MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS
2.1. Chemicals

Nicotinamide adenine dinucleotide phosphate-reduced (NADPH), nitroblue tetrazolium (NBT), 1-chloro 2,4 dinitrobenzene (CDNB), 5, 5’ dithio-bis 2 nitrobenzoic acid (DTNB) and reduced- glutathione (GSH) were purchased from Sisco Research Laboratories, Mumbai. Chloroform, thiobarbituric acid (TBA) was purchased from E-Merck (India), Riboflavin from Loba Chemicals Mumbai, India. All other chemicals and reagents used for the study were of analytical reagent grade.

2.2. Diagnostic kits

<table>
<thead>
<tr>
<th>Test</th>
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<tr>
<td>Cholesterol</td>
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<td>Mumbai</td>
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<tr>
<td>Cholesterol HDL</td>
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<tr>
<td>Triglycerides</td>
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<tr>
<td>Glutamate pyruvate</td>
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<tr>
<td>Transaminase (ALT)</td>
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<tr>
<td>Glutamate oxaloacetate</td>
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<tr>
<td>Transaminase (AST)</td>
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<tr>
<td>Glutathione peroxidase</td>
<td>Randox laboratories Ltd</td>
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<tr>
<td>(GPx)</td>
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<td>Glutathione reductase</td>
<td>Randox laboratories Ltd</td>
<td>United Kingdom</td>
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<td></td>
<td>(G R)</td>
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2.3. Instruments

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<th>Instrument</th>
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<td>(SL 159 and SL 177)</td>
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<td>pH meter</td>
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<tr>
<td>Cold lab</td>
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</table>
2.4. Animals

Sprague Dawley albino rats were obtained from the Small Animal Breeding Station, Veterinary College, Mannuthy, Thrissur, Kerala

2.5. Materials and methods

Adult male albino rats of Sprague Dawley strain weighing (150-200g) were maintained under environmentally controlled conditions with free access to standard food (Lipton, India) and water. Rats were divided into seven groups.

**Group I** - Normal control rats: These groups of rats were kept in the ideal laboratory conditions without any kind of stress.

**Group II** - Fresh water swimming stress: In this type of stress, rats were forced to swim in a small plastic tub for 45 minutes (height; 60cm, diameter; 40cm containing water at room temperature, 28°C). Water depth was always maintained at 30cm. The forced swimming stress was studied for a period of 30 days.

**Group III** - Cold water swimming stress: In this type of stress, rats were forced to swim in the cold water maintained at 10°C. Water depth was always maintained at 30 cm. Cold water swimming stress was studied for a period of 30 days.

**Group IV** - Overcrowding stress: In this type of stress rats were kept in a small cage (mice cage 290 x 220 x 140 mm) in such a way that minimum movement was possible for the rats inside the cages. The overcrowding stress was studied for a period of 30 days.

**Group V** - Isolation stress: Rats were individually kept in a specially designed isolation cage and isolated totally. Isolation stress was studied for a period of 30 days.

**Group VI** - Cigarette smoke exposure: Rats were kept in a polypropylene cage of size 43.5x29.0x16.0 cm and fed with standard diet. The animals were exposed to cigarette smoke keeping a bottomless rectangular metal container on the top of the polypropylene cage containing rats. The metallic container contains 2 holes of about 3 cm diameter. One in front
and other in the back of the container. A burning cigarette was introduced through one hole. Animals were exposed to cigarette smoke for 45 minutes daily for a period of 30 days.

**Group VII**- Alcohol treatment: Male albino rats were given 18% alcohol orally (4gm alcohol/kg body weight) for a period of 30 days.

At the end of the experimental period of 30 days the animals were sacrificed and blood and tissues were collected for various biochemical analysis.

2.6. **Laboratory investigations**

The following investigations were carried out:

2.6.1. **Cholesterol**

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters. In the subsequent enzyme oxidation by cholesterol oxidase, \( \text{H}_2\text{O}_2 \) is formed. This is converted into coloured quinonimine in a reaction with 4-aminoantipyrine and phenol catalysed by peroxidase (Duncan et al, 1982)

The reaction mixture contained 10 µl of serum or plasma or standard (200 mg/dl), 1ml reaction solution, which contained PIPES buffer pH 7.5 (99mmol/L), salicylic alcohol (3.96mmol/L), 4-amino antipyrine (0.5mmol/L), peroxidase \( (\geq 100 \text{U/L}) \), cholesterol oxidase \( (\geq 100 \text{U/L}) \), cholesterol esterase \( (\geq 100 \text{U/L}) \). Mixed well and incubated for 5 minutes at 37°C. Then the absorbance was measured at 565 nm.

2.6.2. **HDL-cholesterol**

HDL-cholesterol was estimated from the supernatant after precipitation by phosphotungstic acid and 8.6mmol/L magnesium chloride (Harris et al 1996). 1ml of the supernatant was mixed with 1ml of reaction solution (same as that for cholesterol). Mixed well and incubated for 5 minutes at 37°C. The absorbance was measured against reagent blank at 546 nm.
2.6.3. **Triglycerides**

Triglycerides in presence of lipase are converted to glycerol and fatty acids. This glycerol by the action of glycerokinase is converted to glycerol-3-phosphate, which is oxidised to form dihydroxyacetone phosphate and H$_2$O$_2$. H$_2$O$_2$ generated in this step will react with aminoantipyrine and 4-chlorophenol in presence of peroxidase (POD) to form chinonimine, which is read at 546 nm. (National Cholesterol Education Programme, 1995).

10μl of serum or plasma was mixed with 1ml of reaction solution which contained Goods buffer pH 7.2 (50mmol/L), 4-chlorophenol (4mmol/L), ATP (2mmol/L), Mg$^{2+}$ (15mmol/L), glycerokinase (≥4KU/L), peroxidase (≥KU/L), lipoprotein lipase (≥KU/L), 4-aminoantipyrine (0.5mmol/L), glycerol-3-phosphate oxidase (≥1.5KU/L). 10 μl of triglycerides (200mg/dl) was used as the standard. Mixed and incubate at 37°C for 10 minutes. The absorbance was measured at 546 nm.

2.6.4. **LDL cholesterol**

LDL-cholesterol was calculated using Friedewald's formula (Friedewald et al, 1972)

\[
LDL-cholesterol \text{ (mg%)} = \text{total cholesterol - (HDL-cholesterol + TG/5)}
\]

2.6.5. **Extraction of tissue for lipid estimation**

The tissues were homogenized with washed powdered glass and extracted with chloroform: methanol (2:1) and 0.5 g of tissue corresponds to 25 ml of the extract in the case of tissues other than aorta. For aorta, aortas from 2 rats (60 mg) were pooled and extract was made up to 10 ml.

2.6.5.1. **Tissue cholesterol**

Total cholesterol was estimated by the method of Abell (Abell et al, 1952). An aliquot from the lipid extract was pipette in to a glass stoppered centrifuge tube and evaporated to dryness. 5ml of ethanolic KOH was added and stoppered and shaken well. It was then warmed in a water bath at 37-40°C for 55 minutes. After cooling to room temperature, 10 ml of
petroleum ether (60-80°C) was added and mixed. 5 ml of water was added to this and shaken vigorously for one minute. It was then centrifuged at a low speed for 5 minutes. 4 ml of petroleum ether layer was pipetted out into a test tube and evaporated to dryness at 60°C. A standard was treated in the same manner. 6 ml of color reagent (20 ml of acetic anhydride+1 ml of con H₂SO₄) was added to acetic acid taken as the blank. After 30-35 minutes, the optical density was read at 620 nm.

2.6.5.2. Tissue triglycerides

Triglycerides were estimated by the method of Van Handel and Zilversmit (Van Handel et al., 1957) with the modification that florisil was used to remove phospholipids. 2 g of florisil were taken in a glass-stoppered tube and 3 ml of chloroform was added. An aliquot of the extract was layered on the top of the florisil and mixed. More chloroform was then added to a total volume of 10 ml. It was then stoppered, shaken intermittently for about 10 minutes and filtered through a filter paper. 1 ml of filtrate was pipetted out into each of three tubes. The solvent was evaporated at 60-70°C. Then 0.5 ml ethanolic KOH (0.4%) was added to 2 out of 3 tubes (saponified sample). The solvent was evaporated at 60-70°C. Then 0.5 ml of ethanol was added to the unsaponified sample. The tubes were closed and kept at 60-70°C for 5 minutes. 0.5 ml of 0.2 N H₂SO₄ was added to each tube and tubes were then placed in a gently boiling water bath for 15 minutes to remove alcohol. They were then cooled to room temperature; 0.1 ml of sodium arsenite solution (0.5 M) was then added. A yellow colour of iodine appeared and vanished within a few minutes. 5 ml of chromotropic acid was added to each tube and mixed. The tubes were closed and then heated in a boiling water bath for 30 minutes. They were cooled and the absorbance was read at 570 nm. Glycerol was used as the standard.
2.6.6. Lipid peroxidation

Lipid peroxidation is a chain reaction initiated by the attack of membrane lipids by free radicals that has sufficient reactivity to abstract a hydrogen atom from the methylene group. This leaves behind an unpaired electron on the carbon atom. The carbon radical is stabilized by molecular rearrangement to produce conjugated diene, which then react with an oxygen molecule to form a peroxy radical. Peroxy radical can form cyclic peroxide and cyclic endoperoxide. Fragmentation of these peroxides leads to the formation of malondialdehyde (MDA). This reacts with thiobarbituric acid to form coloured complex, which is measured at 532 nm. (Yoshioka et al, 1979).

0.2 ml of the serum was mixed with 1ml of 20% trichloroaceticacid (TCA). To the mixture 0.4 ml of 0.67% thiobarbituric acid (TBA) was added, shaken and kept for 30 minutes in a boiling water bath. After cooling to room temperature, 1.6ml of butanol was added and the mixture was shaken. The organic mixture was separated by centrifugation and its absorbance was measured at 532 nm. The breakdown product of 1,1,3,3 tetramethoxy propane was used as the standard

2.6.7. Tissue lipid peroxidation

The lipid peroxide formation was measured by the method of Okhawa (Okhawa et al, 1979). 0.1 ml of tissue homogenate (25%) in tris HCl buffer (pH 7.4) was incubated in a reaction mixture containing KCl (0.1ml), ascorbic acid 0.1 ml and ferrous ammonium sulphate, 0.1ml for one hour at 37°C. The reaction mixture was allowed to cool and centrifuged at 2000 rpm. The incubated reaction mixture 0.4 ml was taken and treated with SDS (0.2 ml, 8%) and acetic acid 1.5 ml (20%, pH 3.5). The total volume was made up to 4 ml by adding distilled water and 5 ml of a mixture of N- butanol- pyridine (15:1) were added and shaken vigorously. The absorbance of the organic layer was measured at 560 nm after centrifugation.
2.6.8. Conjugated dienes

Conjugated dienes estimated by the method of Brein (Brein et al, 1966). 1 ml of aqueous tissue homogenate was mixed thoroughly with 5 ml chloroform:methanol (2:1) followed by centrifugation at 1000 rpm for 5 minutes to separate the phases. The lower layer of chloroform was evaporated to dryness under a stream of nitrogen at 45°C. The lipid residue was dissolved in 1.5 ml of cyclohexane. Optical density at 233 nm was determined against a cyclohexane blank. The amount of conjugated dienes produced can be calculated using the molar extinction coefficient of 2.52x 10^4/ M/cm

2.6.8. Hydroperoxides

Hydroperoxides estimated by the method of Nair R.D (Nair et al, 1971). 1ml of aqueous tissue homogenate was mixed thoroughly with 5 ml chloroform:methanol (2:1) followed by centrifugation at 1000 rpm for 5 minutes to separate the phases. The lower layer of chloroform was evaporated to dryness under a stream of nitrogen at 45°C, while still under a stream of nitrogen. 1 ml of acetic acid:chloroform followed 0.05 ml potassium iodide (1.2g / mlwater) were quickly added and mixed. The samples were placed in the dark at room temperature for 5 minutes followed by addition of 3 ml of cadmium acetate (0.5g /100mlwater). The solution was mixed and centrifuged at 1000 rpm for 10 minutes. The optical density of upper phase was determined at 353 nm against a blank containing the complete assay mixture except the lipid. Standardization of the reaction was done by cumene hydroperoxides as peroxide standard. The molar extinction coefficient of cumene hydroperoxide is 1.73x10^4/M/cm.

2.6.9. Superoxide dismutase (SOD)

SOD is measured by the degree of inhibition of formazan dye, which is formed by the reduction of nitroblue tetrazolium (NBT) in presence of riboflavin (Mc Cord et al, 1969). The assay system consists of 0.6 M phosphate buffer pH 7.4, 0.12 M riboflavin (50 µl), 0.1M
EDTA containing 0.0015% sodium cyanide (200 μl), 1.5 mM nitroblue tetrazolium (100 μl) and various concentrations of test materials, in a total volume of 3 ml. The tubes were illuminated under an incandescent lamp for 15 minutes. Optical density at 530 nm was measured before and after illuminations and the percentage inhibition was calculated using the formula.

\[
\text{Optical density of control tube} - \text{optical density of treated tubes} \times 100
\]

One unit of enzyme activity is defined as the enzyme concentration required to inhibiting optical density measurements at 560 nm of chromogen production by 50% in one minute under assay condition.

### 2.6.10. Catalase

In the UV range, hydrogen peroxide shows a continued increase in absorption with decreasing wavelength. The decomposition of \( \text{H}_2\text{O}_2 \) can be followed directly by the decrease in extinction at 240 nm (\( \text{E}_{240} \)). The difference in extinction (\( \Delta \text{E}_{240} \)) per unit time is a measure of the catalase activity (Aebi, 1983). The estimation was done spectrophotometrically following the decrease in optical density measurements at 230 nm. The assay system consisted of phosphate buffer (50 mM, pH 7) 1 ml, 0.1 ml enzyme solution, 1 ml \( \text{H}_2\text{O}_2 \) solution (30 mM) in test. Control tube contained 2 ml of enzyme solution, the reaction occurred by the addition of \( \text{H}_2\text{O}_2 \). The decrease in extinction was recorded at 240 nm at 15 seconds intervals for 3 minutes.

### 2.6.11. Reduced glutathione (GSH)

The glutathione was estimated by the reaction of GSH with DTNB to give a yellow colored complex with absorption maximum at 412 nm (Moron et al, 1979).

The reaction mixture consisted of 0.5 ml of tissue homogenate. 125 μl of 25% TCA was added to precipitate proteins. The tubes were cooled on ice for 5 minutes and the mixture was further diluted with 0.6 ml of 5% TCA, Centrifuged for 10 minutes and 0.3 ml of the resulting
supernatant was taken for GSH estimation. The volume of aliquot was made up to 1 ml with 0.2 M phosphate buffer pH 8. 2 ml of freshly prepared 0.6 mM DTNB was added to the tubes and the intensity of yellow colour was read at 412 nm. Values were expressed as nmol/mg protein.

2.6.12. **Glutathione peroxidase (GPx)**

Glutathione peroxidase was estimated by the method of Hafemann (Hafemann et al, 1974). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 1 mM sodium azide and 0.2 mM H$_2$O$_2$ in a total volume of 2.5 ml was incubated at 37°C for 6 minutes. After addition of 2 ml of 1.67% phosphoric acid, the mixture was centrifuged at 8000 g for 15 minutes. The supernatant (2 ml) was added to a mixture of 2 ml of 0.4 M Na$_2$HPO$_4$ and 1 ml of 1 mM DTNB. After 10 minutes incubation at 37°C the absorbance of the reaction mixture was measured at 412 nm. One unit of enzyme activity was defined as the decrease in log GSH by 0.001/minute, after subtraction of the decrease in log GSH / minute for the non-enzymatic reaction and expressed as units / mg protein.

2.6.13. **Glutathione-S- transferase (GST)**

Glutathione-S- transferase was estimated by the method of Habig (Habig et al, 1974). The reaction mixture contained 2.79 ml phosphate buffer (0.1 M, pH 6.5), 100 μl of GSH (30 mM), 10 μl of homogenate and 100 μl of CDNB (30 mM). The absorbance was noted for 3 minutes at one-minute interval at 340 nm at 37°C.

2.6.14. **Glutathione reductase (GR)**

Glutathione reductase activity was determined by the method of Racker (Racker et al, 1995). The amount of NADPH consumed in the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The reaction is catalysed by glutathione reductase.

The reaction mixture contained 100 μl of GSSG (2.2 mmol/l), 100 μl of EDTA and
100 µl of NADPH and 680 µl of phosphate buffer (pH 6.6). The reaction commences with the addition of 20 µl sample and a decrease in absorbance/minute was noted and followed at every 1-minute interval for 5 minutes at 340 nm.

2.6.15. Serum Glutamate Oxaloacetate Transaminase (SGOT or AST)

AST was estimated by the method of IFCC (IFCC).

\[ \text{L-aspartate} + \alpha \text{keto glutarate} \xrightarrow{\text{GOT}} \text{Oxaloacetate} + \text{L-Glutamate} \]

\[ \text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + \text{NAD}^+ \]

AST - Aspartate aminotransferase
MDH - Malate dehydrogenase

The reaction mixture contained 1 ml of the reagent 100 µl of the sample, mix well and read the OD at 340 nm. Reagents: MDH (≥ 600 U/L), LDH (≥ 900 U/L), NADH (0.20 mmol/L), α keto glutarate (12 mmol/L), Tris buffer [(pH 7.8) 88 mmol/L], L-Aspartate (260 mmol/L)

2.6.16. Glutamate Pyruvate Transaminase (ALT)

ALT was estimated by the method of IFCC (IFCC).

\[ \text{L-alanine} + \alpha \text{keto glutarate} \xrightarrow{\text{GPT}} \text{Pyruvate} + \text{L-Glutamate} \]

\[ \text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^+ \]

ALT - Alanine aminotransferase
MDH - Malate dehydrogenase

The reaction mixture contained 1 ml of the reagent 100 µl of the sample, mix well and read the OD at 340 nm. (Reagent LDH (≥ 1200 U/L), NADH (0.20 mmol/L), α keto glutarate (16 mmol/L), Tris buffer [(pH 7.5) 110 mmol/L], L-Alanine (550 mmol/L)

2.6.17. HMG CoA reductase

HMG CoA reductase activity of the liver tissue was estimated as described by Rao (Rao et al, 1975) by determining the ratio of HMG CoA to mevalonate.
Equal volumes of freshly prepared 10% tissue homogenate and dilute perchloric acid (50 ml made up to 1 litre) were mixed, kept for 5 minutes and centrifuged at 2000 rpm for 10 minutes. To 1 ml supernatent 0.5 ml of freshly prepared hydroxylamine hydrochloride reagent (alkaline hydroxylamine reagent in case of HMG Co A) was added, mixed and after 5 minutes, 1.5 ml of ferric chloride reagent was added (5.2 g TCA + 10 g FeCl₃ in 50 ml of 0.65 N HCl and made up to 100 ml with latter). After shaking well, readings were taken after 10 minutes at 540 nm against a similarly treated saline arsenate blank. The ratio of HMG Co A to mevalonate is taken as index of activity of the enzyme which catalyses the conversion of β hydroxy β methyl glutaryl CoA to mevalonate, lower the ratio, higher the enzyme activity.

2.6.18. Glucose-6-phosphate dehydrogenase

The enzyme was assayed by the method of Kornberg (Kornberg et al, 1955). The chilled tissue was homogenized with 3 volumes of glycyl glycine buffer pH 7.5. The homogenate was centrifuged at 0°C for 10 minutes. The supernatent was used as the enzyme source. To 1 ml of substrate, (0.02 ml glucose-6-phosphate, 0.1 ml of NADP⁺ (0.0015 M), 0.25 ml of buffer (glycyl-glycine 0.04 M pH 7.5) and 0.2 ml of MgCl₂ (0.1 M) were added and the absorbance was read immediately at 340 nm at one minute intervals for 5 minutes. One unit of enzyme activity is defined as the amount of enzyme which cause an increase of 1.00 in optical density / minute per g protein.

2.6.19. Malic enzyme

The enzyme was assayed by the method of Ochoa (Ochoa et al, 1955). The chilled tissue was homogenized with 3 volumes of glycyl-glycine buffer pH 7.4. The homogenate was centrifuged at 2000 rpm at 0°C for 10 minutes. The supernatant was used as the enzyme source. The reaction mixture consisted of 0.3 ml of 0.25 M glycyl-glycine buffer pH 7.4, 0.06 ml of 0.05 M MnCl₂ (3 mM), 0.1 ml of NADP⁺ (0.135 μmoles), enzyme and water to a final volume of 3 ml. The enzyme assay was carried out at room temperature (23-25°C). The reaction was
started by the addition of either malate or enzyme and optical measurements at 340 nm were taken against a blank cuvette containing all components except NADP⁺ at 15 seconds intervals for two minutes. One unit of enzyme activity is defined as the amount of enzyme, which causes an increase of 0.01 in optical density/minute/g protein.

2.6.20. Isocitrate dehydrogenase

The enzyme was assayed by the method of Ochoa (Ochoa et al, 1955). The reaction mixture consisted of 0.3 ml of glycyl glycine buffer (pH 7.4), 0.1 ml of Mn Cl₂ (1.8 µm), 0.1 ml of NADP⁺ (0.135 M), 0.1 ml of disodium isocitrate (0.006 M), enzyme and water to a final volume of 3 ml. The enzyme assay was carried out at room temperature (23-25°C), the reaction was started by the addition of either isocitrate or enzyme and optical density measurements at 340 nm were taken against a blank containing all components except NADP⁺ at 15 seconds intervals for two minutes.

One unit of enzyme is defined as the amount of enzyme, which causes an initial change in OD of 0.01/minute under the above conditions. Specific activity is expressed as units/mg protein.

2.6.21. Total proteins

Protein was estimated in all enzyme extracts by the method of Lowry (Lowry et al, 1951).

1. Reagent A: 2 g Na₂CO₃ in 100 ml 0.1 N NaOH
2. Reagent B: 5 g CuSO₄ in 100 ml, 2% Rochelle salt (Sodium potassium tartarate) solution.
3. Reagent C: Mix 50 ml of A with 1 ml of B
4. Phenol reagent (Folin Ciocalteu reagent) : (1:1 dilution). It is a mixture of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid.
0.1 ml of enzyme source is taken and made up to 1ml with distilled water. To this 5 ml of reagent C is added and kept for ten minutes. Then 0.5 ml of Folin Ciocalteu reagent is added. Optical density is read after 30 minutes at 670 nm.

2.7. Statistical evaluation

Statistical significance were determined using students ‘t’ test (Lutz N, 1967) according to the formula. To find out the statistical significance between group X and group Y. Value of ‘t’ was found out from the equation

\[
t = \frac{x-y}{\sqrt{S^2(1/n_x+1/n_y)}}
\]

x is the mean value of group (X). y is the mean value of group Y.

nx—number of sample X

ny---- number of sample Y.

S was found out from the equation.

\[
S = \sqrt{\frac{(S_x^2 (nx-1) + S_y^2 (ny-1))}{(nx+ny-2)}}
\]

Sx--- Standard deviation of sample x.

Sy--- Standard deviation of sample y

nx+ny—2 is the degree of freedom, statistical significance (p values ) are found out from ‘t’ distribution table.