NOTE *Laboratory Animal Science*

# Reference Values of Hematological and Biochemical Parameters for the World Smallest Microminipigs

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ABSTRACT. In this study, we demonstrated growth curves and reference values for hematological and serum biochemical parameters of Microminipigs, the world smallest experimental minipigs. In both male and female animals, the body weights (BWs) at 3 and 6 months of age were <5 kg and <10 kg, respectively, and growth curve revealed almost plateau (approximately 20 kg BW) after 18 months of age. Major hematological and serum biochemical parameters showed no gender differences and the values were very similar to those in Göttingen and Yukatan minipigs. The values obtained in this study can serve as fundamental reference, and thereby facilitate the use of Microminipig in life science research.

KEY WORDS: biochemistry, gender difference, growth curve, hematology, swine.

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Swine have been used extensively in biomedical research with a significant increase in recent decades, more than 60,000 pigs having been used in a year in the EU [1, 11]. Because of their physiological and anatomical similarities to humans [5], swine are becoming increasingly attractive animal models in toxicological and pharmacological research. Recently, minipigs defined that they are smaller than domestic swine and their body weight (BW) is <100 kg, have been developed including the strains of Göttingen, Yucatan, Sinclair, Clawn, and others [2, 9, 14]. However, most minipigs are difficult to manage due to their large size, e.g. the Göttingen minipigs of adult are 30–40 kg in BW under conditions of restricted diet [11, 12]. Microminipig (brand name; registered with the Japanese Ministry of Agriculture, Forestry and Fisheries as a novel variety of swine; Fuji Micra Inc., Shizuoka, Japan) has been emerged as a possible experimental animal model for non-clinical pharmacological/toxicological use [4, 8, 10]. The BW of young mature Microminipig is <10 kg, enabling easy handling [5, 7, 13]. The "Eve" of Microminipig (a female minipig named "Catherin") was born by the mating of Pot-bellied pig and another type of minipig [4]. The feature of Microminipig (about a half of body weight of general minipigs) is inherited at the sixth generation. The breeder produces hybrids between six strains of Microminipigs, which correspond to from third to sixth generation. Animals which analyzed in this study were randomly selected from population of hybrids by age. The aim of this study was to establish reference values for growth curves, hematological (23 parameters) and serum biochemical (19 parameters) in the healthy Microminipigs.

All animals were maintained in the same animal unit at 24 ± 3°C and relative humidity at 50 ± 20%, with a 12 hr light/dark cycle in the breeder. The breeding space was 0.5–1.2 $m^2$/an animal. Amount of porcine diet (Marubeni Nisshin Feed Co., Tokyo, Japan) was set to 4–8%, 2–4%, and 1–3% of BW according to one's age, correspond to 1–3, 4–6, and after 7 months, respectively. The diet was composed of >13.0% crude protein, >2.0% crude fat, <8.0% crude fiber, <10.0% crude ash, >1.1% calcium, and >0.9% phosphorus. Tap water was available *ad libitum*. 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 and medication other than vaccination thorough the study. All the data were presented as mean ± SD and statistical analysis of the differences was assessed by F-test and Student's *t*-test or Welch's *t*-test, and considered significant at $P<0.05$.

BWs of animals were measured once monthly (0–12

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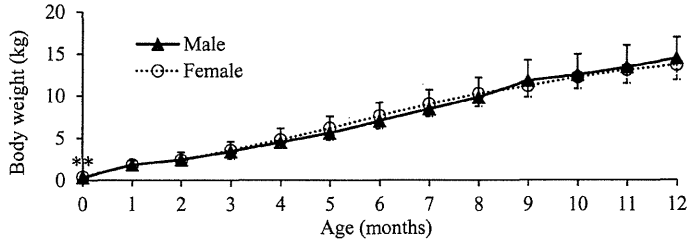


Fig. 1. Body weight of the Microminipig. $^{**}P<0.01$; significantly different for males vs. females.

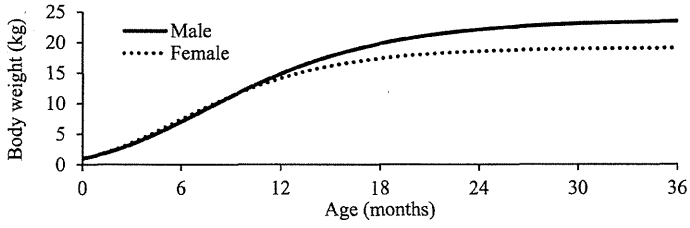


Fig. 2. Growth curve of the Microminipig predicted by Gompertz function.

months of age) by the breeder from 2009 to 2010. Animals aged >13 months could not weigh because of shipment. In total, the original dataset contained 324 and 492 BW measurements of 27 males and 41 females, respectively. Growth curves were fitted by Gompertz function, using KaleidaGraph (4.1J demo version) [6]. BWs in males and females at birth were 336 ± 83 g and 385 ± 65 g, respectively, and showed significant gender differences ($P<0.01$). However, BWs in males and females from 1 to 12 months of age were almost equal and showed no gender differences. The BW in both males and females at 3 and 6 months of age were<5 kg and<10 kg, respectively (Fig. 1). Growth curve revealed almost plateau (approximately 20 kg) over 18 months of age (Fig. 2).

The fasting blood samples were collected from the cranial vena cava of 125 individual conscious animals (58 males and 67 females) aged 0–34 months. The numbers of males in 0–2, 3–5, 6–8, 9–12, 13–24, and 25–34 months of age were 9, 6, 9, 14, 14, and 6, respectively. The numbers of females in 0–2, 3–5, 6–8, 9–12, 13–24, and 25–34 months of age were 11, 8, 17, 13, 10, and 8, respectively. For measurement of 23 hematological parameters except prothrombin time (PT) and activated partial thromboplastin time (APTT) (Table 1), 1 m*l* of blood was collected with an anticoagulant, EDTA-2K and then applied to an automatic analyzer (ADVIA 120, Simens Healthcare Diagnostics Manufacturing Ltd., Dublin, Ireland). For measurement of PT and APTT, 1.5 m*l* of blood was collected with 150 $\mu l$ of 3.8 w/v% sodium citrate solution as an anticoagulant. The plasma was obtained by centrifugation (4°C, 1,710×g, 3,000 rpm, 15 min) and analyzed in an automatic analyzer (CA-7000, Sysmex Corporation, Kobe, Japan). For measurement of 19 serum biochemical parameters (Table 2), the serum was obtained by centrifugation (room temperature, 1,710 × g, 3,000 rpm, 15 min) and applied to an automatic analyzer (JCA-BM8, JEOL Co., Ltd., Tokyo, Japan). As shown in Table 1, major hematological parameters including erythrocyte and leukocyte count showed no gender differences except for the percentage of basophils, lymphocytes and neutrophils. The former 2 were higher in females and the latter 1 was higher in males. As shown in Table 2, major serum biochemical parameters showed no gender differences. Some biochemical values however, showed significant gender differences. The serum levels of alanine aminotransferase, globulin, and total cholesterol were higher in females, and those of urea nitrogen and sodium were higher in males. The major hematological and biochemical parameters in Microminipigs were similar to those in Göttingen and Yucatan minipig, although alkaline phosphatase and albumin showed higher tendency, and APTT and total bilirubin showed lower tendency [2, 3, 9]. These differences on the hematology and biochemistry between Microminipigs and other minipigs may be related to environmental factors such as diets or genetic factors.

Recent development of minipigs and their physiological and anatomical similarities to humans make minipigs a suitable species for toxicological/pharmacological studies; however, despite continuous efforts from breeders, minipigs are not yet widely used in life science research and one possible reason is the lack of reference values [14]. In this study, we provided useful reference values of Microminipigs, and thereby it would facilitate the use Microminipigs in various life science researches.

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Table 1. Reference values of hematological parameters in Microminipig

| Parameter | Unit | Male ($n$=58) | | Female ($n$=66) | | Whole ($n$=124) | |
|---|---|---|---|---|---|---|---|
| | | Mean ± SD | (Range) | Mean ± SD | (Range) | Mean ± SD | (Range) |
| Erythrocyte count | $10^6$/mm$^3$ | 7.85 ± 0.75 | (5.08–9.46) | 7.66 ± 0.89 | (4.28–9.39) | 7.75 ± 0.83 | (4.28–9.46) |
| Leukocyte count | $10^3$/mm$^3$ | 12.41 ± 5.11 | (5.95–35.44) | 12.64 ± 3.92 | (6.66–28.07) | 12.53 ± 4.50 | (5.95–35.44) |
| Hematocrit | % | 45.60 ± 5.48 | (23.20–57.40) | 44.72 ± 5.60 | (21.60–55.90) | 45.13 ± 5.54 | (21.60–57.40) |
| Hemoglobin | g/d*l* | 14.84 ± 1.85 | (8.10–18.80) | 14.69 ± 1.98 | (7.70–18.50) | 14.76 ± 1.91 | (7.70–18.80) |
| Platelet | $10^3$/mm$^3$ | 426.1 ± 126.3 | (87.0–700.0) | 439.5 ± 132.9 | (129.0–786.0) | 433.2 ± 129.5 | (87.0–786.0) |
| Mean corpuscular volume | fL | 58.06 ± 3.85 | (45.60–64.60) | 58.50 ± 4.44 | (47.50–68.80) | 58.29 ± 4.17 | (45.60–68.80) |
| Mean corpuscular hemoglobin | pg | 18.93 ± 1.60 | (14.20–21.80) | 19.22 ± 1.75 | (14.10–22.30) | 19.08 ± 1.68 | (14.10–22.30) |
| Mean corpuscular hemoglobin concentration | g/d*l* | 32.58 ± 1.39 | (28.30–34.90) | 32.84 ± 1.27 | (28.90–36.70) | 32.72 ± 1.33 | (28.30–36.70) |
| Reticulocytes | % | 1.85 ± 3.91 | (0.20–25.20) | 1.44 ± 3.00 | (0.20–22.60) | 1.63 ± 3.45 | (0.20–25.20) |
| Eosinophils | % | 2.53 ± 1.46 | (0.40–6.40) | 2.42 ± 1.48 | (0.30–6.60) | 2.47 ± 1.47 | (0.30–6.60) |
| Basophils | % | 0.91 ± 0.32 | (0.40–2.10) | 1.02 ± 0.30* | (0.40–1.90) | 0.97 ± 0.31 | (0.40–2.10) |
| Monocytes | % | 5.20 ± 1.36 | (1.90–7.80) | 5.18 ± 1.50 | (2.70–10.50) | 5.19 ± 1.43 | (1.90–10.50) |
| Lymphocytes | % | 56.57 ± 13.01 | (23.80–82.50) | 60.88 ± 9.91* | (28.80–82.20) | 58.86 ± 11.62 | (23.80–82.50) |
| Neutrophils | % | 33.29 ± 12.10 | (10.80–63.80) | 28.82 ± 9.81* | (9.40–61.90) | 30.91 ± 11.12 | (9.40–63.80) |
| Large unstained cells | % | 1.51 ± 0.68 | (0.30–3.40) | 1.69 ± 0.93 | (0.30–4.10) | 1.60 ± 0.83 | (0.30–4.10) |
| Eosinophils | $10^3$/mm$^3$ | 0.30 ± 0.21 | (0.03–1.38) | 0.30 ± 0.21 | (0.03–1.05) | 0.30 ± 0.21 | (0.03–1.38) |
| Basophils | $10^3$/mm$^3$ | 0.12 ± 0.08 | (0.03–0.42) | 0.13 ± 0.05 | (0.05–0.29) | 0.12 ± 0.07 | (0.03–0.42) |
| Monocytes | $10^3$/mm$^3$ | 0.63 ± 0.26 | (0.12–1.79) | 0.66 ± 0.33 | (0.24–2.46) | 0.65 ± 0.30 | (0.12–2.46) |
| Lymphocytes | $10^3$/mm$^3$ | 7.04 ± 3.56 | (3.46–19.77) | 7.62 ± 2.53 | (3.99–18.47) | 7.35 ± 3.06 | (3.46–19.77) |
| Neutrophils | $10^3$/mm$^3$ | 4.15 ± 2.50 | (0.94–15.41) | 3.72 ± 1.98 | (1.32–10.65) | 3.92 ± 2.24 | (0.94–15.41) |
| Large unstained cells | $10^3$/mm$^3$ | 0.18 ± 0.11 | (0.02–0.59) | 0.21 ± 0.13 | (0.04–0.63) | 0.20 ± 0.12 | (0.02–0.63) |
| Prothrombin time# | s | 13.05 ± 0.93 | (11.20–16.00) | 12.87 ± 0.88 | (10.60–14.70) | 12.95 ± 0.90 | (10.60–16.00) |
| Activated partial thromboplastin time# | s | 11.79 ± 1.63 | (7.80–17.00) | 11.46 ± 1.19 | (8.90–13.70) | 11.61 ± 1.42 | (7.80–17.00) |

*$P$<0.05; significantly differerent from male. #Male ($n$=55), Female ($n$=64), Whole ($n$=119)

Table 2. Reference values of serum biochemical parameters in Microminipig

| Parameter | Unit | Male ($n$=58) | | Female ($n$=67) | | Whole ($n$=125) | |
|---|---|---|---|---|---|---|---|
| | | Mean ± SD | (Range) | Mean ± SD | (Range) | Mean ± SD | (Range) |
| Aspartate aminotransferase | IU/*l* | 39.72 ± 15.64 | (20.0–124.0) | 41.19 ± 27.54 | (19.0–225.0) | 40.51 ± 22.73 | (19.0–225.0) |
| Alanine aminotransferase | IU/*l* | 42.52 ± 12.46 | (21.0–74.0) | 47.96 ± 17.67* | (16.0–97.0) | 45.43 ± 15.65 | (16.0–97.0) |
| Alkaline phosphatase | IU/*l* | 725.0 ± 708.2 | (176.0–4246.0) | 560.9 ± 373.2 | (175.0–2087.0) | 637.0 ± 558.0 | (175.0–4246.0) |
| Creatinine kinase | IU/*l* | 724.6 ± 929.1 | (134.0–5102.0) | 761.3 ± 1220.8 | (158.0–8446.0) | 744.3 ± 1085.1 | (134.0–8446.0) |
| Total bilirubin | mg/d*l* | 0.031 ± 0.043 | (0.000–0.320) | 0.032 ± 0.040 | (0.000–0.270) | 0.032 ± 0.041 | (0.000–0.320) |
| Total protein | g/d*l* | 7.69 ± 0.97 | (5.50–10.40) | 7.67 ± 0.97 | (4.90–10.30) | 7.68 ± 0.97 | (4.90–10.40) |
| Albumin | g/d*l* | 4.48 ± 0.47 | (3.40–5.50) | 4.30 ± 0.52 | (2.40–5.20) | 4.38 ± 0.50 | (2.40–5.50) |
| Globulin | g/d*l* | 3.21 ± 0.92 | (1.30–5.30) | 3.37 ± 0.96* | (1.40–6.80) | 3.30 ± 0.94 | (1.30–6.80) |
| Albumin-globlin ratio | ratio | 1.53 ± 0.53 | (0.71–3.46) | 1.40 ± 0.47 | (0.51–3.00) | 1.46 ± 0.50 | (0.51–3.46) |
| Total cholesterol# | mg/d*l* | 74.36 ± 16.56 | (43.0–121.0) | 96.70 ± 61.20** | (57.0–533.0) | 86.30 ± 47.20 | (43.0–533.0) |
| Triglycerides | mg/d*l* | 41.71 ± 30.30 | (17.0–236.0) | 47.36 ± 21.84 | (19.0–117.0) | 44.74 ± 26.15 | (17.0–236.0) |
| Glucose | mg/d*l* | 91.02 ± 25.65 | (69.0–224.0) | 90.03 ± 15.66 | (66.0–146.0) | 90.49 ± 20.81 | (66.0–224.0) |
| Urea nitrogen | mg/d*l* | 14.55 ± 3.65 | (6.80–25.40) | 13.06 ± 2.94* | (7.80–24.20) | 13.75 ± 3.36 | (6.80–25.40) |
| Creatinine | mg/d*l* | 0.97 ± 0.34 | (0.49–1.94) | 0.87 ± 0.25 | (0.31–1.78) | 0.91 ± 0.30 | (0.31–1.94) |
| Phosphorus | mg/d*l* | 6.69 ± 1.57 | (4.77–13.21) | 6.92 ± 1.40 | (3.85–11.74) | 6.82 ± 1.48 | (3.85–13.21) |
| Calcium | mg/d*l* | 10.71 ± 0.59 | (9.80–12.30) | 10.63 ± 0.52 | (9.30–12.30) | 10.67 ± 0.56 | (9.30–12.30) |
| Sodium | mEq/*l* | 145.5 ± 3.0 | (136.0–153.0) | 144.1 ± 4.0* | (119.0–151.0) | 144.8 ± 3.6 | (119.0–153.0) |
| Potassium | mEq/*l* | 5.75 ± 0.65 | (4.00–7.50) | 5.73 ± 0.78 | (4.30–7.40) | 5.74 ± 0.72 | (4.00–7.50) |
| Chloride | mEq/*l* | 103.4 ± 3.2 | (93.0–111.0) | 103.3 ± 4.2 | (76.0–108.0) | 103.4 ± 3.8 | (76.0–111.0) |

*$P$<0.05, **$P$<0.01; significantly differerent from male. #Female ($n$=66), Whole ($n$=124).

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# Coagulation Activity and White Thrombus Formation in the Microminipig

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**Abstract.** *Swine are becoming increasingly attractive as animal models for clinical research and the recently developed Microminipig (MMPig) has emerged as a possible experimental animal model. In this study, we demonstrated age-dependent change in hematological parameters and coagulation activity in healthy MMPigs (58 male and 67 females, aged 0-34 months), and investigated white thrombus formation (WTF) using an in vitro microchip flow-chamber system (four males and four females, aged 22-23 months). There was no clear sex or age-dependent difference in any hematological parameter. While activated partial thromboplastin time (APTT) was shorter than prothrombin time (PT), with APTT:PT of 0.88:1, microchip flow-chamber system analysis showed that WTF time was shorter than that in humans, suggesting a possible thrombotic tendency in the MMPig. These results could be useful to life science researchers in the use of the MMPig as an experimental model animal for thrombus formation.*

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*Key Words:* Aging, porcine hematology, coagulation activity, thrombus formation.

Swine have been used extensively in biomedical research, with a significant increase in recent decades, more than 60,000 pigs having been used in a year in the EU (1,2). Because of their physiological and anatomical similarity to humans (3], swine are becoming increasingly attractive as animal models for clinical research. The microminipig (Brand: MMPig; registered with the Japanese Ministry of Agriculture, Forestry and Fisheries as a novel variety of swine; Fuji Micra Inc., Shizuoka, Japan) has emerged as a possible experimental animal model for non-clinical pharmacological/toxicological use (4-6]. A female minipig, "Catherine" (the MMPig "Eve"), was the outcome from mating a pot-bellied pig and another type of minipig (4]. The body weight (BW) of a young mature MMPig is <10 kg, enabling easy handling (3, 7-9). Except for coagulation activity, prothrombin time (PT) and activated partial thromboplastin time (APTT), the major hematological and biochemical parameters in the MMPig are similar to those found in Göttingen and Yucatan minipigs (7).The aim of this study was to measure age-dependent changes in hematological parameters and coagulation activity, and to investigate white thrombus formation (WTF) in healthy MMPigs, using an automated microchip flow-chamber system.

## Materials and Methods

*Animals.* All animals were maintained in the same animal unit at 24±3°C and relative humidity at 50±20%, with a 12 h light/dark cycle, and a maintenance space of 0.5-1.2 m2/animal. The amount of porcine diet (Marubeni Nisshin Feed Co.) provided was set according to age and body weight: 4-8%, 2-4%, and 1-3% of BW corresponding to 1 to 3 months, 4 to 6 months, and 7 months and older, respectively. The diet was composed of >13.0% crude protein, >2.0% crude fat, <8.0%crude fiber, <10.0% crude ash, >1.1%

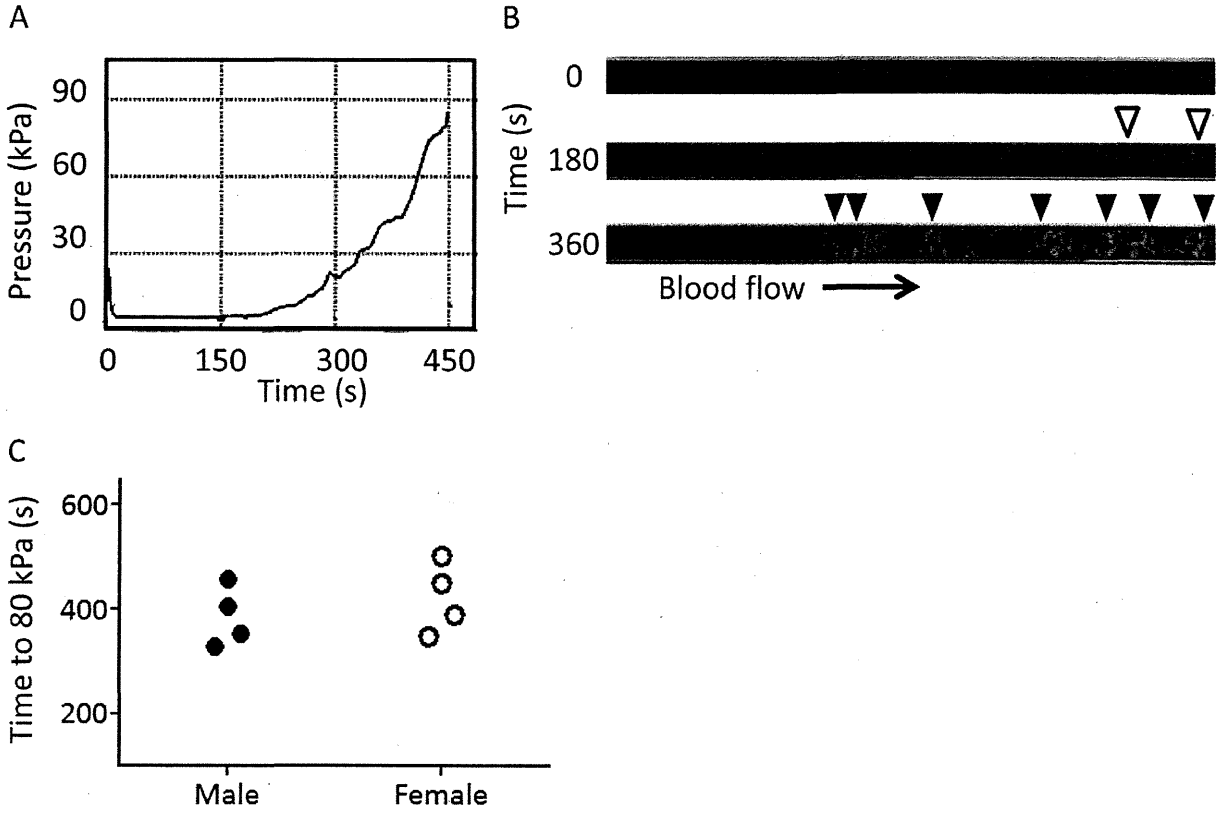


Figure 1. *A: The flow-pressure waveform pattern during white thrombus formation (WTF). The black line represents the increasing pressure resulting from occlusion in the microchip flow-chamber by white thrombus formation. B: A typical image of white thrombus in the microchip flow-chamber. Open arrowheads indicate the initial small thrombus and closed arrowheads indicate mature WTF. C: The lag time for flow pressure to increase to 80 kPa in microminipigs measured by in vitro micro flow chamber system. p-Value is 0.4433 by Student's t-test.*

calcium, and >0.9% phosphorus. Tap water was available *ad libitum*. The animals used in this study were in good health and free of clinical signs of illness. They required no treatment or medication other than vaccination during the study. Data are presented as the mean±SD, and statistical analysis of differences was by F-test, and Student's *t*-test or Welch's *t*-test, at a significance level of $p<0.05$.

*Blood collection.* Blood samples were collected from the cranial vena cava of 125, fasted, conscious animals (58 males and 67 females) aged 0 to 34 months. For measurement of 23 hematological parameters, except PT and APTT, 1 ml of blood was collected with EDTA-2K as an anticoagulant and applied to an automatic analyzer (ADVIA 120, Siemens Healthcare Diagnostics Manufacturing Ltd., Munich, Bavaria, Germany). For measurement of PT and APTT, 1.5 ml of blood was collected with 150 μl of 3.8 w/v% sodium citrate solution as an anticoagulant. Plasma was obtained by centrifugation (4°C, 1,710 × *g*, for 15 min) and analyzed with an automatic analyzer (CA-7000, Sysmex Corporation, Kobe, Japan).

*WTF assay.* WTF assays were performed using an automated microchip flow-chamber system as previously stated (10). Blood (n=4 males and 4 females, aged 22 to 23 months, considered the most likely age for use in life science research) was collected into a tube containing 3.2% sodium citrate and mixed with 20 μl of 0.3 M $CaCl_2$ containing 1.25 mg/ml of corn trypsin inhibitor immediately before application to the microchip. The mixture of blood and corn trypsin inhibitor was perfused over a microchip capillary coated with collagen and tissue thromboplastin at a flow rate of 10 μl/min. The WTF process is monitored by flow pressure changes in the capillary with a pressure sensor. As WTF spreads on the coated surface, the capillary is gradually occluded, increasing the flow pressure (Figure 1A and B). We calculated that the lag time for the flow pressure increase tothe 80 kPa (T80) from baseline, representing almost complete occlusion of the capillary by WTF.

## Results

*Hematological parameters and coagulation activity.* Age-dependent hematological parameters and coagulation activity are listed in Tables I and II. Given statistical significance of $p<0.01$, hemoglobin values at age 3 to 5 months, APTT at age 9 to 12 months, and Red blood cell (RBC) at age 25 to 34 months were significantly lower and shorter respectively in females than those in males. Mean corpuscular

Table I. *Age-specific values in hematology in microminipig.*

| Parameter | Unit | Gender | Age (months) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0-2 (M=9, F=10) | 3-5 (M=6, F=8) | 6-8 (M=9, F=17) | 9-12 (M=14, F=13) | 13-24 (M=14, F=10) | 25-34 (M=6, F=8) |
| RBC | $10^6$/mm$^3$ | M+F | 7.6±1.2 | 7.9±0.8 | 8.0±0.6 | 7.6±0.6 | 7.8±0.8 | 7.6±1.0 |
| | | M | 7.8±1.1 | 8.4±0.8 | 7.9±0.5 | 7.6±0.6 | 7.6±0.8 | 8.3±0.3 |
| | | F | 7.4±1.3 | 7.5±0.6* | 8.1±0.7 | 7.5±0.6 | 8.1±0.8 | 7.0±0.9** |
| WBC | $10^3$/mm$^3$ | M+F | 17.8±6.6 | 14.8±4.6 | 11.8±3.4 | 11.5±2.4 | 10.2±1.8 | 10.5±2.6 |
| | | M | 19.1±8.2 | 13.2±2.7 | 12.4±5.3 | 11.2±2.6 | 10.0±1.8 | 9.8±1.8 |
| | | F | 16.5±5.0 | 15.9±5.5 | 11.5±1.9 | 11.7±2.3 | 10.5±1.9 | 11.0±3.1 |
| Hematocrit | ratio | M+F | 41.8±7.7 | 43.4±5.0 | 45.7±4.5 | 44.2±3.6 | 47.5±5.4 | 48.0±5.0 |
| | | M | 42.9±8.4 | 46.8±4.5 | 45.1±3.7 | 44.2±3.8 | 45.9±5.6 | 51.6±2.4 |
| | | F | 40.9±7.3 | 40.8±3.7* | 46.0±5.0 | 44.1±3.7 | 49.7±4.5 | 45.4±4.8* |
| Hemoglobin | g/dl | M+F | 12.8±2.0 | 14.0±1.6 | 15.1±1.4 | 14.7±1.2 | 15.7±1.8 | 16.1±1.8 |
| | | M | 12.9±2.1 | 15.2±1.2 | 14.8±1.1 | 14.7±1.4 | 15.1±1.8 | 17.3±1.1 |
| | | F | 12.6±2.0 | 13.0±1.3** | 15.3±1.5 | 14.7±1.2 | 16.6±1.5* | 15.2±1.9* |
| Platelet | $10^3$/mm$^3$ | M+F | 541.4±124.6 | 430.9±160.3 | 437.0±119.9 | 417.6±86.2 | 377.1±137.4 | 408.1±107.1 |
| | | M | 511.9±121.6 | 383.8±120.8 | 445.0±122.5 | 419.0±101.6 | 415.0±158.2 | 353.3±69.3 |
| | | F | 567.9±127.5 | 466.1±184.4 | 432.8±122.1 | 416.2±70.1 | 324.0±81.8 | 449.3±115.6 |
| MCV | fl | M+F | 55.0±4.6 | 54.9±2.4 | 57.2±2.9 | 58.5±3.3 | 60.6±2.3 | 63.7±2.8 |
| | | M | 54.5±5.1 | 55.8±1.4 | 57.3±1.9 | 58.2±4.0 | 60.1±2.6 | 61.8±1.9 |
| | | F | 55.5±4.4 | 54.2±2.8 | 57.1±3.4 | 58.9±2.6 | 61.4±1.8 | 65.2±2.5* |
| MCH | pg | M+F | 16.9±1.4 | 17.7±1.0 | 18.9±0.8 | 19.5±1.2 | 20.1±0.9 | 21.3±0.9 |
| | | M | 16.5±1.5 | 18.1±0.5 | 18.8±0.5 | 19.3±1.4 | 19.8±0.8 | 20.7±0.8 |
| | | F | 17.2±1.3 | 17.4±1.2 | 19.0±0.9 | 19.6±1.0 | 20.6±0.8* | 21.8±0.5* |
| MCHC | g/dl | M+F | 30.7±1.9 | 32.2±0.7 | 33.1±0.9 | 33.2±0.6 | 33.1±0.6 | 33.5±0.6 |
| | | M | 30.4±2.2 | 32.5±0.8 | 32.9±0.6 | 33.2±0.6 | 32.9±0.5 | 33.5±0.6 |
| | | F | 31.1±1.8 | 32.0±0.6 | 33.2±1.0 | 33.3±0.6 | 33.5±0.5** | 33.5±0.7 |
| PT$^{\$}$ | s | M+F | 12.5±1.1 | 13.1±0.8 | 13.1±0.9 | 13.4±0.7 | 12.8±0.7 | 12.2±0.9 |
| | | M | 12.4±1.2 | 12.9±0.9 | 13.5±1.0 | 13.5±0.4 | 13.0±0.7 | 12.2±1.2 |
| | | F | 12.6±1.0 | 13.4±0.5 | 13.0±0.8 | 13.4±0.9 | 12.6±0.8 | 12.1±0.8 |
| APTT$^{\$}$ | s | M+F | 11.2±1.1 | 11.5±1.1 | 12.0±1.4 | 12.1±1.0 | 11.6±1.6 | 10.7±1.9 |
| | | M | 11.4±0.9 | 11.2±1.1 | 12.4±2.0 | 12.6±0.9 | 11.7±1.7 | 10.3±2.3 |
| | | F | 11.1±1.3 | 11.8±1.1 | 11.8±0.9 | 11.5±0.8** | 11.5±1.6 | 11.0±1.6 |

RBC: Red blood cell, WBC: White blood cell, MCV: Mean cellular volume, MCH: Mean cellular hemoglobin, MCHC: Mean cellular hemoglobin concentration, PT: Prothrombin time, APTT: Activated partial thromboplastin time. M: males, F: females. *$p$<0.05, **$p$<0.01, significantly differerent from male.

hemoglobin concentration (MCHC) was higher in females aged 13 to 24 months. In the White blood cell (WBC) population, the basophil count was higher in females aged 13 to 24 months, corresponding to a higher basophil ratio and lower neutrophil ratio. No major parameter, including RBC and WBC, showed clear biological sex and/or age differences. APTT was shorter than PT (mean values) and the ratio of APTT to PT was approximately 0.88:1 in males, females, and both at the relevant age points.

*WTF assay.* The lag times for the flow pressure to increase by 80 kPa (T80) for male and female MMPigs was 386.3±50.4 min and 422.8±58.2 min respectively (Figure 1C). There was no significant difference in T80 between the two groups (p=0.4433), indicating similar characteristics in thrombus formation in both males and females.

## Discussion

Since the minipig is physiologically and anatomically similar to man, it is a suitable species for toxicological/ pharmacological studies. However, despite continued efforts by breeders, minipigs are not yet widely used in life science research and one possible reason is the lack of reference values (11). We have reported reference values for hematological parameters in the newly developed MMPig, the world smallest (7). In this study, we analyzed age-dependent changes in hematological and coagulation parameters for the MMPig to provide detailed information. There were no sex or age-dependent changes in hematological parameters during the experimental period. This indicate that there is no major difference in hematological parameters from those previously reported for the minipig (6].

Table II. *Age-specific values in hematology in microminipig.*

| Parameters | Unit | Gender | Age (months) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0-2 (M=9, F=10) | 3-5 (M=6, F=8) | 6-8 (M=6, F=8) | 9-12 (M=9, F=17) | 13-24 (M=14, F=13) | 25-34 (M=14, F=10) |
| Reticulocytes | % | M+F | 6.59±6.97 | 0.87±0.72 | 0.67±0.34 | 0.56±0.34 | 0.84±0.71 | 0.90±0.82 |
| | | M | 7.98±7.56 | 0.45±0.16 | 0.66±0.35 | 0.53±0.22 | 0.94±0.88 | 1.08±0.65 |
| | | F | 5.35±6.55 | 1.19±0.82* | 0.67±0.35 | 0.59±0.44 | 0.70±0.35 | 0.76±0.94 |
| Eosinophils | % | M+F | 1.25±0.99 | 2.71±1.53 | 2.76±1.79 | 2.23±0.94 | 3.03±1.40 | 2.84±1.43 |
| | | M | 1.26±1.23 | 2.42±1.59 | 2.58±2.12 | 2.51±1.02 | 2.94±1.10 | 3.58±1.30 |
| | | F | 1.25±0.78 | 2.94±1.56 | 2.86±1.65 | 1.92±0.78 | 3.16±1.78 | 2.29±1.34 |
| Basophils | % | M+F | 1.19±0.32 | 0.91±0.19 | 0.95±0.35 | 0.84±0.26 | 0.95±0.32 | 1.04±0.30 |
| | | M | 1.21±0.29 | 0.95±0.24 | 0.93±0.49 | 0.77±0.26 | 0.79±0.21 | 0.95±0.19 |
| | | F | 1.17±0.36 | 0.89±0.15 | 0.96±0.27 | 0.92±0.24 | 1.16±0.33** | 1.10±0.36 |
| Monocytes | % | M+F | 3.6±1.0 | 4.3±0.4 | 5.1±1.5 | 5.7±1.2 | 5.4±1.2 | 5.6±1.3 |
| | | M | 4.5±2.0 | 4.5±0.9 | 5.1±1.1 | 6.0±1.0 | 5.4±1.3 | 6.6±0.8 |
| | | F | 5.3±2.3* | 4.7±1.2 | 5.1±1.7 | 5.4±1.3 | 5.5±1.0 | 5.0±1.1* |
| Lymphocytes | % | M+F | 71.5±8.7 | 61.8±10.6 | 58.6±9.9 | 55.7±12.1 | 56.1±12.3 | 53.6±10.1 |
| | | M | 67.3±11.0 | 63.9±8.0 | 54.8±10.9 | 53.2±13.0 | 51.3±12.6 | 51.8±8.7 |
| | | F | 63.5±11.8 | 65.5±5.6 | 60.6±9.1 | 58.4±10.8 | 62.9±8.2 | 55.0±11.5 |
| Neutrophils | % | M+F | 24.5±9.4 | 26.4±8.5 | 31.1±10.7 | 33.8±11.7 | 32.7±11.9 | 35.1±10.4 |
| | | M | 21.2±7.2 | 29.3±11.3 | 35.0±11.8 | 35.9±12.9 | 38.0±11.6 | 35.8±8.7 |
| | | F | 27.5±10.4 | 24.3±5.5 | 29.0±9.8 | 31.6±10.2 | 25.2±8.1** | 34.7±12.1 |
| Large unstained cells | % | M+F | 1.23±0.78 | 1.48±0.58 | 1.53±0.61 | 1.69±0.85 | 1.87±1.02 | 1.76±0.93 |
| | | M | 1.26±0.64 | 1.20±0.47 | 1:58±0.45 | 1.64±0.60 | 1.67±0.97 | 1.40±0.53 |
| | | F | 1.21±0.92 | 1.69±0.59 | 1.50±0.70 | 1.75±1.07 | 2.14±1.09 | 2.04±1.10 |
| Eosinophils | $10^3/mm^3$ | M+F | 0.26±0.31 | 0.43±0.30 | 0.31±0.18 | 0.25±0.11 | 0.31±0.16 | 0.29±0.14 |
| | | M | 0.29±0.43 | 0.33±0.22 | 0.29±0.20 | 0.27±0.11 | 0.29±0.12 | 0.35±0.11 |
| | | F | 0.22±0.18 | 0.51±0.35 | 0.32±0.18 | 0.23±0.10 | 0.34±0.21 | 0.24±0.14 |
| Basophils | $10^3/mm^3$ | M+F | 0.22±0.10 | 0.14±0.06 | 0.11±0.04 | 0.09±0.03 | 0.10±0.04 | 0.11±0.03 |
| | | M | 0.24±0.12 | 0.13±0.05 | 0.10±0.04 | 0.08±0.02 | 0.08±0.02 | 0.09±0.02 |
| | | F | 0.19±0.07 | 0.14±0.06 | 0.11±0.04 | 0.11±0.03* | 0.12±0.04** | 0.12±0.03 |
| Monocytes | $10^3/mm^3$ | M+F | 0.85±0.57 | 0.67±0.25 | 0.60±0.23 | 0.65±0.21 | 0.55±0.13 | 0.58±0.15 |
| | | M | 0.75±0.48 | 0.57±0.15 | 0.62±0.28 | 0.67±0.21 | 0.54±0.14 | 0.63±0.09 |
| | | F | 0.95±0.64 | 0.75±0.29 | 0.59±0.22 | 0.63±0.21 | 0.57±0.13 | 0.54±0.18 |
| Lymphocytes | $10^3/mm^3$ | M+F | 11.7±4.0 | 9.4±3.0 | 6.8±1.4 | 6.2±1.3 | 5.7±1.5 | 5.5±1.3 |
| | | M | 13.3±4.8 | 8.1±1.8 | 6.4±1.4 | 5.7±1.1 | 5.1±1.5 | 5.0±1.0 |
| | | F | 10.2±2.7 | 10.4±3.5 | 6.9±1.5 | 6.7±1.4* | 6.5±1.0* | 5.9±1.4 |
| Neutrophils | $10^3/mm^3$ | M+F | 4.6±2.9 | 3.9±1.6 | 3.9±2.7 | 4.1±2.3 | 3.4±1.4 | 3.8±2.0 |
| | | M | 4.3±3.1 | 3.9±1.9 | 4.8±4.1 | 4.3±2.5 | 3.8±1.5 | 3.6±1.3 |
| | | F | 4.8±2.8 | 3.9±1.5 | 3.4±1.4 | 3.8±2.1 | 2.7±1.2 | 4.0±2.5 |
| Large unstained cells | $10^3/mm^3$ | M+F | 0.21±0.15 | 0.23±0.14 | 0.18±0.08 | 0.20±0.11 | 0.20±0.13 | 0.19±0.13 |
| | | M | 0.24±0.19 | 0.16±0.07 | 0.20±0.09 | 0.19±0.10 | 0.17±0.11 | 0.13±0.04 |
| | | F | 0.18±0.12 | 0.28±0.16 | 0.17±0.08 | 0.20±0.13 | 0.23±0.14 | 0.23±0.15 |

M: males, F: females. *$p<0.05$, **$p<0.01$, significantly differerent from male.

However, the ratio of APTT to PT was different from that in other experimental animals. Although PT and APTT are commonly used as plasma-based assays for the assessment of coagulation activity in experimental animals, the exact time of each assay differs between species (12). In all species, except the rat (F344 strain), APTT is reported to be longer than PT and the ratio of APTT to PT is between 2:1 and 3:1. By contrast, in the MMPig, APTT is shorter than PT and the ratio is 0.88:1. Short APTT (11.2±1.0 s) as observed in the MMPig is a unique characteristic when compared with previously reported results for minipigs; the APTT in the Gottingen minipig is in the range of 26 to 46 s and that for the Yucatan minipig an average of 15.46±1.15 s. APTT is longer than PT in both minipig species, consistent with other species (13, 14). Although it will be necessary to elucidate the biological mechanism of the shorter APTT observed in the MMPig, this hypercoagulable response to the intrinsic pathway may suggest a thrombotic tendency in the MMPig.

APTT is assayed to evaluate the coagulation pathway, but does not fully reflect the interaction of coagulation factors or

platelets *in vivo*. The newly developed microchip-based flow chamber system (WTF assay) mimics *in vivo* blood flow and is influenced by both platelet activation and coagulation reactions over the collagen/tissue thromboplastin-coated surface (10). In this experiment, we used flow rates of 10 μl/min, corresponding to initial wall shear rates of 600 $s^{-1}$, which simulates arterial blood flow in small to medium-sized arteries (15). T80 in the MMPig was 404.5±57.4 min compared with 558±90 in Man, and the WTF assay indicated that white thrombus formation in the microminipig was markedly more rapid than that in human (10). This result further supports the conjecture that the MMPig has a thrombotic tendency, at least by *in vitro* thrombus formation assay.

## Conclusion

In this study, we demonstrated age-dependent changes in hematological and coagulation parameters for MMPigs. All hematological parameters were within the normal range, with no major sex or age difference. APTT in the MMPig was shorter than PT and the ratio of APTT to PT was 0.88:1. We also investigated thrombus formation activity and indicated a thrombotic tendency in the MMPig. These results could be useful to life science researchers in regard to the use of the MMPig as an experimental model animal for thrombus formation.

## Conflicts of Interest

TN and HS are employees of Fujimori Kogyo Co., Ltd. TI and IM hold endowed faculty positions in thrombosis research and have received funds from Medipolis Medical Research Institute, Shin Nippon Biomedical Laboratories, Asahi Kasei Pharma, and Asahi Kasei Medical.

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# Short Communication

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# A Dermal Phototoxicity Study Following Intravenous Infusion Administration of Ciprofloxacin Hydrochloride in the Novel Microminipigs

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Abstract

The authors evaluated dermal phototoxicity using the world smallest minipig (MMPig: Microminipig). MMPigs were administered 100 mg/kg ciprofloxacin hydrochloride with an infusion pump. The dorsal area of each animal was irradiated with ultraviolet-A irradiation. The left dorsal skin was irradiated at intensities of 5, 10, 15, and 20 J/cm$^2$, and the right dorsal back skin was set as a nonirradiated site. Gross and histopathological examinations were conducted before irradiation and from 1 to 72 hr after irradiation. Initial changes in the skin were necrosis of the basal and/or prickle cell layer and cellular infiltration from 24 hr after irradiation. Vesicle formation observed from 48 hr after irradiation was considered similar to bullous eruptions, a known side effect of fluoroquinolones in humans. Therefore, the authors suggest that the MMPig may be a useful experimental animal model for dermal phototoxicity studies.

*Keywords:* ciprofloxacin hydrochloride; dermal; phototoxicity; swine; ultraviolet.

Dermatitis has been found to occur in both laboratory animals and humans exposed to ultraviolet radiation following the administration of photoreactive chemicals and drugs, and antibiotics, psoralens, nonsteroidal anti-inflammatory drugs, and tranquilizers are reported to have induced this undesirable adverse effect (Marrot et al. 2003). Among these, fluoroquinolone antibiotics are recognized as a group associated with drug-induced phototoxicity from interaction with ultraviolet light. *In vivo* models are preferred for estimating fluoroquinolone phototoxicity to humans because they incorporate photoreactivity and toxicity to the skin (Mayne et al. 1997; Owen 1999; Yabe et al. 2005). Therefore, *in vivo* models have been developed in a variety of animals, such as mice, rabbits, guinea pigs, and swine. Among laboratory animals, the pig has relatively hairless skin that enables the clinical evaluation of surface alterations. The skin of minipigs is also considered a good model for human skin because it is morphologically, physiologically, and pharmacologically similar (Yabuki et al. 2007). Recently, the world's smallest minipig (MMPig: Microminipig; registered as a novel variety of swine with the Japanese Ministry of Agriculture, Forestry, and Fisheries) has emerged as a possible experimental animal model for nonclinical pharmacological/toxicological use (Miyoshi et al. 2010; Murayama et al. 2009; Sugiyama et al. 2011). The MMPig is docile with body weight (BW) at young mature of less than 7 kg, a good manageable size for an experimental animal (Kaneko et al. 2011; Kawaguchi et al. 2011; Kawaguchi et al. 2012; Takeishi et al. 2012). However, dermatological investigations have not been conducted in the MMPig and no basic dermal research data are available. In this study, we evaluated the MMPig following intravenous administration of a second-generation fluoroquinolone (CPFX: ciprofloxacin hydrochloride) and showed the possibility of its use as an animal model for assessing phototoxicity.

Five female MMPig (aged 5–7 months, BW approximately 8–15 kg: Fuji Micra Inc., Shizuoka, Japan) were used. Each animal was provided with mashed diet (Kodakara 73; Marubeni Nisshin

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Abbreviations: BW, body weight; CPFX, ciprofloxacin hydrochloride; HE, Hematoxylin–Eosin; MMPig, microminipig; UVA, ultraviolet-A.

Feed Inc., Tokyo, Japan) equivalent to approximately 2% of BW once daily. Tap water was available ad libitum. The animals were singly housed in stainless steel cages [90 (D) × 90 (W) × 80 (H) cm] for beagle dogs in an air-conditioned room (temperature, 22–28°C; humidity, 40–80%; 12-hr light/dark cycle; ventilation 15 times per hour). This study was approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories (Kagoshima, Japan) and was conducted in accordance with the ethics criteria contained in the bylaws of the committee.

The cervical region of the skin was incised and one side of the carotid vein exposed under anesthesia. A polyurethane tube was inserted and located in the sinus venarum cavarum after confirmation of regurgitation of blood. A plug for administration set at the end of the tube was filled with physiological saline with heparin (50–100 U/mL). All pigs were fasted overnight before the procedure. On the day on which the catheter was set in place and on the following 3 days, a postoperative analgesic and an antimicrobial prophylactic were administered for pain relief and infection prevention, respectively.

CPFX (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted to 20 mg/mL in physiological saline. CPFX 100 mg/kg was set as the dose level expected to induce phototoxicity, as previously reported in mice (Yabe et al. 2005).

In a pilot experiment, a female MMPig was dosed at a rate of 5 ml/kg/hr for 60 min to determine the time for CPFX concentration to reach the steady-state level. The plasma concentration level increased in a linear manner immediately after the end of the administration. In simulations at 2.5 ml/kg/hr, half the pilot dosing rate, at least 2 hr were required for the concentration to reach the steady-state level, and it was considered that prolonged continuous dosing would overload the MMPig. Therefore, to shorten the time for the concentration to reach the steady-state level, the initial dosing rate was set at 10 ml/kg/hr for 10 min followed by 2.5 ml/kg/hr for 80 min. To confirm this dosing rate and time, three MMPigs were used to calculate the plasma concentrations of ciprofloxacin. Blood was drawn from the sinus venarum cavarum with a syringe containing heparin sodium. Blood was drawn before dosing, and at 10, 15, 20, 30, 40, 55, 90, 105, and 150 min after dosing. As shown in Figure 1, maximum plasma drug concentration was reached by 10 min after the start of administration in three animals and the mean concentration was 48.8 ± 6.1 μg/mL. Although the plasma concentration level decreased thereafter, it was confirmed that the steady-state level was maintained under continuous dosing. From these results, the initial dosing rate was set at 10 ml/kg/hr for 10 min followed by 2.5 ml/kg/hr for 80 min and the irradiation time of ultraviolet-A (UVA) was set from 15 to 75 min after the start of dosing.

In this study, fur on the back of each animal was shaved off with an electric clipper on the day before administration. The dorsal area of each animal was divided into eight parts (A–H) [1 part; 2.5 cm × 2.5 cm] (Figure 2a). The left back skin (A–D) was irradiated with UVA. The right back skin (E–H), as the control site, was not irradiated. The right back skin (E–H) was covered with a sheet of aluminum foil affixed with

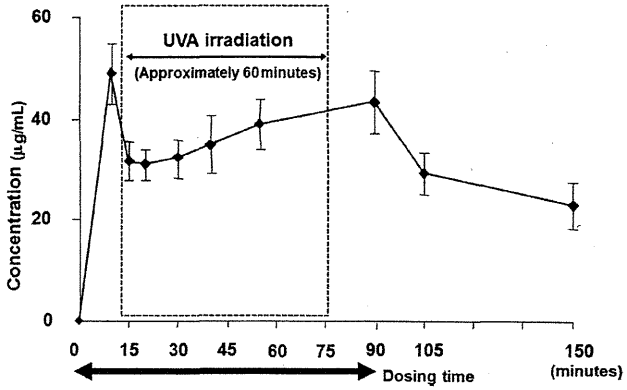


FIGURE 1.—Mean plasma concentrations of CPFX after single intravenous infusion at a dose of 100 mg/kg to three MMPigs in a pilot experiment.

adhesive tape. Each animal was held in a restraint without sedation or anesthesia, and the dosing formulation was administered via the tube, using an infusion pump (Braintree BSP-99 M, Braintree Scientific Inc., MA). Approximately 15 min after the start of administration, when the concentration had reached the steady-state level, irradiation of the animals with UVA at approximately 5, 10, 15, and 20 J/cm$^2$ (5.6 W/cm$^2$, approximately 15, 30, 45, and 60 min) was initiated, using an ultraviolet irradiation apparatus (Dermaray, M-DMR- 50, Eisai Co., Ltd., Tokyo, Japan). The foil was removed sequentially after 0, 15, 30, and 45 min from the 20, 15, 10, and 5 J/ cm$^2$ irradiation sites, respectively (Figure 2b).

The back skin was observed before UVA irradiation and at 1, 24, 48, and 72 hr after UVA irradiation (Animal Nos. 2–5). One animal was observed at 1, 4, 8, 12, and 24 hr (Animal No. 1). Skin reactions at the irradiation sites were evaluated in accordance with Draize's criteria (Draize, Woodard, and Calvery 1944) for erythema and edema.

Histopathological examinations of the back skin were conducted after euthanasia and necropsy. Animal Nos. 2 through 5 were necropsied 1, 24, 48, and 72 hr, respectively, after exposure to ultraviolet radiation. Animal No.1 was not necropsied. The animals were anesthetized and euthanized by exsanguination. The back skin was removed and fixed in 10% neutral buffered formalin for histopathological examination. The specimens were embedded in paraffin, sectioned, stained routinely with Hematoxylin–Eosin (HE) stain, and examined histopathologically.

For immunohistochemical examination, the sections were deparaffinized in xylene and rehydrated through graded alcohol. The primary antibodies and concentrations were as follows: Iba1 (polyclonal, 1:500 dilution; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for macrophage marker, CD3 (F7.2.38; mouse monoclonal, 1:400 dilution; Dako Cytomation Co., Ltd., Kyoto, Japan), and CD79α (HM47/A9; mouse monoclonal, 1:200 dilution; Abcam plc., Cambridge, UK). The sections treated with primary antibody were incubated with the appropriate biotinylated secondary antibody with EnVision (Dako Cytomation Co., Ltd.). Immunoreactivity was visualized

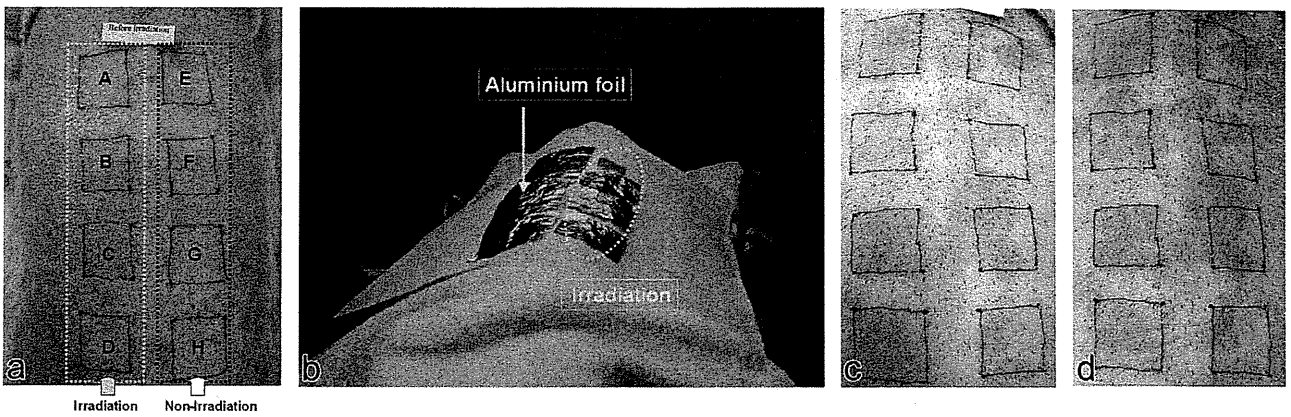


FIGURE 2.—(a) Back skin of the microminipig in the Animal No. 5. Irradiation site is A–D and nonirradiation site is E–H. (b) Irradiation was initiated with all sites other than the 20 J/ $cm^2$ ultraviolet-A (UVA) site covered with aluminum foil. Macroscopic examination. (c) At 24 hr after irradiation, well-defined erythema (score 2) was observed at the 10(C), 15(D), and 20(A) J/$cm^2$ irradiation sites in the Animal No. 5. (d) At 72 hr after irradiation, well-defined erythema (score 2) was observed at the 20(A) J/$cm^2$ irradiation sites in the Animal No. 5.

TABLE 1.—Macroscopic findings from 1 to 72 hr.

| | | | Time (hour) after UVA irradiation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Before irradiation | | 1 | | 24 | | 48 | | 72 | |
| Animal No. | Irradiation site | UVA dose (J/cm2) | Erythema | Edema | Erythema | Edema | Erythema | Edema | Erythema | Edema | Erythema | Edema |
| 2 | A | 5 | 0 | 0 | 0 | 0 | — | — | — | — | — | — |
| | B | 10 | 0 | 0 | 0 | 0 | — | — | — | — | — | — |
| | C | 15 | 0 | 0 | 0 | 0 | — | — | — | — | — | — |
| | D | 20 | 0 | 0 | 0 | 0 | — | — | — | — | — | — |
| | E–H | Nonirradiation | 0 | 0 | 0 | 0 | — | — | — | — | — | — |
| 3 | D | 5 | 0 | 0 | 0 | 0 | 1 | 0 | — | — | — | — |
| | A | 10 | 0 | 0 | 0 | 0 | 2 | 0 | — | — | — | — |
| | B | 15 | 0 | 0 | 0 | 0 | 2 | 0 | — | — | — | — |
| | C | 20 | 0 | 0 | 0 | 0 | 2 | 0 | — | — | — | — |
| | E–H | Nonirradiation | 0 | 0 | 0 | 0 | 0 | 0 | — | — | — | — |
| 4 | C | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | — | — |
| | D | 10 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | — | — |
| | A | 15 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 1 | — | — |
| | B | 20 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 1 | — | — |
| | E–H | Nonirradiation | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | — |
| 5 | B | 5 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| | C | 10 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 1 | 0 |
| | D | 15 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 1 | 1 |
| | A | 20 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 1 | 2 | 1 |
| | E–H | Nonirradiation | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Note.* UVA, Ultraviolet-A.

Draize's criteria, (1) Erythema formation: score 0; no erythema, score 1; very slight erythema (barely perceptible), score 2; well-defined erythema, (2) Edema formation, score 0; no edema, score 1; very slight edema (barely perceptible).

with 0.075% 3,3′-diaminobenzidine tetrachloride. The sections were then washed, counterstained, dehydrated, cleared in xylene, and mounted. The liver of MMPig was set as a positive control specimen to Iba1 and the spleen of MMPig as a positive control specimen to CD3 and CD79α.

At 1, 4, and 8 hr after irradiation, no skin reaction was grossly observed at the 5–20 J/$cm^2$ site. At 12 hr after irradiation, very slight erythema was observed at the 5–20 J/$cm^2$ site, and no edema was observed at the 5–15 J/$cm^2$ site. At 24 hr after irradiation, very slight erythema was observed at the 5 J/$cm^2$ site, well-defined erythema was observed at the 10–20 J/$cm^2$ site and no edema was observed at the 5–20 J/$cm^2$ site. At 48 hr after irradiation, very slight erythema was observed at the 5–15 J/$cm^2$ site, well-defined erythema was observed at the 15–20 J/$cm^2$ site, no edema was observed at the 5–15 J/$cm^2$ site, and very slight edema was observed at the 15–20 J/$cm^2$ site. At 72 hr after irradiation, very slight erythema was observed at the 5–15 J/$cm^2$ site, well-defined erythema was

TABLE 2.—Histopathological findings from 1 to 72 hr.

| Animal No. | 2 | | | | | 3 | | | | | 4 | | | | | 5 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time (hour) after UVA irradiation | 1 | | | | | 24 | | | | | 48 | | | | | 72 | | | | |
| Findings UVA dose (J/cm$^2$) | 0 | 5 | 10 | 15 | 20 | 0 | 5 | 10 | 15 | 20 | 0 | 5 | 10 | 15 | 20 | 0 | 5 | 10 | 15 | 20 |
| Skin (back) | | | | | | | | | | | | | | | | | | | | |
| Necrosis, basal/prickle cell layer, epidermis | − | − | − | − | − | − | ± | + | 2+ | + | − | ± | + | 2+ | 2+ | − | ± | 2+ | 2+ | 2+ |
| Vesicle formation, dermal–epidermal junction | − | − | − | − | − | − | − | − | − | − | − | − | + | 3+ | 3+ | − | − | + | + | 3+ |
| Regeneration, epidermis | − | − | − | − | − | − | − | − | − | − | − | − | − | ± | ± | − | − | − | ± | ± |
| Cellular infiltration, epidermis | − | − | − | − | − | − | − | − | − | − | − | − | ± | ± | ± | − | − | ± | ± | ± |
| Cellular infiltration, vesicle | − | − | − | − | − | − | − | − | − | − | − | − | 2+ | 2+ | 2+ | − | − | 2+ | 2+ | 2+ |
| Cellular infiltration, dermis | − | − | − | − | − | − | ± | ± | ± | ± | − | ± | ± | ± | ± | − | ± | ± | ± | ± |

*Note*: UVA, ultraviolet-A; −, no abnormal changes; ±, very slight; +, slight; 2+, moderate; 3+, marked.

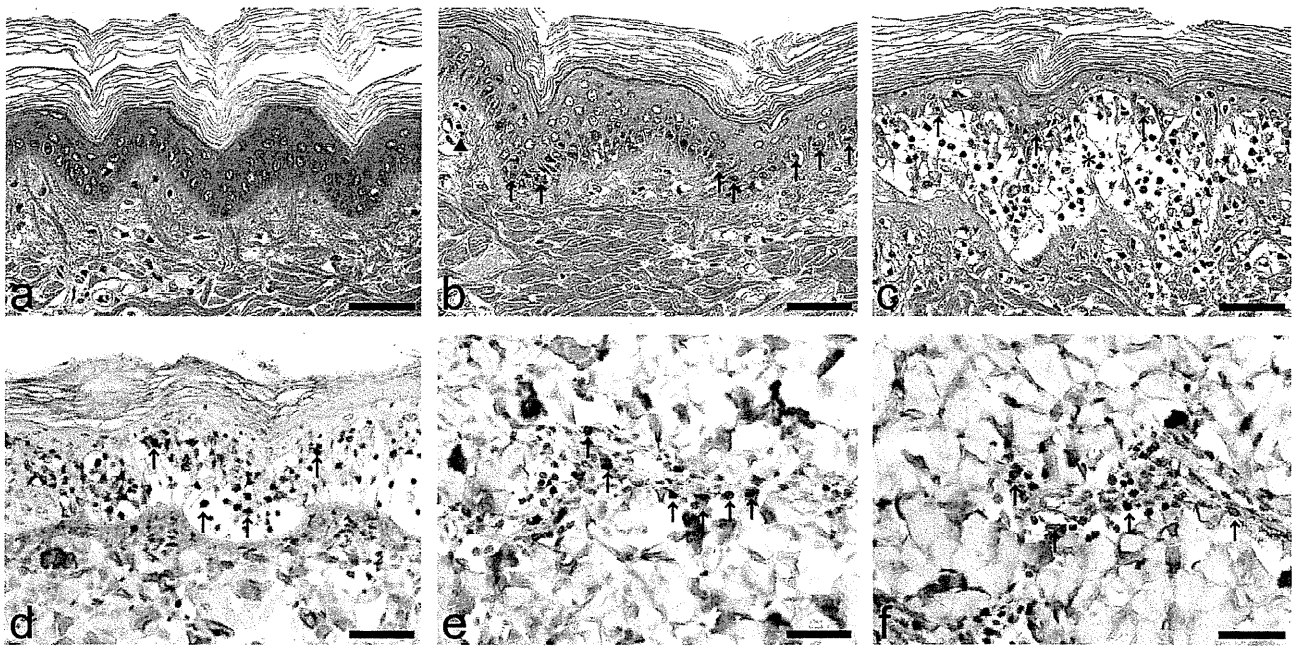


FIGURE 3.—Histopathological examination (a–c: Hematoxylin–Eosin [HE] stain). (a) At 1 hr after irradiation, no abnormal changes were observed at the 20 J/cm$^2$ irradiation sites. (b) At 24 hr after irradiation, necrosis of the basal and/or prickle epidermal cell layer (arrows), and cellular infiltration in the dermis (triangle) were observed at the 20 J/cm$^2$ irradiation sites. (c) At 72 hr after irradiation, necrosis of basal and/or prickle epidermal cell layer (arrows), vesicle formation in the subepidermis (*), and cellular infiltration in the epidermis/dermis were observed at the 20 J/cm$^2$ irradiation sites. Immunohistochemical examination (d–f). At 72 hr after irradiation, (d) Iba-1, (e) CD3, and (f) CD79α (each arrows) were detected, suggesting macrophages, T-cells, and B-cells, respectively. Bars: 50 μm.

observed at the 20 J/cm$^2$ site, no edema was observed at the 5–10 J/cm$^2$ site, and very slight edema was observed at the 15–20 J/cm$^2$ site (Tables 1, Figure 2c and d). Clinical manifestations of erythema and edema in this study were consistent with those reported previously in humans (Lipsky and Baker 1999). Macroscopic examination showed that erythema first appeared from 12 hr after irradiation. While erythema at the low-intensity irradiation sites decreased with time, edema at the high-intensity sites was observed at 48 and 72 hr after irradiation.

Histopathological findings from 1 to 72 hr after irradiation are shown in Table 2. At 1 hr after irradiation, no histopathological changes were observed in any animal (Figure 3a). Necrosis of the basal and/or prickle cell layer of the epidermis was observed from 24 hr after irradiation (Figure 3b). This lesion was considered an initial change from dermal phototoxicity. Vesicle formation in the dermal–epidermal junction and cellular infiltration in the vesicle was observed from 48 hr after irradiation (Figure 3c). These changes tended to be more severe at high-intensity sites than at low-intensity sites. Regeneration of the epidermis observed from 48 hr after irradiation was considered a reactive change against necrosis of the basal and/or prickle cell layer of the epidermis.

Similar histopathological lesions, such as cellular infiltration in the auricle, had been observed in mice that received a single intravenous administration of 100 mg/kg ciprofloxacin at 96 hr after irradiation (Yabe et al. 2005). To our knowledge, time-dependent histopathological examinations of other experimental animals have not been conducted and vesicle formation in the subepidermis has not been reported. It is reported that photosensitivity reactions may generally appear within a few days and these vesicular lesions are similar to bullous eruption, a known dermatological side effect associated with fluoroquinolone in humans (Lipsky and Baker 1999).

Cellular infiltration observed in the epidermis, vesicles, and dermis at the 20 J/cm$^2$ UVA irradiation sites at 72 hr was examined immunohistochemically. Iba-1 positive cell infiltration was observed mainly in the epidermis and vesicles, suggesting mainly macrophages (Figure 3d) because positive staining was detected in macrophages such as Ito cells in the liver. CD3 positive cell infiltration was observed mainly in the dermis, suggesting T-cell (Figure 3e) because positive staining was detected in lymphocytes in the region around splenic lymph nodule and/or white pulp arteries such as sheathed artery. CD79α positive cell infiltration was also observed mainly in the dermis, suggesting B-cells (Figure 3f) because positive staining was detected in lymphocytes in the splenic lymph nodule regions. To our knowledge, immunohistochemical staining techniques for macrophages, T-cells, and B-cells using antibodies against Iba-1, CD3, and CD79α have not been previously reported for the MMPig. Therefore, this immunohistochemical information is considered helpful for further experiments with the MMPig.

In conclusion, this first report of a dermal phototoxicity study in the MMPig demonstrates its potential suitability as a new experimental animal model.

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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

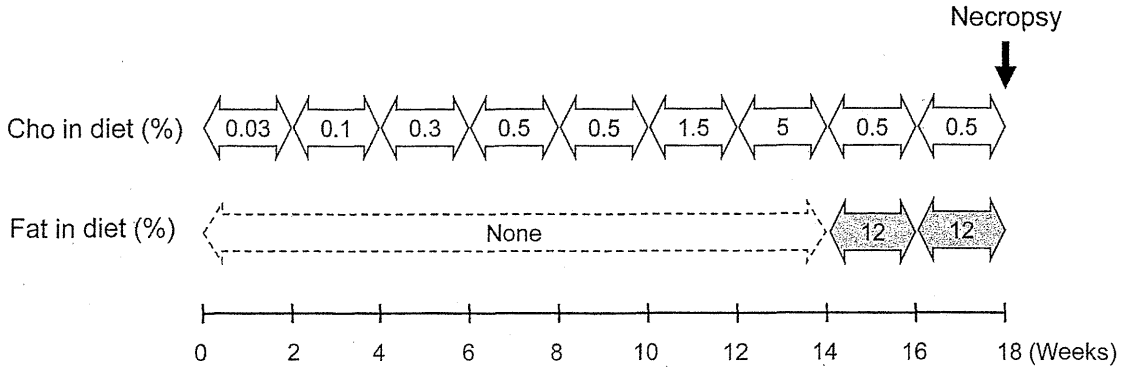


Figure 1. *Experiment B: Study design. Cho: cholesterol.*

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

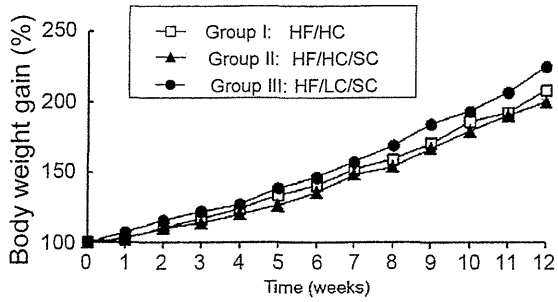


Figure 2. *Experiment A: Body weight gain. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate. Data are percentages, n=2.*

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).

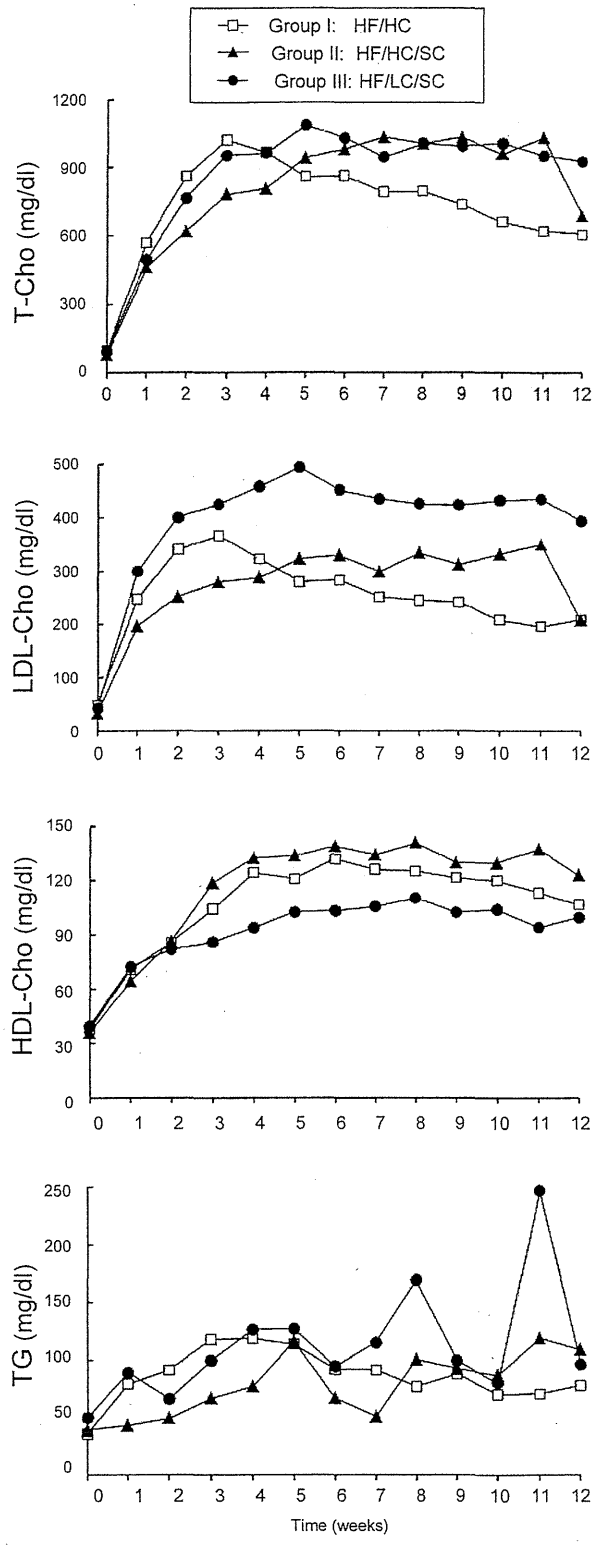


Figure 3. *Experiment A: Biochemical parameters of lipid metabolism. HF: High fat, HC: high cholesterol, LC: low cholesterol, SC: sodium cholate. T-cho: total cholesterol, LDL-Cho: low-density lipoprotein cholesterol, HDL-Cho: high-density lipoprotein cholesterol, TG: triglycerides.*

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

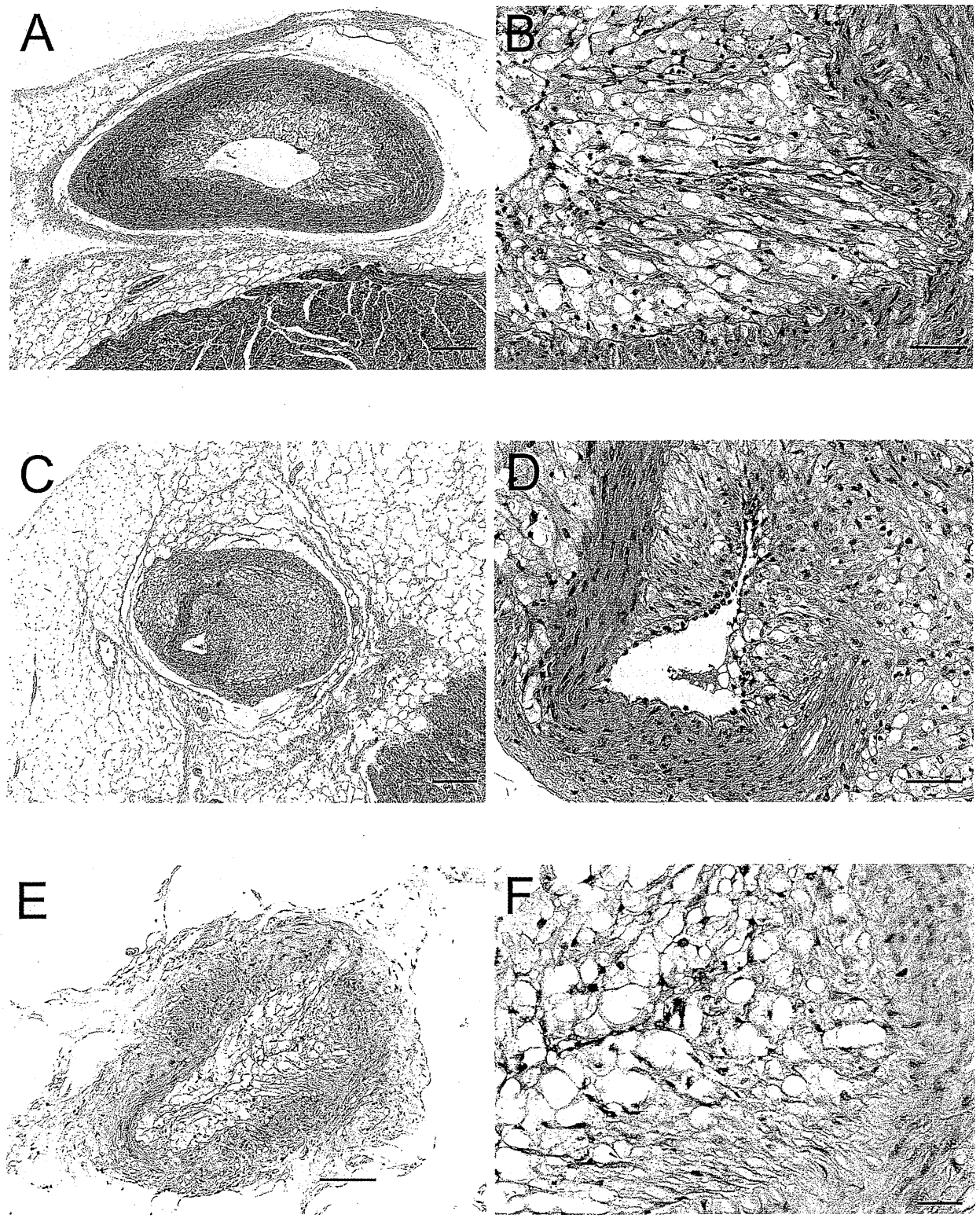


Figure 4. *Experiment A: Microscopic appearance of atherosclerotic lesions. Stenosis (approximately 70%) is shown in the coronary artery (A) and considerable foamy cell infiltration is shown in the thickening site (B). Severe stenosis (approximately 90%) is shown in the coronary artery (C) and considerable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

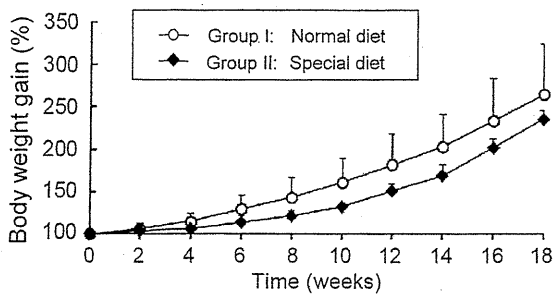


Figure 5. *Experiment B: Body weight gain.*

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.

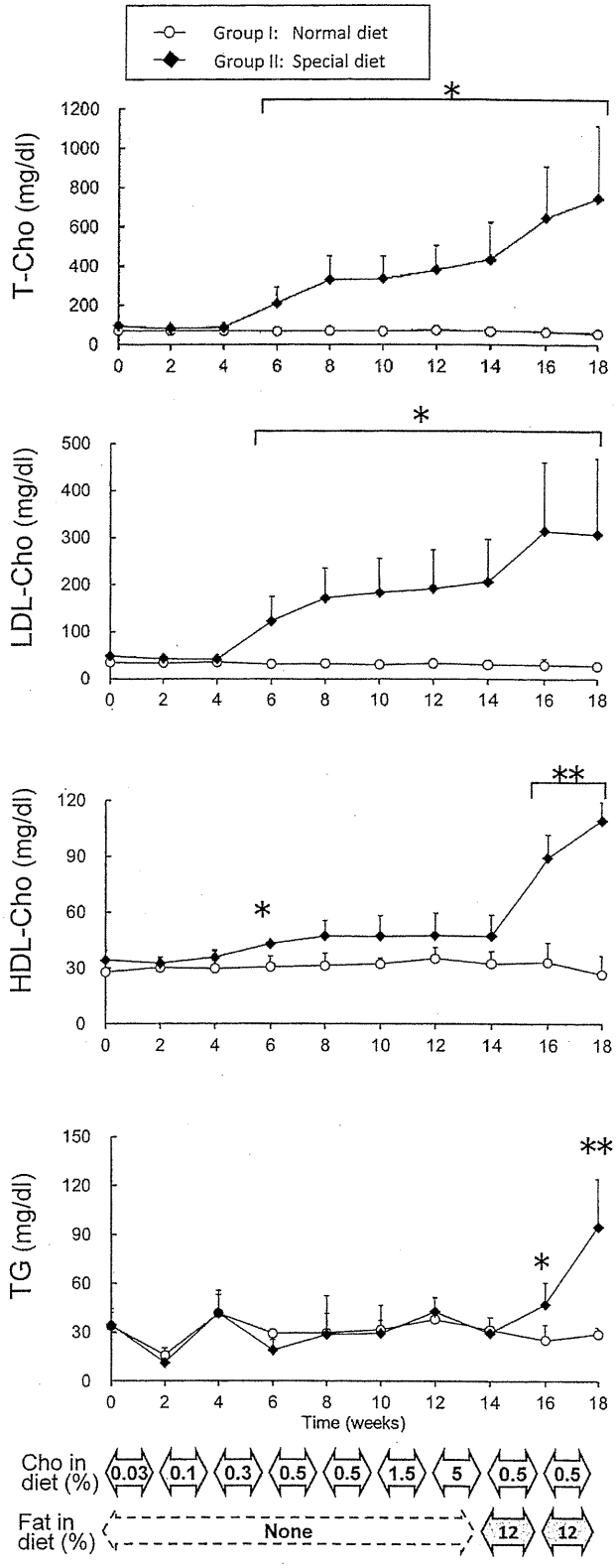


Figure 6. *Experiment B: Biochemical parameters of lipid metabolism. T-Cho: total cholesterol, LDL-Cho: low-density lipoprotein cholesterol, HDL-Cho: high-density lipoprotein cholesterol, TG: triglycerides. *p<0.05, **p<0.01; significantly different from group I.*