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Supplemental Text

Materials and Methods

CETP Assay

Sample Preparation and Western blotting

For lipoprotein fractionation, equal volumes of plasma samples were pooled from six MMPs and six rabbits fed normal chow. The lipoproteins were fractionated using fast protein liquid chromatography (FPLC) with a Superose 6 10/300 GL FPLC column (Amersham Biosciences, Piscataway, NJ). Diluted plasma and aliquots of the FPLC fractions were subjected to SDS-PAGE and transferred unto PVDF membranes. Immunoblotting was performed as previously described. Antibodies against cholesteryl ester (CE) transfer protein (CETP) (mouse monoclonal, designated as "antibody A," and rabbit polyclonal, designated as "antibody B") were purchased from EMD Millipore (Billerica, MA) and Abcam (Cambridge Science Park, Cambridge, UK), respectively. The proteins were visualized using a chemiluminescence method (ECL Plus Western Blotting Detection System; GE Healthcare Piscataway, NJ).

Diagnosis of CETP Deficiency and Determination of the CETP Mass

A 59-year-old man and 54-year-old woman with high HDL-C levels (172 and 191 mg/dL, respectively) were referred to the National Defense Medical College Hospital. Blood samples were obtained, and the sera were isolated with centrifugation. Genomic DNA was prepared using a commercially available kit (Qiagen, Frederick, MA). The CETP mass was measured using a sandwich ELISA with JHC1 and JHC2, two monoclonal antibodies specific to human CETP, as previously described[1]. Common CETP mutations (an intron 14 splicing defect, Int14 + 1 G>A; amino acid substitution from aspartic acid to glycine at position 442, D442G) were identified using an Invader® assay[2].

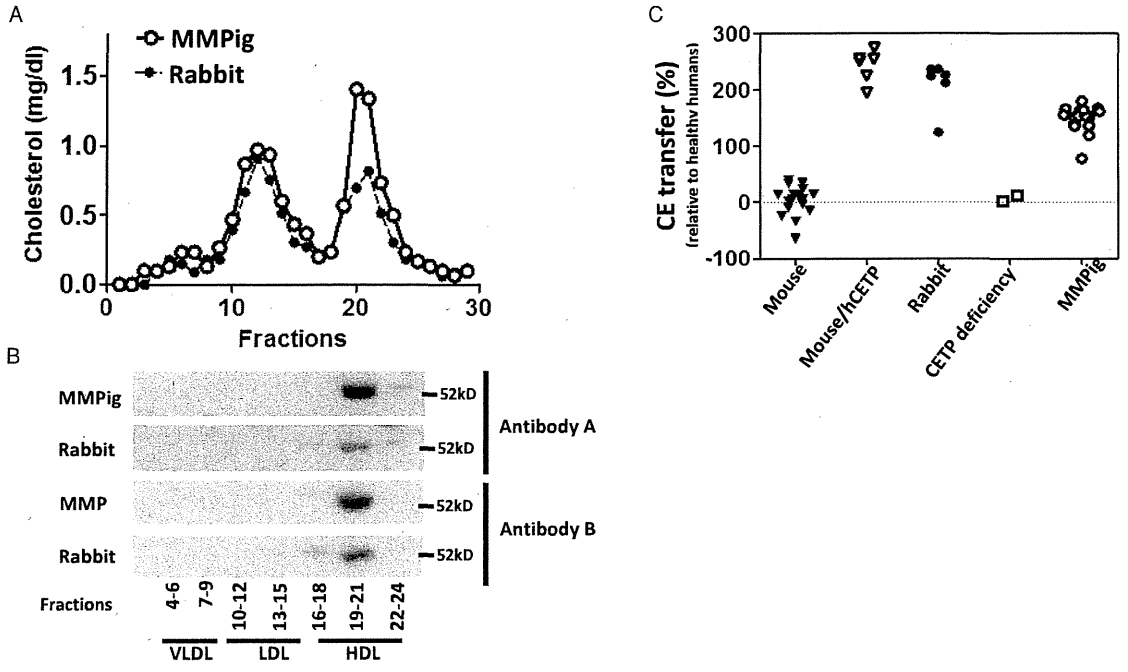
Cloning and Generation of Recombinant Adenoviruses Encoding for Human CETP

A recombinant adenovirus expressing human CETP (Ad-hCETP) was produced using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, an entry clone of the Gateway system (Invitrogen) was generated by cloning the open reading frame into a pENTR/D-TOPO vector (Invitrogen) using first-strand complementary DNA derived from human liver RNA (Clontech, Pao Alto, CA) as a template. The specific primers were as follows: forward: 5'- CAC CAT GCT GGC TGC CAC AGT -3'; reverse: 5'- CTA GCT CAA GCT CTG GAG GAA A -3'. An expression clone for the adenoviral vector was then generated via an LR recombination reaction between the entry clone and a pAd/CMV/V5-DEST (Invitrogen), according to the manufacturer's protocol.

Supplemental Table 1. Genes investigated and primers used for PCR

Gene	Assay ID	Custom made primers	RefSeq	GenBank
LDLr	Ss03374441_u1		NM_001206354.1	AF065990.1
SR-BI	Ss03391104_m1		NM_213967.1	AF467889.2
HMGCR	Ss03390147_m1		NM_001122988.1	DQ432054.1
SREBP-2	Ss03376492_u1			AY493571.1
APOBEC-1		Forward: CCATGGTGTGACCATCCAGAT Reverse: AGTTGACAAAATTCCTCCAGCAGTA Probe: TGGGAGCCCCAGAGTA	XM_003126519.1	
NPC1L1		Forward: CCTGTTCGGAGCGAGTCTCTA Reverse: GAAAGAGGAAATAGTCGAGCAGGTA Probe: CTGCCCAAGGACTC	XM_003134893.1	
GAPDH	Ss03375435_u1		NM_001206359.1	AY307771.1

The assay IDs are listed for the predesigned gene expression assay (ABI). The primers and probes for the APOBEC-1 and NPC1L1 analyses were created based on swine sequences.



Supplemental Fig. 1. MMPig sera activate CE transfer from HDL to LDL

(A) The cholesterol levels in the MMPig and rabbit lipoprotein fractions (pooled from six animals each) isolated using FPLC. (B) A Western blot analysis using two antibodies to detect the CETP expression in the MMPig and rabbit lipoprotein fractions, as described in the Methods. (C) The cholesteryl ester (CE) transfer activity in the sera obtained from C57BL/6 mice (n=18), C57BL/6 mice injected with Ad-hCETP (n=6), rabbits (n=6), subjects with CETP deficiency (n=2) and MMPigs (n=18).

The recombinant adenoviral plasmid was purified and then transfected into 293A cells. The adenovirus was purified using the Adeno-X Virus Purification Kit (Clontech), after a sufficient cytopathic effect was observed in the cells. The multiplicity of infection (MOI) was defined as the ratio of the total number of plaque-forming units to the total number of cells infected.

Injection of the Adenoviral Vector and Blood Sampling

C57BL/6 mice were obtained from Clea Japan (Tokyo, Japan) and handled according to the guidelines of the National Defense Medical College Institutional Animal Care and Use Committee. The mice were injected with Ad-hCETP (5×10^8 pfu) via the tail vein. Five days after injection, the mice were exsanguinated, and blood samples were obtained.

Determination of the CETP Activity

The serum CETP activity was determined according to the method of Cheung *et al*[3]. with a minor modification. In brief, the $d>1.125$ fraction isolated via ultracentrifugation from pooled healthy human plasma (n=6, fasting) was incubated with ^{14}C-cholesteryl oleate (Perkin-Elmer) at 37℃ for 16 hours, after which the $1.125<d<1.21$ fraction was isolated via ultracentrifugation. Following dialysis, the HDL fractions were incubated with human LDL isolated from pooled human plasma (n=6, fasting) in the presence or absence of serum obtained from MMPigs, rabbits, mice or humans. Ten hours after incubation, LDL was precipitated by adding heparin (500 U/mL) and manganese chloride (0.2 μmol/L) to the samples. The radiotracer counts in the supernatants were determined using a scintillation counter. The percentage cholesteryl ester transfer was calculated by dividing the differences between the values obtained in the absence and presence of sera by the values obtained in the absence of sera. The data are expressed as the CE transfer relative to the mean value of sera obtained from eight healthy humans.

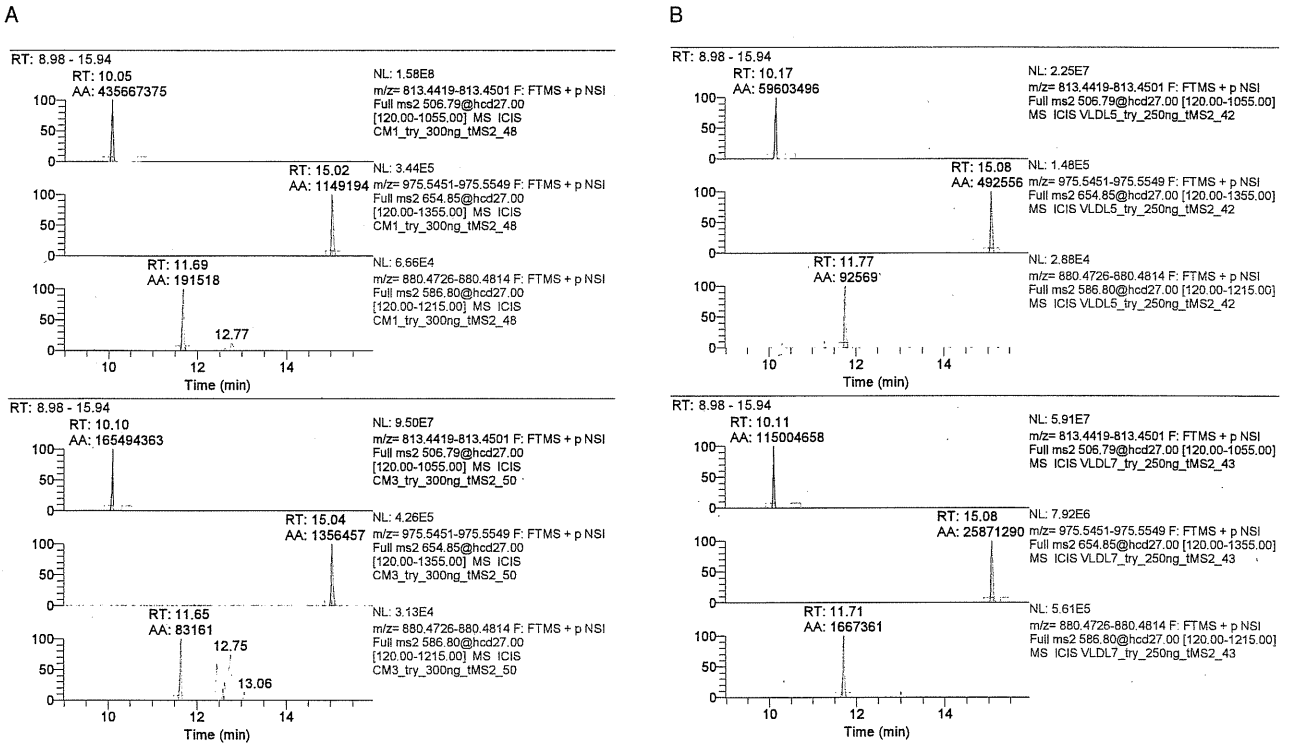
MS/MS Analysis of Apolipoprotein A1 and B

To detect apolipoprotein (apo)A1, B48 and B100 in the plasma, we utilized a HPLC/MS/MS-based method that can be applied across species. A multiple reaction monitoring (MRM) method was developed for apoA1 and apoB48/100[4]. First, the chylomicron

Supplemental Table 2. MRM transitions for the targeted peptides

	m/z (precursor, z=2)	m/z (product)
Apolipoprotein A1		
AKPALEDLR	506.7929	813.446
Apolipoprotein B48/100		
GFEPTLEALFGK	654.8453	975.55
YENYELTLK	586.7953	880.477

Thermo tube lens=120 for all precursor ions. Z=2 for all precursor ions



Supplemental Fig. 2.

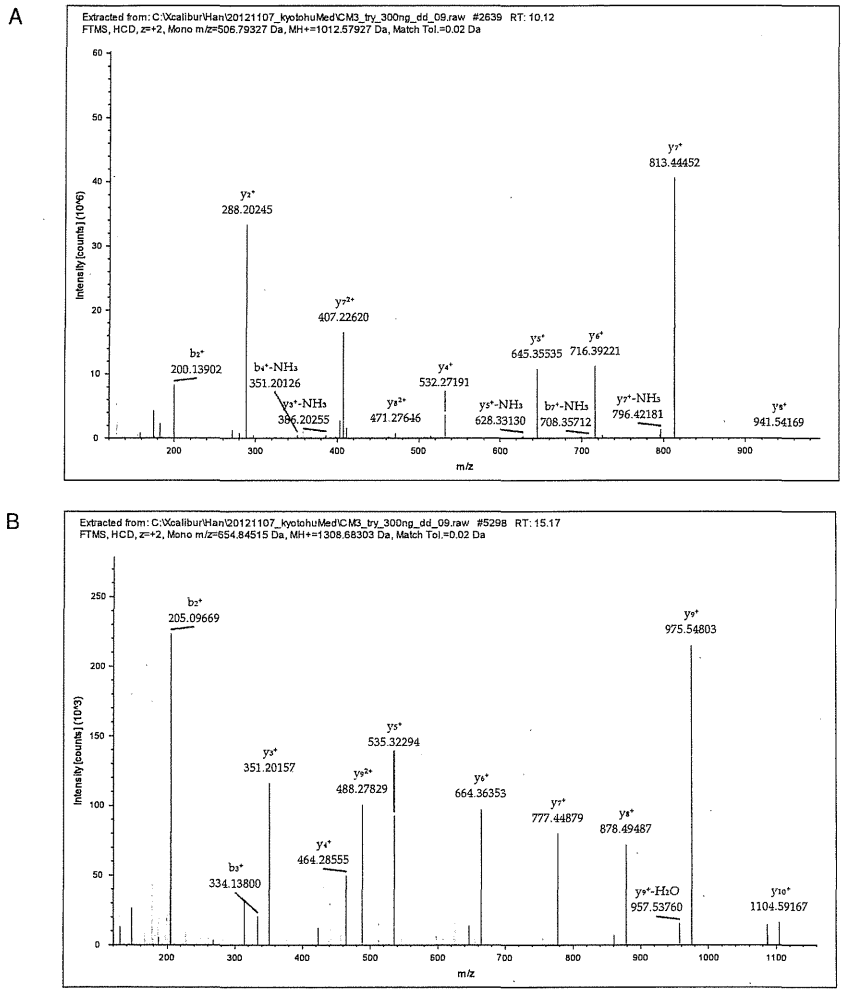
Extracted MRM chromatograms for ApoA1 (AKPALEDLR) and ApoB48/100 (GFEPTLEALFGK, YENYELTLK) from a nanoHPLC-MS/MS analysis of digested human (A) and MMPig (B) plasma fractions, chylomicron (CM) and very-low-density lipoprotein (VLDL).

(CM) and very-low-density lipoprotein (VLDL) fractions were separated from human and MMPig plasma using the Himac Centrifuge (CP70MX, Himac Centrifuge, Hitachi Medical Systems), according to the manufacturer's protocol. The samples were alkylated and digested overnight with trypsin and analyzed on a nano HPLC system (Easy-nLC, ThermoScientific) coupled with a triple quadrupole mass spectrometer (Orbitrap Q Exactive, ThermoScientific). The trap column was the EASY column, C18, 0.1×2 cm, 5 μm (ThermoScientific), and the analytical column was the Tip column, ODS, 0.075×120 mm (Nikkyo Technos). The flow rate was 0.3 mL/min.

Results

CETP Assay

Supplemental Fig. 1A shows the cholesterol distribution in the lipoprotein subfractions isolated using FPLC. The lipoprotein profiles of the MMPs were similar to those of rabbits, except for the higher HDL cholesterol levels. We performed a Western blot analysis using the FPLC subfractions to investigate whether the CETP expression was evident in the MMP. Two antibodies against CETP enabled the visualization of bands of similar molecular size in the HDL fractions obtained from both MMPigs and rabbits, as previ-



Supplemental Fig. 3.

Sequencing of the tandem mass spectrum of (C) a peptide with 506.8 m/z obtained from the tryptic digest of MMPig CM proteins and (D) a peptide with 654.8 m/z obtained from the tryptic digest of MMPig CM proteins.

ously demonstrated in humans[5] and hamsters[6] (**Supplemental Fig. 1B**). We next assessed whether sera obtained from MMPigs activated CE transfer from HDL to LDL. We confirmed that the sera obtained from C57BL/6 wild-type mice do not possess a CE transfer activity and that the intravenous injection of Ad-hCETP results in a 2.4-fold increase in the activity compared with that observed in human sera (**Supplemental Fig. 1C**). To further validate the reliability of our CETP activity assay, we measured the CE transfer activity in the sera obtained from the subjects with CETP deficiency. The CETP mass in the sera of the 59-year-old man (HDL-C, 172 mg/dL) and 51-year-old woman (191 mg/dL) was <0.1 and 0.4 μg/mL, respectively (normal range, 1.04-3.55 μg/mL). A genetic analysis revealed that the man carried the homozygous intronic mutation Int14 +1 G>A, while the woman carried the Int14 +1 G>A/D442G mutation as a compound heterozygote. We also confirmed the absence of the CE transfer activity in the sera of both subjects. Finally, we observed a robust CE transfer activity in the MMPig sera, 1.5-fold higher than that observed in the humans and 0.7-fold lower than that observed in the rabbits, supporting the possible expression of CETP.

Identification of ApoA1- and ApoB48/100-Specific Peptides and MRM Transitions

Supplemental Table 2 shows the identified peptide sequence along with the corresponding experimentally determined MRM transitions. **Supplemental Fig. 2A** and **2B** show MRM chromatograms extracted from both the human and pig plasma fractions, CM and VLDL. The amino acid sequences for apoA1 and B48/100 in the humans and pigs were obtained with Proteome Discoverer software program using a Swiss-Prot-TrEMBL database. The sequencing of the tandem mass spectrum of peptides with 506.8 and 654.8 m/z from the tryptic digest of porcine CM proteins was AKPALEDLR and GFEPTLEALFGK, corresponding to peptides of porcine apoA1 (**Supplemental Fig. 3A**) and apoB48/100 (**Supplemental Fig. 3B**), respectively.

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

KOHEI AKIOKA[1], HIROAKI KAWAGUCHI[1], SAWAKO KITAJIMA[1], NAOKI MIURA[2], MICHIKO NOGUCHI[3], MASAHISA HORIUCHI[4], NORIAKI MIYOSHI[1] and AKIHIDE TANIMOTO[5]

[1]Laboratory of Veterinary Histopathology, [2]Veterinary Teaching Hospital, [3]Laboratory of Domestic Animal Internal Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan; Departments of [4]Environmental Medicine and [5]Molecular and Cellular Pathology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

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.*

Correspondence to: Hiroaki Kawaguchi, DVM, Ph.D, Laboratory of Veterinary Histopathology, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24, Korimoto, Kagoshima 890-0065, Japan. Tel: +81 992853538, Fax: +81 992853538, e-mail: k3038952@kadai.jp and Akihide Tanimoto, MD, Ph.D, Department of Molecular and Cellular Pathology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. Tel: +81 992755263, Fax: + 81 992646348, e-mail: akit09@m3.kufm.kagoshima-u.ac.jp

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

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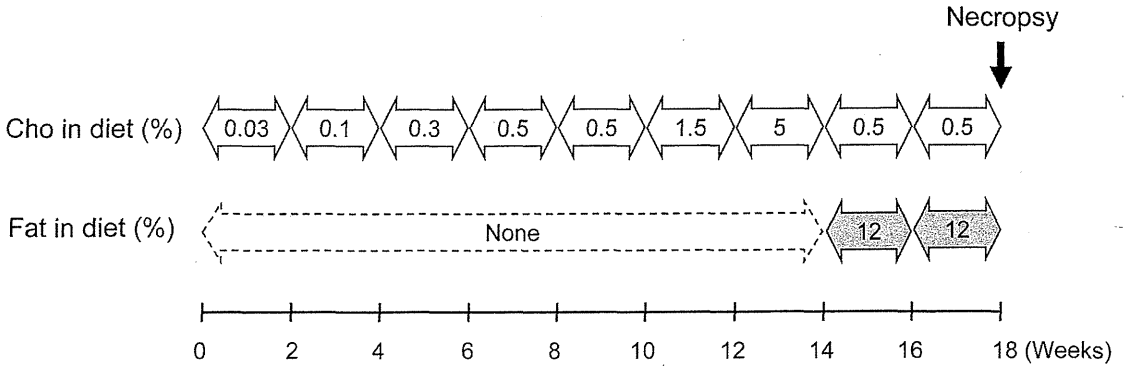

Figure 1. *Experiment B: Study design. Cho: cholesterol.*

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

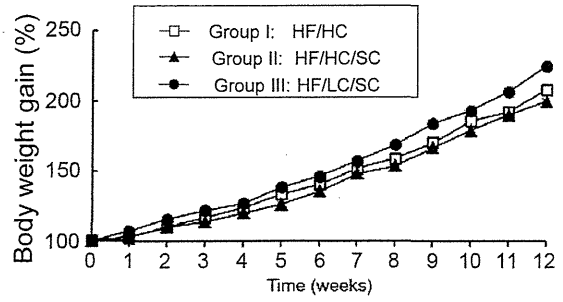

Figure 2. *Experiment A: Body weight gain. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate. Data are percentages, n=2.*

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).

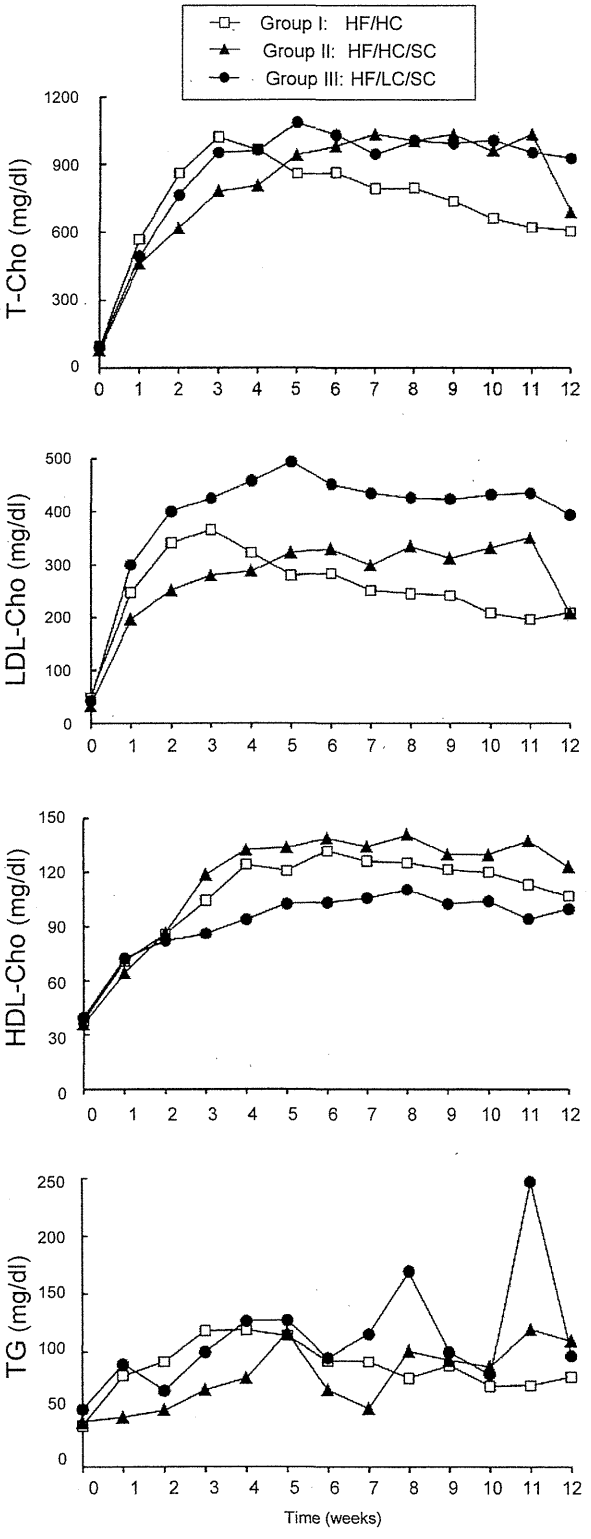

Figure 3. *Experiment A: Biochemical parameters of lipid metabolism. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate. T-cho: total cholesterol, LDL-Cho: low-density lipoprotein cholesterol, HDL-Cho: high-density lipoprotein cholesterol, TG: triglycerides.*

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

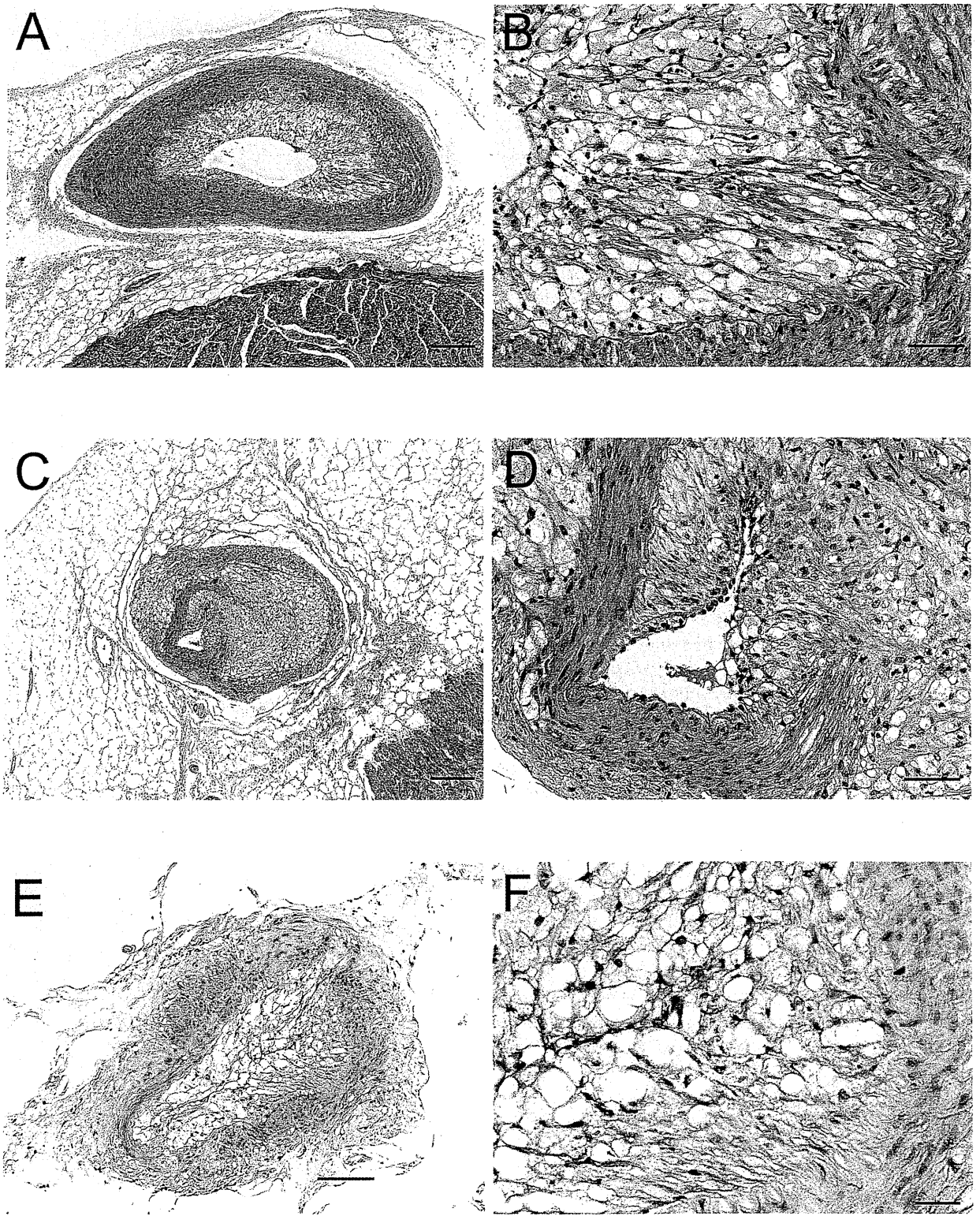

Figure 4. *Experiment A: Microscopic appearance of atherosclerotic lesions. Stenosis (approximately 70%) is shown in the coronary artery (A) and considerable foamy cell infiltration is shown in the thickening site (B). Severe stenosis (approximately 90%) is shown in the coronary artery (C) and considerable 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.*

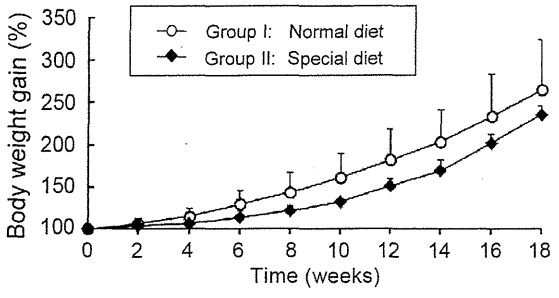

Figure 5. *Experiment B: Body weight gain.*

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.

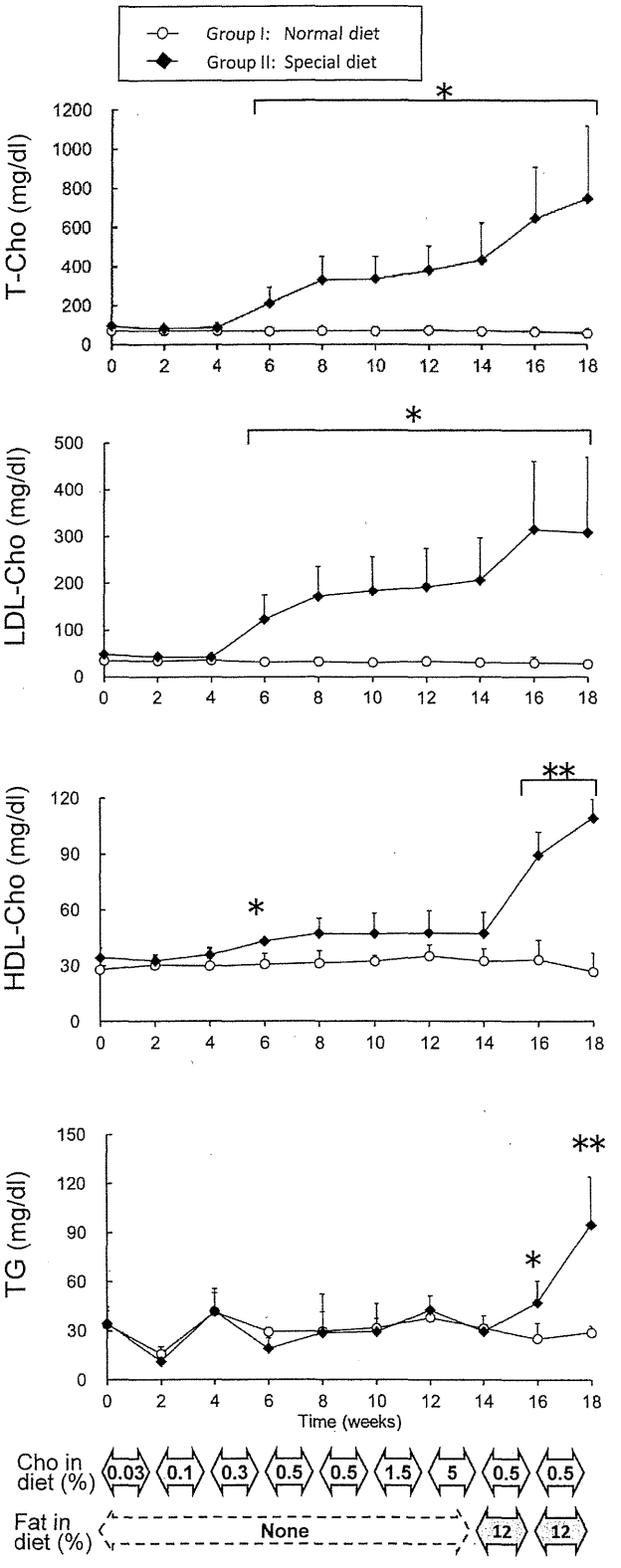

Figure 6. *Experiment B: Biochemical parameters of lipid metabolism. T-Cho: total cholesterol, LDL-Cho: low-density lipoprotein cholesterol, HDL-Cho: high-density lipoprotein cholesterol, TG: triglycerides. *p<0.05, **p<0.01; significantly different from group I.*

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-

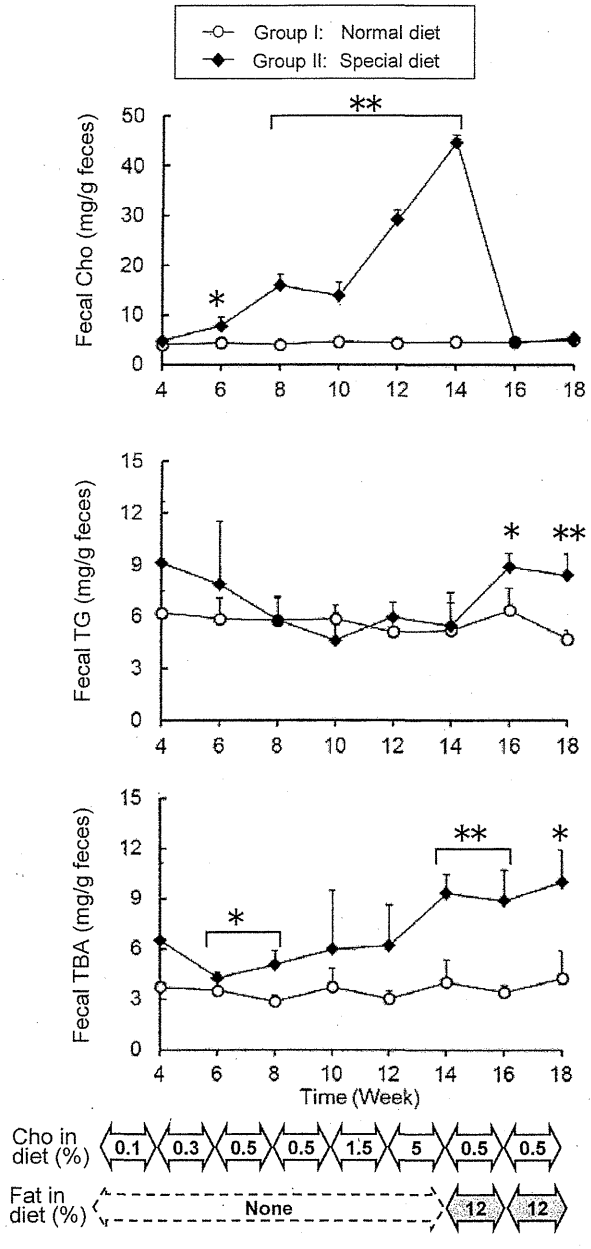

Figure 7. *Experiment B: Fecal analysis. Cho: Cholesterol, TG: triglycerides, TBA: total bile acid.*

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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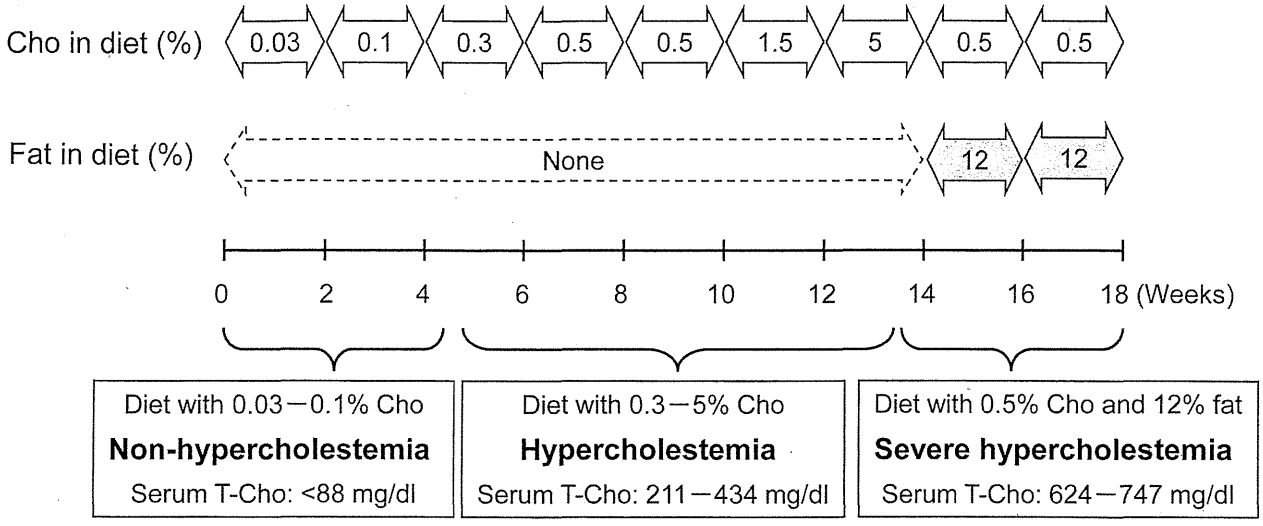

Figure 8. *Experiment B: Study design and result of hypercholestemia. T-Cho: Total cholesterol.*

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Sex Differences of Serum Lipid Profile in Novel Microminipigs

HIROAKI KAWAGUCHI[1*], TOMONOBU YAMADA[2,3*], NAOKI MIURA[4*], MICHIKO NOGUCHI[5], HIROYUKI IZUMI[3], NORIAKI MIYOSHI[1] and AKIHIDE TANIMOTO[2]

[1]Laboratory of Veterinary Histopathology, [4]Veterinary Teaching Hospital, [5]Laboratory of Domestic Animal Internal Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan; [2]Department of Molecular and Cellular Pathology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan; [3]Drug Safety Research Laboratories, Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan

Abstract. *Swine have been used extensively in biomedical research, with a significant increase in recent decades. Minipigs are increasingly becoming an especially attractive animal model in life science research because of their physiological and anatomical similarities to humans. The Microminipig (MMPig) has emerged as a novel and small minipig for non-clinical pharmacological/toxicological use. The MMPig is docile, weighs less than 10 kg in early maturity, and has an easily manageable size. In this study, we report on sex and age patterns in serum biochemistry parameters, including lipid analysis items and lipid profiles in healthy MMPigs. In total, 58 males and 67 females aged 0-34 months underwent serum biochemistry parameter measurements. Most parameters showed no effect of age or sex (although some did). Lipid analyses showed that the serum levels of total cholesterol, but not those of triglycerides (TG), were consistently higher in females at 0-34 months of age. Lipid profiles in 5-month-old MMPigs were investigated in greater detail. Serum low-density lipoprotein-cholesterol (LDL-C) values were higher in females. The percentage of LDL-C against total cholesterol was also higher, although high-density lipoprotein-cholesterol was lower, in females. There were no sex differences in the TG fraction. Although the sex difference in the serum lipid profile remains unexplained, the reference values obtained in this study could help facilitate the use of MMPigs in life science research.*

Swine have been used extensively in biomedical research, with a significant increase in recent decades. More than 60,000 pigs are used for research in a year in the EU (1, 2); however, they are not yet widely used in Japan. Minipigs are increasingly becoming an especially attractive animal model in life science research because of their physiological and anatomical similarities to humans (3, 4). In particular, the number of minipigs used in cardiovascular and skin research is increasing (5, 6). Minipigs can be classified by adult body weight (BW) into a light category weighing 35-70 kg, which includes the Göttingen, Yucatan, and Sinclair strains, and a heavier category weighing 70-90 kg, which includes the Hanford strain (1). The Microminipig (MMPig; Fuji Micra Inc., Shizuoka, Japan) has emerged as a novel and small minipig for non-clinical pharmacological/toxicological use (3, 7). The MMPig is docile, with a BW in early maturity of less than 10 kg, and of a good manageable size for an experimental animal (3, 8, 9). The founder of the MMPig strain was a female (named "Catherin") bred from mating a pot-bellied pig with a minipig of another type (3). The use of MMPigs in pharmacological/toxicological experiments includes: an established atherosclerosis model induced by diet control (high fat and high cholesterol diet) (4, 10), and evaluation in a dermal phototoxicity study (6). Recently, we reported that general hematological and biochemical parameters in MMPigs were similar to those in Göttingen and Yucatan minipigs (8, 11-14). To expand on our previous study, we investigated differences by age and sex in biochemistry parameters and lipid profiles of healthy MMPigs to obtain reference data, which will be essential for future life science research.

*These Authors contributed equally to this study and should be regarded as co-first authors.

Correspondence to: Hiroaki Kawaguchi, DVM, Ph.D. Laboratory of Veterinary Histopathology, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24, Korimoto, Kagoshima 890-0065, Japan. Tel: +81 992858720, Fax: +81 992858722, e-mail: k3038952@kadai.jp and Akihide Tanimoto, MD, Ph.D. Department of Molecular and Cellular Pathology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan. Tel: +81 992755263, Fax: + 81 992646348, e-mail: akit09@m3.kufm.kagoshima-u.ac.jp

Key Words: Lipid profile, porcine serum biochemistry, sex difference, swine.

Table I. *Age-specific values in serum biochemistry in Microminipig.*

Parameter	Unit	Gender	Age (months)						
			0 (M=4, F=3)	1-3 (M=5, F=12)	4-6 (M=7, F=7)	7-9 (M=15, F=22)	10-12 (M=7, F=5)	13-24 (M=14, F=10)	25-34 (M=6, F=8)
Aspartate aminotransferase	IU/l	M+F	43.3±18.9	43.4±11.4	37.9±25.2	42.1±33.3	37.0±7.7	38.5±13.7	40.5±21.9
		M	40.5±14.8	43.6±10.1	43.0±36.1	33.7±7.3	39.7±6.5	42.8±13.3	40.2±13.6
		F	47.0±26.5	43.3±12.3	32.7±4.4	47.9±42.1	33.2±8.3	32.6±12.4	40.8±27.5
Alanine aminotransferase	IU/l	M+F	29.4±5.0	40.9±14.4	54.6±17.8	47.8±18.5	42.8±11.7	45.2±12.0	46.1±12.5
		M	28.8±5.4	38.0±12.7	45.0±16.1	39.1±10.9	44.6±13.3	47.9±9.8	46.2±14.0
		F	30.3±5.5	42.2±15.4	64.1±14.6*	53.7±20.4**	40.2±9.8	41.5±14.3	46.1±12.3
Alkaline phosphatase	IU/l	M+F	2399.3±1138.7	863.9±228.3	584.6±239.8	468.0±191.1	600.7±223.6	455.3±232.1	322.4±137.6
		M	2797.8±1440.6	897.4±249.7	662.4±186.4	546.9±230.7	710.6±228.8	488.9±276.9	285.2±114.2
		F	1868.0±189.7	849.9±228.9	506.7±275.2	414.2±140.1	446.8±90.4*	408.3±150.5	350.3±154.2
Creatinine kinase	IU/l	M+F	1185.7±1027.8	404.6±237.3	677.5±1280.6	863.4±1431.3	827.6±1081.0	557.2±474.0	937.3±1250.2
		M	953.0±412.3	329.2±82.7	990.1±1814.5	656.8±806.6	1033.7±1409.2	589.6±478.2	716.2±697.8
		F	1496.0±1631.5	436.0±275.3	364.9±180.9	1004.2±1739.8	539.0±236.8	511.9±489.9	1103.1±1575.2
Total bilirubin	mg/dl	M+F	0.097±0.136	0.008±0.011	0.021±0.023	0.027±0.023	0.039±0.028	0.034±0.022	0.039±0.027
		M	0.095±0.150	0.012±0.008	0.017±0.017	0.025±0.015	0.047±0.029	0.026±0.019	0.023±0.012
		F	0.100±0.148	0.006±0.012	0.024±0.028	0.029±0.027	0.028±0.026	0.045±0.022*	0.051±0.029*
Urea nitrogen	mg/dl	M+F	10.3±2.5	12.2±2.2	12.6±2.4	13.6±2.9	14.8±4.1	15.3±3.6	15.4±3.6
		M	8.8±2.0	13.0±2.1	13.3±2.5	14.5±3.1	16.6±4.3	16.4±3.8	14.6±2.0
		F	12.4±1.3*	11.9±2.3	11.8±2.3	13.0±2.6	12.1±1.9	13.9±2.8	16.0±4.6
Creatinine	mg/dl	M+F	0.62±0.04	0.88±0.21	0.82±0.25	0.80±0.19	0.89±0.18	1.17±0.40	1.08±0.28
		M	0.61±0.04	0.84±0.10	0.74±0.25	0.87±0.19	0.99±0.15	1.19±0.45	1.27±0.29
		F	0.64±0.04	0.90±0.24	0.89±0.25	0.76±0.19	0.75±0.11*	1.14±0.33	0.93±0.17*
Total cholesterol‡	mg/dl	M+F	202.4±155.8	82.2±8.9	85.9±18.9	74.1±14.6	83.3±12.3	80.3±14.6	77.6±22.9
		M	112.5±8.4	73.8±6.6	74.3±6.4	66.0±12.7	82.0±10.7	74.9±14.0	60.2±14.2
		F	322.3±187.0	85.7±7.3**	97.4±20.5*	79.8±13.3**	85.2±15.5	87.7±12.5*	90.8±19.3**
Triglycerides	mg/dl	M+F	102.6±63.9	42.6±12.9	41.9±13.4	40.9±19.5	41.3±28.0	44.1±14.8	35.4±13.8
		M	116.0±85.2	41.4±8.7	33.7±6.2	33.5±9.4	35.1±14.9	41.6±13.5	30.2±9.8
		F	84.7±22.9	43.1±14.7	50.0±14.1*	46.0±23.0*	49.8±40.8	47.6±16.4	39.4±15.6
Total protein	g/dl	M+F	6.0±0.7	6.3±0.6	7.8±0.6	8.0±0.6	8.0±0.5	8.1±0.8	8.2±0.8
		M	6.0±0.5	6.0±0.3	7.7±0.5	7.9±0.6	8.1±0.5	8.2±0.9	8.0±0.7
		F	6.0±1.0	6.5±0.7	7.9±0.7	8.0±0.7	8.0±0.5	8.0±0.4	8.4±1.0
Albumin	g/dl	M+F	3.9±0.4	4.0±0.6	4.4±0.3	4.3±0.4	4.9±0.3	4.7±0.4	4.5±0.4
		M	3.9±0.4	4.4±0.3	4.5±0.3	4.3±0.5	5.0±0.3	4.6±0.5	4.6±0.4
		F	3.9±0.5	3.9±0.6	4.4±0.4	4.3±0.5	4.7±0.3	4.7±0.3	4.3±0.4
Globulin	g/dl	M+F	2.1±0.3	2.3±0.9	3.4±0.8	3.7±0.8	3.2±0.5	3.4±0.7	3.8±1.0
		M	2.1±0.1	1.7±0.2	3.3±0.6	3.7±0.9	3.1±0.7	3.6±0.8	3.4±0.5
		F	2.1±0.5	2.6±0.9**	3.6±0.9	3.7±0.8	3.3±0.3	3.2±0.5	4.1±1.3
Albumin-globulin ratio	ratio	M+F	1.9±0.2	2.0±0.8	1.4±0.3	1.2±0.4	1.6±0.4	1.4±0.3	1.2±0.3
		M	1.9±0.2	2.7±0.5	1.4±0.3	1.3±0.4	1.7±0.5	1.3±0.3	1.4±0.2
		F	1.9±0.2	1.7±0.7*	1.3±0.3	1.2±0.4	1.5±0.2	1.5±0.3	1.2±0.4
Glucose	mg/dl	M+F	136.9±38.9	98.1±16.4	88.1±12.5	82.5±10.2	84.2±7.2	88.9±20.2	89.6±20.8
		M	148.8±50.7	110.4±18.4	86.7±8.4	84.5±11.2	82.0±5.9	86.5±25.0	78.8±6.1
		F	121.0±5.3	93.0±13.2*	89.6±16.2	81.2±9.5	87.2±8.3	92.2±10.5	97.6±24.6
Phosphorus	mg/dl	M+F	11.3±1.0	8.1±1.0	6.8±0.7	6.5±0.7	6.1±0.6	6.3±0.6	5.5±0.6
		M	11.2±1.4	8.4±0.6	6.7±0.7	6.4±0.5	6.0±0.6	6.1±0.7	5.2±0.3
		F	11.5±0.5	8.0±1.1	6.9±0.7	6.5±0.8	6.3±0.5	6.4±0.5	5.8±0.7
Calcium	mg/dl	M+F	11.8±0.4	10.8±0.5	10.7±0.4	10.5±0.4	11.0±0.4	10.6±0.5	10.3±0.3
		M	11.6±0.3	11.1±0.4	10.8±0.3	10.4±0.4	11.1±0.4	10.6±0.7	10.3±0.4
		F	12.0±0.4	10.6±0.5	10.6±0.4	10.5±0.5	10.9±0.4	10.7±0.3	10.2±0.2
Sodium	mEq/l	M+F	144.6±3.4	143.5±2.3	144.6±2.3	144.3±5.2	146.2±2.4	145.6±2.7	145.1±2.7
		M	144.3±3.0	144.4±1.7	145.7±2.4	144.7±3.3	146.9±2.9	145.9±3.2	146.7±3.4
		F	145.0±4.6	143.2±2.6	143.6±1.9	144.0±6.2	145.2±1.1	145.1±1.8	143.9±1.2
Potassium	mEq/l	M+F	6.1±1.1	5.8±1.1	5.6±0.7	5.7±0.6	5.9±0.5	5.8±0.7	5.4±0.6
		M	6.0±1.3	6.0±1.1	5.9±0.4	5.6±0.4	5.9±0.5	5.8±0.7	5.5±0.6
		F	6.3±1.1	5.8±1.1	5.4±0.8	5.8±0.7	5.9±0.7	5.7±0.7	5.3±0.6
Chloride	mEq/l	M+F	106.6±2.1	106.2±1.8	103.4±3.1	102.3±5.0	102.9±2.9	102.8±2.9	102.3±2.3
		M	107.5±1.7	106.0±2.2	103.9±3.3	102.2±3.2	103.3±3.3	102.4±3.1	103.7±2.2
		F	105.3±2.3	106.3±1.7	103.0±3.0	102.4±6.1	102.4±2.5	103.3±2.7	101.3±2.0

M, Males; F, females. ‡7-9 months of age (M=15, F=21). *$p<0.05$, **$p<0.01$: significantly different from males.

Table II. *Analysis of serum lipid metabolism markers, cholesterol and triglyceride fractions in Microminipigs aged five months.*

Parameter	Unit	Age 5 months of age	
		Males (n=5)	Females (n=5)
T-Cho	mg/dl	77.8±11.3	94.8±3.7*
Free-Cho	mg/dl	16.4±2.5	20.8±1.1**
CE	mg/dl	61.4±8.9	74.0±3.1*
Triglycerides	mg/dl	35.4±6.6	54.4±8.2**
HDL-C	mg/dl	40.8±10.1	39.4±7.5
LDL-C	mg/dl	31.2±5.7	49.2±6.4**
VLDL-C	mg/dl	4.0±1.2	4.0±1.0
CM-C	mg/dl	1.6±0.5	2.2±0.4
Cholesterol fraction			
HDL-C	%	52.2±6.8	41.6±7.3*
LDL-C	%	40.6±6.8	52.2±7.3*
VLDL-C	%	5.2±1.6	4.0±1.0
CM-C	%	2.0±0.0	2.2±0.4
Triglycerides fraction			
HDL Triglyceride	%	13.8±4.1	12.6±2.5
LDL Triglyceride	%	38.8±5.1	40.4±5.3
VLDL Triglyceride	%	30.8±5.0	29.2±5.0
CM Triglyceride	%	16.6±4.7	17.8±8.0

T-Cho: Total cholesterol, Free-Cho: free cholesterol, CE: cholesterol ester, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, VLDL-C: very low-density lipoprotein cholesterol, CM-C: chylomicron cholesterol. *$p<0.05$, **$p<0.01$: significantly different from males.

Materials and Methods

All animals were maintained in the same animal housing unit at 24±3°C and relative humidity at 50±20%, with a 12 h light/dark cycle in the breeder's facility. The dedicated space for each animal was 0.5-1.2 m^2. Restricted feeding of a porcine diet (Marubeni Nisshin Feed Co., Tokyo, Japan) was set as previously reported (8). Tap water was available *ad libitum*. The animals used in this study were found to be in good health and free of clinical signs of illness. They were not given any treatment or medication other than vaccination through the study. All animals were vaccinated against mycoplasmal pneumonia of swine (MPS), porcine pleuropneumonia (APP), and swine erysipelas (SE) at 0, 1-2, and 3 months old, respectively. All protocols were approved by the Ethics Committee of Animal Care and Experimentation, Kagoshima University (A09001) 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).

Blood samples were collected from the cranial vena cava of 125 conscious animals (58 male and 67 females) aged 0-34 months under fasted conditions. Zero month of age was not newborn and over three weeks of age. It was possible for handlers to hold all MMPigs without causing them stress and/or pain while other technicians collected blood from them. For measurement of 19 serum biochemical parameters (Table I), serum was obtained by centrifugation (room temperature, 1710 × *g*, 15 min) and examined with an automatic analyzer (JCA-BM8; JEOL Co., Ltd., Tokyo, Japan). Lipid profiles [high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), very low-density lipoprotein-cholesterol (VLDL-C) and chylomicron] were investigated in 5-month-old MMPigs (five males and five females) by analyzing serum samples with an electrophoresis processing analyzer (Epalyzer 2; Helena Laboratories Japan Co., Ltd., Saitama, Japan). The cholesterol ester (CE) value was calculated as: CE=total cholesterol (T-Cho) – free cholesterol (Free-Cho).

All data are presented as the mean±SD and the statistical significance of any difference was assessed by F-test and Student's *t*-test or Welch's *t*-test, and $p<0.05$ was considered significant.

Results

As shown in Table I, most biochemistry parameters were not affected by sex. A small number of parameters revealed sex differences, although these differences were not consistent at 0-34 months of age. However, lipid analyses showed that the serum levels of T-Cho, but not those of triglycerides (TG), were consistently higher in females. Moreover, the alkaline phosphatase, total bilirubin, T-Cho, TG, and glucose levels in both male and female MMPigs at 0 months of age were higher than those at 1–34 months of age while the alanine aminotransferase level was lower.

As shown in Table II, lipid profile analyses showed that the serum levels of T-Cho, Free-Cho, CE, TG, and LDL-C were higher in females. The percentage of LDL-C against T-Cho was also higher, although that of HDL-C was lower, in females. There were no sex differences in the TG fraction.

Discussion

Most biochemistry parameters were not affected by age or sex. The levels of some parameters, such as aspartate aminotransferase, alkaline phosphatase etc. fluctuated by age as those obtained in Göttingen minipigs (11, 12, 15). In lipid analyses, the levels of serum T-Cho in female MMPigs were consistently high at 0-34 months of age, while serum TG levels were not. This sex difference was similar to that obtained in Göttingen minipigs (11, 12). In addition, the serum levels of T-Cho, total protein, albumin and glucose in MMPigs were also higher than those in Göttingen minipigs. The serum levels of T-Cho, total protein, albumin and glucose in male and female Göttingen minipigs aged six months were 51.4±7.7 mg/dl and 75.7±16.6 mg/dl, 6.1±0.3 g/dl and 6.2±0.4 g/dl, 3.4±0.2 g/dl and 3.3±0.2 g/dl, 57.7±7.2 mg/dl and 57.7±7.2 mg/dl, respectively (11). These phenomena may have been related to nutrition; the MMPigs were provided with feed corresponding to 1-8% of BW compared with usual figure of 2-3% for minipigs (15). The higher T-Cho levels in both male and female MMPigs at 0 months of age than at 1-34 months of age were probably due to the diet in the lactation and weaning periods, when porcine milk, which has a high fat content (about 5-6%), was included in the diet provided (16).

We investigated lipid profiles in greater detail in MMPigs at five months, which is considered the most likely age of use in life science research. The serum levels of T-Cho, Free-Cho, CE, TG, and LDL-C were higher in females. These high levels of lipid metabolism markers in female MMPigs may be related to greater lipolytic sensitivity in females as in humans (17). Women have higher HDL-C levels than men due to female hormones, while a tendency for lower HDL-C levels in female MMPigs was revealed. The tendency for lower HDL-C level in female MMPigs may be due to their young age because in children a similar tendency was revealed (18). This phenomenon may be related to the fact that the percentage of LDL-C against total cholesterol was also higher in female MMPigs, while that of HDL-C was lower. We believe these sex differences in the lipid profile of MMPigs are new findings, since we have not encountered any previous reports of them.

Although breeders have been making efforts to expand their supply, minipigs including MMPigs are not yet widely used in life science research mostly because of a lack of accumulated reference data, which are essential for any field of life science research (19). The reference values for serum lipid analysis items and lipid profiles, including those showing sex differences, obtained in this study could facilitate the use of MMPigs in life science research.

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