm 8^{\#}$
VT (%)	0	100	75	25^{*}	100	75	$50^{*\#}$
Duration of VT [s]	0	52 ± 12	38 ± 9	$14 \pm 5^{*}$	47 ± 10	35 ± 8	$24 \pm 6^{*\#}$

Table III. Changes in the levels of lipid peroxidation (LP) products, superoxide dismutase (SOD) activity, and creatine kinase (CK) activity in the perfusate of the various groups. C, control; IR, ischemia reperfusion; S, saffron; D, doxorubicin. Abbreviations of treatements as in Table I. $^{*}p < 0.05$ *vs.* C; $^{\#}p < 0.05$ *vs.* IR; $^{\dagger}p < 0.05$ *vs.* IRD.

Group	LP [nmol MDA/g tissue]	SOD [U/mg tissue]	CK [IU/mL]
C	30.60 ± 4.95	12.04 ± 1.87	1.12 ± 0.14
IR	$46.93 \pm 5.97^{*}$	$8.72 \pm 1.50^{*}$	$1.68 \pm 0.17^{*}$
SIR	$42.50 \pm 4.27^{*}$	$9.88 \pm 1.76^{*}$	$1.54 \pm 0.20^{*}$
ISR	$38.25 \pm 3.90^{\#}$	$10.92 \pm 1.44^{\#}$	$1.35 \pm 0.16^{\#}$
IRD	$58.10 \pm 5.35^{*\#}$	$5.86 \pm 0.70^{*\#}$	$2.17 \pm 0.32^{*\#}$
SIRD	$50.42 \pm 4.82^{*\#}$	$6.90 \pm 1.12^{*\#}$	$1.84 \pm 0.22^{*}$
ISRD	$43.05 \pm 5.34^{*\dagger}$	$8.04 \pm 1.66^{*\dagger}$	$1.49 \pm 0.17^{*\dagger}$

(LP) as indicated by malondialdehyde (MDA) levels compared to control hearts, and they increased much more when DOX was administered during reperfusion. Saffron treatment prior to ischemia significantly decreased the MDA level when administered during the first minutes of reperfusion. Concerning the superoxide dismutase (SOD) activity, it was significantly decreased following IR, and even more after DOX perfusion. Saffron perfused during the first minutes of reperfusion significantly increased the SOD activity towards control values. Creatine kinase (CK) activity in the perfusate was increased in hearts subjected to IR and DOX and returned towards control values in hearts treated with saffron at reperfusion.

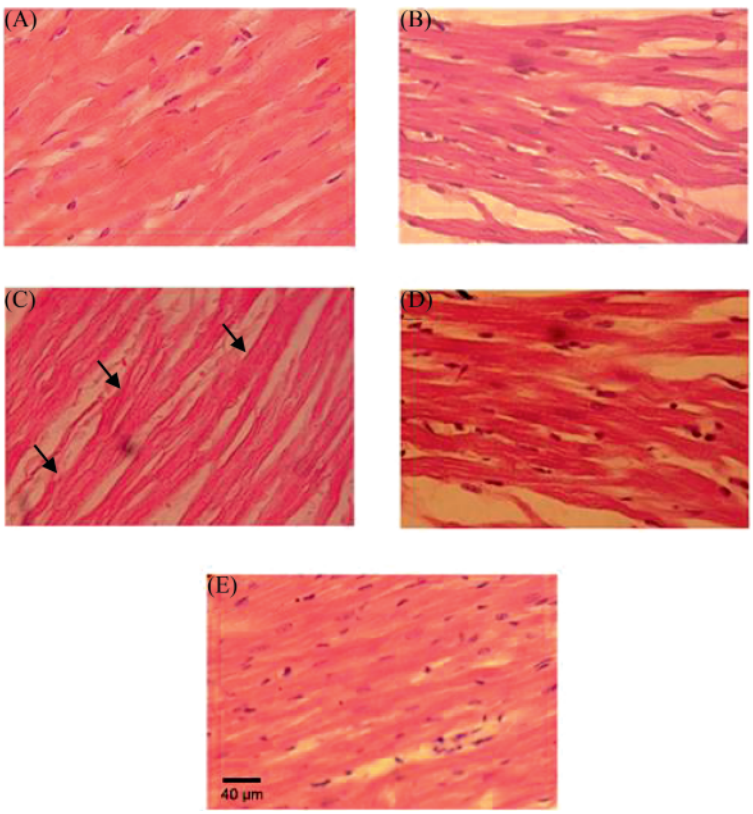

Fig. 3. Light micrographs of cardiac muscle tissue stained with hematoxylin and eosin [original magnification × 400; size bar in (E) is valid for all figures]. (A) Control heart: Muscle fibers are aligned, transverse striations are clear and well-distributed, and no edema or necroses are noted. (B) Heart submitted to 30 min global ischemia/40 min reperfusion: Muscle fibers are irregular with focal loss of striations and increased distance between fibers. (C) Heart submitted to DOX perfusion during reperfusion: Pronounced irregular disposition of fibers, increased inter-fiber distance and focal area of necrosis (arrows). (D) Heart perfused with saffron extract prior to ischemia and reperfused with DOX: Persisting damage. (E) Heart perfused with saffron extract during the first minutes of reperfusion: Some widening of the muscle fibers with no loss in striation and no necrosis.

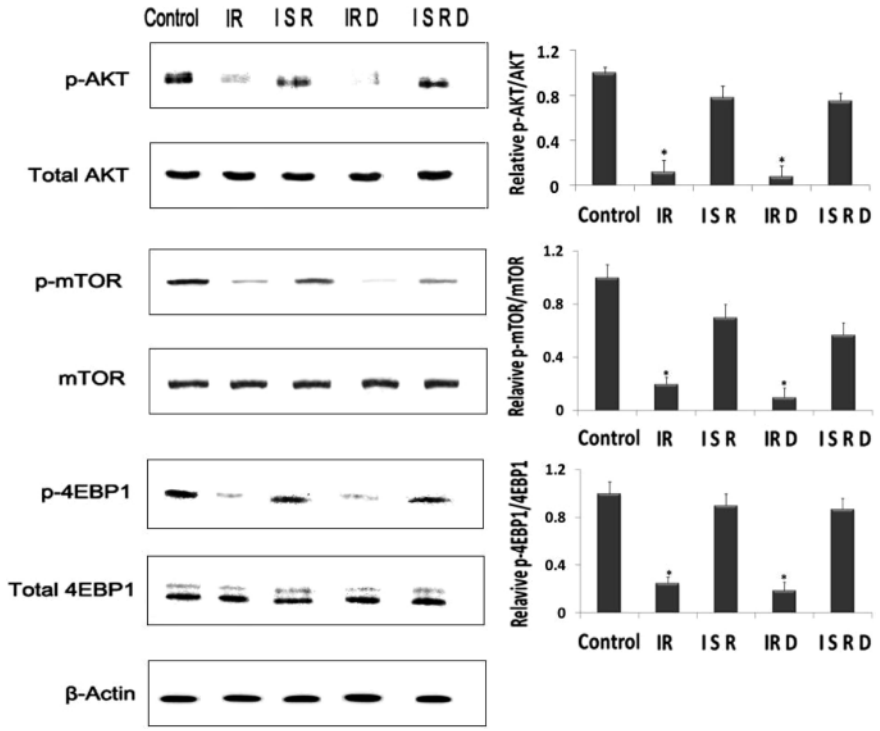

Fig. 4. Representative Western blot analysis and quantification of the levels of phosphorylated AKT (Ser473), mTOR (Ser2448), and 4EBP1 (Ser 65/Thr 70) in isolated rabbit hearts:

- untreated cells (Control);
- cells treated with: doxorubicin (D) (5 μg/ml);
- cells treated with: saffron extract (S) (10 μg/ml) and D (5 μg/ml);
- cells subject to: ischemia/reperfusion (IR) (30 min/40 min) and D (5 μg/ml);
- cells subject to: IR (30 min/40 min), S (10 μg/ml), and D (5 μg/ml).

Anti-β-actin was used as control for the amount of proteins loaded per lane. p-AKT, p-mTOR, and p-4EBP1 were normalized to the total expression of AKT, mTOR, and 4EBP1. Results represent means $\pm$ SD of at least 3 independent experiments. $^{*}p < 0.05$ *vs.* control.

Histopathology

The histology of control hearts was normal: Cardiac muscle fibers were aligned, transverse striation was clear and well distributed, no edema or necrosis were noted (Fig. 3A). Cardiac muscle fibers from hearts submitted to 30 min global ischemia/40 min reperfusion (Fig. 3B) were irregular with focal loss of striation and increased distance between fibers. DOX administered during reperfusion (Fig. 3C) caused a pronounced irregular disposition of fibers, an increase in inter-fiber distance, and focal areas of necrosis. Saffron perfused before ischemia and administered parallel to reperfusion with DOX caused less damage (Fig. 3D), while saffron perfusion during the first minutes of reperfusion exhibited a gentle widening of the muscle fibers with no loss in striations and no myonecrosis (Fig. 3E).

Western blot analysis

Western blot analysis revealed that in IR- and IR D-treated rabbit hearts, the levels of p-AKT, p-mTOR, p-4EBP1, and cTnT were all decreased, while that of p-P38 MAPK was increased in comparison to the control group (Figs. 4 and 5A, B). On the other hand, saffron treatment during reperfusion could significantly reverse these deleterious effects by maintaining the phosphorylation status of these mTOR pathway components.

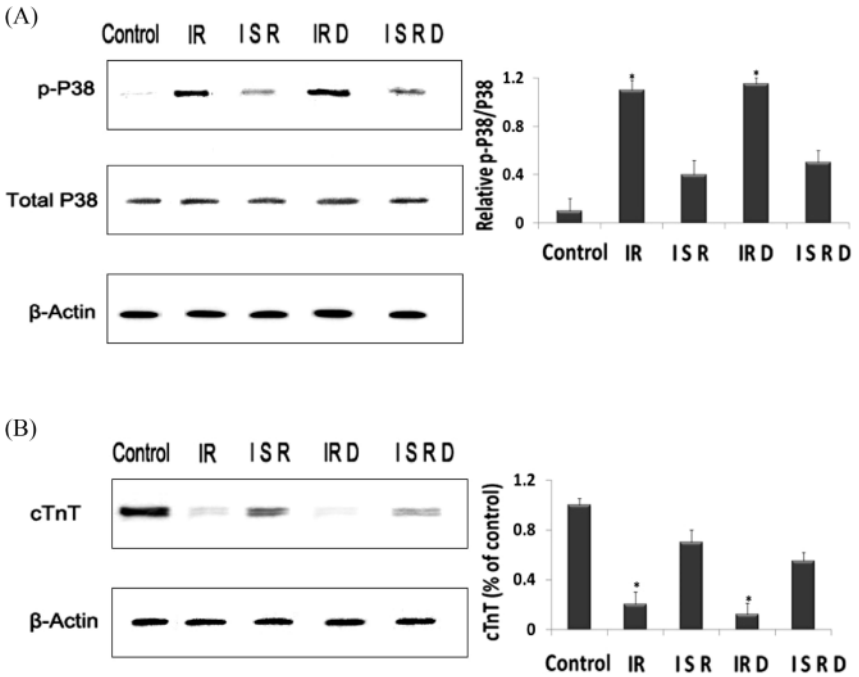

Fig. 5. Representative Western blot analysis and quantification of the levels of (A) phosphorylated P38 (Thr180/Tyr182) and (B) troponin T (cTnT) in isolated rabbit hearts:

- untreated cells (Control);
- cells treated with: doxorubicin (D) (5 μg/ml);
- cells treated with: saffron extract (S) (10 μg/ml) and D (5 μg/ml);
- cells subject to: ischemia/reperfusion (IR) (30 min/40 min) and D (5 μg/ml);
- cells subject to: IR (30 min/40 min), S 10 (μg/ml) and D (5 μg/ml).

Anti-β-actin was used as control for the amount of proteins loaded per lane. p-P38 was normalized with total P38. Results represent means $\pm$ SD of at least 3 independent experiments. $^{*}p < 0.05$ *vs.* control.

Discussion

Due to the importance of DOX in cancer therapy, great efforts have been made to prevent or attenuate the side effects of the administration of this drug. Several approaches have been pursued, such as dosage optimization, synthesis and use of analogues, or combined therapy. However, an optimal therapeutic approach for the protection against DOX-induced cardiotoxicity has not been identified, and the application of various analogues did not show better antineoplastic values or lower toxicity than those of DOX (Volkova and Russell, 2011; Tacar *et al.*, 2013).

The exact pathogenesis of DOX-induced cardiotoxicity is still not clear, although diverse mechanisms have been proposed, including oxidative stress, mitochondrial DNA damage, intra-cellular calcium overload, inhibition of protein synthesis, disturbance of myocardial adrenergic function, cytokine release, myofibrillar degeneration, and cardiomyocyte apoptosis (Singal *et al.*, 2000; Octavia *et al.*, 2012). Among the multiple mechanisms, it is widely accepted that DOX-induced cardiomyocyte apoptosis occurs primarily due to the generation of ROS in the myocardium, triggering intrinsic mitochondria-dependent apoptotic pathways in cardiomyocytes. This mechanism is different from its antineoplastic activity, which occurs primarily through inhibition of topoisomerase II (Stěrba *et al.*, 2013).

Numerous earlier studies indicated that free radical scavengers and antioxidants may combat DOX-induced cardiotoxicity, including probucol, amifostine, dexrazoxane, vitamins (E, C, A, carotenoids), flavonoids, and polyphenols (Hideg and Kálai, 2007; Mokni *et al.*, 2012). However, despite various therapeutic interventions adapted to protect the heart against DOX-induced toxicity, most of the antioxidant ther-

apies remained particularly ineffective and have pronounced clinical disadvantages due to their inability to specifically target cardiac mitochondria (Mazevet *et al*., 2013).

We therefore performed this study with saffron as a cardioprotective natural agent against DOX-induced cardiotoxicity, based on encouraging results we obtained in our laboratory on the protective effects of saffron against free radical generation by electrolysis and DOX in the normal myocardium (Makhlouf *et al*., 2011; Chahine *et al*., 2013). Our hypothesis was encouraged by two reviews reporting the beneficial effects of saffron against multiple pathologies and some cancerous tumours (Abdullaev and Espinosa-Aguirre, 2004; Bathaie and Mousavi, 2010).

While numerous studies exist on DOX-induced cardiotoxicity, only few have been devoted to the understanding of the complications of this cardiotoxicity under ischemic conditions (Schjøtt *et al*., 1996; Bozcali *et al*., 2012), which is frequently of clinical concern during advanced cancer stages. We have not only used saffron against acute DOX toxicity in ischemia-reperfusion injury, but also tried to differentiate between a preventive mechanism and acute trapping of free radicals during reperfusion by administering saffron either before ischemia or during reperfusion.

In the present study, subjecting the isolated heart preparation to 30 min global ischemia followed by 40 min reperfusion was used as a model of acute myocardial ischemia sufficient to cause significant damage yet not cardiac arrest (Kilgore *et al*., 1994; Palmer *et al*., 2004). In this model, the major determinants of left myocard performance, as well as cardiac frequency, coronary flow, and electrocardiogram (ECG) were properly controlled. DOX perfused during reperfusion worsened the hemodynamic parameters and delayed heart recovery. Saffron extract administered before ischemia was not able to improve the recovery, while during reperfusion it significantly improved the recovery of contractility. In this regard, our results are in accordance with those obtained by Hosseinzadeh *et al*. (2009) who used saffron extract and its active constituents (crocin and safranal), thereby affording significant protection against ischemia-reperfusion injury in rat skeletal muscles as well as on cardiotoxicity induced by isoproterenol and diazozin (Mehdizadeh *et al.*, 2013; Razavi *et al*., 2013). In addition, antiarrhythmic effects afforded by saffron have been reported by Joukar *et al.* (2013). Although the isolated perfused heart has certain limitations, this preparation is particularly useful for interventions that may act directly and/or indirectly on the heart.

At the biochemical level, ischemia reperfusion and DOX-induced lipid peroxidation decreased the levels of SOD in the heart tissues and increased that of CK in the perfusate. In fact, reduced supply of oxygen and/or glucose may damage the myocardial cells, and their cell membrane becomes permeable or ruptures – as corroborated by the histopathological studies we obtained – resulting in leakage of enzymes. This suggests that DOX-induced oxidative stress occurs via the generation of ROS in the heart tissue (Hardina *et al*., 2000; Takacs *et al.*, 1992). Heart tissue is especially susceptible to free radical injury because of low levels of free radical-detoxifying enzymes, such as SOD and catalase, as well as of reduced glutathione. Furthermore, DOX also has a high affinity for the phospholipid component of the mitochondrial membrane in cardiomyocytes, leading to accumulation of DOX in the heart tissue. Cellular damage is thus caused by depletion of detoxifying enzymes, which is closely related to lipid peroxidation and disturbance of the Ca^{2+} influx induced by toxic agents (Montaigne *et al*., 2010; Gharanei *et al*., 2013).

When saffron was administered prior to ischemia, a non-significant amelioration of enzyme levels was noted. However, administration of saffron during the first minutes of reperfusion significantly reduced oxidative myocardial damages. It significantly reduced tissue LP, increased the levels of SOD and decreased CK leakage, indicating the protective effects of saffron on DOX-induced cardiotoxicity by scavenging free radicals. Treatment with saffron extract before the ischemic period could have a protective effect as a membrane-stabilizing agent, however during reperfusion, saffron administration maintained the concentration of enzymes at near normal levels, which prevented cell disruption by decreasing free radicals production and Ca^{2+} influx (Sachdeva *et al*., 2012; Razavi *et al*., 2013; González-Salazar *et al.*, 2011).

To investigate at the molecular level how saffron inhibited the IR- and DOX-induced loss of cardiac function, we performed Western blot studies of proteins involved in signaling pathways. AKT/mTOR/4EBP1 and p38 MAPK are components of two essential signaling pathways involved in cardiomyocyte viability, and the phosphoinosite 3-kinase PI3K/AKT pathway promotes survival and growth of cardiomyocytes (Baliga *et al*., 1999). We determined the activation of the mTOR pathway, a major signaling axis downstream of PI3K/AKT which is important

for protein translation (Fingar and Blenis, 2004). IR and DOX decreased the phosphorylation of multiple components of the mTOR pathway, including mTOR (Ser2448) and 4EBP1 (Ser 65/Thr 70). Treatment with saffron extract during reperfusion maintained the phosphorylation of these mTOR pathway components. These results indicate that saffron maintained the activation of the mTOR pathway via PI3K/AKT in IR- and DOX-treated rabbit hearts, which may account for the maintenance of the synthesis of cTnT in the cardiomyocytes.

Troponin is a thin filament-associated complex that regulates the formation of actin-myosin cross-bridges during the contraction-relaxation process in the heart (Zot and Potter, 1987). The release of cardiac troponins is a sensitive and specific indicator of myocardial injury (Wallace *et al.*, 2004). Loss of myofibrillar proteins, *i.e.* troponin I and troponin T, is considered one of the major causes of the DOX-induced decrease in the cardiac contractile function (Ito *et al*., 1990). Loss of cardiac troponin T following IR and DOX administration was demonstrated by Western blot analysis. Saffron administration during reperfusion counteracted this decrease, and increased activation of cardiac phosphorylated AKT and downstream mTOR/4EBP1 caused by saffron administration was associated with the preservation of the troponin T level.

An important cell cycle regulator used by cardiomyocytes to control proliferation is p38 MAPK. It has been shown that this signaling protein induces cell cycle exit. Activated p38 phosphorylates downstream signaling molecules important for cardiomyocyte hypertrophy (Liang and Molkentin, 2003). Saffron reversed the increase in the level of p-P38 caused by IR and DOX, and this resulted in protection against cardiomyocyte apoptosis and contractility depression.

Taken together, these findings demonstrate that saffron extract improved the cardiomyocyte survival and function, preserved cardiac troponin T proteins, inhibited the p38 MAPK pathway, and activated the AKT/mTOR/4EBP1 pathway in IR- and DOX-treated rabbit hearts.

It is debatable whether saffron could be administered at the same time as an anticancer agent to prevent chemotoxicity. This is a function of the dose: At the concentration we used, saffron is a cardioprotective agent, while at high concentrations it possesses anticancer activity (Goel and Aggarwal, 2010; Ziberna *et al*., 2010). In the context of developing therapies providing cardioprotection without antitumour effects, saffron may serve as an adjuvant to DOX therapy by reducing the myocardial toxicity.

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