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Full Length Research Paper

Cardioprotective effects of aerobic regular exercise against doxorubicin-induced oxidative stress in rat

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Doxorubicin (DOX) is an anthracycline antibiotic that is widely used as an anticancer agent. However, the clinical use of DOX is limited due to its cardiotoxic side effects. Few studies have assessed pretreatment effects of chronically exercise against doxorubicin-induced cardiotoxicity. The aim of this study was to determine cardioprotective effects of aerobic regular exercise against doxorubicin-induced oxidative stress in rat. Forty-eight Wistar male rats were randomly assigned to sedentary and trained groups. Training program included treadmill running between 25 to 54 min/day and 15 to 20 m/min, 5 days/week for 6 weeks. The biomarkers related to oxidative stress were assessed in heart tissue after administration of the saline solution (0.9% NaCl i.p) and/or DOX 20 mg/kg and DOX 10 mg/kg. Doxorubicin administration (10 and 20 mg.kg^{-1}) causes an imbalance in the oxidant/antioxidant markers in heart. Six weeks of the aerobic training led to a significant increase of apelin, nitric oxide(NO), superoxide dismutase(SOD) and an insignificant decrease of malondialdehyde(MDA), as compared to sedentary+placebo group. However, after six weeks of aerobic training and DOX treatment with 10 and 20 mg.kg^{-1}, a significant increase in apelin and SOD, and a significant decrease in MDA were detected in comparison to sedentary+DOX 10 and/ or sedentary+DOX 20 groups. However, there was a significant difference between DOX 10 mg.kg^{-1} and DOX 20 mg.kg^{-1} treatments in NO and SOD levels, only. Our study suggests that cardioprotection induced by chronically exercise in DOX treated rats was associated with inhibition of oxidative stress and the up-regulation of antioxidant enzymes.

Key words: Cardiotoxicity, doxorubicin, antioxidant, endurance training, rat.

INTRODUCTION

Cancer is one of the leading causes of death worldwide, accounting for 13% of all deaths, equivalent to 7.4 million people per year (Barbaric et al., 2010). It is treated with various procedures such as: surgery, chemotherapy and radiation (Minghua and Zhi-Gang, 2011). Doxorubicin (Dox) is a powerful and highly efficacious drug and shows a broad range of antitumor activity in many kinds of cancers (Vishwanatha et al., 2012). However, the clinical use of DOX is often limited because of its undesirable serious cardiotoxic side effects on cardiac tissues (Evert et al., 2001; Chatterjee et al., 2010; In Duk et al., 2002; Raschi et al., 2010; Andreadou et al., 2007). Thus, drug-induced cardiotoxicity is emerging as an important issue among cancer survivors and unfortunately, its major adverse effect may limit its use.

Several mechanisms have been proposed to account for the DOX-induced cardiotoxic side effects including free radical induced myocardial injury, lipid peroxidation, mitochondrial damage, vasoactive amine release and cellular toxicity (Evert et al., 2001; Chatterjeeet al., 2010; Vishwanathaet al., 2012, 2011). Oxygen free radicals are apparently involved in all mechanisms proposed (Abdel-Moneim et al., 2009) and increased oxidative stress and release of free radicals as well as endogenous

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antioxidant deficits have been suggested to play a major role in Dox-induced cardiotoxicity and heart damage (Vishwanatha et al., 2012; Andreadou et al., 2007; Hitesh et al., 2011). The heart is particularly vulnerable to injury from free radicals because it has a lower level of protective enzymes such as superoxide dismutase than other tissues (Cecen et al., 2011). Ascensão (2005) reported that the weakness of the heart to oxidative damage may be in part explained by the fact that heart demonstrates a slow turnover and relatively lower levels of antioxidant enzyme activity when compared to most other tissues. Apelin was recently found to be an inotropic polypeptide in isolated rat hearts, and directly activated the vascular L-Arg/NOS/NO pathway, which could be one of the important mechanisms of apelin-regulated vascular function (Jia et al., 2007). Furthermore, Duparc et al. (2011) reported peripheral administration of apelin stimulates glucose utilization and insulin sensitivity through a nitric oxide (NO) pathway. On the other hand, Hitesh et al. (2011) reported that high concentrations of NO participate in cardiomyocyte oxidative damage, apoptosis, and/or necrosis through peroxynitrite formation. DOX promotes the synthesis of NO and ROS, such as the superoxide anion. In contrast, Chatterjee et al. (2010) reported that lack of nitric oxide was associated with enhanced cardiac injury, and mitochondrial injury was attenuated by an increase in manganese superoxide dismutase.

In recent years, by understanding the free radical mechanism of DOX-induced cardiotoxicity, it has become possible to develop effective strategies to prevent or modify expected damage. To date, a number of pharmaceutical agents have been tested to assess their potential to reduce the risk of doxorubicin cardiotoxicity (Raschi et al., 2010; Vishwanatha et al., 2012, 2011; Hitesh et al., 2011; Abdel-Moneim et al., 2009; Cecen et al., 2011). On the other hand, there is a growing interest in the usage of aerobic regular training as a non-drug therapeutics protective strategy against problems related to cardiovascular health such as Dox-induced cardiotoxicity (Ascensão et al., 2012). Although, there is evidence that acute exercise resulted in oxidative stress and cardiac damage (Teixeira et al., 2011), it seems probable that regular endurance exercise training could constitute an excellent tool either to prevent and/or to treat several diseases. Moreover, it provides myocardial protection against many cardiac insults (Ascensão et al., 2006, 2005). The exact mechanisms responsible for this protection continue to be debated. However, it has been argued that they are in part, associated with the decreased free radical production (ROS) and with increased response of the several antioxidant defense systems (Ascensão et al., 2006; Ascensão et al., 2005).

To our knowledge, there are few studies dealing with the preventive effect of moderate-term endurance training on Dox-induced cardiotoxicity and oxidative stress in rats. It was hypothesized that regular exercise attenuated the oxidant/antioxidant imbalance caused by various dosages (10 and 20 mg/kg) of the DOX drug in heart tissue. These new insights would consist in the recognition of regular training as a non-drug therapeutics protective strategy against DOX treatment. Thus, the main purpose of this study was to determine the preventive effects of 6 weeks of aerobic training on biomarkers related to the cardiac oxidative damage including; apelin, malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) in rats that have been acutely exposed to DOX-induced cardiotoxicity.

MATERIALS AND METHODS

Experimental design and laboratory environment

The experimental protocol of the current study approved by department of physiology, university of Mazandaran were performed according to guiding procedures in the care and use of animals, prepared by the Council of the American Physiological Society. The experiments were carried out with forty-eight Wistar male rats, (8-weeks-old, initially weighing 269 ± 4 g), which were obtained from the laboratory of animal bearing and multiplying at the Pasture institute of Iran.

Rats were housed in standard cages of polycarbonate (20 × 15 × 15 cm), made at the Pasture institute of Iran, in a large air-conditioned room with a controlled temperature of 22 ± 2°C, light-dark cycles of 12 : 12 h and humidity of 50 ± 5%. The pollutant standard index (PSI) was in the acceptable range as determined by the Iranian meteorological organization. Rats were fed with a standard rat chow provided by Pars Institute for animals and poultry with a daily regimen of 10 g per 100 g body weight for each rat. Water was available *ad libitum*.

Familiarization and exercise training protocols

Rats in all groups were adapted to the treadmill by running for 5 days. The familiarization protocol was designed as once a day for 10 min/session at a speed of 10 m/min at a slope of 0 degree. Because rats are more active in darkness, the front portion of the treadmill lines was covered with a dark thick paper to darken this area. At the rear of the lines, an electric grid provided a stimulus for running. An electric stimulus (30 V and 0.5 A) was manually turned on for less than 2 s when the animals stayed on the electric grid for longer than 10 s. Rats quickly learned to stay on the belt and avoid shock, except for one rat, which would not stay on the moving belt, and thus was quickly removed from familiarization process. Following this familiarization period, they were randomly assigned into sedentary and trained groups. Exercise training protocol was performed on treadmill with zero slopes between 25 to 54 min/session and 15 to 20 m/min, 5 days/week for 6 weeks (Table 1). We replicated the aforesaid exercise training protocol that was previously reported by Roshan et al. (2011).

Subjects classification

At the end of the exercise training protocol, rats from the sedentary and trained groups were again randomly separated into subgroups; the DOX (10, 20 mg/kg) and placebo treatment. Thus, the sedentary rats were distributed into sedentary + placebo (S + P, n = 8), sedentary + DOX (S + $DOX_{10\ mg/kg}$, n = 8) and sedentary + DOX (S + $DOX_{20\ mg/kg}$, n = 8) groups and the trained rats into trained + placebo (T + P, n = 8), trained + DOX (T + $DOX_{10\ mg/kg}$, n = 8) and trained + DOX (T + $DOX_{20\ mg/kg}$, n = 8) groups.

Table 1. Exercise training protocol in the current study.

Training sessions and variables		Weeks of training					
		1	2	3	4	5	6
1	Speed*	15	16	17	18	19	20
	duration#	25	30	35	40	45	50
2	Speed	15	16	17	18	19	20
	duration	26	31	36	41	46	51
3	Speed	15	16	17	18	19	20
	duration	27	32	37	42	47	52
4	Speed	15	16	17	18	19	20
	duration	28	33	38	43	48	53
5	Speed	15	16	17	18	19	20
	duration	29	34	39	44	49	54

*Meter/min; # min/session.

Doxorubicin treatment

Doxorubicin hydrochloride (EBEWE Pharma Ges.m.b.H.Nfg.KG) was dissolved in saline and administered by i.p injection at two dosages of 10 mg/kg (Karen et al., 2009) and 20 mg/kg (Ascensão et al., 2006), and control animals received saline with comparable volume. Both treatments were carried out at 24 h after the last exercise bout, and animals were sacrificed 24 h after DOX and placebo injections.

Heart tissue collection and preparation

All groups were anesthetized with ketamine and xylozine and decapitated after 10 to 12 h overnight fasting. The Thoracic cavity was opened and the heart was quickly excised from the aortic root. Heart tissues were weighed and it was placed into Petri dishes containing cold isolation medium (0.1 mol/L K_2HPO_4, 0.15 mol/L NaCl, pH 7.4) to remove the blood and were frozen immediately in liquid nitrogen and stored at -80ºC for subsequent analysis of apelin, NO, SOD and MDA. Heart tissue was squashed in liquid nitrogen, homogenized in a lysis buffer (5 ml/g of tissue) with a protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma-Aldrich, St. Louis, U.S.A) 100 ul/1 ml, and 10 m Mtris base (Sigma-Aldrich, St. Louis, U.S.A), pH 7.4 and centrifuged at 1500 g at 4ºC for 15 min. Heart supernatant was diluted 1:30. Plasma was diluted 1:10 and the fluids were used in an Apelin-13 ELISA kits (Phoenix peptides, Burlingame, California, USA), following the manufacturer's instructions.

Biochemical analysis

The assay kit was very specific and detects apelin-13 with 100% cross reactivity. It has an inter-assay variation less than 14% and intra assay coefficient of variation less than 10%. Apelin-13 in the mentioned sample was measured using ELISA kits too (Rat Apelin, ELISA, USCN LIFE Science Inc., Wuhan, P. R. China, USCN, Life Science Inc, Sensitivity 0.128 ng/ml and IntraCV: 5%). Lipid peroxidation (MDA) levels, as important marker in oxidative stress in the heart tissue, were measured with the thiobarbituric-acid reaction using the method of Daniel (Daniel et al., 2004). The quantification of thiobarbituric acid reactive substances was determined at 532 nm by comparing the absorption to a standard curve of MDA equivalents generated by acid catalyzed hydrolysis of 1,1,3,3 tetramethoxypropane. The values of MDA in heart tissue were expressed as nmol/g tissue. The NO concentration was determined by first reducing the nitrate to nitrite using nitrate reductase (Sigma). Superoxide dismutase (SOD) activity was determined spectrophotometrically using the method described by Roshan et al. (2011). In brief, for total SOD (tSOD) activity, the adequate amount of protein (2 mg tissue wet weight) was incubated at 25ºC with 1 m MN, Nbis (2-(bis(carboxymethyl)amino)-ethyl) glycine (DTPA) in 50 m MTris_HCl, pH 8.2, in 1 ml final volume. Reaction was started with 0.3 m mpyrogallol, in which the auto-oxidation rate was recorded at 420 nm.

Statistical analysis

All data have been expressed as mean ± SD. Statistical analysis was performed using a commercial software package (SPSS version 16.0 for Windows). Data of the biomarkers related to the cardiac oxidative damage including; apelin, malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) were normally distributed after log-transformation. A one-way analysis of variance (Statistics software, Stat Soft, Inc., Tulsa, OK) was used to detect statistical differences between groups. A post-hoc test (Tukey test) was performed to determine differences in the various biomarkers between groups. Differences were considered statistically significant at p-value < 0.05.

RESULTS

Mean values of body weight, heart weight and heart-body weight ratio for the six groups are shown in Table 2. At first, no differences existed in the aforesaid characteristic between groups (Table 2). Table 3 shows changes in apelin, NO, MDA and SOD levels following doxorubicin treatment in the various groups. After Doxorubicin administration (10 and 20 mg kg^{-1}), a significant increase in MDA (51 and 96%, respectively), an increase in apelin (41 and 49%, respectively), a significant decrease in SOD (9 and 18%, respectively) and an insignificant increase in NO (8 and 12%) were detected, as compared to S + P group ($P < 0.05$). Although, there was no

Table 2. Effect of aerobic training and DOX treatment on body weight, heart weight and heart-body weight ratio for each group.

Groups	Markers of weight		
	Body weight(g)	Heart weight(g)	Heart-body weight ratio
S+P	333±22.6	1.2±0.1	0.004±0.03
S+DOX_{10}	331±8.7	1.1±0.03	0.003±0.01
S+DOX_{20}	325±14.3	1.1±0.03	0.003±0.01
T+P	339±31.5	1.2±0.1	0.004±0.02
T+DOX_{10}	330±36.8	1.2±0.1	0.004±0.02
T+DOX_{20}	328±34.4	1.1±0.1	0.004±0.01

Data are presented as the Mean ± SD for 8 rats.

Table 3. Effect of aerobic training and DOX treatment on apelin, NO and MDA levels in the various groups.

Markers	Groups					
	S+P	S+DOX_{10}	S+DOX_{20}	T+P	T+DOX_{10}	T+DOX_{20}
Apelin (pg/mg protein)	3.6±0.46	5.1±0.33	5.4±0.44	8.2±0.9	7.1±0.6	6.8±0.8
NO (nmol/mg protein)	0.24±0.02	0.3±0.02	0.3±0.03	0.3±0.03	0.3±0.03	0.4±0.03
MDA (nmol/g protein)	29.6±1.9	44.7±3.7	58.7±8	20.3±4	31.4±7	37.5±6.4
SOD (u/mg protein)	92.3±2.9	84.3±4.4	75.3±6	125.6±2.6	113.4±3	105.7±4.1

Data are presented as the Mean ± SD for 8 Rats, Abbreviations: nitric oxide (NO), malondialdehyde (MDA), Superoxide dismutase (SOD). S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg.kg^{-1}), S + DOX_{20} (sedentary+ doxorubicin 20 mg.kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}).

significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg\text{-}1}$ treatments in apelin and NO levels, there was a significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg}^{-1}$ treatments in MDA and SOD levels.

Six weeks of the aerobic training led to a significant increase of heart apelin, NO and SOD levels (126, 25 and 36%, respectively), and an insignificant decrease in MDA, as compared to S + P group ($P < 0.05$) (Table 3). However, after six weeks of aerobic training and DOX treatment with 10 mg kg^{-1}, a significant increase in apelin and SOD (38 and 34%, respectively), and an insignificant increase in NO (10%), and a significant decrease in MDA (42%) were detected in comparison to S + DOX_{10} group ($P < 0.05$). In contrast, six weeks of aerobic training and DOX treatment with 20 mg kg^{-1} resulted in a significant increase in apelin, NO and SOD (25, 29 and 40%, respectively), and a significant decrease in MDA (36%), in comparison to S + DOX_{20} group ($P < 0.05$).

Data in Figures 1, 2, 3 and 4 show changes in the heart tissue apelin, MDA, NO and SOD levels, respectively, in the six groups. After six weeks of aerobic training and doxorubicin treatment, both 10 and 20 mg kg^{-1}, a significant decrease in apelin (13 and 17%, respectively), a significant decrease in SOD (10 and 16%, respectively), and a significant increase in MDA (54 and 85%, respectively) were detected, as compared to T + P group ($P < 0.05$). Moreover, treatment with $DOX_{20\ mg\ kg}^{-1}$ after six weeks of an aerobic training caused a significant increase in NO level (17%), as compared to T + P group ($P < 0.05$). However, there was no significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg}^{-1}$ treatments in apelin and MDA levels. Furthermore, there was a significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg}^{-1}$ treatments in NO and SOD levels.

DISCUSSION

Doxorubicin, a very potent and often used anti-cancer drug, has a wide spectrum of biological activity (Vishwanatha et al., 2012). The heart is particularly susceptible to free radical injury, because it contains less free radical detoxifying substances, superoxide dismutase, glutathione, and catalase than do metabolic organs such as liver or kidney. Moreover, doxorubicin is known to have a high affinity for cardiolipin, a major phospholipid component of the mitochondrial membrane in heart cells, resulting in selective accumulation of doxorubicin inside cardiac cells (Evert et al., 2001). Several studies have shown that doxorubicin induced cardiotoxicity (Evert et al., 2001; Chatterjeeet al., 2010; In Duk et al., 2002; Raschiet al., 2010; Andreadou et al., 2007; Khositseth et al., 2011). This study was designed to determine the preventive effects of aerobic regular

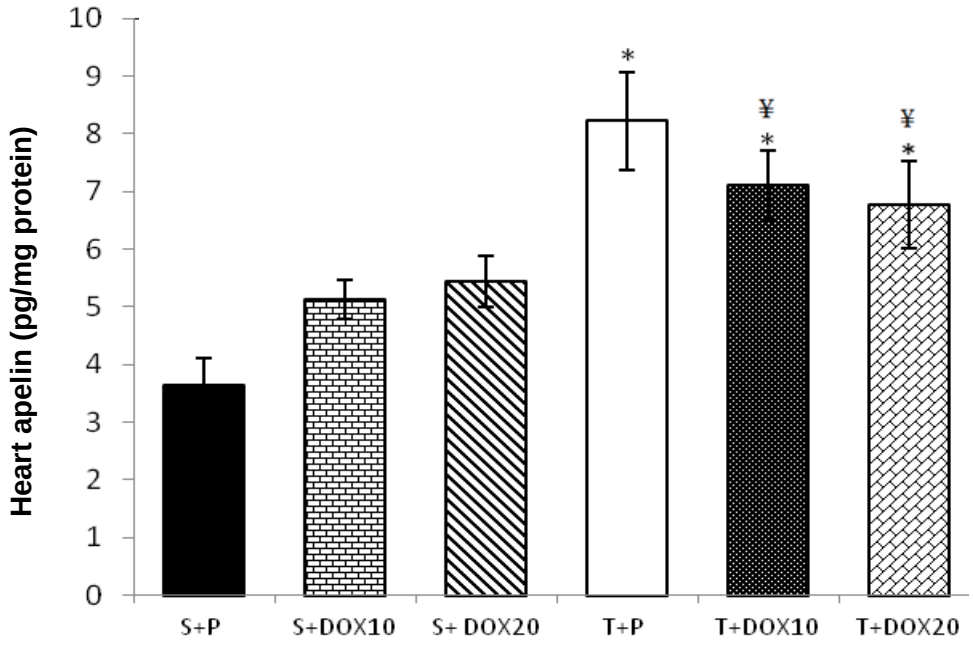

Figure 1. Apelin level after six weeks of aerobic training and DOX treatment. Abbreviations; S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg kg^{-1}), S + DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}). Data are presented as the mean ± SD for 8 Rats. *Significantly different to similar sedentary group (P<0.05), ¥ significantly different to T+P group (P < 0.05).

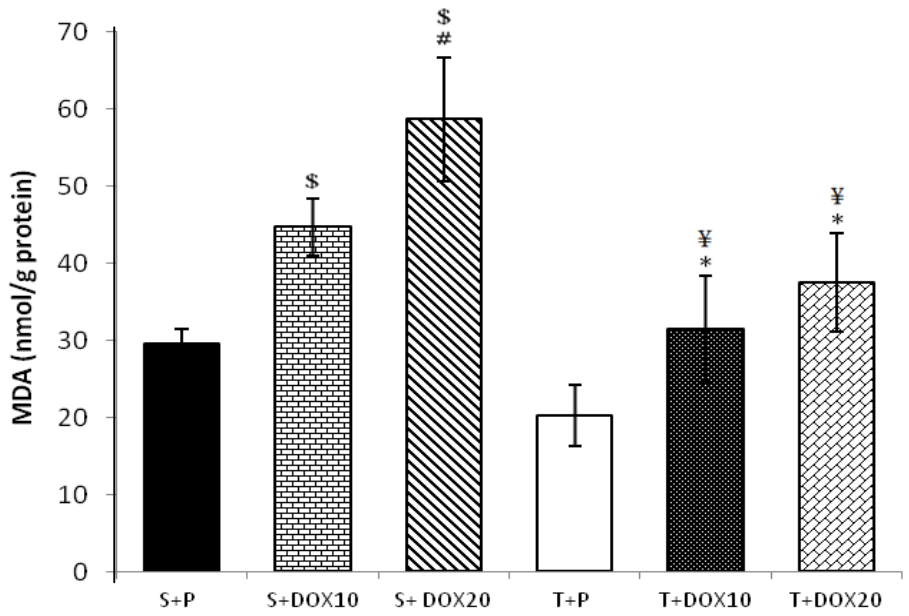

Figure 2. Malondialdehyde (MDA) level after six weeks of aerobic training and DOX treatment. Abbreviations; S+P (sedentary + placebo), S+DOX_{10} (sedentary + doxorubicin 10 mg.kg^{-1}), S+DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T+P (training + placebo), T +DOX_{10} (training + doxorubicin 10 mg.kg^{-1}), T +DOX_{20} (training + doxorubicin 20 mg.kg^{-1}). Data are presented as the mean ± SD for 8 Rats.*significantly different to similar sedentary group (P<0.05), $ significantly different to the S+P group (P<0.05), # significantly different to dose 10 mg kg^{-1}in it's group (P < 0.05), ¥ significantly different to the T + P group (P < 0.05).

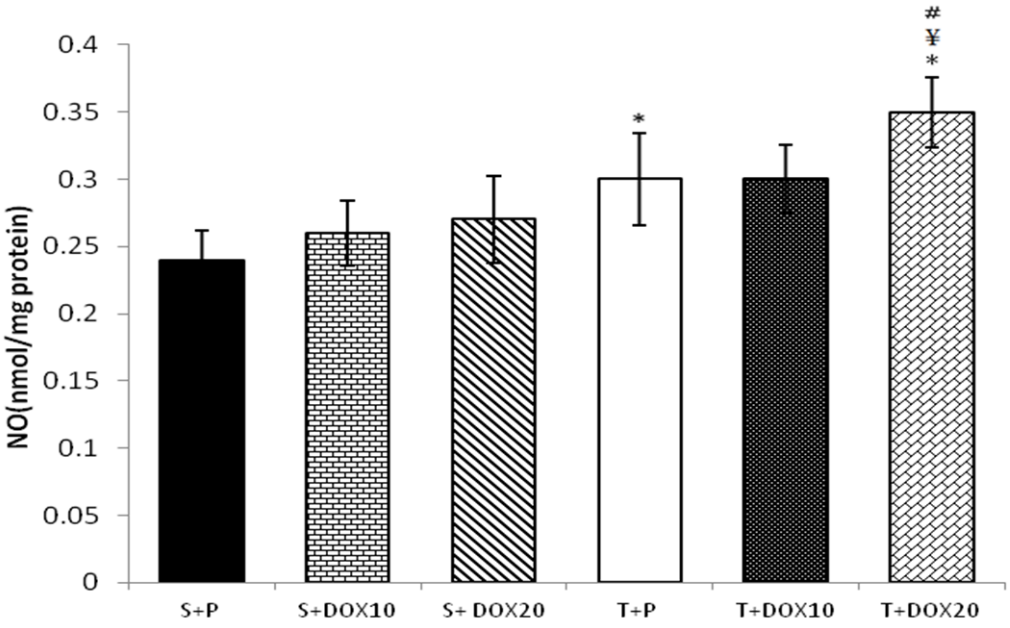

Figure 3. Nitric oxide (NO) level after six weeks of aerobic training and DOX treatment. Abbreviations; S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg kg^{-1}), S + DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}). Data are presented as the mean ± SD for 8 Rats. * significantly different to similar sedentary group ($P < 0.05$), # significantly different to dose 10 mg kg^{-1}in it's group ($P < 0.05$), ¥ significantly different to the T + P group ($P < 0.05$).

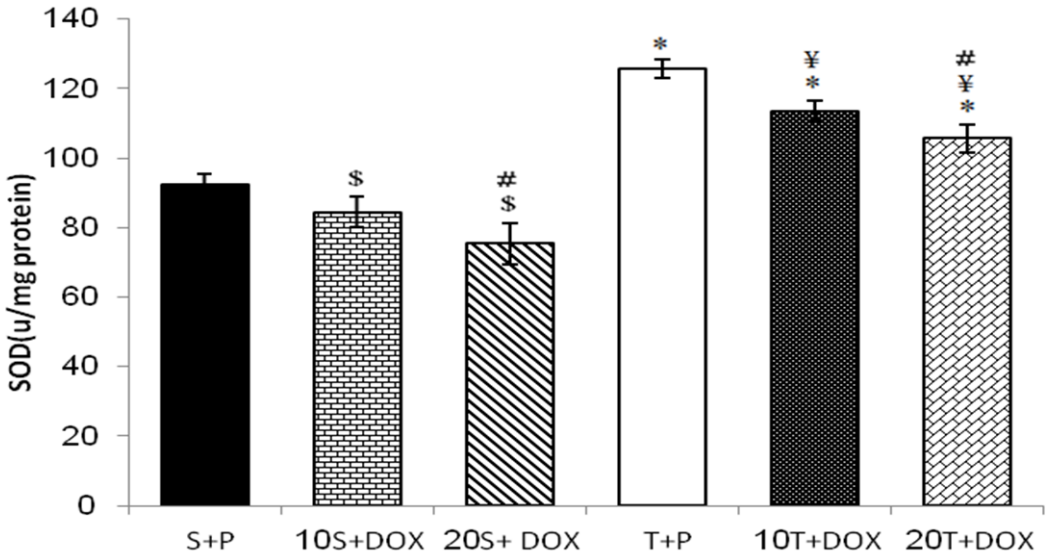

Figure 4. Superoxide dismutase (SOD) level after six weeks of aerobic training and DOX treatment. Abbreviations; S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg kg^{-1}), S + DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}). Data are presented as the mean ± SD for 8 Rats. *Significantly different to similar sedentary group ($P < 0.05$), $ significantly different to the S + P group ($P < 0.05$), # significantly different to dose 10 mg kg^{-1}in it's group ($P < 0.05$), ¥ significantly different to the T + P group ($P < 0.05$).

exercise on Doxorubicin-induced oxidative stress in rats. The primary finding in the present study was that after doxorubicin administration (10 and 20 mg kg^{-1}), a significant increase in MDA, increase in apelin, a significant decrease in SOD and an insignificant increase in NO were detected, as compared to sedentary + placebo (S + P) group.

In the past years, several mechanisms have been suggested to explain the pathogenesis of DOX-induced cardiotoxicity. Chatterjee reported that the mechanisms of therapeutic effects of doxorubicin on tumor cells are different from those of the mechanisms of its cardiotoxicity (Chatterjee et al., 2010). Oxidative stress, ion dysregulation, and alterations of the cardiac specific gene expression program cooperate at inducing cardiotoxicity and oxidative stress (Evert et al., 2001; Raschi et al., 2010; Vishwanatha et al., 2012; Viswanatha et al., 2011). Moreover, decreased levels of antioxidants and sulfhydryl groups, inhibition of nucleic acid and protein synthesis, release of vasoactive amines, altered adrenergic function and decreased expression of cardiac-specific genes are other proposed mechanisms (Chatterjee et al., 2010). However, the proposed principal mechanisms of doxorubicin cardiotoxicity are increased oxidative stress, as evident from increased levels of reactive oxygen species and lipid peroxidation (Chatterjee et al., 2010; Chen et al., 2007).

Raschi et al. (2010) reported that the high reactivity of ROS against cell constituents and the lack of effect of antioxidants against chronic oxidative stress suggest a role of ROS confined to acute cardiac dysfunction. Recent findings also suggest that cellular stress activates a host of kinase pathways that appear important in determining cell fate, and these pathways modulate the response of the heart to DOX exposure (Raschi et al., 2010). Although DOX can readily generate oxygen radicals in several ways, only few free radical scavengers have been reported to protect the heart from doxorubicin induced toxicity. Its mechanism of action appears to be the prevention of free radical formation by doxorubicin, probably through binding of iron. Furthermore, it has been reported that free radical scavengers such as vitamin E and N-acetylcysteine decrease both lethality and severity of cardiac histological lesions in rodents injected with doxorubicin. In contrast, other studies have shown that vitamin E and N-acetylcysteine failed to attenuate doxorubicin-induced cardiotoxicity in rats and dogs. Possible explanations could be the different experimental setups, timing of measurement, etc. Also, the reserve in antioxidant defense plays an important role. However, from the point of view of the free radical hypothesis, the study of Arai et al is noteworthy because this study showed how the formation of hydrogen peroxide and the down-regulation of the sarcoplasmic reticulum calcium pump are connected; explaining the pathway whereby the two could be related (Arai et al., 2000).

In this study, we found out that six weeks of aerobic training leads to a significant increase in apelin and SOD, an insignificant decrease in MDA, as compared to sedentary + placebo (S + P) group. While, treatment with $DOX_{10\ mg\ kg^{-1}}$ after six weeks of aerobic training caused a significant increase in apelin and SOD, an insignificant increase in NO levels and also, a significant decrease in MDA levels, as compared to sedentary + doxorubicin 10 mg kg^{-1} (S + DOX_{10}) group, treatment with $DOX_{20\ mg\ kg^{-1}}$ resulted in a significant increase in apelin, SOD and NO level and also, a significant decrease in MDA level, as compared to sedentary + doxorubicin 20 mg kg^{-1} (S + DOX_{20}). Moreover, a significant difference was detected between $DOX_{10\ mg\ kg^{-1}}$ and $DOX_{20\ mg\ kg^{-1}}$ treatments in apelin, NO, SOD and MDA levels. These data suggest that the increased oxidative stress production by DOX could be blocked by the pretreatment with aerobic regular exercise, with improve antioxidants and dysfunction markers. Data of the current study provided additional support to understand how regular physical exercise, particularly treadmill running training, could contribute to augmentation of cardiac muscle resistance against oxidative stress-based cardiotoxicity induced by DOX administration. Two lines of evidence can be emphasized from the present study. First, and considering cardiac stress marker, namely MDA, aerobic regular training decreased the rise of cardiac disturbances induced by acute single doses of DOX administration, particularly, in dose of 10 mg kg^{-1}. Second, and according to changes observed in cardiac apelin and SOD responses in both sedentary and trained rats hearts treated with DOX, it is likely that these markers might be considered as essential cellular defense against free radical-based cardiotoxicity caused by DOX, providing enhanced tolerance to trained myocardium at least in the first 48 h after the end of training period.

Physical exercise in its various forms has been shown to be an effective intervention that can provide a protective effect against acute and chronic deleterious insults for the myocardium. Moreover, although current data demonstrate that exercise training protects the heart against Dox-induced damage (Kavazis et al., 2010; Chicco et al., 2006), the mechanism(s) by which exercise training protects cardiotoxicity remain unclear. There were three possible pathways to explain the protective effects of regular endurance exercise against DOX-induced cardiotoxicity. At present, the principal mechanism of Dox-induced cardiotoxicity is believed to be increased oxidant production by the mitochondria (Kavazis et al., 2010; Chicco et al., 2006; Ascensão et al., 2005; Chicco et al., 2005). Mitochondria are also the major sites for the production of ROS. Exercise seems to increase the oxygen consumption rate by 10 to 20 folds as reported earlier, and might have released the above factors and thereby induced heart-SOD activity (Husain, 2002). In addition, researchers reported among other cell sources, heart mitochondria electron transport chain,

which has been referred to as one of the major sites of ROS production, through the so-called electron leakage. Whereas, the activity of mammalian cytochrome C oxidase is O_2-saturated at very low O_2 tensions, the rate of electron leakage by mitochondria increases at high O_2 concentrations during certain conditions such as exercise, favoring enhanced ROS production.

Nevertheless, when moderate and systematic, exercise could constitute an excellent tool either to prevent and/or to treat several diseases, providing enhanced parallel resistance to the cardiac muscle tissue. This phenomenon usually referred as cross-tolerance has been demonstrated by several studies in which endurance training seems to up-regulate heart antioxidant systems and mitochondrial function, to reduce the formation of lipid peroxidation by-products and to induce antioxidant defenses such as SOD and heat shock protein after certain stress stimuli (Ascensão et al., 2005). There is now considerable number of studies that suggests daily exercise antagonizes the harmful consequences of *in vivo* and *vitro* DOX treatment on rodent heart, either by preventing, attenuating or reverting the toxicity (Ascensão et al., 2005, 2006, 2012; Teixeira et al., 2011; Kavazis et al., 2010). Considering that DOX-induced cardiac toxicity has a marked oxidative etiology and that chronic exercise ameliorates the cardiac capacity of antioxidant systems to counteract with deleterious ROS effects, it can be suggested that the cardioprotection induced by aerobic exercise training against DOX was attributed at least in some extent to the up-regulation of antioxidant enzymes (Ascensão et al., 2005). Our results also support the concept that oxidant/antioxidant imbalance could be the primary event in DOX-induced cardiotoxicity (Ascensão et al., 2006). The authors reported that aerobic exercise results in many types of physiological (vascular, metabolic and functional) adaptations in the heart. Indeed, several studies have proposed that the increased cardiac function induced by regular exercise can be attributed to the enhanced cell defenses against oxidant production in the re-establishment of redox status.

According to the fact that DOX-induced cardiotoxicity is in part, due to increased free radical generation and hence, to oxidative stress, it is possible that the cardiac redox adaptations induced by aerobic training can contribute to the previously referred tolerance of myocardium to DOX. This up-regulation of glutathione system was expected to be accompanied by an increased activity of antioxidant enzymes. In our study, aerobic regular training induced significant increases in the apelin, NO and SOD levels. Although a significant increase in cardiac glutathione reductase (GR) and glutathione peroxidase (GPx) had been reported elsewhere after endurance swimming training (Childs et al.,2002), other studies demonstrated that swimming training was as such ineffective in raising these and other gene-modulated antioxidant enzymes like SOD and CAT (Ascensão et al., 2006). Some studies reported that exercise related to little but significant increases in myocardial SOD activity may be critical for protection against myocardial injury. The biological rationale for this adaptive response is unclear, but could be associated with the fact that regular exercise induced increases in myocardium ability to eliminate hydrogen peroxide and other organic peroxides resulting from DOX toxicity (Ascensão et al., 2006). In fact, the significant increased activity of SOD in DOX exposed hearts by aerobic regular training (training + placebo group versus training + doxorubicin 10 mg kg^{-1} group and training + doxorubicin 20 mg kg^{-1} group) may be possibly attributed to DOX-induced activation of enzyme activity through protein synthesis, indicating that the adaptations induced by aerobic regular training can be important for increasing the activity of this antioxidant enzyme in the presence of DOX.

Nitric oxide (NO) is a short lived free radical, synthesized from arginine, with extremely high reactivity and a variety of physiological activities. NO cause damage to DNA and is a potential endogenous carcinogen, and its increased production may increase angiogenesis and contribute to tumor progression (In Duk et al., 2002). It has been reported that doxorubicin increases superoxide formation by increasing endothelial nitric oxide synthase, which promotes intracellular hydrogen peroxide formation and recent evidence supports the significant role of nitric oxide synthase (NOS) (Chatterjee et al., 2010; Andreadou et al., 2007). Although, the basal production of NO through constitutive NOS isoforms in cardiomyocytes modulates ventricular contractility and blood flow distribution, higher NO production through iNOS is associated with severe cardiac lesions such as dilated cardiomyopathy and congestive heart failure (In Duk et al., 2002; Andreadou et al., 2007). It has been reported taht high concentrations of NO participate in cardiomyocyte oxidative damage, apoptosis, and/or necrosis through peroxynitrite formation (Evert et al., 2001). Furthermore, overproduction of NO is cytotoxic, and suppresses tumor growth, whereas low NO production may protect cells from apoptosis and promote tumor growth. However, the effects of NO on tumor cells are apparently production dependent, and cell type specific (In Duk et al., 2002). It has also been reported that NO contributes to DOX's antitumor effect (Liu et al., 2008). NO may influence several aspects of tumor biology including cell growth, apoptosis, differentiation, metastatic capability and tumor induced immune suppression (Kavazis et al., 2010).

More recent data support that nitric oxide is involved in DOX-induced toxicity in cardiac (Ascensão et al., 2005) as well as cerebral (Gruber et al., 2010) tissues. Doxorubicin promotes the synthesis of NO and reactive oxygen species, such as the superoxide anion. The reaction of NO and superoxide anion leads to the synthesis of peroxynitrite which is a potent cellular

oxidant (Evert et al., 2001). The effects of DOX on NOS are well established, and show that an increased expression of NOS is associated with DOX-induced apoptosis. However, the effects of DOX on the production of NO by iNOS from studies on rats are not well identified (In Duk et al., 2002).

In summary, our study suggests that the cardiotoxicity induced by DOX may be attributed to oxidant/peroxidant imbalance in heart. Moreover, in this study, we found that cardioprotection induced by aerobic regular exercise in hearts from DOX treated rats was associated with inhibition of oxidative stress and the up-regulation of antioxidant enzymes. Therefore, our study suggests that aerobic regular exercise before administration of DOX may be considered as a potentially useful candidate to limit cardiotoxicity during and after DOX therapy.

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