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Investigation of Necessity of Sodium Cholate and Minimal Required Amount of Cholesterol for Dietary Induction of Atherosclerosis in Microminipigs

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Abstract. *Recently we established a Microminipig (MMPig) model of atherosclerosis induced by high fat/high cholesterol (Cho) diet containing sodium cholate (SC), which is known to cause hepatotoxicity. In the present study, we investigated whether SC is necessary as well as the minimum amount of dietary Cho required to induce atherosclerosis. Experiment A: Six MMPigs were divided into three groups of two, and were fed for 12 weeks as follows: a diet containing 12% fat and 5% Cho with or without 0.7% SC, or the diet including 12% fat and 0.5% Cho with SC. Although each diet induced a similar degree of hypercholesterolemia and atherosclerosis, the liver weights and severity of fatty change in the hepatocytes were maximal in the animals fed 5% Cho and SC. Experiment B: Six MMPigs were divided into two groups of three, and fed for 18 weeks as follows: normal diet, and a diet of increasing dose of Cho (0.03, 0.1, 0.3, 0.5, 1.5 and 5%) for the initial 14 weeks and 0.5% Cho/12% fat diet for the final four weeks. Serum levels of total Cho and low-density lipoprotein-Cho reached a plateau with 0.5% Cho diet, suggesting that the minimum amount of Cho required is 0.5%. The absorption of Cho in MMPigs was enhanced by 0.5% Cho and 12% fat diet compared to the 5% Cho-alone diet. In conclusion, a diet with 0.5% Cho and 12% fat without SC appears to be sufficient to induce atherosclerosis in the MMPig.*

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Key Words: Atherosclerosis, cholesterol, diet control, hepatotoxicity, sodium cholate, swine model.

Atherosclerosis is known to be the predominant risk factor in cardiovascular diseases and is closely-related to serious morbidity and mortality reported in the Western world (1). In Japan, the growing popularity of a Western-style diet may account for the recent increased incidence of coronary and cerebral artery diseases (2-4). These diseases are closely-related to the mechanism of onset of atherosclerosis. Atherosclerosis is induced by both genetic and environmental factors, and models for its investigation should reflect its clinical pathogenesis appropriately.

The development of models of atherosclerosis has been attempted in experimental animals such as genetically-modified mice (5-7), Watanabe heritable hyperlipidemic (WHHL) rabbits (8-10), and transgenic rabbits (11). Swine are more suitable than mice and rabbits for analyzing the influence of environmental factors on atherosclerotic lesions because their feeding habits and biological rhythms are similar to those of humans (12-14), and mice differ from humans in lipid metabolism and some environmental factors (15, 16). Domestic pigs have been used in research into physical treatment for arteries because of their large blood vessels (17), but they are difficult to manage due to their bulky size. The Microminipig™ (MMPig, Fuji Micra Inc., Shizuoka, Japan) has emerged as an experimental animal model for non-clinical pharmacological/toxicological studies (18, 19). The MMPig is the smallest of the general minipigs (*e.g.* Clawn, Göttingen, and Yucatan) for experimental use. We established a model of atherosclerosis in the MMPig by giving animals a high-fat

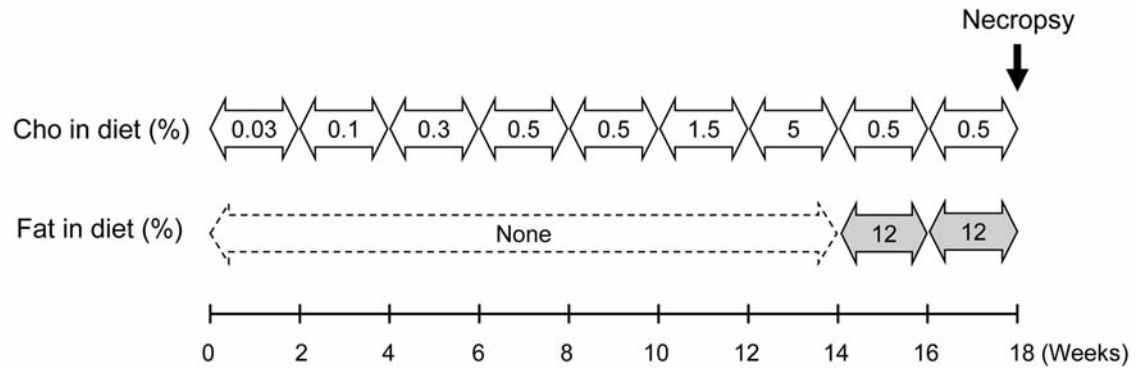

Figure 1. *Experiment B: Study design. Cho: cholesterol.*

Table I. *Fecal excretion of cholesterol, triglyceride and total bile acid at week 12 (experiment A).*

Group	Special diet	Intake of cholesterol (g/day)*	Fecal excretion of cholesterol (mg/g feces)	Intake of fat (g/day)*	Fecal excretion of triglyceride (mg/g feces)	Intake of sodium cholate (g/day)*	Fecal excretion of total bile acid (mg/g feces)
I	HF/HC	10.2	48.0	24.6	14.6	–	8.9
II	HF/HC/SC	9.2	41.9	22.0	10.3	1.3	26.0
III	HF/LC/SC	0.9	22.4	22.3	10.7	1.3	30.6

*Intake is the amount (g) per a day at week 12. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate.

(12% w/w) and high-cholesterol (5% w/w) diet containing sodium cholate (SC; 0.7% w/w, HF/HC/SC) (20). Diet control alone was sufficient to induce atherosclerotic lesions in this model similar to those seen in humans. Because rodents are generally hypo-responsive to dietary cholesterol, hyperlipidemia and atherogenesis can be induced in rats only by HF/HC/SC (21). However, atherosclerosis has been induced in Yucatan and Chinese Bama minipigs and domestic swine by an HF/HC diet alone (22-24), and in the Göttingen minipig by an HF/HC/SC diet (4). There is a known relationship between SC and hepatotoxicity (25). The amount of cholesterol in the diet was higher for the atherosclerosis model in the MMPig than for the other minipig models (14, 20). Accordingly, in the present study, with the aim of establishing an economic and appropriate model, we set out to determine whether SC is actually necessary, as well as the minimum cholesterol requirement for the HF/HC diet in order to induce atherosclerosis.

Materials and Methods

Animal maintenance. Male MMPigs were obtained from a breeder (Fuji Micra Inc., Shizuoka, Japan) and maintained under filtered air laminar flow conditions in a dedicated room at Kagoshima University. The room was maintained at a temperature of 24±3˚C and a relative humidity of 50±20%, with a 12-h light/dark cycle. Tap water was available *ad libitum* and the animals were provided with normal or special diets, with body weight (BW) being measured once a week. All protocols were approved by the Ethics Committee of Animal Care and Experimentation, Kagoshima University (A09029) and the research was performed according to the Institutional Guidelines for Animal Experiments and in compliance with the Japanese Law Concerning the Protection and Control of Animal, (Law No. 105 and Notification No. 6).

Study design. Experiment A. Six MMPigs (3-4 months old; 3.2±0.6 kg BW) were divided into three groups of two and each group fed one of three special diets for 12 weeks. These diets were composed of fat (refined lard; Miyoshi Oil & Fat Co., Ltd., Tokyo, Japan), cholesterol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), with/without SC (Wako Pure Chemical Industries, Ltd.) mixed with a normal diet (ND, Kodakara 73; Marubeni Nisshin Feed Inc., Tokyo, Japan). Groups I, II, and III were provided with a high fat (12% w/w)/high cholesterol (5% w/w) diet without SC (HF/HC), a high fat (12% w/w)/high cholesterol (5% w/w) diet containing SC (0.7% w/w) (HF/HC/SC), and a high fat (12% w/w)/low cholesterol (0.5% w/w) diet containing SC (HF/LC/SC), respectively. After 12 weeks, all MMPigs were anesthetized and then sacrificed by bilateral axillary artery exsanguination.

Experiment B. Six MMPigs (4-5 months old, 4.4±0.4 kg BW) were divided into two groups of three, and fed for 18 weeks as follows: Group I was given a normal diet (ND), and group II was subject to a special dietary regimen (without SC). As shown in Figure 1, under the special dietary regimen, the dietary cholesterol content was incrementally raised from 0.03% to 5% w/w with no supplemental

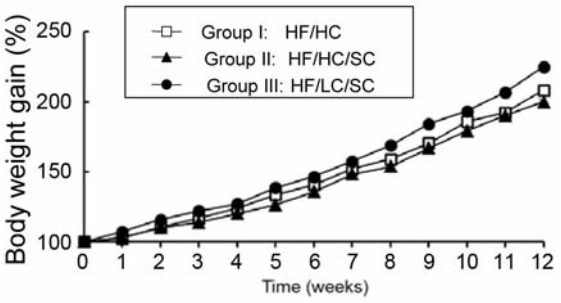

Figure 2. *Experiment A: Body weight gain. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate. Data are percentages, n=2.*

fat over the initial 14 weeks, and cholesterol and fat contents were supplemented at 0.5% w/w and 12% w/w, respectively, for the final four weeks. After 18 weeks, all MMPigs were anesthetized and then sacrificed by bilateral axillary artery exsanguination.

Biochemical analysis. Blood was collected from the cranial vena cava of each MMPig. Blood samples were obtained every week (experiment A) or every second week (experiment B), and analyzed for total cholesterol (T-Cho), low-density lipoprotein cholesterol (LDL-Cho), high-density lipoprotein cholesterol (HDL-Cho), and triglycerides (TG). Samples collected at weeks 0 and 12 were also analyzed for aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, and total bilirubin.

Fecal analysis. Fecal samples (1-2 g) were collected at week 12 (experiment A) or weekly from week 4 to 18 (experiment B) and freeze-dried until use for measurement of cholesterol, TG, and total bile acid (TBA) excretions. Freeze-dried fecal samples (100 mg) were homogenized with 1 ml of 90% ethanol using a Polytron PTMR-2100 Homogenizer (Kinematica, Lutzern, Switzerland) at 2000 rpm for 1 to 2 min. They were then incubated at 60˚C for 1 h and centrifuged at 3,000 rpm for 15 min as previously described (26). Cholesterol, TG, and TBA assays were performed with the supernatants obtained using Cho E-Test Wako, TG-Test Wako, and TBA-Test Wako kits, respectively (all from Wako Pure Chemical Industries, Ltd., Osaka, Japan). All assays were carried out according to the manufacturer's recommendations and absorbance was measured using a SUNRISE microplate reader (Tecan, Salzburg, Austria).

Pathological examination. At necropsy, the arteries, heart, liver and kidney were removed from each animal. All organs removed were fixed in 10% phosphate-buffered formalin (PBF), and embedded in paraffin. Sections of 5 μm thickness were taken for routine hematoxylin and eosin (HE) staining and histopathological examination. Atherosclerotic lesions were graded according to Stary classification (stages I-VIII) (4, 20, 27-29).

Results

Experiment A. The final BW gains (from week 0 to 12) in groups I, II, and III were 208, 200, and 225%, respectively (Figure 2).

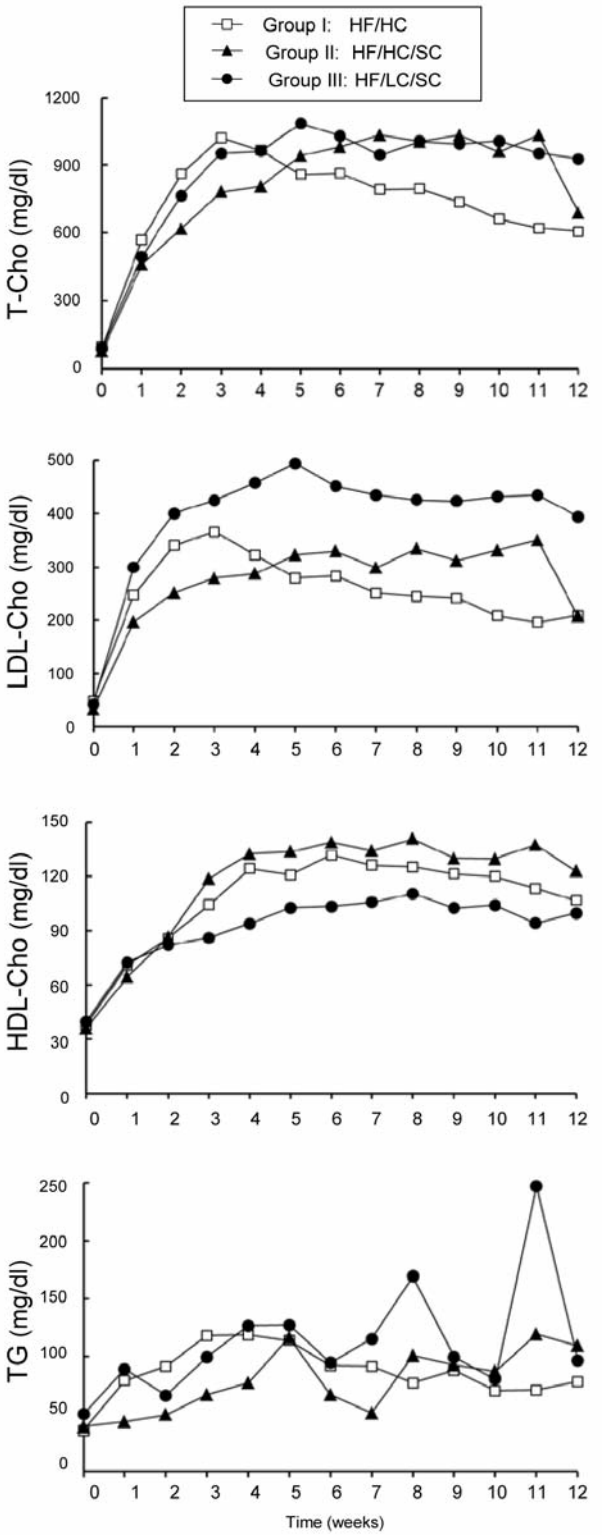

Figure 3. *Experiment A: Biochemical parameters of lipid metabolism. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate. T-cho: total cholesterol, LDL-Cho: low-density lipoprotein cholesterol, HDL-Cho: high-density lipoprotein cholesterol, TG: triglycerides.*

Table II. *Atherosclerosis score according to Stary classification (experiment A).*

Group (diet):	I (HF/HC)		II (HF/HC/SC)		III (HF/LC/SC)	
Animal no.:	1	2	3	4	5	6
LAD artery	II	V[b]	II	V	II	V[a]
LCX artery	–	V[a]	–	II	–	II
RCA	II	V[a,b]	I	VI[a,c]	II	V[a]
Pulmonary artery	II	–	–	I	–	–
Aortic arch	II	II	II	II	II	II
Common carotid artery	–	II	–	II	–	–
Thoracic aorta	II	II	II	II	II	II
Abdominal aorta	V[b]	V[b]	II	V[a]	II	V
External iliac arteries	V	–	II[b]	I	II	–
Internal iliac arteries	I	–	I	II	–	I
Renal artery	–	–	–	–	II	–
Pancreatic artery	–	–	–	II[a]	–	–
Rostral cerebral artery	II	–	–	I	–	–
Internal carotid artery	–	–	–	–	I	–
Caudal communicating artery	I	I	–	I	VI[a]	–
Basilar artery	II[a]	–	–	–	–	–

LAD: Left anterior descending, LCX: left circumflex, RCA: right coronary artery. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate. [a]Stenosis (50%-95%), [b]calcification, [c]hemorrhage.

Table III. *Histopathological examination of the liver (experiment A).*

Group (diet):	I (HF/HC)		II (HF/HC/SC)		III (HF/LC/SC)	
Animal no.:	1	2	3	4	5	6
Liver						
Fatty change in hepatocytes, centrilobular	+/-	+	++	+++	+	+/-
Foamy cells, sinusoid	+/-	+/-	+	+	+	+/-

Change: +/–, very slight; +, slight; ++, moderate; +++, marked. HF: high fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate.

Lipid metabolism parameters in the serum were analyzed (Figure 3). T-Cho and LDL-Cho levels in all groups increased rapidly from week 0 (initiation of diet provision) and approached peak levels at week 2; thereafter, they remained constant until the end of the experimental period in groups II and III and decreased gradually after week 4 in group I. HDL-Cho levels increased gradually from week 0, approached peak levels at week 6, and then remained almost unchanged until the end of the experimental period in all groups. TG levels gradually increased at nearly all points in all groups. The hepatic function parameters showed no marked differences between the three groups and no abnormal changes when compared with the reference data on MMPigs (30, 31).

In fecal analysis (Table I), fecal cholesterol excretion in group III was lower than that in groups I and II. Fecal TG excretion in groups II and III was slightly lower than that in group I. Fecal TBA excretion in Groups II and III was higher than that in group I.

At necropsy, all animals grossly showed plaque formation in the aorta and coronary arteries, and a pale change in the liver, suggesting fatty degeneration. The respective mean absolute and liver weights to relative BW were 171.2 g and 2.3% in group I, 195.8 g and 3.1% in group II, and 171.6 g and 2.6% in group III.

The evaluation of atherosclerosis with Stary classification is shown in Table II. Atherosclerotic lesions in the systemic arteries were histopathologically-observed in all animals, as previously described (20). However, there was no difference in the degree of atherosclerotic lesions between the three groups. The severity of the lesions appeared to be greater in the coronary arteries and abdominal aorta than in the aortic arch and thoracic aorta. Stenosis (50%-95%) was observed in almost all atherosclerotic lesions with higher Stary

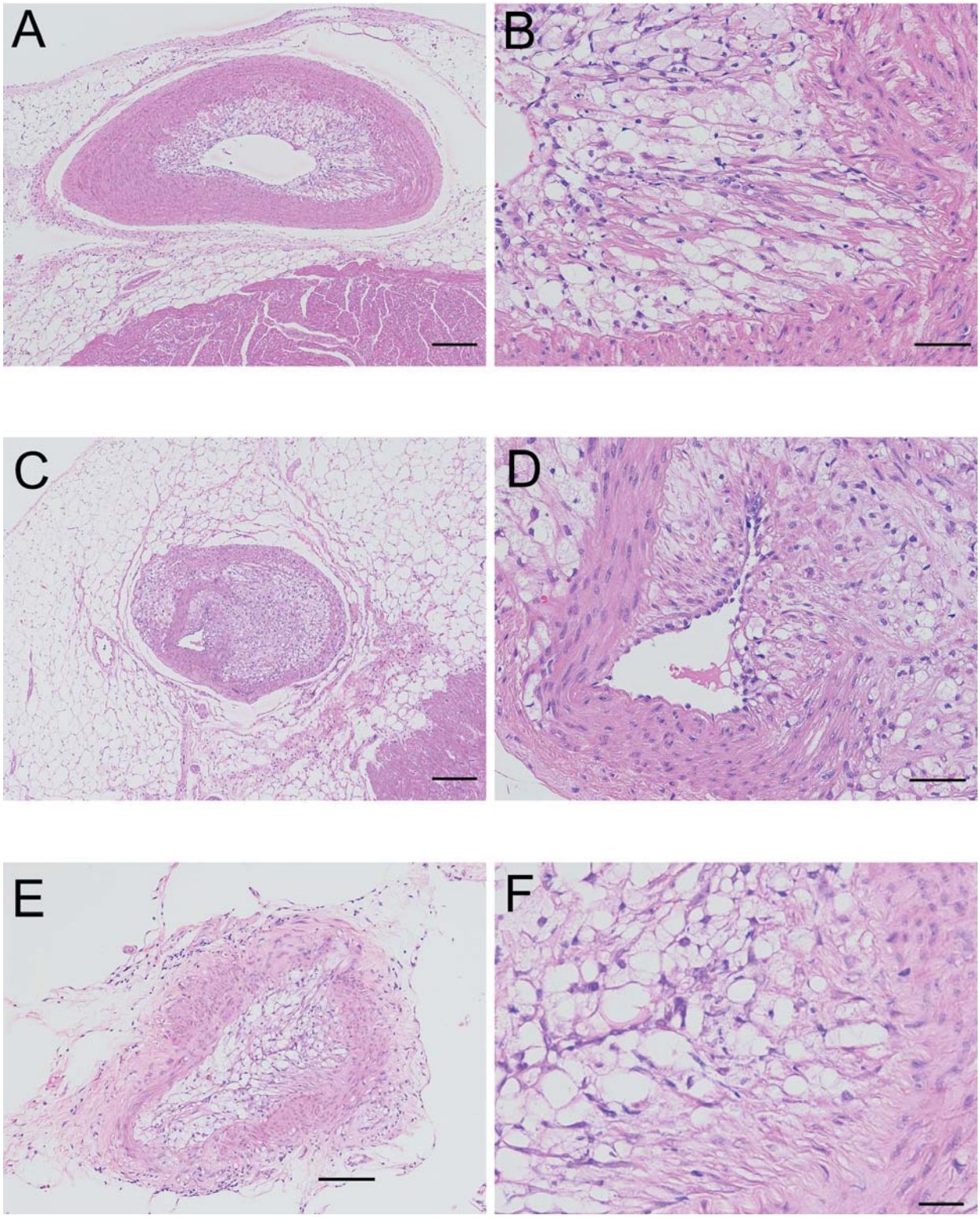

Figure 4. *Experiment A: Microscopic appearance of atherosclerotic lesions. Stenosis (approximately 70%) is shown in the coronary artery (A) and considerable foamy cell infiltration is shown in the thickening site (B). Severe stenosis (approximately 90%) is shown in the coronary artery (C) and considerable foamy cell infiltration is shown in the thickening site (D). Severe stenosis (approximately 95%) is shown in the caudal communicating artery (E) and considerable foamy cell infiltration is shown in the thickening site (F). A, B: Group II (HF/HC+SC); C-F: Group III (HF/LC+SC). Bar=200 μm (A, C), 50 μm (B, D), 100 μm (E), 20 μm (F). HE stain: A-F.*

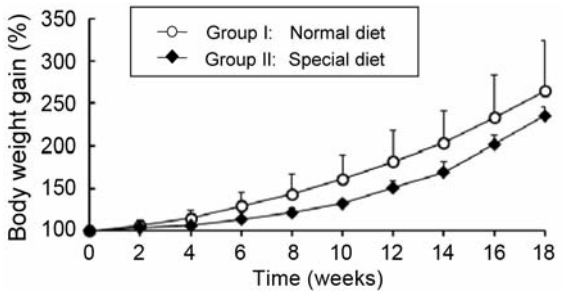

Figure 5. *Experiment B: Body weight gain.*

classification grade (Figure 4A-D). Considerable foamy cell infiltration and extracellular lipid accumulation were observed in the *intima* and *media*. Calcification and hemorrhage were also observed in a small number of atherosclerotic lesions. Fibrous cap formation at the surface of the *intima* containing collagen fiber proliferation and duplicated or disrupted elastic fibers were observed. In particular, the caudal communicating artery in one animal of group III showed atherosclerotic lesions, classified as grade VI, accompanying severe obstruction of the lumen (95% stenosis) (Figure 4E and F).

As shown in Table III, fatty change in the centrilobular hepatocytes and foamy cell accumulation in the sinusoid were observed in all animals, as previously described (20). The severity of these lesions appeared to be greater in group II than in groups I and III.

Experiment B. There were no significant differences in BW gain between groups I and II through the 18-week period (Figure 5).

Lipid metabolism parameters in the serum were analyzed (Figure 6). Serum T-Cho and LDL-Cho levels were significantly higher in group II than group I from week 6 to 18. HDL-Cho levels were significantly higher in group II than group I at weeks 6, 16, and 18. TG levels were significantly higher in group II than group I at weeks 16 and 18. The hepatic function parameters showed no significant differences between the two groups and no abnormal changes when compared with the reference data on MMPigs (30, 31).

In fecal analysis (Figure 7), fecal cholesterol excretions were significantly higher in group II than group I from week 6 to 14. Fecal TG excretions were also significantly higher in group II than group I at weeks 16 and 18. Fecal TBA excretions were significantly higher in group II than group I at weeks 6, 8, and 14-18.

In gross examination at necropsy, moderate plaque formation was observed in the coronary arteries and abdominal aorta in group-II animals. There were no significant differences in mean absolute or relative liver weights between groups I and II.

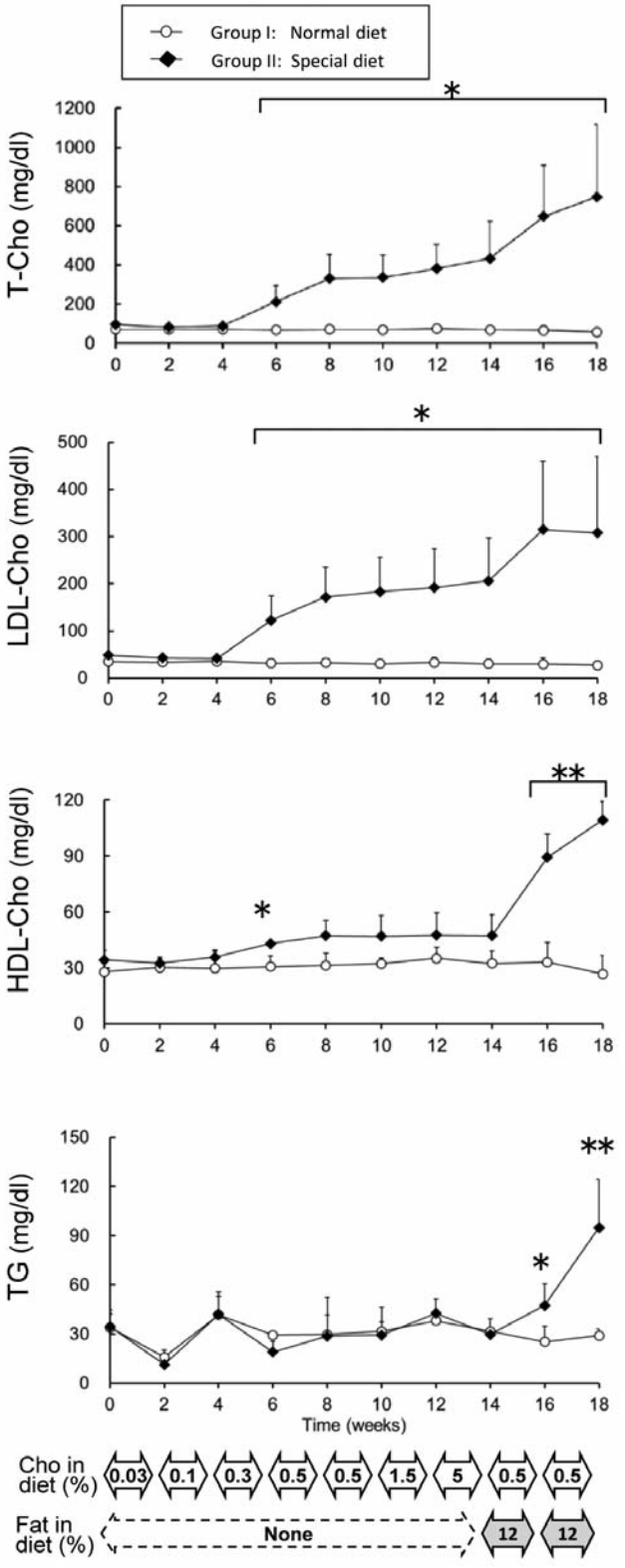

Figure 6. *Experiment B: Biochemical parameters of lipid metabolism. T-Cho: total cholesterol, LDL-Cho: low-density lipoprotein cholesterol, HDL-Cho: high-density lipoprotein cholesterol, TG: triglycerides. *p<0.05, **p<0.01; significantly different from group I.*

Table IV. *Atherosclerosis score according to Stary classification (experiment B).*

Group:	I (Normal diet)			II (Special diet)		
Animal no.:	1	2	3	4	5	6
LAD artery	–	–	–	–	II	–
LCX artery	–	–	–	II	II	II
RCA	–	–	–	II	II	II
Pulmonary artery	–	–	–	–	–	–
Aortic arch	–	–	–	II	II	II
Common carotid artery	–	–	–	–	–	–
Thoracic aorta	–	–	–	–	–	II
Abdominal aorta	–	–	–	II	II	II
External iliac arteries	–	–	–	II	II	II
Internal iliac arteries	–	–	–	–	II	–
Renal artery	–	–	–	II	–	–
Rostral cerebral artery	–	–	–	–	–	–
Internal carotid artery	–	–	–	–	–	–
Caudal communicating artery	–	–	–	–	–	–
Basilar artery	–	–	–	–	–	–
Ventral spinal artery	–	–	–	I	II	II

LAD: Left anterior descending, LCX: left circumflex, RCA: right coronary artery.

As shown in Table IV, the degree of atherosclerosis was evaluated by the Stary classification and no atherosclerotic lesions were observed in the systemic arteries in group I. The lesions in animals of experiment B (group II) were less severe than those in experiment A and no stenosis, calcification, or hemorrhage was observed in this experiment.

In the liver, fewer foamy cells infiltrating the sinusoid were observed as a finding with low severity in one animal in group II without any accompanying fatty change in the hepatocytes.

Discussion

Experiment A revealed that all diets induced similar degrees of hypercholesterolemia and atherosclerosis within a short period of just three months in MMPigs. Serum levels of T-Cho and LDL-Cho reached peaks of approximately 1,000 and 400 mg/dl, respectively, at week 2.

Since fecal cholesterol and TG excretions were slightly lower in animals fed the HF/HC/SC diet than those fed the HF/HC diet, it is suggested that SC slightly stimulated cholesterol and TG absorption in the MMPigs. However, endogenous bile acid including SC was sufficient for cholesterol and TG absorption because the HF/HC and HF/HC/SC diets induced similar degrees of hypercholesterolemia. This suggests that the supplemental dietary SC may not be necessary for the induction of hypercholesterolemia. Actually, an adverse effect of SC, an increase in the severity of the fatty change in the hepatocytes, was highest in the animals fed the HF/HC/SC diet.

Low cholesterol (0.5%) supplementation was considered sufficient for the induction of atherosclerosis in MMPigs because all diets induced similar hypercholesterolemia in the MMPigs, and all these animals showed a similar degree of atherosclerotic lesions. It is considered that the high cholesterol content (5%) may have been excessive because fecal cholesterol excretion in the animals fed HF/HC and HF/HC/SC diets was higher than that in the animals fed HF/LC/SC. This suggests the possibility that a high-fat and low-cholesterol diet without SC may be suitable for an MMPig model of atherosclerosis.

The diet-induced atherosclerotic lesions seen in MMPigs in this study (such as fibrous cap and calcification) were considered to be very similar to those seen in humans because of their location and histopathological characteristics, as previously described (20). Many animals, such as rabbits and swine, have been reported to develop similar atherosclerotic lesions in the coronary arteries, thoracic and abdominal aorta, and other arteries after being provided with a similar diet (3, 4, 7, 14, 20, 32, 33). However, atherosclerosis in the cerebral arterial circle and basilar artery, a finding known to be related to cerebral stroke, was also seen in MMPigs with each of the three diets in this study. This interesting result suggests that the MMPig is potentially suitable as an animal for a cerebral stroke model based on atherosclerosis.

In experiment B, hypercholestemia was induced by supplementation with cholesterol alone (0.3% to 5%) and severe hypercholestemia was induced by cholesterol (0.5%) and fat (12%) supplementation (Figure 8). Serum levels of T-

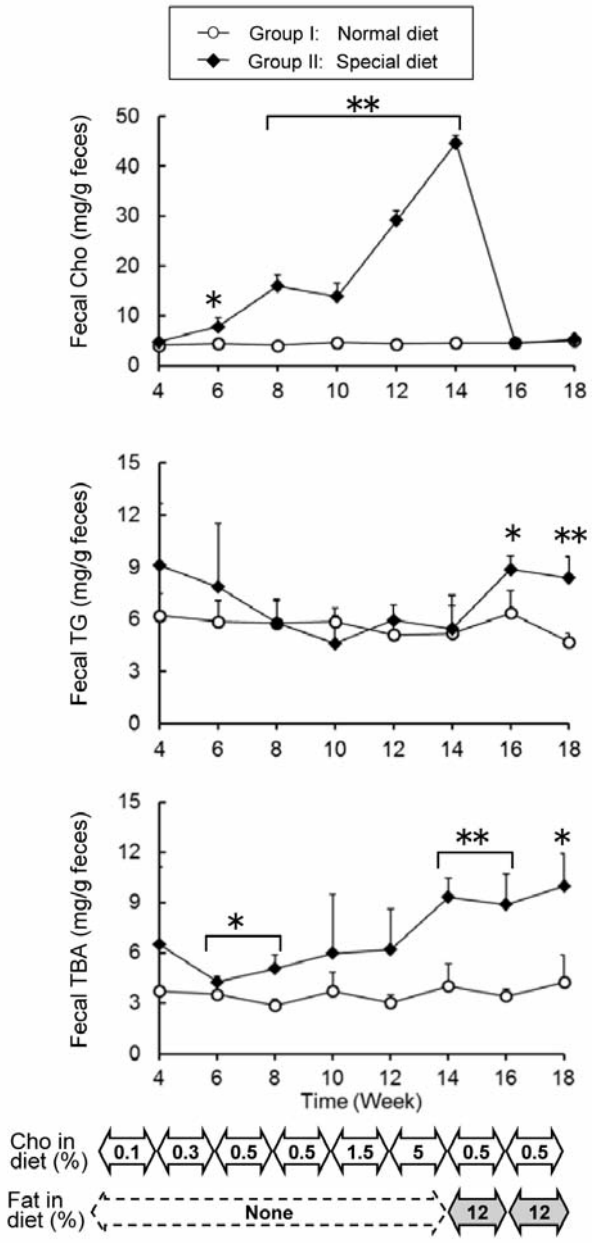

Figure 7. *Experiment B: Fecal analysis. Cho: Cholesterol, TG: triglycerides, TBA: total bile acid.*

Cho and LDL-Cho reached a plateau with 0.5% cholesterol supplementation. The fecal excretion of cholesterol was high in the animals fed 1.5% to 5% cholesterol diets, suggesting there may have been excessive amounts of cholesterol in the diet. Based on these results, it was considered that that the minimal dietary cholesterol content required to induce hypercholestemia in MMPigs is 0.5%. However, the severe hypercholestemia seen in experiment A was not induced when cholesterol alone was dosed to 5%, so the investigation of the dietary regimen was continued with both cholesterol and fat supplementation. Supplementation with cholesterol at 0.5% and fat at 12% proved capable of inducing severe hypercholestemia similar to that seen in experiment A. It is considered that the absorption of cholesterol in MMPigs may be enhanced when both cholesterol and fat are additives (at 0.5% and 12%, respectively), compared with that seen when cholesterol alone was supplemented (at 5%), since severe hypercholestemia was not induced under the latter dietary condition.

The atherosclerotic lesions in animals of experiment B were less severe than those in experiment A, and this was considered to be due to the shorter period of severe hypercholestemia in experiment B; it remains to be determined whether providing a diet with 0.5% cholesterol and 12% fat for 12 weeks can induce atherosclerosis similar to that seen in experiment A.

No fatty changes in the hepatocytes were observed as adverse findings in the liver with the diet of HF/HC alone in experiments A and B, suggesting that such a diet (without SC) may not induce hepatotoxicity.

In conclusion, dietary supplementation of SC was clearly shown nor to be required for the induction of atherosclerosis in the MMPig model, and a diet with cholesterol as the sole additive was judged unable to induce severe hypercholestemia. Moreover, it is suggested that a diet with 0.5% cholesterol and 12% fat may be suitable for the induction of atherosclerosis in the MMPigs. The results of this study show that an appropriate atherosclerosis model can be achieved without hepatotoxicity and demonstrate a cost benefit for research into human atherosclerosis research, for which the MMPig is suggested to be a useful experimental animal.

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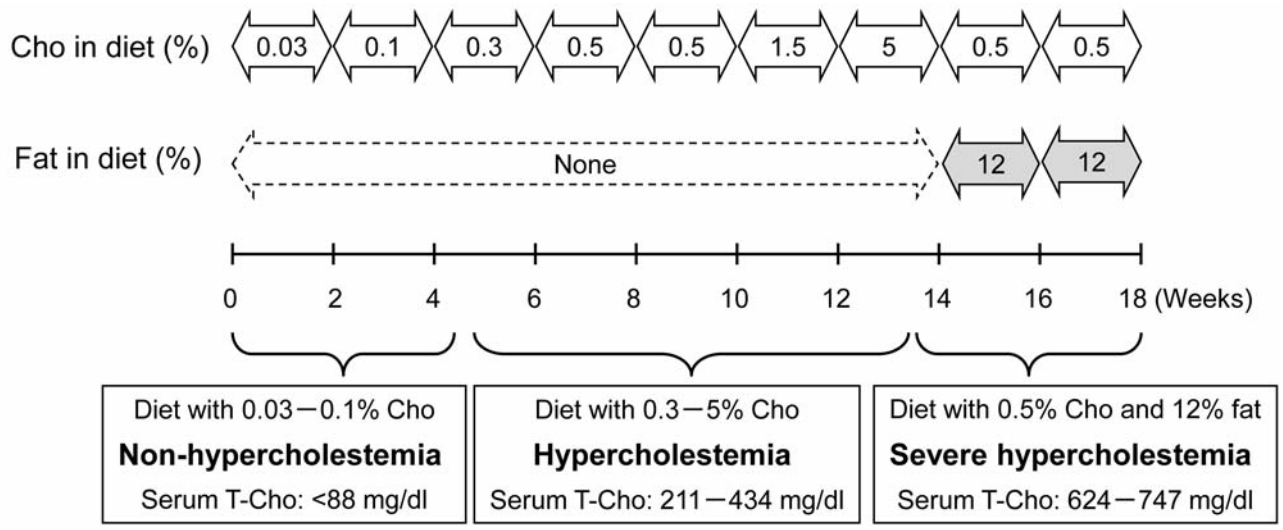

Figure 8. *Experiment B: Study design and result of hypercholestemia. T-Cho: Total cholesterol.*

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