

## Forum Minireview

## Microminipig, a Non-rodent Experimental Animal Optimized for Life Science Research: Novel Atherosclerosis Model Induced by High Fat and Cholesterol Diet

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**Abstract.** Atherosclerotic lesions were observed in male and ovariectomized female Microminipig (MMP) fed a high fat and cholesterol diet with sodium cholate (HFCD/SC) for 3 months. HFCD/SC induced hypercholesterolemia accompanied by an increase in serum total cholesterol (T-Chol), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and cholesterol ester (CE). Unlike the mouse or rabbit, a dominant LDL-C fraction in the intact MMP, similar to that in humans, was observed by serum lipoprotein analysis. HFCD/SC increased body weight gain. At the end of the experiment, computed tomography scans of conscious animals showed that HFCD/SC had decreased liver attenuation values (Hounsfield unit) and increased subcutaneous and abdominal fat, suggesting the induction of fatty liver and obesity. HFCD/SC induced atherosclerotic lesions in systemic arteries, including the external and internal iliac arteries, abdominal aorta, coronary artery, and cerebral arterial circle. Atherosclerosis and pathological findings induced by HFCD/SC in MMP were similar to those in humans. The MMP is a potentially suitable tool for investigating human atherosclerosis.

**Keywords:** atherosclerosis, cholesterol, diet, life-style, Microminipig

### 1. Introduction

Atherosclerosis is known as a predominant risk factor in cardiovascular diseases and is closely related to serious morbidity and mortality reported in the Western world (1). In Japan in recent years, westernization of

life-style, including diet, may account for the increased incidence of coronary and cerebral artery diseases (2 – 4). These diseases are closely related to the onset mechanism of atherosclerosis.

Recently, the Fuji Micra Inc. Microminipig (Brand name, Microminipig: MMP; registered with the Japanese Ministry of Agriculture, Forestry and Fisheries as a novel variety of swine) has emerged as an experimental animal model for non-clinical pharmacological/toxicological studies. The MMP is the smallest of the general minipigs

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(e.g., Clawn, Göttingen, and Yucatan) for experimental use. Very recently, we established an MMP atherosclerosis model by feeding a high fat and cholesterol diet (5).

In this review, we describe pathophysiological similarities between the MMP model and patients with cardiovascular disease.

## 2. Appropriate atherosclerosis animal models

The development of atherosclerosis models has been attempted in experimental animals such as apolipoprotein E deficient (apoE<sup>-/-</sup>) mice (6, 7) and Watanabe heritable hyperlipidemic (WHHL) rabbits lacking low-density lipoprotein receptors (8, 9), which have been reported as atherosclerosis models with genetic abnormalities. The vulnerable plaques of human coronary arteries are histologically characterized by a large lipid core and a thin fibrous cap with inflammatory cells (10). Some plaques in myocardial infarction-prone WHHL rabbits contain a lipid core and a thin fibrous cap (10). As shown in Table 1, however, the rabbit and mouse differ from humans in lipid metabolism and some environmental factors (11, 12). Wild-type mice are quite resistant to atherosclerosis as a result of high levels of antiatherosclerotic high-density lipoprotein cholesterol (HDL-C) and low levels of proatherogenic low-density lipoprotein cholesterol (LDL-C) and very-low-density lipoprotein cholesterol (VLDL-C) (13), while apoE<sup>-/-</sup> mice show morphological evidence of fibrous caps in atherosclerotic lesions (14). Any effort to create an animal model of plaque rupture must presuppose the existence of a fibrous cap overlying a necrotic core; this combination is required for plaque rupture in humans (14). Atherosclerosis is induced by both genetic and environmental factors and should therefore be investigated in an appropriate model that can re-

flect the clinical pathogenesis. Nutritional manipulation by feeding high-fat and -cholesterol diets should be used to develop such models. In the MMP, control of diet (high-fat and -cholesterol diet) alone was sufficient to induce atherosclerotic lesions similar to those in humans, probably due to similarities in both genetic and environmental factors, as shown in Table 1 (5).

## 3. Minipigs in life science research

The wild boar, *Sus scrofa*, is said to be the ancestor of all modern breeds of swine. Although there is evidence of domestication in Europe some 3,500 years ago, the cradle of the domesticated swine has been claimed as being in China about 10,000 years ago (15). Swine have been used extensively in biomedical research, which has increased significantly in recent decades (15). For example, more than 60,000 pigs have been used in a year in the EU (16). Because of their well-accepted physiological and anatomical similarities to humans (Table 1), swine are considered to be increasingly attractive toxicological and pharmacological models. Recently, minipigs such as Clawn, Göttingen, Yucatan, Sinclair, and Hanford, smaller than domestic swine, have been developed (17, 18). For use in pharmacological experiments, hepatic enzyme activities of drug metabolism in the MMP have been measured (19). The skin of the minipig has been reported to be morphometrically similar to that of humans (20). Moreover, we observed that the skin of MMP is histopathologically similar to that of humans.

## 4. Minipigs in atherosclerosis research

Generally, atherosclerosis can be induced in the minipig simply by controlling diet, which is not the case for the mouse or rabbit. Therefore, the minipig is thought

**Table 1.** Comparison of genetic and environmental factors affecting humans and experimental animals

Species	Genetic factors		Environmental factors		
	Lipid metabolism (dominant LDL-C fraction in serum lipoprotein analysis)	Anatomy of coronary arteries (number of arteries)	Diet	Mealtime	Behavior (active period)
Human	Yes	3	polyphagia	schedule	diurnal
Swine (MMP)	Yes	3	polyphagia	schedule	diurnal
Dog	No	2	monophagia	schedule	diurnal
Monkey	Yes	3	monophagia	schedule	diurnal/nocturnal
Rabbit	No	3	monophagia	<i>ad libitum</i>	nocturnal
Mouse	No	4	polyphagia	<i>ad libitum</i>	nocturnal

In the MMP (Microminipig), both genetic and environmental factors are similar to those of humans. However, the dog, rabbit, and mouse differ from humans in lipid metabolism and some environmental factors, and the monkey differs from humans in environmental factors such as monophagia and behavior time.

to be an appropriate experimental animal for research into atherosclerosis (3, 21). Diet is one of the important environmental factors that may develop the pathology of atherosclerosis. Atherosclerosis in rabbit and mouse models showed stable plaque (3, 4, 6, 22, 23), unlike the unstable plaque seen in humans. In humans, the occurrence of organ damage involves a second step, such as the release of plaque and acceleration of inflammation. By using an animal model, the mechanism of organ damage from atherosclerotic lesions can be examined. Related factors include stress from work, stimulation from changes in temperature, and arrhythmia. Swine are more suitable than the mouse or rabbit for analyzing the influence of environmental factors on atherosclerotic lesions because their feeding habits and life rhythms are similar to those of humans (Table 1) (15). The anatomy of the porcine coronary circulation is analogous to that of humans, with three major coronary arteries. In contrast, the dog has essentially a two-vessel system, with a non-dominant right coronary artery supplying only the right ventricle in the vast majority of animals (24). Moreover, the pig has a very limited innate collateral circulation, with only sparse endocardial connections, whereas the dog is endowed with numerous, generally epicardial, innate anastomoses, which are thought to have greater potential for development than those of the pig (24). Swine including MMP are diurnal and are preferable to nocturnal animals for evaluating the influence of night and day on atherosclerotic lesions. As shown in Table 2,

diet control alone was sufficient to induce atherosclerotic lesions in swine such as MMP (5), Göttingen (25), Yucatan (3, 21), Chinese Bama (26), and the domestic pig (27). The shortest period of feeding and lowest percentage of cholesterol-containing diet in swine atherosclerosis models induced by diet control were 3 months and 2%, respectively. The locations of atherosclerotic lesions in these swine studies were the coronary artery and abdominal aorta. Many strains of minipig are difficult to manage due to their large size; for example, the Göttingen has an adult body weight of 30–40 kg even under conditions of a restricted diet (16). The MMP, however, is by far the smallest minipig available, and in our MMP model, only small quantities of a high fat and cholesterol diet were sufficient to induce atherosclerosis (5).

### 5. Atherosclerosis Microminipig model

Animals aged 3 months (body weight:  $4.6 \pm 0.3$  kg) were used (5). Atherosclerotic lesions were induced in male (M) and ovariectomized female (Fx) MMP by feeding a high fat (12%) and high cholesterol (5%) diet with sodium cholate (0.7%) (HFCD/SC) for 3 months, and these were designated M-HFCD/SC and Fx-HFCD/SC, respectively (5). As a control, one male was given a normal diet (ND), designated as M-ND. The body weight of HFCD/SC was greater than that of M-ND, probably due to the difference in calorie intake.

**Table 2.** Comparison of animal characteristics, diet, and location of atherosclerotic lesions in swine atherosclerosis models induced by diet control

Source	Reference No.	Animal characteristics				Diet				Brief location of atherosclerotic lesions
		Breeds	Age (month)	BW (kg)	Sex	Cholesterol	Fat	Others	Period (month)	
Miyoshi N, et al. (2010)	5	Microminipig	3	4.3–4.9	M, Fx	5%	12% Lard	0.7% SC	3	CA, CAC, TA, AA
Takai H, et al. (2008)	25	Göttingen minipig	3	—	M, F	high	—	—	3*	CA, AA
Turk JR, et al. (2005)	3	Yucatan minipig	8–12	25–40	M	2%	17.1% Coconut oil, 2.3% Corn oil	0.7% SC	4–5	CA, AA, BA
Goodrich JA, et al. (2003)	21	Yucatan minipig	6	55	Fx	4%	Lard (40.7% calories)	—	6.5	CA
Zhang C, et al. (2006)	26	Chinese Bama minipig	2	—	M	2%	10% Lard	37% Sucrose	5	AA
Herrmann J, et al. (2007)	27	Domestic pig	3	48–70	F	2%	15% Lard	—	3	CA

The shortest period of feeding in swine atherosclerosis models induced by diet control was three months for the Microminipig, Göttingen, and domestic pig. The lowest percentage of cholesterol-containing diet was 2% for the Yucatan and domestic pig. The locations of atherosclerotic lesions in these swine studies were the coronary artery and abdominal aorta. BW: Body weight, M: male, Fx: ovariectomized female, F: female, SC: sodium cholate, CA: coronary artery, CAC: cerebral arterial circle, TA: thoracic aorta, AA: abdominal aorta, BA: brachial artery. \*All swine were fed a 420 g cholesterol diet (High-cholesterol diet for miniature swine; Nisseiken Co., Ltd., Tokyo) once per day.

### 5.1. Hypercholesterolemia

Serum from the animals fed with HFCD/SC was grossly cloudy-white from the first week. HFCD/SC induced hypercholesterolemia accompanied by an increase in serum total cholesterol (T-Cho), LDL-C, HDL-C, and cholesterol ester (CE) from the first week. Serum T-Cho, LDL-C, and CE levels almost reached maximum at the second week and were maintained throughout the experimental period, and HDL-C gradually increased during the experimental period. Triglyceride (TG) levels in the MMP fed with HFCD/SC showed greater variation during the experimental period (5). Atherosclerosis may have been mainly influenced by cholesterol level and not by TG. This finding is similar to those in human cases (1).

### 5.2. Serum lipoprotein analysis

Serum cholesterol profile was determined by agarose-gel electrophoresis at the end of the experiment. Serum lipoprotein analysis showed a dominant LDL-C fraction in the MMP, as seen in humans, although no dominant LDL-C fractions were seen in the mouse or rabbit (Fig. 1). We designated the ages of 6 months, 40 years, 4 months, and 12 months as mature MMP, human, mouse, and rabbit, respectively. The dominant LDL-C fractions in the animals fed with HFCD/SC shifted to a VLDL-C fraction and the animals fed with HFCD/SC showed greater dominant VLDL-C fractions than M-ND. The precise mechanisms of these findings remain to be resolved.

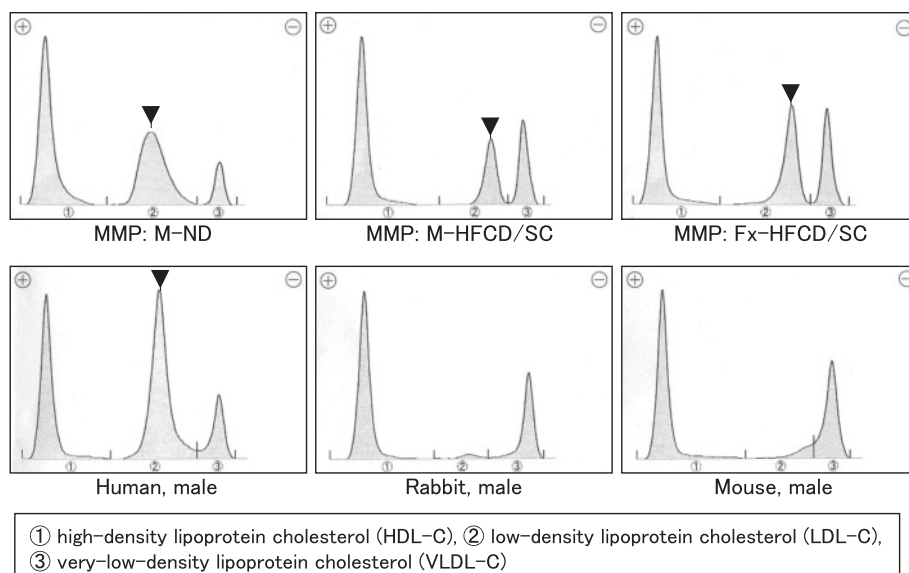
### 5.3. Computed tomography (CT)

After feeding for 3 months (until the day before

necropsy), MMP were examined by CT scanning (Toshiba Medical Systems Corporation, Tochigi), under non-anesthetized conditions due to their mild character and ease of handling. The attenuation values (Hounsfield unit) for the liver in the animals fed with HFCD/SC were decreased (Fig. 2A), suggesting that they were suffering from fatty liver (28, 29). The thickness of subcutaneous fat (Fig. 2B) and the area of visceral fats (fats around the intestines) (Fig. 2C) in the animals fed with HFCD/SC were increased, suggesting the induction of obesity and metabolic syndrome, while no effects on blood pressure and glycemia were observed. No abnormal findings in the brains of the animals fed with HFCD/SC were detected by CT scanning.

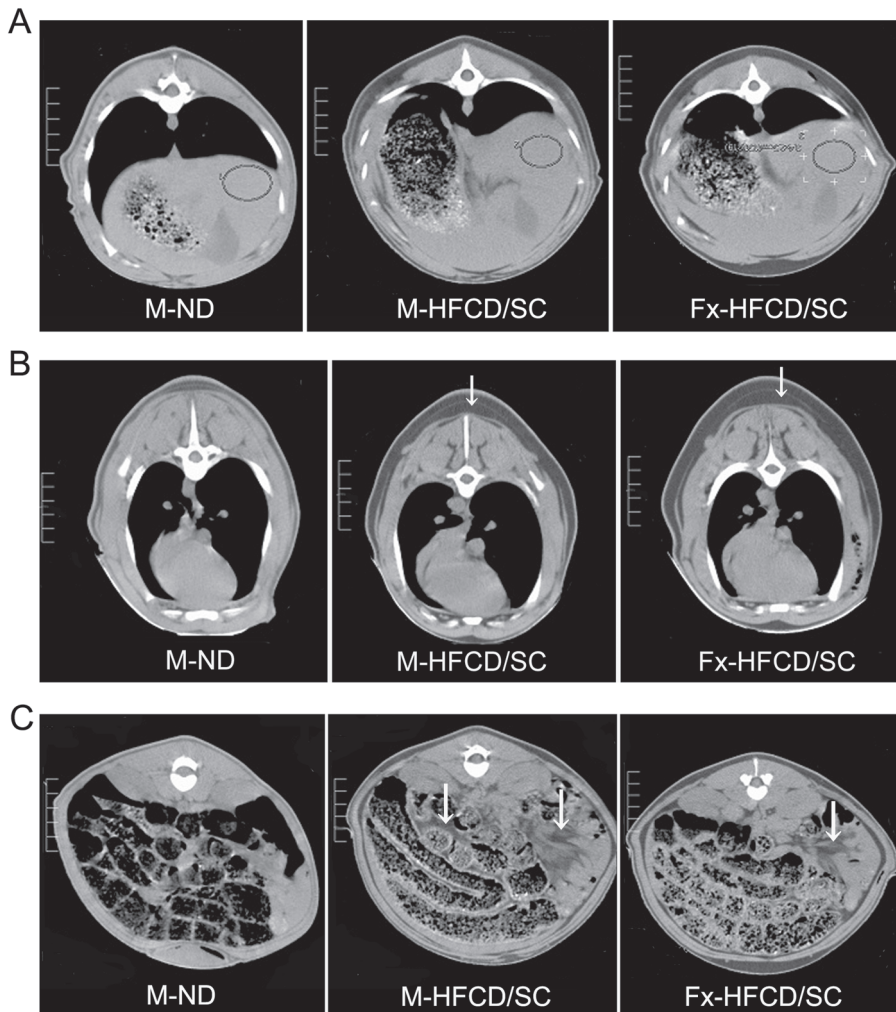
### 5.4. Evaluation of atherosclerotic lesions and pathology

HFCD/SC increased liver, spleen, and greater omentum weights and abdominal fat. The animals fed with HFCD/SC showed grossly white plaque in the abdominal aorta and internal iliac artery. The degree of atherosclerosis was evaluated histopathologically by Stary Stage (I – VIII) (3, 30 – 32). M-ND showed no findings of atherosclerosis in any artery examined. The animals fed with HFCD/SC had an almost similar degree of atherosclerosis in the abdominal aorta, coronary arteries, aortic arch, pulmonary artery, and renal artery. The highest score (VI) for atherosclerosis was shown in the external and internal iliac arteries of M-HFCD/SC, which showed thickening of the intima and arterial media. The thickest sections of the arteries showed considerable foamy cell infiltration, and extracellular lipid accumulation and calcification were observed. A fibrous cap was formed at



**Fig. 1.** Serum lipoprotein analysis. MMP, and humans show dominant LDL-C fractions (closed arrowheads), while the rabbit and mouse do not. The MMP was 6-months-old (upper panels); human male, 40-years-old (lower left panel); male rabbit, 12-months-old (lower middle panel); and male mouse, 4-months-old (lower right panel). MMP: Micromini-pig, M-ND: male-normal diet, M-HFCD/SC: male-high fat and cholesterol diet with sodium cholate, Fx-HFCD/SC: ovariectomized female-high fat and cholesterol diet with sodium cholate.





**Fig. 2.** Computed tomography (CT). A: CT of the liver. The circles are regions of interest for measuring attenuation values (Hounsfield unit). The attenuation values (Hounsfield unit) for the liver in the animals fed with HFCD/SC were decreased, suggesting that they were suffering from fatty liver. B: CT of the thoracic region. Arrows indicate thickened subcutaneous fat at the posterior region of the scapula in the animals fed with HFCD/SC compared with M-ND. C: CT of the abdominal region. Arrows indicate increased area of visceral fats (fats around the intestines) in the animals fed with HFCD/SC compared with M-ND. 1 scale = 1 cm. M-ND: male-normal diet, M-HFCD/SC: male-high fat and cholesterol diet with sodium cholate, Fx-HFCD/SC: ovariectomized female-high fat and cholesterol diet with sodium cholate.

the surface of the intima, at which collagen fiber proliferated and elastic fiber organization was widely damaged. In particular, the cerebral arterial circle in M-HFCD/SC showed atherosclerotic lesions, scored III by Stry classification, in association with severe obstruction of the lumen. Additionally, the Fx-HFCD/SC showed atherosclerotic lesions, suggesting that the MMP may be a suitable animal model for human atherosclerosis in postmenopausal women (33).

Immunohistochemical examinations showed positive expression of lysozyme, HLA-DR, and vimentin in the subintimal areas of atherosclerotic lesions in the animals fed with HFCD/SC, suggesting that the infiltrated cells (foamy cells) originated from macrophages. Severe atherosclerotic lesions corresponding to Stry classification V and VI showed layered cell proliferation having positive expression for  $\alpha$ -SMA, suggesting that the lesions were infiltrated by cells originating from smooth muscle. These findings are similar to those in human cases (34).

Ischemic heart disease and cerebral stroke are promi-

nently involved in atherosclerotic lesions in the relevant arteries (35). An animal model for researching atherosclerosis is required for clinical prevention of atherosclerotic lesions. MMP showed diet-induced atherosclerotic lesions very similar to those seen in humans. However, more suitable fat, cholesterol, and SC content should be evaluated for establishment of atherosclerosis. With a similar diet, many animals, such as rabbits and swine, have been reported to show similar atherosclerotic lesions in the external and internal iliac artery, coronary arteries, abdominal aorta, and common carotid artery (3, 4, 6, 22). However, MMP fed with HFCD/SC showed atherosclerosis in the cerebral arterial circle, which is related to cerebral stroke. This suggests that the MMP may be a suitable animal model for studying a cerebral stroke related to the pathophysiology of atherosclerosis.

### 5.5. Fatty changes in the liver and spleen

The livers of animals fed with HFCD/SC showed histopathologically fatty change around the central veins

and accumulation of particles in the hepatocytes, suggesting that this MMP model may be useful for research into non-alcoholic related fatty liver disease (29). Animals fed with HFCD/SC also showed marked infiltration of foamy cells in the red pulp of the spleen.

## 6. Conclusion

Atherosclerosis induced in the MMP by feeding HFCD/SC for 3 months was very similar in location, pathophysiology, and pathology to that in humans. The MMP is considerably smaller than other minipigs and atherosclerosis formed in a short period. Therefore, the amount of diet required to induce atherosclerosis is relatively small. This represents a cost benefit for such experiments. It is also possible to draw blood and conduct CT scanning under non-anesthetized conditions, indicating the ease of handling and mild character of the MMP. These factors suggest that the MMP will contribute to research into human atherosclerosis.

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