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# Nicorandil ameliorates mitochondrial dysfunction in doxorubicin-induced heart failure in rats: Possible mechanism of cardioprotection

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ABSTRACT

Despite of its known cardiotoxicity, doxorubicin is still a highly effective anti-neoplastic agent in the treatment of several cancers. In the present study, the cardioprotective effect of nicorandil was investigated on hemodynamic alterations and mitochondrial dysfunction induced by cumulative administration of doxorubicin in rats. Doxorubicin was injected i.p. over 2 weeks to obtain a cumulative dose of 18 mg/kg. Nicorandil (3 mg/kg/day) was given orally with or without doxorubicin treatment. Heart rate and aortic blood flow were recorded 24 h after receiving the last dose of doxorubicin. Rats were then sacrificed and hearts were rapidly excised for estimation of caspase-3 activity, phosphocreatine and adenine nucleotides contents in addition to cytochrome *c*, Bcl2, Bax and caspase 3 expression. Moreover, mitochondrial oxidative phosphorylation capacity, creatine kinase activity and oxidative stress markers were measured together with the examination of DNA fragmentation and ultrastructural changes. Nicorandil was effective in alleviating the decrement of heart rate and aortic blood flow and the state of mitochondrial oxidative stress induced by doxorubicin cardiotoxicity. Nicorandil also preserved phosphocreatine and adenine nucleotides contents by restoring mitochondrial oxidative phosphorylation capacity and creatine kinase activity. Moreover, nicorandil provided a significant cardioprotection *via* inhibition of apoptotic signaling pathway, DNA fragmentation and mitochondrial ultrastructural changes. Interestingly, nicorandil did not interfere with cytotoxic effect of doxorubicin against the growth of solid Ehrlich carcinoma. In conclusion, nicorandil was effective against the development of doxorubicin-induced heart failure in rats as indicated by improvement of hemodynamic perturbations, mitochondrial dysfunction and ultrastructural changes without affecting its antitumor activity.



## 1. Introduction

Doxorubicin is still a well-established and a highly effective anti-neoplastic agent that is used to treat several adult and pediatric cancers such as solid tumors, leukemia, lymphomas and breast cancer [1]. However, its clinical use is limited by the risk of severe cardiotoxicity which can lead to progressive irreversible congestive heart failure particularly by multiple cumulative doses [2]. Doxorubicin cardiotoxicity seems to be a multifactorial process which has been associated with increased oxidative stress, decreased mitochondrial function and changes in cardiac gene expression with subsequent cardiomyocyte apoptosis as a terminal downstream event [3]. Mitochondria are believed to be a primary target for doxorubicin. Progression of doxorubicin cardiotoxicity has been associated with mitochondrial swelling that is typical for apoptotic cells. Such ultrastructural changes have been observed in different experimental [4,5] and clinical studies [6].

An important factor which can explain the selective cardiotoxic action of doxorubicin is the presence of mitochondria with their highest density in the heart. Doxorubicin has a high affinity of binding to cardiolipin, an anionic phospholipid in the inner mitochondrial membrane which has been recognized as an essential phospholipid in eukaryotic energy metabolism [7]. Accumulation of redox active doxorubicin in the mitochondrial membrane enhances the production of mitochondrial reactive oxygen species which are considered the main mediator of doxorubicin cardiotoxic action [8]. This represents a particular threat to cellular energy production in the myocardium by affecting the function of numerous crucial mitochondrial integral membrane proteins which depend on cardiolipin in their functions [9]. In addition to mitochondrial energy dysfunction, the accumulation of reactive oxygen species in mitochondria is probably the

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upstream trigger for the cascade that leads to apoptosis [10]. Thus, it is clear that mitochondria play an important role in the pathogenesis of doxorubicin-induced cardiomyopathy and prevention of mitochondrial dysfunction can subsequently affect myocardial alterations and result in better cardiac outcome.

Nevertheless, to date, no single chemical has been applicable to reduce the progressive deleterious action of doxorubicin without affecting the antitumor drug effectiveness. Therefore, the search for an effective and safe drug against doxorubicin-induced heart failure still remains a critical issue in both cardiology and oncology [11]. Nicorandil has attracted keen interest due to its well-documented cardioprotective actions. Nicorandil given orally to rats is preferentially distributed into heart mitochondria [12]. Nicorandil has been demonstrated to inhibit oxidative stress-induced myocyte apoptosis *via* either the opening of mitochondrial $K_{ATP}$ channels or the NO/cGMP-dependent pathway [13]. This agent has revealed potential clinical benefit not only in ischemic syndromes, but also in a broad category of cardiovascular diseases in which apoptosis is involved [14].

In the present study, the protective effect of nicorandil against doxorubicin-induced heart failure was investigated on hemodynamic alterations, mitochondrial oxidative stress, energy dysfunction, apoptosis and ultrastructural changes in rats.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats weighing 170–190 g were obtained from the animal facility of Faculty of Pharmacy, Cairo University. Rats were housed under controlled temperature (25 ± 2 °C) and constant light cycle (12 h light/dark) and allowed free access to a standard rodent chow diet and water. The investigation complies with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

### 2.2. Chemicals

Nicorandil was obtained from Adwia Pharmaceutical Company, Egypt and doxorubicin HCl was purchased from Pharmacia Italia SpA, Milan, Italy.

### 2.3. Experimental design

Rats were randomly divided into four groups. Group I ($n = 8$) served as a normal group. Group II ($n = 8$) received nicorandil (3 mg/kg). The dose of nicorandil used was selected from our previous study [15]. Nicorandil was freshly prepared in saline and orally administered once daily over a period of 2 weeks. Group III ($n = 12$) received doxorubicin (3 mg/kg) dissolved in saline and injected i.p. 3 times weekly (every other day) over a period of 2 weeks to obtain a cumulative dose of 18 mg/kg [16]. In this group, signs of congestive heart failure such as abdominal ascites and liver congestion were observed at the end of 2 weeks. Animals in the fourth group (10 rats) received doxorubicin as in group 3 and nicorandil as in group 2.

### 2.4. Heart rate and aortic blood flow measurements

Animals were weighed 24 h after receiving the last dose of doxorubicin and heart rate and aortic blood flow were recorded. In brief, animals were anesthetized with thiopental (5 mg/kg, i.p.) and kept warmed with a heating lamp to prevent the incidence of hypothermia. The trachea was cannulated for artificial respiration using a small rodent ventilator (Bioscience, UK). Subcutaneous peripheral limb electrodes were inserted for heart rate recording using a polygraph (Letica polygraph 4006, Spain). The chest was then opened by a left thoracotomy and aorta was carefully dissected free from adjacent tissues. Aortic blood flow was measured using a flow probe inserted into the aorta (Transonic Systems Inc., USA), giving an indication of the cardiac output and the left ventricular function. The probe was positioned so that the aorta was in the bottom of the reflector. A syringe with a flexible catheter tip was used to deposit SurgiLube jelly in air spaces of probe to verify good transmission of the ultrasound signal by checking the flowmeter "Test" mode. The probe position was stabilized with a micromanipulator for continuous measurement. At the end of the experiment, animals were euthanized and the heart was rapidly excised, washed with ice-cold saline, dried and weighed. For each group, two sets of experiments were conducted; one for biochemical examination and the other ($n = 3$) for electron microscopic examination.

### 2.5. Isolation of heart mitochondria

A part of ventricle was homogenized in 0.7 M Tris–HCl buffer (pH 7.4) (Sigma–Aldrich Co., USA) containing 0.25 M sucrose (El-Nasr Pharmaceutical Co., Egypt) and centrifuged at 2500 × *g* for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatant fluid was decanted into ependorff tubes and centrifuged at 10,000 × *g* for 10 min at 4 °C to form primary mitochondrial pellet. The supernatant fluid (postmitochondrial fraction) was removed and the pellet was gently resuspended in 0.5 ml Tris–sucrose buffer for washing. The pellet was recentrifuged and the supernatant fluid was decanted. This washing cycle was repeated three times to improve the degree of mitochondrial purity. The final mitochondrial pellet was resuspended in Tris–sucrose buffer. The fresh mitochondrial suspension was used for estimation of mitochondrial oxidative phosphorylation capacity. The remaining mitochondrial fractions were used for assessment of reduced glutathione and lipid peroxidation products as well as the antioxidant enzymes; superoxide dismutase, glutathione peroxidase and catalase activities. The protein content of mitochondrial fractions was determined using the method of Lowry et al. [17].

#### 2.5.1. Oxidative phosphorylation capacity of heart mitochondria

The mitochondrial phosphate utilization was assayed following the method of Hinkle [18] and Banerjee et al. [19]. An aliquot of mitochondrial suspension (100–150 μg of protein) was added to an incubation mixture (500 μl) containing 125 mM KCl (Sigma–Aldrich Co., USA), 75 mM sucrose, 0.1 mM EGTA (Sigma–Aldrich Co., USA), 1 mM $MgCl_2$ (Sigma–Aldrich Co., USA), 10 mM HEPES (Sigma–Aldrich Co., USA), 2 mM $Na_2HPO_4$ (Sigma–Aldrich Co., USA), 0.3% BSA (Sigma–Aldrich Co., USA), 0.5 mM ADP (Sigma–Aldrich Co., USA), 5 mM sodium pyruvate (Sigma–Aldrich Co., USA), 10 mM sodium succinate (Sigma–Aldrich Co., USA), and 10 mM glucose (Merck Chemical Co., Germany). Hexokinase (5 units) (Sigma–Aldrich Co., USA) was added immediately to the incubation mixture following the addition of mitochondrial sample. The incubation was continued at 37 °C for 30 min and terminated by the addition of 3% ascorbic acid (Sigma–Aldrich Co., USA) in 0.5 M HCl (El-Nasr Pharmaceutical Co., Egypt). The amount of inorganic phosphate in the supernatant was estimated spectrophotometrically at 850 nm [20]. A 0 min sample was also assayed for inorganic phosphate content in a similar way. The difference in inorganic phosphate content between 0 and 30 min samples was calculated to estimate the phosphorylation capacity of heart mitochondria and expressed as nmol Pi/min/mg protein.