Materials and Methods
Present study consisted of *in vivo* as well as *in vitro* work and was divided in two phases accordingly. Phase I consisted of *in vivo* studies on high fat diet-induced atherosclerosis using male New Zealand White rabbits as the animal model. Phase II of the work plan included *in vitro* studies on 7β-hydroxycholesterol-exposed mouse peritoneal macrophages.

**MATERIALS:**

Dietary cholesterol was obtained from **Loba Chemie, Mumbai, India.** Monoclonal antibodies against heat shock proteins hsp60, hsp70 and hsp90, ExtrAvidin Peroxidase staining kit, diaminobenzidine (DAB), *in vitro* translation system (using rabbit reticulocyte lysate), diethylpyrocarbonate (DEPC), 3-(N-morpholino)propanesulfonic acid (MOPS), polyvinylpyrrolidone, ficoll (Type 400), dextran sulfate (sodium salt), salmon sperm DNA (type III, sodium salt), DNAase I, PPO, POPOP, cholesterol standard, Dulbecco's modified Eagle's medium (DMEM) without indicator, 7β-hydroxycholesterol (7β-OH), lipopolysaccharide (LPS), MTT, L-glutamine, sodium selenite, N-ethylmaleimide (NEM), O-phthalaldehyde (OPT) were obtained from **Sigma Chemical Co., St. Louis, USA.** Fatty acid free bovine serum albumin, guanidinium thiocyanate, trisodium citrate dihydrate, sodium acetate, ethylenediamine tetracetic acid (EDTA), sarcosyl, 2-mercaptoethanol, redistilled phenol, formamide, formaldehyde, chloroform, isoamyl alcohol, isopropanol, glycerol, all of molecular biology grade, were purchased from **E. merck,**
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Darmstadt, Germany. \[^{35}\text{S}\] methionine (specific activity approx. 1200Ci/mM) was obtained from Board of Radiation and Isotope Technology, Mumbai, India. Peroxidase based in situ apoptosis detection kit was purchased from Oncor Inc., Gaithersburg, MD. Acrylamide, molecular biology grade agarose, sodium dodecyl sulfate, DL-methionine, N-(1-naphthyl) ethylene diamine dihydrochloride, sulfanilamide, L-arginine, reduced glutathione, oxidized glutathione, sodium nitrite were purchased from Sisco Research Labs., Mumbai, India.

All other chemicals used in the present study were of analytical grade obtained from Indian labs and those used for RNA work were of molecular biology grade from E.merck, Darmstadt, Germany.

PHASE I

Male New Zealand White rabbits were divided into four groups namely, group I (control diet fed), group II (high fat diet feeding for one month), group III (HFD feeding for three months) and group IV (HFD feeding for six months). Following parameters were studied in these groups after completion of different diet feeding schedules:

1. Basal serum lipid profile i.e., serum total cholesterol and triglycerides levels.
2. Study of histological changes in the aorta on HFD feeding using light microscopy.
3. Heat shock protein expression studies:
   a) Evaluation of expression and distribution of various heat shock proteins viz., hsp60, hsp70 and hsp90 immunohistochemically on aortic sections.
   
   b) Determination of aortic hsp70 content by western immunoblot assay.

4. Studies of aortic hsp70 mRNA:
   a) Evaluation of aortic expression of hsp70 mRNA by in situ hybridization with oligonucleotide probe specific for its inducible transcripts.
   
   b) Aortic total RNA isolation and its in vitro translation to see the translational efficiency of mRNA. The resulting proteins were analyzed by the following techniques:

   i) Evaluation of total protein synthesis by determining $^{35}$S-methionine incorporation into protein by liquid scintillation counting.
   
   ii) Analysis of resulting proteins by SDS-PAGE and subsequent autoradiography to see hsp70 synthesis.

DETAILED METHODOLOGY

ANIMAL GROUPING AND TREATMENTS

Male New Zealand White rabbits (1.0-1.2 kg body weight) were obtained from Experimental Animal Facility, Institute of Microbial Technology, Chandigarh, India and housed in the Animal House of the
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Department. After acclimatization, these were randomly divided into four groups of six rabbits each, according to different treatments as shown below:

**Grouping of rabbits**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Diet</th>
<th>Treatment period (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control diet</td>
<td>Six</td>
</tr>
<tr>
<td>II</td>
<td>High Fat Diet (HFD)</td>
<td>One</td>
</tr>
<tr>
<td>III</td>
<td>-do-</td>
<td>Three</td>
</tr>
<tr>
<td>IV</td>
<td>-do-</td>
<td>Six</td>
</tr>
</tbody>
</table>

**Diet composition**

<table>
<thead>
<tr>
<th>Component of Diet</th>
<th>Control Diet (g)</th>
<th>High Fat Diet (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powdered rabbit pellet diet</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Table butter</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-</td>
<td>5</td>
</tr>
</tbody>
</table>

Each rabbit was given 80g of respective diet in 24h. Water was available *ad libitum*. On completion of various treatment periods, body weights of rabbits in each group were monitored. Blood was drawn from the marginal ear vein of overnight fasting rabbits and serum prepared from the blood as described by Gupta (1983). All estimations in the serum were performed immediately after separation. After serum analysis, rabbits were
sacrificed by intravenous injection of sodium phenobarbitone (200mg/rabbit), thoracic aortae were removed, washed in chilled buffer and used immediately for various studies.

1. ESTIMATION OF BASAL SERUM LIPID PROFILE

Total cholesterol and triglycerides were estimated in serum from all the four groups of animals at one, three and six months of diet feeding intervals.

SERUM TOTAL CHOLESTEROL: The method of Chiamori and Henry (1959) was used to estimate total cholesterol in serum of rabbits.

To 9.9ml of ferric chloride acetic acid reagent (0.05% FeCl₃·6H₂O in glacial acetic acid), 100μl of serum sample was added and kept at room temperature for 15min after thorough mixing by vortexing. After incubation at 60°C for 2min with occasional shaking, the tubes were cooled and centrifuged at 2000 rpm for 10min. To 5ml of supernatant so obtained, 3ml of concentrated sulphuric acid was added. The tubes were immediately vortexed and kept at room temperature for 10min before taking absorbance at 560nm (UV170, Shimadzu, Japan).

For standard, 5ml of standard cholesterol solution (4mg/100ml in ferric chloride acetic acid reagent) was mixed with 3ml concentrated sulphuric acid. 5ml ferric chloride acetic acid reagent was used as blank.

Values obtained were expressed as mg cholesterol/dl serum.
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SERUM TRIGLYCERIDES: An enzymatic kit, Triglyceride (GPO) Reagent Set (Pointe Scientific Inc., MI, USA) was used to quantitate serum triglycerides. Triglycerides standard was supplied along with the kit. Values obtained were expressed as mg/dl serum.

2. HISTOLOGICAL STUDIES

The thoracic aortae, immediately after removal from the animals, were cleaned of adventitia and washed with phosphate buffer (0.1M, pH 7.2). The aortae were opened longitudinally and cross-sectional portions of normal and lesioned areas were either transferred to 10% neutral buffered formalin for histological studies or cryosections were cut immediately for *in situ* hybridization study. Remaining tissues were either processed for protein analysis by SDS-PAGE or quickly frozen in liquid nitrogen for RNA extraction.

Fixed tissues were washed in several changes of distilled water to remove any traces of fixative and dehydrated in ascending series of alcohol. For embedding in wax, the dehydrated tissues were treated with benzene and then transferred sequentially to 1:3::wax:benzene, 2:2::wax:benzene and then to 3:1::wax:benzene. Finally, tissues were given two changes of pure wax and embedded in wax.

Eight micron thick sections of aortae of control and HFD fed rabbits were cut using manual hand driven microtome and transferred to egg albumin coated glass slides. Sections were dewaxed in xylene, rehydrated
in descending series of alcohol and stained with hematoxylin and eosin. After dehydration in ascending series of alcohol, stained sections were mounted in DPX mountant and viewed under a light microscope for histological examination and photographed.

3. HEAT SHOCK PROTEIN EXPRESSION STUDIES

Expression and distribution of major heat shock proteins- hsp60, hsp70 and hsp90 was seen immunohistochemically in aortae of control and HFD fed rabbits from various treatment groups. Further, overall hsp70 content was evaluated in the aorta by immunoblot assay.

3.1 IMMUNOHISTOCHEMICAL LOCALIZATION OF VARIOUS HEAT SHOCK PROTEINS:

Eight micron thick aortic cross-sections taken on Poly-L-lysine coated glass slides (Sigma Chemical Co., St. Louis, MO, USA) were deparaffinized in xylene and rehydrated in descending series of alcohol. Endogenous peroxidase activity was blocked by incubating sections with 3% H$_2$O$_2$ for 30min at room temperature. Nonspecific staining was blocked by incubation with 2% BSA in phosphate buffered saline (PBS; 10mM, pH 7.2) for 30min at room temperature. Sections were then incubated with monoclonal antibody against either hsp60 (1:100), hsp70 (1:3000) or hsp90 (1:300) diluted in PBS with 1% BSA for 90min in moist chamber at room temperature. For negative control, only diluent (PBS with 1% BSA) was added. Further, after proper washing with PBS-T (PBS with
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0.05% Tween-20), sections were incubated with biotinylated goat anti mouse IgG (1:15) and then with streptavidin-labeled horse radish peroxidase (HRPO; 1:15) provided in the kit. The reaction product was developed using DAB (8mg in 10ml PBS) plus 100μl 3% H₂O₂. Reaction was terminated by washing with distilled water, sections mounted in glycerol jelly (Appendix I) and viewed and photographed with an inverted microscope (Olympus, Tokyo, Japan).

3.2 WESTERN BLOT ANALYSIS OF AORTIC HSP70 CONTENT:

Tissue homogenates (10% w/v) were prepared at 4°C in homogenizing buffer (20mM Tris-HCl buffer, pH 7.2, 1mM EDTA, 0.1% ethanol, 1mM phenylmethylsulfonyl fluoride) using a mechanically driven teflon fitted Potter-Elvejham type homogenizer and was kept at -70°C until analyzed. Protein was estimated by the method of Lowry et al. (1951). Aortic samples from two rabbits were pooled. 15μg of protein was diluted 1:1 with electrophoretic sample buffer (50mM Tris-HCl, pH 6.7, 4% SDS, 4% β-mercaptoethanol, 9.8% glycerol, 6mg% bromophenol blue), incubated in boiling water bath for 2min and resolved on 10% SDS-polyacrylamide gel under reducing conditions (Laemmli, 1970) using a mini-gel system (Mini Protean II, BIO-RAD Systems, USA). BIO-RAD's low molecular weight range prestained markers were used as standard. Proteins were electrophoretically transferred to PVDF membrane.
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(Immobilon-P, Millipore, USA) using 48mM Tris, 39mM glycine, 20% methanol (v/v), 0.4% SDS (w/v), pH 8.3 at 200mA for 2h using electrophoretic transfer apparatus (Pharmacia LKB Biotechnology, Sweden). Membrane was blocked overnight with 3% BSA in PBS at 4°C and incubated with monoclonal anti-hsp70 antibody (diluted 1:3000 in PBS with 1% BSA) with constant shaking for 1h. Hsp70 bands were visualized using peroxidase-based detection system followed by DAB plus H$_2$O$_2$ as done for immunohistochemical study.

4. HSP70 mRNA EXPRESSION STUDIES

For RNA studies, all solutions were prepared in DEPC-treated double distilled water (Appendix Ila) to inhibit RNase activity. All the glassware was baked at 240°C for 6h and separate batches of fresh plasticware were used. Gloves were worn at all times during the experiments to prevent nuclease contamination from hands.

4.1 IN SITU HYBRIDIZATION OF HSP70 mRNA:

_in situ_ hybridization of cellular mRNA is used to determine the cellular localization and the relative expression of specific transcripts within complex cell populations and tissues using biotin or digoxigenin labeled specific oligonucleotide probes. In the present study, a biotin-labeled 30 nucleotide long synthetic oligonucleotide (5'-CGATCTCCTCCTCTTGGTCAGCACCAGCCATGG-3') (Genset Singapore Biotech Pte. Ltd., Singapore) was used. This oligonucleotide sequence is
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complementary to the transcribed mRNA sequence of the human hsp70 encompassing amino acids 21-30 and selectively recognizes induced hsp70 transcripts in the rat (Udelsman et al., 1993).

Ten micron thick cryosections prepared from fresh aortic tissues were set on poly-L-lysine coated slides and stored at -70°C until use. Before hybridization, slides were quickly warmed to room temperature, rinsed once with PBS and then twice with 2X SSC (Stock 20X SSC: 0.3M sodium citrate, 3M sodium chloride, pH 7.0). Endogenous peroxidase activity was blocked with 3% H₂O₂ and sections were dehydrated in graded series of alcohol. Prehybridization was done in prehybridization buffer [50% formamide, 20% dextran sulfate, 5X Denhardt's (Appendix IIb), 10mM sodium phosphate, 5X SSC, 100μg/ml denatured salmon sperm DNA] for 15min at 41°C followed by hybridization with 0.5μg/ml oligonucleotide probe at 41°C for 4h in moist chamber. Hybridized sections were washed sequentially in 50% formamide/2X SSC at 37°C, 2X SSC at 37°C and 1X SSC at room temperature. Non specific staining was blocked with blocking solution (1% BSA in PBS) for 30min at 37°C, followed by 30min incubation with streptavidin-labeled HRPO solution at 42°C. After washing with 0.1% Tween-20/ PBS pre-warmed to 42°C, sections were developed with DAB plus H₂O₂. Reaction was stopped by washing with PBS. Negative controls were carried out following the same protocol, but without adding oligonucleotide probe. Slides were mounted
in 90% glycerol and viewed and photographed with an inverted microscope (Olympus, Tokyo, Japan).

4.2 IN VITRO TRANSLATION STUDY:

Total RNA was isolated from aortae of rabbits of different treatment groups and its translational efficiency assayed by determining $^{35}$S-methionine incorporation into proteins and by analyzing the resulting proteins by SDS-PAGE followed by autoradiography.

4.2.1 RNA isolation:

RNA was isolated using the AGPC (Acid Guanidinium thiocyanate - Phenol - Chloroform) protocol of Chomczynski and Sacchi (1987) that combines guanidinium thiocyanate and phenol-chloroform extraction to give a pure preparation of undegraded RNA in high yield. Aortic tissue from two animals was pooled for each group.

Immediately after removal, aortae from the animal were minced on ice and homogenized at room temperature with solution D (1ml/100 mg tissue; 4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) in a glass-Teflon homogenizer. This was followed by incubation with RNase free pancreatic DNAase I (final concentration 2μg/ml) for 30min at 37°C. Sequentially, 0.1ml of 2M sodium acetate, pH 4, 1ml of phenol (water saturated), and 0.2ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate in 15ml disposable polypropylene tubes (Tarsons Products, Calcutta,
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India), with thorough mixing by inversion after addition of each reagent. After shaking the suspension vigorously for 15 sec, it was centrifuged at 10,000g for 20 min at 4°C. The aqueous phase containing RNA was transferred to a fresh tube and RNA was precipitated with isopropanol at -20°C for 1 h. RNA was sedimented by centrifugation at 10,000g at 4°C and the pellet was redissolved in 0.3 ml of solution D and RNA precipitated again with isopropanol. Pellet so obtained was washed with 75% ethanol and after complete evaporation of ethanol, dissolved in DEPC-treated water. RNA was quantified and its purity determined by taking absorbance at 260 nm and 280 nm and calculating $A_{260}/A_{280}$.

RNA was further checked for its purity by subjecting it to agarose gel electrophoresis. 15 μg RNA of each sample was diluted with sample buffer [5 μl formamide, 1 μl 10X MOPS (Appendix IIc), 1.75 μl formaldehyde, 1 μl ethidium bromide stock (400 μg/ml), 1 μl bromophenol blue stock (25 mg/ml) for 2.25 μl sample]. Samples were incubated at 65°C for 15 min, chilled on ice and separated through 1.2% agarose/1 M formaldehyde gel in 1X MOPS at 60 V. Gel was analyzed for rRNA bands using UV transilluminator (BIO-RAD Systems, USA) and photographed.

4.2.2. In vitro translation:

Total aortic RNA from various treatment groups was translated in vitro using in vitro translation kit (Sigma Chemical Co., St., Louis, USA)
and following the manufacturer's instructions. Briefly, just before translation, RNA was denatured by heating at 75°C for 2min and immediately chilled on ice. Translation reaction mixture (25µl) contained 15µg total RNA, 25µCi [35S] methionine, 65% lysate, 5% minus-methionine labeling mix and 1µl RNase-free water. Incubations were for 1h at 30°C. Water was added in place of RNA for negative control and control RNA provided with the kit (an 1800bp RNA transcript yielding approximately 50.3 kDa protein on translation) was used as the positive control. The end product was evaluated by liquid scintillation counting as well as by electrophoresis.

**Determination of 35S-methionine incorporation into protein:** Aliquots of translation reaction mixture were triturated a few times with water to stop the reaction. It was then incubated with decolorizing solution (1N sodium hydroxide, 1mM DL-methionine, 1.5% H2O2) for 10min at room temperature to deacylate charged tRNA. Protein was precipitated with 25% TCA and precipitates were collected by filtration through glass fiber filters (GF/A; Whatman International Ltd., England). After rinsing tubes and filters twice with 10% TCA, filters were washed with 95% ethanol. Filters were dried under a heat lamp and placed in scintillation vials containing 10ml of scintillation fluid (3g PPO, 1.5g POPOP in 1litre toluene). Counts
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were taken on liquid scintillation counter and results were expressed as counts per minute.

**Protein analysis by SDS-PAGE:** Aliquots (25μl) of translation reaction mixture were treated with 1/10 volume of a 1mg/ml RNaseA solution (Stock: 10mg/ml in 10mM Tris-HCl, pH 7.5, 15mM NaCl) for 10min at 30°C. Samples were heated with 12.5μl reducing protein dye (125mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.3% bromophenol blue) at 95°C for 2min and electrophoresed on 10% SDS-polyacrylamide gel by the method of Laemmli (1970). Gels were then exposed to X-ray films (Kodak X-Omat) at -20°C.

**PHASE II**

In an attempt to simulate *in vivo* atherogenic conditions *in vitro*, mouse peritoneal macrophages were exposed to increasing concentrations of 7β-hydroxycholesterol (7β-OH) and following studies were carried out in control and treated cells:

1. Study of viability of control and treated cells using MTT assay.
2. Determination of cellular redox status in terms of intracellular reduced and oxidized glutathione levels. Influence of selenium, an antioxidant, on the redox status of macrophages exposed to 7β-OH was also studied.
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4. Study of hsp70 expression using western immunoblot assay.

5. Study of translational efficiency of hsp70 mRNA by \textit{in vitro} translation and analysis of the resulting proteins by the following methods:
   i) Determination of $^{35}\text{S}$-methionine incorporation into protein
   ii) Protein analysis by SDS-PAGE and subsequent autoradiography.

6. Study of involvement of apoptotic process by \textit{in situ} detection of DNA fragmentation using terminal deoxynucleotidyl transferase-mediated DIG II-dUTP nick end labeling (TUNEL) assay.

DETAILED METHODOLOGY

Male balb/c mice weighing 25g were obtained from the Central Animal House, Panjab University, Chandigarh, India. Animals were fed on standard pellet diet and water was provided \textit{ad libitum}. Peritoneal macrophages were isolated and cultured with or without different concentrations of 7β-hydroxycholesterol (3.5, 7.5, 15, 25 μg/ml) for the various studies.

ISOLATION AND CULTURE OF PERITONEAL MACROPHAGES:

Each mouse was given 2ml of 3% thioglycollate broth intraperitoneally and sacrificed on third day by cervical dislocation. Macrophages were isolated by peritoneal lavage with 10ml ice cold Ca$^{2+}$, Mg$^{2+}$ free PBS. Cells so obtained were washed with culture medium.
(DMEM without indicator with 1% BSA; appendix III), checked for cell viability with trypan blue and resuspended in the culture medium at a concentration of $1 \times 10^6$ cells/ml using hemocytometer. Cells were allowed to adhere to wells of sterile 96-well tissue culture plates (Corning Inc., NY, USA) for 2h at 37°C in humidified atmosphere of 5% CO$_2$. For RNA studies, macrophages were plated at a concentration of $1 \times 10^7$ cells/ml in 24-well tissue culture plates (Corning Inc., NY, USA). Non-adherent cells were removed and adhered macrophages were incubated for 24h under humidified conditions with LPS (20µg/ml) and with or without different concentrations of 7β-OH in fresh culture medium. Stock solution of 7β-OH (10mg/ml) was prepared in ethanol. It was further diluted in the culture medium to get the final concentrations of 3.5, 7.5, 15 and 25 µg/ml. Final ethanol concentration in the culture never exceeded 0.1% and control wells containing same amount of ethanol were also run.

1. CELL VIABILITY ASSAY

Cell viability was checked after 24h using MTT assay (Alley et al., 1988). MTT solution (10mg/ml in PBS) was added to each of the wells in the tissue culture plate in an amount equal to 10% of the culture medium and incubated at 37°C in humidified atmosphere of 5% CO$_2$ for 3h. The formazan crystals were dissolved in 200µl of organic solvent i.e., dimethyl sulfoxide (DMSO) and the absorbance was taken at 570nm using scanning
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multiwell spectrophotometer (Awareness Technologies Inc., USA). Blanks contained DMSO.

2. STUDY OF MACROPHAGE REDOX STATUS

The redox status of cells is critical in determining its fate as well as the type of cell death - apoptosis or necrosis it undergoes. Macrophage redox status was studied in terms of the ratio of intracellular oxidized and reduced glutathione levels (GSSG/GSH). Total and oxidized glutathione were quantified by fluorimetric method of Hissin and Hilf (1976) that uses O-phthalaldehyde (OPT) as the fluorescent reagent. The method is based on the reaction of reduced glutathione (GSH) with OPT at pH 8 and of oxidized glutathione (GSSG) with OPT at pH 12. GSH is complexed to N-ethylmaleimide (NEM) to prevent interference of GSH with measurement of GSSG.

The influence of selenium, an antioxidant, on the redox status of macrophages treated with 7β-OH was also studied. For this, macrophages were cultured with 7β-OH alone or with sodium selenite, an inorganic form of selenium. Stock solution (20mM) of sodium selenite was prepared in culture medium and was further diluted with the same to get final concentrations of 0.1μM, 10μM and 1mM. Cells were harvested in ice cold Ca²⁺, Mg²⁺ free PBS and lysed in 0.1M sodium phosphate-5mM EDTA buffer (pH 8.0) with 0.1% Triton-X 100. Protein was precipitated
with 10% TCA and supernatant was used for the estimation of total and oxidized glutathione.

2.1 TOTAL GLUTATHIONE:

Total glutathione (GSH+GSSG) was estimated as described below:

\[
\begin{align*}
100\mu l & \quad \text{Supernatant after TCA precipitation} \\
9.4ml & \quad \text{phosphate-EDTA buffer (pH 8.0)} \\
500\mu l & \quad \text{OPT (100 mg% in methanol)}
\end{align*}
\]

This was incubated at room temperature for 15 min and fluorescence was determined at 420nm (excitation at 350nm) with a fluorescence spectrophotometer (ELICO Ltd., Hyderabad, India).

Reduced glutathione was used as standard (10-100 ng). The results were expressed as ng of total glutathione per mg cell protein.

2.2 OXIDIZED GLUTATHIONE:

The supernatant (0.5ml) was incubated with 0.2ml of 0.04M NEM and 4.3ml of 0.1N sodium hydroxide for 30min at room temperature. After incubation, reaction procedure was as given below:

\[
\begin{align*}
500\mu l & \quad \text{Fraction from above mixture} \\
9ml & \quad \text{0.1N sodium hydroxide} \\
500\mu l & \quad \text{OPT (100 mg% in methanol)}
\end{align*}
\]

This was incubated at room temperature for 15 min and fluorescence was determined at 420nm (excitation at 350nm). Oxidized glutathione in
the concentration range of 10-100 ng was used to make standard curve. The results were expressed as ng GSSG per mg cell protein.

Reduced glutathione was calculated by subtracting GSSG values from total glutathione values. The redox ratio (GSSG/GSH) was calculated from the data so obtained.

3. NITRIC OXIDE SYNTHASE ACTIVITY

Nitric oxide synthase activity of macrophages was determined spectrophotometrically as nitrite accumulation in the culture supernatant by the method of Raddassi et al. (1994). Briefly, 100μl Griess reagent (1:1 v/v mixture of 0.1% N-(1-naphthyl)ethylene diamine dihydrochloride in distilled water and 1% sulfanilamide in 2.5% orthophosphoric acid) was mixed with an equal amount of culture supernatant in the wells of an ELISA plate (Nunc, Denmark) and incubated for 20min in dark. Absorbance was read at 540nm with ELISA reader (Awareness Technologies Inc., USA). Sodium nitrite was used as standard and nitrite amount was calculated from the standard curve and expressed as nmol/1x10⁶ cells.

4. HSP70 EXPRESSION BY WESTERN BLOT ASSAY

Cellular hsp70 content of control and treated macrophages was studied by western blot assay. For this, cultured macrophages were detached from culture plate by incubating with ice cold Ca²⁺, Mg²⁺ free PBS for 10min at 4°C and pelleted. Cells were then lysed in 0.1M Tris-
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HCl, pH 6.8, 1.5% SDS, 1mM phenylmethylsulfonyl fluoride by rapid freeze-thaw cycles. Cellular protein was estimated by the method of Lowry et al. (1951). 15μg protein from each sample was separated on to the 10% SDS-polyacrylamide gel (Laemmli, 1970). Proteins were electroblotted on to PVDF membrane and further processed for western immunoblot assay as described for phase I.

5. IN VITRO TRANSLATION STUDY

Translational efficiency of mRNA of macrophages treated with different concentrations of 7β-OH was studied by translating it in vitro and analyzing the resulting proteins for total protein synthesis and hsp70 synthesis specifically.

5.1 RNA ISOLATION:

Total RNA was isolated from cultured macrophages by a modification of the method of Stallcup and Washington (1983) that depends on the differential extraction of RNA by phenol at acid pH.

Cells were washed with ice cold Ca^{2+}, Mg^{2+} free PBS and harvested in 0.4ml lysis buffer (10mM EDTA, pH 8.0, 0.5% SDS). Wells were rinsed with 0.4ml of 0.1M sodium acetate (pH 5.2), 10mM EDTA (pH 8.0) solution which was transferred to cell lysate. This was followed by extraction with 0.8ml water-saturated phenol. The aqueous phase containing RNA was transferred to a fresh tube. To the aqueous phase was added, 88μl ice cold Tris-HCl, pH 8.0, 36μl 5M NaCl followed by two
volumes of ice cold ethanol and stored for 1h at 0°C. RNA was pelleted by centrifugation at 10,000g for 10min at 4°C. Pellet was redissolved in 40µl TE, pH 8.0 and 1µl 5M NaCl. 100µl ice cold ethanol was added and centrifuged again at 12,000g for 5min at 4°C. Ethanol was drained completely and RNA pellet was dissolved in DEPC-treated water. RNA was quantified and its purity checked by UV spectrophotometry as described in Phase I of the study. To further check the purity of isolated RNA, 15µg RNA from each sample was electrophoresed on 1.2% agarose/1M formaldehyde gel as described for phase I studies.

5.2 IN VITRO TRANSLATION:

In vitro translation of RNA isolated from cultured macrophages exposed to different concentrations of 7β-OH and its analysis by liquid scintillation counting and SDS-PAGE were done as described for phase I.

6. IN SITU DETECTION OF DNA FRAGMENTATION BY TdT-MEDIATED DIG II-dUTP NICK END LABELING (TUNEL) ASSAY

To see if 7β-OH causes cell death by apoptosis in macrophages, \textit{in situ} visualization of DNA fragmentation at the single cell level was performed by terminal deoxynucleotidyl transferase-mediated DIG II-dUTP nick end labeling (TUNEL) assay using Apoptag apoptosis kit (Oncor) and according to manufacturer's instructions.
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Oxysterols are known to be cytotoxic and a multistage process of DNA fragmentation is typically associated with morphologic apoptosis. In TUNEL assay, the 3'-OH ends generated by DNA fragmentation are identified with digoxigenin-dUTP which are inserted at these ends by the enzyme Terminal deoxynucleotidyl Transferase (TdT). The signal of TdT-mediated dUTP nick end labeling is detected by anti-digoxigenin antibody conjugated with suitable reporter enzyme system.

For TUNEL assay, cultured macrophages, on the completion of 24h incubation, were harvested in ice cold Ca\(^{2+}\), Mg\(^{2+}\) free PBS and cells were pelleted. Cells (approximately 5x10\(^7\) cells/ml) were fixed in 4% neutral buffered formalin for 10min at room temperature. 50\(\mu\)l cell suspension was dried on poly-L-lysine coated glass slides and washed in 2 changes of PBS. Endogenous peroxidase activity was quenched by incubating with 2% H\(_2\)O\(_2\) in PBS for 5min at room temperature. After washing in PBS, cells were incubated for 15sec with 1X equilibration buffer provided with the kit. This was followed by incubation with working strength TdT enzyme (76\(\mu\)l reaction buffer plus 32\(\mu\)l TdT enzyme) for 1h at 37°C. Reaction was stopped by washing in pre-warmed working strength stop buffer for 30min at 37°C with occasional agitation. After washing slides thrice in PBS, cells were incubated with anti-digoxigenin-peroxidase for 30min at room temperature, washed in PBS and color was developed with 0.05% DAB in PBS plus H\(_2\)O\(_2\). Cells were counterstained with methyl green (0.5% in
0.1M sodium acetate, pH 4.0). Slides were mounted in 90% glycerol and viewed and photographed with an inverted microscope (Olympus, Tokyo, Japan).

**STATISTICAL ANALYSIS OF DATA**

The differences between the mean ± SEM (standard error of means) were compared for the following groups:

i) Control (Group I) \( \text{Vs} \) HFD fed (Groups II, III and IV)

ii) Control (untreated cells) \( \text{Vs} \) treated cells (cells exposed to either 7β-OH or sodium selenite)

iii) Cells treated with 7β-OH and sodium selenite \( \text{Vs} \) cells exposed to similar concentration of 7β-OH alone.

Differences between groups were tested using a Student's \( t \) test for unpaired values. Statistical differences of p-values at the level of 0.05 or less were considered insignificant.