Table 6-76 Blood chemistry in male microminipigs

Study No. SBL703-023

Group	Animal No.	CM-TGL (mg/dL) 25w	28w	33w	37w	41w	45w	49w
1	1	0	1	1	2	2	2	2
	2	1	2					
	3	0	2	1				
	5	2	3	0	1	1	2	2
	Mean	0.8	2.0	0.7	1.5	1.5	2.0	2.0
	±S.D.	1.0	0.8	0.6				
2	6	7	3	3	3	4	2	4
	7	7	3	4	2	3	2	4
	8	2	2	1	1	1	3	
	9	2	3	1	2	3	2	3
	10	3	5	5	1	3	6	4
	Mean	4.2	3.2	2.8	1.8	2.8	3.0	3.8
	±S.D.	2.6	1.1	1.8	0.8	1.1	1.7	0.5
3	11	4	18	2	2	3	9	5
	12	4	3	2	4	4	7	4
	13	2	4	3	3	7	5	4
	14	2	2	1	2	2	3	2
	15	3	1	1	1	1	3	2
	Mean	3.0	5.6	1.8	2.4	3.4	5.4	3.4
	±S.D.	1.0	7.0	0.8	1.1	2.3	2.6	1.3

Note) CM-TGL : Chylomicron-Triglycerides

Table 7 Necropsy in male microminipigs

Study No. SBL703-023

Group	1		2				3				
Findings / Animal No.	1	5	6	7	9	10	11	12	13	14	15
Pericardial cavity											
Retention, pericardial fluid	-	-	-	-	-	-	-	-	-	P	P
Aorta											
White striation, abdominal aorta	-	-	-	-	-	-	-	-	-	P	P
Heart											
Red focus, epicardium	-	-	-	-	-	-	-	-	-	P	P
Carotid artery											
Thickening, vascular wall	-	-	-	-	-	P	-	-	-	-	-
Nodule, intravascular	-	-	-	-	-	-	-	-	P	-	-
Induration, vascular wall	-	-	-	-	-	-	-	-	-	P	P
Kidney											
Cyst, left	-	P	-	-	-	-	-	-	-	-	-

Notes) - : No abnormal changes P : Non-graded changes

Table 8-1 Organ weight in male microminipigs Study No. SBL703-023

Group	Animal No.	Pituit. mg	Thyroid g	Adre.R g	Adre.L g	Adre.R&L g	Test.R g	Test.L g	Test.R&L g	Thymus g	Spleen g	Brain g	Heart g
1	1	204	3.26	1.17	1.08	2.25	26.9	26.2	53.1	11.1	74.0	71.8	114.3
	5	109	1.54	0.72	0.66	1.38	16.6	19.6	36.2	5.7	23.1	62.5	60.0
	Mean	156.5	2.400	0.945	0.870	1.815	21.75	22.90	44.65	8.40	48.55	67.15	87.15
2	6	254	3.31	0.78	0.90	1.68	19.1	20.1	39.2	17.8	95.5	71.1	133.0
	7	205	2.81	0.94	0.96	1.90	24.9	27.9	52.8	16.4	103.2	80.4	97.7
	9	146	2.70	0.77	0.72	1.49	29.2	30.4	59.6	24.7	182.0	67.2	82.5
	10	229	2.59	1.26	1.20	2.46	24.6	25.0	49.6	24.8	152.3	70.5	133.6
	Mean	208.5	2.853	0.938	0.945	1.883	24.45	25.85	50.30	20.93	133.25	72.30	111.70
	±S.D.	46.2	0.318	0.229	0.198	0.420	4.14	4.42	8.49	4.45	41.10	5.67	25.70
3	11	169	2.91	1.11	1.01	2.12	33.0	30.1	63.1	13.9	73.1	64.4	114.9
	12	183	2.39	0.86	0.95	1.81	25.1	25.2	50.3	11.6	67.7	79.2	106.0
	13	162	2.99	0.97	1.05	2.02	38.7	41.9	80.6	17.5	165.2	66.4	110.6
	14	129	2.06	0.87	0.98	1.85	29.0	33.6	62.6	23.0	45.7	66.5	108.8
	15	216	4.63	0.82	0.96	1.78	26.9	29.8	56.7	34.8	61.2	76.1	136.9
	Mean	171.8	2.996	0.926	0.990	1.916	30.54	32.12	62.66	20.16	82.58	70.52	115.44
	±S.D.	31.7	0.990	0.117	0.041	0.147	5.43	6.23	11.30	9.25	47.31	6.65	12.42

Notes) Pituit. : Pituitary
Adre.R : Adrenal (Right)
Adre.L : Adrenal (Left)
Adre.R&L : Adrenal (Right&Left)
Test.R : Testis (Right)
Test.L : Testis (Left)
Test.R&L : Testis (Right&Left)

Table 8-2 Organ weight in male microminipigs Study No. SBL703-023

Group	Animal No.	Liver g	Kidn.R g	Kidn.L g	Kidn.R&L g	Epid.R g	Epid.L g	Epid.R&L g	Sem.V. g	Prost. g	Omentum g	Mes.adip. g
1	1	466.3	61.4	63.9	125.3	13.2	13.2	26.4	114.2	9.9	37.6	129.5
	5	172.2	19.7	19.9	39.6	12.8	13.6	26.4	47.4	8.1	1.3	23.7
	Mean	319.25	40.55	41.90	82.45	13.00	13.40	26.40	80.80	9.00	19.45	76.60
2	6	412.3	63.7	65.9	129.6	10.7	11.7	22.4	75.5	11.3	88.8	296.8
	7	433.1	49.9	51.8	101.7	15.2	17.9	33.1	71.7	9.2	45.7	113.6
	9	323.2	42.9	43.4	86.3	16.0	18.0	34.0	103.7	14.1	18.5	107.2
	10	401.7	67.3	62.1	129.4	15.5	15.7	31.2	305.9	14.6	33.1	132.2
	Mean	392.58	55.95	55.80	111.75	14.35	15.83	30.18	139.20	12.30	46.53	162.45
	±S.D.	48.05	11.49	10.19	21.44	2.46	2.95	5.31	112.05	2.53	30.30	90.19
3	11	490.3	53.6	57.6	111.2	13.9	14.8	28.7	235.7	12.2	32.3	105.7
	12	424.9	44.8	45.5	90.3	13.1	12.5	25.6	106.8	11.2	56.2	86.7
	13	366.6	51.0	50.3	101.3	19.9	20.9	40.8	86.7	14.3	32.3	168.4
	14	268.2	32.9	34.9	67.8	24.4	21.2	45.6	343.4	10.1	13.4	46.0
	15	320.1	51.9	54.0	105.9	13.5	13.4	26.9	296.9	16.6	27.6	88.3
	Mean	374.02	46.84	48.46	95.30	16.96	16.56	33.52	213.90	12.88	32.36	99.02
	±S.D.	86.99	8.47	8.81	17.19	5.01	4.18	9.07	113.78	2.59	15.42	44.54

Notes) Kidn.R : Kidney (Right) Kidn.L : Kidney (Left) Kidn.R&L : Kidney (Right&Left)
Epid.R : Epididymis (Right) Epid.L : Epididymis (Left) Epid.R&L : Epididymis (Right&Left)
Sem.V. : Seminal vesicle Prost. : Prostate Omentum : Omentum
Mes.adip. : Mesenteric adipose tissue (including mesenteric lymph node)

Table 8-3 Relative organ weight in male microminipigs Study No. SBL703-023

Group	Animal No.	B.W. kg	Pituit. mg/kg	Thyroid g/kg	Adre.R g/kg	Adre.L g/kg	Adre.R&L g/kg	Test.R g/kg	Test.L g/kg	Test.R&L g/kg	Thymus g/kg	Spleen g/kg	Brain g/kg	Heart g/kg
1	1	(29.0)	7.0	0.112	0.040	0.037	0.078	0.93	0.90	1.83	0.38	2.55	2.48	3.94
	5	(10.9)	10.0	0.141	0.066	0.061	0.127	1.52	1.80	3.32	0.52	2.12	5.73	5.50
	Mean	(19.95)	8.50	0.1265	0.0530	0.0490	0.1025	1.225	1.350	2.575	0.450	2.335	4.105	4.720
2	6	(38.3)	6.6	0.086	0.020	0.023	0.044	0.50	0.52	1.02	0.46	2.49	1.86	3.47
	7	(29.2)	7.0	0.096	0.032	0.033	0.065	0.85	0.96	1.81	0.56	3.53	2.75	3.35
	9	(21.2)	6.9	0.127	0.036	0.034	0.070	1.38	1.43	2.81	1.17	8.58	3.17	3.89
	10	(30.0)	7.6	0.086	0.042	0.040	0.082	0.82	0.83	1.65	0.83	5.08	2.35	4.45
	Mean	(29.68)	7.03	0.0988	0.0325	0.0325	0.0653	0.888	0.935	1.823	0.755	4.920	2.533	3.790
	±S.D.	(6.99)	0.42	0.0194	0.0093	0.0070	0.0159	0.365	0.378	0.741	0.318	2.662	0.560	0.497
3	11	(26.6)	6.4	0.109	0.042	0.038	0.080	1.24	1.13	2.37	0.52	2.75	2.42	4.32
	12	(24.5)	7.5	0.098	0.035	0.039	0.074	1.02	1.03	2.05	0.47	2.76	3.23	4.33
	13	(24.5)	6.6	0.122	0.040	0.043	0.082	1.58	1.71	3.29	0.71	6.74	2.71	4.51
	14	(20.7)	6.2	0.100	0.042	0.047	0.089	1.40	1.62	3.02	1.11	2.21	3.21	5.26
	15	(29.5)	7.3	0.157	0.028	0.033	0.060	0.91	1.01	1.92	1.18	2.07	2.58	4.64
	Mean	(25.16)	6.80	0.1172	0.0374	0.0400	0.0770	1.230	1.300	2.530	0.798	3.306	2.830	4.612
	±S.D.	(3.23)	0.57	0.0242	0.0060	0.0053	0.0109	0.273	0.338	0.601	0.330	1.945	0.371	0.386

Notes) Pituit. : Pituitary
Adre.R : Adrenal (Right)
Adre.L : Adrenal (Left)
Adre.R&L : Adrenal (Right&Left)
Test.R : Testis (Right)
Test.L : Testis (Left)
Test.R&L : Testis (Right&Left)

Table 8-4 Relative organ weight in male microminipigs Study No. SBL703-023

Group	Animal No.	Liver g/kg	Kidn.R g/kg	Kidn.L g/kg	Kidn.R&L g/kg	Epid.R g/kg	Epid.L g/kg	Epid.R&L g/kg	Sem.V. g/kg	Prost. g/kg	Omentum g/kg	Mes.adip. g/kg
1	1	16.08	2.12	2.20	4.32	0.46	0.46	0.91	3.94	0.34	1.3	4.5
	5	15.80	1.81	1.83	3.63	1.17	1.25	2.42	4.35	0.74	0.1	2.2
	Mean	15.940	1.965	2.015	3.975	0.815	0.855	1.665	4.145	0.540	0.70	3.35
2	6	10.77	1.66	1.72	3.38	0.28	0.31	0.58	1.97	0.30	2.3	7.7
	7	14.83	1.71	1.77	3.48	0.52	0.61	1.13	2.46	0.32	1.6	3.9
	9	15.25	2.02	2.05	4.07	0.75	0.85	1.60	4.89	0.67	0.9	5.1
	10	13.39	2.24	2.07	4.31	0.52	0.52	1.04	10.20	0.49	1.1	4.4
	Mean	13.560	1.908	1.903	3.810	0.518	0.573	1.088	4.880	0.445	1.48	5.28
	±S.D.	2.023	0.273	0.183	0.451	0.192	0.224	0.418	3.769	0.173	0.62	1.69
3	11	18.43	2.02	2.17	4.18	0.52	0.56	1.08	8.86	0.46	1.2	4.0
	12	17.34	1.83	1.86	3.69	0.53	0.51	1.04	4.36	0.46	2.3	3.5
	13	14.96	2.08	2.05	4.13	0.81	0.85	1.67	3.54	0.58	1.3	6.9
	14	12.96	1.59	1.69	3.28	1.18	1.02	2.20	16.59	0.49	0.6	2.2
	15	10.85	1.76	1.83	3.59	0.46	0.45	0.91	10.06	0.56	0.9	3.0
	Mean	14.908	1.856	1.920	3.774	0.700	0.678	1.380	8.682	0.510	1.26	3.92
	±S.D.	3.106	0.199	0.190	0.380	0.301	0.245	0.544	5.234	0.057	0.64	1.79

Notes) Kidn.R : Kidney (Right) Kidn.L : Kidney (Left) Kidn.R&L : Kidney (Right&Left)
Epid.R : Epididymis (Right) Epid.L : Epididymis (Left) Epid.R&L : Epididymis (Right&Left)
Sem.V. : Seminal vesicle Prost. : Prostate Omentum : Omentum
Mes.adip. : Mesenteric adipose tissue (including mesenteric lymph node)

II　研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hiroaki KAWAGUCHI, Tomonobu YAMADA, Naoki MIURA, Yoshihiro TAKAHASHI Tsuyoshi YOSHIKAWA, Hiroyuki IZUMI, Tatsuo KAWARASAKI, Noriaki MIYOSHI, Akihide TANIMOTO	Reference Values of Hematological and Biochemical Parameters for the World Smallest Microminipigs	J. Vet. Med. Sci.	74(7)	933–936	2012
Tsuyoshi YOSHIKAWA, Yoshihiro TAKAHASHI, Hiroaki KAWAGUCHI, Shinji UTSUNOMIYA, Naoki MIURA, Hiroyuki IZUMI, Noriaki MIYOSHI, Akihide TANIMOTO	A Dermal Phototoxicity Study Following Intravenous Infusion Administration of Ciprofloxacin Hydrochloride in the Novel Microminipigs	Toxicol. Pathol.	41	109-113	2013
Naoki MIURA, Hiroaki KAWAGUCHI, Tomoka NAGASATO, Tomonobu YAMADA, Takashi ITO, Hiroyiki IZUMI, Hisayo SHAMESHIMA, Noriaki MIYOSHI, Akihide TANIMOTO, Ikuro MARUYAMA	Coagulation Activity and White Thrombus Formation in the Microminipig	in vivo	27	in press	2013

III　研究成果の刊行物・別刷

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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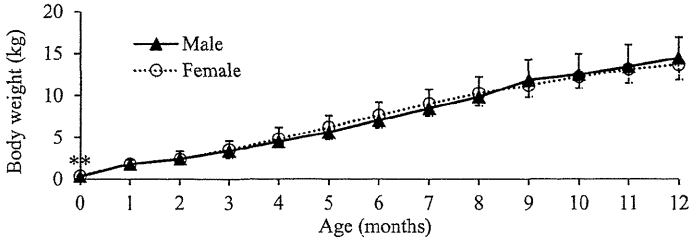

Fig. 1. Body weight of the Microminipig. $^{**}P<0.01$; significantly different for males vs. females.

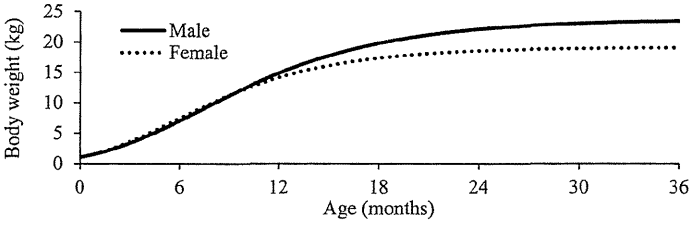

Fig. 2. Growth curve of the Microminipig predicted by Gompertz function.

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 μl 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.

ACKNOWLEDGMENTS. This work was partly supported by Health Labour Sciences Research Grant (No. 33361105) from the Ministry of Health, Labour and Welfare of Japan

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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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

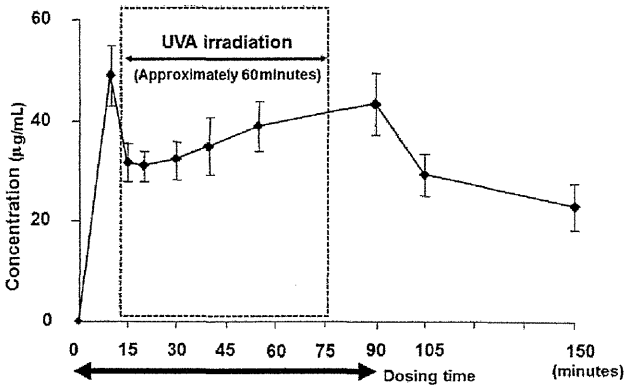

FIGURE 1.—Mean plasma concentrations of CPFX after single intravenous infusion at a dose of 100 mg/kg to three MMPigs in a pilot experiment.

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

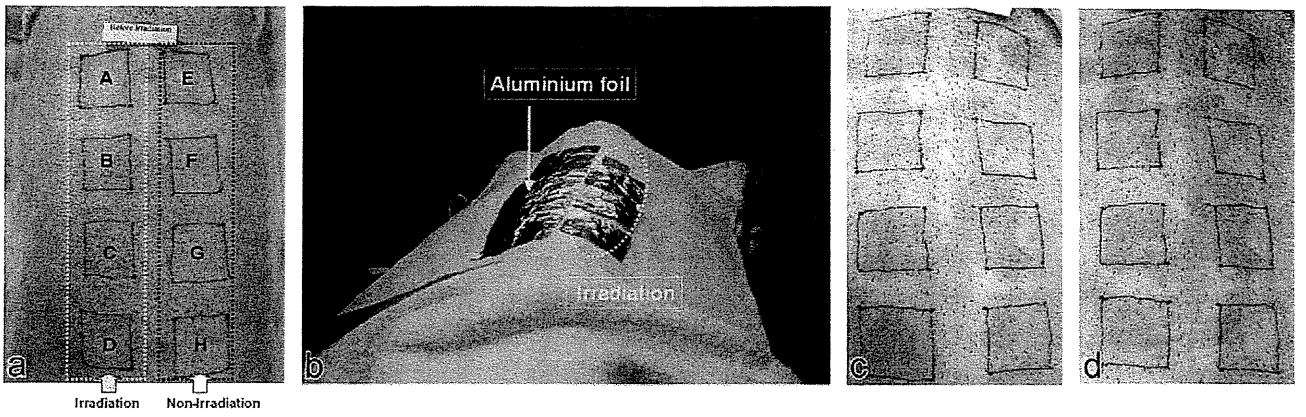

FIGURE 2.—(a) Back skin of the microminipig in the Animal No. 5. Irradiation site is A–D and nonirradiation site is E–H. (b) Irradiation was initiated with all sites other than the 20 J/ cm^2 ultraviolet-A (UVA) site covered with aluminum foil. Macroscopic examination. (c) At 24 hr after irradiation,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

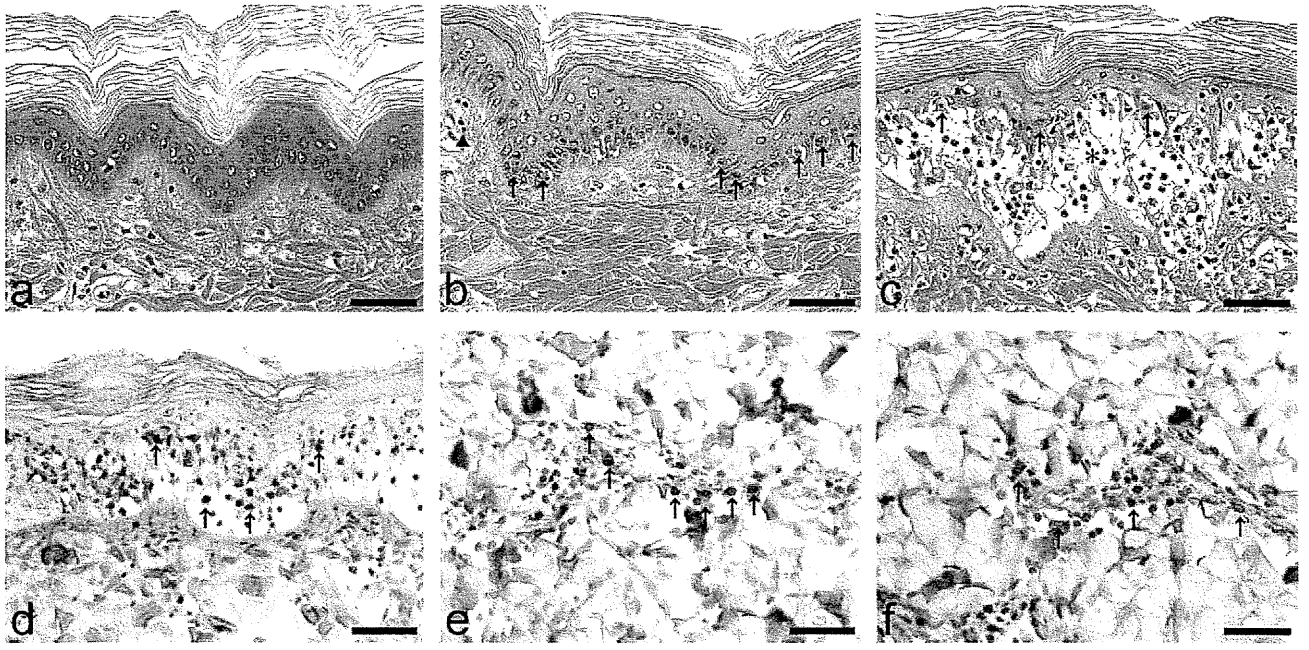

FIGURE 3.—Histopathological examination (a–c: Hematoxylin–Eosin [HE] stain). (a) At 1 hr after irradiation, no abnormal changes were observed at the 20 J/cm^2 irradiation sites. (b) At 24 hr after irradiation, necrosis of the basal and/or prickle epidermal cell layer (arrows), and cellular infiltration 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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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%

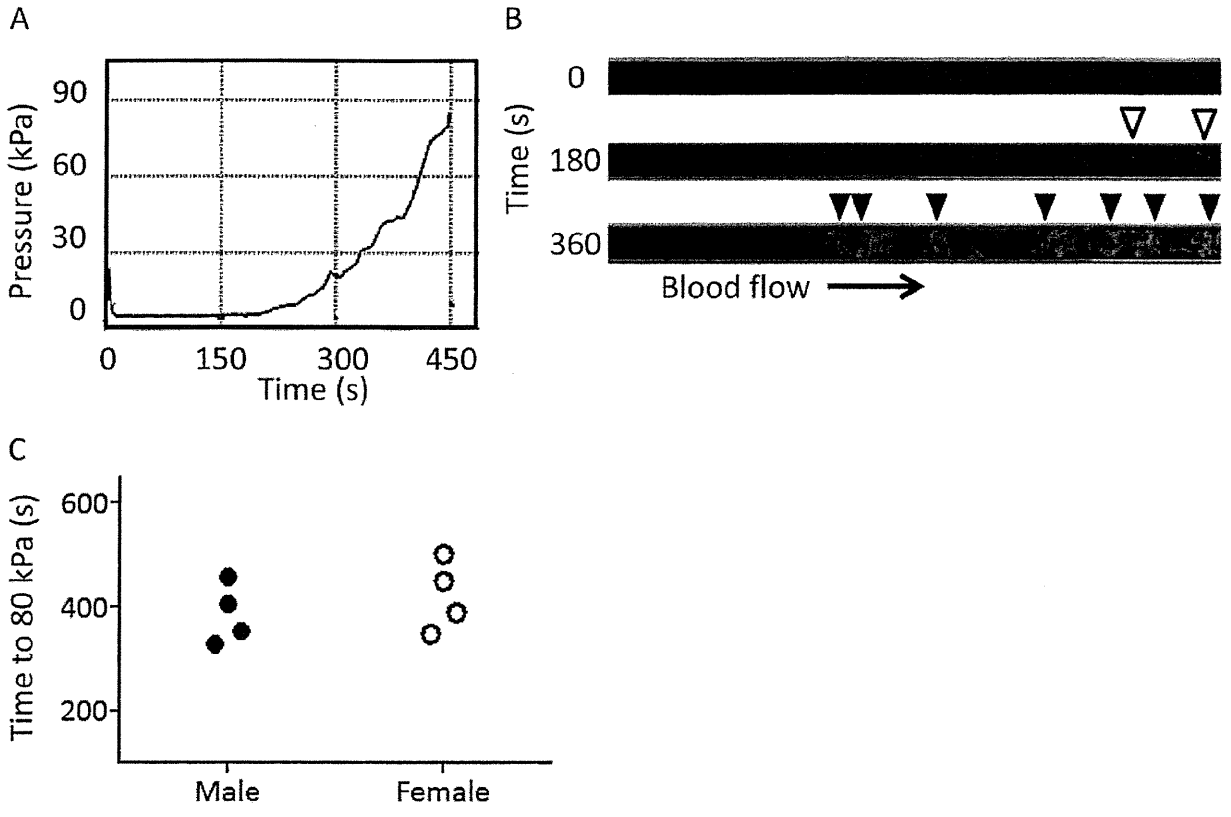

Figure 1. *A: The flow-pressure waveform pattern during white thrombus formation (WTF). The black line represents the increasing pressure resulting from occlusion in the microchip flow-chamber by white thrombus formation. B: A typical image of white thrombus in the microchip flow-chamber. Open arrowheads 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].