Introduction
Atherosclerosis, the principal cause of heart attack, stroke and gangrene of the extremities, is responsible for 50% of mortality in the USA, Europe and Japan. Indians, both native and immigrant, are predisposed to develop a cluster of risk factors, and consequently are more prone to the disease as compared to several other ethnic groups. Atherosclerosis is a highly complex disorder with multiple genetic and environmental influences that affects man in the later part of his life.

The process, in normal circumstances a protective response to insults to the endothelium and smooth muscle cells of the arterial wall, consists of the formation of atheromatous and fibrous lesions, preceded and accompanied by inflammation. The advanced lesions of atherosclerosis result from excessive inflammatory-fibroproliferative response to numerous different kinds of insult.

Atherosclerosis has a multifactorial etiology, yet the various hypotheses that have been outlined in order to explain the lesion formation can be integrated into the so called 'response to injury' hypothesis (Ross, 1993). Several proatherogenic factors, like oxygen radicals (Quinn et al., 1987), oxidized derivatives of cholesterol (Aupeix et al., 1995; Lizard et al., 1999), cytokines (Quinn et al., 1987), modified lipoproteins (Steinberg et al., 1989), and mechanical stress (MacMahon et al., 1990) are cytotoxic; therefore, they might activate some mechanisms of cellular defense.
Cells facing toxic stimuli rapidly and preferentially synthesize a family of cytoprotective proteins, the heat shock proteins (hsp) (Welch, 1990), leading to heat shock response. From prokaryotes to man, the heat shock response represents a universally conserved cellular defense program. The heat shock response and heat shock proteins protect against thermal stress and other non-thermal cytotoxic agents, including tumor necrosis factor-α, oxidants, and endotoxin (Roma and Catapano, 1996).

Evidence is growing for an involvement of stress proteins in atherosclerosis. The possible relevance of stress proteins in this pathological condition is consistent with the fact that several of the factors contributing to the etiology of the disease can also activate a stress response viz. inflammation, immune response and endothelial injury caused by mechanical/chemical stress.

Wick et al. (1995) proposed a 'two-stage' model for the formation of atherosclerotic lesions. According to their hypothesis, the first stage, essentially mediated by the immune system would lead to the formation of reversible lesions, containing monocytes, lymphocytes and smooth muscle cells. The second stage, characterized by the appearance of foam cells and the development of persistent lesions, would only occur when hypercholesterolemia develops, for instance as a consequence of lipid-rich diet. Various stimuli potentially associated with the induction of a stress response, might be involved in the initiation of the first stage by activating
an immune response and by causing endothelial dysfunction. Lipoprotein overload and/or calcification of the vessel wall would lead to the second stage of the process.

Stress proteins form a large family, which includes polypeptides of low, intermediate and high molecular sizes. In the field of atherosclerosis, hsp60 and hsp70 (hsp60 and hsp70) are the most widely investigated stress proteins. Hsps are present in the unaffected vessel walls and at the sites of atherosclerotic lesions. A number of studies indicate that hsp60 may act as an autoantigen (Xu et al., 1992, 1993b and 2000; Heng and Heng, 1994; Schett et al., 1997; George et al., 1999) and has recently been suggested to be a marker for early cardiovascular disease (Pockley et al., 2000).

Hsp70 has been detected in areas of atherosclerotic lesions of human aortae (Berberian et al., 1990; Johnson et al., 1993) suggesting that hsp70 localization changes during atherosclerotic evolution while the overall aortic hsp70 content remains unchanged (Johnson et al., 1993). Hsp70 is considered to be cytoprotective. Exogenous hsp72/73 in low concentration (10 μg/ml) is shown to increase survival of smooth muscle cells isolated from both normal and atherosclerotic macaques at all temperatures (Johnson et al., 1990). Hsp70 is reported to improve cardioprotection in vivo (Yellon et al., 1992) and in vitro (Iwaki et al., 1993).
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Not much work has been done on hsp90 in relation to atherosclerosis though Johnson et al. (1993) demonstrated hsp90 expression in atherosclerotic lesions of humans. Excess of hsp90 is shown to be responsible for increased apoptosis in macrophages, and thus suggested to play a role in controlling the part played by mononuclear phagocytes in immunopathology (Galea-Lauri et al., 1996).

While there are a few studies regarding the expression of major hsps in atherosclerotic lesions, none of the reporters have investigated the expression of these hsps at various stages of the development of atherosclerosis. It, therefore, seemed important to see the expression and distribution of major hsps during atherosclerotic progression i.e. at a very early stage when hyperlipidemia is not accompanied by histological changes and at later stages in less developed and advanced lesions.

Further, the expression of hsps is mainly regulated at the level of transcription in mammalian cells. It is mediated through one or more of a family of heat shock transcription factors (HSFs) (Morimoto, 1993). Though many studies have been done on the regulation of hsp gene expression under different conditions and in various cell models, such as experimental hypertension model where hsp70 has been shown to have transcriptional regulation (Kohane et al., 1990), no study has so far been reported regarding the regulatory response of hsp in atherogenic model.
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Much research is channeled these days into two aspects of pathogenesis of atherosclerosis. First, the role of macrophages and second, the role of oxidative events and the oxidized forms of lipids in the vessel wall. In lesions of atherosclerosis, at all stages of lesion development, the presence of monocytes and macrophages is a common feature (Jonasson et al., 1986; Emeson and Robertson, 1988; van der Waal et al., 1989; Capron, 1993; Stary et al., 1995). One of the initial events in the development of atherosclerotic lesions is the adherence of blood monocytes to the arterial endothelium. Lipid uptake by macrophages, leading to foam cell formation is observed throughout the lesion development (Raines and Ross, 1997). The accumulation of foam cells and the subsequent formation of necrotic core due to macrophage death, significantly contribute to lesion mass, alter structural properties of the vessel wall and can destabilize the plaque (Davies et al., 1993).

Further, lipid uptake may contribute to monocyte/macrophage activation. In atherosclerotic lesions, monocytes/macrophages have been shown to express cytokines (Kishikawa et al., 1993) and immune-modulating factors, enzymes and inhibitors, extracellular proteins that modulate adhesion, regulators of lipoprotein metabolism, stimulators of growth and migration, adhesion molecules and factors that alter coagulation and thrombosis (Raines et al., 1995). Macrophages also play a
critical role in the fibroproliferative response in lesions (Leibovich and Ross, 1975).

Growing evidence suggests that atherosclerosis is either caused by or accompanied by oxidative events in the vessel wall. These oxidative events have been implicated in the proatherogenic modification of proteins, alteration of gene expression, promotion of inflammation, remodeling of vessels and perturbations of vascular tone (Schultz and Harrison, 2000). Oxidation is now considered to be a prerequisite for the atherosclerotic process (Steinbrecher et al., 1990; Ross, 1993). Oxidative modification of low density lipoproteins (LDL) by activated macrophages in the subintimal space is an important event in atherogenesis.

Oxidation of LDL converts these lipoproteins into atherogenic molecules (Ox-LDL) that are viewed as key factors in endothelial injury (Steinberg et al., 1989; Steinberg and Witztum, 1990). Oxidative modification of LDL leads to the formation of a number of bioactive molecules like oxidized sterols, oxidized fatty acids and phospholipids and protein derivatives. Among the principal components of oxidized LDL responsible for cellular injury, oxysterols play a critical role both in vivo and in vitro, particularly those oxidized at C7 such as 7β-hydroxycholesterol and 7-ketocholesterol (Lizard et al., 1997).
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Sterols oxygenated at the 7-position have been shown to predominate in Cu$^{2+}$-oxidized LDL as well as in human atherosclerotic plaque (Brown et al., 1997). Increased levels of 7β-hydroxycholesterol have been found in plasma and atheromatous plaques of humans (Addis et al., 1989; Carpenter et al., 1993) and hypercholesterolemic rabbits (Hodis et al., 1991). Plasma 7β-hydroxycholesterol has recently been linked with an increased risk of atherosclerosis (Salonen et al., 1997; Ziedén et al., 1999).

Ox-LDL have been shown to have a pleiotropic effect on cellular functions (Witztum and Steinberg, 1991; Witztum, 1993; Navab et al., 1994); products of Ox-LDL are chemotactic for monocytes and T cells and induce one or more endothelial-cell adhesion molecules specific for these cell types; Ox-LDL can stimulate monocytes to secrete interleukin-1, which in turn incites smooth muscle cell proliferation; Ox-LDL is also immunogenic and antibodies to epitopes of Ox-LDL are found in plasma and in lesions associated with immune complexes; Ox-LDL or its products can profoundly impair the nitric oxide-mediated vasorelaxation of coronary arteries in response to agents such as acetylcholine.

Ox-LDL have also been shown to induce hsp70 expression in endothelial (Zhu et al., 1994) and smooth muscle cells (Zhu et al., 1995) and low molecular weight hsp, -34kDa and -23kDa proteins, in mouse
peritoneal macrophages (Yamaguchi et al., 1993). The component of Ox-LDL responsible for this induction of hsp expression is still unknown.

Oxysterols like Ox-LDL, have a wide spectrum of biological activities, including inhibition of DNA and cholesterol synthesis, immune response modulation, cytotoxicity and others (Smith and Johnson, 1989). But these, including 7β-hydroxycholesterol, were unable to induce hsp70 expression in cultured endothelial cells (Pirillo et al., 1999). In the context of the above reports available in literature, it seemed logical to evaluate hsp70 expression in the presence of 7β-hydroxycholesterol in macrophages.

Apart from hsp70, another cellular protective mechanism against oxidative stress involves glutathione, an essential tripeptide, and related anti-oxidative enzymes, etc. It participates in the maintenance of the reduced thiol groups in intracellular proteins/enzymes/aminoacids and protects cells against oxidative damage (Meister and Tate, 1976; Meister and Anderson, 1983). Reduced glutathione (GSH) can effectively detoxify reactive oxygen species (ROS) in the presence of superoxide dismutase (SOD) (Meister and Anderson, 1983; Pichoner et al., 1995). GSH is also required for the preservation of the reduced active form of the selenoenzyme glutathione peroxidase (GSH-Px), that catalyzes the reduction of organic hydroperoxides to corresponding nontoxic alcohols (Flohe et al., 1979).
A weak glutathione-related enzymatic antioxidant shield is present in human atherosclerotic lesions (Lapenna et al., 1998). Oxidized LDL has been shown to increase glutathione levels in macrophages and endothelial cells (Gotoh et al., 1993; Cho et al., 1999). Further, fractions of oxidized LDL containing oxysterols have been demonstrated to have a larger cytotoxic effect than their effect on GSH depletion in endothelial cells (Therond et al., 2000). But, the effect of oxysterols like 7β-hydroxycholesterol on intracellular GSH levels and thus the cellular redox status in macrophages has not been explored till now.

Further, nitric oxide (NO), an important biological product of mammalian cells, is a potent vasodilator and a potent modulator of monocyte interaction with the vessel wall (Bath et al., 1991). The impairment of NO activity has been shown to be an early event in the development of atherosclerosis (Sorenson et al., 1994; Kari et al., 1997). NO is suggested to abrogate the ability of macrophages to oxidize LDL (Mao et al., 1992). Hence, NO is considered to be anti-atherogenic. Also, NO has been shown to exert either pro- or anti-apoptotic effects depending on the cell type and stimulus (Mannick et al., 1997). Although Ox-LDL (Yang et al., 1994) and oxidized cholesterol in Ox-LDL (Liu et al., 1998) have been shown to inhibit lipopolysaccharide-induced NO production by macrophages, NO release has not been monitored in macrophages in the presence of 7β-hydroxycholesterol as yet.
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Since, free radicals are known to play an important role in initiation of plaque formation, involvement of antioxidants as the preventive agents in atherosclerosis has been implicated. The protective action of selenium against atherosclerosis is well documented (Wojcicki et al., 1991; Poltronieri et al., 1992; Kang et al., 1998). Selenium is an integral structural part of the enzyme, glutathione peroxidase (Shamberger, 1983) containing selenium in the form of selenocysteine (SeCys). Further, seleno compounds have been shown to inhibit NO production by immunostimulated or asbestos-induced macrophages (Southan et al., 1996; Fan et al., 1997).

In the light of the above position, the effect of 7β-hydroxycholesterol on cellular redox status and NO production in macrophages in the presence of an antioxidant, sodium selenite, was investigated.

Further, apoptosis is increasingly being recognized as the mode of cell death in the vessel wall, thus contributing towards formation and enlargement of the lipid core of the advanced lesions (Isner et al., 1995; Hegyi et al., 1996; Kockx et al., 1996; Kockx and Hermann, 1998; Kang et al., 1999). Ox-LDL has been shown to be toxic for macrophages (Reid and Mitchinson, 1993; Marchant et al., 1995) leading to apoptosis (Reid et al., 1993; Hardwick et al., 1996). Oxysterols, including 7β-hydroxycholesterol have been reported to induce apoptosis as well as necrosis in various cell
types (Aupeix et al., 1995, Nishio and Watanabe, 1996; Lemaire et al., 1998; Lizard et al., 1999).

On the other hand, heat shock proteins are well recognized to play a protective role in cell survival and downregulation of hsp70 expression is shown to result in apoptosis (Mosser et al., 1997). Thus, keeping these reports in mind, it seemed important to study the status of apoptosis in relation to hsp expression in the presence of oxysterols in macrophages.

Keeping all these reports in view, the present study was designed to evaluate the expression of heat shock proteins under atherogenic conditions in vivo and in vitro. Rabbits with high fat diet-induced hyperlipidemia were used as the atherogenic model for in vivo studies and mouse peritoneal macrophages exposed to 7β-hydroxycholesterol were employed as the in vitro cell model.

Though diet-induced hyperlipidemia in rabbits is a well-established model for experimental atherogenesis and a lot of work has been done on this model, the study of heat shock proteins at such an extensive level has not been carried out earlier. Further, considering the fact that macrophages play a critical role in the initiation, formation as well as progression of atherosclerotic lesions and that 7β-hydroxycholesterol, an oxysterol, is found in significant amounts in vivo under atherogenic conditions, in this study, an attempt was made to examine the changes in hsp70 expression and other related metabolic activities of macrophages in the presence of
7β-hydroxycholesterol. Also, the aspect of cell death induced by 7β-hydroxycholesterol in macrophages was explored.

**OBJECTIVES OF THE STUDY**

1. Induction of experimental atherosclerosis in rabbits by high fat diet feeding for one, three and six months. Characterization of atherogenic state by studying serum lipid profile i.e., serum total cholesterol and triglycerides levels, and examining histopathological changes in the aorta.

2. Identification of major heat shock proteins viz. hsp60, hsp70 and hsp90 and study of their distribution at various stages of high fat diet-induced atherosclerosis in the rabbit aorta.

3. Evaluation of the aortic content of hsp70, expression of stress-inducible hsp70 mRNA and its translational efficiency under atherosclerotic conditions in rabbits.

4. To investigate the changes in hsp70 expression and translational efficiency of its mRNA, in cultured murine peritoneal macrophages in the presence of 7β-hydroxycholesterol, an important oxysterol in atherosclerosis.

5. To study changes in intracellular glutathione levels, cellular redox status, and nitric oxide synthase activity of macrophages in the presence
of 7β-hydroxycholesterol and during selenium supplementation and also to investigate the status of apoptosis.