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Exercise training with ageing protects against ethanol induced myocardial glutathione homeostasis

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Abstract
Glutathione plays a central role in the maintenance of cellular antioxidant defense. The alterations in the glutathione and associated recyclic enzymes caused by both exercise training and ethanol are well documented; however, their interactive effects with age are not well understood. Therefore, the influence of ageing and the interactive effects of exercise training and ethanol on the myocardial glutathione system in 3 months and 18 months old rats were examined. The results showed a significant ($p < 0.01$) reduction in GSH content, Se and non-Se GSH-Px, GR and GST activities in the myocardium of rat with age. A significant increase ($p < 0.05$) in the activities of these enzymes was observed in both age groups of rats in response to exercise training. This exercise-induced elevation of Se and non-Se GSH-Px and GR activities was more pronounced in the 18 months old rats when compared to 3 months old rats. Ethanol consumption significantly ($p < 0.05$) reduced the GSH content, Se and non-Se GSH-Px and GR activities in both age groups of rats. In contrast, ethanol consumption significantly ($p < 0.05$) increased the activity of GST. The combined action of exercise plus ethanol significantly ($p < 0.05$) elevated the GSH content, Se and non-Se GSH-Px, GR and GST activities when compared to the ethanol treated rats in both age groups, indicating the suppression of ethanol-induced oxidative stress by exercise training. In conclusion, there was a compensatory myocardial response lessening ethanol-induced oxidative stress by exercise training, which seemed to result from the higher activity of glutathione recycling and utilizing enzymes, which may be critical for preventing chronic oxidative damage to the myocardium during ageing and even due to ethanol consumption.

Keywords: *Exercise training, ethanol, ageing, glutathione system, myocardium, male rat*

Introduction

The glutathione-dependent antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species [1]. It consists of reduced glutathione (GSH) and an array of functionally related enzymes, of which γ-glutamyl cysteine synthetase and glutathione reductase (GR) are responsible for the synthesis and regeneration of GSH, respectively, whereas glutathione peroxidase (GSH-Px) and glutathione s-transferase (GST) work together with GSH in the decomposition of hydroperoxide or other organic hydroperoxides [2]. Ageing has been hypothesized to be caused by the deleterious and cumulative effects of reactive oxygen species (ROS) taking place throughout the life span [3]. During organism's ageing, the production of ROS is increased as a result of the functional deterioration in mitochondria [4]. There is strong evidence that oxidative stress may be the underlying mechanisms for several age-related pathogenesis. Thus, the cellular

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antioxidant defense system plays a crucial role at advanced age. Ageing has been shown to be associated with declined capacities of both enzymatic and non-enzymatic antioxidant systems in many important organs such as the liver, brain, heart and kidney [5,6].

Chronic ethanol ingestion leads to impaired cardiac function, including depressed cardiac contraction, ventricular hypertrophy and electrophysiologic abnormalities [7]. Oxidative stress derived from alcohol metabolism has been a major focus in the study of alcohol-induced tissue injury [8]. The pro-oxidant effect of ethanol depends on the induction of a major isoform of the cytochrome P450 family CYP2E1, which has been reported to lead to the formation of ROS [9]. Accumulation of ROS along with a compromised antioxidant capacity contributes to excess damage to cellular carbohydrates, proteins, lipids and nucleic acids [10]. Among the endogenous antioxidant systems, reduced glutathione (GSH) plays multiple roles in the detoxification of toxic chemicals [11]. Several studies have observed a selective decrease in the GSH content in the mitochondria due to partial inhibition of the specific mitochondrial carrier that translocates GSH from the cytosol into the mitochondrial matrix [12]. The decrease in mitochondrial GSH may represent an important step in the development of alcohol-induced myocardial oxidative stress and injury.

The GSH levels and the antioxidant enzyme activities in the heart are relatively lower than in other tissues [13]. As a result, heart tissue may be more susceptible to oxidative damage. In addition, post-mitotic tissues such as the myocardium have a lesser ability to up-regulate antioxidant defenses and/or to repair accumulated oxidative damage than tissues with greater proliferation capacity [14]. Ageing causes prominent oxidative damage to various cellular components, especially in tissues with high oxidative capacity [15]. Free radical mechanisms could be involved in the adverse ethanol effects, even in tissues poorly metabolizing ethanol, such as the heart [16]. Acetaldehyde, the metabolite of ethanol, promotes GSH depletion, free radical mediated toxicity and lipid peroxidation. Severity of intoxication, withdrawal and release of gamma-amino butyric acid following chronic ethanol consumption have been shown to be associated with age.

Strenuous physical activity results in an increased production of free radicals and ROS and it is well known to induce oxidative stress in individuals subjected to intense exhaustive exercise [17]. However, it has been consistently observed that individuals undergoing regular exercise training have high levels of antioxidant enzymes and contain increases in non-enzymatic antioxidants in skeletal and cardiac muscles; and demonstrate greater resistance to exercise-induced or imposed oxidative stress [18,19]. Several studies have shown that protein and non-protein thiols are essential in the protection against the deleterious effects of ROS [20,21]. Exercise is known to increase the cell's oxidative capacity and to improve endurance factors that generally decline with age and ethanol-toxicity. Thus far a comprehensive picture regarding the interactive effects of exercise and ethanol on the glutathione system of myocardium with reference to ageing is not known. Therefore, the purpose of the present study was to determine (1) GSH content and its recycling enzyme activities with ageing in rat myocardium; and (2) ethanol-induced changes in the myocardial GSH and its recycling enzyme activities and the role of exercise training in both young and old age rats.

Materials and methods

Animal care and training protocol

Wistar male albino rats were used in the current investigation. This was approved by the Institutional Animal Ethics Committee of the University, Tirupati. Cao and Cutler [22] reported ageing physiological changes occur from 6 months onwards in rats. The maximum life span of Wistar strain rats ranges from 24–36 months [23]. For the young animals, the puberty of the rat is reached between 50–60 days (i.e. 2 months). So, any time after 2 months is considered an adult matured age. Hence, in the present study, 3 months age group was used as young and 18 months age group was used as mid-age in the experimental design. The rats were fed with a standard rat pellet diet and water *ad libitum*. The animals of each age (young/mid) were divided into four groups. Each group consists of six animals and the division of groups is as follows:

- Group I. Sedentary Control (SC): The rats were put on the treadmill belt for 5 min for equivalent handling and were treated with sucrose via orogastric tube for equivalent caloric intake because ethanol gives extra calories
- Group II. Exercise training (Ex): The rats were made to run at a time on the six channel treadmill for 30 min at a speed of 23 m/min/5 days in a week for a period of 2 months utilizing an incremental belt speed. The running programme was scheduled between 6.00 am and 8.00 am and the rats were treated with sucrose via orogastric tube.
- Group III. Ethanol (Et): The rats were administered with 20% ethanol (2 g/kg body weight) daily via orogastric tube for 2 months.
- Group IV. Exercise Training and Ethanol (Ex+Et): The rats were exercised on the treadmill as described in group II and 5 min after exercise, the animals were given ethanol as described in group III daily for 2 months.

In this experiment administration was given after the exercise, since ingestion of ethanol prior to exercise may have an effect on the animal's neuronal balance.

The batches of rats selected for exercise training were acclimatized for 1 week on a treadmill belt prior to experiment. Initially they were exercised on the treadmill 5 min/day at a belt speed of 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 period of 2 months of training with a constant gradient of 7.5%. Gentle hand prodding and mild electric shock (20 mV, 1.67 Hz) was combined to encourage the animals to run throughout the study.

Tissue processing. The animals were sacrificed after 24 h of the last exercise session by cervical dislocation. The heart was excised at 4°C, washed with ice cold saline and blotted. After the atria and blood vessels were trimmed, the hearts were immediately immersed in liquid nitrogen and stored at −80°C for biochemical analysis and enzymatic assays.

Analytical procedures

Chemicals and solvents. Standard chemicals and enzymes, GSH, GSSG, CDNB (1-Chloro 2,4-Di Nitro Benzene), DTNB (Dithio-bis-Nitrobenzoic acid), cumene hydroperoxide, NADPH and glutathione reductase (GR) were obtained from Sigma chemicals (St Louis, MO). All organic solvents were of spectral grade and general chemicals were of reagent grade.

Biochemical assays

Glutathione (GSH). Glutathione content was estimated according to the method of Theodorus and Sies [24]. The heart tissue was homogenized (5% W/V) in 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.0) and protein was precipitated with 1 ml of 5% sulphosalicylic acid and the contents were centrifuged at 5000 g for 15 min at 4°C. The resulting supernatant was used as the tissue extract. 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) and 0.41 ml of tissue extract. The reaction was initiated by adding tissue extract and change in absorbance was recorded at 425 nm against the reagent blank. The glutathione content was expressed in μ moles of glutathione oxidized/mg protein.

Preparation of tissue extract. Exactly 5% (W/V) tissue homogenate was prepared in cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and centrifuged at 10 000 rpm for 10 min at 4°C in cold centrifuge and the supernatant was used as an enzyme source for the estimation of selenium (Se-GSH-Px) and non-selenium dependent glutathione peroxidase (non-Se GSH-Px) and glutathione reductase (GR).

Selenium (Se-GSH-Px) and non-selenium dependent glutathione peroxidase (non-Se GSH-Px) (EC. 1.11. 1.9). The Se-GSH-Px and non-Se GSH-Px activities were assayed following the NADPH oxidation by glutathione reductase using cumene hydroperoxide (CHP) for Se-GSH-Px and hydrogen peroxide (H_2O_2) for non-Se GSH-Px as substrates as per the modified method of Flohe and Gunzler [25]. The reaction mixture consisted of 500 μl of phosphate buffer, 100 μl of 0.01 M GSH (reduced form), 100 μl of 1.5 mM NADPH and 100 μl of GR (0.24 units). The tissue extract (100 μl) was added to the reaction mixture and incubated at 37°C for 10 min. To 450 μl of tissue reaction mixture, 50 μl of 12 mM cumene hydroperoxide was added to measure Se-GSH-Px and 50 μl of H_2O_2 was added to 450 μl of tissue reaction mixture to measure non-Se GSH-Px. Later the activity was read at 340 nm for 180 s. The molar extinction coefficient of 6.22×10^3 M/cm was used to determine the activity. One unit of activity is equal to the μ moles of NADPH consumed/min/mg protein.

Glutathione reductase (GR, EC. 1.6.4.2). Glutathione reductase (GR) was determined by a slightly modified method of Carlberg and Mannervik [26] at 37°C. NADPH (50 μl; 2 mM) in 10 mM Tris buffer (pH 7.0) was added to the cuvette containing 50 μl of GSSG (20 mM) in phosphate buffer. Later tissue extract (100 μl) was added to the NADPH-GSSG buffered solution and measured at 340 nm for 3 min. The molar extinction coefficient of 6.22×10^3 M/cm was used to determine GR activity. One unit of activity is equal to the μ moles of NADPH oxidized/min/mg protein.

Glutathione s-transferase (GST, EC. 2.5.1.18). Glutathione s-transferase (GST) activity was measured with its conventional substrate 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm as per the method of Habig et al. [27]. The tissues were homogenized (5% W/V) in 50 mM ice-cold Tris-HCl buffer (pH 7.4) containing 0.2 M sucrose and centrifuged at 16 000 g for 45 min at 4°C and the resulting supernatant was again centrifuged at 105 000 g for 1 h at 4°C. The pellet was discarded and the supernatant was used as the enzyme source. The reaction mixture in a volume of 3 ml contained 2.4 ml of 0.3 M potassium phosphate buffer (pH 6.9), 0.1 ml of 30 mM CDNB, 0.1 ml of 30 mM GSH and 0.4 ml of enzyme source. The reaction was initiated by the addition of glutathione and the absorbance was read at 340 nm against reagent blank and the activity was expressed in μ moles of thioether formed/mg protein/min.

Protein. Protein was estimated by the method of Lowry et al. [28] using bovine serum albumin as standard.

Statistical analysis

Comparison of estimated parameter values among treatments (SC, Ex, Et and Ex+Et (4 levels)) and age (Young and Old (2 levels)) has been carried out using Two-way Analysis of Variance (ANOVA) with multiple (six) observations in each combination. The *F*-value due to age, due to treatment and due to interaction is obtained from the ANOVA, run with the help of SPSS 11.5. Multiple comparisons using Dunnet's test have been carried out to compare the mean of each treatment with control separately for younger and mid-aged groups.

Results

The results showed a significant ($p < 0.01$) reduction of GSH content, Se and non-Se GSH-Px, GR and GST activities in the myocardium of rat with age in all treatment groups (Figures 1–5). In striking contrast, a significant increase ($p < 0.05$) in the activities of these enzymes was observed in both the age groups of rats in response to exercise training (Figures 1–5). However, this exercise-induced elevation of Se and non-Se GSH-Px and GR activities was more pronounced in the 18-month old rats when compared to 3 month old rats. Moreover, ethanol consumption significantly ($p < 0.05$) lowered the GSH content (Figure 1), Se and non-Se GSH-Px (Figures 2 and 3) as well as GR (Figure 4) activities in both age groups. What is more, ethanol consumption significantly ($p < 0.05$) increased the activity of GST in both age groups (Figure 5). Importantly, exercise training attenuates the ethanol induced decreases in GSH content, Se and non-Se GSH-Px, GR and GST activities in both age groups. This strongly suggests a protective effect of exercise training against ethanol-induced oxidative stress.

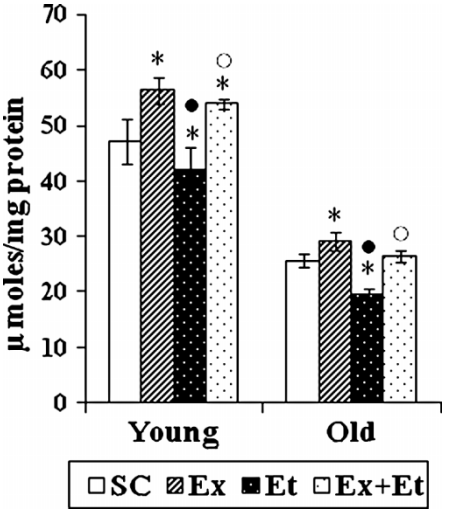

Figure 1. Effect of exercise (Ex), ethanol (Et) and combination of the two (Ex+Et) for a period of 2 months on Glutathione (GSH) content in myocardial (Heart) tissue of male albino rats of Young (3 month) and Old (18 month) age groups. Values represent mean ± SD, $n = 6$ rats. The values are significantly different ($p < 0.05$) compared to sedentary control (SC) (*), exercise (●) and ethanol (○).

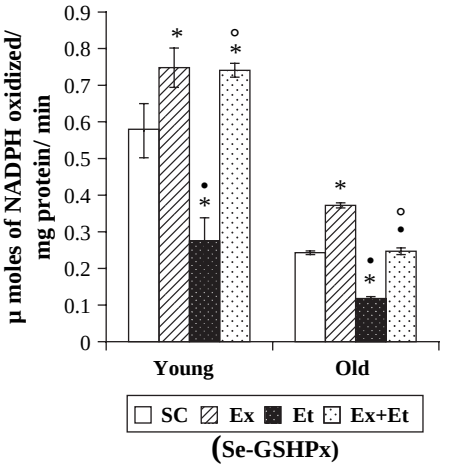

Figure 2. Effect of exercise (Ex), ethanol (Et) and combination of the two (Ex+Et) for a period of 2 months on Selenium dependent Glutathione Peroxidase (Se-GSHPx) activity in myocardial (Heart) tissue of male albino rats of Young (3 month) and Old (18 month) age groups. Values represent mean ± SD, $n = 6$ rats. The values are significantly different ($p < 0.05$) compared to sedentary control (SC) (*), exercise (●) and ethanol (○).

Discussion

The GSH content was decreased in the myocardium of 18 month old rats when compared to those of

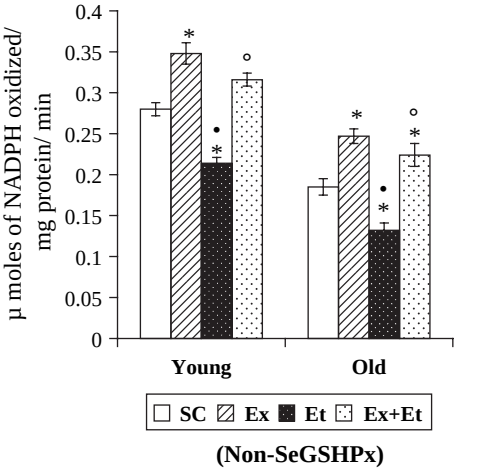

Figure 3. Effect of exercise (Ex), ethanol (Et) and combination of the two (Ex+Et) for a period of 2 months on non-Selenium dependent Glutathione Peroxidase (non-Se GSHPx) activity in myocardial (Heart) tissue of male albino rats of Young (3 month) and Old (18 month) age groups. Values represent mean ± SD, $n = 6$ rats. The values are significantly different ($p < 0.05$) compared to sedentary control (SC) (*), exercise (●) and ethanol (○).

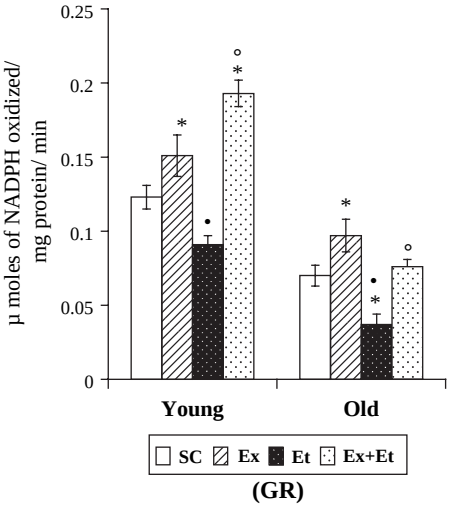

Figure 4. Effect of exercise (Ex), ethanol (Et) and combination of the two (Ex+Et) for a period of 2 months on Glutathione Reductase (GR) activity in myocardial (Heart) tissue of male albino rats of Young (3 month) and Old (18 month) age groups. Values represent mean ± SD, $n = 6$ rats. The values are significantly different ($p < 0.05$) compared to sedentary control (SC) (*), exercise (●) and ethanol (○).

3 month old rats. Decreased concentrations of myocardial GSH have been reported in several diseased states and are associated with an increased risk to oxidative stress [29]. Reduced levels of GSH content in the present study confirm an increased susceptibility to oxidative damage with age. Low GR activity may also contribute to the lower levels of myocardial GSH. Kakarla et al. [6] reported decreased levels of GSH in the myocardium of female albino rats with age. An age-dependent decrease was

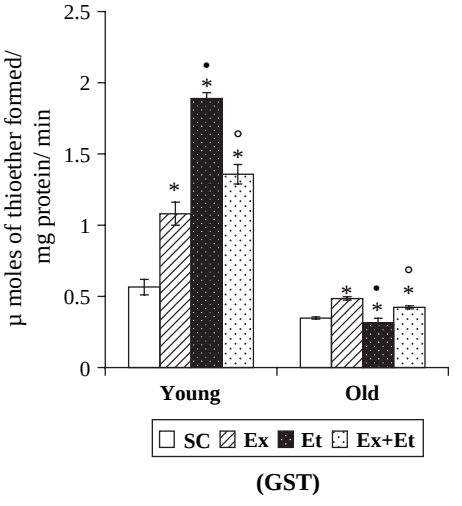

Figure 5. Effect of exercise (Ex), ethanol (Et) and combination of the two (Ex+Et) for a period of 2 months on Glutathione s-transferase (GST) activity in myocardial (Heart) tissue of male albino rats of Young (3 month) and Old (18 month) age groups. Values represent mean ± SD, $n = 6$ rats. The values are significantly different ($p < 0.05$) compared to sedentary control (SC) (*), exercise (●) and ethanol (○).

observed in the activities of both Se-GSH-Px and non-Se GSH-Px in the myocardium of rats. Hazelton and Cang [30] reported that in male C 56/BLI/6J mice the Se and non-Se GSH-Px activity of the heart decreases between 10–36 months of age. A significant decrease in specific activity of GR in heart of rats during ageing can be attributed to increased oxidation or decreased synthesis of GSH. The reduced availability of NADPH may also cause a decrease in GR activity. In conjunction, a decreased enzyme activity level of G-6-PDH (glucose-6-phosphate dehydrogenase) associated with age results in the reduced synthesis of NADPH. The decreased activity of GST in the myocardium of 18 month old rats when compared to 3 month old rats indicates the age-dependent alterations in the activity of the enzyme. A similar age-dependent decrease in the myocardial GST activity was reported by Kakarla [31].

In the present experiment, training resulted in a large and significant increase in myocardial non-protein thiols. Because glutathione is known to be the most important non-protein thiol in cells, we interpret this finding as evidence that endurance training resulted in elevated levels of myocardial (reduced) glutathione status and the function of the antioxidant enzyme GSH-Px [32]. Non-Se GSH-Px is much more efficient with organic hydroperoxide substances than with H_2O_2. The activity levels of non-Se GSH-Px increased significantly in the myocardium of rat during exercise training. The induction of non-Se GSH-Px activity could be aimed at reducing the endoperoxides produced by exercise-induced oxidative stress. Inconsistent with the above results, GR activity was elevated in the current experiments with exercise training, this may be due to the increased GSH-Px activity. An interesting finding in this study was that myocardial Se and non-Se GSH-Px and GR activities were associated to the exercise training in the 18 month old rats when compared to 3 month old rats. It has been demonstrated that generation of free radicals and oxidative tissue damage is more pronounced in muscle of aged experimental animals [33] and, therefore, it was suggested that enhanced activity of antioxidant enzymes might be a compensatory response to the higher oxidative stress imposed on the aged muscle [34]. The results of the present study showed elevated activity of myocardial GST with exercise training in both age groups of rats, indicating the possible activation of the enzyme or its synthesis in response to exercise training, since GST is an inducible enzyme. The hyperoxic condition by the enhanced respiratory rate during exercise training might be the causative factor for the induction of GST with exercise training. Hussain [35] reported similar increase in the activity levels of GSTs in the plasma of rat with exercise training.

In the current experiment, chronic ethanol consumption significantly reduced myocardial GSH content. A variety of mechanisms have been proposed to account for the decrease in this key cellular antioxidant, including the unrelenting production of ROS, the inhibition of GSH transport or synthesis and the direct interaction of GSH with acetaldehyde [16,36]. Acetaldehyde binds with cysteine, one of the three amino acids of glutathione, and may contribute to a decrease of GSH [37]. Glutathione is the most abundant non-protein thiol in cells which participates in detoxification functions. As a substrate of GST and GSH-Px, GSH constitutes one of the most important lines of cellular defense in the protection and metabolism of xenobiotics and ROS [38,39]. Therefore, the activities of glutathione peroxidase and reductase were monitored to examine the changes in glutathione utilization efficiency caused by chronic ethanol feeding. Chronic ethanol administration resulted in the lower activity of glutathione peroxidase and glutathione reductase. Similar decrease in the GSH-Px activity was reported in the liver of ethanol-treated rat [40]. In this study, chronic ethanol consumption resulted in a significantly higher activity of myocardial GST. The relatively easy inducibility of this enzyme by ethanol indicates its important role in detoxification of xenobiotics such as ethanol [41]. Therefore, the enhancement of GST activity in myocardium of ethanol-treated rats could be considered an adaptive response protecting the tissue against ethanol-induced oxidative damage. A similar increase in the GST activity of ethanol-treated rats was observed by Oh et al. [21].

The combination of exercise plus chronic ethanol ingestion enhanced the myocardial GSH level in both 3 month and 18 month old rats, indicating a training induced adaptive response in the myocardium and providing protection due to maintenance of interactive effects of ethanol and exercise. A significant increase in GR activity in the myocardium due to the combination indicated that the heart responded in order to recycle and to maintain the GSH levels or in response to insufficient absolute level of NADPH. GST activity was also elevated due to the interactive effects of exercise training and ethanol in the rat myocardium [42]. This elevation was more in the 3 month old rats when compared to 18 month old rats suggesting the adaptive response against ethanol in the younger rats. Increased loss of both GST and GSH from the liver into plasma alters the enzyme activity during ethanol administration [43].

In conclusion, there was a remarkable compensatory myocardial response lessening ethanol-induced oxidative stress by exercise training, which seemed to result from the higher activity of glutathione recycling and utilizing enzymes, which may be critical for preventing chronic oxidative damage to myocardium during ageing and even due to ethanol consumption.

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