Effects of age increment and 36-week exercise training on antioxidant enzymes and apoptosis in rat heart tissue

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Abstract
This study investigated the onset of age-related changes in the myocardial antioxidant enzymes and apoptosis and the vulnerability of the myocardium to oxidative stress following exercise training. Few studies have investigated the influence of the most prevalent life-prolonging strategy physical exercise, on the age increment alterations in the myocardial antioxidant enzymes and apoptosis at mid age and to determine whether exercise-induced antioxidant defense system could attenuate lipid peroxidation. Thirty six male Wistar rats were randomly assigned to exercise-trained (n = 18) and sedentary (n = 18) groups. The rats in the training group went under 12, 24 and 36 weeks of moderate exercise trainings (25 m·min\(^{-1}\) for 60-min with a 0% slope). Six sedentary controls were killed together with each exercise group at the end of the training programs. Levels of thiobarbituric acid-reactive substances (TBARS) and catalase (CAT) activity in myocardial homogenates were unchanged by training irrespective of the protocol duration. However, an increased content of the TBARS was detected in hearts from both the 24 and 36-week trained and sedentary control rats when compared with their corresponding 12-week groups (p<0.01). The activity of superoxide dismutase (SOD) remained unchanged after the 12-week training period whereas a significant increase was observed in heart homogenates of 24-week trained animals as compared with their sedentary controls (p<0.05). The activity of glutathione peroxidase (GPX) remained unchanged. The rates of apoptosis which was detected by ELISA assays, were significantly modified after 24 and 36-week of training (p<0.05). These results demonstrate that a long-term endurance training (24 weeks) induced increases in SOD activities in rat myocardium and elicited a marked reduction in apoptosis rate. However, a shorter training program (12 weeks) was not effective in increasing heart antioxidant defenses.

Key words: Oxidative stress, superoxide dismutase, apoptosis, heart, exercises training.

Introduction
In resting conditions, for every 25 O\(_2\) molecules reduced by normal mitochondrial respiration in aerobic organisms, one incomplete O\(_2\) reduction occurs (McCord, 1979), that generate reactive oxygen species (ROS), such as superoxide, hydrogen peroxide or hydroxyl radical which are highly reactive molecules that can attack and damage cellular structures. The myocardium may be especially vulnerable to oxidative damage because it has a high VO\(_2\) due to continuous working, and low antioxidant defences (Leeuwenburgh et al., 1999). In addition, post-mitotic tissues such as the myocardium have a lesser ability to up-regulate antioxidant defences (Ji, 2000).

Much controversy exists concerning the effects of endurance training on the oxidative status and antioxidant defences systems of the myocardium as decrease, increase, or even remain unchanged (for a comprehensive review see Ji, 1999). Some controversy might arise from the different methodologies used for determinations, and differences in the models employed (running vs. swimming, rats vs. mice, male vs. female). In this regard, it is worthy to note that total training volume (duration and intensity) could be a critical factor to induce adaptations as previously proposed by Powers et al. (1993). Most studies employ moderate intensity protocols which have duration no longer than 12 weeks. If intensity, duration of the daily sessions, and/or total duration of training program are not prolonged, the total training volume could be insufficient to elicit adaptations given the aforementioned low ability of the myocardium to up-regulate its antioxidant defences. In this regard, total duration of training could influence the type of heart response against exercise-linked oxidative stress.

On the other hand, no study has shown conclusive justification for the functional or physiological role of apoptosis in postmitotic muscle cells in young and adult healthy individuals and to what extent apoptosis takes place in these tissues. However, the influence of regular moderate physical activity in apoptosis is still unknown. There is evidence showing that oxidative stress contributes to the mediation of apoptosis (Beere et al., 2000; Yuan et al., 2003). Increased oxidative stress has been shown to be related to the activation of apoptosis (Gorman et al., 1996; Pollack et al., 2002; Yuan et al., 2003). Because exercise training has been consistently shown to increase the antioxidant defence capacity of cardiac muscles (Locke, 1997; Powers et al., 2002), it seems plausible to hypothesize that exercise training is able to decrease the level of apoptosis.

Therefore, the purpose of this study was to determine modulation of the enzymatic antioxidant system and apoptosis rate during age increment in rat heart tissue and the effects of both a short (12 weeks) and a long-term (24 and 36 weeks) endurance training program on these factors.

Methods

Animals

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Thirty six male Wistar rats (12-week old and initial body mass of 235 ± 27 g) were obtained from laboratory animal house of Tabriz University of Medical Sciences. They were housed in an animal room at 22-24 ºC and given free access to commercial rat chow and tap water. The animals were adapted to an inverse 12:12 h light: dark cycle (light period 08:00–20:00 hours) before the beginning of the exercise program. All the experimental procedures employed, as well as rat care and handling, were in accordance with guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Committee of the Tabriz University of Medical Sciences.

Training program
Rats were randomly divided into sedentary (n = 18) and exercise-trained groups (n = 18). Each group was divided into three subgroups (12, 24 and 36 weeks sedentary life and trained rats). All the rats in the trained group ran on a rodent motor-driven treadmill (Danesh Yakhteh Co, Tabriz, Iran) and performed weekly six exercise sessions of 60-min duration at 25 m min⁻¹ with a 0% slope. From the beginning of the training protocol until the 4th week, the workload (from 10-min duration at 15 m min⁻¹ with a 0% slope) was progressively increased in intensity and duration, so that in the 5th week, the rats could run anticipated workload after a warm-up period of 5 min at 15 m min⁻¹. After 12-week training, 12-week trained (n = 6) and sedentary rats (n = 6) were killed (12-week or short-term protocol animals). The remaining rats in both 24 and 36-week trained and sedentary groups were killed after 12 and 24 additional weeks of training (n = 6) or sedentary lifestyle (n = 6). While trained rats were undergoing the training programs, sedentary control rats were placed on the treadmill for 10 minutes daily to familiarize themselves with treadmill and handling.

Tissue processing and homogenate preparation
At the end of the training programs, 48 h after the last exercise-training session, the rats were weighed, anaesthetized with ether, and killed by decapitation. The heart was quickly removed, washed with ice-cold saline, and blotted. After the atria and great blood vessels were trimmed, the ventricles were weighed, and the apex was cut and quickly frozen in liquid N₂. Duration of the process was less than 2 min. Cardiac homogenates were prepared at 0–4 ºC as previously described (Rothermel et al., 2000). In brief, fifty milligrams of ventricle muscle were homogenized on ice in 1 mL of ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, pH 7.4). The homogenates were centrifuged at 1000 rpm for 1 min at 4ºC. The supernatants contained the cytoplasmic protein fraction were collected and protease inhibitor cocktail (104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64) (P8340, Sigma-Aldrich, St Louis, MO) was added to them and stored at 80 ºC until use. The protein concentration of the homogenates was determined using Total Protein kit (Randox labs. Crumlin, UK).

Lipid peroxidation measurement
Lipid peroxidation was analyzed by measuring thiobarbituric acid-reactive substances (TBARS) in homogenates, as previously described by Draper and Hadley (1990). Briefly, the samples were mixed with 1 mL 10% trichloroacetic acid (TCA) and 1 mL 0.67% thiobarbituric acid. Then the samples were heated in a boiling water bath for 15 min, butanol (2:1:v:v) were added to the solution. After centrifugation (800g, 5 min), TBARS were determined from the absorbance at 535 nm.

Antioxidant enzymatic activities
Superoxide dismutase (SOD) activity was determined using a RANSOD kit (Randox labs. Crumlin, UK) according to Delmas-Beauvieux et al. (1995). SOD activity was measured at 505 nm on a spectrophotometer on supernatant. In this method, xanthin and xanthin oxidase was used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (ITN) to form a red formazan dye. Concentrations of substrates were 0.05 mmol-L⁻¹ for xanthin and 0.025 mmol-L⁻¹ for INT. SOD was measured by the degree of inhibition of this reaction.

Glutathione peroxidase (GPX) activity was determined using a RANSEL kit (Randox labs.), according to the method of Paglia and Valentine (1967). GPX catalyses the oxidation of glutathione (at a concentration of 4 mmol-L⁻¹) by cumene hydroperoxide. In the presence of glutathione reductase (at a concentration ≥0.5 units/L) and 0.28 mmol/L of NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NAD⁺. The decrease in absorbance at 340 nm was measured using a spectrophotometer.

Catalase activity (CAT) was measured as previously described by Aebi (1984). The decomposition of H₂O₂ was followed directly by the decrease in absorbance at 240 nm and 20 °C. Previously, myocardial homogenate aliquots were centrifuged at 1000 g and 4 °C for 10 min. The adequate amount of supernatants (60 µL equivalent to 1.5 mg tissue wet weight) was added to a reaction mixture that contained 0.002% Triton X-100, 0.1 mm EDTA, 0.5 m potassium phosphate buffer, pH 7.0, and 15 mm H₂O₂ in 1 mL final volume. Activity was calculated with the initial 30s decomposition rate.

Quantification of apoptosis
Cell death detection ELISA kit (1544675, Roche Molecular Biochemicals, Mannheim, Germany) was used to quantitatively detect the cytosolic histone-associated DNA fragmentation, according to manufacturer’s instructions (Siu et al., 2004). Briefly, the extracted cytoplasmic fractions of ventricle muscle were used as an antigen source in a sandwich ELISA with a primary antimouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2’-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 10 min at 20°C. The change in color was measured at a wavelength of 405 nm. All measurements were performed in duplicate, with CON and TR samples analyzed on the same microtiter
plate in the same setting. The OD405 reading was then normalized to the total amount of protein in the sample. The data were reported as an apoptotic index (OD405·mg protein⁻¹) to indicate the level of cytosolic mono and oligonucleosomes.

Data analysis
All determinations were performed at least in duplicate. Data were expressed as mean ± SD and were analyzed by a two-way ANOVA. To test for the two main effects (exercise training and protocol duration) and for the interaction between them, using a standard computerized statistical program, SPSS13.0 for windows software (SPSS INC, Chicago, IL, USA). When a significant P-value was obtained, the LSD Tukey post-hoc test was employed to determine the differences between groups. A level of p < 0.05 was selected to indicate statistical significance.

Results
Oxidative stress marker
There was a significant increase in TBARS, a lipid peroxidation biomarker myocardial content when comparing the 24 and 36-week groups of trained and sedentary, with their respective 12-week counterparts Figure 1, (p < 0.01), But failed to show a significant main effect for the training factor.

Antioxidant enzymes
There was significant increase in SOD activity between 24-week trained animal and their sedentary control (P < 0.05, Figure 2a). SOD activity tended to increase in all trained animals compared with their 12-week counterparts but the difference did not reach statistical significance.

Figure 2b shows that CAT activity tended to decrease in all animals during protocol compared with their 12-week counterparts but only 36-week trained rats group was reached to statistical significance with their 12-week counterparts (p < 0.05).The GPX was unaffected either by training or protocol duration (Figure 2c).

Figure 1. Effects of 12, 24 and 36-week training protocols on levels of thiobarbituric acid-reactive substances (TBARS) in rat myocardium. Results are mean ± SD of independent preparations.
** Significant differences between the 24 and 36-week groups and the corresponding 12-week groups (p < 0.01).

Figure 2. Effects of 12, 24 and 36-week training protocols on antioxidant enzymes activity in rat myocardium. (a) Superoxide dismutase (SOD), (b) Catalase (CAT), and (c) glutathione peroxidase (GPX).
# p < 0.05 compared with trained group. * p < 0.05 between the 24 and 36-week groups and the corresponding 12-week groups.
Apoptosis
ELISA estimated rate of apoptosis in heart homogenates shows that the rate of apoptosis were significantly decrease after 24 and 36-week of training rats compared with their sedentary controls (Figure 3, p < 0.05).

Discussion
The main finding of this study was that increase of myocardial SOD activity during a long-term treadmill training which was more apparent during 24 weeks. Furthermore rate of apoptosis was decreased in heart homogenates of long term trained animals. In contrast, a shorter (12 weeks) training protocol with the same intensity did not significantly modify these parameters. It should be pointed out that the mild electrical stimulation used to encourage the rats to run did not likely cause an additional stress to exercise for trained animals. Both 12- and 24-week training protocols are moderate-intensity training programs for rodents and workload was progressively increased during the initial 4 weeks. Therefore, the rats which are very young at the beginning of the training period were able to cope easily with the conditions of the daily exercise session, and electrical stimulation was almost unnecessary.

Previous research has analyzed the effects of exercise training on myocardial antioxidant systems but the overall results are controversial (for a comprehensive review see Atalay and Sen, 1999 and Ji, 1999). Only two previous studies (Kanter et al., 1985, Morán et al., 2004) have evaluated the adaptive response of these systems to a long-term training protocol (21 weeks and 24 weeks, respectively) and shown increase in CAT, GPX and SOD activities. But to our knowledge no other study has used a training program with duration as long as 36 weeks. The aforementioned modifications suggest that maturing of the rats throughout the experimental period could induce oxidative stress in heart enough to elicit on one hand lipid peroxidation, and on the other hand, an adaptive response of some antioxidant enzymes to avoid more cellular oxidative damage. ROS generation has been reported to increase with age (Bejma et al., 2000) and elevated levels of TBARS and augmented SOD activity have been detected in old rats (Ji, 1993, Fiebig et al., 1996).

Training on its own did not alter cardiac levels of TBARS. The absence of variations in these parameters in response to exercise training is in agreement with previous research (Bejma et al., 2000; Fiebig et al., 1996; Husain and Somani 1997; Liu et al., 2000; Powers et al., 1998). This result suggests that both training programs were insufficient to raise the basal levels of oxidative dant defences. In this regard, Delgado et al. (1999) and Morán et al. (2003) have shown that the Ca\(^{2+}\) regulatory systems involved in the excitation-contraction coupling and relaxation processes remained unaltered in rat heart after a 12-week treadmill training whereas adaptive changes were found following a 24-week training program similar to used in this study. The results of this study support the idea that a prolonged training protocol is required to increase heart antioxidant defences. However, a second possibility which could be considered is that the rats from the 24-week groups were more prone to suffer oxidative stress because their older age in turn, would increase the need for improved antioxidant defences to cope with the stress associated to exercise sessions. Our data are consistent with this hypothesis that levels of oxidative stress biomarkers and antioxidant enzymatic activities were higher in 24 and 36-week than in 12-week animals independently of their training status, i.e. these changes were also apparent in sedentary rats (Irrespective of Significance). Increased TBARS content in 24 and 36-week rats points to an enhanced myocardial lipid peroxidation. Also SOD activity was also increased in 24-week rats compared with the 12-week animals. The aforementioned modifications suggest that maturing of the rats throughout the experimental period could induce oxidative stress in heart enough to elicit on one hand lipid peroxidation, and on the other hand, an adaptive response of some antioxidant enzymes to avoid more cellular oxidative damage. ROS generation has been reported to increase with age (Bejma et al., 2000) and elevated levels of TBARS and augmented SOD activity have been detected in old rats (Ji, 1993, Fiebig et al., 1996).

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![Figure 3. Effects of 12, 24 and 36-week training protocols on apoptosis in rat myocardium. Results are mean ± SD of independent preparations.

# Significant difference between the trained and their sedentary groups (p < 0.05).](image-url)
stress in lipids. Other authors have reported increased ROS production after a single bout of exercise (Bejma et al., 2000; Kumar et al., 1992; Ohkuwa et al., 1997), and increases in lipid peroxidation or oxidized proteins in adult and old rat hearts (Bejma et al., 2000; Kumar et al., 1992; Leeuwenburgh et al., 1999). Nevertheless, our animals were killed 48 h after the last exercise session, and hence the response to exercise analyzed is an adaptive response. Therefore, the absence of alterations in TBARS content in trained animals could reflect an adaptation that leads to minimize the transitory oxidative stress occasioned by a single exercise session.

The training-induced increase in myocardial SOD activity shown in the present investigation likely represents an important defense mechanism against oxidative stress. The enhancement in SOD activity suggesting that probably its activation results from augmented exercise-linked mitochondrial O$_2$ production. It is interesting to note that the training-induced increase in SOD activity only reached statistical significance in the 24-week trained rats; hence exercise-linked mitochondrial superoxide generation may be greater in the 24-week than in the 12-week trained rats due to their older age. However, it is possible that because our low samples the difference in 36-week trained rats do not reach to statistical significance. As SOD provides the first line of defense against produced mitochondrial superoxide, the increase in SOD activity shown in our long-term trained animals could reduce the exposure to superoxide and even to the hydroxyl radicals formed via the Haber–Weiss reaction.

The CAT activity in myocardium homogenate was unchanged by training irrespective of the protocol duration. However, the CAT activity of trained and sedentary animals tended to decrease during protocol, only a decreased activity of CAT in 36-week trained reach to significance as compared with their corresponding 12-week groups (p < 0.05). The meanings of these changes are unclear at present. Previous investigations have shown decreases (Hong and Johnson 1995; Kihlstrom, 1990; Kihlstrom et al., 1989; Moran et al., 2004; Wilson and Johnson, 2000), no changes (Demirel et al., 2001; Fiebig et al., 1996; Husain and Hazelrigg, 2002; Powers et al., 1993; 1998) or even increases (Husain and Hazelrigg, 2002; Somani et al., 1995) in CAT activity after training. Harris and Starnes (2001) points to a temporal evolution of myocardial antioxidant levels as a function not only of the duration of the training program, but also of the state of adaptation to exercise stress and core temperature. Differences in these factors might explain the controversial results about CAT activity in response to training.

In this study GPX activity was not affected by training. Results from previous studies analyzing the response of both enzymes to training are disparate. Increases in GPX activity (Husain and Hazelrigg, 2002; Husain and Somani, 1997; Somani et al., 1995), and decreases in this parameter have been described (Hong and Johnson, 1995; Wilson and Johnson 2000). Nevertheless, most authors have reported absence of variations, including works performed with high volume protocols (Bejma et al., 2000; Demirel et al., 1998; Fiebig et al., 1996; Kanter et al., 1985; Leeuwenburgh et al., 1997; Powers et al., 1993; 1998).

Therefore, it seems reasonable to hypothesize that probably no further adaptation is needed in this enzyme to assist the heart in its defence against the oxidative stress associated to exercise and increase in SOD activity might be enough to protect myocardium from exercise-related oxidative stress; However the protective role of SOD can be doubtful when increase in SOD activity has not been coupled with catalase or GPX activities.

The present study provides evidence that exercise training is capable of influencing apoptosis in cardiac muscle in adult rats during age increment. We showed that exercise training attenuates the extent of apoptosis in cardiac muscle when measurements are taken at 48 h after the last exercise bout. The influence of exercise training on apoptosis has not been fully defined, and only a few studies have attempted to study the relationship of acute strenuous exercise with apoptosis in skeletal muscle (Podhorska et al., 1999; Sandri et al., 1995). Activation of apoptosis in skeletal locomotion muscles following acute bouts of exercise is not fully understood; It is unclear whether the proposed increase in muscle apoptosis after intense exercise plays a protective role in improving the muscle’s recovery process or accelerates muscle deterioration. Nonetheless, increased production of ROS and glucocorticoids during intense exercise has been suggested to be the possible reason for increased apoptosis after acute intense exercise (Phaneuf and Leeuwenburgh, 2001). In contrast to acute strenuous exercise, the effect of exercise training on apoptosis is unknown. Furthermore, few data exist to describe the influence of exercise training in cardiac tissue. For example Siu et al. (2004) have showed that the protein content of Mn-SOD increases (39%) and apoptosis rate decreases in the TR animals ventricle muscles relative to the CON animals after 8 wk of endurance training and they suggested that exercise training may attenuate muscle apoptosis. In the present study, we have demonstrated that long-term exercise training reduced DNA fragmentation of cardiac muscle when compared with sedentary control animals. This is the first study to show a relationship between long-term regular moderate-intensity exercise (36-week). Our data are suggests that an increased antioxidant capacity due to exercise training may be associated with reduced apoptosis.

Conclusion

In conclusion, the present investigation demonstrates that a long-term endurance training program of moderate, constant intensity, induced increases in the activity of myocardial SOD and decreases the rate of apoptosis, whereas a shorter (12 weeks) training protocol of similar characteristics did not enhance antioxidant defences. It should be taken into account is that 24 and 36-week animals are more prone to suffer oxidative stress because of their older age, condition that would increase the need for improved antioxidant defences to cope with the stress imposed by the exercise sessions.

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References


Key points
- Exercise training induces activity of myocardial SOD.
- Long-term regular moderate-intensity exercise decreases the rate of myocardial apoptosis.
- Short-term regular moderate-intensity exercises do not change the rate of myocardial anti oxidant capacity and apoptosis.

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