Summary and Conclusions
Atherosclerosis is increasingly being viewed as an inflammatory disease linked to an abnormality in oxidation-mediated signals in the vasculature. The presence of oxidative and other kinds of stress in the vasculature induces stress response in the cells leading to the formation of heat shock proteins (Lindquist, 1986) which constitute a universal cellular defense program (Morimoto, 1993).

Oxidized LDL, a key proatherogenic factor has been shown to induce hsp70 expression in cultured human endothelial cells and smooth muscle cells (Zhu et al., 1994 and 1995). Levels of 7β-hydroxycholesterol, one of the sterols oxidized in C7 and considered to be one of the principal components of oxidized LDL responsible for cellular injury, are increased in hypercholesterolemic humans and rabbits (Addis et al., 1989; Hodis et al., 1991; Carpenter et al., 1993).

Another important fact is that early lesions composed primarily of lipid-loaded macrophages show the highest concentration of 7β-hydroxycholesterol relative to cholesterol (Carpenter et al., 1993). Macrophage is considered to be the principal, inflammatory mediator of cells in the atheromatous plaque microenvironment. Infact, monocytes and macrophages are a common feature in atherosclerotic lesions at all stages of lesion development.

Further, glutathione and glutathione-dependent enzyme GSH-Px, represent a coordinately regulated defense against oxidative stress and
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Nitric oxide, an important biological product of mammalian cells, is critical for normal vessel wall homeostasis. The protective action of selenium, a biological trace element and an antioxidant, against atherosclerosis is well documented. Also, apoptosis is increasingly being recognized as the mode of cell death in vessel wall.

In the light of the above mentioned reports, the present study was undertaken with the following objectives:

1. To evaluate atherogenic status of animals after HFD feeding for one, three and six months.

2. Whether hsp expression and distribution is affected in very early stages of atherosclerosis when histoarchitectural changes do not appear in aorta. How are expression and distribution of major hsps altered in the aorta during later stages of atherosclerosis?

3. If alterations in the distribution are accompanied by a change in the aortic content of hsp70 expression and if hsp70 expression is regulated at transcriptional or translational level during experimental atherosclerosis.

4. If hsp70 expression in macrophages is altered in response to 7β-hydroxycholesterol, an important component of oxidized LDL and if yes, whether it is regulated at the translational level?
5. How 7β-hydroxycholesterol affects normal macrophage metabolic functions like redox cycle and iNOS activity and how selenium, an antioxidant, influences these.


Keeping these objectives in mind, the study in hand was divided into two phases. In phase I, high fat diet-induced atherosclerosis in rabbits was used to study hsp expression and in phase II, murine peritoneal macrophages exposed to 7β-hydroxycholesterol were analyzed for hsp70 expression and other related metabolic activities. The experiments revealed that:

1. HFD feeding led to generation of hyperlipidemic state manifested as increased serum lipid profile in terms of serum total cholesterol and triglycerides. While aortic histology remained unaffected on HFD feeding for one month, prolonged HFD feeding for three and six months induced changes in the histoarchitecture of aorta in the form of fatty streaks and atheromatous plaques followed by fibrous plaques.

2. Though expression of major hsps- hsp60, hsp70 and hsp90, as observed by immunohistochemical localization studies, after HFD feeding for one month was same as in aorta from control group, these were found to be elevated and their distribution too
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was heterogeneous on prolonged HFD feeding for three and six months. Hsps were seen to localize preferentially in the plaque areas, particularly the foam cells, the endothelial and subendothelial space and the fibrous cap. Apart from changes in the distribution and expression of hsp70, its aortic content, as seen by immunoblot assay, though similar in aortae from normal and one month treated group, increased after HFD feeding for three and six months.

3. HFD-generated atherogenic state resulted in the expression of inducible hsp70 mRNA in the rabbit aorta, which was not detected in the normal aorta, as observed by in situ hybridization studies. Hsp70 mRNA was observed to be localized in the plaque area and to a lesser extent in the media.

4. In vitro translation studies in aortae from all the groups revealed that the translational efficiency of RNA and overall protein synthesis were unaffected during atherosclerosis as shown by determination of $^{35}$S-methionine incorporation into proteins. Specifically, synthesis of hsp70, as analyzed by subjecting proteins obtained by in vitro translation to polyacrylamide gel electrophoresis, too, was unchanged after HFD feeding for one, three and six months.
5. *In vitro* studies revealed that while lower concentrations of 7β-hydroxycholesterol (3.5 and 7.5 μg/ml) did not affect cell viability, the higher concentrations of 15 and 25 μg/ml decreased cell viability significantly. While exposure to 3.5 μg/ml of 7β-hydroxycholesterol did not affect the cellular redox ratio, higher concentrations (7.5-25 μg/ml) increased the redox ratio in macrophages revealing thereby that macrophages treated with 7β-hydroxycholesterol were in a higher oxidative state.

6. Supplementation of selenium, an antioxidant, to macrophages treated with 7β-hydroxycholesterol decreased the redox ratio significantly by decreasing GSSG levels and increasing GSH levels. Selenium restored the redox status of macrophages treated with lower concentrations (3.5 and 7.5μg/ml) of 7β-hydroxycholesterol to normal. At higher concentrations of 7β-hydroxycholesterol too, redox ratio was lowered by selenium supplementation significantly as compared to 7β-hydroxycholesterol treated cells.

7. 7β-hydroxycholesterol downregulated the LPS-induced nitric oxide synthase activity of macrophages in a dose-dependent manner as increased nitrite levels were observed in the culture
supernatant. Selenium supplementation, however, further decreased the nitric oxide production by macrophages.

8. Hsp70 levels in macrophages, evaluated by immunoblot assay, decreased progressively on treatment with increasing concentrations of 7β-hydroxycholesterol. Suppression of hsp70 expression by 7β-hydroxycholesterol even at its noncytotoxic concentrations indicated that it is not an effect of decreased cell viability.

9. The translational efficiency of RNA from macrophages treated with 7β-hydroxycholesterol as shown by $^{35}$S-methionine incorporation into proteins though unaffected at the noncytotoxic concentration of 7β-hydroxycholesterol, decreased at its higher concentrations. Hsp70 synthesis, however, as seen by SDS-PAGE analysis of proteins obtained by in vitro translation decreased at all concentrations of 7β-hydroxycholesterol.

10. 7β-hydroxycholesterol induced apoptosis in macrophages in a dose-dependent manner at its cytotoxic concentrations as revealed by in situ detection of DNA fragmentation by TUNEL assay.

HFD feeding induces hyperlipidemia by increasing LDL and VLDL levels in blood, thus triggering the onset of atherosclerosis. Continued
hyperlipidemia leads to histoarchitectural changes in the aorta manifested as atherosclerotic lesions—fatty streaks/atheromatous plaques at early stage and later on as fibrous plaques. Oxidative stress due to elevated LDL levels and release of ROS and mechanical stress due to endothelial injury and presence of intra- and extracellular lipid deposits in atherosclerotic plaques induce a stress response in the cells of the vascular wall in the form of various hsps. The study indicates that hyperlipidemia alone is not enough to cause alteration in hsp expression as hsp levels were increased only after structural changes appeared in the aortae.

While presence of hsp60 in the foam cells of atheromatous plaques might account for increased necrosis in plaques, elevated hsp70 levels might be responsible for cellular protection against various stresses, thus aiding in cell survival. Increased hsp90 levels in fatty streaks/atheromatous plaques composed of macrophage foam cells on the other hand, suggest increased apoptosis in the cells, thus contributing towards cell debris and also plaque stabilization by avoiding the implications of necrotic death of macrophages in the plaque. While hsp70 expression is generally enhanced in atherosclerotic aorta, some plaque components might inhibit hsp70 expression leading to decreased hsp70 synthesis observed in the present study in certain plaque areas.

The effect of which of the three hsps predominates, will decide the fate of the developing lesion—whether it will develop into an advanced
lesion with a relatively acellular core stabilized by macrophage death by apoptosis or it will evolve into a lesion with a necrotic core leading finally to plaque rupture and the complications accompanying it.

Highlights of the present study include the depiction of a change in the aortic hsp70 content along with alteration in hsp70 distribution and that of the expression of inducible hsp70 mRNA in the atherosclerotic aorta. In situ hybridization of hsp70 mRNA with oligonucleotide probe specific for its inducible transcripts revealed a site – specific induction of hsp70 in atherosclerotic lesions. Absence of any detectable signal in normal aorta proved the specificity of the probe towards inducible transcripts of hsp70 mRNA. Hsp70 in the plaque area as seen by immunohistochemical techniques, colocalized with inducible hsp70 mRNA as seen by in situ hybridization, revealed that increased hsp70 levels are due to expression of inducible hsp70. The present study also points towards regulation of hsp70 expression at the transcriptional level under atherosclerotic conditions rather than translational level.

In order to simulate in vivo atherogenic conditions in vitro, murine peritoneal macrophages were exposed to 7β-hydroxycholesterol. The study shows that higher concentrations of 7β-hydroxycholesterol are cytotoxic. This also indicated that 7β-hydroxycholesterol impairs the cellular redox buffer system thus subjecting macrophages to severe oxidative stress.
Selenium supplementation regulates the GSH redox cycle, thus resulting in lower oxidative state of the cells. 7β-hydroxycholesterol impairs iNOS activity of macrophages suggesting that it is one of the components involved in the oxidized LDL induced inhibition of NO production by macrophages.

The major outcome of the study is the depiction of inhibition of hsp70 levels in macrophages in response to 7β-hydroxycholesterol even at its noncytotoxic concentrations. This reveals that 7β-hydroxycholesterol impairs the cellular defense program in macrophages involving hsp70. Low levels of hsp70 contribute towards 7β-hydroxycholesterol-induced apoptosis in macrophages.

Not only does the study indicate that concentrations of 7β-hydroxycholesterol necessary to downregulate the hsp70 expression in macrophages in vitro could be present in vivo in advanced lesions, but it also points towards 7β-hydroxycholesterol, one of the components of oxidized LDL and of atherosclerotic plaques, being responsible for inhibition of hsp70 synthesis in certain plaque areas, as observed in the present study, thus contributing towards apoptotic cell death in atheroma.

The present study of hsp expression under atherogenic conditions in rabbits shows that the cells of the vascular wall are subjected to extreme stress during atherosclerotic plaque development. It also implies that
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several hsps expressed in response to this stress, act simultaneously pro- or anti-atherogenically, rather than one specific hsp acting alone. In conclusion, the *in vivo* and *in vitro* models used in the present study revealed that hsp expression is altered under atherogenic conditions and indicated towards transcriptional regulation of hsp70 expression during atherogenesis and that the uptake of 7β-hydroxycholesterol by macrophages in atherosclerotic plaques might be responsible for areas of decreased hsp70 expression.