MATERIALS AND METHODS
CARE AND MAINTENANCE OF EXPERIMENTAL ANIMALS:

Male, pathogen-free Wistar strain albino rats aged 3 months (younger) and 18 months (older) were housed in clean polypropylene cages, six in each, in a temperature controlled room (27°C) with photo-period of 12 hrs light and 12 hrs dark cycle. The rats were fed with standard laboratory chow (Hindustan Lever Ltd., Mumbai) and water ad libitum.

SELECTION OF AGE AND GROUPING OF ANIMALS:

In the book entitled, "International care and treatment of rabbits, mice, rats, guinea pigs and Hamsters" published by W.B. Saunders Co., Philadelphia, USA Schuchman (1989) given a detailed table regarding age and life span of different strains of laboratory animals. The maximum life span of a rat is 3 years. Cao and Cutler (1995) studied aging process from 6 months age through 12 and 24 months. Jang et al. (2001) studied age related changes in 2.5, 5, 10 and 23 months of Wistar strain rats. In their study 12 months age group rats were considered as the second highest age group, which is considered as old age. Indira Sriram and Jhansi Lakshmi (2001) also reported endurance exercise induced alterations in antioxidant enzymes of aging rats, which were of 12 months age. Thus, the literature pertaining to selection of age groups in the field of 'exercise science and aging' is variable in various studies.
Maintenance of animals for three years long period to attain maximum aging in the laboratory is practically difficult. Investigating studies involving very old animals and doing exercise training with these animals has 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 change with the very old animals. The puberty of the rat reaches in between 50-60 days (i.e., 2 months). So, any time after two months is considered as matured age. Hence, in the present study, 3 months age group was considered as “young” and 18 months age group was considered as “old” for effective comparison of aging process in relation to exercise.

The sub-maximal exercise training and ethanol administration was followed as per the protocols given by Somani and Husain (1997). The animals of each age (Young/Old) were divided into four groups. Each group consists, 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 minutes for equivalent handling and were treated with normal saline via orogastric tube.
Group II. **Exercise training (Ex):** The rats were made to run on the treadmill for about 30 minutes 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 program was scheduled between 6.00 AM and 8.00 AM. The rats were treated with normal saline via orogastric tube.

Group III. **Ethanol (Et):** The rats were administered daily with 20% ethanol (2 g/kg body weight) 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 minutes after exercise, the animals were given ethanol as described in group III daily for 2 months.

The animals were sacrificed after 24hr of the last exercise session by cervical dislocation. The myocardial (heart) tissue was excised at 4°C. After washing the tissues with ice cold normal saline, immediately immersed in liquid nitrogen and stored at -80°C. Selected parameters were estimated by employing standard methods. Before assay the tissues were thawed, sliced and homogenized in ice cold conditions.

**PROCUREMENT OF CHEMICALS:**

All the chemicals used in the present study were of Analar (AR) grade and obtained from the following scientific companies: Sigma (St. Louis, MO, USA), Fisher (Pittsburg, PA, USA), Merck (Mumbai, India), Qualigens (Mumbai, India).
Orogastric administration of ethanol to the Rat

Exercising of Rats on Treadmill
CONTEMPLATE METHOD OF APPROACH:

LACTATE DEHYDROGENASE ACTIVITY (L-LACTATE: NAD\textsuperscript{+} OXIDOREDUCTASE (EC. 1.1.1.27)

Lactate dehydrogenase (LDH) activity of the myocardium was assayed by the method of Nachlas et al. (1960) as modified by Prameelamma and Swami (1975).

Ten percent homogenates of the heart tissue was prepared in ice cold 0.25 M sucrose solution and centrifuged at 1000g for 15 min. The supernatant fraction was used for enzyme assay. The incubation mixture in a final volume of 2.0 ml contained 100 µ moles of phosphate buffer (pH 7.4), 40 µ moles of sodium lactate, 0.1 µ mole of NAD and 4 µ moles of INT (2-P-iodophenyl 3-P-nitrophenyl 5-phenyl tetrazolium chloride). The reaction was initiated by adding 0.2 ml of the homogenate containing 20 mg of tissue in each tube. The incubation was carried out at 37° C for 30 minutes. The reaction was stopped by the addition of 5.0 ml of glacial acetic acid. The formazan formed was extracted overnight into 5.0 ml of toluene at 5° C. The intensity of the color developed was measured at 495 nm against toluene blank in a spectrophotometer and the activity of LDH was expressed as µ moles of formazan formed/ mg protein/ hour.

ISOCITRATE DEHYDROGENASE ACTIVITY (ISOCITRATE: NADP\textsuperscript{+} OXIDOREDUCTASE (EC. 1.1.1.42)

Isocitrate dehydrogenase (ICDH) was assayed by the method of Korenberg and Pricer (1951) as modified by Mastanaiah et al. (1978).
Ten percent homogenate of heart tissue was prepared in 0.25 M ice cold sucrose solution and centrifuged at 1000g for 15 minutes. The supernatant was used for the enzyme assay. The reaction mixture in a final volume of 2.0 ml contained 40 μ moles of DL-isocitrate, 100 μ moles of magnesium chloride, 100 μ moles of sodium phosphate buffer (pH-7.4), 4 μ moles of INT (2-P-iodophenyl 3-P nitrophenyl 5-phenyl tetrazolium chloride), 0.2 μ mole of ADP and 0.2 μ mole of NADP (for NADP⁺ - ICDH).

The reaction was initiated by the addition of required volume of the supernatant and the contents were incubated at 37° C for 30 minutes. After incubation the reaction was stopped by adding 5.0 ml of glacial acetic acid and the formazan formed was extracted overnight at 5° C into 5.0 ml of toluene. The color was measured at 495 nm in a spectrophotometer against toluene blank. The enzyme activity was expressed as μ moles of formazan formed/mg protein/hour.

SUCCINATE DEHYDROGENASE ACTIVITY (SUCCINATE ACCEPTOR OXIDOREDUCTASE (EC. 1.3.99.1))

Succinate dehydrogenase (SDH) activity was estimated by the method of Nachlas et al. (1960) with slight modifications as suggested by Prameelamma and Swami (1975).

The tissue homogenates (10% w/v) were prepared in ice cold 0.25 M sucrose solution and centrifuged at 1000g for 15 min at 4° C. The supernatant fraction was
used for the assay of the enzyme. The reaction mixture in a final volume of 2.0 ml contained 40 μ moles of sodium succinate, 100 μ moles of phosphate buffer (pH 7.4) and 4 μ moles of INT. The reaction was initiated by the addition of 0.2 ml of enzyme source. The incubation was carried out for 15 min at 37°C and the reaction was arrested by the addition of 5.0 ml of glacial acetic acid. The formazan formed was extracted overnight into 5.0 ml of toluene at 4°C. The intensity of the color was measured at 495 nm in a spectrophotometer against toluene blank. The activity was expressed in μ moles of formazan formed/ mg protein/ hour.

MALATE DEHYDROGENASE ACTIVITY (L-MALATE: NAD⁺ OXIDOREDUCTASE (EC. 1.1.1.37))

Malate dehydrogenase (MDH) activity was estimated by the method of Nachlas et al. (1960) with slight modifications as suggested by Prameelamma and Swami (1975).

10% homogenate of heart tissue was prepared in ice cold 0.25M sucrose solution and centrifuged at 1000g for 15 minutes. The supernatant fraction was used for enzyme assay. The incubation mixture in a final volume of 2.0 ml contained 100 μ moles of phosphate buffer (pH 7.4), 40 μ moles of sodium malate as substrate, 0.1 μ mole of NAD and 4 μ moles of INT (2-P-iodophenyl 3-P nitrophenyl S-phenyl tetrazolium chloride). The reaction was initiated by adding 0.4 ml of the homogenate containing 40 mg of tissue. The incubation was carried out at 37°C for 30 minutes. The reaction was stopped by the addition of 5.0 ml of glacial acetic acid. The
formazan formed was extracted overnight into 5.0 ml of toluene at 5° C. The intensity of the color developed was measured at 495 nm against toluene blank in a spectrophotometer and the activity of MDH was expressed as μ moles of formazan formed/mg protein/hour.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY (EC. 1.1.1.49)

Glucose-6-phosphate dehydrogenase (G-6-PDH) activity was assayed by the method of Lohr and Waller (1965) as modified by Mastanaiah et al. (1978). The tissue homogenates were prepared in 0.25 M ice cold sucrose and centrifuged at 1000g for 15 min at 4° C. The reaction mixture in a volume of 2 ml contained 100 μ moles of sodium phosphate buffer (pH 7.4), 20 μ mole of glucose-6-phosphate, 2 μ moles of INT and 0.3 μ mole of NADP. The reaction was initiated by adding 0.5 ml of enzyme source. The reaction mixture was incubated at 37° C for 30 min and the reaction was stopped by the addition of 5 ml of glacial acetic acid. The formazan formed was extracted overnight into 5 ml of toluene at 4° C. The optical density of the formazan was read at 495 nm against the toluene blank. The activity was expressed in μ moles of formazan formed/mg protein/hour.

ASCORBIC ACID

Ascorbic acid was estimated by the method of Omáye et al. (1971). Tissue homogenates were prepared with ice cold TCA and centrifuged for 20 min at 3500 g. 1.0 ml of supernatant was mixed with 0.2 ml of DTC solution (diphenyl hydrazine-
thiourea-copper sulfate solution) and incubated for 3 hours at 37°C. Then 1.5 ml of ice cold 65% H₂SO₄ was added, mixed well and the solution was allowed to stand at room temperature for an additional 30 min. The developed color was read at 520 nm against the reagent blank. The ascorbic acid content was expressed in mg of ascorbic acid/gram wet weight of the tissue.

URIC ACID

Uric acid was estimated by the method of Martinek (1970). Tissues were homogenized in lithium carbonate solution, 0.5 ml of 0.66N H₂SO₄ and 0.5 ml of 10% (w/v) tungstate were added. The contents were centrifuged at 1000 rpm for 5 min. To the supernatant, 1 ml of sodium hydroxide solution and 1 ml of uric acid reagent were added and the developed color was read at 680 nm in a spectrophotometer against the reagent blank. The uric acid content was expressed in μ moles of uric acid/gram wet weight of the tissue.

XANTHINE OXIDASE ACTIVITY (EC. 1.2.3.2)

Xanthine oxidase (XOD) activity was estimated by the dye reduction method of Srikanthan and Krishnamurthy (1955). The assay mixture contained 100 μ moles of sodium phosphate buffer (pH 7.4), 50 μ moles of xanthine, 0.1 μ mole of NAD, 0.4 μ moles of INT and the enzyme source. The reaction was initiated by the addition of 20 mg of enzyme source and incubated at 37°C for 30 min. The reaction was stopped by the addition of 5 ml of glacial acetic acid and the formazan formed was extracted.
into toluene and read at 495 nm against toluene blank. The activity was expressed in µ moles of formazan formed/mg protein/hour.

**LIPID PEROXIDATION**

This assay is used to determine malondialdehyde (MDA) level as described by Ohkawa et al. (1979). The tissue homogenate (200 µl) was added to 50 µl of 8.1% sodium dodecyl sulfate (SDS), vortexed and incubated for 10 min at room temperature. Twenty percent acetic acid (375 µl; pH 3.5) and 375 µl of thiobarbituric acid (0.6%) were added and placed in a boiling water bath for 60 min. The samples were allowed to cool at room temperature. A mixture of 1.25 ml of butanol: pyridine (15:1) was added, vortexed and centrifuged at 1000 rpm for 5 min. The colored layer (500 µl) was measured at 532 nm using 1, 1, 3, 3-tetraethoxy propane as a standard.

**SUPEROXIDE DISMUTASE ACTIVITY (EC. 1.15.1.1)**

Superoxide dismutase (SOD) activity was determined according to the method of Misra and Fridovich (1972) at room temperature. Tissue extract (100 µl) was added to 880 µl (0.05 M, pH 10.2, 0.1 mM EDTA) carbonate buffer; 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and measured at 480 nm for 4 minutes. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit.
CATALASE ACTIVITY (EC. 1.11.1.6)

Catalase (CAT) activity was determined by a slightly modified version of Aebi (1984) at room temperature. EtOH (10 μl; 100%) was added per 100 μl of tissue extract and then placed in an ice bath for 30 minutes. After 30 min the tubes were kept at room temperature followed by addition of 10 μl of Triton X-100 RS. In a cuvette containing 200 μl of phosphate buffer and 50 μl of tissue extract, 250 μl of 0.066 M \( \text{H}_2\text{O}_2 \) (in phosphate buffer) was added and decrease in optical density was measured at 240 nm for 60 seconds. The molar extinction coefficient of 43.6 M cm\(^{-1}\) was used to determine catalase activity. One unit of activity is equal to the μ moles of \( \text{H}_2\text{O}_2 \) degraded/ min/ mg protein.

GLUTATHIONE

Glutathione (GSH) content was estimated according to the method of Theodorus et al. (1981). The heart tissue was homogenized in 0.1M phosphate buffer containing 0.001M EDTA (pH 7.0) and protein is precipitated with 1 ml of 5% sulfosalicylic acid 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 0.1M 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 required amount of tissue source. The reaction was initiated by adding enzyme source and change in absorbance was recorded at 425 nm against the reagent blank. The glutathione content was expressed in μ mol/ gram wet weight.
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 (H₂O₂) for Non-Se GSH-Px as substrates as per the modified method of Flohe and Gunzler (1984). The reaction mixture consisted of 500 μl phosphate buffer, 100 μl 0.01 M GSH (reduced form), 100 μl 1.5 mM NADPH and 100 μl GR (0.24 units). The tissue extract (100 μl) was added to the reaction mixture and incubated at 37°C for 10 minutes. Then 50 μl of 12 mM t-butyl hydroperoxide was added to 450 μl tissue reaction mixture and measured at 340 nm for 180 seconds. The molar extinction coefficient of 6.22 X 10³ M cm⁻¹ was used to determine the activity. One unit of activity is equal to the μ moles of NADPH Oxidized/ min/ mg protein.

GLUTATHIONE REDUCTASE ACTIVITY (EC. 1.6.4.2)

Glutathione reductase (GR) was determined by a slightly modified method of Carlberg and Mannervik (1985) 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. The tissue extract (100 μl) was added to the NADPH-GSSG buffered solution and measured at 340 nm for 3 minutes. The molar extinction coefficient of 6.22 X 10³ M cm⁻¹ 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 ACTIVITY (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. (1974).

The tissues were homogenized in 50 mM ice-cold Tris-HCl buffer (pH 7.4) containing 0.2 M sucrose and centrifuged at 16,000g for 45 min at 4°C and the resulting supernatant was again centrifuged at 1,05,000g 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.

VALIDITY OF EXPERIMENTAL PROCEDURES

General

For all the enzymes studied in the present investigation, the assays were standardized in both experimental and sedentary control tissues by conducting preliminary tests to determine the optimal pH, temperature, enzyme and substrate concentrations and these optimal conditions were subsequently followed for each enzyme assay. These preliminary tests were carried out for all the enzymes in the
myocardial (heart) tissue of all treatment groups. Appropriate control (sedentary control) animals maintained. Any change in the enzyme activities of experimental tissues were compared with their respective control ones.

**Aliquots for Assay**

Aliquots were selected such that initial rates were approximated as nearly as possible yet providing sufficient product to fall in a convenient range of spectrophotometric measurement.

**Enzyme Units**

Enzyme activities were expressed in standard units i.e., μ moles of product formed or substrate cleaved/ mg protein/ minute or hour.

**Substrate Requirement**

All the enzyme assays were done under the conditions following zero order kinetics unless otherwise stated.

**Lambert-Beer Law**

All most all the products of the reactions were measured by the spectrophotometric procedures in which the optical density (absorbance) of the resulting colored complex was proportional to the concentration of the reaction products.
Enzyme Nomenclature

The nomenclature of the enzymes used in the present context is according to the report of the commission of enzymes of the "International Union of Biochemistry".

Assay of Dehydrogenases Using INT

Tetrazolium salts are unique class of oxidation-reduction indicators in the study of dehydrogenases. The advantages of using tetrazolium salts as electron acceptors are:

i) The tetrazolium salts give a stable color on reduction

ii) They are highly soluble in aqueous solutions.

iii) They can be reduced both aerobically and anaerobically.

iv) They have high redox potential which makes the reduction easier.

v) They are freely permeable through membranes.

The first developed tetrazolium salt was triphenyl tetrazolium chloride (TTC). Following the application of TTC, new tetrazolium salts were developed. Various tetrazolium salts receive electrons from various sites of electron transport system (Oda et al., 1958; Nachlas et al., 1960) which is due to the inherent difference in the redox potentials of various tetrazolium salts. The phenyl ring was observed to increase its redox potential. Karmarker et al. (1959) reported that INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) was superior to the tetrazolium salts as electron acceptor for the assay of various dehydrogenases.
STATISTICAL TREATMENT OF THE DATA:

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 null hypotheses are:

1) The mean values due to the four treatments do not differ significantly.

2) The mean values of young and old do not differ significantly and

3) There is no interaction effect between age and treatment.

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. It is observed that age as well as treatments does have significant effect on the response. Age Vs treatment interaction is also found to be significant. Multiple comparisons using Dunnet’s test have been carried out to compare the mean of each treatment with control separately for younger and older groups. This module is available in SPSS one-way ANOVA under the option post Hoc. Significant differences at $p<0.05$ level have been marked with ‘*’. The means, standard deviations, percent changes and the results of ANOVA are given in the tables.