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Koike, Tomonari
Tamura, Shiori
Yu, Ying
Kuniyoshi, Nobue
Shiomi, Masashi

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High susceptibility of atherosclerotic coronary arteries to the onset of vasospasm and angina pectoris-like symptoms due to coronary spasm in WHHLMI rabbits

Tomonari KOIKE[1)], Shiori TAMURA[2)], Ying YU[1)], Nobue KUNIYOSHI[1)], and Masashi SHIOMI[1,2)]

[1)]*Institute for Experimental Animals, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan*
[2)]*Division of Comparative Pathophysiology, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan*

Abstract: We examined the relationship between atherosclerosis and the provocation of coronary spasm as well as the influence of coronary spasm on the onset of acute ischemic myocardial disease. Coronary spasm was provoked in anesthetized normal Japanese white (JW) rabbits and myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits, an animal model for coronary atherosclerosis and myocardial infarction, by injecting ergonovine during the infusion of norepinephrine through a marginal ear vein. A decrease in contrast flow in the left circumflex artery was observed on coronary angiograms. Ischemic changes were observed on the electrocardiograms of 29% (2/7) of JW and 79% (27/34, P=0.007) of WHHLMI rabbits. The frequency of coronary spasm was significantly high in rabbits with severe coronary plaques showing diffuse lesions. Left ventricle motility in vasospasm-positive rabbits, which was evaluated with echocardiograms, was decreased by 29% following the ergonovine injection (P<0.001), and every serum ischemic marker markedly increased 4 h after the provocation of vasospasm. These results demonstrate that atherosclerotic coronary arteries are positively related to the provocation of vasospasm, and vasospasm in severe atherosclerotic coronary segments evokes angina pectoris-like findings and/or non-fatal myocardial infarction. WHHLMI rabbits may be a novel animal model for angina pectoris and acute ischemic heart disease.
Key words: angina pectoris, animal model, coronary atherosclerosis, coronary spasm, WHHLMI rabbit

Introduction

Based on clinical observations, coronary spasm has been implicated as one of the causes of acute ischemic coronary events [5], such as sudden cardiac death [15], lethal arrhythmia [3], variant angina [6], and acute myocardial infarction [17, 20]. Due to its relationship with coronary heart disease (CHD), the gravity of coronary spasm was reconfirmed in the onset of acute ischemic coronary events in Western countries [8]. However, limited evidence is available for the causal link between coronary spasm and CHD. The development of suitable animal models may promote a clearer understanding of the role of coronary spasm in the provocation of acute ischemic heart disease. Several animals have been used as models in the study of coronary spasm. In a swine model fed a cholesterol diet, constrictive responses induced by constrictors were significantly augmented [2,

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Address corresponding: M. Shiomi, Division of Comparative Pathophysiology and Institute for Experimental Animals, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

4, 9, 10]. Although these findings have contributed to clarifying the mechanisms involved in the provocation of vasospasm [9], the extensive denudation of arterial endothelial layers does not occur under physiological conditions. Previous studies [4, 7] examined the relationship between coronary spasm and the onset of acute coronary syndromes, but did not evaluate clinical findings.

We developed a myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic (WHHLMI) rabbit [12, 14] by selective breeding [12, 18], and this rabbit develops coronary atherosclerosis and hypercholesterolemia because of a genetic defect in low-density lipoprotein receptors. We previously provoked coronary spasm in WHHLMI rabbits and identified relationships between coronary spasm and plaque disruption as well as ischemic myocardial damage [11]. However, we did not sufficiently examine the relationship between coronary plaques and the provocation of coronary spasm, or the influences of coronary spasm on the development of ischemic heart disease, such as angina pectoris. In the present study, we analyzed stored specimens from our previous study [11] and additional four rabbits in order to examine the suitability of the WHHLMI rabbit as an animal model for coronary spasm and spastic angina.

Materials and Methods

Animals

We used 34 WHHLMI rabbits aged 12–29 months in experiments on the provocation of coronary spasm. WHHLMI rabbits were bred at the Kobe University Graduate School of Medicine. As a control, 7 male Japanese white (JW) rabbits (Kitayama Labes, Co., Ltd., Ina, Japan) aged 8 months were used in the coronary spasm provocation test and examination of left ventricular function. Rabbits resided individually in metal cages (width 550 mm, depth 600 mm, and height 450 mm) with a flat floor, and were given standard rabbit chow (LRC4, Oriental Yeast Co., Ltd., Tokyo, Japan) at 120 g/day and water *ad libitum*. Animal rooms were maintained under a constant temperature (22 ± 2°C), relative humidity (50–60%), ventilation rate (15 cycles/hour), and lighting cycle (12 h light/dark). This study was approved by the president of Kobe University after being reviewed by the Kobe University Animal Care and Use Committee (approval numbers: P080606 and P091101), and animal experiments were conducted in accordance with the Regulations for Animal Experimentation of Kobe University, and Japanese regulations, such as the Act on the Welfare and Management of Animals (Law No. 105; 1973, revised 2006), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88, 2006), and Fundamental Guidelines for the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No.71, 2006).

Anesthesia and euthanasia

Rabbits were anesthetized with an intravenous injection of ketamine hydrochloride (15 mg/kg, Daiichi-Sankyo Co., Ltd., Tokyo, Japan) plus midazolam (1 mg/kg, Dormicum, Astellas Pharma Inc., Tokyo, Japan), and anesthesia was continued by the intravenous infusion of ketamine hydrochloride at 60 mg/kg/h. During experiments, oxygen was supplied through a face mask (2.0 l/min for rabbits), and rabbits were warmed with a heating pad. Rabbits were euthanized with exsanguination under the intravenous administration of sodium pentobarbital (30 mg/kg).

Provocation of coronary spasm

The provocation and evaluation of coronary spasm were performed as previously described [11], and the study design was shown in Fig. 1. We analyzed the left circumflex artery (LCX) because it is a major coronary artery in rabbits [1, 13], and severe atherosclerotic lesions were observed in LCX; however, the degree of lesions was shown to markedly vary and the frequency of atherosclerotic lesions was low in the anterior descending artery [14].

Evaluation of ventricular contractile dysfunction and myocardial ischemia

Echocardiograms were performed on 13 WHHLMI rabbits and 7 JW rabbits using the Philips Envisor C echocardiograph (Philips Inc., Eindhoven, the Netherlands) [11]. Left ventricular function was evaluated by fractional shortening, which was calculated as 1 − [systolic left ventricular diameter (LVDs)] / [diastolic left ventricular diameter (LVDd)]. In our previous study [11], we examined serum markers for ischemic myocardial damage (heart-type fatty acid-binding protein [H-FABP], cardiac troponin-I [cTroponin-I], and myoglobin) in 6 WHHLMI rabbits. In order to ensure the onset of ischemic myocardial damage after the provocation of coronary vasospasm, assays were performed using the sera of 20 WHHLMI

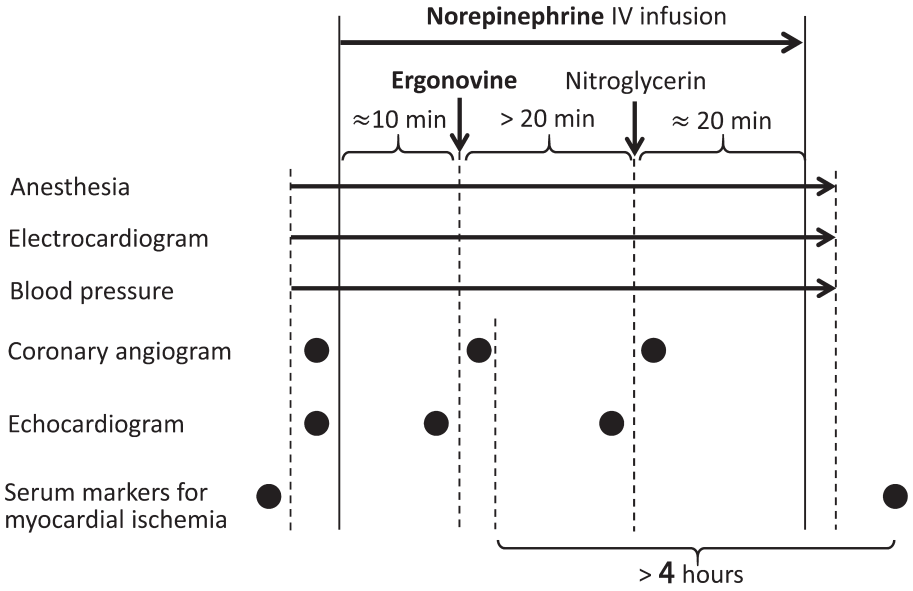


Fig. 1. Study design for the provocation of coronary spasm. Coronary spasm was provoked with an intravenous injection of ergonovine maleate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) at a dose of 0.45 μmol/kg during the infusion of norepinephrine (Daiichi-Sankyo Co., Ltd., Tokyo, Japan) at a rate of 12 nmol/kg/min through a marginal ear vein. Nitroglycerin (Hikari Pharmaceutical Co., Ltd., Tokyo, Japan) was injected intravenously (10 μg/kg) 20–30 min after the onset of ischemic changes on electrocardiograms (ECG) in order to reverse coronary spasm. The occurrence of coronary spasm was monitored with coronary angiograms using an X-ray apparatus (OPESCOPE PLENO; Shimadzu Corporation, Kyoto, Japan), and ECG were measured using an amplifier (AB-621G; Nihon Kohden, Tokyo, Japan) and PowerLab/8SP (ADInstruments Pty Ltd., Bella Vista, Australia) or an electrocardiograph (CARDIOSUNY α6000 AX-D, Fukuda M-E Kogyo, Co., Ltd., Tokyo, Japan). Blood pressure was also monitored invasively at the femoral artery.

rabbits stored at −80°C. These serum biomarkers for ischemic myocardial damage were assayed using ELISA kits (Life Diagnostics Inc., West Chester, PA, USA).

Preparation of coronary sections

Rabbits were euthanized after being examined under anesthesia. Hearts were excised, immersion-fixed with 10% neutral buffered formalin solution, and embedded in paraffin. Coronary arterial segments were prepared as reported previously [13]. Sections were stained using Elastic van Gieson staining. Coronary stenosis was evaluated using cross-sectional narrowing (%) in the LCX, which was calculated by dividing the lumen area by the area surrounded by an internal elastic lamina. In the present study, branches of the LCX were not examined.

Assay of serum lipid levels

Serum total cholesterol and triglyceride levels were assayed enzymatically with kits when animals were 12 months of age using sera obtained after 15 h of fasting.

Statistical analyses

Data are represented as the mean ± standard error of the mean (SEM). Statistical analyses were performed for mean values with the signed Wilcoxon test, Mann-Whitney U-test, or Student's *t*-test, and for frequency with the chi-squared test. In order to compare mean values among multiple groups, we performed the Bonferroni test. A value of $P<0.05$ was considered to be significant.

Results

Baseline data of WHHLMI rabbits

Table 1 shows the baseline data of WHHLMI rabbits used in this study. We added data on blood pressure, heart rate, and animals with more than 75% coronary stenosis to our previous findings [11], and updated data on 30 rabbits from our previous study [11] for 34 rabbits in the present study. As shown in Table 1, all WHHLMI rabbits showed hypercholesterolemia and had atherosclerotic lesions in the coronary arteries. Maximum stenosis was

Table 1. Baseline data of WHHLMI rabbits provoked with coronary spasm

Examined rabbits	34
Gender (female : male)	11:23
Age (months)	18.0 ± 0.8
Body weight (kg)	3.32 ± 0.07
Serum lipid levels at 12 months old (mg/dl)	
Total cholesterol	845 ± 31
Triglyceride	364 ± 34
Coronary plaques	
Examined segments	21.6 ± 1.0
Segments with lesions	14.4 ± 1.4
Animals with more than 75% coronary stenosis	67.6% (23/34)
Segments with more than 75% stenosis	9.7 ± 1.6
Maximum stenosis (%)	78.2 ± 3.3
Blood pressure at the femoral artery (n=13, mmHg)	
Systolic	131 ± 4.6
Diastolic	68 ± 3.5
Heart rate (beats/min)	243 ± 5.4

Data are represented as the mean ± SEM.

78.2 ± 3.3%. Coronary stenosis (evaluated as cross-sectional narrowing) of more than 75% was observed in 67.6% of rabbits (23/34).

Occurrence of coronary spasm

Figure 2 shows the ECG results of rabbits. In analyses, we added data obtained from JW rabbits (Fig. 2B) to our previous findings [11], and updated the ECG results of 30 WHHLMI rabbits in our previous study [11] for 34 WHHLMI rabbits in the present study. Furthermore, a 12-lead ECG (Fig. 2A) was presented instead of the 9-lead ECG in the previous study [11]. During the vasospasm provocation test, ECG showed ischemic changes in WHHLMI rabbits (Fig. 2), e.g. ST depression/elevation, T-wave elevation/inversion, poor R-wave progression, a deep Q-wave, and the ventricular premature complex. The frequency of these abnormalities was markedly higher in WHHLMI rabbits (27/34, 79.4%, P=0.007) than in normal JW rabbits (2/7, 28.6%).

Figure 3 shows the provocation of angiographical coronary spasm in WHHLMI rabbits *in vivo*. We did not analyze the relationship between coronary stenosis and the provocation of coronary spasm in our previous study [11]. Contrast flow was markedly decreased after the injection of ergonovine (Fig. 3A), and the area with decreased perfusion in the LCX corresponded to segments with plaques (sections 7–16) (Fig. 3B). Regarding the relationship between coronary plaques and the development of coronary spasm (Fig. 3C), maximum coronary stenosis was significantly larger in vasospasm-positive rabbits (76 ± 4%, P=0.034) than in vasospasm-negative rabbits (57 ± 10%). The average of coronary stenosis in each atherosclerotic lesion was significantly larger in vasospasm-positive rabbits (65 ± 4%, P=0.023) than in vasospasm-negative rabbits (33 ± 6%). In addition, the frequency of coronary segments with more than 75% stenosis was significantly higher in vasospasm-positive WHHLMI rabbits (32 ± 6%, P=0.036) than in vasospasm-negative WHHLMI rabbits (8 ± 6%). These results demonstrated that atherosclerotic coronary arteries were highly susceptible to the provocation of vasospasm.

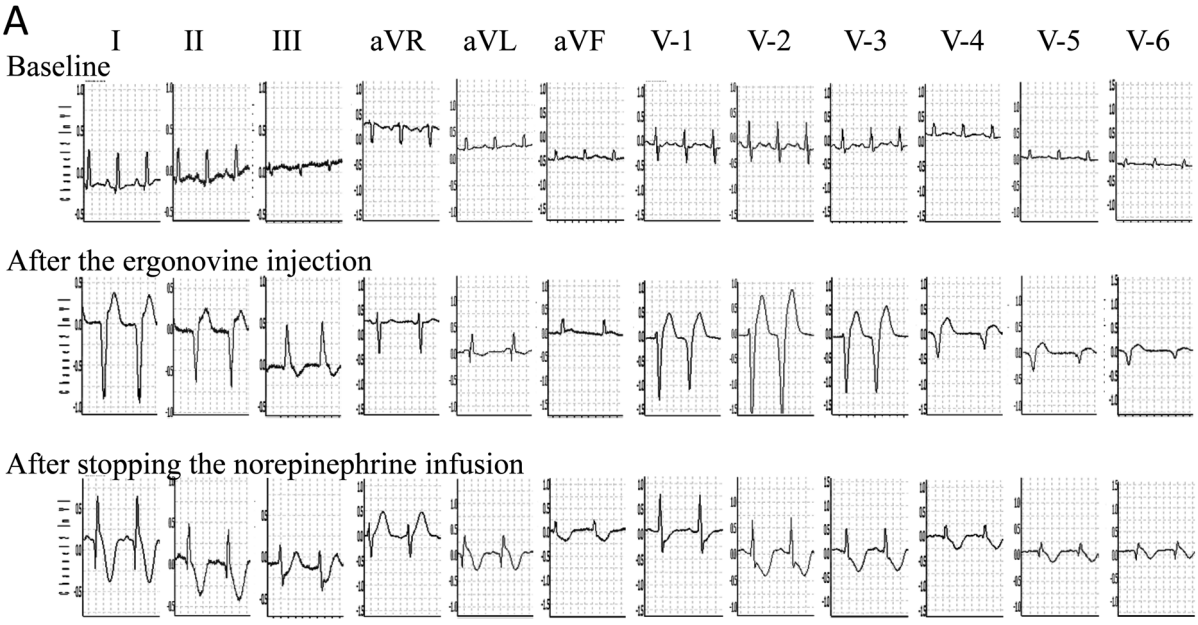
Changes in the motility of the left ventricular wall

Table 2 shows the results of echocardiograms during the vasospasm provocation test. In this examination, we added data from JW rabbits to our previous findings [11], and updated data on 10 WHHLMI rabbits in the previous study [11] for 13 WHHLMI rabbits. Although fractional shortening was already significantly lower at the baseline in WHHLMI rabbits (32.5 ± 1.2, P<0.004) than in normal JW rabbits (41.5 ± 3.1), it decreased further after the spasmogen treatment (23.0 ± 0.6, P<0.001). The reduction observed in fractional shortening was mainly due to the depression of left ventricular contractions (11.6 ± 0.5 vs. 9.8 ± 0.6, P=0.009). These results indicate that coronary spasm leads to cardiac dysfunction in WHHLMI rabbits.

Evaluation of ischemic damage to the left ventricular wall

Table 3 shows changes in serum markers for ischemic myocardial injury in WHHLMI rabbits. In this examination, we updated data on 6 WHHLMI rabbits in our

A



B

Frequency of ischemic ECG changes after the administration of spasmogens

	JW rabbits	WHHLMI rabbits	*P*-value
Rabbits examined	7	34	
ST-depression	2 (28.6%)	26 (76.5%)	0.013
ST-elevation	0	9 (26.5%)	0.123
T-wave inversion	0	18 (52.9%)	0.010
High T-wave	0	8 (23.5%)	0.153
Q-wave	0	10 (29.4%)	0.099
Poor R-wave progression	0	8 (23.5%)	0.153
Ventricular premature complex	2 (28.6%)	16 (47.1%)	0.369
Left bundle branch block	0	5 (14.7%)	0.279
Any ischemic changes	2 (28.6%)	27 (79.4%)	0.007

Fig. 2. Electrocardiograms (ECG) of rabbits treated with an ergonovine bolus injection under the norepinephrine infusion. (A) Representative ECG changes in a WHHLMI rabbit during the experiments. (B) Frequency of ischemic patterns on ECG in normal rabbits (n=7) and WHHLMI rabbits (n=34). Statistical analyses were performed with the chi-squared test.

previous findings [11] for 20 WHHLMI rabbits in order to ensure the development of ischemic myocardial injury after the provocation of coronary spasm. These serum markers were within normal human ranges at baseline. However, they markedly increased after the provocation of vasospasm, which confirmed that coronary spasm induced ischemic injury in the myocardium.

Discussion

The present study demonstrated that vasospasm was frequently induced in coronary arteries with diffuse atherosclerotic plaques showing more than 75% coronary stenosis, and cardiac dysfunction and ischemic myocardial injury developed in these rabbits. These results suggest that WHHLMI rabbits are a suitable animal model for coronary spasm, angina pectoris-like findings, and/or non-fatal myocardial infarction.

In the present study, the frequency of coronary spasm in WHHLMI rabbits with severe coronary stenosis and diffuse atherosclerotic plaques was significantly higher than that in WHHLMI rabbits with less stenosis and focal plaques in spite of similar serum cholesterol levels (851 ± 37 mg/dl vs. 824 ± 58 mg/dl, P=0.735). Previous *ex vivo* studies using endothelial-denudated coronary strips demonstrated that atherosclerotic coronary strips

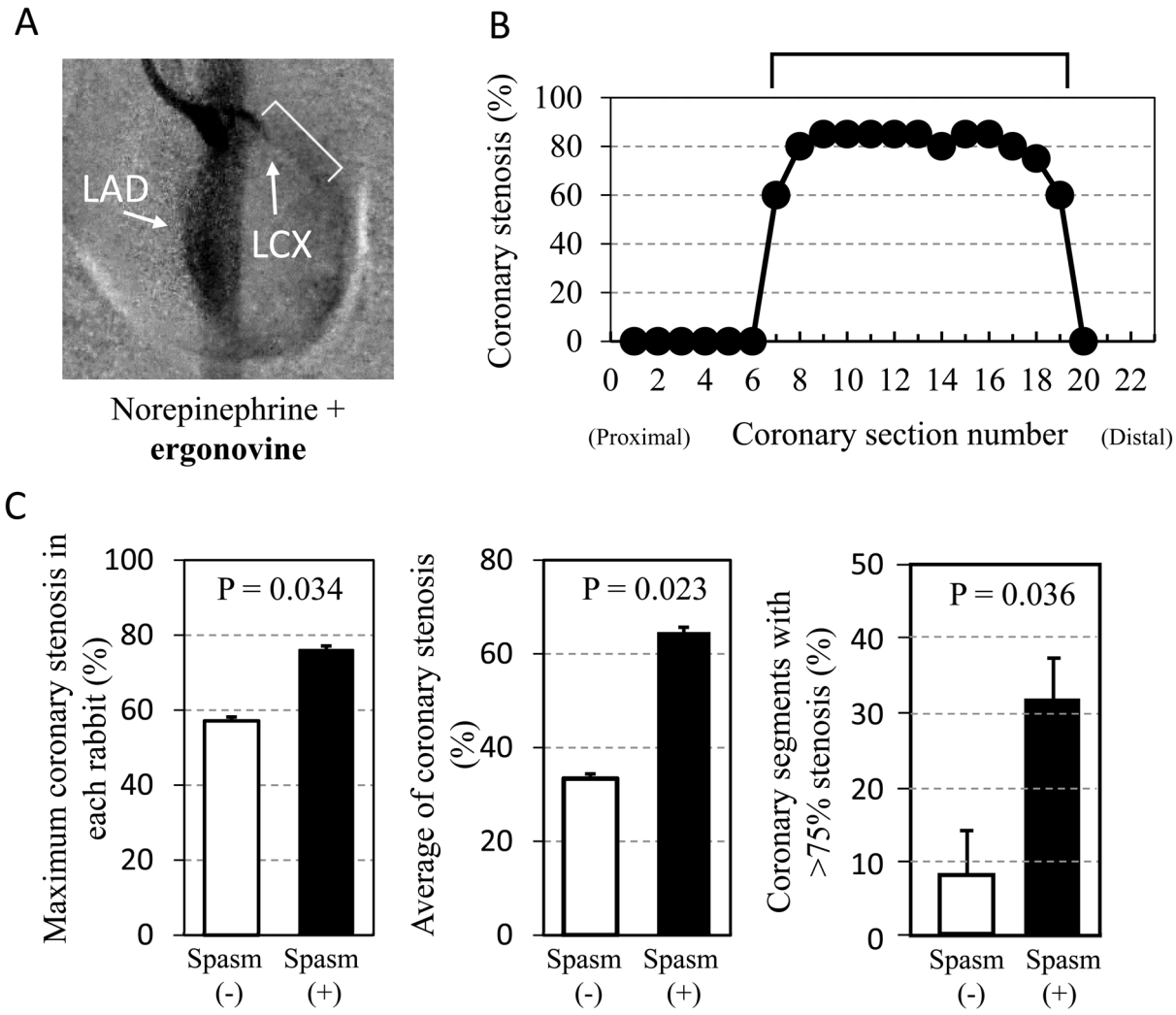


Fig. 3. Relationship between coronary plaques and coronary spasm in WHHLMI rabbits. (A) Coronary angiograms after the ergonovine injection during the norepinephrine infusion. (B) Coronary stenosis evaluated as cross-sectional narrowing of the left circumflex artery as indicated on the angiogram. (C) Relationship between coronary plaques and the provocation of coronary spasm. Data are represented as the mean ± SEM. Statistical tests were performed with the Mann-Whitney U-test. LAD, left anterior descending artery, LCX, left circumflex artery.

showed markedly greater sensitivity and reactivity against vasoconstrictors than normal coronary strips [21]. The present results are consistent with these findings. Since the 1990s, atherosclerotic lesions have been detected at the site of focal vasospasm in the coronary arteries of patients [19], and the existence of atherosclerotic lesions is considered to be related to the onset of vasospasm [15]. Therefore, we considered coronary arteries with severe atherosclerotic plaques to be related to the onset of coronary spasm. Regarding gender differences in coronary spasm, all females (11/11) developed coronary spasm, whereas its incidence was 70% (16/23) in males. One of the causes for this gender difference may be differences in the degree of coronary stenosis. Coronary atherosclerosis was more severe in females than in males (data not shown).

After the provocation of coronary spasm, left ventricle motility in rabbits with coronary spasm evaluated with echocardiograms was decreased, and serum markers for ischemic myocardial injury were markedly increased, which is consistent with our previous findings [11]. Wang *et al*. also observed elevations in serum cardiac troponin-I levels in patients after the provocation of coronary spasm [17]. The present results suggest the onset of angina pectoris-like symptoms and/or non-fatal myocardial infarction; however, the frequency of occlusive

Table 2. Left ventricular wall motility in WHHLMI rabbits with coronary spasm

	Left ventricular diameter (mm)		Fractional shortening
	Diastole	Systole	
Normal JW rabbits (n=7)	13.2 ± 1.0	7.8 ± 0.8	41.5 ± 3.1
WHHLMI rabbits (n=13)			
Baseline	14.5 ± 1.0	9.8 ± 0.6	32.5 ± 1.2*
Norepinephrine infusion	15.1 ± 0.5	10.6 ± 0.4	29.7 ± 1.3
Norepinephrine + Ergonovine	15.1 ± 0.7	11.6 ± 0.5$^{\#}$	23.0 ± 0.6$^{\#\#}$

Data are represented as the mean ± SEM. Statistical analyses were performed with the Student's *t*-test for differences between normal and WHHLMI rabbits, and with the Bonferroni test for WHHLMI rabbits in the coronary vasospasm provocation test. *, P=0.004 (JW vs. WHHLMI); $^{\#}$, P=0.009 (baseline vs. the combination treatment); $^{\#\#}$, P<0.001 (baseline vs. the combination treatment).

Table 3. Changes in serum markers for ischemic myocardial damage in WHHLMI rabbits with coronary spasm (n=20)

	H-FABP (ng/ml)	cTroponin-I (ng/ml)	Myoglobin (ng/ml)
Baseline	2.07 ± 0.48	0.02 ± 0.02	67.8 ± 21.9
4 hours after the treatments	28.0 ± 4.9	2.71 ± 0.83	907 ± 127
P-value	<0.001	<0.001	<0.001

Human normal ranges were < 6.2 ng/ml in H-FABP, <0.04 ng/ml in cTroponin-I, and 20–80 ng/ml in myoglobin. Data are represented as the mean ± SEM. Statistical analyses were performed with the Wilcoxon signed rank test. H-FABP, heart type fatty acid-binding protein; cTroponin-I, cardiac troponin-I.

thrombi after the disruption of coronary plaques was very low [11]. Previous studies reported that coronary spasm plays an important role in the pathogenesis of not only variant angina, but also ischemic heart disease, including other forms of angina, acute myocardial infarction, arrhythmias, and ischemic sudden death [3, 5, 6, 16, 17, 20]. These findings are consistent with the results of the present study; therefore, we speculated that the WHHLMI rabbit is useful as an animal model of experimentally provoked coronary spasm and subsequent myocardial ischemia.

Limitations of the study

In patients, angina pectoris is defined as unstable chest pain and ischemic ECG changes. Since rabbits do not have the ability to express chest pain similar to humans, it is difficult to diagnose the present results obtained after a spasmogen injection as the provocation of angina pectoris.

The Japanese Circulation Society reported that it is possible to establish a diagnosis even in patients without a chest pain by recording a 12-lead ECG, and more than half of patients had asymptomatic attacks [15]. In the present study, coronary spasm caused ischemic changes in ECG, cardiac dysfunction, and ischemic injury to the myocardium. These results suggest that angina pectoris-like findings and/or non-fatal myocardial infarction developed due to the provocation of coronary spasm.

In conclusion, the present results suggest that atherosclerosis in coronary arteries contributes to the development of coronary spasm, and coronary spasm in severe atherosclerotic lesions results in acute ischemic myocardial damage, which resembles angina pectoris in humans. The WHHLMI rabbit is a good animal model for coronary spasm and the related ischemic heart disease accompanying cardiac dysfunctions.

Conflict of Interests

The authors have declared that no competing interests exist.

Sources of Funding

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