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An Experimental Model for Studying Atherosclerosis

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ABSTRACT

The aim of the present study was to clarify if old N-MRI rat, a strain which is easily available in Iran and cheap to maintain, is a suitable alternative to those previously reported. In this model, we compared three quantifiable parameters between old and young rats: biochemically, involving measurement of differences in the lipid profile. Histologically, the differences of the thickness of the wall of the aorta, the apparent degree of splitting within the aortic media, and the formation of foamy lipid layers on the outermost layer of the aorta. Pharmacologically, *in vitro* contractile responses/mg wet tissue weight to submaximal concentration of phenylephrine (1 µM) and the rate of relaxation min^{-1} mg^{-1} tissue weight following administration of 0.1 mM of acetycholine. The proposed model was validated using lovastatin as test drug known for its lipid lowering and anti-atherosclerosis actions. The results showed that this model to be reliable, quantifiable and capable of detecting the effect of orally administered lovastatin. We recommend this model as an easy and accessible experimental model for various atheroscleorosis investigations. *Iran. Biomed. J. 7 (2) 65-71, 2003*

Keywords: Atherosclerosis, Model, N-MRI rat, Lipids, Foam cells

INTRODUCTION

Experimental models to investigate the process of atherosclerosis has been described in both man [1] and experimental animals [2]. The aims of such studies were twofold. They attempted to gain an understanding of atherosclerosis process and to develop models in which the effectiveness of various interventions could be evaluated for their potential effects on this process. In addition, experimental atherosclerosis studies in man by necessity and ethics, are limited. On the other hand, it is recognized that the extrapolation of results from animals to man should be guarded.

Most investigations carried out for studying atherosclerosis involve the use of a classical administration of high fat, high cholesterol diet, and an agent that produce artherosclerotic plaques in the arteries over a period of 3 to 6 months [2]. On the other hand, investigations involving experimentally induced hyperlipidaemia may use the same methodology can commence after a few weeks rather months [2]. Still a more rapid method for studying hyperlipidaemia is the triton-induced model [3]. In this model, an intraperitoneal administration of 400 mg/kg of isooctylpolyoxyethlenephenol (triton) produced within 48 h, an established and a measurable hypercholesterolaemia in rats [2]. These methods carry an inherent weakness in that they do not truly represent the natural process of atherosclerosis. Since the process of atherosclerosis is characterized by its silent and slow progress of deposition of lipids in the major arteries, including the aorta, over a prolonged period. In order to overcome this limitation, other researchers [4], have used genetically modified strains of animals that have an inherent tendency to develop atherosclerosis. Still, more recent introduction is diabetes-accelerated atherosclerosis porcine [5] and rodent models [6]. In these models, the effect of diabetes on the development of atherosclerosis and hyperliproteinaemia are investigated. All these studies clearly reflect that there is a lack of a consensus on the animal model to use. With these limitations in mind, other researchers considered more natural approach. They used old animals from different stains not available in Iran, and are proposed to reflect more the natural phenomenon of this disease [7].

Once an experimental model was found, the next stage was to evaluate the effectiveness of various treatment protocols. Different parameters have been measured as an indication of the degree of

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atherosclerosis progression or regression. These included the extent of lipid lowering, rate of blood flow [8], reduced thickness of the arteries [7], relaxation of major arteries following acetycholine [1], or on follow up treatment for the rate of mortality in human studies [9]. These various studies clearly reflect the fact that a universally accepted consensus on what to measure as an indication of extent of progression or regression of atherosclerosis is also lacking.

It has been known that aged animals from various strains develop the characteristic features of atherosclerosis [7]. It was therefore of interest to investigate if old N-MRI rats are suitable alternatives to others proposed for such studies. In this study, we provide evidences that *old* N-MRI rats, a strain of rats which is widely breed in Iran and cheap to keep under normal laboratory conditions, is also a suitable alternative to other not easily available strains. The model has an additional advantage that it simultaneously takes into account of three quantifiable parameters that are thought to reflect the measure of the severity of atherosclerosis.

The studies were divided into two phases: first, to determine and evaluate the parameters which are related to atherosclerosis and show the differences between young and aged rats from the biochemical, histological and pharmacological viewpoints, and second, to validate the usefulness of the chosen model by its response to lovastatin, a cholesterol-lowering drug known to be effective in treatment of atherosclerosis [10].

MATERIALS AND METHODS

Animals. Young and aged rats of N-MRI strain (2 and 11 months old) were utilized. These were purchased from Tehran Animal Centre. The animals were housed in groups of three in PVC cages, had free access to water and standard laboratory chew, and were maintained at 27 ± 5°C and 60 ±10% humidity. The lighting cycle was on 12 hourly day/night bases and lighting conditions were between 7 a.m. to 7 p.m.

In the initial phase of investigation, 16 rats (8 young, less than 2 months old, and 8 aged, 11 months old) were used. The average weight of the old and young rats used was 230 and 50 g, respectively.

In the second stage of work, another 16 rats were used (9 months old when started the experiments). The animals were divided into two groups of 8. The first group was used as controls to which 1 ml of normal saline was administered orally, daily for 8 weeks. The second group was administered orally, via a blunt-head feeding syringe, 4 mg/Kg lovastatin (Sermic, Mexico) using 10 mg/ml suspension in normal saline, daily for 8 weeks [11].

Experimental Procedure. Following the injection of an anaesthetizing dose of pentobarbital sodium, a blood sample was withdrawn from the tail of each animal for biochemical evaluation of serum lipids. The animals were then sacrificed by exsanguination. The thoracic aorta was removed, and sections were used for both pharmacological and histological tests.

Biochemical investigations. A 2-ml blood sample was withdrawn from the tail of the anaesthetised rats and centrifuged at 600 ×g and then the serum was separated for the measurement of the lipid profile: LDL, HDL, VLDL, total cholesterol and triglyceride levels, using standard enzymatic based laboratory kits (Man, Iran).

Pharmacological investigations. Utilizing the classical endothelium-mediated vasomotor relaxation response to acetycholine inphenylephrine -induced pre-contracted aorta [12]. In order to perform the pharmacological evaluations, approximately a 4-mm long aortic ring was removed from the thoracic part, weighed and placed in an oxygenated Tyrode solution maintained at 37°C. The Tyrode solution had the following composition (% w/v): NaCl 0.9, KCl 0.02, $MgCl_2$ 0.01, $CaCl_2$ 0.02, NaH_2PO_4 0.005, $NaHCO_3$ 0.01, Glucose 0.1 [13].

The aortic rings were then connected to an isometric transducer (model F60, USA) and to a 4-pen Darco chart recorder. The aortic rings were suspended under 1 g tension, by a stainless steel triangular wire, which was slowly and carefully inserted into the lumen without damaging the endothelial layer. The following protocol was adopted: following the equilibration period of 1 h and washing with fresh Tyrode solution every 15 min, the aortic rings were exposed to a dose of 1 µM final bath concentration of phenylephrine hydrochloride. The plateau level of contraction produced was recorded for all groups, from which the level of contraction/mg wet tissue weight was calculated and statistically compared. This concentration was the submaximal concentration that produced 75% of maximum contraction in the

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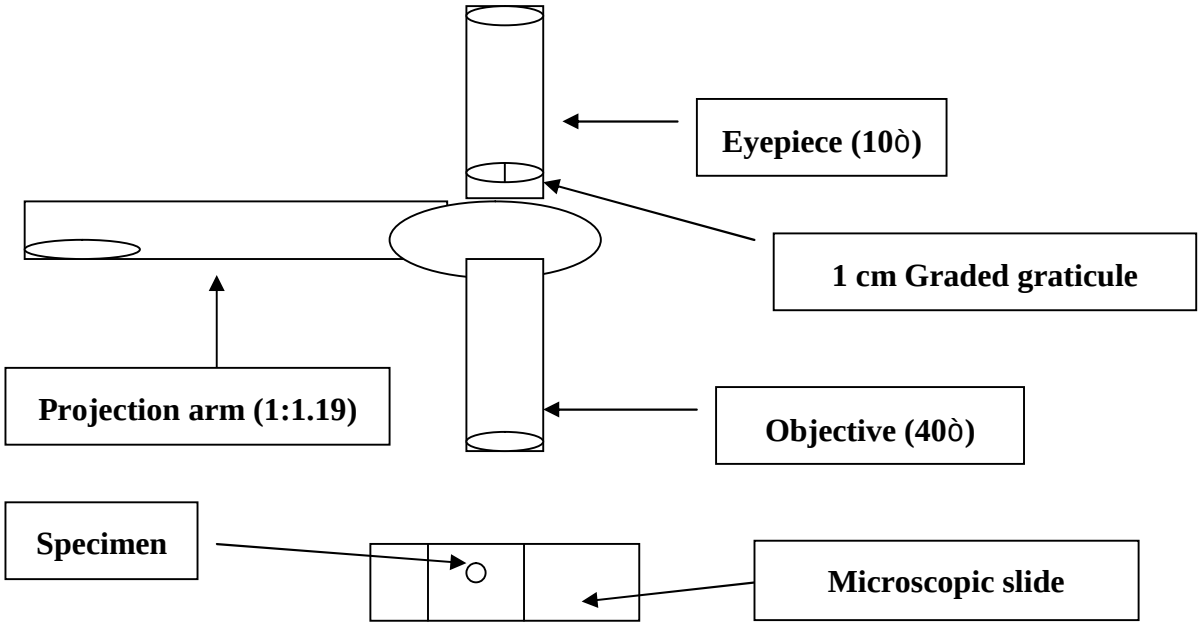

Fig. 1. A simplified diagrammatic representation of the microscopic arrangement for measurement of the thickness of rat aorta samples. Note that the magnification for the sample projected to the eye piece is 400×, which is reduced by the projection arm by a factor of 1: 1.19, providing a final magnification at the projection end 336× that of the actual thickness of the sample.

majority of aorta tested in aorta taken from both young and old rats (results not shown).

Endothelium-mediated relaxation was induced by the addition of acetycholine (0.1 mM). The time taken for relaxation to the baseline tension was measured, and from which the rate of relaxation min^{-1} mg^{-1} tissue weight was calculated and statistically compared.

Histological investigations. The remaining section of the aorta was immersed in a phosphate buffered solution for histological examination. Using 4 µm thick sections, the samples were stained with haematoxylin and eosin. Three histological samples were prepared from each animal. The thickness of the aorta were recorded using a simple bench microscope (Olympus CH-2), equipped with a projection arm and with aid of a graded graticule.

The projection arm allowed section image to be projected onto a flat surface for measuring the thickness of the aorta with aid of a simple ruler. For this purpose, the thickness of ten randomly selected sites from each aorta was measured. Using 40× objective and 10× eyepiece, and with aid of a ruler and the graded graticule, the magnification that was projected from the projection arm was calculated. A simplified diagrammatic representation illustrating the set up is drawn in Figure 1. The resulting measurement obtained from this arrangement was divided by a factor of 336, to give the value of the thickness for each sample, which was finally statistically compared. In addition, histological observations that characterize atherosclerosis were recorded, namely: the apparent splitting within the intima, and extent of extracellular foamy layer on the outer most layer of the aorta [12].

Statistical analysis. The data obtained were expressed as mean ± standard error of the means (SEM). The means between the different groups were compared using analysis of variance (ANOVA) for multiple-group comparison. Where ANOVA showed significance ($P<0.05$), between two groups, then further comparisons was carried out using student's *t*-test. All statistical tests were performed using Excel software.

RESULTS

Biochemical findings. The results from serum lipids values are summarized in Table 1. The mean level of LDL, VLDL, triglyceride and total cholesterol in the serum from young rats were 47.4 ± 6.3, 7.85 ± 1.24, 39 ± 2.7 and 118.25 ± 3.8 mg/dl respectively. These values were found to be highly significantly greater in the old rats (90.7 ± 5.3, 10.2 ± 0.58, 51 ± 2.9 and 81.8 ± 2.1mg/dl respectively,

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Table 1. Summary of the concentrations of lipids in both young and old N-MRI rats, and following treatment with lovastatin.

Rat Tested	LDL	Lipid Type in mg/dl (s.e.m.)			
		HDL	VLDL	TG	T. Chol.
Young	47.40 ***(6.3)	63*** (3.08)	7.85**(1.24)	39.0***(2.77)	118.25*** (3.8)
Old	90.75 (5.3)	42 (0.7)	10.20 (0.58)	51.0 (2.9)	142.95 (3.1)
Lovastatin-treated old rats	48.30***(4.35)	54** (4.5)	6.50 ***(0.54)	32.3***(2.7)	108.00** (1)

$^{**}P$<0.01 and $^{***}P$<0.001 relative to old control rats, n = 8 for both young and 16 for old control rats, ANOVA followed by unpaired student's *t*-test. LDL (low-density lipoprotein); HDL (high-density lipoprotein); VLDL (very low-density lipoprotein); TG (triglyceride); T. Chol. (total cholesterol).

P<0.001). However, the mean HDL serum level in old rats were significantly lower (42 ± 0.7 mg/dl) compared to that in young rats (63 ± 3.08 mg/dl) (*P*<0.001) The results in the lovastatin treated group, showed that lovastatin daily (10 mg/Kg orally, for 8 weeks) reduced significantly (*P*<0.001) serum LDL, VLDL, triglyceride and total cholesterol to 48.3 ± 4.35, 6.5 ± 0.54, 32.3 ± 2.7 and 108 ± 1 mg/dl respectively. Conversely, serum HDL were significantly increased from 42 ± 0.7 in old control to 54 ± 4.5 mg/dl in lovastatin-treated old rats (*P*<0.01).

Pharmacological findings. The general observation from the pharmacological investigations showed that the aorta taken from the young rats had greater contraction and more rapid rate of relaxation per mg wet weight relative to aorta taken from old rats. The mean submaximal contraction recorded with 1 µM phenylephrine for aorta taken from the young rats was significantly greater (448 ± 47 mg $min^{-1}.mg^{-1}$ weight), compared to 171 ± 14 mg min^{-1} mg^{-1} weight in the aorta taken from old rats (*P*<0.001) (Fig. 2). On the other hand, the rate of relaxation in the aortic samples taken from young rats was significantly faster (13.1 ± 0.8 mg.$min^{-1}mg^{-1}$ wet weight), relative to 5.4 ± 0.21 mg.$min^{-1}mg^{-1}$ wet weight in the aorta taken from old rats (*P*<0.001) (Fig. 3). Both means for the submaximal contraction and the rate of relaxation recorded for the aorta taken from the lovastatin-treated group showed significant increase relative to control aged rats, 202 ± 9 mg/mg wet tissue weight (*P*<0.05), and of 6.7 ± 0.1 mg $min^{-1}mg^{-1}$ wet tissue weight (*P*<0.001) respectively (Figs. 2 & 3).

Histological findings. The general observation for the aorta taken from the young rats showed an organized circular structure with convoluted elastic bands intervened with muscular fibers and little or no splitting between the layers (Fig. 4A). While, the aorta sample taken from the old rats showed similar circular arrangement, but there was a thicker medial layer.

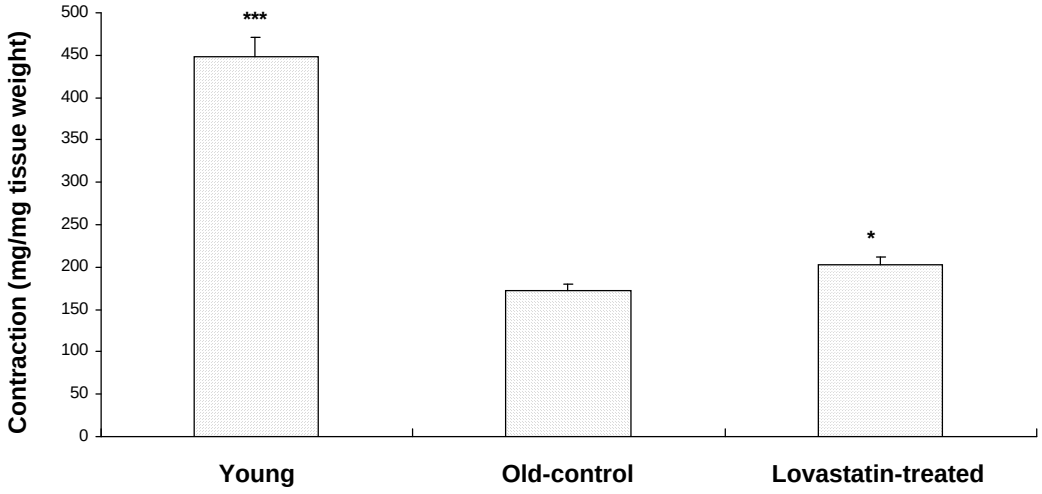

Fig. 2. A graphic representation showing the degree of aortic contraction (in mg tension/mg wet tissue weight) in old and young N- MRI rats and following treatment with lovastatin (4 mg/kg daily for 8 weeks) in response to submaximal concentration of phenylephrine hydrochloride (1 µM). $^{*}P$<0.05 and $^{***}P$<0.001 relative to old rats, ANOVA followed by unpaired student's *t*-test, n = 8.

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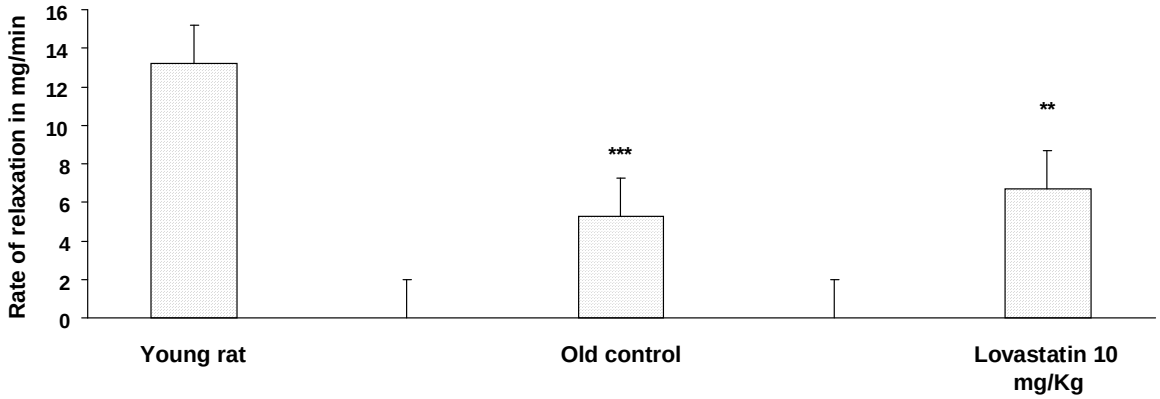

Fig. 3. A graphic representation showing the *in vitro* rate of relaxation (in mg tension min^{-1}. mg^{-1} wet tissue weight) following administration of 0.1 mM of acetylcholine to phenylephrine-induced pre-contracted aortic rings of old and young N-MRI rats and following treatment with lovastatin (4 mg/kg orally, daily, for weeks). ***P*<0.01 and ****P*<0.001 relative to old rats, ANOVA followed by unpaired student's *t*-test, n = 8.

In addition, there were distinct areas of splitting due to intracellular lipid deposits within the intimal layer and extracellular foaming cells in the outermost layers (Fig. 4B). The mean thickness of the aorta of the old rats was 0.0954 ± 0.017 mm. This was thicker and highly significant (*P*<0.001), compared to 0.0667 ± 0.012 mm in aorta taken from the young rats. While in the lovastatin group the mean thickness was 0.0885 ± 0.016 mm, showing a significant reduction relative to aorta taken from old untreated rats (*P*<0.001). (Fig 4C). The observed splitting within the aorta and extracellular foaming cells on the outermost layer were reduced and were intermediate between those observed in the aorta taken from old and young rats (Fig. 4C).

DISCUSSION

The present studies demonstrated that N-MRI rat is a suitable strain for studying changes associated with the phenomenon of atherosclerosis. The results from all the three parameters were found to be closely correlated with changes in the features related to atherosclerosis. The pharmacological results showed that the aortic rings taken from aged rats produced less contraction per mg wet tissue weight and had a slower rate of relaxation. The histological observations showed sings of atherosclerosis in the old rats: increased thickness of the aorta, the presence of intimal splitting, and presence of extracellular foaming lipid layer. Furthermore, the biochemical changes correlated with increase in LDL, VLDL, triglyceride and cholesterol and lower HDL lipid profile relative to that of young rats.

The aim of the present studies was to establish a simple, reproducible, predictable and quantifiable animal model of atherosclerosis. The results suggest that aged N-MRI rats, methodologically tested for the first time, is a suitable strain of animals to use for such studies. It was shown that the animal of this strain has a natural course of developing characteristics of atherosclerosis and hyper-cholesterolaemia similar to those in humans [10]. In contrast to other studies [2-5], an aged animal model has a more realistic approach, in that it reflects more closely the process of development of atherosclerosis which by its nature is a slow progressing process developing over years [7].

The suitability of this model and the techniques used to assess atherosclerosis were found to be correlated with the features considered important in similar studies in both animal and human [7, 8]. The differences between the young and aged rats, suggest that the old rat have characteristic hardening and thicker aorta, and relatively higher LDL/HDL ratio and therefore were atherosclerotic. Furthermore, the three suggested parameters can be studied simultaneously and the effectiveness of various interventions on the process of this disease can be assessed and correlated.

In order to limit the influence of other confounding factors such as age, sex and other housing conditions, we used aged male N-MRI rats, (11 months of age) as the end point [7]. This was considered an essential protocol to use, in order to limit the effect of these confounding factors on the final conclusions that may be drawn from such studies.

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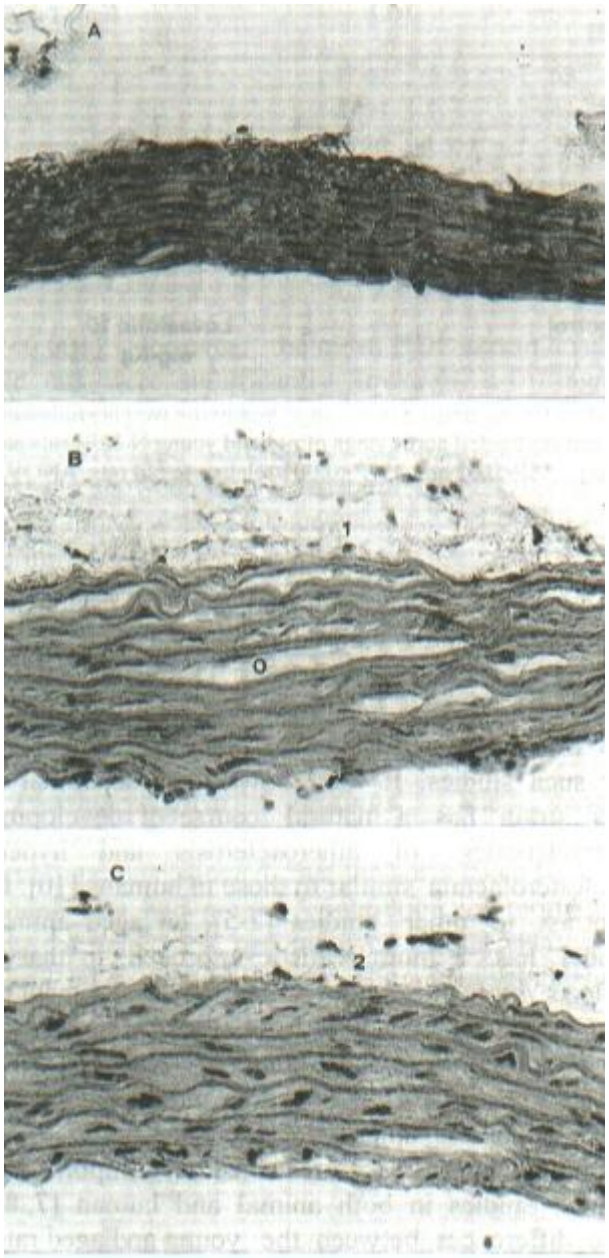

Fig. 4. A photographic illustration of Haematoxylin-Eosin stained aortic samples taken from young **(A)**, old control; **(B)**, lovastatin-treated; **(C)**, N-MRI rats. Note the prominent splitting within the media (0), and extracellular foam cell deposits (1) on the outermost layer of the aorta taken from old rats which were absent in aorta from the young rats (a). Treatment with lovastatin (c, 4 mg/kg orally daily for after 8 weeks) reduced the splitting and the foaming cells were mostly replaced with connective tissues with its associated cells (2).

A model, which is merely reproducible, may be insufficient to investigate modifications to artherosclerosis process, therefore, must also be capable of detecting the effect of a potential treatment or intervention. Prudently, this is an important aspect of such studies which need to be undertaken. To this end, we used lovastatin. Lovastatin at dose of 4 mg/Kg administered orally, daily for 8 weeks, showed to be effective in modifying the lipid dysfunction seen in old control rats, the rate of relaxation was also increased significantly. In addition, it reduced the thickness of the aorta, and the degree of splitting and extracellular lipid deposits. All these observations agree with the reported effectiveness and usefulness of lovastatin in modifying the sings of atherosclerosis [14], and that this model is accurate enough to detect these effects.

Our observations suggest that a number of important considerations that any study on atherosclerosis must contain. Firstly, the change in lipid profile, which has been one of the standards taken into account by various studies. Secondly, the histological observations, carried out concurrently, provide yet other evidences to support previous observations. Finally, the pharmacological interventions demonstrated that the aorta taken from old rats were less responsive to acetycholine. This observation is consistent with the notion that resistance of smooth muscle cells to nitric oxide, [12] released via acetycholine, contributes to abnormal endothelium-dependent vasodilatation during hypercholesterolemia and can play an important role in the pathogenesis of atherosclerosis [15-17].

What are the significant clinical applications of such studies? Even though we recognise that extrapolation of data from animal studies to man should be very guarded, nevertheless the results from reliable animal models together with future pharmacological interventions may yield potentially useful information on the mechanisms underlying the process of atherosclerosis and may prove to have a wider clinical application.

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