Ginkgo biloba extract 761 reduces doxorubicin-induced apoptotic damage in rat hearts and neonatal cardiomyocytes

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Aims The objective of this study was to investigate whether a cytoprotective herb-derived agent, Ginkgo biloba extract (EGb) 761, could have a beneficial effect on doxorubicin-induced cardiac toxicity in vitro and in vivo.

Methods and results Primary cultured neonatal rat cardiomyocytes were treated with the vehicle, doxorubicin (1 μM), EGb761 (25 μg/mL), or EGb761 plus doxorubicin. After 24 h, doxorubicin upregulated p53 mRNA expression, disturbed Bcl-2 family protein balance, disrupted mitochondrial membrane potential, precipitated mitochondrion-dependent apoptotic signalling, induced apoptotic cell death, and reduced viability of cardiomyocytes, whereas EGb761 pretreatment suppressed all the actions of doxorubicin. Similarly, rats treated with doxorubicin [3 mg/kg intraperitoneally (i.p.) three doses every other day] displayed retarded growth of body and heart as well as elevated apoptotic indexes in heart tissue at both 7 and 28 days after exposure, whereas EGb761 pretreatment (5 mg/kg i.p. 1 day before each dose of doxorubicin) effectively neutralized the aforementioned gross and cellular adverse effects of doxorubicin.

Conclusion Doxorubicin impairs viability of cardiomyocytes at least partially by activating the p53-mediated, mitochondrion-dependent apoptotic signalling. EGb761 can effectively and extensively counteract this action of doxorubicin, and may potentially protect the heart from the severe toxicity of doxorubicin.

1. Introduction

Doxorubicin has long been one of the most effective chemotherapeutic agents for the treatment of various types of cancer. Clinical use of this drug is, however, greatly limited by its serious adverse cardiac effects that may ultimately lead to cardiomyopathy and heart failure. Since cellular apoptosis is at least partially responsible for the pathogenesis of doxorubicin cardiac toxicity, in vitro and in vivo studies have been conducted employing anti-apoptotic remedies to manage this devastating complication. Nevertheless, to date, no single chemical has proven to be able to reduce the deleterious action of doxorubicin. Therefore, the search for an effective and safe antagonist of doxorubicin cardiac toxicity remains a critical issue in both cardiology and oncology. Ginkgo biloba is one of the oldest plants in the world. This so-called ‘living fossil’, which has intrinsic anti-biotic and anti-fungal properties, has been existing on earth for 250 million years. Utilization of the leaves of this plant as a herbal medicine for treating a variety of diseases can be traced back to thousand of years in ancient China. Recently, a standardized chemical prepared from Ginkgo biloba leaves, or Ginkgo biloba extract (EGb) 761, has been used widely for the treatment of several nervous system diseases because of its proven anti-platelet and anti-angiogenic effects. Additional studies further demonstrate that EGb761 can attenuate ischaemic-reperfusion injury in rat hearts, modulate mitochondrial function by scavenging peroxyl radicals, and ameliorate cell apoptosis in gossypol-treated...
human lymphocytes, implying that EGB761 might be a promising cytoprotective agent against various extrinsic toxic stimuli. To determine whether EGB761 could also protect against doxorubicin-induced apoptotic actions in heart, this study was designed to examine the impact of EGB761 on doxorubicin-induced apoptotic abnormalities in rat neonatal cardiomyocytes and heart tissue. The results of the present study could clarify the role of this herbal drug in the prevention of doxorubicin cardiotoxicity, and may shed light on a possible solution to this very serious cardiac complication of doxorubicin.

2. Methods

2.1 Materials

Doxorubicin was purchased from Pfizer Italia S.R.L. (Milan, Italy). Standard EGB761 preparation containing two major functional constituents (24% flavonoid glycosides and 6% terpenoids) was from Dr. Willmar Schwabe Pharmaceuticals (Karlruhe, Germany). Anti-cytochrome c antibody was purchased from Lab Vision Corp. (Fremont, CA, USA). JC-1 was obtained from Molecular Probe (Eugene, OR, USA). All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and other chemicals from Sigma (St. Louis, MO, USA).

2.2 Animals and in vivo pharmacological treatment

Male Sprague–Dawley rats weighing 270–330 g (8 weeks of age) were randomly assigned to four groups. Control group rats (Cont, n = 15) were given intraperitoneal (i.p.) injections of normal saline (the solvent for doxorubicin and EGB761). EGB group animals (Egb, n = 15) received EGB761 at a dose of 5 mg/kg i.p. every 2 days for a total of three injections (cumulative dose 15 mg/kg). Doxorubicin group rats (Dox, n = 15) were treated with doxorubicin i.p. at a dose of 3 mg/kg every 2 days for a total of three injections. This cumulative dose (9 mg/kg) was equivalent to 630 mg for a 70 kg man, just above the threshold at which doxorubicin cardiomyopathy is expected to occur clinically. EGB-doxorubicin group animals (EgbDox, n = 15) received EGB761 followed by doxorubicin (each injection was given 1 day after EGB761). Ten of the 15 animals from each group were euthanized on day 7 and the remaining 5 were euthanized 28 days after the first administration of medication (normal saline, doxorubicin, or EGB761) for morphological and cellular studies. The body and heart weight was measured and compared with their baseline heart weight was measured and compared with their baseline.

2.6 Flow cytometric analysis of cardiomyocyte apoptosis

Cardiomyocytes were washed with PBS, centrifuged at 800 g for 6 min, resuspended in ice-cold 70% ethanol/PBS, centrifuged at 800 g for a further 6 min, and resuspended in PBS. Cells were then incubated with Ribonuclease A (0.1 mg/mL) and propidium iodide (PI) solution (40 µg/mL) for 30 min at 37 °C. The stained cells were filtered through a 40 µm nylon mesh and then differentiated using flow cytometry (FACSscan, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The fluorescent emission of the PI-stained cells excited by 488 nm argon ion laser was measured at a wavelength of 610 nm in a FACS Vantage flow cytometer. The associated cell sorter was interfaced to a Macintosh operating system equipped with CellQuest Software (Becton & Dickinson, San Jose, CA, USA). The proportion of apoptotic cells in each sample was estimated from the sub-G1 peak in the DNA histogram. At least 10,000 events were used in calculations for each sample.
fields in each sample were calculated and represented the surrogate marker of loss of mitochondrial ∆Ψm.

2.8 Immunoblocking
Heart tissue or cardiomyocytes were lysed on ice with T-PER Tissue or Cell Protein Extraction Reagent (Pierce Chemical Co., Rockford, IL, USA) containing a 0.1 mM diethiothreitol and protease inhibitor cocktail. The lysates were then centrifuged at 10 000 g for 10 min to obtain solubilized cellular proteins. Supernatant cell concentrations were determined by Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). An equal amount of protein (50 μg) from each sample was resolved by SDS-PAGE, transferred to PVDF membranes, reacted with primary antibodies (Bcl-2, Bcl-xL, Bax, Bad, cytochrome c, prohibitin, and caspase-3; 1:1000 dilutions) at 4°C, washed three times with PBS buffer containing 0.1% Tween 20, incubated with HRP-conjugated secondary antibodies (1:5000 dilutions), and detected with ECL Plus (Pierce, IL, USA). The density of each protein band was determined using ScienceLab 2001 Image Guage 4.0 Software (Fujifilm, Tokyo, Japan) and compared with densitometry.

2.9 Abundance of mitochondrial and cytosolic cytochrome c in cardiomyocytes
Cytosolic and mitochondrial protein fractions of cardiomyocytes were extracted as previously described.16 The purity of mitochondrial and the cytosolic fractions was verified by immunoblotting for a reliable mitochondrial marker, prohibitin. Next, 50 μg of cytosolic and mitochondrial extracts from each sample was resolved by 15% SDS-PAGE and analysed by immunoblotting for cytochrome c. The ratio of cytosolic to total (cytosolic plus mitochondrial) abundance of cytochrome c was determined using densitometry. Average values of this ratio from ≥10 measurements represented the degree of cytochrome c release from mitochondria to cytosol.16

2.10 Determination of caspase-3 activity in heart tissue and cardiomyocytes
Activity of caspase-3 in heart tissue and cardiomyocytes was measured using the CaspACE™ Assay System according to the manufacturer’s instructions (Promega, Madison, WI, USA). Heart tissue or pelleted cardiomyocytes were centrifuged at 450 g for 10 min, washed with ice-cold PBS and resuspended in Cell Lysis Buffer. After being lysed by repeat freeze-thaw, tissue or cells were incubated on ice for 15 min and centrifuged at 15 000 g for 20 min. The protein concentrations of the supernatants were determined. Equal amounts of protein (50 μg) were added to the Caspase Assay Buffer containing 20 mM of caspase-3 substrate Ac-DEVD-pNA and the mixture was incubated at 37°C for 4 h. The yellow colour generated from cleavage of the substrate by caspase-3 was monitored with a SPECTRmaxx340 spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA, USA) at an excitation wavelength of 405 nm. Reaction mixture without cardiac extracts was used as a control.

2.11 Real time (RT)-PCR analyses of p53, Fas, and Fasl in cultured cardiomyocytes
Total RNA was extracted from cultured cardiomyocytes using the TRizol reagent. RNA samples were reverse-transcribed into cDNA using a random hexamer and a Superscript III first strand cDNA-synthesis kit (Invitrogen, Carlsbad, CA, USA). RT-PCR analysis was then performed by StepOne™ RT-PCR system (Applied Biosystems, Foster City, CA, USA) with each amplified primer set (actin-F, TCC TGTGGCCCTCAGGAAAAC; actin-R, GGAGGAATGCTCTGAACTTT; p53-F, CAGCCAAGTCTGTATAGCTG; p53-R, ACCATCAGAAGCAAGCCCTAT; FasL-F, CTCAGACTCTGGCCAG; FasL-R, GCCTCTGAGTCGGCA; Fas-F, CGAAGTCGAGGACTGA; Fas-R, AAGTCCACAGGAGTGGCA) under formulated conditions (Applied Biosystems, Warrington, UK). Amplification data were analysed and displayed by StepOne™ software. mRNA expression of p53, FasL, and Fas was then quantified in comparison with actin as their relative expression levels.

2.12 Statistical analysis
All experiments were repeated ≥3 times, and one representative from ≥3 similar results is provided. Continuous variable data are presented as mean ± SEM. The statistical significance was tested by ANOVA for primary assessment of inter-group differences and Student’s t test for special between-group comparisons (Dox vs. Cont and EGbDox vs. Dox) whenever needed. A P-value < 0.05 was considered statistically significant.

3. Results
3.1 EGb761 suppressed the deleterious effects of doxorubicin on the growth of body and heart
Among the four groups of animals, only doxorubicin-treated rats developed early physical lethargy and later progressive ascites. These animals also had gained significantly less body weight than controls at 7 and 28 days (Figure 1A) unless pretreated with EGb761, indicating the growth-impeding effect of doxorubicin and the counteracting action of EGb761 against doxorubicin. Though the absolute heart weight at euthanasia was significantly lower in doxorubicin-treated rats yet was considerably preserved by EGb761 pretreatment (EGbDox, Figure 1B), the relative heart weight index (heart weight to body weight ratio) was similar among all four groups at both 7 and 28 days (Figure 1C), indicating the parallel impact of doxorubicin and EGb761 on growth of both the heart and the whole body.

3.2 EGb761 ameliorated doxorubicin-induced cytotoxicity and cardiomyocyte apoptosis
To determine how doxorubicin cardiotoxicity is caused and whether EGb761 prevents from it, viability of cardiomyocytes was assessed with an MTT assay. As shown in Figure 2, doxorubicin significantly reduced cardiomyocyte viability to 68% of the control level, whereas pretreatment with 25 μg/mL of EGb761 maintained viability at 93%, indicating the cytotoxic effect of doxorubicin and the cytoprotective effect of EGb761. A higher concentration of EGb761 (up to 50 μg/mL) provided no additional benefit to cell viability, so the dose of 25 μg/mL was selected for subsequent in vitro experiments.

Apoptosis is one of the most detrimental side effects of doxorubicin.3 To evaluate whether the impact of doxorubicin and EGb761 on cardiomyocyte viability involves the process of cell apoptosis, heart tissue and cardiomyocytes of all four groups were assessed by TUNEL and/or flow cytometric assays. In TUNEL results (Figure 3A and B), doxorubicin treatment significantly increased the percentage of positively stained (i.e. apoptotic) cells from 1.25 to 23.8% in heart tissue at papillary muscle level and from 0.62 to 41.2% in cultured cardiomyocytes, whereas EGb761 pretreatment reduced the proportion of apoptotic cells to 5.6% in heart tissue and 14.4% in cardiomyocytes (Figure 3C). In flow cytometry results (Figure 3D and E), the percentage of the subG1 peak in DNA distribution histograms, or the apoptotic cell subpopulation,17 was significantly increased to 12.5% in doxorubicin-treated cells as compared with the controls (2.8%) (Dox vs. Cont, P = 0.006), but was reduced to 4.5%
This finding indicates the pro-apoptotic effect of doxorubicin and the alleviative action of EGb761 pretreatment on doxorubicin-mediated apoptosis.

3.3 EGb761 suppressed doxorubicin-induced disruption of mitochondrial membrane potential $\Delta \psi_m$

Maintenance of intact mitochondrial membrane potential ($\Delta \psi_m$) is fundamental to cell survival, and loss of $\Delta \psi_m$ triggers a cascade of reactions leading to cell apoptosis.15 To determine whether doxorubicin induced apoptosis through disrupting $\Delta \psi_m$, while EGb761 sustained it, cardiomyocytes were stained with the mitochondrial potential indicator JC-1, which accumulates to form J-aggregates and emits red fluorescence in mitochondria with higher $\Delta \psi_m$, but dissociates into monomers and emits green fluorescence in those losing $\Delta \psi_m$. The results showed that control cardiomyocytes exhibited a normal pattern of mitochondrial staining with high red and low green fluorescence intensity (Figure 4A, Cont), while doxorubicin-treated cells emitted overwhelming green over red fluorescence symbolizing depolarized $\Delta \psi_m$ (Figure 4A, Dox), unless pretreated with EGb761 (Figure 4A, EGbDox). Quantitative analysis displayed that doxorubicin significantly elevated the JC-1 green/red fluorescence ratio yet EGb761 pretreatment depressed this apoptotic index (Figure 4B), confirming the disruptive effect of doxorubicin and the preservative effect of EGb761 on mitochondrial $\Delta \psi_m$.

3.4 EGb761 mitigated the pro-apoptotic effects of doxorubicin on Bcl-2 family proteins, mitochondrial cytochrome c release, and caspase-3 activation

Bcl-2 family proteins are upstream regulators of mitochondrial $\Delta \psi_m$.16 Since doxorubicin depolarized $\Delta \psi_m$, while EGb761 maintained it, whether these two chemicals also influenced the equilibrium of Bcl-2 family proteins was investigated. Immunoblotting studies demonstrated that doxorubicin downregulated the anti-apoptotic (Bcl-2, Bcl-xL) and upregulated the pro-apoptotic (Bax, Bad) Bcl-2 proteins in cardiomyocytes (Figure 5A) and in heart tissue at both day 7 (Figure 5B) and day 28 (Figure 5C), whereas EGb761 pretreatment effectively repressed these doxorubicin-evoked pro-apoptotic events. Quantitative analysis ascertained...
that doxorubicin significantly decreased the Bcl-2 to Bax ratio, but EGb761 pretreatment preserved this anti-apoptotic index (Figure 5, bar graphs).

Cytochrome c is located in the mitochondrial intermembrane space in the resting state. Stimuli that disrupt mitochondrial potential \( \Delta \psi_m \) induce cytochrome c release from mitochondria to the cytosol and trigger downstream cell signalling events that lead to apoptosis. To investigate whether doxorubicin modulated this apoptotic factor while EGB761 repressed it, distribution of cytochrome c in mitochondrial and cytosolic compartments of cardiomyocytes was assessed. The immunoblotting study showed that cytosolic cytochrome c abundance increased (Figure 6, left) from 10 to 88% of total cellular content in doxorubicin-treated cardiomyocytes (Figure 6, right), but remained limited to 53% when pretreated with EGb761. These findings indicate that EGB761 pretreatment can counteract doxorubicin-triggered cytochrome c release in cardiomyocytes.

Caspase-3 is a key factor in the execution of mitochondrial apoptosis. To examine whether doxorubicin and EGB761...
ultimately influence this factor to modulate apoptosis, activity of caspase-3 in both heart tissue and cardiomyocytes was measured. The results showed that doxorubicin significantly upregulated caspase-3 activity by 1.47-fold (at 7 days) to 1.85-fold (at 28 days) in heart tissue (Figure 7B), whereas EGb761 pretreatment depressed this activity of doxorubicin and inhibitory action of EGb761 on caspase-3 activity. The activation of caspase-3 in cardiomyocytes was confirmed by immunoblotting analysis illustrating that doxorubicin increased the amount of cleaved caspase-3, a product and indicator of caspase-3 activation, whereas EGb761 pretreatment inhibited its generation (Figure 7C).

3.5 EGb761 repressed doxorubicin-activated p53 mRNA expression in cardiomyocytes

p53 and Fas are initiators of the intrinsic and the extrinsic apoptotic pathways, respectively.19 The cellular factor p53 senses DNA-damaging stresses and activates downstream mitochondria-dependent apoptotic signalling,20 while the cell membrane receptor Fas binds to its ligand FasL and triggers mitochondrion-independent apoptosis when receiving death signals.2 To clarify whether doxorubicin and EGb761 impact these upstream apoptotic mediators, mRNA expression of p53, Fas, and FasL in cardiomyocytes was assessed. Quantitative RT-PCR analysis demonstrated that p53 expression was significantly increased by doxorubicin but was neutralized by EGb761 pretreatment (Figure 8A), implying the p53 apoptotic signalling to be a possible target object of doxorubicin and EGb761. In contrast, expression of Fas and FasL was not influenced by either doxorubicin or EGb761, indicating that the Fas-mediated extrinsic pathway is not involved in the apoptotic actions of doxorubicin or the protective effects of EGb761 (Figure 8B).

4. Discussion

The mechanism of doxorubicin cardiomyopathy has been studied rigorously since this complication was first discovered, but the solution to this potentially fatal complication has not been determined. The present study, exploring the possible counteracting effect of a pleiotrophic herbal drug EGb761 against doxorubicin cardiotoxicity, demonstrates that doxorubicin activates a cascade of signalling messengers to induce cardiomyocyte apoptosis, whereas EGb761 pretreatment acts to stabilize these apoptotic effects and effectively protects cells. These results provide adequate evidence that EGb761 possesses additional beneficial properties against doxorubicin toxicity, and raise this herbal agent a potential therapeutic adjuvant that may prevent the heart from the serious side effect of doxorubicin.

4.1 Doxorubicin cardiotoxicity involves p53-mediated cardiomyocyte apoptosis

The primary cytotoxic action of doxorubicin is accomplished through intercalating the agent itself into the DNA backbone of neoplastic cells and inhibiting DNA topoisomerases.16 The adverse effects of this chemotherapeutic agent on non-neoplastic tissue are, in contrast, mediated through distinct pathogenic processes.21 In the heart, many previous investigations have designated cardiomyocyte apoptosis as the most direct cause of doxorubicin cardiotoxicity,4,15 in which pro-apoptotic Bcl-2 members, mitochondrial effectors, and caspase proteins are activated to provoke apoptotic cell death.14,18 This study demonstrates that, in addition to widespread activation of a series of key apoptotic factors in the mitochondrion-dependent apoptotic pathway, doxorubicin also upregulates mRNA expression of p53 but not the death receptor Fas or its ligand FasL, suggesting p53-mediated, or intrinsic, rather than Fas-mediated, or extrinsic signalling to be the dominant mechanism involved in doxorubicin-induced cardiomyocyte apoptosis. Since stresses that activate p53 include DNA damaging signals and free radicals,22 the typical cytotoxic and the adverse free-radical-generating actions of doxorubicin23 could be the relevant triggers for actuating this apoptosis-inducing factor in cardiomyocytes. This concept may also explain why the severity of doxorubicin cardiotoxicity is in a dose-dependent manner.2 The demonstration of this apoptotic process highlights the need for meticulous titration of this chemotherapeutic agent to avoid untoward effects in non-neoplastic tissue, and justifies manipulation
of p53-mediated signalling as a potential strategy for management of doxorubicin cardiotoxicity. Importantly, as apoptosis of myocardial cells is evoked shortly after a rat is exposed to doxorubicin, treatment should be preventive or as early as possible before a considerable portion of myocardial cells succumb to irreversible apoptotic death.

4.2 Ginkgo biloba extract 761 suppresses apoptotic effects of doxorubicin in heart

The therapeutic value of EGb761 ever demonstrated in previous research falls mainly in the category of neurological disorders but rarely on heart diseases. The findings in the
present study that EGb761 alleviates doxorubicin-induced cardiomyocyte apoptosis through stabilizing a cascade of mitochondrial-signalling effectors from p53, Bcl-2 proteins, mitochondrial \( \Delta \Psi_m \), cytochrome \( c \) to caspase-3 implicate the additional counteracting action of EGb761 against doxorubicin apoptotic cardiotoxicity at multiple cellular levels. This cytoprotective effect was similarly higher by 4.1-fold in doxorubicin-treated cardiomyocytes (Dox) compared with the controls (Cont) \((P = 0.0005)\), whereas remained at 2.3-fold of the control level in those pretreated with \( Ginkgo \) biloba extract 761 (EGBDox) \((EGBDox \text{ vs. } Dox, P = 0.036)\). (C) Immunoblotting study. The cellular amount of cleaved caspase-3, a product and indicator of caspase-3 activation, was significantly increased in doxorubicin-treated cardiomyocytes (Dox) yet was maintained less so abundant in cells pretreated with \( Ginkgo \) biloba extract 761 (EGBDox). Data are from 15 animals in each in vivo study group \((n = 10 \text{ for 7 day and } n = 5 \text{ for 28 day experiments})\) and three independent experiments performed in triplicate in the in vitro study.

In conclusion, this in vivo and in vitro study demonstrates that doxorubicin impairs survival of cardiac muscle cells at least partially through triggering p53-mediated, mitochondrial-dependent cell apoptosis, whereas EGb761 ameliorates nearly all of these apoptotic actions of doxorubicin and preserves cell viability. As doxorubicin plays an important role guardian that promotes DNA repair in cells encountering genotoxic stress and induces apoptosis of those with serious DNA damage, inhibition of this intrinsic protective mediator may carry a risk of uncontrolled overgrowth of genetically defective cells and ensuing tumorigenesis. Fortunately, the findings in this study that EGb761 only suppresses doxorubicin-stressed but not steady-state p53 expression, as similar to another p53 inhibitor pifithrin-\( \alpha \) reported to repress p53 exclusively in UV-irradiated but not in un-irradiated cells,\(^{26}\) may well preclude the possibility of EGb761 becoming a carcinogenic agent in non-stressed cells. Finally, whether the antagonistic effects of EGb761 could possibly interfere with the antitumour activity of doxorubicin remains an issue of concern. Nevertheless, as EGb761 itself carries potential anti-cancer properties\(^{11} \) and the anti-tumour effects of doxorubicin were not altered by EGb761 administration in mice bearing H22 hepatoma or gastric carcinoma,\(^ {27} \) clinical application of EGb761 for prevention of doxorubicin cardiomyopathy should not outweigh the anti-tumour effects of this potent anti-neoplastic agent.

In conclusion, this in vivo and in vitro study demonstrates that doxorubicin impairs survival of cardiac muscle cells at least partially through triggering p53-mediated, mitochondrial-dependent cell apoptosis, whereas EGb761 ameliorates nearly all of these apoptotic actions of doxorubicin and preserves cell viability. As doxorubicin plays an important role
in the treatment of various tumours, and yet its application carries a risk of serious cardiac toxicity, our results provide a window for reduction of the serious cardiac complication by adjuvant administration of EGb761, which could be a possible solution to this very serious and potentially fatal complication of doxorubicin.

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