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Vulnerability of the mid aged rat myocardium to the age-induced oxidative stress: Influence of exercise training on antioxidant defense system

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Abstract
This study investigated the onset of age-related changes in the myocardial antioxidant defense system (ADS) 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-dependent alterations in the myocardial antioxidant enzyme system of female rats at mid age and to determine whether exercise-induced ADS could attenuate lipid peroxidation. Two age groups young (3 months old) and mid age (12 months old) Wistar strain female albino rats were given chronic exercise training for a period of 12 weeks. We found a striking decrease ($p < 0.01$) in the activity levels of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) in the myocardium of mid aged rats when compared to young rats by 36, 50 and 29%, respectively, suggesting the onset of age-dependent decrease in the myocardial ADS. A similar age-related decrease ($p < 0.01$) was observed in the reduced glutathione (GSH) content (36%). Despite the reduction in ADS, lipid peroxidation (LPO) (20%) was also decreased. In contrast, exercise training significantly elevated ($p < 0.01$) these antioxidant enzyme activities and the content of GSH. The increase in SOD and CAT activities were more pronounced in the mid aged rats when compared to younger rats, but increased the level of lipid peroxidation to higher levels in the mid-age group following the training regimen. The findings of the present study suggest that, although the activity levels of the myocardial antioxidant enzymes were elevated with the 12 weeks of exercise training, the changes were not sufficient enough in attenuating oxidative stress in the myocardium of female rats during this short period of exercise training.

Keywords: *Exercise training, aging, oxidative stress, antioxidant defense system*

Introduction

The heart is a highly aerobic organ, characterized by high oxygen consumption rate, energy out put (ATP turn over) and a high mitochondrial population and density among all body tissues [1]. The electron transport associated with the mitochondrial respiratory chain is considered to be the major metabolic process leading to reactive oxygen species (ROS) production at rest and during exercise [2]. Increased production of ROS is a feature of most, if not all, human diseases, including cardiovascular disease and cancer [3]. Exercise increases the oxygen consumption rate by 20-fold, resulting in increased production of ROS, which could be harmful to the cells [4–7]. However, the cells are equipped with a host of enzymes that are directly or indirectly involved in the antioxidant defense to protect against ROS damage.

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The antioxidant defense system (ADS) consists of free radical scavenging enzymes like superoxide dismutase (SOD), catalase (CAT) and antioxidants such as reduced glutathione (GSH), vitamin C, E etc.

It is known that the heart is metabolically active and may generate more superoxide anions ($O_2^{\bullet -}$). Heart tissue has four times less antioxidant enzyme activity and GSH levels compared to liver and other tissues [8]. Therefore, this tissue may be more vulnerable to peroxidative damage due to oxidative stress. An imbalance caused by increased generation of free radicals and decreased functional efficiency of ADS has been suggested to be one of the primary factors that contribute to the aging process [9]. As a result of aging, there is a general decline in antioxidants. Exercise is known to increase the cell's oxidative capacity and to improve endurance factors that generally decline with age. Exercise brings about specific physiological adaptations that involve subtle cellular as well as gross physiological changes and enhances the antioxidant defense [10]. Although, the impact of exercise on ADS has been studied in detail in different age groups, few studies have investigated the adaptations of antioxidant enzymes to exercise training during mid age, the stage in the life span of rats which is most reflective of humans, at which time they are engaged in intense exercise activities.

Investigative studies involving the exercise training of very old animals have a major limitation in that, it is exceptionally difficult to train very old animals without causing minor and/or major leg injuries. Moreover, it is unclear if changes are due to training and/or inflammation. The intensity and duration of exercise training may also vary with the old animals when compared to young animals. The additional rationale to take up this study is that few studies have investigated the effects of exercise training on antioxidant enzymes in the mid age female rats. Hence, the present study was undertaken to test two hypotheses (1) it is believed that the onset of age-related changes especially disturbances in the anti-oxidant enzyme system and vulnerability to oxidative stress is during the mid age and (2) oxidative damage leading to the aging process would be challenged by amelioration of antioxidant system by exercise training.

Materials and methods

Animal care and training protocol

Wistar strain female albino rats were used in the current investigation. Approved by the Institutional Animal Ethics Committee (Regd No. 438/01/a/CPCSEA/dt.17.7.2001) in its resolution number 9/IAEC/SVU/ZOOL/dt.4.3.2002. Cao and Cutler [11] reported aging physiological changes occurs from 6 months onwards in rats. The maximum life span of Wistar strain rats ranges from 24 to 36 months [12]. Hence, in the present study, the 3 months age group was considered young and the 12 months age group as mid age. Female Wistar strain albino rats weighing 160–185 g were used in the present study and were fed with a standard rat pellet diet and water *ad libitum*.

The female rats of each age group (3 months and 12 months) were divided into two batches of six each. One batch of rats from each age group was subjected to treadmill exercise with constant gradient of 7.5% at a speed of 23 m/min, 30 min/day, 5 days/week for a period of 12 weeks. The second batch of animals from each age group served as control rats. The batch of rats selected for exercise training was given 1 week of treadmill acclimatizing before experimentation. Initially, they were made to exercise 5 min/day at 23 m/min, with a progressive increase to 30 min/day over a period of 1 week and thereafter for 30 min/day at 23 m/min for a total training period of 12 weeks. Gentle hand prodding and mild electric shock (20 mV, 1.67 Hz) was combined to encourage the animals to run throughout the study. The running program was scheduled between 6.00 and 8.00 AM. The animals were sacrificed after 24 h of the last exercise session by cervical dislocation and heart tissues were isolated. The tissues were washed with cold saline and immediately immersed in liquid nitrogen and stored at −80°C for biochemical analysis and enzymatic assays. Before assay, the tissues were thawed, sliced and homogenized under ice cold conditions.

Analytical procedures

Chemicals and solvents. Riboflavine, Nitroblue tetrazolium, Triton X-100, GSSG, NADPH, Dithio-bis-Nitrobenzic acid, Glutathione reductase, Thiobarbituric acid were obtained from Sigma Chemicals (St Louis, MO). All organic solvents were of spectral grade and general chemicals were of reagent grade.

Biochemical assays

Superoxide dismutase (SOD, EC 1.151.1). SOD activity was measured as the inhibition of photoreduction of nitroblue tetrazolium (NBT) by the enzyme described in Beauchamp and Fridovich [13]. The tissue homogenate (10% w/v) was prepared in ice cold potassium phosphate buffer (pH 7.5), containing 1% polyvinyl pyrolidone (w/v) and centrifuged at 16,000*g* for 15 min at 4°C. The supernatant was used as enzyme source. The total reaction mixture consisted of 100 mM phosphate buffer (pH 7.5), 10 mM EDTA, 130 mM methionine, 750 mM NBT, 60 mM riboflavine and 0.025 mg of enzyme source. The reaction was initiated

by the addition of riboflavine and the samples were placed under a tube light (fluorescent) for 30 min and the resulting color was read at 560 nm against the reagent blank kept in dark place. The amount of enzyme that results in 50% inhibition of the NBT photoreduction is defined as one unit.

Catalase (CAT, EC 1. 11. 1.6). Catalase activity was measured following the method of Chance and Machly [14]. The tissue was homogenized (10% w/v) in ice cold 50 mM phosphate buffer (pH 7.0) and centrifuged at 16,000*g* for 45 min at 4°C and again the supernatant was centrifuged at 1,05,000*g* for 1 h at 4°C and the resulting supernatant was used as the enzyme source. The reaction mixture contained 2 ml of phosphate buffer (pH 7.0), 0.45 ml of hydrogen peroxide (30 mM H_2O_2) in buffer and 2.5 mg of enzyme source. The absorbance was read at 240 nm against the blank. The enzyme activity was expressed in μmoles of H_2O_2 metabolized/mg protein/min.

Glutathione reductase (GR, EC 1. 6. 4. 2). The GR activity was assayed by the method of Carlberg and Mannervik [15]. Tissue was homogenized in ice cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% Triton X-100 and the contents were centrifuged at 16,000*g* for 45 min at 4°C. The resulting supernatant was used as the enzyme source. The reaction mixture in a total volume of 3 ml contained 1.5 ml of 0.2 M sodium phosphate buffer (pH 7.6), 0.3 ml of 10 mM GSSG, 0.3 ml of 1 mM NADPH and 0.9 ml of enzyme source and distilled water. The reaction was initiated by adding enzyme source and oxidation of NADPH was monitored at 340 nm against the reagent blank. The enzyme activity was expressed in μmoles of NADPH oxidized/mg protein/min.

Glutathione content (GSH). Glutathione content was determined according to the method of Akerboom and Sies [16]. The tissue was homogenized in 0.1 M ice cold phosphate buffer containing 0.001 M EDTA (pH 7.0) and protein is precipitated with 1 ml of 5% sulfosalycylic acid (w/v) and the contents were centrifuged at 5000*g* for 15 min at 4°C. The resulting supernatant was used as the enzyme source. The reaction mixture in a volume of 2.5 ml contained 2.0 ml of 0.1 M potassium phosphate buffer, 0.05 ml of NADPH (4 mg/ml of 0.5% $NaHCO_3$), 0.02 ml of DTNB (1.5 mg/ml), 0.02 ml of glutathione reductase (6 units/ml). The reaction was initiated by adding 0.41 ml of tissue source and change in absorbance was recorded at 425 nm against the reagent blank. The glutathione content was expressed in μmoles/g wet weight of the tissue.

Lipid peroxidation. The lipid peroxides were determined by the Thiobarbituric acid (TBA) method of Ohkawa et al. [17]. The tissue was homogenized in ice cold 1.5% KCl (w/v). To 1 ml of tissue homogenate, 2.5 ml of 20% TCA (w/v) and 2.5 ml of 0.05 M sulphuric acid were added. To this, 3 ml of TBA was added and the samples were kept in a hot water bath for 30 min. The samples were cooled and malondialdehyde (MDA) was extracted with 4 ml of n-butanol and read at 530 nm in a spectrophotometer against the reagent blank. The trimethoxy pentane (TMP) was used as external standard. The values were expressed in μmoles of malondialdehyde formed/ gram wet weight of the tissue.

Protein was estimated by the method of Lowry et al. [18].

Statistical analysis

Statistical analyses were made using two-Factor analysis of variance (ANOVA) with age (Young/Mid) as factor-1, treatment (Exercise/Control) as factor-2. The ANOVA was run using SPSS (version 10.0) statistical package. Results are reported as the mean ± SD of six observations, with the level of significance set at $p < 0.01$.

Results and discussion

SOD activity was significantly decreased ($p < 0.01$) in the mid aged rats when compared with younger ones (Figure 1). Exercise training significantly elevated the enzyme activity in both age groups of rats (Figure 1).

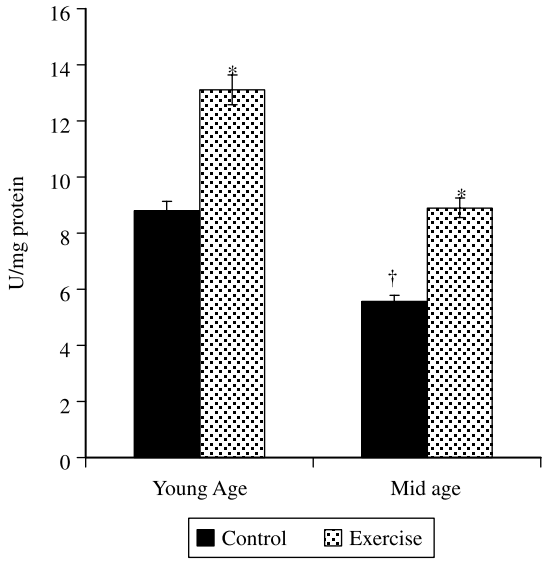

Figure 1. Effects of exercise on the myocardial total superoxide dismutase (SOD) activity in young (3 months) and mid age (12 months) rats. Values represent means ± SD; $n = 6$ rats. †Age effect (on young control rats): values are significant ($p < 0.01$). ⋆Exercise effect (on age matched control rats): values are significant ($p < 0.01$).

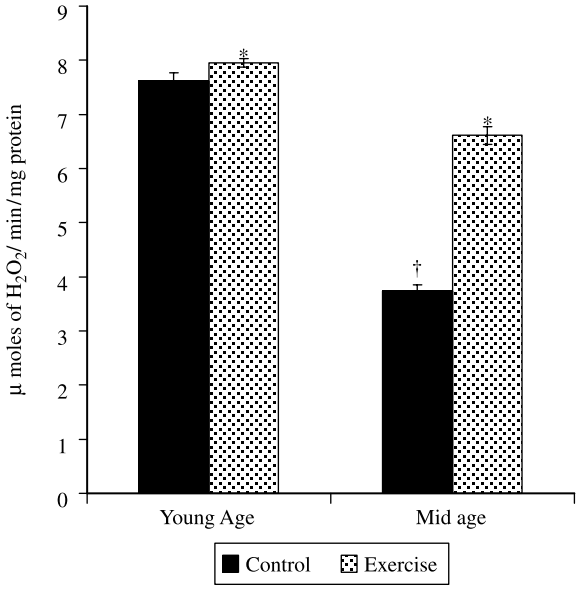

Figure 2. Effects of exercise on the myocardial catalase (CAT) activity in young (3 months) and mid age (12 months) rats. Values are means ± SD; $n = 6$ rats. †Age effect (on young control rats): values are significant ($p < 0.01$). ⋆Exercise effect (on age matched control rats): values are significant ($p < 0.01$).

Similarly, CAT activity was decreased in the mid aged rats compared to younger rats and with exercise it was increased significantly (Figure 2). However, this increase was more pronounced in the mid aged rats (76%) than in the younger (4%) animals (Figure 2).

GR activity was decreased with age and the exercise training significantly elevated ($p < 0.01$) the GR activity

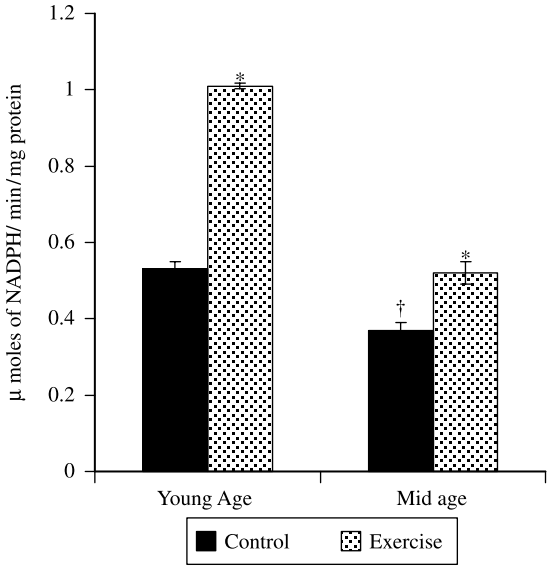

Figure 3. Effects of exercise on the myocardial glutathione reductase (GR) activity in young (3 months) and mid age (12 months) rats. Values are means ± SD; $n = 6$ rats. †Age effect (on young control rats): values are significant ($p < 0.01$). ⋆Exercise effect (on age matched control rats): values are significant ($p < 0.01$).

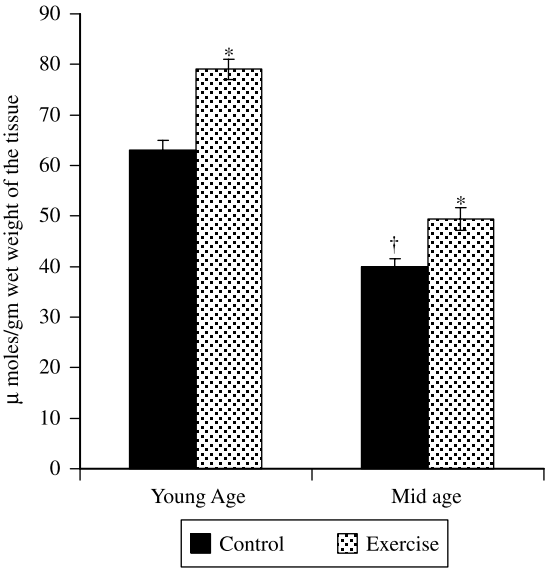

Figure 4. Effects of exercise on the myocardial reduced glutathione (GSH) level in young (3 months) and mid age (12 months) rats. Values are means ± SD; $n = 6$ rats. †Age effect (on young control rats): values are significant ($p < 0.01$). ⋆Exercise effect (on age matched control rats): values are significant ($p < 0.01$).

in both age groups (Figure 3). The levels of GSH which were also decreased in the myocardium of mid aged rats when compared to younger age were elevated significantly with exercise training (Figure 4). This increase was observed more in mid age exercised rats when compared to young age exercised rats (Figure 4).

MDA levels were significantly decreased ($p < 0.01$) in the mid aged rats than the younger rats, indicating the decreased lipid peroxidation in the myocardium of mid aged rats (Figure 5). Following the 12-week

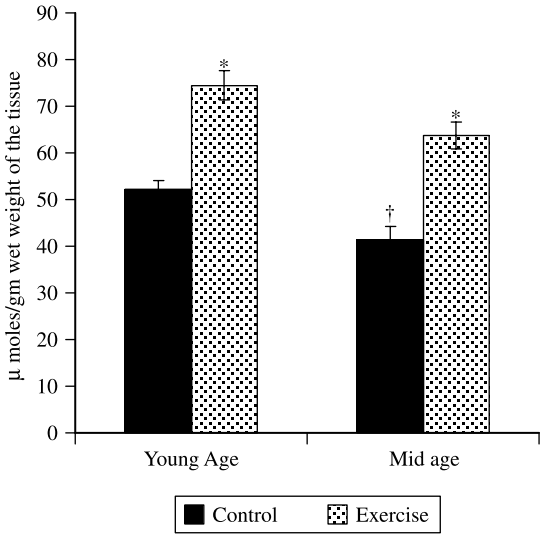

Figure 5. Effects of exercise on the myocardial lipid peroxidation (LPO) level in young (3 months) and mid age (12 months) rats. Values are means ± SD; $n = 6$ rats. †Age effect (on young control rats): values are significant ($p < 0.01$). ⋆Exercise effect (on age matched control rats): values are significant ($p < 0.01$).

exercise training period, MDA levels were significantly increased in both age groups of rats. However, the percent increase was more (54%) in mid aged rats compared to younger rats (42%).

SOD is involved in dismutation of the superoxide anion ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and oxygen and the results of the present study demonstrate a decrease in the SOD enzyme activity in the mid aged rats than the younger rats. This decrease in the activity of SOD, observed in the present study indicates either reduced synthesis of the enzyme or elevated degradation or inactivation of the enzyme during the onset of aging. It has been reported that in male Fischer 344 rats, the cytoplasmic SOD activity levels of the brain, heart, hepatocyte, intestinal mucosa and kidney decreased between 6 and 24–26 months of age and a large decrease in the SOD activity of hepatocytes occur between 6 and 16 months of age [19]. It has also been observed that, in Wistar rats the total cytoplasmic and mitochondrial SOD levels of the brain, liver, lung, heart and kidney remain unchanged or decreased during aging [20]. In contrast to the above findings, it has been reported, that the activity of SOD enzyme increases in skeletal and heart muscles of rats with advancing age [21]. These results contain many conflicting findings which may arise from differences in the strains of rats and maintenance conditions used, because SOD is thought to be readily induced by oxidative stress [22]. It is quite possible that heart may generate more ROS, which in turn, would inhibit SOD activity. Similar to our results, reports are available in the heart, brain and liver tissues of male rats of 12 months age group [23] and in the liver of female rats [24]. In response to treadmill exercise training, SOD activity in the myocardium of mid aged rats increased to levels comparable to those of unexercised controls. The SOD activity is sensitive to tissue oxygenation and its biosynthesis has been reported to be elevated in rats subjected to high oxygen tension [25]. The oxygen utilization has increased during exercise training, therefore, SOD activity was elevated with exercise. Earlier studies demonstrated that exercise training and exhaustive exercise increases SOD activity in the tissues of liver, lung and muscle of rat [26,27].

Catalase is inhibited by $O_2^{\bullet -}$ [28]. Superoxide anion served to convert catalase to the ferroxy and ferryl states, which are inactive forms of the enzyme [29]. Hence, the lower activity of CAT observed in mid aged rats may be due to lower levels of SOD, which was similar to the reported results of the present study or may be due to inactivation of catalase owing to excess production of ROS. Rao et al. [19] reported that in male Fisher rats, the CAT activity levels in brain, liver and kidney decreased with advancement of age. Similar results were observed by Indira Sriram and Jhansi Lakshmi [23] in the liver, heart and brain of male Wistar rats of 12 months age. In the present study, CAT activity was significantly increased with exercise training. The elevated activity of catalase in the exercised rats of mid age indicates its active involvement in the decomposition of H_2O_2 produced during exercise. A change in the binding characteristics of enzyme to membranes or their release from peroxisomes has been proposed as a possible mechanism for increased activity levels of CAT [30]. CAT and SOD are considered to be indispensable for the survival of the cell against deleterious effects of hydroperoxides. The combined action of SOD and CAT provide an efficient mechanism for removal of free radicals from the cell [31].

GR activity, which controls endogenous level of reduced glutathione (GSH), was decreased in the mid aged rats when compared to younger rats, suggesting an inadequate level of reducing equivalents (NADPH) and a failure to maintain GSH levels. The decrease in enzyme activity also suggests the possible free radical mediated oxidative stress and consequent damage to the myocardium. Sohal [32] reported that the younger age group rats exhibit lesser oxidative damage and this increased with the advancement of age. The activity of GR enzyme that converts GSSG to GSH was increased in exercise-trained rat heart mitochondria [33]. Similar results observed in the present study indicate the sub cellular adaptive response of the heart during exercise training. It was reported that endurance exercise is known to increase the GR activity in the skeletal muscles of rat [34]. GR activity elevated in the present study is to maintain GSH turnover in the rat heart under exercise stress condition.

GSH serves as a sensitive marker of oxidative stress and it plays an important role in maintaining the integrity of the cell system. Age-related alterations in the levels of reduced glutathione seems to be very complicated. In the present investigation, the myocardium of mid aged rats showed lower levels of GSH as compared to those of younger rats. The lower levels of GSH content in the mid aged rat myocardium observed in the present study indicate the greater participation of reduced glutathione in detoxifying the peroxides formed during age-related changes. Because the myocardium utilizes a significant amount of GSH during the metabolic activity, the GSH status in the myocardium may be subjected to a delicate balance between GSH consumption and uptake. GSH is synthesized primarily in the liver and then it is transported to different organs of the body via blood [35]. It is hypothesized that exercise increases the delivery of GSH to this organ. This enhanced intracellular transport of GSH seems to be essential in maintaining the redox state and to cope with the oxidative stress during exercise training. Myocardial GSH has been reported to be augmented after swim training in rats [36]. In mice also the GSH levels were increased significantly by exercise [37]. The results of the present study are inconsistent with the above results.

In the present study, we observed a decline in MDA content in the heart tissue of mid aged female rats. Lipid peroxidation decreases during the period of increased mitotic activity such as regeneration [38] or in transformed cells [39]. Zhan et al. [40] reported that there was an age-related decline in $O_2^{\bullet -}$ production. In their study, free radical generation in the liver, testis and ovary of Wistar rats significantly declined from 3 to 24 months of age. Sawada and Carlson [41] quantified mitochondrial superoxide production and showed that free radical production in mitochondria is organ-dependant. Exercise training significantly elevated the lipid peroxidation levels in the myocardium of young and mid aged rats. The increase in lipid peroxidation with exercise training was more pronounced in the myocardium of mid aged rats. ROS generated under increased oxygen consumption during exercise training, interact with membrane lipids causing lipid peroxidation. The increased lipid peroxidation in this study may be due to increased metabolic rate, activation of xanthine oxidase and consequent increased oxidative stress with exercise. Data of our lab, which also showed elevated levels of xanthine oxidase attests these results [42]. Increased lipid peroxidation levels were also observed in the hypothalamus of rat after exercise training [43]. Gunduz et al. [44] reported elevated levels of TBARS in the tissues of heart and soleus muscle after one year's swimming exercise. Kakarla et al. [24] reported that exercise training elevated lipid peroxide levels in the hepatic tissue of 3 and 12 months old female rats. In contrast to this, several studies reported a significant decrease in treadmill exercise training in liver, heart and plasma [27,45,46], where as, Husain and Somani [47] reported that exercise training did not alter the MDA level in the heart. These disparate results may be due (sex differences?) to type, intensity and duration of exercise.

In summary, the findings of the present study suggests that the activity/concentration levels of enzymatic and non-enzymatic antioxidants in the myocardium of female Wistar rats tend to decline during mid age when compared to the younger age. Since, the antioxidant enzymes can detoxify the deleterious reactive oxygen species, exercise-induced elevation in the activity levels of these enzymes could be a defense mechanism against the free radicals. The antioxidant enzyme levels are elevated with exercise training to cope with the tremendous increase in the production of the reactive oxygen metabolites under the age-related oxidative stress. To conclude, though the activity levels of the myocardial antioxidant enzymes were elevated with 12 weeks exercise training, the activity levels were not sufficient enough in minimizing the age-induced oxidative stress in the myocardium of female Wistar rats during this short period of exercise training.

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