Protective effects of remote ischemic preconditioning against cisplatin-induced hepatotoxicity in rats

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Background

As cisplatin (CP) remains one of the most effective antineoplastics used in chemotherapy, strategies to protect tissues against CP toxicity are of clinical interest. A major dose-limiting side effect in CP-based chemotherapy is hepatotoxicity. Remote ischemic preconditioning (rIP) represents a noninvasive model for organ protection. The present study was designed to examine, *in vivo*, the CP-induced hepatic injury and to find the protective probability of rIP in this model in relation to an inflammatory mechanism and the hepatic energetic activity.

Materials and methods

Twenty-four adult male albino rats were divided equally into three groups that were treated as follows: (i) control group, (ii) CP group (single intraperitoneal injection of CP 7 mg/kg body weight), and (iii) preconditioned group. rIP was induced with three 10-min ischemia/10-min reperfusion cycles of the right hind limbs just before CP injection. The animals were killed 14 days after the treatment. Among all groups, the gene expression of *nuclear factor κB (NF-κB)*, *coenzyme Q10 (Mito.Q10)*, an autophagy marker *LC3* and fatty acid-binding protein *L-FABP* was assessed by real-time reverse transcription-PCR in the rat liver tissue, in addition, the serum levels of liver enzymes alanine aminotransferase and aspartate transaminase were measured.

Results

CP induced an increase in hepatic NF-κB and mitochondrial dysfunction as reflected by the decrease in *Mito.Q10* and a significant reduction in the mitochondrial clearance mechanism: mitochondrial autophagy, which is known as mitophagy. Further, CP significantly decreased the expression of the main protein involved in fatty acid transport, *L-FABP*, which is also considered an effective endogenous antioxidant. However, these alterations were ameliorated in preconditioned rats.

Conclusion

We can assume that the alleviative outcome of rIP in CP-induced hepatotoxicity could be because of induction of anti-inflammatory and antioxidant responses associated with the upregulation of mitochondrial function.

Keywords:

autophagy, cisplatin, coenzyme Q10, fatty acid-binding protein, hepatotoxicity, ischemic preconditioning, mitophagy

Introduction

Cisplatin (CP) is a platinum-based chemotherapeutic drug used commonly for the treatment of a wide variety of human malignancies. Recent studies worldwide suggested that hepatotoxicity is one of the major dose-limiting side effects in CP-based chemotherapy [1,2]. It is suggested that the defects induced in mitochondria by oxidative damage play a pivotal role in CP-induced apoptosis [3].

Autophagy is an evolutionarily conserved process that is responsible for the degradation of components in the cytoplasm through the lysosomal pathway [4]. When the cell receives a signal to initiate autophagy, a membrane called the phagophore is formed. Primary phagophore formation, or nucleation, requires the assembly of a complex consisting of BECLIN1,

vacuolar protein sorting (VPS)34, and VPS15 [5]. Subsequent expansion of the membrane is mediated by two ubiquitin-like conjugation systems, microtubule-associated protein 1 light chain 3 (LC3), and autophagy protein (ATG)12–ATG5, which promote assembly of the ATG16L complex and the conjugation of LC3 with phosphatidylethanolamine [6]. The phagophore expands until its edges fuse around its target(s), forming a double-membrane structure called the autophagosome. Next, the autophagosome fuses with a lysosome and the contents are degraded by lysosomal

enzymes. Autophagy can specifically target organelles such as mitochondria and endoplasmic reticulum [7,8]. Thus, autophagy constitutes a very important quality-control mechanism in cells, particularly in postmitotic cells such as hepatocytes.

Although the mitochondrion is the bioenergetic and metabolic center of eukaryotic cells, it can rapidly transform into death-promoting organelles. In response to changes in the intracellular environment, mitochondria become producers of excessive reactive oxygen species (ROS) and release prodeath proteins, resulting in disrupted ATP synthesis and activation of cell death pathways [9]. Mitochondrial dysfunction is the rate-limiting step for hepatocyte cell death.

Interestingly, cells have developed a defense mechanism against aberrant mitochondria that can cause harm to the cell. Induction of mitochondrial autophagy, mitophagy, results in selective clearance of damaged mitochondria in cells, ensuring mitochondrial quality control [10,11].

Parkin has been found to play an important role in clearing mitochondria in ischemic preconditioning. The E3 ubiquitin ligase Parkin is predominantly cytosolic under basal conditions, but rapidly translocates to mitochondria upon loss of mitochondrial membrane potential [10,11]. Parkin then ubiquitinates mitochondrial proteins, which serves as a signal for mitophagy.

Biochemical measures of mitochondrial proteins, enzyme activities, and lipids are often used as markers of mitochondrial content and muscle oxidative capacity (OXPHOS) [12]. Coenzyme Q10 (Co.Q10 or Mito.Q10) is an integral part of the mitochondrial electron transport chain that transports electrons and acts as a natural antioxidant. Mito.Q10 can also be found in much of the human diet. Beneficial effects of Mito.Q10 supplementation have been noted for most of the symptoms of metabolic syndrome – for example, hypertension, diabetes, liver diseases, insulin resistance, and obesity [13]. Therefore, we will consider its expression as an index of mitochondrial function.

One of the intracellular fatty acid-binding proteins (FABPs) family is the L (liver) type, which plays a primary role in regulating fatty acid metabolism with other members of the family [14]. L-FABP binds fatty acids and transports them to the mitochondria or peroxisomes, where they are metabolized through β-oxidation and provide energy to cells. Furthermore, L-FABP can mediate fatty acid metabolism through the nuclear protein peroxisome proliferator activated receptor (PPAR), which initiates the transcription of enzymes involved in β-oxidation of long-chain fatty acids [15]. It was assumed that PPARα activation correlates positively with intracellular L-FABP concentrations [16].

Interestingly, L-FABP has a high capacity to bind fatty acid peroxidation products with high affinity and causes their excretion into urine; thus, L-FABP may be an effective endogenous antioxidant [17] and may be useful in states of extreme oxidative stress when intracellular antioxidants such as superoxide dismutase, glutathione, and catalase cannot quench excessive quantities of ROS [18].

As CP remains one of the most effective antineoplastic drugs used in chemotherapy, strategies to protect tissues against CP toxicity are of clinical interest. Recently, remote ischemic preconditioning (rIP) notably has shown numerous health benefits. rIP is initially defined as transient brief episodes of ischemia at a remote site before a subsequent prolonged ischemia/reperfusion (I/R) injury of the target organ; it is an adaptational response that protects against ischemic and reperfusion insult [19].

Ischemic preconditioning has primarily been applied to the myocardium as a target organ, but subsequent studies showed that brief ischemia induced in non target tissue, most commonly in a limb, confers protection at a remote site such as the brain, lung, kidney, intestine, or skeletal muscle [20]. The underlying mechanisms of rIP are very complex and not yet fully distinct.

An open question is what role mitophagy plays in the liver cell injury after CP-based chemotherapy: a condition reported to result in elevated ROS levels. Here, we investigate the importance of mitochondria and mitophagy in rIP of CP-induced hepatotoxicity in relation to a novel antioxidant index, L-FABP, and provide an understanding of how these processes are regulated.

Materials and methods
Experimental animal
Our work was carried out on 24 adult male albino rats, an inbred strain matched for age and weight (6–9 months and 150–200 g). Rats were inbred in the Experimental Animal Unit, Faculty of Medicine, Cairo University. They were kept at room temperature and normal dark–light cycles in groups of four per cage and were maintained according to the standard guidelines of Institutional Animal Care and Use Committee and after Institutional Review Board approval. They

had free access to laboratory rat chow and tap water throughout the experimental protocol.

Animal groups

Rats were divided equally into three groups that were treated as follows:

(i) Control group (saline solution, 1 ml/100 g body weight, intraperitoneal),
(ii) CP group [single intraperitoneal injection of CP (Sigma, St. Louis, MO, USA) 7 mg/kg body weight] [21], and
(iii) Preconditioned group, in which rIP was induced as mentioned below just before a CP injection at the same dose as for group 2.

At the planned time, 14 days after treatment, blood samples were collected from all rats before they were killed for measurement of serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST). The liver tissues were collected for the measurement of quantitative real-time PCR (qRT-PCR) gene expressions of LC3, Mit.Q10, nuclear factor κB (NF-κB), and L-FABP in all groups.

Induction of remote ischemia preconditioning
Anesthesia
Before the experiments, the animals were anesthetized with thiopental (50–60 mg/kg) administered intraperitoneally together with heparin (500 IU). Then, an area of the right hind limb was shaved.

Remote ischemic preconditioning
rIP was induced by a brief occlusion by tourniquet (a rubber band) applied on the right hind limb. The blood circulation of the limb was stopped by increasing pressure on the trochanter with the rubber band. Cessation of the blood flow in descending branches of the femoral artery was indicated by the appearance of clear signs of ischemia (cyanosis and cold skin).

The protocol for rIP consisted of three cycles of 10 min limb ischemia, followed by 10 min of reperfusion [22].

Serum ALT and AST assay
ALT and AST levels were determined using conventional laboratory methods.

Detection of *NF-κB, Mito.Q10, LC3,* and *L-FABP* gene expression by qRT-PCR.
RNA isolation and reverse transcription
Total RNA was isolated from tissue homogenate using the high pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany) and then quantified on a Nano Drop 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). The OD 260 nm/280 nm ratio was 1.9–2.0. RNA samples were further assessed by electrophoresis on 1.5% agarose gels and then visualized under UV light after ethidium bromide staining. RNA samples were stored at -80°C in aliquots until use. cDNA was synthesized with 1 mg total RNA and 1 ml oligo(dT)18 using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher scientific, Waltham, USA) according to the manufacturer's instruction.

qRT-PCR
qPCR was performed in an optical 96-well plate using an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Carlsbad, California, USA) and under universal cycling conditions: 95 C, 40 cycles of 15 s at 95 C, and 60 s at 60 C. Each 10 ml reaction contained 5 ml SYBR Green Master Mix (Applied Biosystems), 0.3 ml gene-specific forward and reverse primers (10 µmol/l), 2.5 ml cDNA, and 1.9 ml nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table 1. Data were analyzed using the ABI PRISM Sequence Detection System Software and quantified using the v1.7 Sequence Detection Software (PE Biosystems, Foster City, California, USA). The relative expression of the studied genes was calculated using the comparative threshold cycle method. All values were normalized to the glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) genes [23].

Statistical analysis
The data were coded and entered using the statistical package SPSS (version 15; SPSS Inc., Chicago, Illinois, USA). The data were summarized using descriptive statistics: mean ± SD for all variables. Statistical

Table 1 Primer sequences used for real-time PCR

Primers	Sequence
NF-κB	Forward: 5′-GCTTACGGTGGGGATTGCATT-3′
	Reverse: 5′-TTATGGTGCCATGGGTGATG-3′
Mito.Q10	Forward: 5′-GGGGAGGAAGATGTCAAAGC-3′
	Reverse: 5′-GCCTGAGACCCAATTGAAGG-3′
LC3	Forward: 5′-CCTGCTGCTGGCCGTAGT-3′
	Reverse: 5′-CGCTGTACGAGGAACACCCCAGCT3′
L-FABP	Forward: 5′-GACTGTTGCTCCCCCTTACC-3′
	Reverse: 5′-ACAAGTCACATCGCCTTCGT-3′
GAPDH	Forward: 5′-ATGGAAATCCCATCACCATCTT-3′
	Reverse: 5′-CGCCCCACTTGATTTTGG-3′

GAPDH, glyceraldehyde 3 phosphate dehydrogenase; LC3, microtubule-associated protein 1 light chain 3; L-FABP, L-fatty acid-binding protein; Mito.Q10, coenzyme Q10; NF-κB, nuclear factor κB.

differences between groups were tested using analysis of variance for quantitative normally distributed variables. When a significant *F* was obtained, multiple-comparison after tests were used to determine which groups were significantly different. *P*-values less than or equal to 0.05 were considered statistically significant.

Results

Results of serum liver enzymes: ALT and AST
These are summarized in Table 2. There was a statistically significant increase (*P* < 0.001) in both ALT and AST in CP-treated rats compared with the controls.

In terms of the effect of rIP on these enzymes, there were significant comparable reductions of both (*P* < 0.001 for both) in the preconditioned group compared with the CP rats.

Changes in the level of hepatic mRNA expression of *NF-κB, Mito.Q10, LC3,* and *L-FABP* in the studied groups
These are summarized in Table 3. The liver of CP rats showed a notably statistically significant decrease (*P* < 0.001) in *NF-κB*, *Mito.Q10*, *LC3*, and *L-FABP* gene expression. These were significantly increased in rIP (*P* < 0.001) compared with CP, but they were still significantly higher than those in the control animals (*P* < 0.001).

Discussion
Despite the recent considerable progress in the cancer chemotherapy, hepatotoxicity still remains the leading cause of mortality with CP-based chemotherapy. In our study, we provide evidence that rIP, a noninvasive almost cost-free intervention, has a hepatoprotective strategy and can improve liver function because of CP-induced injury.

To date, no optimal technique for induction of rIP of the liver has been proposed. In our study, we used the protocol of preconditioning with three 10 min cycles of occlusion, each followed by 10 min of reperfusion of descending branches of the femoral artery on the right hind limb of the rat. This protocol was evaluated by researchers as the most suitable on the basis of evaluation of biochemical and functional parameters [22]. The three-cycle model of rIP described in the 'Materials and methods' section also seems to be effective for clinical trials [24,25].

The increase in serum ALT and AST in the CP group in our study indicated that CP induced acute liver injury. This may be because of the release of these enzymes from the cytoplasm into the blood rapidly after cellular damage, where variable forms of hepatocytes degeneration, apoptosis, and necrosis were observed histopathologically [26]. This response was noted to be ameliorated under rIP in our study.

In the current work, CP treatment led to sustained induction of hepatic expression of NF-κB as indicated by its significant increase in CP rats compared with the control rats. This finding was in agreement with the study of Al-Malki and Sayed [26], which showed a significant activation of the NF-κB–p65 electromobility shift assay in the rat liver injected with CP.

rIP has been proven to be a valuable technique to reduce I/R injury in several organs [27,28], and recent studies also suggest rIP-mediated protective effects in systemic inflammatory situations [29]. However, the nature of the organ-protective molecules and how they are transferred to the target organ are still a subject of debate, and the current knowledge can be condensed into three main hypotheses [30–32]:

Table 2 Level of serum ALT (U/l) and AST (U/l) in the studied groups (*n* = 8 in each group)

Groups	Parameters (mean ± SD)	
	ALT	AST
Control	22.4125 ± 5.94581	21.8500 ± 3.09516
Cisplatin	74.8875 ± 8.97480*	61.7750 ± 15.34031*
Preconditioned	40.9875 ± 9.35513*#	36.3750 ± 3.98022*#

ALT, alanine aminotransferase; AST, aspartate transaminase, *Statistically significant compared with the control group, #Statistically significant compared with the cisplatin group.

Table 3 Levels of hepatic relative expression of *NF-κB, Mito.Q10, LC3,* and *L-FABP* in the studied groups (*n* = 8 in each group)

Groups	Parameters (mean ± SD)			
	NF-κB	*Mit.Q10*	*LC3*	*L-FABP*
Control	1.0438 ± 0.076151	1.0613 ± 0.07240	10.1075 ± 1.86586	10.8812 ± 0.06702
Cisplatin	13.1338 ± 2.92344*	0.2075 ± 0.10977*	2.0163 ± 1.00054*	2.6825 ± 1.53971*
Preconditioned	5.3925 ± 1.15379*#	0.7812 ± 0.25815*#	5.4213 ± 1.22550*#	7.9325 ± 1.65983*#

L-FABP, L-fatty acid-binding protein; LC3, microtubule-associated protein 1 light chain 3; NF-κB, nuclear factor κB. *Significant compared with the control group at *P* ≤ 0.05. #Significant compared with the cisplatin group at *P* ≤ 0.05.

(i) Humoral factors are produced by rIP in the distant organ or tissue, released into the systemic circulation, and act on the target organ;

(ii) Neural pathways transfer the rIP stimulus to the target organ; and

(iii) The rIP stimulus induces a systemic anti-inflammatory and antiapoptotic response leading to organ protection, although the protective mechanisms may differ according to the therapeutic time window [33].

In the current work, we showed that the hepatic expression of *NF-κB* significantly decreased in rIP-treated rats compared with the non treated CP rats. Our results were confirmed by the finding of Gassanov *et al.* [19], who found that rIP suppressed all three key kinases involved in tumor necrosis factors (TNF) synthesis.

In response to cytotoxicity stress, prosurvival and prodeath pathways are concomitantly activated. Thus, there is a delicate balance between life and death in the hepatocyte during stress and the final outcome depends on the complex cross-talk between these pathways. Damage to the mitochondria often results in activation of both mitophagy/permeabilization in the same cell.

Mitophagy functions as an early protective response, favoring adaptation to stress by selectively removing damaged mitochondria. In an effort to prevent cell death, damaged mitochondria are sequestered by autophagosomes and degraded before apoptosis or necrosis can be triggered. The identification of the autophagy adaptor protein p62 has provided important insights into the process of mitophagy [34]. The p62 protein binds to ubiquitinated proteins through its ubiquitin-associated domain and to *LC3* on the phagophore through its LC3-interacting region [35]. Thus, the binding of p62 to ubiquitinated mitochondrial proteins tethers the mitochondrion to the LC3-positive phagophore for engulfment. However, it is possible that other autophagosomal proteins can participate in binding proteins on mitochondria to ensure docking and removal.

In our work, we found that the expression of the autophagy marker LC3 was reduced markedly under CP therapy, whereas it was improved by rIP treatment. This finding indicated that there was a dysfunction in the major mitochondrial clearing mechanism. Therefore, we can assume that the hepatoprotective mechanisms triggered by rIP involved an increased hepatic LC3 expression and thus it will provide cellular protection from dysfunctional mitochondria and maintain cellular energetic.

Otherwise, if mitophagy fails to remove dysfunctional mitochondria, the mitochondria loaded with ROS and peroxynitrite undergo mitochondrial membrane permeability transition and collapse of its membrane potential, which in turn leads to uncoupling of oxidative phosphorylation, energetic failure, ATP depletion, and ultimately cell death [36–38].

Moreover, we observed that the expression of *Mito. Q10* was significantly reduced in CP-injured livers compared with the control one, and these results improved under rIP treatment. These findings were in agreement with those of the work of Sourris *et al.* [39], who found that Mito.Q10 supplementation prevents renal mitochondrial dysfunction in an experimental model of type 2 diabetes. They suggested that a deficiency in mitochondrial oxidized Mito.Q10 may be a likely precipitating factor for diabetic nephropathy. Moreover, Fouad and Jresat [40] concluded that Mito.Q10 protects rat liver against acute acetaminophen hepatotoxicity, most probably through its antioxidant, anti-inflammatory, and antiapoptotic effects.

Recently, Yamada *et al.* [41] innovated an *in-vivo* delivery system of Mito.Q10 to mitochondria using a MITO-Porter to evaluate its effects in a mouse liver I/R injury model. They found that it prevents I/R injury in mice livers. This indicates the potential use of such a delivery system in mitochondrial therapies.

We observed an improvement in the expression of *L-FABP* under rIP compared with the CP group. Thus, we can suggest that CP suppresses hepatic mitochondrial fatty acid oxidation by reducing the expression of the main transporter protein L-FABP [14–16].

It was assumed that a lack of L-FABP or interference with its activity may significantly inhibit both hepatocellular proliferation and hepatic regeneration as it plays a role in mitosis [42].

Conclusion

Our study presents evidence that the endogenous protective mechanisms triggered in the liver by rIP also related to hepatic mitochondria in addition to an anti-inflammatory mechanism. A significant protective effect of rIP on the liver mitochondria is reflected in a partial improvement in Mito.Q10, LC3, and antioxidant L-FABP. These findings suggest that rIP might have the potential to reduce tissue damage in vital organs at risk of clinical cytotoxic injury.

Conflicts of interest
There are no conflicts of interest.

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