

## Age-Related Changes in Doxorubicin-Induced Oxidative Damage: Protective and Pretreatment Effects of Short-term Aerobic Exercise

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### ABSTRACT

**Background and Objective:** Doxorubicin (DOX) is an effective anticancer drug. It has been shown that a short-term exercise performed prior to DOX-treatment has no effect on cardiotoxicity in young rats. In the present study, old and young rats were evaluated to determine the protective effects of pre-treatment with short-term exercise on DOX-induced oxidative damage in cardiac tissue.

**Methods:** Forty-eight male Wistar rats were randomly divided into two groups of young and old, and later divided into three sub-groups of young+DOX, young+training+DOX, young+training+salin, old+DOX, old+training+DOX and old+training+salin. The training protocol included treadmill running for 25-39 min/day at 15-17 m/min, 5 days/week for three weeks. All treatments were carried out 24h after the last exercise bout. The rats were sacrificed 48h after DOX administration.

**Results:** Although DOX injection significantly affected the cardiac tissue of old rats compared to young rats, pretreatment with endurance training in DOX-treated rats caused an increase in Heat shock protein (3.02% vs. 23.36) and superoxide dismutase (30.12% vs. 31.12), and a decrease in malondialdehyde (10.92% vs. 19.60) in both old and young rats.

**Conclusion:** Although DOX-induced production of free radicals and cardiotoxicity in aged rats is more than that in young rats, the short-term aerobic exercise reduced the damaging effects of free radicals in the old rats more than in young ones. The concentration of antioxidant enzymes also increases with exercise in the old rats compared to young rats.

**Keywords:** Cardiotoxicity, Doxorubicin, Oxidative Stress, Aerobic Exercise.

## INTRODUCTION

Doxorubicin (DOX) is one of the most effective chemotherapeutic factors used in the treatment of various types of cancer (1, 2). However, the clinical use of DOX is often limited because of its serious toxic side effects on different organs such as the heart, liver, etc.(3). Most studies confirm that oxidative stress plays an important role in the pathogenesis of DOX-induced toxicity (4, 5).

Several mechanisms have been proposed to be attributed for the DOX-induced cardiotoxicity including free radicals, lipid peroxidation, mitochondrial damage and cellular toxicity (6,7). The overproduction of free radicals and oxidative stress in violation of anti-oxidants plays a main role in the development of DOX-induced tissue damage (8,9). Malondialdehyde (MDA) is a marker of lipid peroxidation. Evidence suggests that the increased oxidative stress due to increased free radical production and decreased myocardial endogenous antioxidants play important roles in the pathogenesis of heart failure.

Aging is caused by accumulation of molecular damage in DNA (10, 11). Oxidative stress increases with age due to macromolecular damage and/or an imbalance between reactive oxygen/nitrogen species production and antioxidant defense (12, 13). In addition, the incidence of cancer is high among the elderly (14, 15), demonstrating the role of oxidative stress in aging-associated diseases (16, 17). Therefore, it is necessary to find the strategies that reduce the adverse effects of oxidative stress and DOX treatment in this age group, and cancer-treated elderly in particular. In contrast, several studies have reported that an organism is generally protected from free radicals damage via its antioxidant defense system, which is modulated by administration of DOX (18). The cellular antioxidant defense system operates mainly via antioxidant enzymes, such as superoxide dismutase (SOD), and independent from non-enzymatic antioxidants such as heat protein shock (HSP) (19). Induction of HSP<sub>70</sub> is known to occur in myocardial tissue following exercise training,

which is associated with the exercise-induced preservation of cardiac function during states of oxidative stress (19, 20). Primary functions of HSP include protection against apoptosis and oxidative damage (17, 21). According to previous studies, the main function of antioxidants is the detoxification of reactive species and toxic oxygen metabolites generated by the endogenous and exogenous metabolism pathways (22).

Heart is one of the most affected organs in DOX therapy (23). Therefore, it is necessary to seek for complementary and alternative strategies against DOX-induced cardiotoxicity. Although, there is evidence that acute exercise results in oxidative stress and cardiac damage (24), it seems that regular endurance training could be helpful for prevention and/or treatment of several diseases. Moreover, while most recent studies have focused on the beneficial effect of endurance exercise in DOX-induced cardiotoxicity among young adults, most elderly cancer patients have been relatively neglected. Thus, the increased quality of life in this group of people is essential (25). A new insight could consist of recognizing regular training as a non-pharmaceutical therapeutic strategy for treatment with DOX. There is a hypothesis that DOX-induced cardiotoxicity may be related to free radical formation and oxidative stress, and enhanced antioxidant/oxidation ratio after regular endurance exercise may protect against DOX-induced toxicity in cardiac tissue. Therefore, this study investigated the protective effects of pretreatment with short-term aerobic exercise on oxidative damage, HSP<sub>70</sub>, SOD and MDA of young and old rats acutely exposed to DOX.

## MATERIAL AND METHODS

The male Wistar rats were divided into two groups based on age (3 and 32 months). The animals were then randomly separated into subgroups of control+DOX (20 mg/kg, n=8), training+DOX (T+DOX20mg/kg n=8), and

training+saline (T+S). The subjects were habituated to treadmill running for 5 days (10 min exercise/day at 10 m/min, 0% grade). The exercise training program was performed on treadmill with zero slopes for 25-39 min/session at 15-17 m/min, 5 days/week for three weeks. The mentioned exercise training was performed according to the protocol described by Ashrafi et al. (Table 1) (3). DOX was obtained from EBEWE Pharma Ges.m.b.H.Nfg, KG (A-4866 unterach, Austria) as a vial of ph.Eur. DOX was dissolved in 0.9% saline to prepare a concentration of 20 mg/kg, which is the clinical dose pharmacologically scaled for use in rats (16).

The rats in all groups were anesthetized with ketamine (30–50 mg/kg of body weight, ip) and xylazine (3–5 mg/kg of body weight, ip) after 10-12 hours of overnight fasting. The subjects were placed supine, the abdominal cavity was opened to expose left ventricle, and 2-ml blood samples were collected in tubes. Then, hearts were quickly excised, rinsed, carefully dried, weighed and placed into Petri dishes containing cold isolation medium (0.1 mol/L K<sub>2</sub>HPO<sub>4</sub>, 0.15 mol/L NaCl, at pH 7.4) to remove the blood. They were frozen immediately in liquid nitrogen and stored at -80 °C (22,23). The heart samples were homogenized in a homogenization buffer (0.05 M Tris, 0.03 M Lserine, and 0.06 M boric acid, pH 7.6; 100 mg of tissue/ml of buffer). HSP<sub>70</sub> was measured using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Zellbio Co., Germany). First, 100µl of standard, blank and sample were added to each well. The liquid was removed from each well and 100µl of biotin-antibody working solution were added to each well. Each well was aspirated and washed three times. Then, 100µl of HRP-avidin working solution was added to each well. The aspiration and washing process were repeated five times in the fourth step. Moreover, 90 µl of 3,3',5,5'-Tetramethylbenzidine substrate was added to each well. Then, 50 µl of stop solution was added to each well, while the first four wells containing the highest concentration of standards showed an obvious blue color.

Finally, the optical density of each well was determined within 30 minutes at 450nm using a microplate reader. Later, SOD activity and MDA content of the supernatants were evaluated using commercial assay kits (Zellbio Co., Germany) according to the method described by Chularojmontri et al. (24, 25). One-way analysis of variance (Stat Soft, Inc., Tulsa, OK) was used to identify significant differences among the groups. A post-hoc test (Scheffe's test) was performed to determine differences in levels of different biomarkers among the groups. The P-value less than 0.05 was considered statistically significant.

## RESULTS

Mean levels of HSP<sub>70</sub>, SOD and MDA in tested rats that have been acutely exposed to DOX (20 mg/kg) are shown in Table 2. Administration of 20 mg/kg DOX in the old rats caused insignificant increases in MDA (5.55%) and HSP<sub>70</sub> (17.57%) levels, while causing an insignificant decrease in SOD levels (3.86%) when compared to the young rats. Moreover, three weeks of aerobic exercise in the old group caused a significant increase (16.89%) in the MDA, an insignificant decrease (1.81%) in the HSP<sub>70</sub> and an insignificant decrease (4.20%) in SOD level compared to the young group.

After three weeks of aerobic exercise, HSP<sub>70</sub> level increased in the young and old groups (23.36% vs. 3.02%). There was a significant increase in SOD level (31.12% vs. 30.60%) and a significant decrease in MDA level (19.60% vs. 10.64%) in comparison with the control+DOX group.

Moreover, three weeks of pretreatment with aerobic exercise caused a significant and an insignificant increase in HSP<sub>70</sub> level of young rats and old rats in the training+salin group, respectively. The SOD level increased significantly in young rats and insignificantly in old rats. MDA levels of young and old rats in the training+salin group decreased significantly compared to training+DOX and control+DOX groups.

Table1- Exercise training protocol used in the present study

Training sessions and variables	Weeks of training			
	1	2	3	
1	speed *	15	16	17
	duration #	25	30	35
2	speed	15	16	17
	duration	26	31	36
3	speed	15	16	17
	duration	27	32	37
4	speed	15	16	17
	duration	28	33	38
5	speed	15	16	17
	duration	29	34	39

\*Meter/min; # min/session

Table2- Effect of pretreatment with aerobic exercise and DOX treatment on oxidative stress-related biomarkers in the cardiac tissue of subjects in different groups.

Markers	Groups	Y +DOX	Y+T+DOX	Y+T+S	E +DOX	E+T+DOX	E+T+S
HSP <sub>70</sub> (ng/mg protein)		21.4 ±2.44	26.4 ±1.98	30.2±2.86	25.16 ±3.6	25.92 ±4.74	27.99±5.55
SOD(μ/mg protein)		75.31 ±6	98.75 ±2.75	112.23±3.06	72.43±4.84	94.6 ±6.83	102.87±6.46
MDA(nm/mg protein)		58.66±7.97	47.16±10.15	22.35±4.25	61.89±4.93	55.13±6.97	27.61±3.95

Data are presented as the Mean±SD for 8 rats; Y+ DOX (young + 20 mg.kg<sup>-1</sup>DOX), Y+T+ DOX (young+training+ 20 mg.kg<sup>-1</sup> DOX), Y+T+S (young+training+salin), E+DOX (old + 20 mg.kg<sup>-1</sup> DOX), E+T+DOX (old + training + 20 mg.kg<sup>-1</sup> DOX), E+T+S (old+training+salin).

## DISCUSSION

Previous studies have focused on the role of physical activity as a non-pharmaceutical strategy for treatment of cancer. To the best of our knowledge, we are the first to investigate the effect of pretreatment with short-term endurance training before DOX administration on markers related to cardioprotection and cardiac stress in old and young rats. The results indicated a potential relationship between aged-associated oxidative stress and DOX-induced cardiac stress. In addition, the data suggested that pretreatment with regular short-term aerobic exercise could prevent increased oxidative stress caused by DOX administration by improving antioxidants' activity. These findings helped in understanding how regular physical exercise, particularly running on a treadmill, could contribute to augmentation of cardiac muscle against DOX-induced cardiac stress (26). In this regard, Kavazis, Chicco and Ascensao reported that intraperitoneal injection

of 20 mg/kg of DOX to rats significantly increases HSP<sub>70</sub>, cardiac malfunction, and coronary blood flow decreases SOD expression and increases lipid peroxidation (MDA) (17, 22, 23).

The use of DOX is limited for chemotherapy due to its toxicity (26, 27). It disrupts the oxidant-antioxidant systems, resulting in tissue damage caused by lipid peroxidation and protein oxidation (28). The present study revealed severe biochemical changes as well as an oxidative damage in cardiac tissue after treatment with 20mg.kg<sup>-1</sup> of DOX. However, the exact mechanism of DOX-induced cardiac stress remained unclear. Nevertheless, a large body of evidence indicates the formation of oxygen free radicals, which can damage cells through lipid peroxidation (7, 29). Free radicals are continuously produced in vivo, and there are a number of protective antioxidant enzymes (SOD, catalase, etc.) for dealing with toxic substances. The delicate balance between production and catabolism of oxidants is

critical for maintaining biological functions (6, 30). According to the results of the present study, regular endurance training decreased the level of MDA and cardiac disturbances induced by administration of acute single dose of DOX. Considering the changes observed in SOD (partially) and HSP<sub>70</sub> of rats treated with DOX in response to training, these markers might be taken into account as essential cellular defenses against free radical-based DOX-induced cardiotoxicity, providing enhanced tolerance to trained myocardium at least in the first 48 h after the end of training period. Moreover, the three weeks of aerobic training and DOX administration (20mg.kg<sup>-1</sup>) significantly increased HSP<sub>70</sub> and SOD, while causing a significant decrease in MDA compared to the control+DOX group (20mg.kg<sup>-1</sup>). However, the findings demonstrated that the exercise could protect the heart against DOX-induced damage (3, 31). These findings are consistent with the findings of our previous study that showed the cardioprotective role of endurance training in DOX-treated rats (22). Since DOX-induced cardiotoxicity has a marked oxidative etiology (32), and chronic exercise improves cardiac capacity of antioxidant systems to counteract with adverse ROS effects (33), it can be suggested that the exercise training-induced protection against DOX could be partially attributed to improvement in the cardiac antioxidant systems. There were three possible pathways to explain the protective effects of regular endurance exercise against DOX-induced cardiac stress. Aging is a multifactorial process resulting in damage of molecules, cells, and tissues. It has been demonstrated that the expression and activity of antioxidant systems (SOD, HSP) are modified in aging, with reducing cell ability to counteract the oxidant molecules, and consequent weak resistance to ROS accumulation. Physical activity is one of the best methods in treatment of age-related chronic-degenerative diseases and in prevention of changes associated with aging. It also increases the expression and activity of

antioxidant enzymes, with consequent reduction of ROS. Positive effects of physical exercise on antioxidant activity could be ascribable to a greater expression and activity of SOD enzymes and HSP<sub>70</sub> (17, 25). Currently, the principal mechanism of DOX-induced cardiotoxicity is believed to be increased oxidant production by the mitochondria (22, 23), which is a main site of ROS production. Exercise seems to increase the oxygen consumption rate by 10-20 fold, and might have released the above factors and thereby induced heart-SOD activity (3). The phenomenon usually referred as cross-tolerance has been demonstrated by several studies in which endurance training seems to up-regulate cardiac antioxidant systems and mitochondrial function, to reduce the formation of lipid peroxidation by-products and induce antioxidant defenses such as SOD and HSP after certain stress stimuli (22, 23). Our results also support the concept that oxidant/antioxidant imbalance could be the primary event in DOX-induced cardiotoxicity (22). The study of Rinaldi et al. showed that a physical training program increased the level of SOD and HSP<sub>70</sub> in trained old rats compared to sedentary old and young rats. Physical activity is able to reduce the generation of oxidants during ischemia-reperfusion injury and plays a calcium-protective role via activation of the ROS scavenger enzyme (MnSOD). This improved oxidative status consequent to a correct physical activity program is partially responsible for some benefits such as decreased arterial stiffness, improved endothelial function, metabolic and clotting setting, and reduced body weight (31, 34). An interesting finding that may provide further insight into the effects of DOX on the myocardium was a slight decrease in HSP<sub>70</sub> protein content in the heart of DOX-treated rats (36). Induction of HSP<sub>70</sub> is known to occur in myocardial tissue following exercise training and is associated with the exercise-induced preservation of cardiac function

During oxidative stress regardless of its cardioprotective role. Over-expression of HSP could be interpreted as an acute sign of cellular stress (25). Hence, given the vast protective properties of HSP<sub>70</sub>, we hypothesized that exercise-induced increases in myocardial HSP<sub>70</sub> level played an important role in exercise-induced cardioprotection against DOX-mediated cardiac injury (11, 35). Therefore, regular endurance training caused a significant increase in the HSP<sub>70</sub> and SOD activity and a decrease in MDA of cardiac tissue of subjects in the training+DOX groups.

## CONCLUSION

The present study suggests that DOX treatment be associated with oxidant/antioxidant imbalance in cardiac

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tissue. The present study provides new insights into the biochemical mechanisms through which pre-treatment with endurance exercise training exerts its potential antioxidant properties, and protects cardiac tissue against the DOX-induced toxicity.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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