Studies of Apoptosis and bcl-2 in Experimental Atherosclerosis in Rabbit and Influence of Selenium Supplementation

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Abstract. Apoptosis is a ubiquitous physiological mechanism of cell death regulating tissue mass and architecture. An attempt was made in the present study to see the occurrence of apoptotic cell death in three different treatment groups of rabbits viz. Control, HFD fed and HFD + Selenium fed. Apoptotic activity as checked by *in situ* DNA end labelling (TUNEL Assay) revealed excessive staining, mostly concentrated in plaque region both in fibrous as well as atheromatous plaque in HFD fed animals. However, in selenium (Se) supplemented animals, very little TUNEL staining could be seen, and even that confined to endothelial cells only. The control group on the other hand was totally devoid of any staining. Transmission Electron Microscopic (TEM) study also depicted the occurrence of apoptosis in aortic cells of HFD fed animals and very little in Se supplemented animals. Apoptotic activity has been discussed in relation to oxidative stress in HFD fed group. bcl-2, though an antiapoptotic oncoprotein, was found to be expressed more in atherogenic group as compared to control/HFD + Se treatment. On the whole, the study highlighted the occurrence of apoptotic process in atherosclerosis and the role of Se, a potent antioxidant, in inhibition of apoptotic process in HFD fed animals.

Key words: Experimental Atherogenesis — High fat diet — Rabbit — Selenium — Apoptosis — bcl-2

Introduction

In the recent experiments in this laboratory, it was found that selenium (Se) supplementation along with high fat diet (HFD) feeding inhibited the induction of hypercholesterolemia in rabbits and rats (Kang et al. 1997, 1998). Lipid peroxidation and nitric oxide synthase (NOS) activity induced by HFD feeding was also reported to decrease on Se supplementation. On the basis of these studies, the protective effect of Se on HFD induced alterations was concluded to be through its antioxidant action.

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Lipid rich core containing extracellular lipid deposits, cholesterol crystals, foam cells and dead cells, characterizes the atheromatous plaque (Stary 1989) whereas advanced atherosclerotic lesions mainly consist of fibrous cap, smooth muscle cells, necrotic debris, inflammatory infiltrates, foam cells and calcium deposits. Apart from the necrotic cell loss, recent studies have shown that death of vascular cells may occur also in a more physiological manner as a result of programmed cell death (PCD) or apoptosis (Bennett et al. 1995; Geng and Libby 1995). PCD has been recognized as an important pathophysiological event in the remodeling of atherosclerotic lesions (Thompson 1995). It is well evident that PCD is induced by various stimuli e.g., radiation, oxidative stress, hyperthermia, glucocorticoids etc. (Magno and Joris 1995). PCD is also very well shown to be regulated by specific gene products e.g., bcl-2, P53, ced-9 etc. (Yang et al. 1997). Of these, bcl-2 proto-oncogene has been well documented to block apoptosis in an antioxidant way (Hockenbery et al. 1993).

Selenium, an antioxidant, has been used experimentally for trials on the preventive aspect of atherogenesis induced by HFD feeding. However, there are no reports of occurrence of apoptosis or bcl-2 expression as well as measure of oxidative stress in Se supplemented HFD feeding. Present study therefore was designed to study the apoptosis, bcl-2 expression and level of oxidative stress in three different treatment groups of rabbits viz. Control, HFD fed and HFD fed + Se supplemented.

Materials and Methods

Materials

Cholesterol was obtained from LOBA chemicals (Bombay, India). Monoclonal antibodies to bcl-2, apoptosis detection kit and sodium selenite were from Sigma Chemical Co. (USA). All other reagents and chemicals were obtained from SISCO Research Laboratories (India).

Treatment Protocol

Eighteen male New Zealand white rabbits (900–1100 g bw) obtained from the Animal House (Hisar, India) were randomly divided into three groups. Group I rabbits were fed with control basal diet as supplied by Hindustan Lever Ltd. (India). Animals in other two treatment groups were given HFD or HFD + Se (1 ppm as sodium selenite) supplementation diet respectively. HFD was prepared by dissolving cholesterol in table butter and then mixing it with ground rabbit food. Final diet contained 1 % cholesterol and 100 g butter/kg diet. Selenium was added to diet as sodium selenite and fed to animals in pellet form in the morning. The treatment protocol was kept for three months.

Before sacrifice, blood was withdrawn from ear vein of overnight fasting rabbits. Serum was prepared using standard procedure and used for further assays. After sacrifice of the animals, aorta was removed for experimental studies. For the histochemical examination, the thoracic aorta was fixed in 4 % neutral formalin. 5 μm thick transverse sections of paraffin embedded tissues were cut.

Serum total cholesterol and triglycerides

Serum total cholesterol was estimated according to the method of Chiamori and Henry (1959). Triglyceride levels in serum were quantified using glycerol phosphate oxidase-peroxidase (GPO-POD) based Enzokit supplied by Ranbaxy Diagnostic Ltd. (India).

Serum selenium levels

Serum Se levels were quantified by the fluorimetric method described by Hasunuma et al. (1982).

Selenium dependent glutathione peroxidase activity

Activity of glutathione peroxidase (GSH-Px) was assayed by the coupled enzyme procedure with glutathione reductase using hydrogen peroxide (H_2O_2) as a substrate (Paglia and Valentine 1967). The enzyme activity was determined in the post-mitochondrial fraction (PMF) of 10 % homogenates of liver and aorta and the activity was expressed as μmol of NADPH oxidized *per* min *per* mg protein.

Total and oxidized glutathione

Total and oxidized glutathione were quantified by another fluorimetric method (Hisin and Hilf 1976). Proteins from 10 % tissue homogenates were precipitated with trichloroacetic acid (TCA) to the final concentration of 5 % and the supernatant obtained after centrifugation at 10,000 $\times$ g for 10 min at 4 ℃ was used for the assay.

bcl-2 expression

This was studied on paraffin sections using immunohistochemical detection system. bcl-2 protein was localized by immunoperoxidase reaction using 3,3′-diaminobenzidine as a chromogen and H_2O_2 as a substrate. Unlabelled primary monoclonal antibody and biotinylated secondary antibody were used according to the standard protocol of ExtrAvidin kit (Sigma, USA).

Apoptotic activity

Presence of apoptotic cells in aorta from all three treatment groups was studied by *in situ* programmed cell death assay (TUNEL staining), as per Boehringer Biochemicals, Germany, on paraffin sections and also by observing the ultrastructural cellular changes (characteristics of apoptotic process) in aortic cells by transmission electron microscope (Kerr et al. 1994).

For TUNEL staining, fragmented DNA was end labelled with Dig 11-dUTP at 3′ end using the enzyme terminal deoxinucleotidyl transferase and this *in situ* labelling was detected by alkaline phosphatase based detecting system, using levamisole as an inhibitor of endogenous alkaline phosphatase activity.

For TEM, the small fragments of aorta were fixed in 1 % glutaraldehyde in 0.1 mol/l sodium cacodylate buffer, pH 7.4. They were postfixed in 1 % (v/v) osmium tetraoxide in 0.1 mol/l sodium cacodylate buffer, pH 7.4. After dehydrating in

ethanol gradients, they were embedded in ERL 4206 medium. Ultrathin sectioning was carried out using a diamond knife attached to ultramicrotome (RMC, MTXL). Only gray to silver sections (60 nm thick) were transferred to copper grids and were double stained, first with ethanolic uranyl acetate for 20 min as according to Epstein and Holts (1963) and then with Reynolds lead citrate stain (Reynolds 1963). Stained specimens were viewed under transmission elecron microscope (Jeol EK transmission electron microscope).

Total protein estimation

Protein estimation in the tissue homogenates was done using the modified method of Lees and Paxman (1972).

Statistics

Data are expressed as mean ± S.E.M. Differences between groups were tested using Student's t-test for unpaired values.

Results

To evaluate the extent of hyperlipidemia, serum total cholesterol and triglycerides were estimated in all the three groups. Rabbits fed on HFD showed a highly significant increase ($p < 0.001$) in the concentration of total cholesterol (nine folds) as well as triglycerides (two folds) in the serum, when compared to control rabbits (Table 1). Administration of selenium along with HFD feeding however showed a significant suppression ($p < 0.001$) of the total cholesterol as well as triglycerides in animals of group III when compared to those of group II.

Serum selenium levels

A significant decrease ($p < 0.05$) in serum Se levels was seen in animals fed on HFD (group II) in comparison to controls (Table 1). Further, group III showed an apparent significant increase ($p < 0.001$) in serum selenium levels in comparison to both group I and II values.

Selenium dependent GSH-Px activity

HFD feeding in group II showed a significant increase ($p < 0.001$) in the GSH-Px levels in the aorta in comparison to control animals. Activity of GSH-Px in aorta was seen to further increase in HFD + Se fed group in a significant manner ($p < 0.01$) in comparison to animals in group II where HFD alone was given. Liver GSH-Px showed the results in similar pattern to that of aorta (Table 1).

Total and oxidized glutathione

In aorta as well as liver, HFD feeding was seen to cause a significant increase in the values of total glutathione in comparison to that of group I (Table 1). When HFD was co-administered along with selenium, statistically nonsignificant change was observed in the both organs in comparison to the group II.

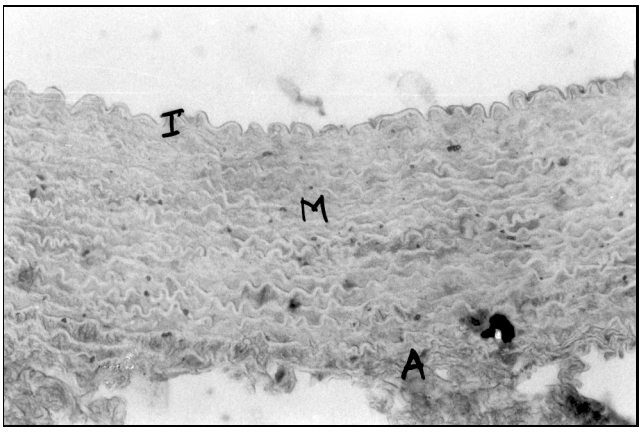
Table 1. Total cholesterol, triglycerides, selenium, glutathione peroxidase and glutathione levels in rabbit after three months of diet feeding schedule

	Control (I)	HFD fed (II)	HFD + Se fed (III)
Total cholesterol (mg/dl)	63.57 ± 2.41 (6)	554.81 ± 26.92*** (6)	394.34 ± 18.30$^{\#\#\#}$ (6)
Triglycerides (mg/dl)	108.40 ± 4.54 (6)	245.99 ± 9.99*** (6)	215.27 ± 8.32$^{\#\#\#}$ (6)
Serum selenium levels (μg/l)	77.85 ± 1.16 (5)	73.49 ± 0.83* (5)	98.59 ± 1.11$^{\#\#\#}$ (5)
Glutathione peroxidase (μmol NADPH oxidized/min/mg protein)			
Aorta	195.67 ± 10.32 (5)	287.67 ± 11.87*** (5)	358.50 ± 17.39$^{\#\#}$ (5)
Liver	375.83 ± 15.29 (5)	505.50 ± 13.81*** (5)	560.50 ± 17.63$^{\#}$ (5)
Total glutathione (ng/mg protein)			
Aorta	151.66 ± 6.16 (5)	199.11 ± 11.37** (5)	189.65 ± 5.95 (5)
Liver	173.22 ± 6.33 (5)	207.75 ± 7.12** (5)	212.77 ± 10.97 (5)
Oxidized glutathione (GSSG, ng/mg protein)			
Aorta	31.01 ± 1.46 (5)	36.37 ± 1.88 (5)	44.16 ± 2.10$^{\#}$ (5)
Liver	37.49 ± 1.64 (5)	41.48 ± 2.59 (5)	49.23 ± 1.25$^{\#}$ (5)
Ratio (GSH/GSSG)			
Aorta	3.90 ± 0.10 (5)	4.47 ± 0.16* (5)	3.32 ± 0.20$^{\#\#}$ (5)
Liver	3.64 ± 0.18 (5)	4.05 ± 0.19 (5)	3.31 ± 10.15$^{\#}$ (5)

Data are represented as mean ± standard errors of means (S.E.M.). HFD represents High Fat Diet. Ratio was calculated as (Total glutathione – oxidized glutathione)/Oxidized glutathione. Values in parenthesis represent the number of observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ represent the comparison between group I and II. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ represent the comparison between group II and III.

Group II rabbits on HFD showed a very small increase but not significant in oxidized glutathione (GSSG) levels in both organs when compared to group I values. When selenium was co-administered with HFD (group III), there was a significant increase ($p < 0.05$) of GSSG in aorta as well as in liver in comparison to group II rabbits.

A significant increase ($p < 0.05$) was observed in GSH/GSSG ratio from group II aorta as compared to that of control group. Further, combined feeding of selenium and HFD (group III) lowered the above ratio significantly ($p < 0.01$) as compared to the ratio observed in group II rabbits (Table 1). However, no significant change in the ratio was observed in liver of group II as compared to group I. On Se supplementation along with HFD, liver showed significant decrease ($p < 0.05$) in this ratio versus group II.



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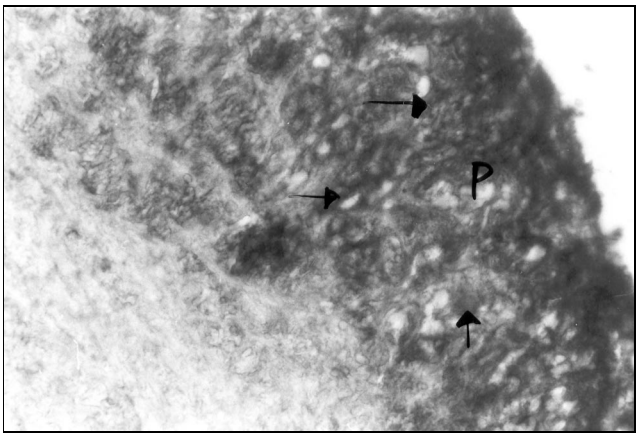

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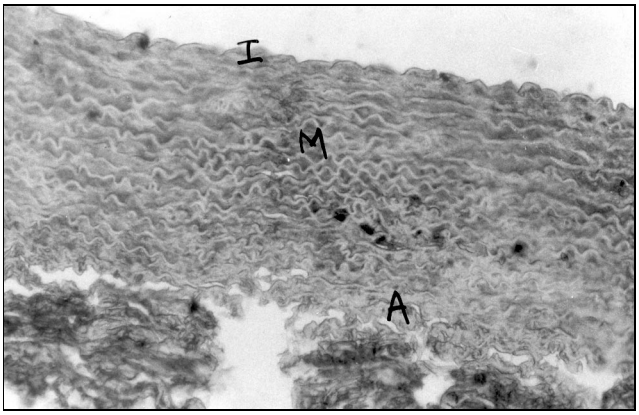


c

Figure 1. Immunohistochemical staining for bcl-2 in rabbit aortic sections from a) Control, b) HFD fed, c) HFD + Se supplementation. I, Intima; M, Media; A, Adventitia; P, Plaque; Arrows indicate staining for bcl-2.

bcl-2 expression

bcl-2 positive cells were more numerous in atherogenic aorta as compared to aorta from control or Se supplemented animals (Fig. 1a–c). Thus indicating the expression of bcl-2 was higher in cells of atherogenic aorta versus that of control/HFD + Se fed.

Apoptotic process in atherogenesis

Aorta from control diet fed rabbits did not show any apoptotic activity (Fig. 2a). None of the cells were TUNEL stained in either intima or media. Aorta from group II rabbits (HFD fed) showed excessive apoptosis, mostly concentrated to plaque region, as most of the cells were stained positively by TUNEL (Fig. 2b,c). Most of the foam cells present in fibrous plaque (Fig. 2b) showed apoptotic activity. Atheromatous plaque (Fig. 2c) also showed extensive apoptotic cell death, since large number of TUNEL positive cells were seen in that area. Animals fed on HFD + Se (group III) showed very little apoptotic cell death. As seen in aortic section of group III rabbits, only little TUNEL staining was seen, and even that confined to endothelial layer only (Fig. 2d).

In the rabbits fed on control diet, all the structural organelles, nucleus as well as nuclear and cell membrane of both endothelial and smooth muscle cells (Fig. 3a,b) showed normal ultrastructure indicating lack of apoptotic process. The results are similar to that obtained by TUNEL staining.

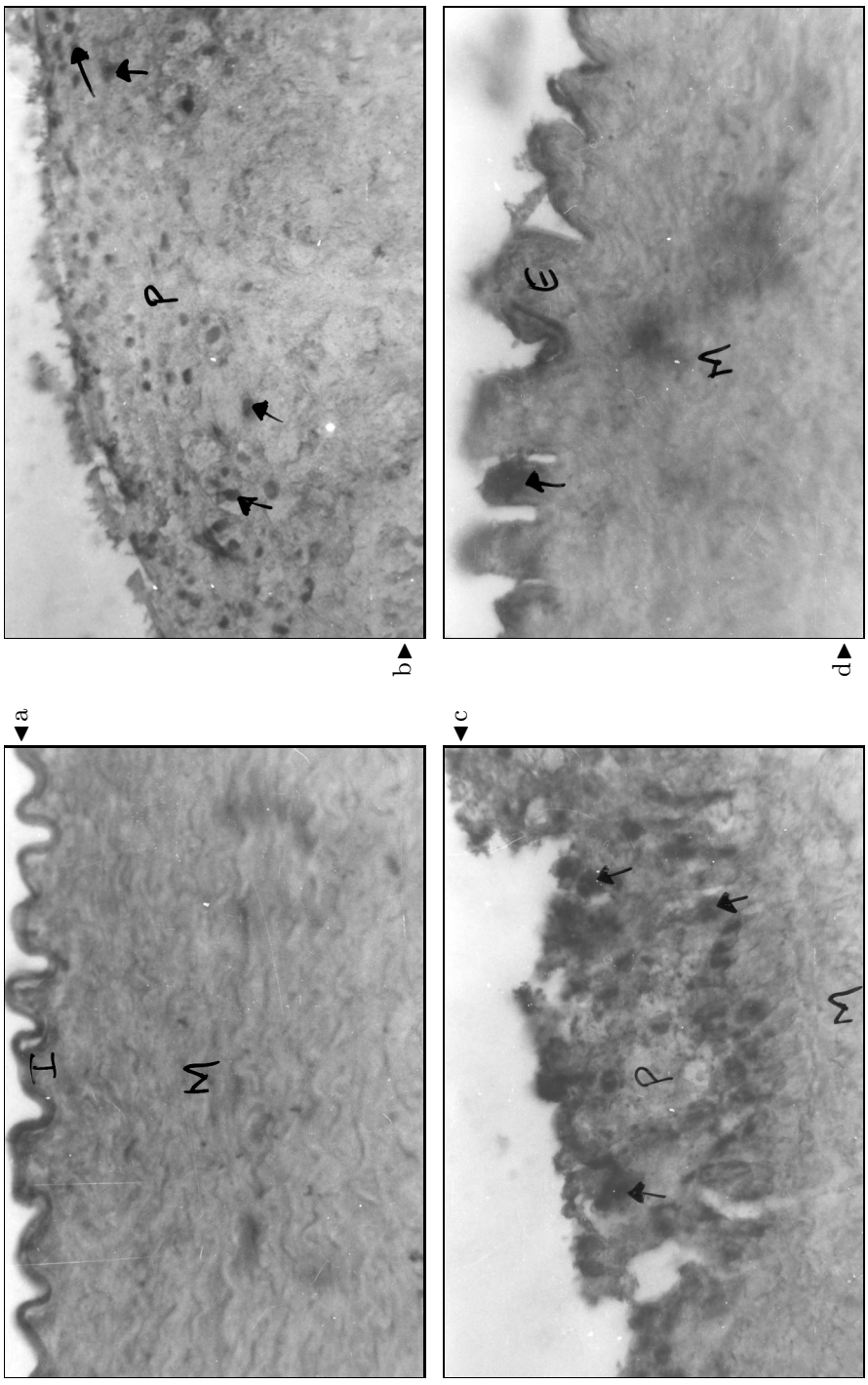
Ultrastructural change in aortic cells from group II (HFD fed) rabbits showed the extensive apoptotic activity in the endothelial and smooth muscle cells. Chromatin condensation and margination, a feature typical of initial stages of apoptotic process, can be seen in endothelial cells (Fig. 3c,d). Further, fragmentation of nuclear and cell membrane can also be seen in both of these micrographs. Some of the smooth muscle cells also showed cell membrane blabbing leading to the formation of apoptotic bodies (Fig. 3d).

Aorta from group III animals showed most of the endothelial as well as smooth muscle cells to be normal. In some of the endothelial cells, structural changes similar to group II were also seen (Fig. 3e). However, smooth muscle cells showed more or less normal ultrastructure (Fig. 3f).

Discussion

In atherogenic studies reported earlier and also in the present studies, it is observed that as the atherosclerotic lesions become more advanced, region of low

Figure 2. TUNEL staining of rabbit aortic sections. a) Control, No TUNEL positive cell seen in either intima or media; magnification ×400. b) HFD fed fibrous plaque, arrows indicate TUNEL positive cells indicating apoptosis; ×200. c) HFD fed atheromatous plaque, arrows indicate TUNEL positive cells; ×400. d) HFD + Se fed, TUNEL stained endothelial cell. Most of the endothelial cells (E) and medial layer (M) are however TUNEL negative; ×600.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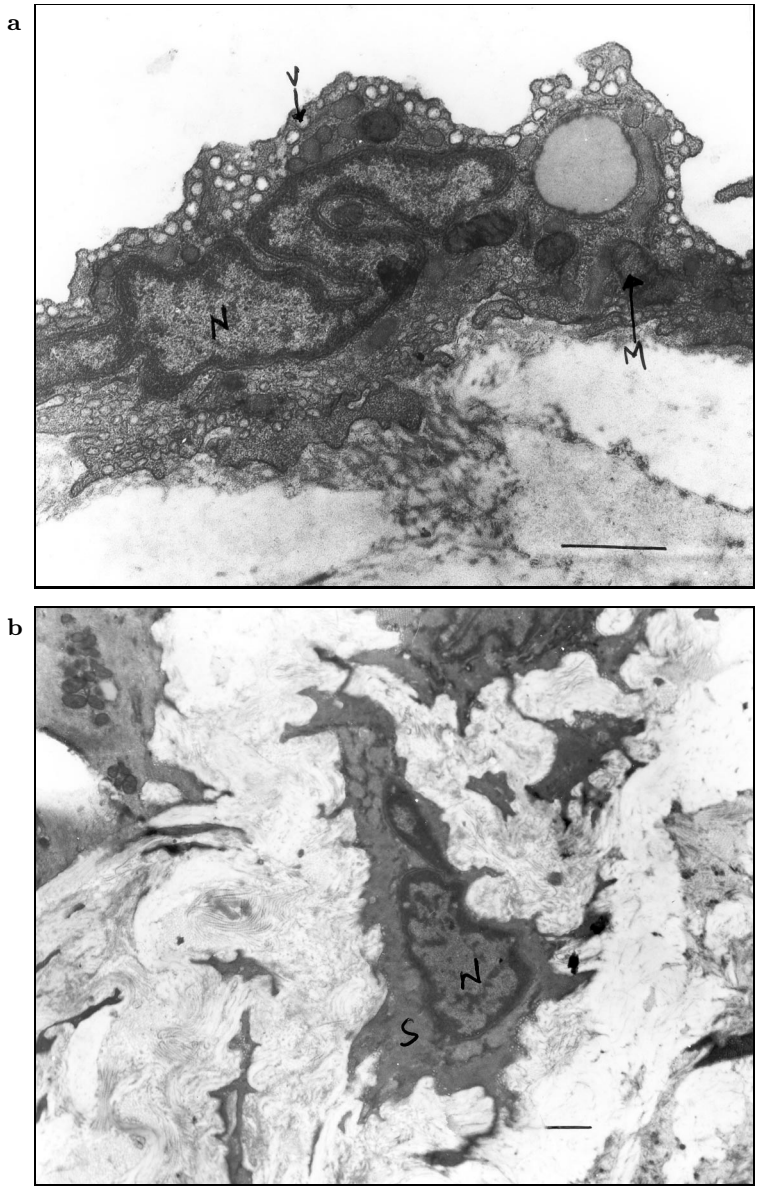

Figure 3a,b. Transmission electron micrographs of aortic sections of rabbits.
a) Control: shows an endothelial cell with well defined nucleus (N), mitochondria (M), pinocytotic vesicles (v) at the cell perifery and other cytoplasmic organelles can be seen; × 15,000 (Bar: 500 nm).
b) Control: shows a smooth muscle cell (s) with well defined nucleus (N); × 5,000 (Bar: 1 μm).

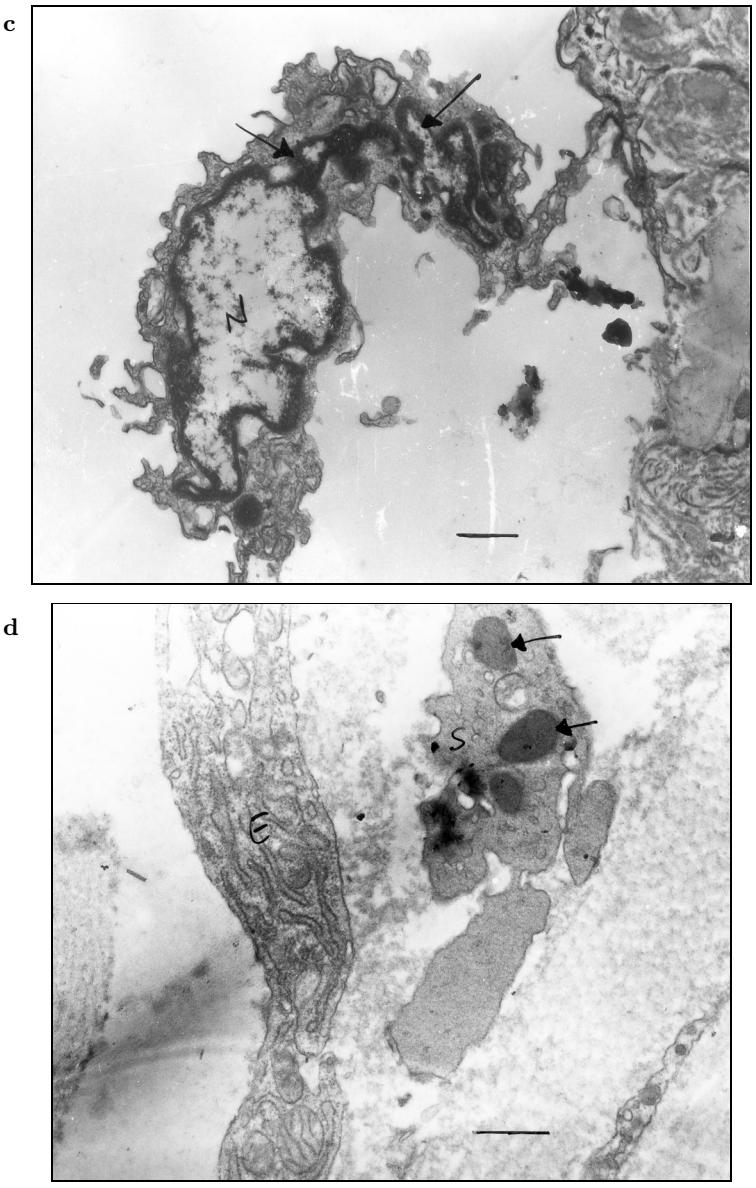

Figure 3c,d. Transmission electron micrographs of aortic sections of rabbits.
c) HFD fed: nuclear chromatin organization as well as cytoplasmic condensation can be clearly seen. Arrow heads point to the detached part of nucleus to form apoptotic bodies; × 10,000 (Bar: 500 nm).
d) HFD fed: shows a smooth muscle cell (S). Invegination of cell membrane detached itself from the cell seen. Arrows indicate the fragmented and condensed nuclear parts. An endothelial cell (E) can also be seen in the micrograph; × 12,000 (Bar: 500 nm).

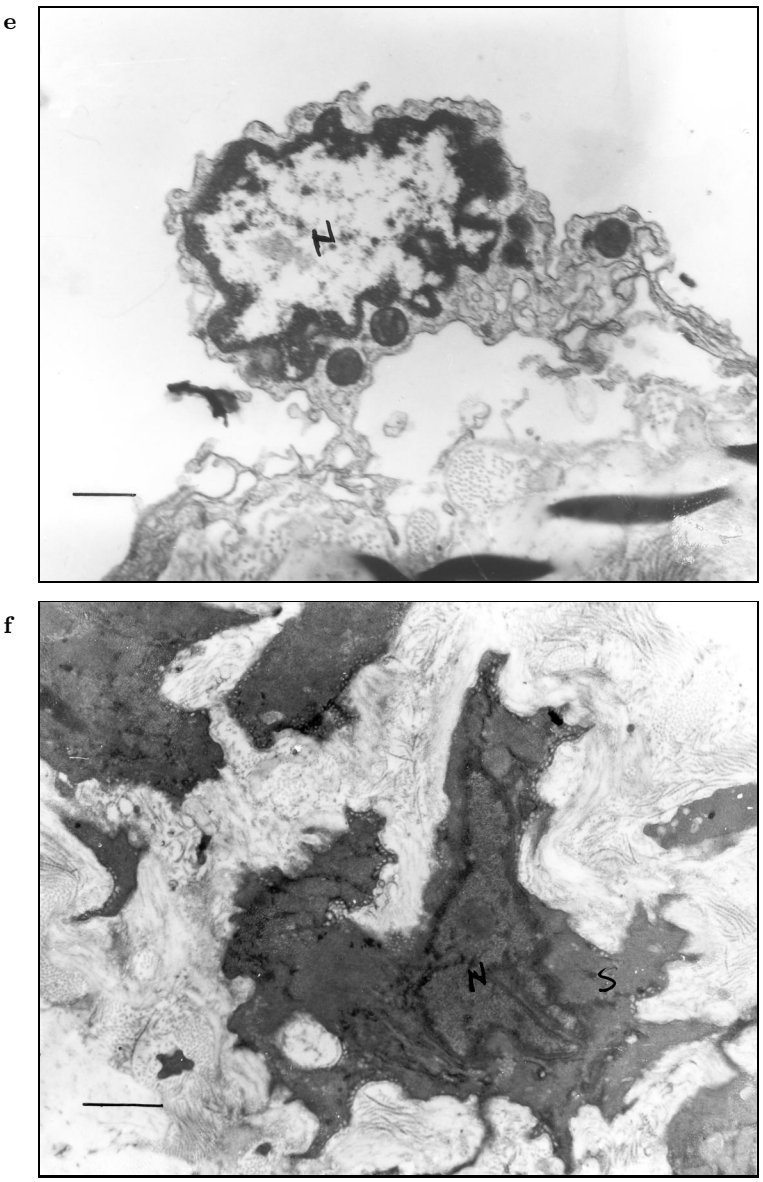

Figure 3e,f. Transmission electron micrographs of aortic sections of rabbits.
e) HFD + Se fed, shows apoptotic endothelial cell condensation and margination of chromatin can be seen in the nucleus (N). Cytoplasmic components and cell membrane are well preserved; × 10,000 (Bar: 500 nm).
f) HFD + Se fed, shows smooth muscle cell (S). Nucleus is well preserved. Nuclear membrane and number of pinocytic vesicles can be seen along the perifery of the inner side of the cell membrane; × 7500 (Bar: 1 μm).

cellular density with little appearance of cellular debris was seen (Han et al. 1995; Kockx et al. 1996). This process by which a cellular lesion evolves into the acellular sclerotic lesion, leading to plaque instability, rupture and further complications can be attributed to cell death followed by phagocytotic removal. Cell death in atherosclerotic lesions has been well reported as the process of necrosis (Garratt et al. 1991). However, involvement of programmed cell death (PCD) in apoptosis has been highlighted only recently (Kockx et al. 1996; Crisby et al. 1997).

In the present studies, animals fed on HFD showed high incidence of apoptosis in the plaque region of aorta from rabbit as studied by TUNEL and transmission electron microscopy (TEM). Control groups of animals were devoid of any apoptotic cells. Present results are in agreement with some recently reported studies (Geng and Libby 1995; Han et al. 1995; Kockx et al. 1996; Crisby et al. 1997).

Different reasons have been given for the onset of apoptosis in atherosclerosis lesions. Cai et al. (1997) have demonstrated the role of FAS/APO1, a transmembrane protein, whereas Geng and Libby (1995) supported the role of over expressed interleukin-1B converting enzyme (ICE) gene. In addition to these factors, in context with present studies, oxidative stress may also be playing a vital role in the apoptotic process. Supporting our results, oxidative stress has been reported in a very recent publication to be an early event in apoptosis (Estene et al. 1999).

Our earlier studies (Kang et al. 1997) of increased lipid peroxidation on HFD feeding clearly indicate the increased oxidative stress on HFD feeding. Increase in GSH-Px as observed in the present studies is also attributed to increased lipoperoxidative stress associated with HFD feeding. This is in accord with the literature where elevation of GSH-Px activity is reported to be associated with a small increase in oxidative stress (Frank and Messaro 1980; Oie et al. 1982). Supplementation of Se with HFD further elevated the activity of GSH-Px in liver and aorta, and this is in agreement with the well known fact that Se induces GSH-Px (Sunde and Evenson 1987). The oxidative stress of HFD feeding makes it even more pronounced.

Increase in tissue Se-dependent enzyme, GSH-Px, on HFD feeding also substantiates the observed decrease in serum Se levels, even though the diet contains adequate level of Se (0.2 ppm). Since overexpression of GSH-Px, a selenoenzyme, is reported to alter the apoptotic cell death (Hockenbery et al. 1993), present results show that levels of GSH-Px are not sufficient to maintain the glutathione redox cycle, since GSH/GSSG ratio indicates the oxidatively stressed condition in aorta, leading to the onset of apoptotic process. However, supplementation of Se to the animals along with HFD has shown occasional apoptotic involvement, which is restricted to endothelial cells only as evident from TUNEL as well as TEM. From the results it becomes apparent that apoptotic process gets initiated only after lesion is formed. Since glutathione redox pair is an index of oxidative stress (Ving 1990), thus, decreased ratio of GSH/GSSG as compared to that of group II further explains the negligible/occasional apoptosis in the group III.

Buttke and Sandstrom (1994) have also suggested that antioxidants, mainly enzymes and substances of glutathione redox cycle, play an inhibitory role on

cell death due to apoptosis. Further, atherogenic lesion is reported to be rich in macrophages, which are known to secrete tumor necrosis factor (an apoptosis-inducing factor, Old 1985) thus justifying enhanced apoptosis in lesion area only. Moreover, superoxide anion (O_2^-) radicals are known to oxidatively modify the LDL, especially during atherogenic conditions. Ox-LDL can also be thought to induce apoptosis in *in vivo* conditions since direct addition of Ox-LDL is reported to induce apoptosis in cultured foam cells (Christ et al. 1993).

Free radical nitric oxide (NO) has recently also been implicated as an inducer of apoptosis. Lopez-Collazo et al. (1997) has demonstrated the involvement of $NO^{\bullet}$ and calcium mobilization in the induction of apoptosis in adrenal vascular endothelial cells. Results of present study are supported by our recently reported (Kang et al. 1997, 1998) findings of increased NOS activity along with increased basic lipid profile on HFD feeding. Further, regarding the expression of bcl-2 in the atherogenic aorta, it was seen increased in spite of the evidence that bcl-2 protects cells from apoptosis. Selenium supplementation resulted in bc1-2 expression similar to that of control. Supporting our results, Olivetti et al. (1997) observed 1.8 times higher bc1-2 expression as compared to normal one in the hearts of cardiac failure patients along with very high rate of apoptosis.

On the whole, the study depicted the occurrence of apoptosis in the atherogenic lesions, in spite of the increased expression of bc1-2. These phenomena may contribute to progression of aortic wall degeneration.

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