4. MATERIALS AND METHODS

4.1 Institutional Animal Ethics Committee (IAEC) approval:

Institutional Animal Ethics Committee approval for the present study was obtained, and
the study was done according to the directives given by the Committee for the Purpose of
Control and Supervision of Experiments on Animals (CPCSEA).

4.2 Animals and their maintenance:

4.2.1 Animals used for the study:

Present study was done on experimentally naïve rabbits that were procured from the In
Vivo Research Facility, Central Animal House, Dr. D. Y. Patil Medical College, Hospital
and Research Centre, Pimpri, Pune-18 (Regn. No.: 619 / PO / ReB I / S / 02 / CPCSEA),
that also served as the site of the study. Male New Zealand White rabbits belonging to
Oryctolagus Cuniculus species and weighing 1.5-2.5 kg were selected.

4.2.2 Animal feed:

The animals were fed the normal rabbit chow comprising of green leafy vegetables,
lettuce, carrots, cucumber etc. Provision of the drinking tap water that was supplied by
Pimpri Chinchwad Municipal Corporation was made for the rabbits. Daily replenishment
of food and water was done.

4.2.3 Animal housing:

Each rabbit was housed individually in separate standard stainless-steel cages at 24°C,
relative humidity (55 ± 10%) and 12-hour light: dark cycle. The rabbits were left
undisturbed, apart from the daily replenishment of food and water,
4.3 Study Drugs/Chemicals:

4.3.1 Lycopene

Route of administration: Oral

Source: Zenith Nutrition, Bengaluru


4.3.2 High Fat Diet (HFD)

Route of administration: Oral

Source of Coconut Oil: Marico Industries (Mumbai)

Source of Vanaspati Ghee: Hindustan Lever Ltd. (Mumbai)
4.4 Methodology:

4.4.1 Method to prepare the high fat diet:

The mixture of edible coconut oil and Vanaspati ghee, in 2:3 ratio respectively v/v as described by Shyamala MP. 102

4.4.2 Method of inducing hyperlipidaemia:

High fat diet, made up of coconut oil and Vanaspati ghee, in 2:3 ratio v/v respectively in 5 ml/kg dose, was administered orally with a feeding tube, once daily, for six weeks, in addition to the normal rabbit chow, to all rabbits in all three treatment groups.

4.4.3 Administration of lycopene:

Lycopene was administered orally, by using a feeding tube, once daily, for six weeks, in the dose of 10 mg/kg body weight to the second group of six rabbits, and in the dose of 20 mg/kg body weight to the third group of six rabbits, in addition to the above diet.
4.5 Study plan:

After one week period of acclimatization, the collection of blood samples was done after overnight fasting from all rabbits, for estimation of the baseline levels i.e. Serum TC, HDL, LDL, TG, VLDL, SOD, BSL and NO. Rabbits were divided into three treatment groups, six rabbits in each group, and fed one of the following diets orally by using an oral feeding tube, once daily, in addition to the normal rabbit chow, for six weeks period.

<table>
<thead>
<tr>
<th>Group Number (n=6)</th>
<th>Drugs and Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HFD (5 ml/kg)</td>
</tr>
<tr>
<td>II</td>
<td>HFD (5 ml/kg) + Lycopene (10 mg/kg)</td>
</tr>
<tr>
<td>III</td>
<td>HFD (5 ml/kg) + Lycopene (20 mg/kg)</td>
</tr>
</tbody>
</table>

After six weeks, the collection of blood samples was done after overnight fasting from all rabbits, for estimation of the levels i.e. Serum TC, HDL, LDL, TG, VLDL, SOD, BSL and NO.
4.6 Withdrawal of blood from the marginal ear vein of the rabbit

For the purpose of withdrawal of blood from the marginal ear vein, placing and restraining the rabbit in a rabbit restrainer was done. After that, removal of the hair from the ear was done gently by using a clipper. Gentle stroking and tapping of the ear lobe were done, for the purpose of making the ear veins visible. The marginal ear vein was located. By using a spirit gauze the site was disinfected. The ear was pulled taut by the operator by using the non-dominant hand. By using the dominant hand, the operator inserted into the marginal ear vein a 22-gauge, 1-inch long needle with an attached syringe and withdrawal of 5 ml blood sample was done. Care was taken not to withdraw any more blood than was necessary. After the withdrawal of blood, a sterile gauze pad was pressed gently on the withdrawal site for some time until the bleeding had stopped. After ensuring that the bleeding was stopped, the rabbit was returned for recovery to its cage. This procedure was repeated, one by one, in all rabbits belonging to the three treatment groups.
4.7 Methods of estimations:

All the reagents used for the estimations were of analytical grade and were purchased from Erba Mannheim.

4.7.1 Estimation of lipid profile

Estimations of the levels of serum TC, HDL and TG were done manually on spectrophotometer. Estimations of the levels of LDL and VLDL were done according to Friedewald’s equation\textsuperscript{103} that is as follows:

$$LDL = TC – HDL – \frac{\text{TG}}{5}$$

$$\text{VLDL} = \frac{\text{TG}}{5}$$

4.7.1.1 Estimation of serum total cholesterol levels

Estimation of serum total cholesterol levels was done manually on spectrophotometer by CHOD-PAP method (modified Roeschlau’s method).\textsuperscript{104}

Principle:

Following enzyme catalysed reactions are involved in the estimation of serum total cholesterol levels.

Cholesterol esterase

1. Cholesterol ester $\xrightarrow{\text{Cholesterol esterase}}$ Cholesterol + Fatty acids

Cholesterol oxidase

2. Cholesterol + O$_2$ $\xrightarrow{\text{Cholesterol oxidase}}$ Cholest-4-en-3-one + H$_2$O$_2$

Peroxidase

3. 2H$_2$O$_2$ + 4-Aminoantipyrine + Phenol $\xrightarrow{\text{Peroxidase}}$ 4H$_2$O + Quinoneimine

The absorbance of quinoneimine thereby formed is directly proportional to the serum cholesterol concentration.
Table-4.7.1: Assay procedure for estimation of serum total cholesterol levels

<table>
<thead>
<tr>
<th>Pipette into the tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>20 µl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>20 µl</td>
<td>--</td>
</tr>
<tr>
<td>Test</td>
<td>--</td>
<td>--</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

After mixing well, incubation was done for the period of ten minutes at 37º C. Aspiration of the Blank was done followed by Standard and Tests. The absorbance readings of standard and each test tube were noted against Blank at 505 nm.

**Calculation:**

\[
TC = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}
\]

4.7.1.2 Estimation of serum high density lipoproteins levels

Serum high density lipoproteins levels were estimated manually on spectrophotometer by phosphotungstic acid method as described by Burstein M.\(^{105}\)

**Principle:**

In the presence of divalent cations like magnesium, phosphotungstic acid causes the precipitation of chylomicrons, LDL and VLDL from the serum. However, HDL cholesterol does not undergo precipitation, remains in the supernatant, and is estimated with the help of cholesterol reagent.

\[
\text{Phosphotungstic Acid} \\
\text{Serum} \rightarrow \text{HDL} + \text{(LDL + VLDL + Chylomicrons)}
\]

\[
\text{Mg}^{2+} \quad \text{(Supernatant)} \quad \text{(Precipitate)}
\]
Table-4.7.2: Precipitation of LDL, VLDL and Chylomicrons

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>250 µl</td>
</tr>
<tr>
<td>Precipitating reagent</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

Mix well. Let the reaction mixture stand for the period of ten minutes at the room temperature (15-30°C). Centrifuge for 10 minutes at 4000 RPM to obtain a clear supernatant. This supernatant is used for determining HDL cholesterol concentration in the sample.

Table-4.7.3: Assay procedure for estimation of serum high density lipoproteins levels

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol Working Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>50 µl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>HDL Standard</td>
<td>--</td>
<td>50 µl</td>
<td>--</td>
</tr>
<tr>
<td>Supernatant</td>
<td>--</td>
<td>--</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

After mixing well, incubation was done for the period of ten minutes at 37°C. Aspiration of the Blank was done followed by Standard and Tests. The absorbance readings of standard and each test tube were noted against Blank at 505 nm.

Calculation:

\[
HDL = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard} \times \text{Dilution factor}
\]
4.7.1.3 Estimation of serum triglycerides levels

Serum triglycerides levels were estimated manually on spectrophotometer by GPO-Trinder method.\textsuperscript{106} The reagent is based on the method of Wako\textsuperscript{107} and the modifications done by McGowan MW\textsuperscript{108} and Fossati P.\textsuperscript{109}

**Principle:**

Following enzyme catalysed reactions are involved in the estimation of serum triglycerides levels.

Lipoprotein lipase  
1. Triglyceride + H\textsubscript{2}O \rightarrow Glycerol + Free fatty acids

Glycerol kinase  
2. Glycerol + ATP \rightarrow Glycerol -3-phosphate + ADP

Glycerol phosphate oxidase  
3. Glycerol -3-phosphate + O\textsubscript{2} \rightarrow Dihydroxyacetone phosphate + H\textsubscript{2}O\textsubscript{2}

Peroxidase  
4. H\textsubscript{2}O\textsubscript{2} + 4 Aminoantipyrine + 3,5-Dichloro-2-hydroxybenzene sulfonate \rightarrow Quinoneimine + 2H\textsubscript{2}O
Table-4.7.4: Assay procedure for estimation of serum triglycerides levels

<table>
<thead>
<tr>
<th>Pipette into the tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>10 µl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>10 µl</td>
<td>--</td>
</tr>
<tr>
<td>Test</td>
<td>--</td>
<td>--</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

After mixing well, incubation was done for the period of ten minutes at 37º C. Aspiration of the Blank was done followed by Standard and Tests. The absorbance readings of standard and each test tube were noted against Blank at 505 nm.

The absorbance of quinoneimine so formed is directly proportional to the concentration of serum triglycerides.

**Calculation:**

\[
TG = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}
\]

4.7.1.4 **Estimations of serum low density lipoproteins and very low density lipoproteins levels:**

Estimations of serum Low Density Lipoproteins and serum Very Low-Density Lipoproteins levels were done as per the Friedewald's equation\(^{103}\) that is as follows:

\[
LDL = \text{Total Cholesterol} - \text{HDL} - \left(\frac{\text{TG}}{5}\right)
\]

\[
VLDL = \frac{\text{TG}}{5}
\]

4.7.2 **Estimation of serum superoxide dismutase levels**

Estimation of serum superoxide dismutase levels was done manually on the spectrophotometer by using the method described by Marklund S.\(^{110}\)
Table-4.7.5: Assay procedure for estimation of serum superoxide dismutase levels

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>3.0 ml</td>
<td>2.95 ml</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0.3 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>------</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

After mixing well, absorbance is read uninterrupted for the period of 4 minutes at 420 nm at the interval of 30 seconds.

**Calculation:**

The absorbance reading at 3.5 minutes minus that at 0.5 minutes is noted for calculation. (Initial 1.5 minutes are required for the stabilization of assay mixture). If A is the reading of control, whereas B is reading of test:

\[
\text{SOD} = \frac{A - B}{A \times 50} \times \frac{100}{0.05} \text{ Units per ml}
\]

**4.7.3 Estimation of blood sugar levels**

Estimation of blood sugar levels was done by using the optimum Xceed glucometer, for which, test strip on which blood drop was placed, was inserted in glucometer.

**Basis for the glucometer blood glucose test:**

Measurement of electric potential, generated by reaction of blood glucose with glucose oxidase and potassium ferricyanide, reagents on the strip’s electrode, producing electrons current proportional to glucose concentration in the sample, is the principle underlying this glucometer blood glucose test. Display of glucose concentration in the sample is seen after a specific time that is the reaction time required for the glucometer.
4.7.4 Estimation of serum nitric oxide levels

Estimation of serum nitric oxide levels was done manually on the spectrophotometer by using the method of Cortas and Wakid.

Principle:

Nitric oxide, an extremely unstable gas, is rapidly degraded by undergoing oxidation into nitrate and nitrite, that are stable breakdown products. Modified cadmium reduction method is used to assay the serum levels of nitrate and nitrite.

As per this method, deproteinisation of serum samples is done by using Somogyi reagent. The deproteinised serum then is carried out by the use of copper coated cadmium in the glycine NaOH buffer (2.5-3 g for 4 ml mixture).

Reduction of nitrate to nitrite is determined by means of diazotisation of sulfanilamide and subsequent coupling to naphthalene diamine. The resultant colour formed by nitrite diazotisation is thereby measured at 545 nm on spectrophotometer.

Reagents used for the estimation of serum nitric oxide levels:

Preparation of all reagents is done as follows in the deionised water that is nitrate free.

1. Cadmium granules: Are broken into small pieces with a wire cutter and are stored in 0.1 M H₂SO₄ in Erlenmeyer flask.

2. Glycine NaOH buffer: Glycine in the quantity of 15 g is dissolved in 1000 ml deionised distilled water. The pH is adjusted to 9.7 by the use of 2N NaOH solution.

3. Sulfanilamide: Sulfanilamide powder in the quantity of 5 g is dissolved in warm 500 ml (3 mol/L) HCl solution, that is then let to cool.

4. N-naphthyl ethylene diamine: 50 mg N-naphthyl ethylene diamine powder is dissolved in 250 ml of deionised distilled water.

5. Standard: Sodium nitrate or Potassium nitrate (10 µmol/L).

6. Zinc sulfate solution (75 mmol/L): Prepared by adding 225 mg of Zinc sulfate powder into 10 ml of deionised distilled water.
7. Sodium hydroxide (55 mmol/L): Prepared by dissolving 220 mg of anhydrous NaOH powder into 100 ml of deionised distilled water.

**Method for estimation of serum nitric oxide levels:**

1. **Deproteinisation of the serum sample:** Done by addition of serum in 0.5 ml quantity into 2 ml of Zinc sulfate solution (75 mmol/L), and subsequently mixing with 2.5 ml of sodium hydroxide (55 mmol/L). After allowing it to let it stand for ten minutes period it is then centrifuged.

2. **Activation of the stored cadmium granules:** Acid from the cadmium granules is rinsed thrice by using deionised distilled water. After that, they are swirled in 5 mmol/L copper sulfate solution in glycine NaOH buffer for the period of two minutes. These copper coated cadmium granules are drained, copper sulfate is rinsed three times by using the glycine buffer and then they are used in ten minutes. After the use, these granules are rinsed and stored in 0.1 M H₂SO₄ solution in Erlenmeyer flask, so as to enable their regeneration and reactivation later on, by means of repeating the above steps.

3. **Reduction of nitric oxide:** It is done in Erlenmeyer flasks as per the following table.

   **Table-4.7.6: Reduction of nitric oxide**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test (ml)</th>
<th>Blank (ml)</th>
<th>Standard (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine NaOH buffer</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Deproteinised sample</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard NaNO₃ solution (10 µmol/L)</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Deionised water</td>
<td>2.0</td>
<td>3.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

   The flasks are then stirred and kept aside for the period of 90 minutes.

4. **Addition of the reagents:** Take 2.0 ml solution from each of the above three flasks and put it into each of the following three labelled test tubes as given below for nitrite estimation.
Table-4.7.7: Reagents used for estimation of serum nitric oxide levels

<table>
<thead>
<tr>
<th>Pipette into the tube marked as</th>
<th>Test (ml)</th>
<th>Blank (ml)</th>
<th>Standard (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent from the above mixture</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Sulphanilamide</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>N-naphthalene diamine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Contents of each test tube are mixed well. The absorbance is read against blank at 545 nm after 20-60 minutes.

5. Calculations: µmol of nitrite formed per litre serum

\[
\text{Effective volume of serum} = \frac{\text{O.D. of Test} - \text{O.D. of Blank} \times \text{Concentration of Std} \times 1000}{\text{O.D. of Std} - \text{O.D. of Blank}}
\]

Standardization:

Stock standard:

Sodium nitrate 85 mg is dissolved in Sodium tetraborate (Na₂B₄O₇) 100 ml so as to prepare sodium nitrate (NaNO₃) 10 mmol/L.

Working standard:

Dilution of the stock standard is done for which 2.0 ml of the standard (10 mmol/L) is taken and deionised water is added to it so as to make the volume up to 50 ml. The concentration of NaNO₃ that is obtained is 400 µmol/L. This is diluted still further as follows so as to obtain the concentration of the working standard.

Table-4.7.8: Standardization

<table>
<thead>
<tr>
<th>Volume of 400 µmol/L standard (ml)</th>
<th>Volume of the deionised water (ml)</th>
<th>Standard concentration (400 µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>17.5</td>
<td>5</td>
</tr>
<tr>
<td>5.0</td>
<td>15.0</td>
<td>10</td>
</tr>
<tr>
<td>7.5</td>
<td>12.5</td>
<td>20</td>
</tr>
<tr>
<td>10.0</td>
<td>10.0</td>
<td>40</td>
</tr>
<tr>
<td>12.5</td>
<td>7.5</td>
<td>80</td>
</tr>
<tr>
<td>15</td>
<td>5.0</td>
<td>160</td>
</tr>
</tbody>
</table>
4.8 Statistical Analysis

Statistical analysis of this study was done by using the statistical package Graph Pad Prism Version 8.0.1 (2018) for Windows.

Student’s ‘t’ test (paired ‘t’ test) was used for comparison between the baseline measurements and measurements taken at 6 weeks, within each group.

Results were expressed as mean ± SD and statistical significance between means was analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s test of multiple comparisons.

Value of p < 0.05 was considered statistically significant.

Value of p < 0.01 was considered statistically highly significant.