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Impact of Ivabradine on Reactive Nitrogen and Oxygen Radicals in Doxorubicin Induced Acute Cardiotoxicity in Mice

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Doxorubicin (DOX) has been used in variety of human malignancies for decades. Despite its efficacy in cancer, clinical usage is limited because of its cardiotoxicity, which has been associated with oxidative stress. The possible protective mechanisms of ivabradine, a selective inhibitor of the $I_{(f)}$ channel, against acute doxorubicin cardiotoxicity were investigated in mice. Cardiac toxicity was induced by a single intraperitoneal injection of doxorubicin (15 mg/kg). Ivabradine treatment (10 mg/kg/day, orally) was started 5 days before doxorubicin administration. Ivabradine significantly reduced the elevated heart rate and the serum cardiac enzymes resulted from DOX administration. Also, ivabradine reversed DOX-induced deficits in the antioxidant defense mechanisms, decreased lipid peroxidation in cardiac tissue and attenuated the production of nitric oxide levels by induced nitric oxide synthase enzyme. In addition, DOX-induced cardiac tissue damage observed by histopathological examination was markedly ameliorated with ivabradine. In conclusion, the beneficial effects of ivabradine against DOX induced cardiotoxicity are mediated by the reduction of heart rate with inhibition of oxidative stress and nitric oxide detrimental effects.

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Keywords: Cardiotoxicity; doxorubicin; ivabradine; iNOS; nitric oxide; oxidative stress.

1. INTRODUCTION

Anthracyclines are anticancer compounds that were originally derived from *Streptomyces* and their anti-tumor activities were established in the 1960s. Doxorubicin (Adriamycin) belongs to the anthracyclines that held promise as a powerful drug in the fight against cancer. However, reports of fatal cardiotoxic effects of doxorubicin have subdued enthusiasm for this drug [1].

The need of searching for novel cardioprotectants to be used in treatment of doxorubicin cardiotoxic effects depends on the molecular mechanisms of anthracycline cardiotoxicity. Anthracycline antibiotics are able to induce complex changes in cell homeostasis as a result of dysregulation of survival signals involved in the preservation of cardiomyocyte integrity, such as free radical-dependent lipid oxidation, mitochondrial dysfunction and calcium overload [2]. Also, the nitric oxide (NO) has been implicated in the etiology of doxorubicin-induced cardiotoxicity because it regulates many aspects of cellular function in the normal heart as well as in ischemic and nonischemic heart failure, septic cardiomyopathy, cardiac allograft rejection, and myocarditis [3]. However, the precise mechanism of myocardial impairment remains unclear [4].

The pacemaker current $I_{(f)}$ plays a central role in determining spontaneous activity of the sinus node. Ivabradine, a selective inhibitor of the $I_{(f)}$ channel, reduces resting and exercise heart rates without affecting cardiac contractility or blood pressure. Also, ivabradine exerts antianginal and anti-ischemic effects in patients with stable coronary disease [5]. Ivabradine given orally to mice (10 mg/kg body weight per day) reduces heart rate without influencing left ventricular contractile function [6].

Therefore, the aim of the present study was to evaluate the potential role of ivabradine on nitric oxide and oxygen free radicals release in doxorubicin (DOX) induced acute cardiotoxicity in mice.

2. MATERIALS AND METHODS

2.1 Chemicals

Doxorubicin (Adriblastina10mg vials, Pharmacia Italia, S.P.A Italy), Ivabradine (procoralan 5 mg tablets, Servier Egypt Industries Limited, 6th October City, Giza, ARE)

2.2 Animals

Forty eight male Balb-C mice (Urology and Nephrology Center, Mansoura University, Egypt), weighing 30-40 gm±5 gm were housed under conditions of controlled temperature and a12 h lighting cycle and fed with standard diet *ad libitum.* The study was approved by Institutional Ethics Committee for the use of laboratory animals.

The animals were divided into four groups of 12 animals each.

Control animals received 0.5 mL of sterile double distilled water, orally once daily for 5 consecutive days.

Ivabradine-treated group received 10 mg/kg of ivabradine dissolved in 0.5 ml sterile double distilled water orally once daily for 5 consecutive days [7].

DOX-treated group received sterile double distilled water, orally once daily for 5 consecutive days. One hour after the last treatment, the animals of this group received IP injection of doxorubicin hydrochloride at a dose of 15 mg/kg [8].

Ivabradine + DOX-treated group received the same dose of ivabradine orally once daily for 5 days. One hour after the last treatment, the animals of this group received the same dose of doxorubicin hydrochloride IP.

Thirty hours after DOX injection, the animals were anaesthetized with ether and the heart rate and blood pressure were monitored with a tail blood pressure analyzer (Pressure Meter LE 5001, Panlab, Letica SA, Barcelona, Spain). A digital display showed systolic, diastolic and mean arterial blood pressure (MAP) was calculated every 2 min as the average of 10 measurements during the light cycle. Mice were then sacrificed and blood samples were taken by heart puncture. Serum was separated and used for measurement of cardiotoxicity indices and NO. Hearts were dissected from all animals, and divided into three parts; one part was stored at -80ºC for analysis by reverse transcriptase polymerase chain reaction (RT-PCR). The other

part was homogenized and used for the measurement of myocardial oxidative stress parameters and the third part was processed for light microscopic study.

3. BIOCHEMICAL PARAMETERS

3.1 Measurement of Serum Indices of Cardiotoxicity

Serum lactate dehydrognase (LDH), creatine phosphokinase andisoenzyme (CK-MB) activities troponin-I (cTn-I) were measured as markers for cardiac muscle damage.

LDH and CK-MB activities were determined kinetically at 340 nm using acommercially available kits (Stanbio laboratory, INC. USA). Troponin-I was measured spectrophotometrically at 450 nm using a solid phase enzyme-linked immunosorbent assay kits (DRG International, Inc., USA).

3.2 Measurement of Cardiac Oxidative Stress Parameters

The heart samples were dried, weighed, homogenized in 50 mM ice cold Phosphate-Buffered Saline (pH 7.4) and centrifuged for 5 min at 5000 g. The samples were stored at −70°C for biochemical estimations. The product of lipid peroxidation, malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA). The amount of lipid peroxides was measured as the production of MDA, which in combination with TBA forms a pink chromogen compound whose absorbance at 532 nm was measured. The result was expressed as nmol/mg protein [9]. The superoxide dismutase (SOD) activity was determined spectrophotometrically according to the reported method [10]. The method is based on the ability of SOD to inhibit the reduction of cytochrome *c* in the presence of xanthine and xanthine oxidase. One unit was defined as the amount of enzyme that inhibits the reduction of cytochrome *c* by 50% and activity was expressed as units/mg protein. Protein was determined by method of Lowry et al. [11].

The level of glutathione (GSH) was determined according to Beutler et al. [12]. The reaction mixture contained 0.1 ml of supernant, 2.0 ml of 0.3 ml phosphate buffer (PH-4.8), 0.4 ml of double distilled water and 0.5 ml of DTNB (5,5 dithiobis 2-nitrobenzoic acid). The reaction mixture was incubated for 10 minutes and the absorbance was measured at 412 nm. The level of glutathione (GSH) was determined from the standard curve with commercially available GSH (sigma chemical). GSH is expressed as µ mol per gm tissue.

3.3 Measurement of Cardiac NO

Cardiac NO level was assessed in the supernatant indirectly by measuring the nitrite/ nitrate concentration using the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% napthyl ethlenediamine dihydrochloride, in a ratio of 1 : 1). The concentration of nitrite/nitrate in the samples was determined spectrophotometrically at 540nm. For every NO assay, a standard curve was performed, using sodium nitriteas a NO source [13].

3.4 Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from the cardiac tissue using a modificationof the method of Chomczynski and Sacchi [14]. The RNA concentrationwas determined using spectrophotometry (OD_{260}). The reversetranscriptase (RT) reaction was performed using a QIAGEN one-stepRT-polymerase chain reaction (PCR) kit (Hilden, Germany) aspreviously described. One microgram of total RNA was reverse transcribed into cDNA using Omniscript RT, Sensiscript RT, and primers. The sense primer sequence for iNOS was 5'-TTGGGTCTTGTTAGCCTAGTC-3' and the antisense was 5'-TGTGCAGTCCCAGTGAGGAAC-3'.

Amplification was initiated at 50ºC for 30 minutes, followed by 30 cyclesconsisting of denaturation at 94ºC for 1 minute, annealingat the appropriate primer-pair annealing temperature for 1minute, and extension at 72ºC for 1 minute, and then a finalextension step of 10 minutes at 72ºC. ß-actin(sense: 5'-TCTACAAT GAGCTGCGTGTG-3' and antisense: 5'-GGTCAGGATCTTCATGAGGT-3') was used as an internal control and standard.The RT-PCR products were electrophoresed on a 1.5% agarosegel and visualized by staining with ethidium bromide.

3.5 Histopathology of Heart

Tissues for histology were harvested from anesthetized mice after fixation via transcardial perfusion with 10% neutral buffered formalin. Subsequent paraffin processing, embedding, and

sectioning were performed by standard procedures. Sections (5 µm) were stained with hematoxylin and eosin and were examined under light microscope [15].

3.6 Statistical Analysis

Dataare presented as mean±SEM. Differencesamong groups within an experiment were analyzed by the one-way ANOVA analysis of data followed by post hoc test of Tukey HSD. A *P* value of <0.05 value was considered significant.

4. RESULTS

4.1 The Heart Rate and Map

Treatment with ivabradine resulted in a significant decrease in the heart rate while DOX induced significant increase in the heart rate compared with the control. In the ivabradine-DOX group, the heart rate was significantly decreased nearly similar to that of control group (Fig. 1). The MAP was not significantly affected by either DOX or ivabradine.

4.2 The Cardiotoxic Indices

In comparison to the control group, DOX-treated group showed significant increase in the serum CK-MB, LDH and troponin-I. Pre-treatment with ivabradine in the DOX induced acute cardiotoxicity animals showed significant decrease in the serum cardiotoxic indices as compared to DOX-treated group (Figs. 2a and b).

4.3 The Cardiac Oxidative Stress Parameters

DOX-treated group showed significant elevation of MDA with significant reduction of the antioxidant activity; SOD andGSH in the cardiac tissues as compared to those of the control group. Ivabradine-DOX group showed significant decrease in the cardiac MDA and NO significant increase of the cardiac SOD and GSH in comparison to those of DOX-treated group (Table 1).

4.4 The Cardiac NO and Inos RNALevels

Cardiac iNOS RNA and NO were significantly elevated in DOX-treated group in comparison to control group. Pre-treatment with ivabradine showed significant decrease in the iNOS RNA and NO levels as compared to the DOX-treated group (Figs. 3 and 4).

4.5 The Histopathology of Heart

Histopathology of heart treated with doxorubicin showing severe congestion, myocyte loss, myofibrillar degeneration, and extensive vacuolization. Pre-treatment with ivabradine revealed almost similar myocardial histological profile to control group except for few degeneration of some cardiac muscle (Fig. 5).

5. DISCUSSION

Our results showed that a single dose (15 mg/Kg) of DOX caused a significant increase in the heart rate, serum cardiotoxic indices, cardiac oxidative stress markers as well as iNOS RNA and NO levels. These biochemical changes were parallel to the histopathological changes noticed in the cardiac tissue. Pre-treatment of mice with ivabradine for 5 days before DOX treatment produced a significant decrease in the heart rate with significant improvement in either the biochemical or the histopathological features of cardiotoxicity.

Table 1. Effect of pretreatment of ivabradine (10 mg/kg) on the mean levels of cardiac oxidative stress parameters in DOX-treated mice

Treatment	MDA (nmol/mg protein)	SOD (U/ mg protein)	GSH (µ mol/gm tissue)
Control group	125.14±9.81	30.31±0.25	3.55±0.12
Ivabradine-treated group	133.90± 8.75	33.72±0.21	3.94±0.05
DOX-treated group	345.67±12.44 *+	13.56±0.71*+	1.90±0.01*+
Ivabradine+DOX- treated group	169.74±11.35$	27.88±0.52$	2.95±0.1$

*Statistical analysis was carried by one-way ANOVA followed by Tukey–HSD; multiple comparison test. All values are presented as means of 12 mice ± SEM; *Indicates significant change from control values at p < 0.05*
+ Indicates significant change from ivabardine treated groups at p < 0.05.; $ Indicates significant change from DOX treated groups at p < 0.05

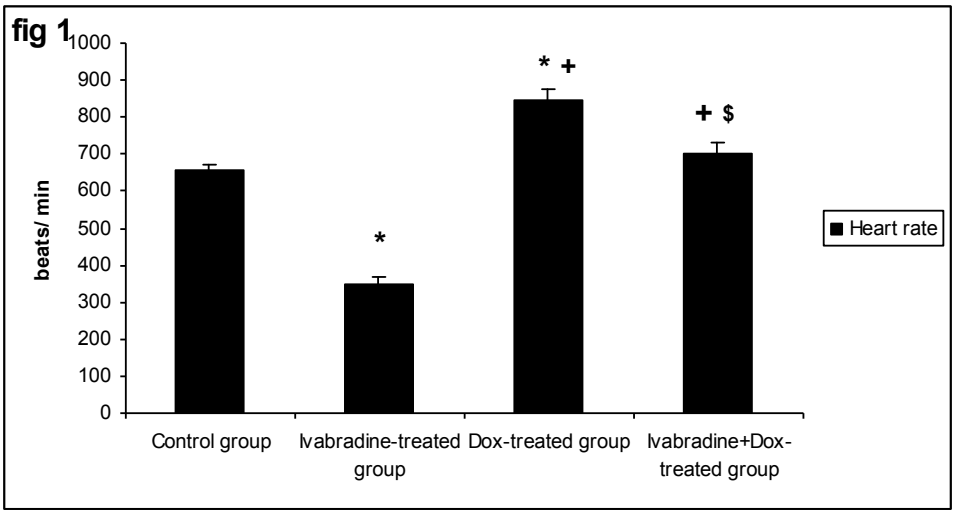


Fig. 1. Effect of pretreatment of ivabradine (10 mg/kg) on the heart rate in DOX-treated mice
Statistical analysis was carried by one-way ANOVA followed by Tukey–HSD; multiple comparison test. All values are presented as means of 12 mice ± SEM;**Indicates significant change from control values at p < 0.05*
+ Indicates significant change from ivabradine treated groups at p < 0.05;*$ Indicates significant change from DOX treated groups at p < 0.05*

Co-administration of ivabradine with DOX therapy resulted in decrease of the elevated HR. This finding is in harmony with Colak et al. [16] who reported that ivabradine application after DOX treatment significantly reduced HR levels in rats. The HR reduction with ivabradine improves energy metabolism and mechanical function of isolated ischemic rabbit heart [17]. Since HR is a major determinant of myocardial oxygen consumption and of cardiac work, reduction of HR may represent an important strategy for the treatment of patients with a wide range of cardiac disorders [18]. This may explain the improvement of the serum cardiac indices in the DOX-Ivabradine-treated group in our study.

The significant elevation of the cardiac enzymes LDH, CK-MB and cTn-I has been reported to be a reliable indicator of DOX induced cardiotoxicity in either mice [19] or rats [20]. O'Brien [21] reported that the biomarker of myocardial injury, including CK-MB, LDH does not come close to cTn in effectiveness. Cardiac troponin has been widely used for clinical assessment and monitoring of cardiac toxicity in humans being treated for cancer. It is gradually being reverse translated from human into animal use as a safety biomarker. Its use is especially merited whenever there is any safety signal indicating potential cardiotoxicity [22].

The improvement of heart rate by ivabradine was also associated with restoration of the cardiac oxidative stress markers induced by DOX in the present research. DOX-treated group showed rise of the lipid peroxidation product; MDA and reduction of the antioxidant activity; SOD andGSH in the cardiac tissues. The DOX mediated oxidative stress is quite compatible with previous studies [23]. Heart tissue is rich in mitochondria, which occupy about forty percent of the total intracellular volume of cardiomyocytes [24]. DOX has high affinity for cardiolipin, a negatively charged phospholipid abundant in the mitochondrial inner membrane, leading to mitochondrial accumulation of DOX [25]. Under clinically relevant plasma DOX concentrations, the heart becomes a site of redox reactivity. The quinone functionality of DOX is transformed, in the presence of NADH, into a semiquinone via one-electron reduction by complex I of the electron transport chain [26]. The semiquinone form reacts with O_2 to produce a superoxide radical, whereby DOX returns to the quinoneform. The cycling of DOX between quinone and semiquinone generates large amounts of O_2, which further give rise to a variety of ROS species [27]. ROS can damage membrane lipids and other cellular components and consequently lead to cardiomyocyte apoptosis or death [28].

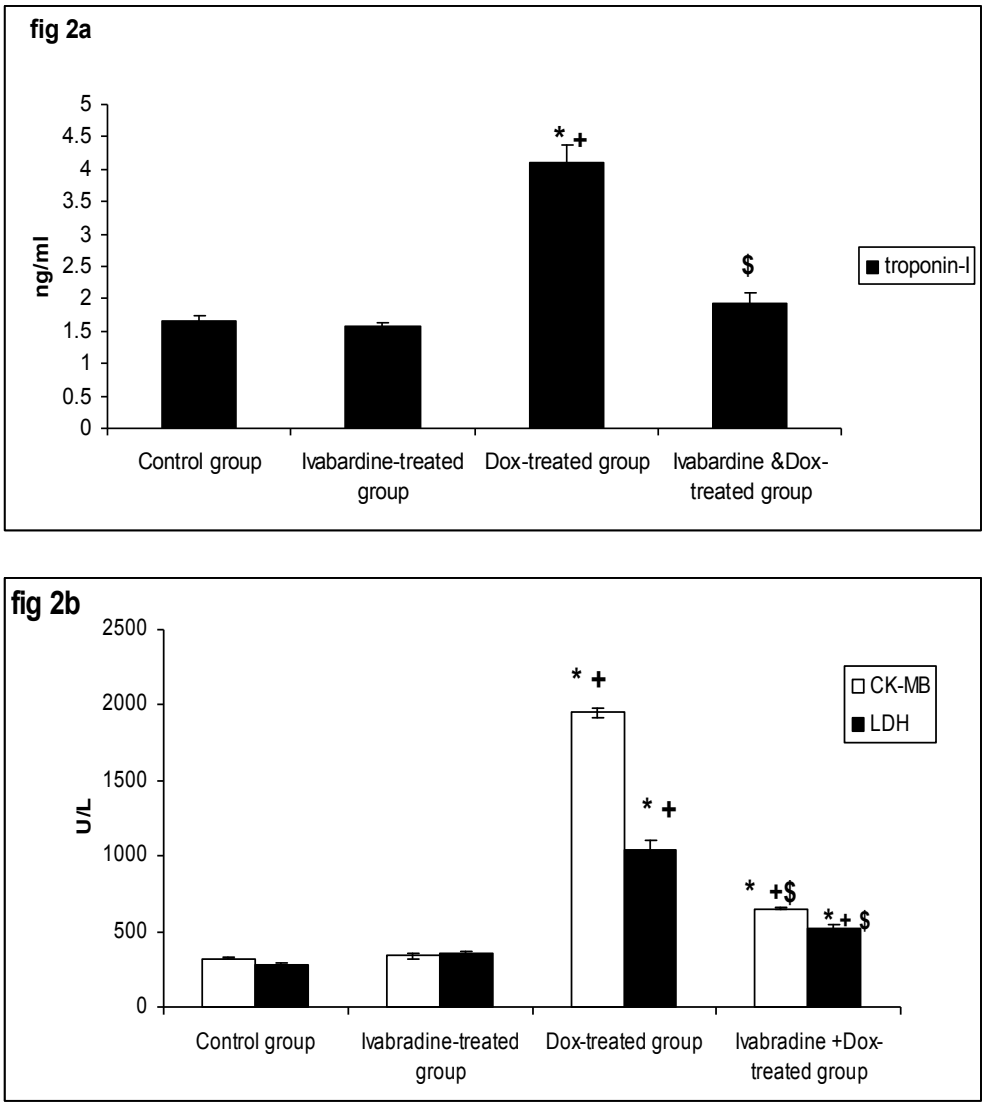


Fig. 2. Effect of pretreatment of ivabradine (10 mg/kg) on the mean serum levels of cardiotoxicity indices in DOX-treated mice

*Statistical analysis was carried by one-way ANOVA followed by Tukey–HS; multiple comparison test. All values are presented as means of 12 mice ± SEM; *Indicates significant change from control values at $p < 0.05$; $^{+}$ Indicates significant change from ivabradine treated groups at $p < 0.05$; $^{\$}$ Indicates significant change from DOX treated groups at $p < 0.05$*

Redox signalling potentially plays a role in the processes of apoptosis and programmed necrosis, which are thought to contribute to cell death in heart [29]. ROS can activate an Apoptosis signal-regulating kinase (ASK) 1 which was demonstrated to be important in causing apoptosis in an in vivo model of heart failure [30]. Other mechanisms by which oxidative stress can result in cardiomyocyte apoptosis include the down regulation of an anti-apoptotic protein called the apoptosis repressor with caspase recruitment domain (ARC), which is associated with subsequent sarcoplasmic reticulum calcium release, caspase-3 cleavage, and cardiomyocyte apoptosis [31]. ROS were shown to mediate cardiomyocyte apoptosis by down regulating the class III histone deacetylase, SIRT3, potentially involving downstream Bcl-2/Bax signalling [32].

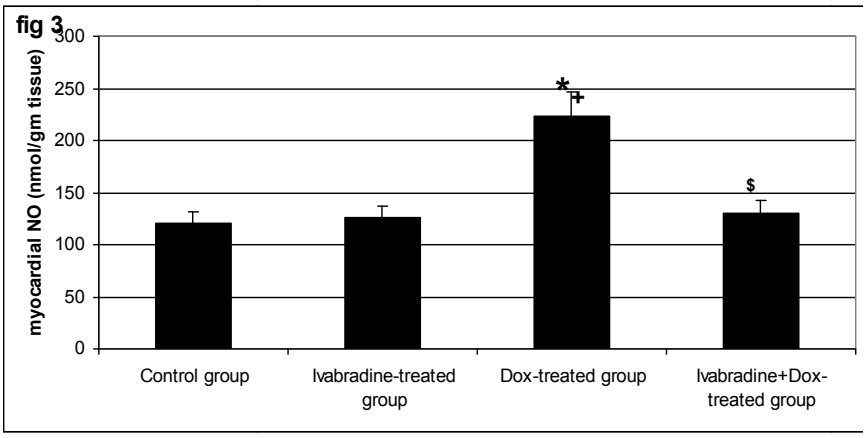
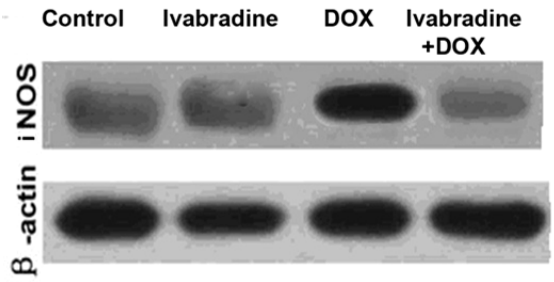
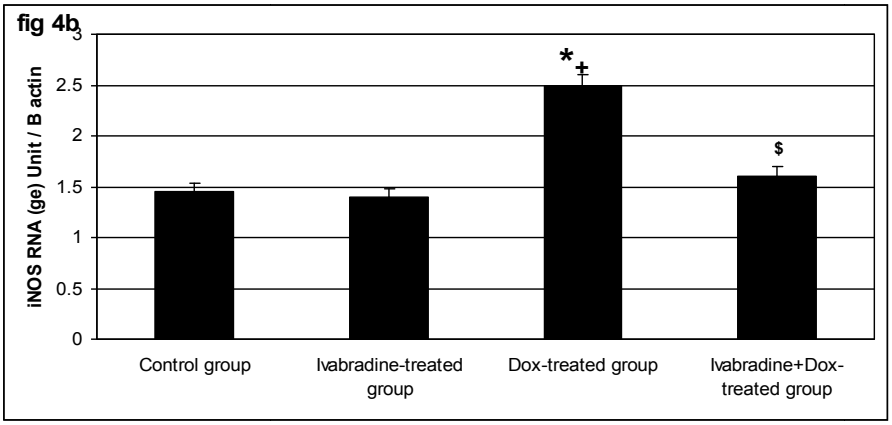


Fig. 3. Effect of pretreatment of ivabradine (10 mg/kg) on the mean levels of cardiac NO in DOX-treated mice

*Statistical analysis was carried by one-way ANOVA followed by Tukey–HSD; multiple comparison test. All values are presented as means of 12 mice ± SEM; *Indicates significant change from control values at p < 0.05 +Indicates significant change from ivabradine treated groups at p < 0.05; $Indicates significant change from DOX treated groups at p < 0.05*



(4a)



(4b)

Fig. 4. Effect of pretreatment of ivabradine (10 mg/kg) on the mean levels of the cardiac inosin DOX-treated mice. Fig (4a) Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis demonstrates expression of inos (ratio to-B-actin) in the cardiac muscle. Fig (4b) Quantitative analysis of cardiac inos

*Statistical analysis was carried by one-way ANOVA followed by Tukey–HSD. multiple comparison test. All values are presented as means of 12 mice ± SEM;*Indicates significant change from control values at p < 0.05 + Indicates significant change from ivabradine treated groups at p < 0.05;$ Indicates significant change from DOX treated groups at p < 0.05*

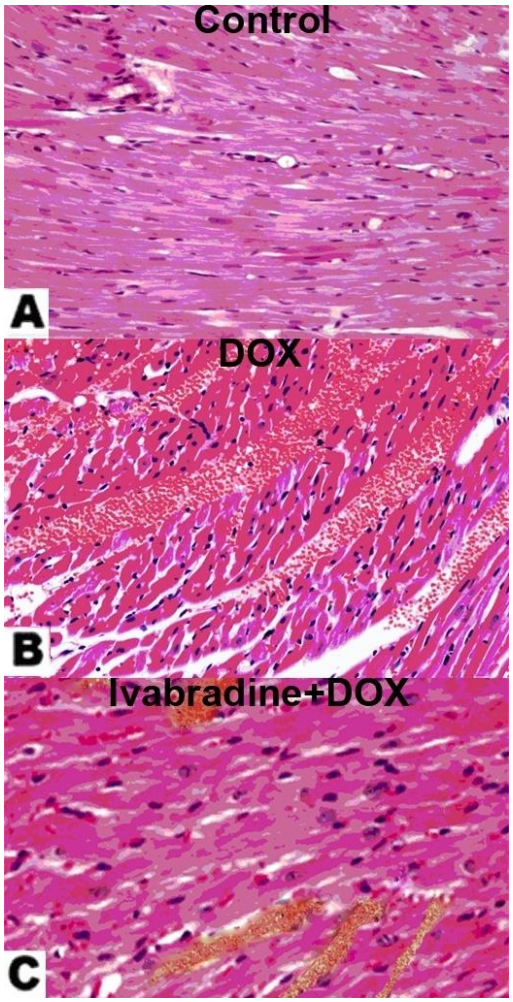


Fig. 5. Photomicrographs of heart showing (A)normal cardiac muscle of control group; (B) severe congestion and occasional degeneration of cardiac muscle of DOX treated- group; (C) little congestion and muscle degeneration of ivabradine+DOX-treated group (H&Ex200)

The overproduction of ROS, caused by DOX administration, can account for this decrease in GSH and SOD activity, as these species are detoxified by endogenous antioxidants causing their cellular stores to be depleted [33]. The decrease of cardiac GSH content may also be attributed to the enhanced activities of GSH metabolizing enzymes by DOX administration [34]. Custodis et al. [5,35] reported that heart rate reduction by ivabradine reduces oxidative stress through down regulation of NADPH oxidase activity and superoxide release. However Colak et al. [16] found that, ivabradine administration could not reduce the lipid peroxidation product but it caused rise in the antioxidant enzyme activity in DOX treated rats.

Additionaly, ivabradine attenuated NO synthesis by iNOS in the DOX-treated mice heart in the present work. These results are in agreement with that of [36] who reported that ivabradine attenuated myocardial lesions through inhibition of NO synthesis by iNOS in murine viral myocarditis model. The significant increase of the NO and mRNA levels of iNOS in cardiac tissue after DOX treatment in the present study has also been reported by others [37,38]. Increased iNOS expression formation has been observed after a single dose of DOX in mice cardiomyocyte [39]. The influence of anthracyclines on the NO signaling pathway has been studied in experimental models. In vivo echocardiographic measurements in mice showed that the marked reduction in cardiac contractility in animals given doxorubicin at 20 mg/kg i.p., as assessed by fractional shortening and stroke volume 5 days post dose, was associated with a statistically significant increase in the immunopositivity of myocardial iNOS as compared with the control group [40]. It is known that purified recombinant NOSs are able to trigger doxorubicin redox cycling to produce reactive oxygen species, including superoxide anion and hydrogen peroxide [3]. The maximum velocity of iNOS for doxorubicin was 5 to 10-fold higher than that of nNOS and eNOS [41], thus confirming the important role of iNOS in the pathogenesis of acute anthracycline cardiotoxicity. The reaction of NO and superoxide anion leads to the synthesis of peroxynitrite which is a potent cellular oxidant that contributes significantly to DOX-induced cardiac dysfunction [42]. Owing to the importance of NO as a key regulator of vascular tone and an important mediator in the myocardial contractile response [43], the resulting acute change in NO homeostasis may account at least in part for the early electrocardiographic changes that occur upon administration of anthracyclines in rats [44] and mice [40]. Also severe cardiac lesion such as dilated cardiomyopathy and congestive heart failure was observed in human [45]. On the other hand, Cole et al. [46] found that NO produced by iNOS contributes to protection of normal cardiac tissue injury by adriamycin in vivo. They explained their idea that NO radical can react with super oxide anion, therefore the level of NO radical can alter the fate of super oxide anion. Nitric oxide may aid SOD in the removal of excess super oxide anion to prevent generation of hydroxyl radical [47].

The improvement of the clinical and biochemical data by ivabradine was parallel to the improvement of the cardiac histopathology of DOX-treated mice. Since severe congestion, myocyte loss, myofibrillar degeneration, and extensive vacuolization were observed. These morphological changes have been previously recorded in mice by Saad et al. [48] who recognized marked interstitial edema, chronic inflammatory cells infiltration, focal subendocardial fibrosis, marked myocardial fibrosis, marked myocardial fibers swelling and disorganization with perinuclear vacuolation and myocardial necrosis induced by DOX. Also Mukherjee et al. [49] found the same histopathological changes in rats in the form of focal as well as subendocardial myocytolysis with infiltration of macrophages, lymphocytes and edema.

6. CONCLUSION

It was concluded that ivabradine, through its ability to reduce the heart rate and its antioxidant activity and reduction of nitric oxide levels, is a potential candidate to protect against acute doxorubicin cardiotoxicity, a major and dose-limiting clinical problem.

CONSENT

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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