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Atheromatous Plaque Formation in Rabbit Aorta Fed with High Cholesterol Diet

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ABSTRACT

Atherosclerosis, the cholesterol deposition in and around cells of the intimal layer of the aorta, has been recognized as one of the main causative factors for cardiovascular diseases. Intensive research has been carried out throughout the world but the precise atherogenesis has yet to be fully understood, though hypercholesterolaemia is considered to be the prime risk factor. The aim of the study was to evaluate the effect of high cholesterol diet consumption on the formation of atherosclerosis *in vivo*. Three groups of adult White New Zealand male rabbits (six animals per group) were used in this study. Except for one group which acted as a control (K), the other two groups were given 1% and 2% high cholesterol diet respectively for 10 weeks. At the end of the experiment, blood samples were taken from the marginal ear vein for plasma cholesterol estimation. The animals were sacrificed and the aorta was excised for histomorphometric analysis. The result shows that despite no significant differences in plasma cholesterol levels being observed between the groups treated with 1% and 2% cholesterol, high cholesterol consumption was able to induce hypercholesterolaemia significantly ($p<0.01$) compared to the control group. The atheromatous plaque formation in the group given 2% cholesterol diet was significantly higher than the group given 1% cholesterol ($p<0.05$), indicated by increased thickness of the intimal layer of the aorta. There was disruption of the intima-media junction in hypercholesterolaemic groups but no atheromatous plaque formation was observed in the control group.

INTRODUCTION

Atherosclerosis, characterised by thickening and hardening of artery walls, is a condition in which fatty material is deposited along the walls of arteries. This fatty material thickens, hardens, and may eventually block the arteries. Eventually, this fatty tissue can erode the wall of the artery, diminish its elasticity (stretchiness) and interfere with blood flow (Stary, 1987; Stary, 1990).

An artery is made up of three layers. The innermost layer known as tunica

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intima, consists of endothelial cells typically the simple squamous cells and associated to connective tissue. Beneath the connective tissue, there will be the internal elastic lamina, which delimits the tunica intima with the tunica media. The tunica media is formed by a layer of circumferential smooth muscle cells and variable amounts of connective tissue. A second layer of elastic fibers, the external elastic lamina, is located beneath the smooth muscle. It delimits the tunica media from the tunica adventitia, which consist mainly of connective tissue fibres. The tunica adventitia blends with the connective tissue surrounding the vessel.

Atherosclerosis is a chronic immune-inflammatory disease in which the interaction of monocytes with activated luminal endothelium is a crucial event leading to atherosclerotic alteration of the arterial intima (Hansson, 2005; Libby, 2002; Wick, Knoflach & Xu, 2004). Monocytes migrate into the subendothelial layer of the intima where they differentiate into macrophages or dendritic cells (Ross, 1993; Bobryshev, 2005). The subendothelial space is enriched with atherogenic lipoproteins (Skalen *et al.,* 2002; Williams & Tabas, 2005), which most macrophages were transformed into foam cells (Ross, 1993). Foam cells aggregate to form the atheromatous core and as this process progresses, the atheromatous centres of plaques become necrotic, consisting of lipids, cholesterol crystals and cell debris (Stary *et al.,* 1995 and Takahashi *et al.,* 2002). When fully advanced, these plaques restrict the flow of blood through the vessel and this often results in tissue ischaemia. Atherosclerotic plaques can also rupture, causing debris to migrate downstream within an artery, causing embolisme (Stary, Blankenhorn & Chandler, 1992). This is a common cause of heart attack and stroke (Tintut *et al.,* 2002).

Atheroslerosis is one of the main causative factors for cardiovascular disease (CVD). Previous studies showed that the risk of developing atherosclerosis is closely related to high levels of cholesterol in the blood. Apart from high cholesterol intake, atherosclerosis and CVD have been shown also to have direct correlations with smoking, excessive alcohol consumption, diabetes, sedentary lifestyle and obesity.

Cholesterol, a waxy substance present in fatty foods that predominantly arise from animal sources, is an essential part of a healthy body and is a precursor for steroid hormone biosynthesis and an important component of cellular membrane. However, despite its beneficial role in most eukaryotes, increased cholesterol levels in the circulatory blood system, known as hypercholesterolaemia, has been well documented to be positively correlated to various cardiovascular diseases (Witztum & Steinberg, 1991; Castelli, 1984). Increased plasma concentration of cholesterol, mainly the low density lipoprotein (LDL), triggers the biochemical chain reaction mediated by free radicals, transforming the LDL molecules into its oxidised form. Next, macrophages engulf oxidised LDL via chemoactractant-signalling effect and the products eventually reside at the intimal layer of the arteries as foam cells (Castelli, 1984). These mechanisms were considered to be the starting point of the consequences in the pathogenesis of atheroslerosis (Tintut *et al.,* 2002; Marie, 1998).

Many efforts have been made to identify unstable atheromatous plaques. Some of the parameters suggested to be important in rendering a plaque unstable are size, lipid content, inflammation, calcification, and hemorrhage into the plaque. The pathogenetic basis for these have been suggested to be mechanical (size), the physical composition of the plaque (with lipid rendering the plaque less solid), oedema and proteolytic digestion of the fibrous cap (inflammation), mechanical rigidity (calcification), and increase in size (intraplaque hemorrhage) (Farb, Burke & Tang, 1996; Dickson & Gotlieb, 2003).

This study sought to assess the effect of cholesterol in the progression of athero formation of the aortic vessel in animal models that are fed with high cholesterol diet.

MATERIALS AND METHODS

Experimental design

Three groups of adult White New Zealand male rabbits (six animals/group) at average body weight of 2.5 – 3.0 kg were randomly segregated into individual cages. The animals were acclimatised for one week with intervals of equal light-dark exposure and free access to drinking tap water and normal rabbit chow (Golden Hope, Malaysia). Following acclimatisation, one group acted as a control (K), while the other two groups were given 1% and 2% high cholesterol diet (ICN Biomedicals, USA) respectively in replacing normal chow. The experimental period was designed for 10 weeks. Prior to the experiment, 10 mL of marginal ear vein blood was withdrawn for plasma cholesterol estimation. One animal from each group was sacrificed and the aorta removed for baseline reference. At the end of the experimental period, similar procedure was governed; the animals were fasted for at least 12 hrs before blood sampling. Following blood withdrawal, the animals were sacrificed via exsanguinations through common carotid artery. The experiment protocol and animal handling throughout the study were in accordance with guidelines approved by the institution ethics committee where the study was conducted.

Biochemical analyses

The blood sample obtained was placed into a tube containing ethylenediamine tetra acetic acid (EDTA) and centrifuged at 3000 rpm for 10 mins. The plasma obtained was subjected to plasma cholesterol estimation by enzymatic colorimetric methods using commercial kits (Boeheringer Mannheim, Germany).

Histological examination and assessment of atherosclerosis

The aorta, from the ascending region to the common iliac artery was excised for histology evaluation. In brief, following removal of the tissue from the carcass, 3mm of ascending aorta from the aortic trunk was sectioned and fixed in 10% buffered formalin overnight for fixation. Following fixation, the washed tissue was dehydrated in the descending grades of isopropanol and finally cleared in xylene. The tissue was then embedded in molten paraffin wax. 3 µm in thickness was sectioned using a kryostat and the slide preparation was stained with hematoxylin–eosin (H&E) staining. The samples were first examined under light microscopy in order to identify the degree of lipid deposition. Then the specimens were analysed by an image-analyser system interfaced to a Zeiss Axioscop microscope for the average thickness of the atheromatous plaque formation. The image analysis system consisted of a Macintosh lix computer (Apple) equipped with a Frame Grabber Card (Quick Capture, Data Translation), a Sony high-resolution video camera, and Trinitron Super Mac 21 in colour monitor.

Statistical analysis

The values are expressed as mean ± S.D. One-way ANOVA was used to test for differences in blood cholesterol level and the athero-formation between groups. When significant differences were detected, individual mean values were compared by Tukey post test. A value of $p<0.05$ was used to denote statistical significance.

RESULTS AND DISCUSSION

Plasma cholesterol level estimation

Figure 1 shows the results of plasma cholesterol levels at week 10 for control (K) group, 1% and 2% cholesterol-fed groups. This experiment demonstrated that prolonged consumption of high cholesterol diet causes retention of cholesterol in the blood leading to hypercholesterolaemia. Both groups treated with 1% and 2% cholesterol exhibit a significantly higher level of cholesterol ($p<0.01$) (33.16 ± 4.03mmol/L and 34.61 ± 1.49 mmol/L respectively) compared with that of the group that was not given cholesterol (K) (5.65 ± 0.10 mmol/L). No significant difference in plasma cholesterol level was observed between 1% and 2% cholesterol group. High levels of plasma cholesterol have been clearly identified as a risk factor for atherosclerosis and coronary heart disease (Kannel, Castelli & Gordon, 1979). Hypercholesterolaemia, with elevated levels of cholesterol-rich low density lipoprotein, is one of the most important risk factors for the disease; cholesterol, accumulating in the atherosclerotic lesions (Meydani, 2001).

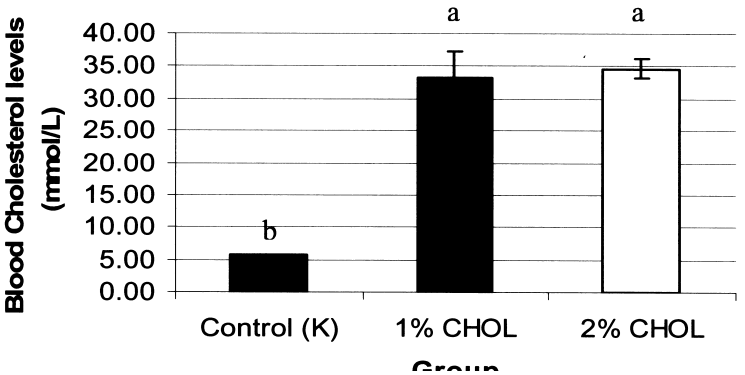

Figure 1. Plasma cholesterol levels at week 10. Values with same letter are not significantly different ($p<0.05$) between groups

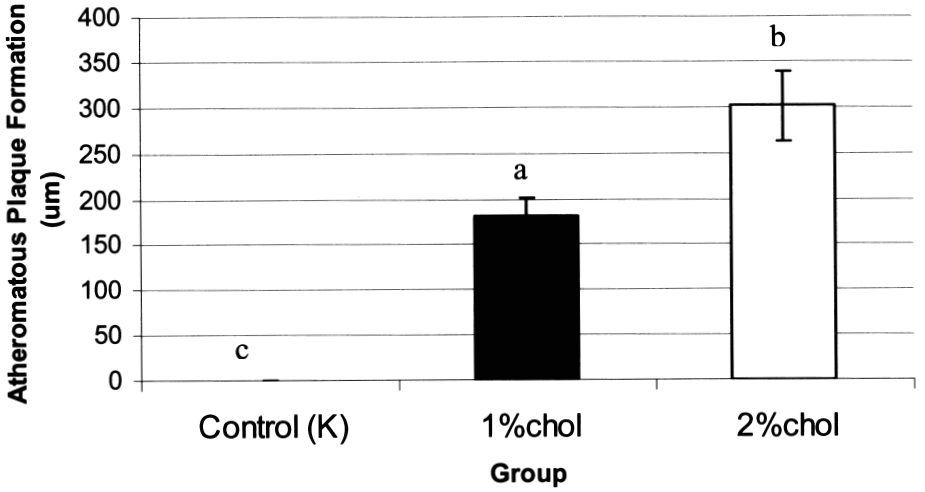

Figure 2. Atheromatous plaque thickness (in μm). Values with same letter are not significantly different ($p<0.05$), between groups

Quantitative Analysis of Atheromatous Plaque Formation

Figure 2 depicts the athero formation in groups treated with cholesterol. 1 and 2 % cholesterol added in the diet were demonstrated to not only cause hypercholesterolaemia, but also caused the atheromatous plaque formation in the intimal layer of the aorta of the respective groups. The thickness of atheromatous plaque in the group given 2% cholesterol was 306.70 ± 20.37μm and was significantly higher than that of e given 1% cholesterol (196.30 ± 11.07 μm) ($p<0.05$). No atheromatous plaque formation was observed in the control (K) group. Foam cell formation is a characteristic feature of atherosclerotic lesions, which are formed mainly by uncontrolled uptake of oxidised LDL by smooth muscle cells and macrophages. Common models of atherosclerosis by the formation of foam cells were indicated to be an early event in the onset of atherosclerosis (Keaney, Simon & Freedman, 1999).

Histology of Aorta Tissue Sample of Control (K) Group

Cells of the aorta tissue samples from the control (K) group were arranged with no differences to that of the arrangement of cells from the baseline reference (figure of baseline reference not shown) (Figure 3). The intima was not clearly distinguished from the medial layer. The flattened cells lining the lumen are the single squamous endothelium. The subendothelial layer of connective tissue is characterised by a lower density of cells, i.e. fewer nuclei, a fibrous appearance of the tissue and the absence of well-defined elastic layers (Stary, 1987). The intimal layer is separated from the medial by a piece of elastin tissue known as internal elastic laminae (IEL), which can clearly be seen.

Histology of Aorta Tissue Sample of Group with 1% and 2% Cholesterol

The aorta tissue samples of groups fed with high cholesterol diet clearly showed a remarkable thickening of the intimal layer

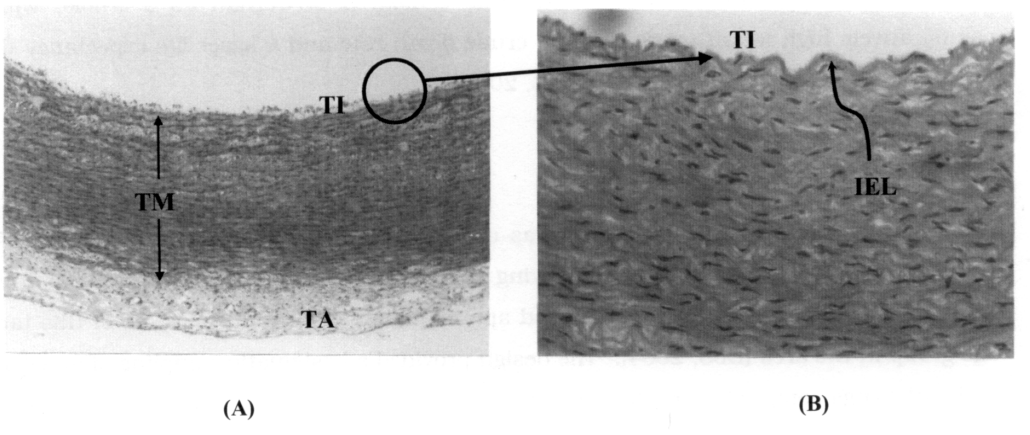

Figure 3. Cross section of aorta tissue specimen from control (K) group stained with H&E at LM x40 (A) and LM x400 (B) revealing the three layers of tunic and proper organisation of cells and connective tissues (TI = tunica intima; TM = tunica media; TA = tunica adventitia; IEL = internal elastic laminae).

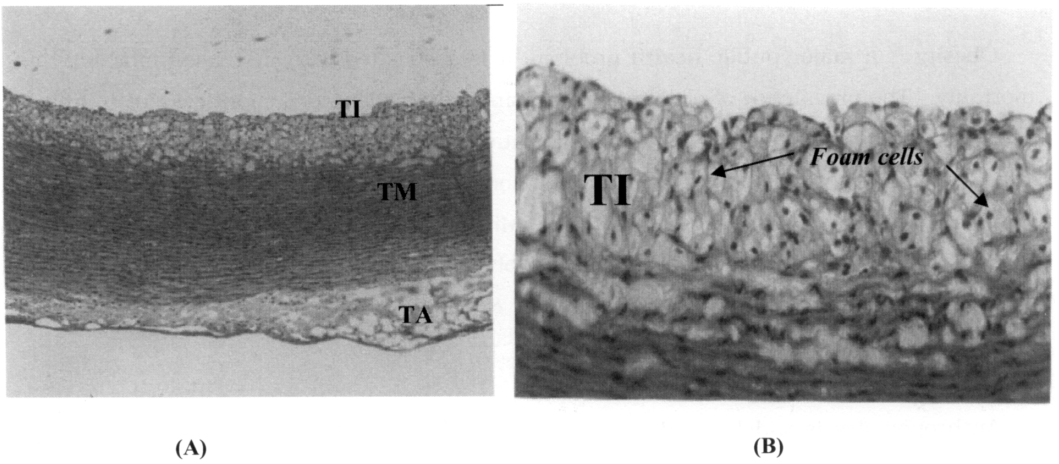

Figure 4. Cross section of aorta tissue specimen from group fed with 1% cholesterol, stained with H&E at LM x100 (A) and LM x400 (B) revealing the deposition of cholesterol and thickening of the intimal layer of the aorta. Notice the disruption of intima-medial junction and no prominent IEL can be seen in that region indicated that infiltration process of macrophages and smooth muscle cells across the media to the intima has occurred. (TI = tunica intima; TM = tunica media; TA = tunica adventitia; IEL = internal elastic laminae).

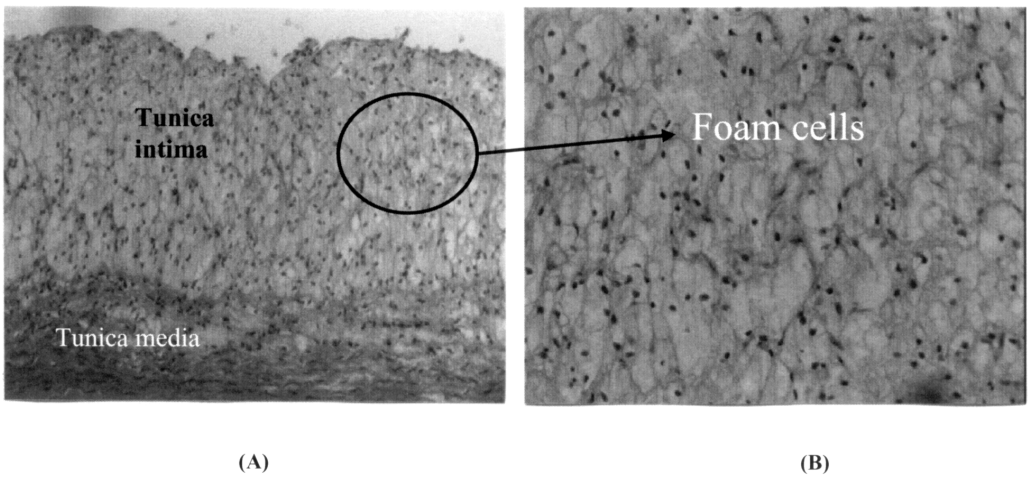

Figure 5. Cross section of aorta tissue specimen from group fed with 2% cholesterol, stained with H&E at LM x100 (A) and LM x400 (B) revealing the more severe deposition of cholesterol and thickening of the intimal layer of the aorta. Notice the disruption of intima-medial junction and no prominent IEL can be seen in that region indicated that infiltration process of macrophage and smooth muscle cells across the medial to the intima has occurred. The foam cells are differentiated with other lipid cells by its nucleus which reside at the center of the cell and stringy substances occupy the sytoplasm (TI = tunica intima; TM = tunica media; TA = tunica adventitia; IEL = internal elastic laminae).

and much different to that of the respective baseline reference (Figure 4 & Figure 5). Most of the cells in the tunica media are smooth muscle cells and were stained with intensed eosinophilic. Smooth muscle cells and collagen fibres are found between the layers of elastic fibres. The IEL was not clearly seen as the intima-media junction was disrupted by infiltrated circulatory macrophages and smooth muscle cells from the media into the intima. Deposition of cholesterol in the intima was represented by lipid laden foam cells which originally derived from circulating monocytes and also from the tunica media's smooth muscle cells. Isolated macrophages are reported to be present in the nondiseased intima and in the intima of animals that are not hypercholesterolaemic (Stary, 1990; Stary *et al.,* 1992). Studies in animals indicate that many more monocytes from the circulation enter the intima under conditions of hypercholesterolaemia (Keaney, Simon & Freedman, 1999). This movement may be a response to the increased presence of oxidised LDL, which has been shown to be chemotactic for monocytes *in vitro*. This study clearly provided the evidence that high cholesterol consumption may cause an increase in blood cholesterol level which triggers the formation of athero fatty streaks in the blood vessel, predominantly in the intimal layer of the aorta.

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