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Morphological and Biochemical Evaluation of the Induction of Atherosclerosis in Japanese Quails

Hirosuke OKU[1], Takayoshi TODA[2], Yushi HAMADA[2]
Masaya KIYUNA[2], Isao CHINEN[1], Mari TOYOMOTO[1]
and Akihisa SHINJO[3]

Department of Agricultural Chemistry, College of Agriculture[1],
Department of Clinical Laboratory, University Hospital, School of Medicine[2],
and Department of Animal Science, College of Agriculture[3],

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SUMMARY : A total of 77 birds were divided into 7 groups which were fed the following diets : Group I, basal ; Group II, 5% corn oil (CO) + 0.5% cholesterol (CH) ; Group III, 5% CO + 2% CH ; Group IV, 5% CO + 4% CH ; Group V, 15% CO + 0.5% CH ; Group VI, 15% CO + 2% CH ; Group VII, 15% CO + 4% CH. Significant increase of serum lipid, accumulation of lipid in the liver, and lipid-rich aortic lesions were produced in Groups IV, VI and VIII. However, hyperlipidemia correlated well with the extent of hepatic lipid accumulation and severity of aortic atherosclerosis in Group VI. Proliferating intimal cells showed positive reaction to antibodies for vimentin and alpha-1-antichymotrypsin implicating an important role of phenotypical transformation of intimal cells from the medial fibroblastic cells in the development of aortic atherosclerosis. These results suggest that Japanese quail is highly susceptible to atherosclerosis, and the optimal dietary level of cholesterol and corn oil is 2% and 15%, respectively to induce lipid-rich aortic lesions in Japanese quail.

INTRODUCTION

Numerous studies have been reported that the ischemic heart disease frequently occurs in people who consume higher amount of dietary cholesterol and culinary fats (11). Animal experiment offers valuable information in the study of atherogenesis because clinical experiment with human may not be acceptable to solve the complex influence of culinary fats on atherogenesis. Various avian species such as pigeon (3) turkeys (21) and chikens (17) have been demonstrated to be the convenient experimental animals for the induction of atherosclerosis. The Japanese quail seems to be an ideal laboratory animal for the long-term experiment because it is small in size, short in life cycle, low in feed consumption (20). The present experiment was carried out to determine the dietary levels of cholesterol, and culinary fat and oil which are the factors in the induction of the atherosclerotic lesions, and to characterize morphologically the atherosclerotic lesions in Japanese quail.

MATERIALS AND METHODS

Japanese quail, 40-day-old, were purchased from the commercial supplier (Kyudo Ltd). As shown in **Table 1**, seven different groups were fed with test diets which differ in levels of cholesterol and corn oil. The basal diet (Kyoei Co., Ltd., Okinawa) contained 18 percent protein,

Table 1. Experimental Design

Dietary Components	Groups						
	I	II	III	IV	V	VI	VII
Number of birds	15	10	10	10	10	13	9
Corn Oil (%)	—	5	5	5	15	15	15
Cholesterol (%)	—	0.5	2.0	4.0	0.5	2.0	4.0

3.8 percent fat, 6.3 percent ash, 3.5 percent fiber, and 2,842 Kcal/Kg. Cholesterol was purchased from Kyowa Co. Ltd. Corn oil was donated from NIHON YUSHI Co. Ltd. and fatty acid composition of which was as follows : 14 : 0-0.1, 16 : 0-14.3, 16 : 1-0.7, 18 : 0-4.9, 18 : 1-31.9, 18 : 2-41.1, 18 : 3-7.0. The test diets were given ad libitum. The whole aorta and its large branches with the heart was obtained from the quails fed with experimental diets for 3 months. Formalin-fixed, praffinembedded blocks of aortic tissue samples were cut into 4 micrometer thickness, then stained by hematoxylin eosin (H. E.), Mallory azan (M. A.) and elatica van Gieson (E. V.). The intimal thickness was measured from the representative three to four arteries including ascending aorta and its large branches in each quail using an ocular micrometer. The atherosclerotic index was expressed as the average degree of intimal thickness of the examined cases. For immunohistochemical examination with PAP method (23), antibodies for beta-lipoprotein, alpha-1-antichymotrypsin, vimentin, and desmin were purchased from Dakopatts. The serum cholesterol concentrations were determined by the method of Sperry and Webb (22). The serum triglyceride concentrations were determined by the method of Fletcher (6). The aliquots of lipid were extracted by the method of Folch *et al.* (7), and the liver cholesterol and triglyceride concentrations were determined by the method of Sperry and Webb (22), and Fletcher (6), respectively. The fatty acid composition of test diets, sera and liver tissues was analyzed by gas-liquid chromatography (24). The serum hydroperoxide value was determined by the method of Yagi (29).

RESULTS

I. Gross and Chemical Findings. The fatty acid composition of test diets is shown in **Table 2**. All the diets contained enough amounts of essential fatty acids for the animal requirement of essential fatty acids. **Table 3** lists body and liver wieghts in each experimental group. The mean body weights were similar among all

Table 2. Fatty Acid Composition of Test Diet

Group	14 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	18 : 3	Others
I	1.3	17.2	1.3	2.4	21.2	51.4	2.0	3.2
II	0.3	13.7	0.2	1.8	29.7	52.9	1.4	—
III	0.3	12.6	0.5	2.1	30.0	52.1	2.4	—
IV	1.0	14.6	1.9	6.9	28.4	38.1	9.2	—
V	0.2	11.5	0.2	1.9	33.0	50.9	2.4	—
VI	0.1	11.5	0.2	1.9	32.3	52.4	1.6	—
VII	0.8	12.9	0.2	3.9	37.9	42.7	1.7	—

Table 3. Body and Liver Weights in Each Experimental Group

Group	I	II	III	IV	V	VI	VII
Body Weight	96 ± 4	109 ± 4	104 ± 4	97 ± 1	103 ± 3	103 ± 3	100 ± 2
Liver Weight (g/100g BW)	1.4 ± 0.2	1.7 ± 0	1.7 ± 0	2.3 ± 0.2	1.8 ± 0.1	2.4 ± 0.2	2.6 ± 0.5

Table 4. Serum Lipid (mg/dl) and Hydroperoxide (n mol/ml) Profile in Each Experimental Group

Parameters	Groups						
	I	II	III	IV	V	VI	VII
TG	77 ± 12	87 ± 14	109 ± 18	110 ± 29	66 ± 9	134 ± 31	88 ± 34
TC	196 ± 12	651 ± 123	678 ± 231	980 ± 191	586 ± 155	1094 ± 225	763 ± 227
FC	63 ± 2	162 ± 26	168 ± 49	269 ± 74	151 ± 34	264 ± 46	210 ± 58
CE%	70	74	74	73	73	75	71
HP	7	8	8	7	7	8	7

TG : Triglyceriede, TC : Total cholesterol, FC : Free cholesterol, CE : Cholesterol esterification, HP : Hydroperoxide (TBA) value.
Data are expressed as mean ± standard error.

Table 5. Fatty Acid Profile of Serum Lipid in Each Experimental Group

Group	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:6	Others
I	1.0	19.4	3.8	16.9	22.4	20.9	0.4	7.4	0.6	5.2	2.0
II	1.0	11.8	3.8	12.4	20.7	24.6	1.5	11.2	3.3	8.0	1.7
III	0.7	13.5	2.9	14.7	22.0	28.1	1.2	8.7	2.5	4.7	1.0
IV	0.6	14.1	5.2	13.3	23.6	22.6	1.9	11.2	1.6	4.8	1.2
V	0.6	12.4	2.2	14.7	21.8	30.3	1.2	9.8	1.7	4.3	1.0
VI	0.7	11.4	2.5	13.4	20.3	26.0	2.0	13.5	1.9	6.2	2.2
VII	0.6	12.4	3.1	13.9	20.0	23.1	2.1	14.9	1.2	6.3	2.4

groups, but the liver weight increased in Groups IV, VI and VII. The serum lipid profile in each experimental group is shown in **Table 4**. No significant difference was noted in serum triglyceride level among all the experimental goups. However, the serum cholesterol level was different in each experimental group and was in the following order: Group VI, Group IV, Group VII, Group III, Group II, Group V, Group I. The percentage of serum cholesterol esterification and hydroperoxide value showed no significant difference in all the experimental groups. **Table 5** indicates fatty acid profile of serum lipid in each experimental group. In the fatty acid composition of serum lipid of each experimental group (**Table 5**), linoleic acid was the main fatty acid and its level was highest in Group V which was fed with 0.5% cholesterol and 15% corn oil. However, linoleic acid level tended to decrease with proportion to increase of dietary cholesterol. **Table 6** indicates liver lipid profile in each experimental group. The lipid content of the liver was increased by feeding cholesterol and corn oil, and it was highest in Group VI which was fed with 2% cholesterol and 15% corn oil. The total cholesterol content of the liver was also highest in Group VI. The cholesterol esterification was almost equal in other groups except for Group I. The fatty acid composition of liver lipid in all the experimental groups is shown in **Table 7**. Feeding of corn oil increased content of linoleic acid in the liver, while supplementation of cholesterol induced reciprocal reduction of the content of linoleic acid in the liver.

Table 6 Effect of Cholesterol and Oil Level on Liver Lipld Concentration (mg/g liver tissue)

Parameters	Groups						
	I	II	III	IV	V	VI	VII
TL	55	154	143	131	182	218	169
	±	±	±	±	±	±	±
	3	20	10	15	15	19	31
TG	11	33	22	8	16	27	9
	±	±	±	±	±	±	±
	2	9	9	2	6	7	1
TC	3	38	39	43	46	61	45
	±	±	±	±	±	±	±
	0	7	6	4	4	3	6
FC	2	4	5	6	6	6	6
	±	±	±	±	±	±	±
	0	1	0	0	1	1	1
CE (%)	26	88	88	85	87	89	86

TL: Total lipid, TG: Triglyceride, TC: Total cholesterol, FC: Free cholesterol, CE: Cholesterol esterification.
Data are expressed as mean ± standard error.

II. Light Microscopic Findings: Intimal thickening lisions were more prevalently seen in the ascending aorta and its large branches than the abdominal aorta. Therefore, we choose

Table 7. Fatty Acid Profile of Liver Lipid in Each Experimental Group

Group	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:6	Others
I	0.8	17.9	3.9	18.8	19.4	19.1	0.3	8.4	0.5	9.8	0.9
II	1.2	9.6	5.1	10.8	17.6	18.0	3.8	11.0	2.6	16.1	4.4
III	1.2	9.8	4.3	11.7	18.5	18.9	2.8	12.0	2.2	15.7	2.8
IV	0.8	10.1	5.4	11.2	17.9	15.8	2.7	13.8	1.8	14.9	5.6
V	0.6	9.6	3.0	12.2	20.7	20.8	2.4	12.6	1.1	14.9	2.1
VI	0.9	9.9	3.6	11.9	22.4	21.6	3.0	11.4	1.2	11.1	3.1
VII	0.7	10.5	4.0	12.3	20.0	18.1	2.8	13.6	1.0	11.6	5.4

the ascending aorta and its large branches as the site to measure atherosclerotic index. The incidence of intimal lesions of ascending aorta and its large branches, and athrosclerotic index are listed in **Table** 8. No significant intimal lesions were observed in Group I (**Fig. 1**) while the other group developed arterial intimal lesions, and the severity of which was various among experimental groups. The atherosclerotic index in each experimental group was in the following order: Group VII, Group IV, Group VI, Group III, Group II, Group I, Group V. The most severe arterial lesions accurred in group VIII (**Fig. 2**), which was fed with 4% cholesterol and 15% corn oil.

III. Immunohistochemical Findings: Beta-lipoprotein was observed diffusely in the thickened intima and inner media of the ascending aorta and its large branches. These beta-lipoprotein positive intimal cells in the inner layer of the aortic wall strongly reacted with OKM1 (**Fig. 3**). The fibroblastic cells in the thickened intima did not reacted with antibody to desmin. The medial smooth muscle cells of the aorta from all 8 groups reacted with antibody to desmin and vimentin (**Fig. 4**). The fibroblastic cells of the tunica media of the aorta from a basal group showed slight positive reaction with antibody to vimentin. The staining reaction of alpha-1-antichymotrypsin and vimentin was more intense for fibroblastic cells in both deeper portion of the thickened intima and the inner layer of the tunica media of the aortic wall (**Fig. 5**) than for those in the middle layer of the aortic wall.

Table 8. Incidence of Intimal Thickening of Ascending Aorta and Brachiocephalic Arteries and Atherosclerotic Index in Each Experimental Group

Parameter	Groups						
	I	II	III	IV	V	VI	VII
Incidence of Intimal thickening (%)	15.0	22.8	43.2	68.0	41.7	39.0	48.3
Atherosclerotic Index	1.4	2.0	2.8	6.7	1.2	4.6	11.8

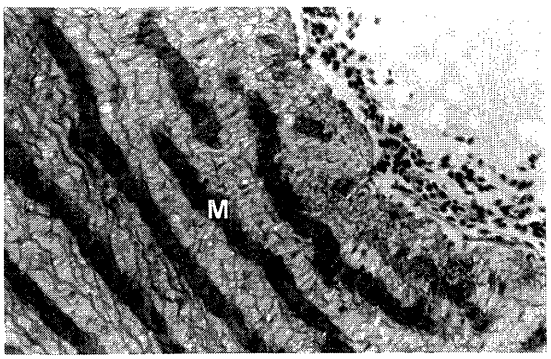

Fig. 1 Ascending aorta from a quail fed a basal diet. No discernible intimal thickening is noted. Muscular (M) and nonmuscular lamellar structure is observed in the tunica media (EV ×170).

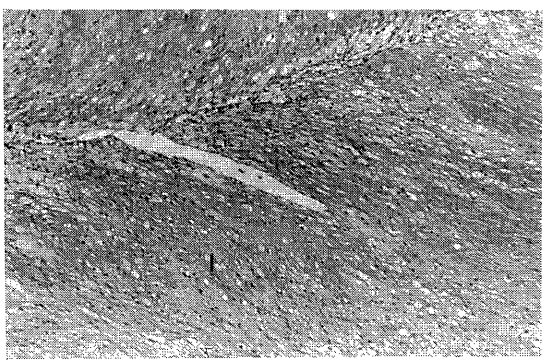

Fig. 2 Ascending aorta from a quail fed a diet containing 15% corn oil and 2% cholesterol. The lumen is remarkably obliterated by severe intimal thickening (I) (HE ×170).

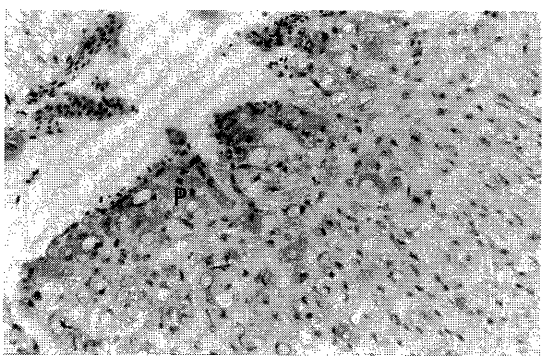

Fig. 3 Positive reaction of OKM1 is demonstrated in the lipid-containing cells (P) in the superficial area of the thickened intima (PAP stain ×170).

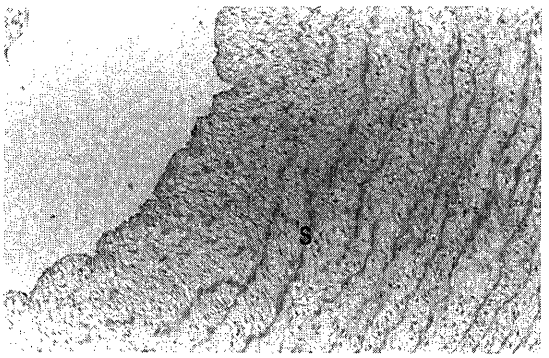

Fig. 4 Immunoreaction of desmin is demonstrated in medial smooth muscle cells (S), but not in fibroblastic cells in the tunica media (PAP stain ×170).

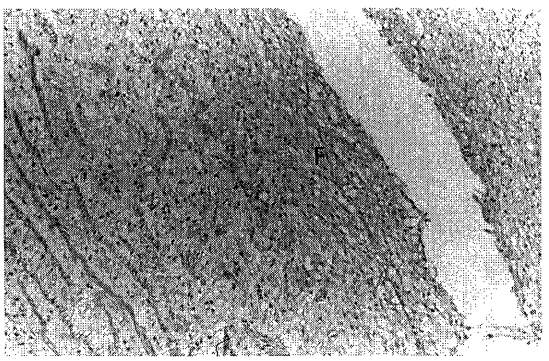

Fig. 5 Immunostaining of vimentin represents a strong reaction with fibroblastic cells (F) in the thickened intima (PAP stain ×170).

DISCUSSION

Wexler (28) reported that both the male and female Japanese quails developed spontaneous arteriosclerosis at 2 years of age. No spontaneous arteriosclerotic lesions were found in our 5 month-old quails fed with a basal diet. Feeding of cholesterol and fat or oil has been the conventional way to induce atherosclerotic lesions in the various experimental animals (3, 17, 21). The present results also demonstrate that dietary feeding of cholesterol and oil can induce typical atherosclerotic lesions more frequently in the ascending aorta and its large branches than in the abdominal aorta in Japanese quail as in the chickens (25). However, the experimental conditions were various in different laboratories. Day *et al.* (4) reported that atherosclerosis was induced by feeding with 2% cholesterol and 0.5% cholic acid for 15 weeks. Morrissey *et al.* (16) reported that atherosclerosis was induced by feeding with 1% cholesterol and 10% fat for 10 weeks. Lipid-rich aortic lesions were produced in Groups IV, VI, and VIII. However, the serum cholesterol, cholesterol ester in the liver, and the atherosclerotic index showed parallel rise in Group VI. Consequently, the optimal term and levels of dietary feeding of atherogenic diets were determined to be 3 months and 2% cholesterol and 15% corn oil, respectively in this commercially available Japanese quail.

Glavind *et al.* (9) reported that atherosclerotic aorta contained more abundant oxidized lipid than normal aorta. It has been well known that malondialdehyde is an endproduct of lipid peroxidation which occurs under various conditions such as radiation damage, hypoxia, oxygen toxicity, and vitamin deficiencies (14, 18). In the previous report, the subcutaneous injection of malondialdehyde induced degenerated cells with or without lipid droplets in the aorta of the chickens (26). In this experiment, however, there was no significant correlation between the degree of aortic atherosclerosis and serum levels of hydroperoxide.

Proliferation of intimal smooth muscle cells is considered to play an important role in the development of atherosclerosis (10). Previously we reported that fibroblasts rather than smooth muscle cells are the main cellular component in the development of atherosclerois in Japanese quail as in chicken (25, 27). Foam cells characterize the atherosclerotic lesions and it is believed that they are derived from hematogenous macrophage and smooth muscle cell (15). The present immunohistochemical findings suggest that foam cells in the luminal side of the thickened intima were derived from hematogenous macrophage because they react with antibody of OKM1 which is the marker of macrophage (1). However, most foam cells in the deeper portion of the thickened intima are considered to be fibroblast origin. Most cells have been thought to contain cytoplasmic filaments (13, 19). Recently, several investigators (8, 12) have reported the cytoskeletal differences of smooth muscle cells between the normal

aortic media and the thickened intima and that only vimentin-positive medial smooth muscle cells participate in the intimal thickening. In the present study, most proliferating intimal cells probably of fibroblast origin here show an intense reaction with antibody for vimentin and these intimal cells in turn have shown intense reaction with alpha-1-antichymotrypsin which is one of useful markers of histiocytic tumors(5). Thus, observation in the present study suggests phenotypic transformation of intimal cells migrating from the tunica media to play an important role in the initiation and the development of atherosclerosis.

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