MATERIALS AND METHODS

Work Plan

Male Sprague Dawley (SD) rat was the animal model used for the study. Animals were obtained from the Central Animal House, Panjab University, Chandigarh.

Chemicals Procurement: All the molecular biology grade chemicals for RNA isolation like chloroform, isopropanol, ethanol and formaldehyde were purchased from Amresco, Ohio, USA. Diethyl pyrocarbonate (DEPC), thyroxine, sodium selenite, 2,3-diaminonaphthalene (DAN), bovine serum albumin (BSA), sephadex G-25, agarose, ethidium bromide, EDTA, sodium acetate, chloramine-T, NADPH, glutathione reductase, dithiothreitol were purchased from Sigma Aldrich, St. Louis, USA. Oil red ‘O’ (C.I. 26125) and glycerol were obtained from BDH Chemicals Ltd., Poole, England. Formamide, methanol, hydroxyl ammonium chloride and cyclohexane (HPLC grade) and sucrose were purchased from E. Merck (India) Ltd., India. Cholesterol for the animal diet was procured from LOBA CHEMIE, Bombay, India.

All other chemicals and reagents used in the present study were of analytical grade and were procured from Indian manufacturers.

Treatment Protocol: Male SD rats of approximately 100g-body weight were used in the present study. Animals were kept in polypropylene cages and acclimatized to the laboratory animal room and divided into three groups initially, group I (selenium deficient diet fed), group II (selenium adequate diet fed) and group III (selenium excess diet fed). Feed and water were given *ad libitum*. Initially respective selenium (Se) status diets were given to the animals in these three groups for 10 days so as to achieve the required Se status. After that animals in each group were further divided into two each viz.: Group Ia (selenium deficient control diet fed), Group Ib (selenium deficient + high cholesterol diet fed); Group IIa (selenium adequate control diet fed), Group IIb (selenium adequate + high cholesterol diet fed).
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cholesterol diet fed); Group IIIa (selenium excess control diet fed); Group IIIb (selenium excess + high cholesterol diet fed).

Diet Preparation

Selenium deficient diet: Yeast based synthetic Se deficient diet (supposed to contain 0.02ppm Se) was prepared in the laboratory itself according to the composition given by Burk (1987)

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>56.99</td>
</tr>
<tr>
<td>Baker's Yeast (inactivated)</td>
<td>30.00</td>
</tr>
<tr>
<td>Corn oil</td>
<td>6.67</td>
</tr>
<tr>
<td>Mineral mixture (USP XIV)</td>
<td>5.00</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.00</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.30</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Vitamin mixture: The vitamins were weighed individually as given below

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>10.00g</td>
</tr>
<tr>
<td>Inositol</td>
<td>1.10g</td>
</tr>
<tr>
<td>Para aminobenzoic acid</td>
<td>1.10g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>16.50g</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1.00g</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.22g</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>0.22g</td>
</tr>
<tr>
<td>Calcium pentothenate</td>
<td>0.66g</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>0.22g</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.02g</td>
</tr>
<tr>
<td>d-Biotin</td>
<td>4.40mg</td>
</tr>
<tr>
<td>Vitamin B_{12}</td>
<td>0.3mg</td>
</tr>
</tbody>
</table>
The above vitamin mixture was made 100g with sucrose and the final concentration of it in the diet was 1% as stated above.

Oil soluble vitamins were dissolved directly in the corn oil to be added in the diet as per following scheme: Vitamin A - 20,000 IU; Vitamin K - 5mg; Vitamin E - 100mg and Vitamin D - 1000 U for 1 kg diet.

_Selenium supplemented diet:_ Selenium adequate and excess diets were prepared from Se deficient diet by supplementing it with 0.2ppm and 1ppm of Se as sodium selenite. This 1ppm selenium dose was so chosen that the level is in excess to the adequate level of selenium but well below the sub toxic limits (2ppm).

All the three control groups received the respective Se status diet, whereas the high cholesterol diet (HCD) groups were fed with the respective Se status diet having 2% cholesterol. Treatment protocol was for 1, 2 and 3 months. Throughout the study, body weight of the animals was monitored so as to check the growth of the animals.

After completion of diet feeding schedule, blood was drawn from the retro orbital sinus of the ether anaesthetized overnight fasting rats. Serum was prepared from blood. After bleeding, the animals were sacrificed under ether anesthesia followed by cervical dislocation. Tissues (liver, aorta and thyroid) were removed immediately, washed with normal saline and stored at -20°C to analyze the following parameters;

1. Selenium estimation in liver and serum after 1, 2 and 3 months of diet feeding schedule.
2. Glutathione peroxidase (GSH-Px) activity in liver at all the treatment intervals.
3. Estimation of basic lipid profile i.e. cholesterol and triglycerides levels in serum after 1, 2 and 3 months of respective diet feeding.
4. Estimation of low-density lipoprotein (LDL) levels in serum in all the treatment groups.
5. T₃ and T₄ levels were estimated in serum at all the treatment intervals.
6. Type-I 5'-iodothyronine deiodinase (5'-DI) activity in liver, aorta and thyroid in all the treatment groups.
7. Estimation of in vivo low-density lipoprotein receptor (LDL-R) activity after 1, 2 and 3 months of diet feeding schedule.
8. Apolipoprotein B (ApoB) levels by ELISA in all the treatment groups after 1, 2 and 3 months of diet feeding schedule.
9. Apolipoprotein B (ApoB) expression by western immunoblot analysis after 1, 2 and 3 months of diet feeding schedule.
10. 5'-DI mRNA expressions in liver, aorta and thyroid by RT-PCR at different time intervals.
11. LDL-R mRNA expression in liver by RT-PCR at different time intervals.
12. HMG-CoA reductase mRNA expression was studied by RT-PCR after 3 months of diet feeding schedule.

**Selenium Estimation**

Selenium levels were estimated by following the method of Hasunuma et al. (1982) based on the principal that selenium content in the serum or liver on acid digestion is converted to selenous acid. The reaction between selenous acid and aromatic-O-diamines such as 2,3-diamino naphthalene (DAN) leads to the formation of 4,5-benzopia-zselenol which displays brilliant lime green fluorescence when excited at 366nm in cyclohexane.

![4,5 benzopia-zselenol](image)

All the reagents were prepared in double distilled water.

*Preparation of DAN:* 50mg of DAN was dissolved in 50ml of 0.5% hydroxyl ammonium chloride in 0.1M HCl in brown bottle and kept for 1hr. DAN solution
was washed with equal volume of cyclohexane by vigorous shaking in separating funnel with stopper and kept for 2–3 min till the two layers separated. Lower aqueous layer of DAN was collected. This washing procedure was repeated twice and the final DAN solution was stored in dark bottle.

**Digestion of Samples:** Samples were digested in modified kjeldahl type flasks with reflux condensers attached to the neck to prevent any loss of selenium as volatile selenides.

5ml of concentrated HNO₃ was added to 0.3ml of serum sample or 200mg of liver tissue in digestion flasks. Mixture was heated on sand bath at approximately 100°C. Heating was continued till brown fumes ceased that takes approximately 2-3hrs. Temperature of sand bath was lowered and 1.0ml of perchloric acid (HClO₄) was added directly to flasks without removing the condensers. After heating for 30min again at 100°C, the flasks were removed from sand bath and allowed to cool. Removed the condensers and volume of digested mixture was measured for each sample. This volume was termed as digest.

**Estimation:** 1ml of digested sample was taken for assay. To this added 1ml of 6M HCl, 1ml of 50% formic acid, 2ml of stabilizing solution (0.025M EDTA in 2.5% hydroxyl ammonium chloride), 2.5ml of ammonia solution and 5ml of DAN solution to it.

After incubation at 50°C for 20min in water bath, tubes allowed to cool to room temperature. 3ml of cyclohexane was added to each tube and mixed on vortex mixer for 1 min. Tubes were allowed to stand to separate the cyclohexane layer. Cyclohexane layer (upper) was taken out using a pasteur pipette and collected in a separate tube. Again added, 2ml of cyclohexane to the tubes and vortexed for 30sec. Cyclohexane layer was collected similarly and pooled with previously separated layer. This step was repeated again using 2ml of cyclohexane.
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Fluorescence emission in extracted cyclohexane was read on fluorescence spectrophotometer (Perkin Elmer, USA) using 366nm as excitation wavelength and 520nm as emission wavelength.

One ml of working standard solution and 1ml of distilled water were added to respective standard and blank tubes.

**Working Standard Solution:** Stock solution (1mg Se/ml) was prepared by adding 2.3 mg of dried sodium selenite in 0.1 ml of HClO₄ and final volume was made up to 1 ml with distilled water. This solution was diluted to 0.1µg Se/ml, and used as working standard solution.

Calculations:

\[
\frac{\text{Test reading}}{\text{Standard reading}} \times \frac{\text{Concentration of standard}}{\text{Tissue wt./Serum vol.}} \times \frac{\text{Total volume of digest}}{\text{Total volume of digest}}
\]

Selenium levels were expressed as µg/g liver and as µg/L serum.

**Glutathione Peroxidase**

Glutathione peroxidase (GSH-Px) activity was assayed in post mitochondrial fraction (PMF) in liver by the coupled enzyme procedure with glutathione reductase using hydrogen peroxide as substrate (Paglia and Valentine, 1967). Tissue homogenates (10% w/v) were prepared in cold 20mM Tris-HCl (pH 7.4) buffer using mechanically driven teflon fitted potter elvejham type homogenizer for one minute under ice cold conditions. Homogenates were centrifuged at 10,000g for 30 min at 4°C to get PMF.

**Reaction mixture consisted of:**

- 0.08M NADPH 37.50µl
- Glutathione Reductase (10U/ml) 37.50µl
- 0.15M glutathione 37.50µl
- 0.125M sodium azide 3.75µl
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0.05M potassium phosphate buffer (pH 7.0) 820.00μl
0.002M H$_2$O$_2$ 37.50μl

The reaction was started by the addition of 20μl of PMF diluted with 0.05M potassium phosphate buffer (pH 7.0). Oxidation of NADPH was recorded at 340nm in spectrophotometer (UV 160A, Shimadzu). Enzyme activity was expressed as μmoles of NADPH oxidized/min/mg protein.

**Total Cholesterol Levels**

Serum total cholesterol was estimated by enzymatic colorimetric test kit obtained from Human Diagnostic (Germany) based on CHOD-PAP method.

**Principle:** The kit is based on the principle that cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase. The free cholesterol is then oxidized by cholesterol oxidase to cholestren-4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield a colored complex (Quinoneimine dye), which is read at 505nm. The intensity of the color produced is directly proportional to the concentration of total cholesterol in the sample.

**Procedure:** Mixed 1ml of cholesterol working reagent and 10μl of serum sample or cholesterol standard (provided in the kit) and incubated the mixture at 37°C for 10min. Read the absorbance at 505nm in spectrophotometer against reagent blank.

$$\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}$$

**Triglycerides Levels**

Serum triglycerides levels were estimated by enzymatic test kit obtained from Accurex Biomedical (India) based on GPO method.

**Principle:** Triglycerides in the body are hydrolyzed to glycerol by lipase. Glycerol is converted to glycerol-3-phosphate by glycerol kinase, which is oxidized to dihydroxyacetone phosphate and hydrogen peroxide is formed. Hydrogen peroxide oxidizes 4-aminoantipyrine in the presence of peroxidase to form a red
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colored complex (Quinoneimine dye). The absorbance of the colored complex is measured at 505nm, which is directly proportional to triglycerides concentration.

Procedure: Mixed 1ml of triglycerides working reagent and 10μl of serum sample or triglycerides standard (provided in the kit) and incubated the mixture at 37°C for 5min. Read the absorbance at 505nm in spectrophotometer against reagent blank.

\[
\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Concentration of Standard}
\]

Estimation of LDL Levels

LDL level in the serum was estimated by enzymatic kit obtained from E. MERCK Dianostics (Germany).

Procedure: 100μl of serum sample was mixed with 1000μl of LDL reagent (provided in the kit), incubated it at room temperature for 15 minutes and then centrifuged at 2500g for 20minutes. Added 100μl of the supernatant or cholesterol standard (1:10) to 1000μl of cholesterol reagent (provided in the kit), incubated for 5 minutes at 37°C and then measured the OD at 505nm. This value was subtracted from the total cholesterol content measured in the serum to get the LDL content.

Triiodothyronine (T3) Levels

Serum T3 estimation was done by radioimmunoassay (RIA) kit procured from Bhabha Atomic Research Centre (BARC), Mumbai, India (Cat. No. RIAK-4/4A).

Principle: Kit was based on the principle that unlabelled endogenous T3 competes with radiolabelled T3 for the limited binding sites on the antibody (Ab1) made specifically for T3. The antibody is in the form of complex with secondary antibody (Ab2). At the end of incubation period, the T3 (Ag) bound to antibody secondary antibody complex (Ag-Ab1-Ab2) and free T3 were separated by the addition of polyethylene glycol (PEG). The amount bound to the antibody
complex in the assay tube was compared with the values of the known standards and the \( T_3 \) concentration in the patient sample was calculated. 8-anilino 1-napthalene sulphonic acid (ANS) is used in this kit for displacing \( T_3 \) bound to TBG.

Assay was performed strictly according to the instruction manual supplied with the kit. 50μl of serum was used in the assay procedure. Standard curve was prepared using standards of different concentrations (0-240 ng/dl). Hormone free rat serum was added to all the standard tubes to provide the appropriate matrix to the standard tubes.

**Thyroxine (\( T_4 \)) Levels**

Serum \( T_4 \) estimation was done by radioimmunoassay (RIA) kit procured from BARC, Mumbai, India (Cat. No. RIAK-5/5A).

*Principle:* Kit was based on the principle that endogenous \( T_4 \) competes with radiolabelled \( T_4 \) for the limited binding sites on the antibody made specifically for \( T_4 \). At the end of incubation period, the \( T_4 \) (Ag) bound to antibody (Ag-Ab) and free \( T_4 \) were separated by the addition of polyethylene glycol (PEG). The amount bound to the antibody complex in the assay tube was compared with values of known standards and the \( T_4 \) concentration in the patient sample was calculated. 8-anilino 1-napthalene sulphonic acid (ANS) is used in this kit for displacing \( T_4 \) bound to thyroxine binding globulin (TBG).

Assay was performed strictly according to the instruction manual supplied with the kit. 10μl of serum was used in the assay procedure. Standard curve was prepared using standards of different concentrations (0-20 μg/dl). Hormone free rat serum was added to all the standard tubes to provide the appropriate matrix to the standard tubes.

*Preparation of Hormone Free Rat Serum:* Collected 10ml of rat serum, to this added 1g of activated charcoal. Mixed it on shaker for 2hrs. Centrifuged it at 3000rpm for 20min. Took the supernatant, centrifuged it for 1h at 10,000rpm.
Collected the clear supernatant. It was used for adding into different standards (T₃ & T₄ estimation).

**Type-I 5’-Iodothyronine Deiodinase (5’-DI) Activity**

5’-DI activity was estimated in tissues (liver, aorta and thyroid) by following the modified method of Behne et al. (1990).

*Principle:* The estimation was based on the principle that when known amount of protein containing 5’-DI enzyme was incubated with T₄ to convert it into T₃, the activity of 5’-DI can be estimated in terms of T₃ produced in the reaction mixture.

Each sample (1mg of protein) was incubated in a final volume of 0.5 ml of 100mM tris-HCl buffer (pH 7.4) containing 1µM T₄ and 1mM dithiothreitol at 37°C for 60 min. 1ml of chilled ethanol was added to each sample at the end of the incubation period. Samples were vortexed and kept at 20°C, overnight. After centrifugation, *in vitro* T₃ production was determined in the ethanolic extracts by radioimmunoassay.

Heat denatured protein samples were used as background controls and each sample had its own respective control.

**LDL Receptor (LDL-R) Activity**

LDL-R activity was estimated *in vivo* after 1, 2 and 3 months of diet feeding schedule. LDL was isolated from overnight fasting human plasma. Purity of the isolated LDL was checked by performing the agarose gel electrophoresis for purified LDL as well as for whole plasma sample. The isolated LDL was radiolabeled with Na¹³¹I and injected to the rats intravenously. Percent decrease in counts in blood at increasing time interval was taken as a measure of clearance of LDL from animal blood and in turn the LDL-R activity.

*Isolation of Plasma LDL:* Plasma lipoprotein subspecies were isolated using a single vertical spin density gradient ultracentrifugation as described by Chung et al. (1986).
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Plasma was adjusted to a density of 1.3g/ml by adding dried solid potassium bromide (KBr) to it (0.4946g/ml). Chylomicrons were removed by Pasteur pipette after centrifugation of plasma at 30,000rpm for 30min at 10°C. A discontinuous KBr density gradient was then formed in 5ml vertical spin tubes (Backman, USA) with density adjusted plasma (1.5ml) at the bottom, it was then overlaid with normal saline having 0.01% EDTA (pH 7.4). Sudan Black B (0.1% in ethylene glycol) was added in the reference tube to localize the band positions in tubes with test samples. Tubes were sealed (Beckman quick sealer) and centrifuged at 7°C at 80,000rpm (VTi-80 rotor, Beckman, USA) for 45 min. with slow acceleration and deceleration mode.

**Separation of LDL Layer:** After centrifugation, LDL layer was directly aspirated with syringe by puncturing the tube at the layer position with syringe needle. Samples were pooled from different tubes. Excess of KBr in the separated LDL solution was removed by washing the LDL solution with normal saline and then in 0.05M potassium phosphate buffer (pH 7.5) using centricon (Amicon, USA) with 10kD molecular weight cut off. Finally, LDL was suspended in 0.05M potassium phosphate buffer (pH 7.5). After protein estimation in concentrated sample, it was stored at 4°C under nitrogen environment till further use.

**Electrophoretic Separation of Plasma Lipoprotein:** To check the purity of plasma LDL isolated by ultracentrifugation, agarose gel electrophoresis was performed for both purified LDL as well as whole plasma sample by following the method of Noble (1968).

Prepared 50ml of 1% agarose in tris borate EDTA buffer (TBE buffer; 0.05M, pH 8.6) in an erlenmeyer flask in the microwave oven. The gel was allowed to cool to 60°C, to this added 1.0ml of BSA (25% BSA in normal saline) mixed it thoroughly. The gel was poured into horizontal plastic tray (both the ends sealed with scotch tape). Comb was already positioned in the tray before pouring the agarose solution. The gel was allowed to stand at room temperature for 30min.
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After the gel was completely polymerized, removed the comb and the scotch tape carefully and mounted the gel tray in the electrophoresis tank.

Separating buffer (0.05M TBE) was added in the tank. 50μg of sample, mixed with the sample buffer (Bromophenol blue + glycerol) was applied to each well and electrophoresis was carried out at constant voltage of 100V (EPS-1001, Amersham Biosciences, USA) for 1hr.

After completion of electrophoresis, gel was fixed for 30min in fixing solution (5% glacial acetic acid in 75% ethanol) and then stained with staining solution (1% oil red ‘O’ in 60% ethanol) for 6hrs with continuous shaking. Gel was destained in 30% ethanol solution for 15min and washed thoroughly in running tap water. Finally the bands were viewed and photographed using Gel Doc (Uvitech, England).

Radioiodination of LDL with Na$^{131}$I: LDL was radioiodinated with Na$^{131}$I using chloramine-T method as described by Salahuddin and Singh (1983). Na$^{131}$I was purchased from BARC, Mumbai, India. Radioiodination was done as follows:

2mCi of Na$^{131}$I was dispensed into 1.5ml polystyrene microfuge tube. To this added, 20μl of 0.5M potassium phosphate buffer (pH 7.5), 20μg of purified LDL and 10μl of chloramine-T solution (4mg/ml) in 0.5M buffer (freshly prepared before use). Reaction was allowed for about 60seconds with thorough mixing by bubbling air through fine tip of pasteur pipette. To this added 25μl of sodium metabisulphite solution (4mg/ml in distilled water; freshly prepared before use), then 135μl of potassium iodide solution (4mg/ml in distilled water) was added.

Separation of LDL bound and unbound radioiodide was performed by gel filtration through sephadex G-25 column (1x15cm). Column was saturated by passing 1ml of BSA solution (5% in 0.05M potassium phosphate buffer, pH 7.5) through it. Sample was loaded in the column and then eluted with normal saline. 0.5ml fractions were collected in polystyrene tubes and counted simultaneously in
Materials and Methods

![Image](materials_and_methods.png)

Liquid scintillation analyzer (Perkin Elemer, USA). Fractions were collected till both the iodinated LDL and the unbound radioiodide peaks were eluted. Fractions containing iodinated LDL were pooled and stored at 4°C under nitrogen environment after filter sterilization through 0.2μm nitrocellulose syringe filter (Sartorius AG, W-3400 Goettingen, Germany).

**LDL Receptor Activity Estimation:** LDL-R activity was measured by the method of Brown and Goldstein (1984). 0.5ml (specific activity 112μCi/μg) of filter sterilized 131I-LDL was injected (i.v) to each rat through penile vein. Counts per ml blood after 2hrs. of i.v. injection were considered as counts at zero time or total initial counts in the blood. One ml of blood was withdrawn from the retroorbital sinus of each rat after every 24hrs and counts were measured. Blood was taken until the counts in any of group were reduced to 10% of the total initial counts. Percent decrease in counts at increasing time interval was used as a measure of clearance rate of LDL from blood and in turn LDL-R activity.

**Apolipoprotein B (ApoB) Levels by ELISA**

Apolipoprotein B concentration was estimated in liver by ELISA. Tissue homogenates (10%) from different groups were prepared at 4°C in homogenizing buffer (20mM Tris-HCl, pH 7.4) using mechanical driven teflon fitted Potter-Elvejham type homogenizer. The homogenates were centrifuged at 3000g for 10min. suprnatants were used for assay. Protein content was estimated by the method of Lowry et al., (1951) The assay was standardized by titrating the different concentrations of antigen and antibodies. Finally, each well was coated with 2.5μg of protein (antigen) in 100μl of 0.05M carbonate buffer (pH 9.6) and kept overnight at 4°C in a moist chamber. Removed the unbound antigen by decanting the plate solution and wells were blocked with 1% BSA in 0.1M phosphate buffered saline (pH 7.2) for 1hr at 37°C. Flicked and washed wells thrice with 200μl of PBS containing 0.05% (v/v) tween-20. Wells were then incubated with polyclonal primary antibody, anti-ApoB (Santa Cruz
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Biotechnology, Inc. USA) diluted 1:1000 in PBS (containing 0.05% tween-20 and 1% BSA) and kept for 2 hr at 37°C. Plate was again washed and incubated with peroxidase labeled secondary antibody (1:20,000), anti-goat IgG (Sigma-Aldrich, USA) for 2 hr at 37°C. Wells were washed further three times as described above and color was developed by addition of 100µl of 2,2'-azino-di-(3-ethylbenzothiazolin sulfonic acid) reagent in each well for 30 min. Finally optical density of each well was measured at 405nm in ELISA reader (Stat Fax: Awareness Technology Inc., USA).

Apolipoprotein B Expression by Western Immunoblot Analysis

Western blot for apoB was done in liver. Tissue homogenates (10% w/v) prepared in 20mM Tris-HCl buffer (pH 7.4) at 4°C using mechanically driven teflon fitted Potter-Elvejham type homogenizer. The homogenates were centrifuged at 3000g for 10 min and the supernatants were used in the assay. Protein content was estimated by the method of Lowry et al., (1951). Protein samples (30µg) from each treatment group were separated on SDS-PAGE explained as follows:

**Polyacrylamide Gel Electrophoresis (SDS-PAGE):** The protein samples were separated by 7.5% SDS-polyacrylamide gel electrophoresis by the method of Laemmli, (1970) using minigel apparatus (BIORAD, UK). Following reagents were prepared

1. Buffer A
   1.5M Tris-HCl (pH 8.9)
2. Buffer B
   0.5M Tris-HCl (pH 6.8)
3. Acrylamide stock
   Acrylamide 30g
   Bis-acrylamide 0.8g
   Final volume was made to 100ml with double distilled water.
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4. Ammonium persulphate (APS): 10% (Prepared fresh)

5. Plug gel
   Acrylamide: 1 ml
   APS (10%): 40 μl
   TEMED: 4 μl

6. Separating gel 7.5% (Prepared fresh)
   Acrylamide stock: 1.25 ml
   Double distilled water: 2.42 ml
   Buffer A: 1.25 ml
   APS (10%): 25 μl
   SDS (10%): 50 μl
   TEMED: 2.5 μl

7. Stack gel (Prepared fresh)
   Acrylamide stock: 330 μl
   Double distilled water: 1.4 ml
   Buffer B: 250 μl
   APS (10%): 20 μl
   SDS (10%): 20 μl
   TEMED: 2 μl

8. Sample buffer (2X, prepared fresh)
   SDS (40%): 100 μl
   Beta-mercaptoethanol (β-Me): 40 μl
   0.5M Tris-HCl (pH 6.8): 100 μl
   Glycerol: 100 μl
   Bromophenol blue (33 mg%): 160 μl

9. Running buffer (Prepared fresh)
   Tris: 3 g
   Glycine: 14.4 g
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SDS 1g
Final volume was made to 1000ml with distilled water.

10. Coomassie blue stain
Methanol 250ml
Glacial acetic acid 50ml
Coomassie brilliant blue (R-250) 250mg
Final volume was made to 500ml with double distilled water.

11. Destaining solution
Methanol 62.5ml
Glacial acetic acid 17.5ml
Final volume was made to 250ml with double distilled water.

Casting the gels: Thoroughly cleaned the glass plates, spacers, comb, and the buffer reservoir of the gel apparatus with detergent and then rinsed well with water. Assembled the gel casting apparatus and poured the plug gel first to seal the bottom of the chamber so formed between the two plates. Determined the height to which the separating gel was to be poured by inserting a well-forming comb between the glass plates and marking the outer plate 1-2 cm below the teeth of the comb. Separating gel solution was then poured and it was overlaid with water saturated 2-butanol. Then allowed the plates to stand undisturbed for one hour to polymerize the gel. After the polymerization of the gel, thoroughly rinsed the top of the separating gel with water and dried with whatmann sheet and then stack gel was cast and comb was placed. The gel was allowed to stand for another half an hour at room temperature to polymerize.

Sample preparation: The proteins samples (30μg) were mixed with the sample buffer (2X) in 1:1 ratio and heated the samples at 95°C for 4min, cooled and centrifuged at 1000g for 2min.
Electrophoresis: Assembled the electrophoresis cell, filled the upper and lower reservoirs with running buffer and removed the comb from the stacking gel. Loaded the samples into the wells in the stacking gel by layering them under the buffer using micropipette. Glycerol in the samples provided necessary density for them to sink to the bottom of the wells. Bromophenol blue dye enabled the samples to be seen during loading. Finally attached the leads to the unit and connected them to the power supply (Power Rack 300, BIO-RAD, UK). The electrophoresis was carried at a constant current of 10mA through the stack gel and 15mA through the separating gel. As the dye front reached the plug gel, removed the gel assembly, the gel was then processed for western immunoblot as follows:

Western Immunoblot Procedure: Protein on the gel was transferred to PVDF membrane (Immobilon-P, Millipore, USA). Following reagents were prepared.

1. Transfer buffer (Tris-glycine, pH 8.3; One litre):
   - Tris 5.81g
   - Glycine 2.93g
   - SDS 1.0g
   - Methanol 200ml
   - H₂O 800ml

2. Phosphate buffer saline (0.01M, pH 7.4, One litre):
   - NaH₂PO₄ 0.132g
   - Na₂HPO₄ 1.452g
   - NaCl 9.0g
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*Electroblotting procedure:* For this four sheets of filter paper, and two foam pads of the same size as the gel were soaked in transfer buffer. The PVDF membrane was first soaked in 100% methanol and then in transfer buffer. The transfer stack was built in the following order: cathode side of the unit, foam pad, two sheets of filter paper, gel, membrane, two sheets of filter paper, foam pad, anode side of the unit. Tightened the transfer stack unit and put it into the transfer chamber (BIO-RAD, UK) filled with chilled transfer buffer and connected the power supply (EPS-1001, Amersham Biosciences, USA). Transfer was done at 300mA for 3hrs.

*Immunoblotting procedure:* Immediately after the protein transfer was completed, placed the membrane in a dish and incubated with blocking solution (5% non-fat milk in phosphate buffered saline) for 1hr with constant shaking. The membrane was then incubated with polyclonal primary antibody anti-ApoB (Santa Cruz Biotechnology, Inc. USA) diluted 1:500 with blocking solution, for 90min at room temperature. The membrane was sequentially washed with PBS, 0.05% Tween–20 in PBS and PBS each for 5 min. Then resaturated the membrane with 5% non-fat milk for 5min. The membrane was then incubated with peroxidase labeled secondary antibody (1:2000), anti–goat IgG (Sigma–Aldrich, USA) for 45 min each at room temperature. Again repeated the washing steps with PBS, 0.05% Tween–20 in PBS and then with PBS for 5 min each and the bands were developed by addition of diamino benzidine (8mg/10ml PBS + 12µl H₂O₂). The reaction was terminated by rinsing the membrane with double distilled water.

*Staining the gel:* One of the SDS-PAGE gel was placed in the coomassie brilliant blue stain overnight with constant shaking. Then destained the gel in destaining solution with gentle shaking. The destain solution was changed as needed until the background was clear. The gel was then dried in a gel wrap.
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Protein Estimation

Protein estimation was done by the method of Lowry et al., (1951). To the 10µl of samples from different treatment groups was added 3ml of copper carbonate [50:1 mixture of 2% sodium carbonate in 0.1N NaOH and (0.5% CuSO₄ and 1% Na-K-Tartarate in distilled water)] solution. Incubated it for 10minutes at room temperature. Added 300µl of 1N Folin’s phenol reagent to each tube, mixed it, incubated it for 30minutes at room temperature. Then measured the optical density at 620nm in spectrophotometer (UV 160A, Shimadzu). Bovine serum albumin (BSA) was used as standard (10-100µg).

mRNA Expression Studies

mRNA expression analysis was done for 5'-DI (liver, aorta and thyroid), LDL-R (liver) and HMG-CoA reductase (liver) genes at different time intervals by RT-PCR (QIAGEN, Germany). For this total RNA was isolated from different tissues and then RT-PCR was done

Total RNA Isolation

Total RNA isolation from different tissues was done using TRI REAGENT (Molecular Research Centre, Inc. Ohio, USA). TRI REAGENT combines phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate and most effective inhibition of RNase activity. TRI REAGENT isolates whole spectrum of RNA molecules. Protocol included the following steps:

Homogenization: 30mg of tissue samples from different treatment groups were homogenized in 0.5ml of TRI REAGENT in 1.5ml polystyrene microfuge tubes using hand homogenizer.

Phase separation: Kept the samples (homogenate) for 5 minutes at room temperature (20-25°C) to permit the complete dissociation of nucleoprotein complexes. Added 0.1ml chloroform to it and mixed it vigorously for 15seconds. Kept the samples at room temperature for 10 minutes. Then centrifuged it at 12000g for 15 minutes at 4°C. Following centrifugation, the mixture separated into
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a lower red phenol chloroform phase, interphase, and the colorless upper aqueous phase. The volume of aqueous phase should be about 60% of the volume of TRI REAGENT.

RNA precipitation: Transferred the aqueous phase to fresh tubes and added 250pl of isopropanol to precipitate the RNA. Mixed and kept the samples at room temperature for 10min. Centrifuged at 12000g for 10 minutes at 4°C. RNA precipitates formed white pallet on the bottom of the tube.

RNA wash: Removed the supernatant, washed RNA pallet with 0.5ml 75% ice-cold ethanol using centrifugation at 7500g for 5min at 4°C.

RNA solubilization: Removed the ethanol wash, briefly air dried the RNA pallet (not completely) then dissolved the pallet in 10μl of DEPC treated water.

Estimation of Purity and Concentration of RNA

Purity: Purity of isolated RNA was checked by determining the ratio of absorbance values at 260 and 280nm. The ratio for all the samples was approximately 1.9.

Concentration: Concentration of RNA was estimated by measuring the absorbance at 260nm (A_{260}) in spectrophotometer (UV-160A, Shimadzu) using A_{260}=1 \equiv 40\mu g/ml.

Integrity of RNA

Integrity and size distribution (quality) of isolated total RNA was checked by denaturing agarose-gel electrophoresis as follows:

Electrophoresis of RNA Through Agarose Gel Containing Formaldehyde: The method was adopted from Lehrach et al., (1977).

Preparation of 10X MOPS: Dissolved 10.46g of 3-(N-morpholino) propanesulfonic acid (MOPS) in 225ml of diethyl pyrocarbonate (DEPC) treated water and to this added 4.17ml of 3M sodium acetate. Adjusted the pH to 7.0 with 10M NaOH and then made up the volume to 250ml. Autoclaved, added 5ml of 0.5M EDTA (pH 8.0), and stored it in the dark bottle.
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*Preparation of gel:* Prepared 40ml of 1.2% agarose in DEPC treated water in a erlenmeyer flask in microwave oven. Then allowed it to cool to 60°C, to this added 5ml of 10X MOPS and 1.5ml of formaldehyde, mixed it. The gel was poured into horizontal gel electrophoresis chamber with comb and allowed to stand at room temperature for 30min (covered with aluminium foil).

*Preparation of samples:* Prepared the samples by mixing the following in sterile microfuge tubes:

- RNA (5µg) 4.5µl
- 10X MOPS 1.0µl
- Formaldehyde 3.5µl
- Formamide 8.0µl
- Ethidium Bromide 1.0µl (1mg/ml)

Mixed the samples, incubated at 65°C for 15min then chilled on ice and centrifuged for 30sec. To all the samples then added 2µl of 10X RNA formaldehyde gel loading buffer, mixed it.

*10X RNA formaldehyde gel loading buffer:*

- 50% glycerol
- 1mM EDTA (pH 8.0)
- 0.25% bromophenol blue
- 0.25% xylene cyanol

Running buffer (1X MOPS) was added in the electrophoretic chamber after removing the comb and then samples were loaded in different wells. Electrophoresis was carried out at 60V. Finally the bands were viewed and photographed using Gel Doc (Uvitech, England).
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**Deoxyribonuclease (DNase) Treatment**

Isolated RNA was treated with DNase before using it for RT-PCR, so as to make it free from DNA contamination. For this purpose DNase kit i.e. Deoxyribonuclease I (RNase free) procured from Fermentas (USA) was used.

Protocol: To the 5μg of RNA sample was added 5μl of 10X reaction buffer with MgCl₂, 45μl of DEPC-treated water and 5μl of Deoxyribonuclease I (1U/μl), incubated it at 37°C for 30min. To inactivate the deoxyribonuclease I, heated the above mixture at 65°C for 10 min in the presence of 5μl of 25mM EDTA (provided in the kit). Then used the prepared RNA as a template for reverse transcriptase.

**Primer Designing and Synthesis**

Primers for all the genes were designed with the help of software “Gene Runner” and got synthesized from Sigma Aldrich (USA). Length of the primers chosen was ~20bp. Primer sequence for different genes and product size was as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Expected product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-DI Sense</td>
<td>5'-TCTGGGATTTCAATTCAAGGC-3'</td>
<td>346bp</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-TAGAGCCTCTCAGGCAGAGC-3'</td>
<td></td>
</tr>
<tr>
<td>LDL receptor</td>
<td>5'-ACCGCCATGAGGTACGTAAG-3'</td>
<td>341bp</td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GGGTCTGGACCCTTTCTCTC-3'</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-GGGTCTGGACCCTTTCTCTC-3'</td>
<td></td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>5'-CATGATTTCCAAGGTTACGG-3'</td>
<td>323bp</td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GGGCACATGCAATGTAGATG-3'</td>
<td></td>
</tr>
<tr>
<td>Rat β-actin</td>
<td>5'-AGAGCTATGAGCTGCTGAC-3'</td>
<td>236bp</td>
</tr>
<tr>
<td>Sense</td>
<td>5'-CTGCATCTGTCAGCTGAC-3'</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-CTGCATCTGTCAGCTGAC-3'</td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

RT-PCR

One Step RT-PCR kit was used to carry out the RT-PCR for different genes (QIAGEN Inc., Germany). Reverse transcription and PCR were carried out sequentially in the same PCR tube (Labware Scientific Inc., USA).

Protocol: 2µg of RNA template was used in RT-PCR reaction from different groups. To this added all the reagents as per the instructions given by the manufacturer i.e. 10µl of 5X QIAGEN OneStep RT-PCR buffer (to provide a final concentration of 2.5mM MgCl₂ in the reaction mix), 2µl of dNTP mix (containing 10mM of each dNTP), 5µl of each sense and antisense gene specific primers (from 10µM stock) and 2µl QIAGEN One Step RT-PCR Enzyme Mix, 1µl RNAase inhibitor (1U/µl), finally PCR grade RNase-free water (provided in the kit) was added to make the total volume 50µl. Mixed it gently by vortex and centrifuged it to collect all the components at the bottom of the PCR tubes. Programmed the thermal cycler (Techne Inc. UK) as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>50°C</td>
<td>50min</td>
</tr>
<tr>
<td>Initial PCR activation</td>
<td>95°C</td>
<td>15min</td>
</tr>
<tr>
<td>3-step cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58.8°C</td>
<td>45sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1min</td>
</tr>
<tr>
<td>Number of cycles 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>68°C</td>
<td>10min</td>
</tr>
<tr>
<td>Final hold</td>
<td>10°C</td>
<td>10min</td>
</tr>
</tbody>
</table>
Materials and Methods

Started the RT-PCR program while PCR tubes were still on ice. Waited until the thermal cycler reached 50°C. Then placed the PCR tubes in thermal cycler. PCR products formed were analyzed on agarose gel electrophoresis.

**Agarose Gel Electrophoresis for PCR Products**

*Preparation of 50X TAE buffer (100ml):*

- 24.2g Tris base
- 5.71ml glacial acetic acid
- 10.0ml of 0.5M EDTA (pH 8.0)

*Preparation of gel:* 30ml of 1.5% agarose was prepared in 1X TAE (Tris-acetate) buffer in a erlenmeyer flask in the microwave oven. The gel was allowed to cool to 60°C and ethidium bromide was added to a final concentration of 0.5µg/ml and mixed thoroughly. The gel was poured into the horizontal plastic tray (both the ends sealed with scotch tape). Comb was already positioned in the tray before pouring the agarose solution. The gel was allowed to stand at room temperature for 30min. After the gel was completely polymerized, removed the comb and the scotch tape carefully and mounted the gel tray in the electrophoresis tank.

*Preparation of samples:* 5µl of PCR product from each PCR tube was taken and mixed with DNA gel loading buffer, mixed it by vortex and centrifuged it.

**DNA gel loading buffer:**

- 0.25% bromophenol blue
- 0.25% xylene cyanol FF
- 30% glycerol in water

Then running buffer (1X,TAE) was added in the electrophoresis tank. The samples were loaded into the wells of the submerged gel and electrophoresis was carried at constant current of 70mA for 50 minutes, the bands were viewed and photographed with Gel Doc. (Uvitech, England).

**Densitometric Analysis of Bands**

Densitometric analysis of the bands was done by UviBandMap software (Uvitech, England).

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**Statistical Analysis of the Data**

The difference between means ± Standard Deviations (SD) for control and treated groups were examined by using the Student’s t-test for unpaired values. Statistical differences of p-value at the level of 0.05 or less were considered significant.