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Effects of dietary hypercholesterolemia on plasma lipids levels male and female Japanese white and heterozygous Kurosawa-Kusanagi hypercholesterolemic rabbits

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Abstract

High plasma cholesterol levels constitute a major risk factor for coronary heart diseases. In this study, we compared the response to dietary hypercholesterolemia with respect to plasma lipids levels in male and female Kurosawa-Kusanagi hypercholesterolemic (KHC) rabbits, which have Low density lipoprotein (LDL) receptor deficiency. Male and female Japanese white (JW) and heterozygous KHC rabbits were given a standard milled rabbit diet or diets with different concentrations of cholesterol. All the male and female JW and KHC rabbits given a standard milled rabbit diet did not show increased plasma cholesterol levels over the 24-week experiments. The plasma cholesterol levels in male JW rabbits fed a 0.1 % cholesterol-containing diet did not increase, but the levels in male heterozygous KHC rabbits fed the same diet transiently increased for 4-8 weeks (to 300 mg/dL) and then gradually decreased till the initial level. In contrast, in female JW rabbits fed the same diet, the plasma cholesterol

levels increased for 4-24 weeks (to 300 mg/dL), and in heterozygous KHC rabbits, the levels gradually increased (to 675 mg/dL after 24 weeks) after consumption of the 0.1 % cholesterol-containing diet. In ovariectomized JW and KHC rabbits fed the 0.1 % cholesterol-containing diet, the plasma cholesterol levels were half value the levels in non-ovariectomized rabbits

after 24 weeks. Gene expression of the LDL receptor in the liver significantly increased in male JW and KHC rabbits, but significantly decreased in female KHC rabbits. These results indicate that female rabbits had a greater response to the cholesterol diet than male rabbits and that the responsiveness to dietary hypercholesterolemia was predominantly genetic.

Key words: heterozygous hypercholesterolemic rabbits; cholesterol feeding; plasma cholesterol levels, hypercholesterolemia.

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Introduction

Hypercholesterolemia is a major risk factor for coronary heart disease.1),2) Many clinical studies have indicated that plasma hypercholesterolemia cause a severe heart diseases. Familial hypercholesterolemia (FH) has the highest prevalence among genetic metabolic diseases, as it is present in 1 per 300 to 500 heterozygous patients in the general opulation.3).4)

Coronary artery disease mortality in heterozygous FH is several times higher than that in the general population.5).6) Women have a higher risk for heart disease after menopause than men. Premenopausal women have a lower incidence of coronary artery disease than men of the same age, whereas after menopause the incidence in women approaches that in men.7)-9) Estrogen deficiency may be responsible for this phenomenon.10) In deed, recent studies have documented that estrogen replacement is associated with a reduction of approximately 50 % in the incidence of coronary artery disease in postmenopausal women.11).12) In contrast, a recent report released by the American Heart Association demonstrated not only a higher prevalence of coronary artery disease in men than in women, but also a similar pattern of difference for the disease.13) Therefore, in this study, we compared the effects of dietary

hypercholesterolemia on plasma lipid levels in male and female JW rabbits and heterozygous KHC rabbits.

Materials and Methods

1. Animals

Twelve-week-old JW and heterozygous KHC rabbits were purchased from Japan Laboratory Animals Inc. (Tokyo, Japan). They were maintained in an animal facility (room temperature: 23 ± 2 °C, relative humidity: 55 ± 10 %, all fresh air ventilation: 15-20 times/h, 12 h light and 12 h dark) and subjected to the experiment after a 1-week quarantine period.

2. Experimental design

Male JW rabbits were divided into 2 groups: one group received a standard milled diet (MR-stock, Nihon Nosan Kogyo, Yokohama, Japan), and another was fed a 0.1 % cholesterol-containing diet. The non-ovariectomized female JW rabbits were divided into 5 groups: the female control group received a standard milled diet, the other group were fed a 0.05 % or 0.1 % cholesterol-containing diet. The ovariectomized rabbits were fed a standard milled diet or 0.1 % cholesterol-containing diet. Heterozygous male KHC rabbits were divided into 3 groups; the control group received a standard milled diet, and the 2 groups were fed the 0.1 % or 0.25 % cholesterol-containing diet. The non-ovariectomized female heterozygous KHC rabbits were divided into 5 groups: the control group received a standard milled diet, the other groups were fed the 0.01 %, 0.05 % or 0.1 % cholesterol-containing diet. The ovariectomized KHC rabbits were fed a 0.1 % cholesterol-containing diet. The diet intake as 100 g once a day, and water was given ad libitum.

3. Cholesterol levels in the plasma and lipoprotein fractions Blood samples were collected every 4 weeks from an ear artery. Plasma was separated by centrifugation at 3,000 x g for 15 min at 4 °C. Fresh plasma samples were stored at -20 °C and used for analysis of lipids levels. Lipoprotein fractions were separated by centrifugation as described by Bronzert.14) Total cholesterol levels in plasma and lipoprotein fractions were measured enzymatically by using test kits (Wako Chemical Co., Osaka, Japan) in an auto-analyzer (Hitachi 7070, Hitachi Co., Tokyo, Japan).

4. Liver lipid levels and Apo-B, Apo-E and LDL receptor mRNA expression in the liver After the experimental period, rabbits were anesthetized with pentobarbital sodium. The liver tissue was then exsanguinated and excised, blotted to remove excess blood and immediately stored at -80 °C until the assay. The lipids in the liver were separated by the Folch method15) and measured using enzymatic test kits (Kyowa Medix, Tokyo, Japan). Total RNA was isolated by the guanidium thiocyanate method described by Chirgwin et al.16) The RNA obtained was then treated with 1 M glyoxal at 50 °C for 1h. After treatment, the mRNA was electrophoresed on a 1 % agarose gel in 10 mM phosphate buffer. The RNA was transferred to a nylon membrane and northern blot hybridization using 32P-labeled c-DNA probes.17) The mRNA expression was measured using densitometer to evaluate band intensity in the autoradiogram.

5. Pathological evaluation aorta

The thoracic aorta was opened longitudinally, and the percentages of the area of the aorta containing an atheromatous plaque was calculated according to Kita's method.18)

6. Statistical evaluation

The results have been expressed as mean ± S.E. The differences among the groups were analyzed using a one-way ANOVA. The differences were considered significant at $P<0.05$.

7. Ethics

All of the experimental procedures were conducted according to the guidelines of the Animal Care and Use Committee of Nihon Pharmaceutical University.

Results

Plasma cholesterol levels Fig.1 shows the plasma cholesterol levels in JW (A & B) and KHC (C & D) rabbits fed different concentrations of the cholesterol-containing diet. The plasma cholesterol levels of the male JW rabbits showed in A and female in B, respectively. An increase in total cholesterol level was not seen in male and female rabbits fed the standard milled diet over the 24 weeks of the experiment. When male JW rabbit were fed the 0.1 % cholesterol-containing diet, the total cholesterol levels did not increase relative to those for the standard milled diet group. In female JW rabbits, plasma cholesterol levels increased to 250 mg/dL between 4 weeks and 24 weeks. Moreover, the maximal plasma cholesterol level was 130 mg/dL in ovariectomized JW rabbits. The plasma cholesterol levels of male and female JW rabbits fed the 0.1 % cholesterol-containing diet for 24 weeks were 69.3 mg/dL and 236.4 mg/dL, respectively. The plasma cholesterol levels of the heterozygous male and female KHC

rabbits are shown in C and in D, respectively. The plasma cholesterol levels did not increase until 24 weeks in male and female KHC rabbits fed the standard milled diet. In male KHC rabbits fed the 0.1 % cholesterol diet, the plasma cholesterol levels significantly increased from 4 to 8 weeks and then gradually decreased, reaching the control level by 24 weeks. However, in the

group that received the 0.25 % cholesterol-containing diet, the plasma cholesterol levels reached 1,200 mg/dL and then gradually decreased to 530 mg/dL within 24 weeks. In contrast, when heterozygous female KHC rabbits were fed the 0.1 % cholesterol-containing diet, plasma cholesterol levels significantly increased from 4 weeks to 24 weeks, and the levels after 24 weeks was 674.4 mg/dL. When the 0.05 % cholesterol-containing diet was administered to these rabbits, the plasma cholesterol was maintained at approximately 300 mg/dL for 4-12 weeks and then gradually decreased. The group that received the 0.01 % cholesterol-containing diet did not show increased plasma cholesterol levels for the 24 weeks of the experiment. In

addition, the plasma cholesterol level of ovariectomized KHC rabbits fed the 0.1 % cholesterol-containing diet increased from 350.4 mg/dL at 4 weeks to 317.9 mg/dL at 24 weeks.

Liver lipid levels

As shown in Table 1, liver cholesterol levels were significantly increased in male and female KHC rabbits fed the 0.1 % cholesterol-containing diet. No significant changes were observed in Japanese white rabbits even administered the 0.1 % cholesterol-containing diet for 24 weeks.

Cholesterol levels in the lipoprotein fractions for female rabbits

The total cholesterol levels in the lipoprotein fractions for female rabbits at 24 weeks are shown in Table 2. The VLDL and LDL cholesterol levels significantly increased in non-ovariectomized JW and KHC rabbits fed the 0.1 % cholesterol-containing diet. Moreover, the levels in LDL significantly increased in the ovariectomized KHC rabbits fed the 0.1 %cholesterol-containing diet. In contrast, the HDL level significantly increased in ovariectomized KHC rabbits fed the 0.1 % cholesterol-containing diet. No significant changes were observed in the other rabbits.

Apo-E, apo-B and LDL-receptor mRNA gene expression in the liver The expression ratios of apo-B, apo-E and LDL-receptor mRNA to GAPDH mRNA in the liver are presented in Table 3. The mRNA expression of the LDL –receptor significantly increased in the male Japanese white and KHC rabbits fed the 0.1 % cholesterol-containing diet. In contrast, the LDL-receptor mRNA expression of female KHC rabbits fed the 0.1 % cholesterol-containing diet significantly decreased. The level of apo-E and apo-B mRNA expression did not change in the other groups.

Pathological evaluation of the aorta

The intimal surface area of the thoracic aorta in female JW and heterozygous KHC rabbits after consumption of the 0.1 % cholesterol-containing diet for 24 weeks is shown in Table 4. Japanese white and KHC rabbits fed the standard milled diet did not show histopathological changes in the aortic arch. Half of the female Japanese white rabbits fed the 0.1 % cholesterol diet showed lesions at 24 weeks, and all the female KHC rabbits showed lesions. The other animal groups did not show lesions. In addition, the area of the lesions with respect to the total area of the aortic arch was 28.2 ± 16.3 % in female Japanese white rabbits and 32.7 ± 4.6 % in female KHC rabbits, respectively.

Discussion

In this study, we investigated the response to dietary hypercholesterolemia in male and female JW and heterozygous KHC rabbits. The plasma cholesterol levels of JW female rabbits fed a 0.1 % cholesterol-containing diet for 24 weeks were 3 times higher than those in male rabbits, similar results were observed for KHC rabbits. When male heterozygous KHC rabbits were administered the 0.25 % cholesterol-containing diet, the plasma cholesterol level greatly increased from 4 to 24 weeks. These data show that plasma cholesterol levels increased

when LDL-deficient rabbits were fed a diet containing a high concentration of cholesterol. In male rabbits fed the 0.1 % cholesterol-containing diet, plasma cholesterol levels did not increase. In contrast, when female rabbits were fed the same 0.1 % cholesterol-containing diet, the plasma cholesterol level gradually increased from 4 to 24 weeks. These results suggest that

genetic factors and environmental factors (such as fat-rich food intake) are important risk factors in hypercholesterolemia. After ovariectomy, the plasma cholesterol levels in the female rabbits decreased to half of the level seen in non-ovariectomized rabbits. These results suggest that the male sex hormones may have cholesterol-lowering effects in rabbits. These data indicate that the response to dietary cholesterol differs between JW and KHC rabbits. Moreover, KHC rabbits are more responsive to dietary cholesterol than JW rabbits. Sex hormones are thought to play an important role in coronary artery diseases. Estrogen treatment can reduce low-density lipoprotein (LDL) cholesterol and total cholesterol levels,19) although the effects of post-menopausal estrogen use upon overall cardiovascular disease risk are still controversial.20) Estrogen is thought to reduce LDL cholesterol levels by up regulation of the LDL receptor, thus enhancing the hepatic clearance of LDL from plasma.21) Early studies in animals showed that pharmacological doses of 17-ethinyl estradiol significantly reduced LDL cholesterol levels as a result of an increase in hepatic LDL receptor expression.22)-24) However, it has been reported that the effect of estrogens on serum cholesterol is dependent not only on the dose, but also on the length of treatment and the type of estrogen.25) In humans, the response of hepatic LDL receptor activity to estrogens has been less well documented. The cholesterol-lowering effect of diets high in phytoestrogens may result from their ability to increase hepatic LDL receptor activity.26) Pharmacologic doses of estrogen increase the concentration of hepatic mRNA for the LDL receptor in rabbits. Thus, the pharmacological effects of estrogen include induction of the expression of hepatic LDL receptor.27),28) The mechanism underlying the onset of hypercholesterolemia in KHC rabbits, similar to that in Watanabe heritable hypercholesterolemia (WHHL) rabbits, involves deficiency of the LDL

receptor.29) In our study, the cholesterol levels in VLDL and LDL fractions significantly

increased in JW and KHC rabbits fed the cholesterol-containing diet. Moreover, the LDL -receptor mRNA levels in the liver increased in male JW and heterozygous KHC rabbits fed the 0.1 % cholesterol diet. Only female KHC rabbits showed a significant decrease when fed the same cholesterol-containing diet. These data suggest that LDL receptor mRNA expression caused the high plasma cholesterol level in the female rabbits. The LDL receptor in the liver plays the most important role in cholesterol uptake from the plasma. In the thoracic aorta, all female KHC rabbits showed plaques, however, the number of plaques was half that in female JW rabbits. We speculate that the incidence of plaques increases above a plasma cholesterol cut-off of 300 mg/dL. Therefore, we conclude that genetic influence (especially LDL receptor

expression in the liver) is the most important factor underlying hypercholesterolemia. Moreover, female rabbits are more responsive to the cholesterol than male rabbits in the case of dietary cholesterol. Thus, this rabbit model of heritable hypercholesterolemia is useful for investigating atherosclerosis and the lipidological and physiological mechanisms underlying human lifestyle-related diseases. The relative importance of these different mechanisms warrants further investigation.

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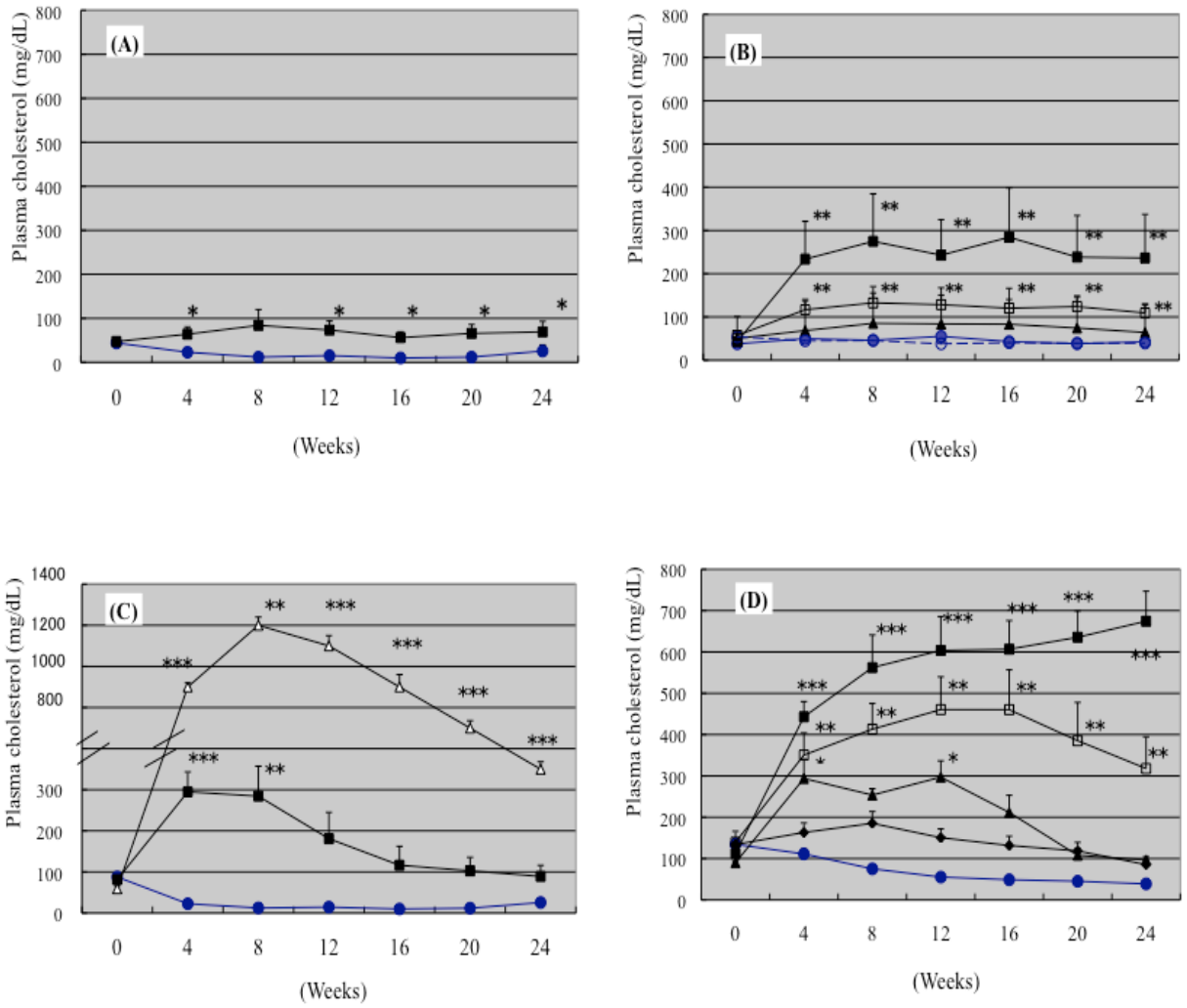
Figure legends

Fig.1 Plasma cholesterol levels in JW (A & B) and KHC (C & D) rabbits fed different concentrations of cholesterol in their diet.



Plasma cholesterol levels of male and female JW rabbits are shown in A and B, respectively.Plasma cholesterol levels of heterozygous male and female KHC rabbits are shown in C and in D, respectively. ●: Standard milled diet group, ■: 0.1 % cholesterol-containing diet group, □: 0.0.1 % cholesterol-containing diet group, □:0.25% cholesterol-containing diet group comprising non-ovariectomized rabbits. ○: A standard milled diet group for ovariectomized rabbits. □: 0.1 % cholesterol-containing diet group comprising ovariectomized rabbits.

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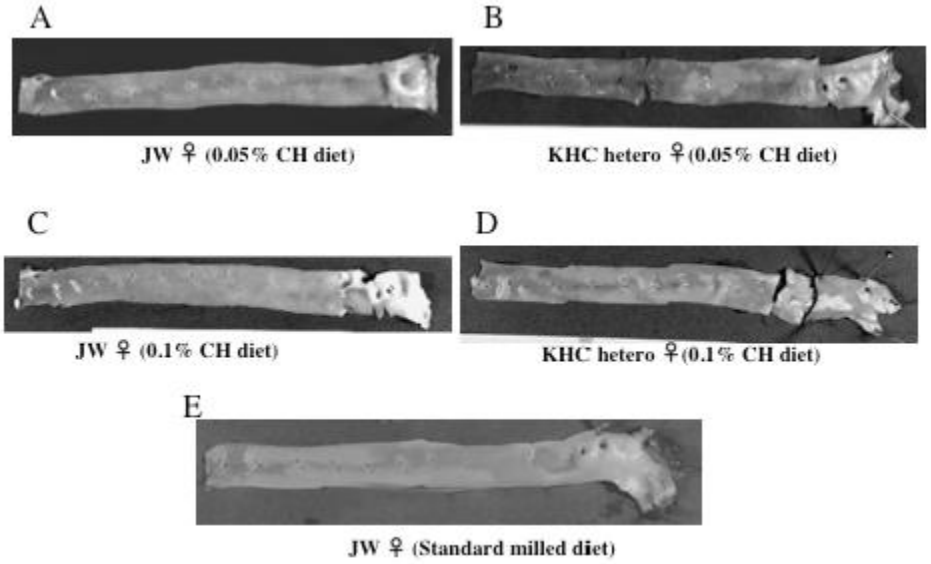
Fig. 2 Photograph of the aortas of JW and KHC rabbits.



A: JW rabbits fed the 0.05 % cholesterol-containing diet; B: heterozygous KHC rabbits fed the 0.05 % cholesterol-containing diet; C: JW rabbits fed the 0.1 % cholesterol-containing diet; D: heterozygous KHC rabbits fed the 0.1 % cholesterol-containing diet; E: JW rabbits fed a standard milled diet; for 24 weeks.

Table 1 Liver cholesterol levels in JW and KHC rabbits fed the several concentrations of cholesterol containing diet

		JW (mg/dL)	KHC (mg/dL)
Male	Standard milled diet	5.1 ± 0.6	4.5 ± 1.1
	0.1% CH diet	8.0 ± 1.8	7.2 ± 0.3 *
Female	Standard milled diet	ND	4.9 ± 0.7
	0.1% CH diet	5.2 ± 1.2	8.0 ± 0.9*
	0.25% CH diet	6.0 ± 1.0	ND
Ovx.	0.1% CH diet	ND	7.1 ± 0.2*

(ND: Not done)

Each value represents the mean ± S.E. (N= 3-8)

Significantly different from the standard milled diet group at *p<0.05.

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Table 2 Ratio to GAPDH of Apo-B,-E and LDL-r mRNA expression in the liver

			Apo B (%)	Apo E (%)	LDL-r (%)
JW	Male	Standard milled diet	100 ± 6.6	100 ± 5.7	100 ± 9.2
		0.1%CH diet	115.6 ± 12.7	106.4 ± 10.7	130.0 ± 5.4*
	Female	Standard milled diet	100 ± 0.6	100 ± 0.6	100 ± 0.6
		0.1%CH diet	64.7 ± 17.6	148.7 ± 10.4	105.3 ± 16.4
KHC	Male	Standard milled diet	100 ± 12.1	100 ± 1.6	100 ± 6.8
		0.1%CH diet	102.6 ± 3.2	86.0 ± 9.4	151.4 ± 2.3***
	Female	Commercial diet	100 ± 9.0	100 ± 5.0	100 ± 9.3
		0.1%CH diet	83.8 ± 12.1	114.6 ± 9.5	70.5 ± 4.6*

Each value represents the mean ± S.E. (N= 4-8)

Significantly different from the standard milled diet group at *p<0.05 and ***p<0.001.

Table 3 Total cholesterol levels in the lipoproteins in female rabbits for 24 weeks

			in VLDL	in LDL	in HDL
JW	Non Ovx.	Standard milled diet	15.9 ± 4.5	14.2 ± 1.1	12.4 ± 1.1
		0.05%CH diet	17.1 ± 3.4	30.7 ± 9.7	16.3 ± 2.1
		0.1%CH diet	98.4 ± 47.9*	123.7 ± 54.2**	14.4 ± 0.9
	Ovx.	Standard milled diet	18.0 ± 3.0	10.8 ± 1.5	10.9 ± 1.3
		0.1%CH diet	46.2 ± 11.4	44.3 ± 10.1	18.8 ± 1.5**
KHC	Non Ovx,	Standard milled diet	19.5 ± 3.8	17.5 ± 4.8	15.7 ± 2.9
		0.01%CH diet	20.4 ± 3.5	48.4 ± 6.4	16.4 ± 0.8
		0.05%CH diet	18.2 ± 1.8	62.6 ± 6.9	15.8 ± 0.8
		0.1%CH diet	327.8 ± 11.1***	299.9± 27.5***	14.4 ± 1.4
	Ovx.	0.1%CH diet	114.2 ± 36.2	193.2 ± 47.5***	10.4 ± 1.4*

Each value represents the mean ± S.E. of mg/dL. (N= 4-8)

Significantly different from the standard milled diet group at *p<0.05,**p,0.01 and ***p<0.001.

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Table4 Intimal surface area of thoracic aorta in female JW and heterozygous KHC rabbits after feeding of 0.1% cholesterol containing diet

	Aortic arch (%)	Total aorta (%)	Number of animals with lesions/Total number of animals
JW	28.2 ± 16.3	17.8 ± 10.8	2/4
KHC	32.7 ± 4.6	17.9 ± 2.6	4/4

Each intimal surface area of thoracic aorta value represents the mean ± S.E. (N=4)