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Effect of high cholesterol diet on aortic hydroxyproline and collagen content in rabbits

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ABSTRACT

Collagen represents the chief structural vertebral protein. The most important function of collagen is to withstand tensile stress. The aorta is one of the important sources of collagen. The present study was undertaken to investigate the effect of high cholesterol diet on the hydroxyproline fractions and collagen content in the aorta of rabbits. 12 Weeks old New Zealand white male rabbits were divided into control group and cholesterol-fed group. The control group (n=10) was fed on 100g/day of normal diet, ORC-4 (Oriental Yeast Co. Ltd., Tokyo, Japan) for 15 weeks. The cholesterol-fed groups (the experimental groups; n=15) were fed on high cholesterol and saturated fat diet of ORC-4 containing 1 % cholesterol plus 1 % olive oil (100 g/day) for periods of 5 weeks (group 1), 10 weeks (group 2) and 15 weeks (group 3). The rabbits were sacrificed after the mentioned time period and their serum was used to determine total cholesterol, low-density lipoprotein cholesterol and triglycerides. The aortae were used to determine their hydroxyproline fractions and collagen content. Feeding rabbits on high cholesterol diet caused significant increase in serum levels of total cholesterol, low-density lipoprotein cholesterol and triglycerides. High cholesterol diet also caused a significant decrease of collagen from the aorta which was supported by histological studies.

KEYWORDS

Aorta;
Collagen;
Hydroxyproline;
Rabbits.

INTRODUCTION

Collagen represents the chief structural protein accounting for approximately 30% of all vertebrate protein. In majority of the tissues the most important function of collagen is a mechanical one- to withstand tensile stress. The hydroxyproline (Hyp) is a post translational product of proline hydroxylation catalyzed by the enzyme prolylhydroxylase (EC 1.14.11.2)[1]. The occurrence of this amino acid is thought to be confined exclusively to collagen, where it is present in the Y position of the Gly-X-Y repeating tripeptide[2]. Consequently, the presence of hydroxyproline (Hyp) in tissues or serum can be used as a measure of collagen or collagen degradation products[3]. In our previous studies[4,5] we have shown that $HgCl_2$ treatment to rats

damages the collagen which is reflected by increased levels of Hyp in serum and an increased excretion of Hyp in urine.

Gregory[6] has postulated that serum hypercholesterolemia accelerates atherogenesis by augmenting cholesterol accumulation in the arterial intima. High total cholesterol (TC) and low-density lipoprotein cholesterol (LDLC) have been correlated with the increased risk of atherosclerosis[7]. Hypertriglyceridemia is associated with an increased risk of coronary heart disease[8]. Abdelhalim et al.[9] reported that cholesterol feeding has a general tendency to induce softening of the arterial wall due to denaturation of collagen and elastin which exist in the media of the arterial wall and is known to play a dominant role in governing mechanical properties of blood vessels. In the present study an attempt was made to study the effect of high cholesterol diet on aortic collagen content and various hydroxyproline fractions in rabbits.

MATERIALS AND METHODS

Animals

12-Weeks old, New Zealand white male rabbits, were purchased from Kitayama Lab. Ltd., Kyoto, Japan, individually caged, and divided into either control group or cholesterol-fed group. The control group (n=10) was fed on 100g/day of normal diet, ORC-4 (Oriental Yeast Co. Ltd., Tokyo, Japan) for 15 weeks. The high cholesterol diet fed groups (the experimental groups; n=15) were fed on high cholesterol and saturated fat diet of ORC-4 containing 1% cholesterol plus 1% olive oil(100g/day) for feeding periods of 5 weeks (group 1), 10 weeks(group 2) and 15 weeks(group 3).

Collection of blood and preparation of serum

About 2 ml of blood was drawn from the rabbits via venepuncture of an antecubital vein and collected into polypropylene tubes. Serum was prepared by allowing the blood to clot at 37^0C and centrifugation at 3000 revolutions per minute for ten minutes.

Dissection of thoracic and abdominal aortae

The rabbits were sacrificed by injecting an overdose of pentobarbital into the auricular vein. The chest and abdomen were opened through a middle incision. While carefully separating tissues surrounding the aorta and its branched vessels, the thoracic and abdominal aortae were removed with great care so as to avoid any damage to the tissues surrounding the aorta, and were placed in 10% buffered neutral formalin. The aortae were stored in a refrigerator at a temperature of 4^0C for a period less than 48 hours until the staining was performed. A part of the aorta was preserved in liquid nitrogen and used to determine the Hyp concentration.

Staining specimens of thoracic and abdominal aorta

According to the routine procedures, thoracic and abdominal aortic specimens were stained by Masson trichrome staining for examination of fatty streaks, fibrous plaques, and degenerative change of collagen and elastin of the arterial wall. Determination of total cholesterol and low-density lipoprotein cholesterol Serum TC and LDLC levels were analyzed by the clinical laboratory centre of King Khaled Hospital. LDLC concentrations were determined by the previously reported method[10,11].

Preparation of the sample for hydroxyproline estimation

Dissected aortae were homogenized in normal saline (0.8 percent g ml^{-1}) using a stainless steel Omni-Mixer homogenizer (Omni International, Inc, Gainesville, VA, USA). The homogenate was used for determination of Hyp concentrations. Further details about sample collections have been previously reported[12]. Total collagen content was calculated from Hyp concentration assuming that Hyp consitutes12.5% collagen[13].

Extraction of free, peptide-bound and protein-bound hydroxyproline

Free, peptide-bound and protein-bound hydroxyproline was extracted by the method of Varghese et al.[14] with a slight modification as described by Siddiqi et al[12]. 0.5ml of the homogenate was treated with 3×5ml of rectified absolute alcohol and centrifuged at 3000 rpm for 10min. The supernatant was pooled and kept at 40ºC till the evaporation of ethanol. The residue was dissolved in 0.5ml of distilled water and 50µl of the extract was used for estimation of free hydroxyproline. The peptide-bound hydroxyproline was determined after alkaline hydrolysis of the ethanol extractable fraction. The pellets of all the samples were dissolved in an aliquot of distilled water and 50µl of the extract was used for determination of protein-bound hydroxyproline. The precipitate obtained upon ethanol treatment of the plasma was subjected to alkali hydrolysis to determine protein-bound hydroxyproline.

Regular Paper

TABLE 1: Effect of high cholesterol diet on the concentration of total cholesterol, low density lipoprotein cholesterol and triglycerides in the serum of rabbits

Group	Control 15 weeks (10)	Cholesterol-fed rabbits 5 weeks (5)	Cholesterol-fed rabbits 10 weeks (5)	Cholesterol-fed rabbits 15 weeks (5)
Total cholesterol (TC; mg/dl)	54.63±16.4	841.3±45.4**	837.4±21.5**	708.7±14.8**
Low density lipoprotein (LDLC; mg/dl)	36±10.1	677.7±38.5**	677.2±11.01**	608.7±24.1**
Triglycerides (TG; mg/dl)	50.1±3.6	161.5±4.2**	410.2±54.9**	406.0±120.03**

Data are expressed as mean±SD, n=5 animals. **P<0.01, Tukey's multiple comparision test. The cholesterol-fed groups(the experimental groups; n=15) were fed on high cholesterol and saturated fat diet of ORC-4 containing 1% cholesterol plus 1 % olive oil (100g/day) for feeding periods of 5 weeks (Group 1), 10 weeks (Group 2) and 15 weeks (Group 3)

TABLE 2 : Concentrations of various hydroxyproline fractions in aorta of control and rabbits fed on high cholesterol diet

Groups	Free hydroxyproline (µg/g fresh tissue)	Peptide-bound hydroxyproline (mg/g fresh tissue)	Protein-bound hydroxyproline (µg/g fresh tissue)	Total hydroxyproline (mg/g fresh tissue)
Control	147.3±34.22	5.24±1.43	235.3±55.14	7.32±3.46
Group 2	132.0±31.92^{ns}	1.58±0.63**	ND	1.71±0.62*
Group 3	136.5±32.15^{ns}	1.84±0.62**	ND	1.96±0.59*

Data expressed as mean±SD, n=5 animals. ns not significant (P>0.05), ND–Not Detectable, *P<0.05, Tukey's multiple comparision test, **P<0.01, Tukey's multiple comparision test. The cholesterol-fed groups (the experimental groups; n=15) were fed on high cholesterol and saturated fat diet of ORC-4 containing 1 % cholesterol plus 1 % olive oil (100g/day) for feeding periods of 10 weeks (Group 2) and 15 weeks (Group 3).

Determination of hydroxyproline concentration

Hydroxyproline was measured by the modified alkaline hydrolysis method of Reddy and Enwemeka[3]. Briefly to 50µl of homogenate sample was added NaOH (2 N final concentration) and the mixture was hydrolyzed by heating in boiling water bath for about 3-4 hours. Approximately 900µl of 56 mM chloramines T reagent was added to the hydrolyzed sample and oxidation was allowed to proceed at the room temperature for 25 minutes. Then 1.0ml 1M Ehrlich's reagent (P-dimethylaminobenzaldehyde) was added to the oxidized sample and the chromophore was developed by incubating the samples at 65ºC for 20min. The absorbance was read at 550 nm. The hydroxyproline concentration in the samples was calculated from the standard curve of hydroxyproline. More details about the optimization, linearity, specificity, precision and reproducibility have been previously reported[12].

Statistical analysis

Each sample was run in duplicate. The Hyp concentration and collagen content were expressed as mean±SDµg or mg/g wet weight tissue for n=5 animals. The Hyp concentration and collagen content in various tissues were compared using one-way ANOVA analysis followed by Tukey's test for multiple comparison test. Bartlett's test was used for homogeneity of variances. Spearman correlation analysis was used to examine the association between variables. Values were considered significant if P<0.05. Statistical analysis was performed by means of InStat® package for personal computers (GraphPad™ Software, Inc., San Diego, USA).

RESULTS

TABLE 1 shows the effect of high cholesterol diet in the serum levels of total cholesterol, LDLC and triglycerides in the serum of control rabbits and rabbits fed on high cholesterol diet. The total serum cholesterol showed a significant increase of 1440%, 1433% and 1197% (P<0.001) in groups 1, 2 and 3 respectively when compared to the control rabbits. Similarly LDLC also showed a significant increase of 1782%, 1781% and 1591% (P<0.001) in groups 1, 2 and 3 when compared to control group. Serum triglycerides also showed a significant increase of 222%, 719% and 710% (P<0.001) in groups 1, 2 and 3 respectively when control rabbits.

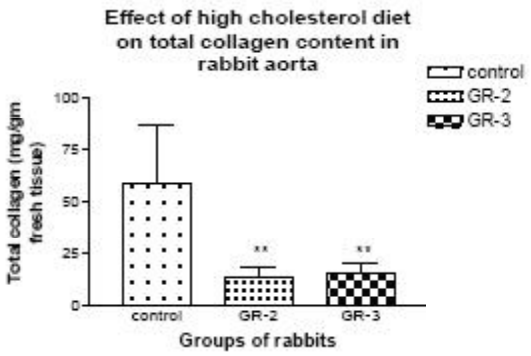
TABLE 2 shows the concentration of various Hyp fractions in the arotha of control and rabbits fed on high cholesterol diet. There were no significant changes in all the Hyp fractions of group 1 rabbits (unpublished data). There was also no significant change in free Hyp fractions of group 2 and 3 rabbits (P>0.05) when compared to control group. Peptide-bound Hyp showed a significant decrease of 70% and 65% in group 2 and 3 respectively (P<0.01) when compared to control rabbits. The concentration of protein-bound Hyp in the control group of rabbit was 235.3±55.14µg/g fresh tis-

Regular Paper

sue. However protein bound Hyp fraction was not detected in groups 2 and 3. Total Hyp showed a significant decrease of 77% (P<0.05) and 73% (P<0.05) in groups 2 and 3 respectively when compared to control rabbits.

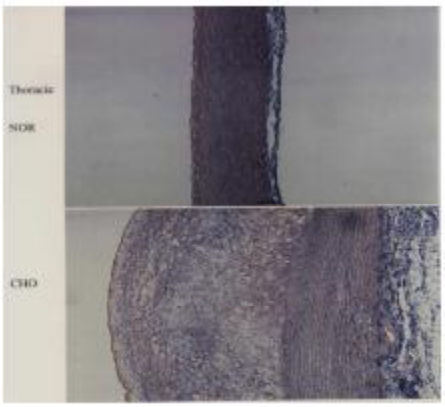
Figure 1 shows the concentration of total collagen in control rabbits and rabbits fed on high cholesterol diet. Total collagen showed a significant decrease of 77% (P< 0.05) and 73% (P <0.05) in groups 2 and 3 respectively when compared to control rabbits.

The results of histological examination of thoracic aorta are shown in figure 2. Figure 2 represent photomicrographs of the Masson trichrome stained thoracic aorta obtained from a normal rabbit (NOR) and a high cholesterol diet fed rabbit (CHO). The lower panel (CHO) shows a marked intimal thickening, smooth muscle proliferation and connective tissue formation together with focal loss of normal medial architecture. Tunica media underlying plaques shows a marked disruption with loss of collagen and elastin fibers. The elastin and collagen fibers were found less condensed and fragmented near the innermost and outermost boundary of the media and within the central portion of the intima.



Values are expressed as±SD, n=5 animals. **P < 0.01, Tukey's multiple comparision test. The cholesterol-fed groups (the experimental groups; n=15) were fed on high cholesterol and saturated fat diet of ORC-4 containing 1 % cholesterol plus 1 % olive oil (100 g/day) for feeding periods of 10 weeks (Group 2) and 15 weeks (Group 3)

Figure 1



Photomicrographs of the Masson trichrome stained thoracic aorta obtained from a normal-fed rabbit (NOR) and a cholesterol-fed rabbit (CHO)

Figure 2

DISCUSSION

In the present study, rabbits were fed a high cholesterol and saturated fat diet containing 1 % cholesterol for periods of 5, 10, and 15 weeks. The accompanying changes in TC and LDLC levels in serum and hydroxyproline fractions/collagen concentration in aorta of rabbits during the feeding periods of 5, 10, and 15 weeks were studied. Thoracic and abdominal aortic specimens were stained by Masson trichrome for examination of fibrous plaques and any degenerative change in collagen and elastin of the arterial wall. It is suggested that cholesterol feeding has a general tendency to induce increase in TC and LDLC levels in serum which may be deposited in the atheromatous lesions. Studies of Sloop[15], have shown that a high-cholesterol diet elevated level of plasma TC and LDLC which are incorporated into atherosclerotic plaques. Another possible mechanism by which a high-cholesterol diet may accelerate atherogenesis is by increasing blood viscosity and disturbing the mechanical fragility of atherosclerotic plaques making them vulnerable to rupture and thrombosis. It is has been suggested that when LDLC is oxidized by macrophages in lesions, it becomes toxic to the endothelium, and thereby could injure endothelial cells. Thus, the effects of high cholesterol diet are not only confined to deposition of lipids in atheromatous lesions, but may also contribute to primary endothelial injury. In the present study, intima of the aortae of cholesterol fed rabbits demonstrated a marked increase in thickness and smooth muscle cell proliferation. In addition, lipid laden cells were observed near the basement of the lesion. The tunica media underlying plaques showed a marked disruption with a focal loss of collagen, elastin, and smooth muscle cells. The focal loss of collagen and elastin induce softening of the arterial wall which is known to play a dominant role in governing mechanical properties of blood vessels[9].

These results were further confirmed by biochemical analysis which showed that feeding rabbits with a high cholesterol diet caused a significant decrease of

Regular Paper

total collagen and Hyp concentration in the aorta. Type I and type III collagen play an important role in arterial physiology by preventing arterial expansion beyond physiologic limits. Smooth muscle cells in the atherosclerotic arteries synthesize new extracellular matrix components, including collagen[16]. In atherosclerotic arteries collagen is crucial for plaque stability and its removal from the plaque's fibrous cap area may result in plaque rupture[17]. In addition to providing the extracellular matrix stability, the extracellular collagen network functions as a framework for the migration of smooth muscle cells into the intima where they proliferate and synthesize new extracellular matrix components. Since collagen plays key roles in plaque stability and cell migration properties, a comprehensive understanding of collagen expression and organization during the progression of atherosclerosis is essential. The aorta is among the most abundant tissue sources of collagen XVIII[18] and also contains Type I and Type III collagen. Karen et al.[19], hypothesized that collagen XVIII is degraded during atherosclerosis and that loss of this vessel wall proteoglycan promotes the proliferation of vasa vasorum into the intima of atheromas. There studies further provide genetic evidence that loss of collagen XVIII promotes atherosclerosis. Loss of collagen XVIII increases plaque angiogenesis and vascular permeability to lipids by distinct mechanisms that develop at different gene doses. Studies of Karen et al., 2004 [19] also demonstrate that the function for collagen XVIII in basement membranes is to maintain vascular permeability. In the present study there was no significant change in the concentration of free Hyp in the aorta of rabbits from the experimental groups. This shows that the processes which contribute to free Hyp pool viz., mature collagen, newly synthesized collagen dietary collagen etc. were not affected by high cholesterol diet. There was a significant decrease in peptide- bound Hyp in experimental groups though protein bound Hyp was not detected in the aorta of rabbits fed on high cholesterol diet.

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REFERENCES

[1] T,Pihlajaniemi, R.Myllya, K.Krivirikko; Journal of Hepatology, **13**, S2-S7 **(1991)**.
[2] G.Nemethy, H.A.Scheraga; Biochemistry, **152**, 267-273 **(1986)**.
[3] K.Reddy, C.S.Enwemeka; Clinical Biochemistry, **29**, 225-229 **(1996)**.
[4] N.J.Siddiqi, A.S.Alhomida; Molecular and Cellular Biochemistry, **271**, 159-165, **(2005)**.
[5] N.J.Siddiqi, A.S.Alhomida; Medical Science Monitor, **12**, BR95-101 **(2006)**.
[6] D.S.Gregory; Atherosclerosis, **142**, 265-268, **(1999)**.
[7] M.J.Martin, S.B.Hulley, W.S.Browner, L.H.Kuller, D.Wentworth; Lancet, 2, 933-936 **(1986)**.
[8] M.Miller, A.Seidler, A.Moalemi, T.A.Pearson; Journal of American College of Cardiology, **31**, 1252-1257 **(1998)**.
[9] M.A.Abdelhalim, M.Sato, N.Ohshima; JSME International Journal, 37 series A, **1**, 79- 86 **(1994)**.
[10] A.J.Lee, P.Mowbray, G.D.Lowe, A.Rumley, F.G. Fowkes, P.L.Allan; Circulation, **97**, 1467-1473 **(1998)**.
[11] W.Koenig, M.Sund, B.Filipiak, A.Doring, H.Lowel, E.Ernst; Arteriosclerosis Thrombosis Vascular Biology, **18**, 768-772 **(1998)**.
[12] N.J.Siddiqi, A.A.Al-Jafari, A.S.Alhomida; Cell Biochemistry and Function, **18**, 243-248 **(2000)**.
[13] C.A.Edwards, W.D.Jr O'Brien; Clinica Chimica Acta, **104**, 161-167 **(1980)**.
[14] Z.Varghese, J.F.Moorhead, M.R.Wills; Clinica Chimica Acta, **110**, 105-111 **(1981)**.
[15] G.D.Sloop; Medical Hypothesis, **47**, 321-325 **(1996)**.
[16] K.Pietila, T.Nikkari; Atherosclerosis, **37**, 11-19 **(1980)**.
[17] M.J.Barnes, R.W.Farndale; Experimental Gerontology, **34**, 513-525 **(1999)**.
[18] N.Miosge, T.Sasaki, R.Timpl; FASEB Journal, **13**, 1743-1750 **(1990)**.
[19] S.M.Karen, R.O.Bjorn, B.A.Silvia Sonn, N.Fukai, D.Zurakowski, X.Xiaokun Zeng; Circulation, **110**, 1330-1336 **(2004)**.